Structural rearrangements during tRNA transcription initiation

Ewan Phillip Ramsay & Alessandro Vannini*

[1]The Institute of Cancer Research, London SW7 3RP, UK.

* Correspondence to A.V. (alessandro.vannini@icr.ac.uk)

Abstract

RNA polymerase III catalyses the synthesis of tRNAs in eukaryotic organisms. Through concerted biochemical and structural characterisation, multiple auxiliary factors have been identified alongside the polymerase as critical in both facilitating and regulating this transcription. Together, this machinery forms dynamic multi-protein complexes at tRNA loci required for polymerase recruitment, DNA opening and elongation of the tRNA transcript. Central to the function of these complexes is their ability to undergo multiple conformational changes and rearrangements that regulate each step. Here, we discuss the available data on the auxiliary factors and their regulation, focussing on the dynamic interactions between them and the polymerase as well as the current model for transcriptional initiation. Elaborating, we summarize the current understanding of polymerase re-initiation and its role in tRNA production. Increasingly, structural information is becoming available for both RNA polymerase III and related complexes, which are allowing for a deeper understanding of tRNA transcriptional initiation.

Highlights

- A summary of tRNA transcription initiation in eukaryotes
- New mechanistic insights for the structure of RNA polymerase III
- Novel redox regulation of tRNA transcription

- A summary of the current understanding of polymerase facilitated-recycling

Keywords

TFIIIC; TFIIIB; Brf2; RNA Polymerase III; Transcription Initiation; Facilitated Recycling

1. Introduction

In eukaryotes, the transcription of nuclear DNA is carried out using three distinct multi-subunit complexes, namely RNA Polymerase (Pol) I, II and III. Identified more than forty years ago by chromatographic separation from sea urchin preparations [1], Pols have since been characterised as highly functionally distinct enzymes, transcribing different classes of RNAs. The Pol III enzyme is devoted to the transcription of the entire pool of transfer RNAs (tRNAs) and other non-coding RNA transcripts such as 5S rRNA, U6 small nuclear RNA (snRNA), micro RNAs (miRNA), RNase P RNA and the 7SL RNA, amongst others [2, 3]. The high efficiency of the specialised Pol III machinery allows for high levels of transcription, with as many as 3-6 million tRNA molecules produced per population doubling in *S. cerevisae* [4].

Recently, significant progress has been made in the understanding of the structure and mechanism of initiation of the Pol III enzyme. In this review, we summarise the different promoter architectures and their associated auxiliary factors that are essential for tRNA transcription, with a particular emphasis with the best-characterised model organism *S. cerevisiae*. We describe the structural rearrangements involved during the process of polymerase recruitment, DNA promoter opening and transcriptional

initiation. Finally, we discuss the models of facilitated recycling which may be behind the extraordinary ability of RNA Pol III to produce high levels of tRNAs.

2. A Diverse Set of Promoters are Recognised by TFIIIC to Initiate tRNA Transcription

tRNAs represent the most populated class of genes, known as class II, transcribed by the Pol III enzyme, with all members in *S. cerevisae* sharing a consensus gene structure [2, 5]. Early studies suggested an intragenic architecture of tRNA promoters, with no observable sequence conservation in the 5' flanking regions, with deletions leaving as little as 6 base pairs 5' of the transcribed gene still supporting transcription [6-9]. Deletion or mutation of internal gene sequences was found to severely disrupt Pol III transcription, with sequences located both within the 5' and 3' regions of the tRNA coding region required for efficient transcription [10, 11]. Subsequent deletion and sequence analysis in *Xenopus laevis* identified two highly conserved consensus sequences in the 5' and 3' of tRNA genes, named the 'A-box' and 'B-box' respectively [12]. These regions, encoding the D and T loops of the tRNA, were found to be essential for transcription of many tRNAs, suggesting a common mechanism of regulation [13, 14].

The A and B boxes of the tRNA gene promote transcription through recruitment of the transcription factor TFIIIC in the first stage of tRNA transcription [15, 16] (Fig. 1A). In *S. cerevisae* TFIIIC is composed of six subunits arranged into two large globular sub-complexes termed τ_A (composed of subunits τ_{131}(Tfc4), τ_{95} (Tfc1) and τ_{55} (Tfc7)) and τ_B (composed of subunits τ_{138} (Tfc3), τ_{91} (Tfc6) and τ_{60} (Tfc8)) [5, 17-19]. TFIIIC architecture and composition appears to be conserved, with homologues for each yeast subunit identified in the human TFIIIC [20]. Together, the sub-

complexes τ_A and τ_B contain the DNA binding activity to the conserved A- and B-boxes, respectively [21-23]. Photochemical crosslinking and antibody-based interference experiments have identified the subunits mediating these interactions, with τ_{95} responsible for binding the A-box and τ_{138} binding the B-box [24, 25]. Whilst both these interactions are necessary for Pol III transcription, τ_B has been observed to have significantly higher DNA binding affinity than τ_A suggesting that this may the principle interaction required for TFIIIC recruitment [21]. Indeed, mutations of either τ_{138} or the B-box that abrogate this interaction significantly reduce TFIIIC-promoter affinity [26, 27]. Subsequent binding of τ_A to the A-box has been suggested to allow for correct selection of the transcriptional start site (TSS) [28, 29].

Although both the A and B-boxes are present at fixed positions from the 5' and 3' boundaries of the tRNA gene, insertion of introns (of between 7 to 71 nucleotides) or variation in the length of the variable arm in the tRNA sequence between them can alter their relative positions with separations of between 31 to 93 base-pairs frequently observed [30, 31]. This diverse promoter spacing is accommodated by a highly elastic TFIIIC structure. Early experiments using scanning electron microscopy and partial proteolysis suggested a highly flexible hinge between τ_A and τ_B, which could alter the relative positioning of the DNA-binding assemblies, forming a 'dumb-bell' shape [18, 19]. Recently, using cross-linked mass spectrometry (XL-MS), Male *et al.* identified this linker region as the tetratricopeptide (TPR) motif of τ_{131} contacting a disordered region of τ_{138} termed the τ Interacting Region (τIR) [32]. The disordered nature of this region is suggested to allow for a highly flexible structure that can dynamically recognise different promoter spacings and direct efficient tRNA transcription.

