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Short communication

THE TRANSCRIPTION REINITIATION PROPERTIES OF RNA POLYMERASE III IN THE ABSENCE OF TRANSCRIPTION FACTORS

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Abstract: Transcription reinitiation by RNA polymerase (Pol) III proceeds through facilitated recycling, a process by which the terminating Pol III, assisted by the transcription factors TFIIIB and TFIIIC, rapidly reloads onto the same transcription unit. To get further insight into the Pol III transcription mechanism, we analyzed the kinetics of transcription initiation and reinitiation of a simplified *in vitro* transcription system consisting only of Pol III and template DNA. The data indicates that, in the absence of transcription factors, first-round transcription initiation by Pol III proceeds at a normal rate, while facilitated reinitiation during subsequent cycles is compromised.

Key words: RNA polymerase III, Transcription reinitiation, TFIIIB, G-less cassette

INTRODUCTION

Transcription reinitiation accounts for the bulk of RNA synthesis in living cells [1]. In eukaryotes, the RNA polymerase III (Pol III) transcription system (devoted to the production of very abundant, untranslated RNAs such as the tRNAs and the 5S rRNA) attains the highest known reinitiation efficiency, due to the existence of a facilitated Pol III recycling mechanism that dramatically increases the

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Abbreviations used: NTP – ribonucleoside triphosphate; PIC – pre-initiation complex; Pol – RNA polymerase; TF – transcription factor

transcription reinitiation rate [2]. The Pol III auxiliary transcription factors, TFIIIB and TFIIIC, play a fundamental role in facilitated recycling by favouring Pol III reloading onto the same transcription unit after each transcription cycle [3]. These transcription factors should thus be viewed as acting on at least two levels. First, during the initial assembly of a functional transcription complex, they assemble with the promoter, forming a stable pre-initiation complex (PIC) that allows for specific Pol III recruitment and initiation at the correct transcription start site. Then, during subsequent transcription cycles, the TFIIIB/TFIIIC-containing PIC is required for iterated recapture of Pol III by the same transcription unit. It is reasonable to assume that Pol III possesses functional and structural features that allow it to be handed back to the same transcription start site upon transcription termination, as suggested in a recent paper [4]. Such unique features might in principle influence the transcription initiation and reinitiation properties of Pol III even in the absence of transcription factors. To address this issue, we analyzed the kinetics of Pol III transcription initiation and reinitiation using a simplified *in vitro* system lacking any transcription factor and consisting solely of RNA polymerase, template DNA, a dinucleotide primer and the four ribonucleoside triphosphates (NTPs).

MATERIALS AND METHODS

For the construction of the 3'-overhanged G-less template used in the factor-free transcription experiments, equimolar amounts of the two oligonucleotides Gless_34mer_fw (5'-GAGCTCTTCCCCTCCATACCCTTCCTCCATCTAT) and Gless_34mer_rev (5'-ATAGATGGAGGAAGGGTATGGAGGGGAA GAGCTC) (Sac I site underlined) were annealed, and the resulting double-stranded 34-bp fragment was inserted into the Stu I site of the I(TAT)LR1 tDNA of *S. cerevisiae*, contained in the pBluescript KS-I(TAT)LR1 plasmid [5]. The resulting plasmid was digested with the SacI restriction endonuclease, yielding a ~3000-bp linear DNA molecule that was gel-purified using the QIAquick gel extraction kit (Qiagen), and used for the transcription experiments.
In vitro transcription of the Sac I-digested, 3'-overhanged template was carried out in 25-μl reaction mixtures containing 150 mM KCl, 8% glycerol (v/v), 5 mM $MgCl_2$, 1 mM DTT, 160 μg/ml bovine serum albumin, 400 μM CpU dinucleotide primer, 500 μM each of ATP, GTP and CTP, 25 μM cold UTP and 10 μCi of [α-^{32}P]UTP (Amersham Biosciences, 800 Ci/mmol), in the presence of 10 units of SUPERase-In (Ambion) as an RNase inhibitor. In a typical reaction (30 min at 37ºC), the template DNA (varying amounts of the gel-purified, ~3000-bp SacI fragment described above) was pre-incubated for 20 min with *S. cerevisiae* Pol III, purified as described [3], in the presence of the CpU dinucleotide primer, then NTPs were added for an additional 10 min. In the reinitiation time course experiment (Fig. 2B), only ATP, CTP and UTP were added at this stage for 10 min, followed by GTP addition and additional incubation to allow for the completion of the first transcription cycle and

transcription reinitiation. All the transcription reaction products were fractionated on 6% polyacrylamide, 7 M urea gels, which were then dried and phosphorimaged with a Personal Imager FX (Bio-Rad). Bands corresponding to transcription products were then quantified using the MultiAnalyst PC software (Bio-Rad).