3. A Sequentially Assembled Complex of Transcription Factors Recruits Pol III to tRNA Genes

Once bound to the DNA, TFIIIC recruits an additional transcription factor known as TFIIIB [16, 33, 34]. TFIIIB was initially characterised as two chromatographically separable factors, B' and B'' that could be separated by high salt concentrations, and together were required to drive Pol III-mediated transcription [35, 36]. Early studies identified first the factor Brf1 (TFIIIB Related Factor-1) as part of the TFIIIB complex due to its ability to repress A-box mutations [37]. Brf1 is a paralogue of the Pol II general transcription factor TFIIB, which is required for Pol II transcription [38]. Indeed, architectural and functional homology is observed between Brf1 and TFIIB, with both proteins containing an N-terminal zinc-ribbon domain and two conserved N-terminal cyclin repeats (forming the B-core domain), essential for faithful transcription [39, 40]. This conserved N-terminal region is then followed by a unique C-terminus in Brf1 consisting of three Brf homology regions [5]. Through both genetic and biochemical experiments, Brf1 was also shown to interact with TATA-Box Binding Protein (TBP), which together were able to reconstitute the B' activity known to be part of the TFIIIB transcription factor [40-43]. The B' complex is able to bind the B'' factor, identified as the 90 kDa protein Bdp1 (B-Double Prime 1). Bdp1 was shown to contain a large conserved SANT (SWI–SNF, ADA complexes, N-Cor, and TFIIIB) domain in the C-terminus that was required for Bdp1 recruitment to form the TFIIIB complex [5, 41, 44-47]. The crystal structure of a Brf1 C-terminus-TBP complex revealed how the C-terminus of Brf1 (encompassing the Homology II region) straddled TBP, with site-specific mutations confirming that this formed a 'two-sided' adhesive surface which was able to bind Bdp1 via the SANT domain and assemble TFIIIB [48-50]. However, the N-terminus of Brf1 is also

capable of assembling a transcriptionally active TFIIIB assembly *in vitro* [51], suggesting an interaction between this region and TBP. Mutational cross-linking analysis further suggested this interaction, specifically suggesting binding of the Brf1 N-terminus to the C-terminal stirrup of TBP in a mode similar to TFIIB-TBP binding in the Pol II system [52]. Indeed, fusion of the Brf1 N- and C-termini separately either side of TBP produced a chimera, which bound DNA and facilitated transcription *in vitro* in a similar manner to the wild-type assembly [53]. This suggested that Brf1 makes many interactions with the TBP component, effectively 'sandwiching' the subunit between the N- and C-termini, with the interaction between the Brf1 C-terminus and TBP contributing to the major tethering interaction [53].

Whilst TFIIIC is crucially important in the recruitment of TFIIIB, with abrogation of the B-box-TFIIIC interaction preventing TFIIIB recruitment [34], TFIIIB appears to represent the minimal machinery required to direct multiple rounds of Pol III tRNA transcription. Following recruitment, TFIIIB can interact stably with the DNA upstream of the TSS forming a heparin-resistant assembly, with TFIIIC remaining labile [33, 54]. In this context TFIIIC acts as an assembly factor facilitating this complex formation and is dispensable for further transcription, with its heparin-mediated removal not affecting the kinetics of tRNA transcription *in vitro* [33].

Early work established the recruitment of TFIIIB as a dynamic, sequential interaction in which stepwise recruitment of TFIIIB components led to structural rearrangement within the multi-protein complex. Insights from both cross-linking and DNase I protection assays observed rearrangements in the association of TFIIIC complex with DNA following Brf1 and TBP association, producing an intermediate complex in which the upstream footprint was markedly different and internal gene regions became accessible for DNase I digestion [35, 41]. The expected upstream DNA

footprint for TFIIIB-DNA binding was only observed upon association of Bdp1, suggesting further conformational changes upon binding of this subunit and pointed towards a model of sequential conformational rearrangements in both TFIIIC and TFIIIB during complex assembly [35, 41].

The initial, rate-limiting step of TFIIIB recruitment is the binding of Brf1 [55-57]. Brf1 is recruited first to the promoter by the highly conserved N-terminus of the τ_{131} subunit of TFIIIC (Fig. 1B) [55, 58-61]. Early experiments had suspected this region to be important in TFIIIB recruitment as it was the only TFIIIC region that could be cross-linked from the A-box to upstream of the TSS, where TFIIIB was positioned [25, 35]. An interaction was also observed between human Bdp1 and TFIIIC102, the human homologue of τ_{131} suggesting conservation of this recruitment mechanism in humans [62]. The N-terminus of τ_{131} possesses a distinct structure, consisting of a protease-sensitive N-terminal region which immediately precedes 11 tetratricopeptide (TPR) repeats that are split into two clusters by a 134 amino acid intervening region (IVR) between TPRs 5 and 6 [63]. Consistent with the widespread role of TPRs in mediating protein-protein interactions [64], both high affinity (encompassing the N-terminus and TPRs 1-5) and low-affinity (formed by TPRs 6-9) sites for Brf1 binding have been identified in these regions [60, 65]. Yeast two-hybrid and competition analyses have suggested that the second cyclin repeat and C-terminal Brf homology II regions in Brf1 contact the N-terminal region up to the fifth TPR (Nt-TPR1-5) of τ_{131}, suggesting a dominant role for this τ_{131} region [66]. However, τ_{131} fragments including the later TPRs 6-9 showed lower affinity for Brf1, suggesting an auto-inhibitory interaction that was regulating the level of Brf1 recruitment [65]. Indeed, mutations in TPRs 1 and 2 were found to increase TFIIIB recruitment to pre-formed TFIIIC-DNA complexes and the formation of heparin-resistant TFIIIB-DNA complexes [55, 67].

Interestingly, the affinity for the N-terminus coupled to TPRs 1-5 was largely unchanged upon mutation of TPR1, with an increase in TFIIIB binding affinity seen in the mutant only for fragments incorporating the additional TPR6-9 region [67]. This was consistent with the incorporation of Brf1 peptides (corresponding to the C-terminal region in the full-length protein) that bound to, and competed for, TPRs 6-9 during complex assembly, relieving auto-inhibition and increasing TFIIIB binding [66]. Moreover, the activating mutation in TPR2 was found to have higher TFIIIB recruitment than the wild-type even in the presence of saturating levels of Brf1, suggesting loss of intrinsic inhibition upon mutation [55]. Together these studies suggest the mutations were able to overcome the internal auto-inhibition through potentially stabilising an association-competent conformation, implying a conformational change that must occur prior to Brf1 binding that may regulate the level of transcription (Fig. 1B).

Following Brf1 recruitment, TBP binds via interactions with the C-terminus of Brf1 and τ_{60} (Fig. 1C) [68], forming the B'-TFIIIC-DNA complex [41]. Introduction of TBP induces a DNA bend at tRNA promoters, as seen in the Pol II system [69, 70] (Fig. 1C). This, in turn, drives the association of Bdp1 to fully assemble the general transcription factor (GTF) complex on the tRNA gene, which stabilises the TFIIIB-driven DNA bending, kinetically trapping and stabilising the bound TFIIIB [54, 69, 71]. Initial observations suggested that TPRs 2 and 6-8 were important for Bdp1 binding [63, 72]. Indeed, D468K and L469K mutations in TPR 8 reduced Bdp1- τ_{131} binding *in vitro* and Bdp1 recruitment to TFIIIC complexes. The observation that these mutations also reduced Brf1 recruitment to TFIIIC complexes and association with τ_{131} in pull-down experiments led Liao *et al* to suggest that both Brf1 and Bdp1 share overlapping binding sites in TPRs 6-8 [63]. More recently, despite being able to

confirm TPR8 as the Bdp1 binding site using isothermal titration calorimetry (ITC), Male *et al* were unable to observe the reduction in Brf1 binding to TPR8 mutants using ITC with a purified Brf1-TBP fusion protein, suggesting distinct binding sites for each component [32]. This raises the intriguing possibility that binding of TBP to Brf1 could favour Brf1 association with the N-terminus, leaving Bdp1 free to bind at TPR8. However, crosslinking analysis has also shown the region around TPR8 to be the site of τ_{138} interaction (via the τIR). Mutation of D468K and L469K reduced τ_{131}-τ_{138} interaction in a manner similar to Bdp1. Indeed, both τ_{138} and Bdp1 competed directly for τ_{131} binding, suggesting a shared interaction interface on τ_{131} [32] (Fig. 1C). From this, a model has emerged for the complex process of TFIIIB recruitment to tRNA loci. Initial binding by Brf1 is driven by the τ_{131} N-terminus through overcoming of the auto-inhibitory interaction driven by TPRs6-9 in the TFIIIC subunit, possibly by binding of the C-terminal region of Brf1 [66] (Fig. 1B). This is followed by TBP binding and finally recruitment of Bdp1. Binding of Bdp1 displaces the τIR of τ_{138} from TPR8 of τ_{131} instigating a conformational change in TFIIIC which generates the highly stable TFIIIB-TFIIIC-DNA complex [32] (Fig. 1C). In this model, the relief of auto-inhibition in Brf1-τ_{131} binding and the subsequent rearrangement of τ_{138} upon Bdp1 binding may elicit the structural rearrangements which give rise to the alterations in DNase I protection and DNA-crosslinking of the complex during assembly seen previously [41].

4. Selenocysteine tRNA Transcription is Driven by a distinct Class of Pol III Promoters

While all tRNA transcription is carried out using TFIIIB and TFIIIC binding to the class II intragenic promoter in *S. cerevisae* [73], in higher eukaryotes an additional

extragenic promoter, termed the class III promoter, drives expression of the selenocysteine tRNA, essential in the production of selenoproteins [5, 74]. The Class III promoter has a markedly different architecture than the more commonly observed class II promoter, with the control elements placed upstream of the coding sequence. Immediately upstream of the TSS is the TATA box element, followed by a proximal sequence element (PSE) and then an additional enhancer sequence termed the distal sequence element (DSE) placed much further upstream (Fig. 2) [5]. In these promoters, TFIIIC does not bind and TFIIIB is recruited by binding directly to the DNA via interactions between TBP and the TATA box [75, 76]. Alongside the altered mode of TFIIIB recruitment at these promoters, the TFIIIB bound to class III promoters has an altered composition with incorporation of a Brf variant not seen in *S. cerevisae* known as Brf2 (previously called BRFU or TFIIIB50) [77, 78]. Despite sharing the same conserved TFIIB-like N-terminus with both zinc-ribbon and cyclin repeats, Brf2 differs in the C-terminal region compared with Brf1, with no homology regions [77]. Recently, structural analysis of assembled Brf2-containing TFIIIB complexes suggested a pivotal role of this region in complex assembly. The unique Brf2 C-terminus was shown to have an extended conformation, forming a 'molecular pin' structure at the interface between the Brf2 second cyclin repeat, TBP and the DNA that is essential for stabilisation of the TFIIIB complex [74]. Additional contacts are made with TBP by the extreme C-terminus, in a region termed the TBP 'anchor domain', which structurally resembles Brf1 homology region II and similarly directly binds TBP. The TBP anchor domain, together with TBP, also forms an interface with Bdp1 in the full TFIIIB complex, binding via the conserved SANT domain as seen previously for the yeast TFIIIB [49, 69].

Uniquely, the stability of the Brf2-containing TFIIIB complexes appears to be redox-regulated [79]. The conserved cysteine 361 residue, part of the molecular pin, is surface exposed and can cycle through both oxidised and reduced states, with the oxidised state showing reduced complex assembly [74]. Such redox regulation allows for the modulation of slenocysteine tRNA synthesis during oxidative stress. Through reduction of TFIIIB complex assembly, prolonged oxidative stress reduces selenoprotein production and induces apoptosis [74]. Such regulation is crucial for normal cellular growth, with overexpression of Brf2 (which overcomes this oxidative stress induced apoptosis) associated with carcinogenesis [74, 80].