Factor-dependent *in vitro* transcription of the I(TAT)LR1 tDNA was carried out under previously described conditions [3]. In addition to pure Pol III, the reaction mixtures contained recombinant TBP and Brf1 proteins together with partially purified TFIIIC and B" fractions, all prepared as previously described [6].

RESULTS

In this study, we exploited the ability of Pol III to autonomously initiate transcription at a 3' overhang of linear DNA molecules [7]. The template used for the factor-free transcription experiments (referred to as G-less tDNA^{Ile}) contains part of the coding region (approximately from the B-block to the terminator) of the *Saccharomyces cerevisiae* I(TAT)LR1 gene, coding for

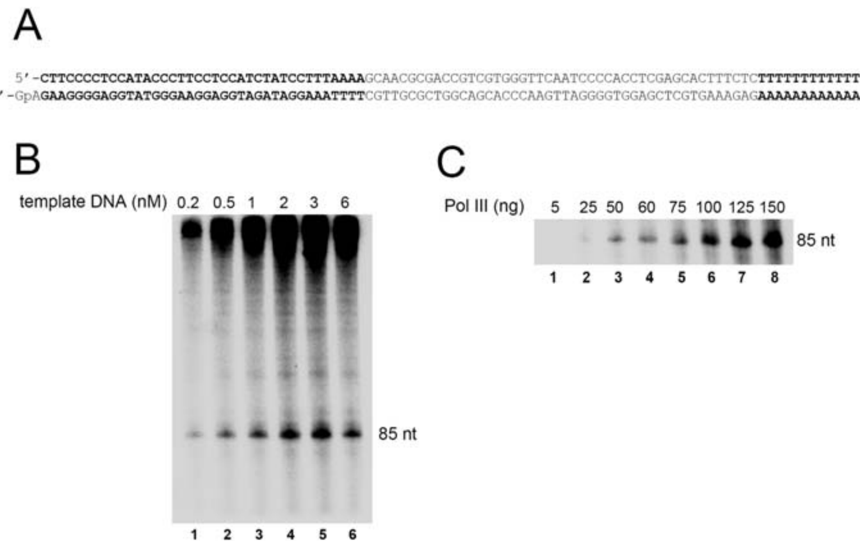

Fig. 1. Template and RNA polymerase III titration assays with a 3'-overhanged template. A – The structure of the 3'-overhanged template used in this study. The G-less fragment and the terminator region are in bold characters. B – Purified Pol III (125 ng), the four NTPs and the CpU dinucleotide primer were incubated with increasing amounts of the 3'-overhanged template (as indicated above the gel image) following the incubation protocol described in the Materials and Methods section. The migration position of the ~85 nt RNA resulting from termination at the T_{12} termination signal is indicated on the right. C – Increasing amounts of purified Pol III (as indicated above the gel image) were incubated with the 3'-overhanged template (3 nM), the four NTPs and the CpU dinucleotide primer. The migration position of the ~85 nt RNA is indicated on the right.