The recruited TFIIIB also binds via the TBP subunit to a large, multi-protein complex termed $SNAP_c$ (snRNA activating complex), which also interacts weakly with the PSE. $SNAP_c$ is also used by Pol II in the transcription of small nuclear RNAs (snRNAs) where this PSE binding is the sole signal required for GTF recruitment. The presence of a TATA box for TFIIIB binding specifies Pol III transcription of the gene [81, 82] (Fig. 2). $SNAP_c$ consists of five subunits: SNAP190, SNAP50, SNAP45, SNAP43 and SNAP19, with DNA recognition carried out by the Myb repeats found in the largest subunit SNAP190 [5, 83] (Fig. 2). $SNAP_c$ appears central to the assembly of the GTF complexes found at class III promoters. The DSE elements, found much further upstream bind to two additional enhancer factors known as Oct-1 (also known as POU2F1) and ZNF-143 [5]. Alongside interactions with TFIIIB and the PSE, $SNAP_c$ also contacts Oct-1 with mutational analysis suggesting an interaction between SNAP-190 and the N-terminal POU domain in Oct-1 [84, 85]. In order to juxtapose these two sites to allow for the enhancer interaction, the intervening DNA (with a length of approximately 150 base pairs) houses a nucleosome, rendering the intervening chromatin sequences resistant to both DNase I

and micrococcal nuclease digestion [86] (Fig. 2). This juxtaposition is essential for efficient transcription from class III promoters. *In vitro* transcription assays revealed a significant loss of transcriptional activity in the absence of nucleosomes [86]. This nucleosomal transcriptional dependence could be alleviated by progressively shortening the length of the intervening DNA [86], suggesting that the nucleosome deforms the DNA to facilitate a direct protein interaction in order to enhance the level of transcription [86, 87].

5. RNA Polymerase III

Each of the diverse promoter-GTF assemblies form interfaces which recruit RNA Pol III to the promoters for tRNA transcription. Pol III is a large (approximately 700 kDa), 17 subunit enzyme which contains a highly conserved ten subunit core. At the centre of the core are the C160 and C128 subunits in the *S. cerevisae* enzyme (RPC1 and RPC2 in the human polymerase) [88, 89]. These are highly conserved across the three polymerases (corresponding to A190 and A135 in Pol I and Rpb1 and Rpb2 in Pol II) and form the active centre for RNA synthesis [38]. This is bound by another heterodimeric subassembly in Pol III called the polymerase stalk (consisting of subunits C25 and C17 in Pol III) [90, 91] and C11, a subunit sharing homology with the Pol II GTF TFIIS that has RNA cleavage activity [38, 92]. The remainder of the core consists of small, highly conserved subunits, all of which are essential for polymerase function.

In Pol III, this core is bound by additional sub-assemblies that are specific to the Pol III enzyme. These include namely a heterotrimer (consisting of the subunits C82, C34 and C31) and a heterodimer (formed of subunits C53 and C37), which can be disassociated from the polymerase core as stable sub-complexes [93, 94]. Sequence

analysis has revealed that these stably incorporated subunits are homologous to the TFIIE and TFIIF GTF assemblies required for Pol II transcription initiation. Whilst TFIIE and TFIIF remain separated from Pol II they are incorporated in the Pol III system [95]. Recent structural analysis has complemented previous cross-linking studies showing that the heterotrimer and heterodimer assemble on C160 and C128 respectively, either side of the DNA binding cleft [89]. This incorporation is suggested to increase transcriptional efficiency. Pol III transcription is characterised by multiple rounds of re-initiation, as will be discussed in later sections, which may underlie the exceptionally high levels of transcription observed for this enzyme [96]. Incorporation of essential GTFs therefore may allow for rapid re-engagement and initiation without the requirement to reassemble the entire complex from individual factors as in the Pol II system [4]. Indeed, this may be a general feature of highly efficient polymerase complexes. The RNA polymerase I enzyme, which transcribes the rRNA precursor contributing to 60% of total cellular transcription [97], also has TFIIF (A49 N-terminal domain and A34.5) and TFIIE (A49 C-terminal domain) sequences incorporated into the polymerase enzyme [38].

6. Assembly of the RNA Polymerase III Pre-Initiation Complex at tRNA Genes

In order to transcribe the tRNA gene, bound TFIIIC and TFIIIB recruit the Pol III enzyme to form the pre-initiation complex (PIC), which directs transcription initiation (Fig. 1D). In the *S. cerevisae* PIC, numerous interactions are responsible for polymerase recruitment. The best characterised of these involve the Pol III specific subunit C34 (Fig. 1D). Initial yeast-two hybrid analysis suggested an interaction between this Pol III subunit and Brf1 [98]. A direct interaction was subsequently confirmed between the two components, with the N-terminal cyclin repeats of Brf1

observed to interact with the C-terminus of C34 [59]. Surprisingly, despite the high homology of this N-terminal region of Brf1 with TFIIB, this interaction was specific for the Pol III factor, suggesting an interaction specific for driving Pol III-mediated transcription [98]. Indeed, mutation of C34 led to loss of Pol III recruitment to assembled TFIIIC-TFIIIB complexes and a reduction in *in vitro* transcription, implying that this interaction may be the dominant binding event during polymerase recruitment [99]. Recently, employing crosslinking analysis, the nature of this interaction has been probed further. Both cross-linking and C34-directed hydroxyl-cleavage again displayed involvement of the N-terminal cyclin repeats in C34 binding [100]. This was at odds with earlier mutational analyses that had suggested a role for the C-terminus of Brf1 in binding C34 [101]. However, it is true that this region is also important for TBP and Bdp1 binding [49, 101, 102] so it is unclear if mutations in the C-terminus specifically targeted the recruitment interaction or caused a more general alteration of the TFIIIB complex, which affected pol III recruitment. Using this crosslinking approach, Khoo *et al* were able to also identify binding sites on the C34 partner. The C34 sequence consists of three winged-helix (WH) domains thought to be important in DNA binding. Brf1 was found to cross-link C34 at a conserved region between WH2 and WH3, which could also potentially alter C34 conformation and initiate Pol III-mediated transcription [100]. Indeed, it was shown previously that C34 has additional roles following recruitment during initiation [99], which will be described in later sections.