a $tRNA^{Ile}$(TAT) [5], preceded by a 32-bp G-less cassette starting with a Sac I restriction site (Fig. 1A). Sac I cleavage generates a 3'-overhang at which Pol III can initiate transcription in the presence of a CpU dinucleotide primer. RNA polymerase can be halted at position +38 by GTP omission: a stable ternary complex is formed in this case, containing template DNA, Pol III and a 38-nt nascent transcript. In the presence of all four NTPs, transcription termination is expected to take place at the natural $tDNA^{Ile}$ terminator, a stretch of 12 T residues. We initially performed titration reactions in the presence of all four of the NTPs to define balanced concentrations of template DNA and Pol III to be used in subsequent experiments. In the DNA titration experiment (Fig. 1B), the template DNA was titrated in the presence of a fixed concentration (5 ng/μl) of purified Pol III. Transcription initiation at the 3' overhang, followed by termination at the $tDNA^{Ile}$ T_{12} termination signal, generated an ~85 nucleotide (nt) transcript, the levels of which increased linearly with increasing DNA concentration, reaching a plateau in the presence of 3 nM template DNA (Fig. 1B). A parallel increase was observed in the levels of longer transcripts, resulting from T_{12} read-through followed by termination at downstream T runs in the plasmid sequence. Significant terminator read-through has previously been reported for Pol III forced to initiate transcription on poly(dC)-tailed DNA templates [8]. In the Pol III titration experiment (Fig. 1C), the Pol III concentration was varied, while the template DNA concentration was kept constant at 3 nM (the saturating template concentration in the previous experiment). A Pol III concentration of 3 ng/μl (75 ng total amount) was found to be clearly sub-saturating, and was used for subsequent experiments. The choice of a sub-saturating Pol III concentration in reinitiation experiments increases the probability that the observed transcription reinitiation is performed by the same Pol III molecules that first initiated.

The time course of first-round transcription initiation on the G-less $tDNA^{Ile}$ template in the presence of sub-saturating amounts of Pol III is shown in Fig. 2A. RNA chain initiation, monitored by the accumulation of a 38-nt G-less transcript, was completed in 10 min, with 50% of the initiation events being completed after ~5 min (Fig. 2A, see quantitation plot below the gel image). To estimate the rate of transcription reinitiation on G-less $tDNA^{Ile}$, we compared the outputs of multiple- versus single-round *in vitro* transcription reactions, performed with a limiting Pol III concentration [2, 9]. In the experiment in Fig. 2B, stalled ternary complexes were formed upon incubation of template DNA with Pol III, the CpU dinucleotide primer and an NTP mixture lacking GTP. GTP was then added, alone (lanes 2, 4, 6, 8) or in association with heparin at a concentration that completely inhibits reinitiation (lanes 1, 3, 5, 7). As shown in Fig. 2B, in this factor-free system, reinitiation proceeded slowly, with only two transcription cycles completed after 15 min (cf. lanes 5 and 6) and 3 cycles after 20 min (lanes 7 and 8). By comparison, Fig. 2C shows that, when the reinitiation time course was performed in the presence of transcription

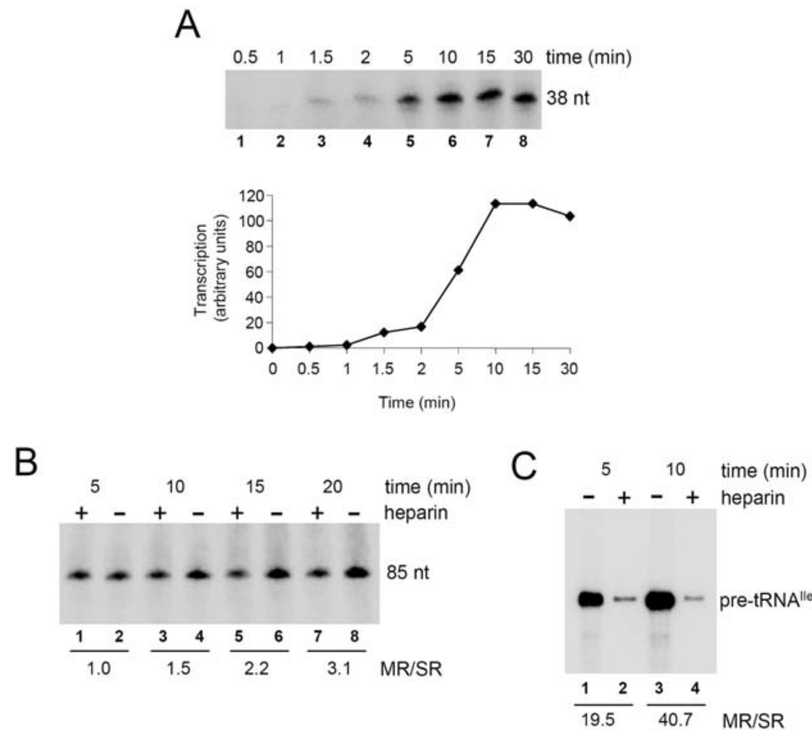

Fig. 2. Transcription initiation and reinitiation by Pol III in the absence of transcription factors. A – Stalled elongation complexes were assembled in reaction mixtures containing 3'-overhanged G-less $tDNA^{Ile}$ template, purified Pol III, the CpU dinucleotide primer and an NTP pool lacking GTP. Aliquots were taken and the reaction was stopped at the times indicated above each lane. The migration position of the 38-nt long nascent RNA, representing initiated complexes, is indicated on the right. A plot derived from phosphorimaging quantification is given below the gel image. B – Stable ternary complexes assembled as described above were chased by the addition of GTP alone (*lanes 2, 4, 6* and *8*) or GTP plus heparin to 200 μg/ml (*lanes 1, 3, 5* and *7*), and transcription was allowed to proceed for the time periods indicated above the lanes. The migration position of the correctly terminated, ~85-nt long transcription product is indicated on the right. For each time point, the ratio between the transcriptional output of multiple-round and single-round transcription reactions (*MR/SR*) is reported below the lanes. C – Factor-assisted transcription reinitiation on the I(TAT)LR1 template. Stable PICs, containing both TFIIIB (reconstituted from recombinant TBP, recombinant Brf1, and a crude B" fraction) and partially purified TFIIIC were formed on the I(TAT)LR1 tDNA template. Pol III (20 ng) was then added together with an NTP mixture lacking CTP, to allow for the formation of stalled ternary complexes containing a 7-nt nascent RNA transcript [5]. Elongation was then resumed by the addition of CTP alone (lanes 1 and 3) or in association with 200 μg/ml heparin (lanes 2 and 4), and multiple transcription cycles were allowed to occur for the indicated time periods. For each time point, the ratio between the transcriptional output of multiple-round and single-round transcription reactions (*MR/SR*) is reported below the lanes. The migration position of the I(TAT)LR1 primary transcript is indicated on the right (*pre-tRNA^Ile^*).

factors, ~20 transcription cycles were completed by Pol III in 5 min (40 in 10 min) on the natural tDNA^{Ile}(TAT) template. Thus, in the absence of TFIIIB and TFIIC, the rate of reinitiation (Fig. 2B) is very similar to the rate of first-round transcription initiation (Fig. 2A), with no sign of facilitated Pol III recycling.

DISCUSSION

By exploiting the ability of purified Pol III to autonomously initiate transcription at a restriction-generated 3'-overhang of linear DNA molecules [7], we were able to study the intrinsic (i.e. factor-independent) transcription initiation and reinitiation properties of yeast RNA polymerase III. By comparing the time course of first-round transcription initiation (50% completion of initiation events in ~5 min) with reinitiation frequency (1 cycle every 7 min), we could conclude that, in the absence of transcription factors, the initiation rate of the first transcription cycle is maintained by Pol III during subsequent cycles, meaning that facilitated reinitiation is abolished. This result is in perfect agreement with the previously demonstrated involvement of TFIIIB and TFIIIC in Pol III recapture during reinitiation [3]. A form of Pol III missing the C11, C37 and C53 subunits (referred to as Pol IIIΔ) was recently shown to be impaired in facilitated reinitiation, even in the presence of TFIIIC and TFIIIB [4]. These observations, together with the results of this study, support the conclusion that the subunits missing in Pol IIIΔ (in particular C11) must act in concert with basal transcription factors to promote facilitated reinitiation. By contrast, the Pol III behaviour in first-round initiation was somehow unexpected. Pol III was found to be able to find a 3'-overhanged, artificial initiation site at approximately the same rate characterizing PIC recognition and transcription initiation by Pol III under natural conditions (see [3]). This observation suggests that, during the first transcription cycle, TFIIIB and TFIIIC mainly contribute to the selectivity, and not to the rate, of Pol III association to the template, while their stable association with the promoter in subsequent cycles strongly contributes to the high rate of transcription reinitiation by Pol III. By exploiting the simplified transcription reinitiation system described here, it should be possible to systematically evaluate the individual contributions of basal (e.g. TFIIIC, [3]) and stimulatory (e.g. Nhp6, [10]) Pol III transcription factors to the reinitiation reaction.

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