Despite the apparent dominant role of the Brf1-C34 interaction, additional contacts between the polymerase and promoter-bound GTFs have been identified. Indeed, the essential stalk subunit C17 has been shown to directly contact the N-terminal cyclin repeats of Brf1 [90] (Fig. 1D), with other cross-links recently identified between Brf1,

C160 and C128 [100]. Further to this, other components of the TFIIIB complex have also been implicated in Pol III recruitment. For instance, both human TBP and Bdp1 have been observed to interact with the human equivalent of C34, RPC6 (hRPC39) [94]. Indeed, this is consistent with crosslinks identified between Bdp1 and the C-terminus of C37 [103], which itself also interacts with C34 and RPC2 placing the N-terminus of Bdp1 as part of a network close to the polymerase active site [104] (Fig. 1D). Aside from TFIIIB contacts, it appears that TFIIIC may also bind Pol III at the promoter. Pol III subunits ABC10α and C53 have been found to contact τ_{131} [105, 106]. It is interesting to speculate that this TFIIIC subunit, which also contacts TFIIIB, may allow for additional crosstalk between the polymerase and TFIIIB in the PIC. Together, all these contacts likely present a large interaction interface to the polymerase enzyme, underlining the complex nature of polymerase recruitment to GTF complexes.

Recruitment of Pol III also requires loading of DNA in the active site cleft of the enzyme. Recent cryoEM structures of both apo and elongating pol III have revealed a high conformational flexibility that facilitates this DNA binding [89]. The flexibility resides in the clamp region of C160, which in the apo state forms a widened cleft to facilitate DNA loading. Contrasting this structure with that of the elongating pol III, there is a significant narrowing of the cleft caused by closing of the clamp, which secures the DNA into the active site. This motion also causes a rearrangement of the stalk and heterotrimer structures bound to C160, which move closer together in the elongating state [89].

7. A Sequential, Two-Step Model for Promoter Opening

RNA polymerase initiation proceeds in the PIC in a process which involves melting the DNA template and the initiation of RNA synthesis. In the first step of this initiation, the duplex DNA is loaded into the active site cleft of the recruited polymerase, forming a closed complex (CC) (Fig. 1D). In order to initiate tRNA transcription, the promoter is then melted into its constituent single strands, which are engaged by machinery in the polymerase active site. This forms the open complex (OC), which, upon synthesis of the first complementary RNA bases forms the initially transcribing complex (ITC). The polymerase then escapes the promoter, forming the elongating complex (EC), which transcribes the DNA template strand into RNA [107].

The precise mechanism of Pol III-PIC promoter opening following polymerase recruitment has been the subject of intense biochemical investigation. Early experiments alluded to a two-stage promoter opening, with Pol III promoter sequences showing two regions that opened at different temperatures. An upstream segment, around -9 to -5 melted at a lower temperature than sequences around the TSS (positions -3 to +11), suggesting an upstream region that is more readily opened during initiation [108]. This opening is spontaneous upon Pol III association, however requires the apparently sequential action of Bdp1 and Brf1 to induce DNA melting [108-110]. Using a series of N- and C-terminal truncations of each component [51, 109, 110], important regions required for this promoter opening were identified. A combination of Brf1 N-terminal truncation (of the first 68 residues) with a small deletion in Bdp1 (between residues 355 and 421) led to a complete loss of promoter opening capability of correctly assembled PICs [111]. Interestingly, singly substituting either truncation with the wild-type version restored transcriptional activity of the PICs for supercoiled but not linear DNA templates, suggesting a

deficiency in DNA melting [111]. Following this, Kassavetis *et al* demonstrated that N-terminal deletions in Bdp1 could be rescued by pre-opening the linear DNA at positions -9 to -5, with a single N-terminal truncation of Brf1 rescued by a DNA bubble near the TSS, between positions +2 to +6. These regions agreed with the previously observed promoter opening sequences, suggesting two distinct events during promoter opening [108, 110, 111]. From this, a model was proposed in which Bdp1 N-terminal region (encompassing residues 355 to 421) first opens an upstream bubble (Fig. 1E), which is then extended by the zinc-ribbon domain in the N-terminus of Brf1 (Fig. 1F) to complete promoter opening [111]. Indeed, recent structural observations have supported this model. The crystal structure of the assembled human TFIIIB in complex with the U6 snRNA promoter showed a highly extended N-terminal linker immediately before the SANT domain which inserts between the two cyclin domains of Brf2 and makes contact with the major groove and DNA backbone using highly conserved aromatic residues. Deletion of residues in this region generated promoter-opening defective mutants, as previously seen in the yeast homologue [111]. As a result, the linker region was suggested to correctly position the N-terminal linker domain for promoter opening [69].

However, despite the observations that the Bdp1 N-terminus extends into the polymerase active site and forms DNA contacts [69, 103], photo-crosslinking studies have suggested that both Bdp1 and Brf1 drive promoter opening through inducing conformational changes in pol III. In particular, Bdp1 is thought to alter the arrangement of the heterotrimer subcomplex, which has been long implicated in promoter opening [94, 99, 112], consistent with the homology shared between C34 and C82 with TFIIE that serves this function in the pol II system [38]. Indeed, mutation of C34 generated mutants which could efficiently recruit Pol III to the

promoter but were unable to facilitate transcription, forming inactive complexes despite the presence of wild-type Brf1 and Bdp1 [99]. Recent structural analysis has also observed that the DNA-binding WH domains of C34 extend over the polymerase DNA binding cleft, likely allowing for interaction with the DNA [89]. The binding partner of C34, C82 also similarly extends into the binding cleft and contacts the downstream DNA via its WH domains at two sites around +7 and +15 in the elongating state [89]. Furthermore, transition to the elongating state positions the heterotrimer closer to the bound DNA, presumably to facilitate this contact [89]. This is consistent with crosslinking analysis, which observed extensive C82-DNA crosslinking following DNA opening [113]. Together, these data suggest that the heterotrimer is important in the stabilisation of the opened DNA bubble through forming multiple DNA contacts, and may serve as an efficient site for TFIIIB to regulate in order to control DNA melting. Carrying out crosslinking analysis at both 0^{o}C and 30^{o}C, Brf1, Bdp1, C160, C128, C34 and C82 were all observed to be close to the DNA irrespective of promoter opening [113]. However, both Brf1 and Bdp1 cross-linked the DNA at sites distinct from the regions previously observed to be required for DNA melting, suggesting an indirect mechanism of TFIIIB action. Indeed, incorporation promoter-opening deficient Bdp1 and Brf1 mutants led to a dramatic alteration of C82, C34, C160 and C128 DNA crosslinking compared to the wild-type, suggesting that the presence of the deleted regions possibly influences the conformation of the active site in order to favour initiation [113].

8. Promoter Escape Likely Induces Further PIC Rearrangements

For the majority of tRNA genes, transition of Pol III to the EC and elongation of the tRNA transcript must be able overcome the unique nature of the intragenic promoter

in a process that is currently not well understood. TFIIIC, which is bound to part of the transcribed region, presumably acts as a 'block' on transcription with removal of this 'block' likely involving further conformational rearrangements in the auxiliary factors. This marks a transition from recruitment to a processive transcription and re-initiation state, which produces most of the tRNA molecules present in the cell [4]. Such rearrangement may lead to the complete removal of the TFIIIC in single or sequential steps or could induce conformational changes that render the template strand available to the polymerase without the need for complete TFIIIC removal. Indeed, TFIIIC appears dispensable following polymerase recruitment [33] with early genome wide studies suggesting its removal in active, transcribing Pol III genes [114]. *In vitro* experiments also suggested this removal of TFIIIC during Pol III transcription [115]. Indeed, during genome-wide chromatin immunoprecipitation (CHiP) experiments, removal of nutrients from cultures of *S. cerevisae* reduced Pol III and TFIIIB gene occupancy whilst increasing TFIIIC gene association. Re-introduction of the nutrients led to a reversal of this effect, with loss of TFIIIC from the active genes [114]. However, the system may not be a simple, binary response, with other CHiP analyses suggesting a constant low level of TFIIIC occupancy (albeit at a low level compared to TFIIIB and Pol III) of Pol III genes irrespective of transcriptional activity [116]. Soragni *et al* reported the occupancy of the Pol III locus ZOD1, which shows low levels of transcription, possessed a similar TFIIIC occupancy compared to more active genes. Furthermore during this study introduction of an artificial substrate during the CHiP experiment, which showed a six-fold reduction in transcription compared to the wild-type reference construct, had a markedly reduced TFIIIC occupancy despite this construct able to form TFIIIC-TFIIIB-DNA complexes efficiently using *in vitro* electromobility shift assays

(EMSAs) [116]. Therefore, it seems that regulation of TFIIIC levels may be multi-faceted and could also have some degree of gene variability. Indeed, Harismendy *et al* observed such variability in *S. cerevisae.* In this study, CHiP analysis identified eight loci for which TFIIIC occupancy did not change with others which even decreased upon transition from exponential to stationary phase growth, a transition characterised by a reduction in Pol III gene association [117]. This could potentially point to additional factors or gene sequences that regulate the dynamics of TFIIIC association during transcription.

The nature of TFIIIC removal and potential interactions with the Pol III machinery has also been investigated. Recent studies have observed unequal Pol III distributions across tRNA coding units [118]. Pol III appears to be concentrated in two regions found on the 5' and 3' end of the genes. The 5' peak showed a higher intensity than the 3', suggesting that build-up of polymerase here may be due to promoter escape representing a major rate-limiting step during transcription [118]. Interestingly, these Pol III rich regions also corresponded to the A and B-boxes bound by TFIIIC. This suggested that TFIIIC was forming a transient barrier, which could also potentially regulate transcription kinetics. The observation of the two peaks also suggests that Pol III may be sequentially removing first the τ_A (Fig. 1G) and then the τ_B module (Fig. 1H) in a sequential removal model. With the documented interactions between the polymerase and TFIIIC [105, 106] and the intrinsic flexibility of this auxiliary factor [18, 32], it is intriguing to speculate that the elongating Pol III may induce conformational changes in TFIIIC throughout the transcription cycle. Such changes may regulate the DNA binding affinity for each module to facilitate polymerase passage, allowing for tRNA transcription. However, currently, the conformational changes that occur during Pol III promoter escape remain elusive.

9. Faciltated Recycling – Termination-Linked Re-initiation

Following transcript elongation, Pol III transcription is terminated by a single stretch of at least five thymidine nucleotides on the non-template strand [119]. Consistent with the homology shared with the TFIIF factor, the Pol III-specific C53/C37 heterodimer serves this function through C37 by extending a loop region into the active site to recognise this termination signal on the 3' end of the tRNA gene [89, 119, 120]. The use of such a minimal terminator appears to allow for efficient termination of tRNA transcripts and facilitate the observed high transcription levels. However, a remarkable feature of this termination mechanism, which is crucial for the observed tRNA high yields, is its linkage to rapid re-initiation from the stably bound TFIIIB complex, bound just upstream of the TSS [33, 54] (Fig. 1I).

The model of facilitated recycling was first proposed by Dieci *et al* following observations of the yeast system *in vitro* that multiple round transcription proceeded 5- to 10-fold more rapidly that initial transcription and had a much reduced sensitivity to heparin. Pol III also displayed a higher preference for association with templates on which it was transcribing in competition assays following introduction of a competing template with GTF complexes pre-assembled for Pol III recruitment. This is conserved from yeast to humans, with template commitment of the human enzyme *in vitro* protecting it from Maf1-mediated inhibition [121]. Together, these data suggested that Pol III, once stably associated was not released from the gene and instead was recycled through many rapid cycles of transcription, observed to be as rapid as 16 seconds per cycle for some tRNA genes in yeast [96, 122] (Fig. 1I). For short genes, such as those transcribed by Pol III, consistent with its apparent function as an assembly factor [33], TFIIIC was dispensable for rapid re-initiation, suggesting

that this process is regulated by TFIIIB components. Interestingly, increasing the gene size to greater than 300 base pairs revealed a requirement for these genes of TFIIIC to support re-initiation [122]. However, this is still poorly understood, with current suggestions that TFIIIC is removed by transcribing polymerase apparently at odds with a role for TFIIIC in such a re-initiation mechanism.

Consistent with the commitment of Pol III to a single template during transcription, Pol III is thought to maintain constant association with the target gene. As a result, re-initiation requires linkage to transcriptional termination. Indeed, *in vitro* the level of termination runoff was inversely proportional to the re-initiation efficiency [96]. Therefore, it has been suggested that the terminator maybe somehow juxtaposed to the stably-bound TFIIIB complex at the TSS, facilitating a polymerase 'hand-over' which allows for the rapid recycling of the enzyme [96]. This termination-linked re-initiation requires the C11 subunit bound to both the C53/C37 heterodimer and the C128 subunit [89]. Deletion of C11 from Pol III did not affect terminator recognition or transcript release, but prevented re-initiation [120]. This C11-mediated re-initiation did not require the RNA cleavage activity found in this subunit, with the catalytically dead E92H mutant still facilitating polymerase recycling. Instead, the cleavage-independent C11 activity was proposed to be due to a C11-initiated conformational change in the enzyme over the terminator, which switched it from an elongating to a terminating state [92, 120]. Indeed, a recent study observed a stable, paused pre-termination complex (PTC) over the fourth thymidine nucleotide in the termination signal. These PTCs were distinct from stalled ECs in their transcript release and extension properties. Furthermore, the formation of these PTCs required the C53/C37 dimer and the C11 subunit, with the fifth thymidine in the sequence promoting release of the RNA transcript from these PTCs [119]. The distinct nature of the PTCs from

the ECs and their reliance on C11 alongside the heterodimer for formation suggests that this complex may potentially represent the previously suggested conformational change from the elongating to the terminating polymerase state. Such a conformational change may render Pol III competent for re-engagement with TFIIIB and allow facilitated recycling. However, currently, the precise mechanism by which Pol III re-initiation occurs is unknown and is likely to represent an interesting field for future study.

10. Conclusions and Future Perspectives

From the initial discovery of Pol III almost fifty years ago, a number of mechanistic details of transcription initiation, elongation and termination have been elucidated, giving a window into how Pol III achieves its remarkably efficient and precise transcription of tRNAs. Despite these remarkable achievements, a number of key questions remain which would provide a deeper understanding of the Pol III system. One such critical question is how the PIC is able to catalyse promoter opening and allow for initiation of transcription followed by promoter escape. Although a number of studies have highlighted factors such as Brf1 and Bdp1 and the Pol III C82/34/31 heterotrimer as key players and have suggested a two-step model for promoter opening [99, 110, 111, 113], the precise mechanism by which this is achieved is currently unknown. Mechanistic detail is also elusive for how Pol III can be rapidly reloaded and recycled on a single engaged transcript. This appears to underline the impressive yields of, in particular, tRNAs and may involve a complex set of conformational changes and interactions, which could also be the targets of novel Pol III regulatory mechanisms. Answers to these questions would allow for an unprecedented insight into Pol III transcription and tRNAs synthesis, whose

deregulation has been linked to to cancer [123] and neurodegenerative diseases [124], opening avenues for future studies into therapeutic interventions.

The enormous promise of new structural biology techniques may provide the means to finally arrive at such a mechanistic understanding of Pol III transcription. In particular cryo-EM, which has undergone what has been described as a ‘resolution revolution’ in recent years [125], has been useful in uncovering details of both Pol I and Pol II transcription. In the last few years, combining cryo-EM structures with other structural and biochemical analyses of Pol I and Pol II PICs in multiple states of initiation together with structures of both elongating and unbound enzymes have been invaluable in uncovering detailed mechanisms and important interactions involved in transcription [126-134]. Furthermore, the recent structures of unbound and elongating Pol III have started to shed light on the specific features of this enzyme [89]. However, a full understanding of initiation and the associated conformational changes will require structural analysis of the PIC in multiple assembly and initiation states. This would allow for characterisation of the dynamic assembly of the complex and potential conformational changes induced in the polymerase upon initiation. Also, structural analyses of polymerase termination and its link to TFIIIB at the TSS would allow for an understanding of how the enzyme is recycled on the tRNA template. Overall, applying a modern structural biology approach, the transcription cycle could be finely dissected, giving a mechanistic understanding of Pol III transcription and tRNA synthesis.

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Fig. 1

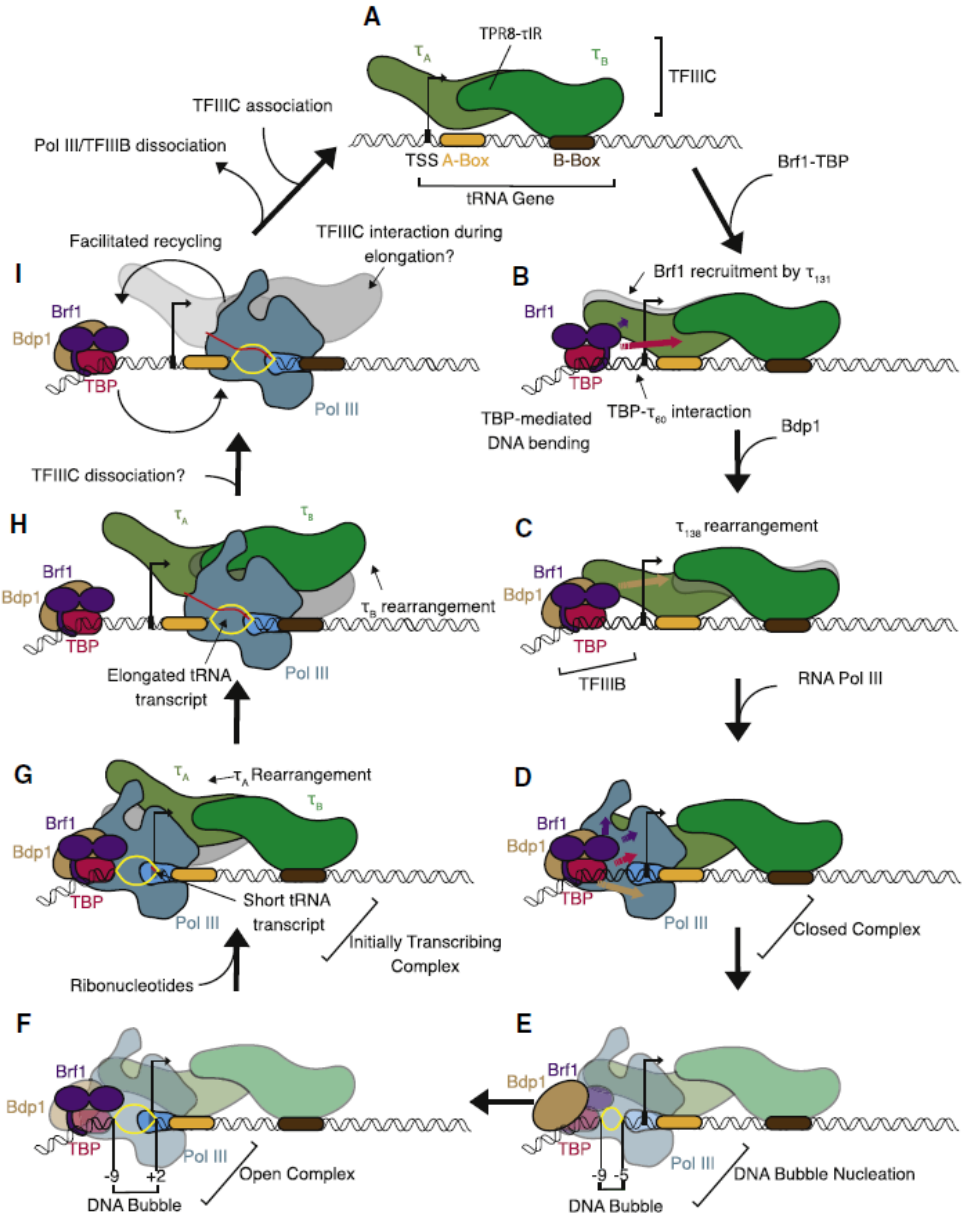

Fig. 1. Model of Pol III-mediated tRNA transcription. (A) TFIIIC recognises the A and B-box elements within the tRNA gene sequence via the τA and τB modules. (B) Brf1-TBP is recruited by the N-terminus of the τ131 subunit of TFIIIC (purple arrow). TBP association is facilitated via interactions with τ60 (pink arrow) and causes DNA bending, forming the B′-TFIIIC complex. (C) Bdp1 association competes for τ131 binding with τ138 (gold arrow), leading to structural rearrangement and formation of the stable TFIIIB-TFIIIC complex at the promoter. (D) The TFIIIB-TFIIIC assembly then recruits Pol III to the promoter via interactions between Brf1 and TBP and the C34/C17 Pol III subunits (purple arrows). Additional interactions between Bdp1 and C37 are also observed (gold arrow). The polymerase cleft closes around the DNA, forming a closed pre-initiation complex at the tRNA promoter. (E) The N-terminus of Bdp1 initiates promoter opening upstream of the transcriptional start site (TSS) between positions −9 to −5. (F) The DNA bubble is extended by the action of the N-terminal zinc ribbon domain of Brf1, forming the full DNA bubble, which extends to the TSS in the polymerase active site producing the open complex. (G) Initial synthesis of the complementary tRNA transcript on the template strand forms the initially transcribing complex. Subsequent promoter escape of this assembly is a rate-limiting step and likely requires τA rearrangement at the promoter. (H) Promoter escape leads to the formation of the elongating complex, which synthesises the tRNA transcript. The elongating polymerase assembly may rearrange the τB module, potentially removing TFIIIC from the active gene whilst leaving the stably bound TFIIIB upstream of the TSS. (I) TFIIIB directs multiple rounds of re-initiation on the same template through facilitated recycling, allowing for high yields of tRNA. Dissociation of these components requires TFIIIC re-association to reassemble the general transcription factor-polymerase complex and allow for further rounds of transcription.

Fig. 2

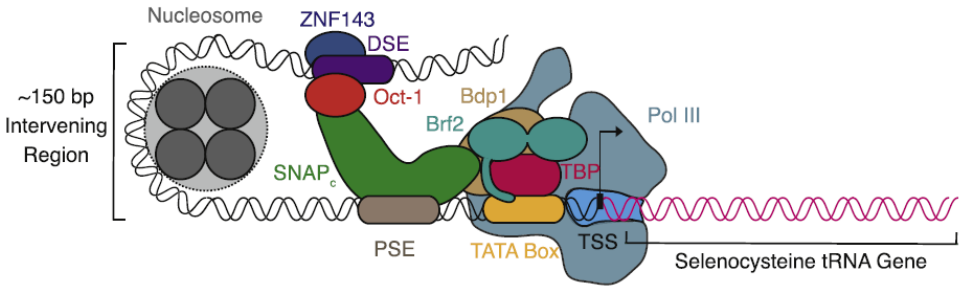

Fig. 2. The selenocysteine tRNA promoter. In vertebrates, selenocysteine tRNAs are transcribed using the class III promoter. In this class of promoters, TFIIIB is formed as a heterotrimer of TBP, Bdp1 and Brf2, as opposed of Brf1 in class I and III promoters, forming a redox-sensitive complex. This binds the DNA directly via TBP binding to the TATA box found upstream of the TSS. TFIIIB also binds the multi-subunit factor SNAPc which binds immediately upstream at the proximal sequence element (PSE). SNAPc binding is stabilised via binding of the Oct-1 and ZNF-143 proteins that bind at the distal sequence element (DSE) enhancer. These components are juxtaposed via nucleosome binding which loops the intervening DNA sequence. This assembly recruits Pol III via TFIIIB, fully assembling the pre-initiation complex at this class of promoters.