The composition of the RNA polymerase I transcription machinery switches from initiation to elongation mode

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Abstract The amounts of RNA polymerase I (Pol I) and basal rDNA transcription factors were determined in yeast whole cell extracts. A 17-fold excess of Pol I was found compared to the Pol I-specific initiation factors upstream activating factor (UAF) and core factor (CF) which underlines that both initiation factors interact with a minor fraction of Pol I when rDNA transcription is active. Surprisingly, Rrn3p, another Pol I-specific initiation factor, is more abundant in cell lysates than UAF and CF. Our analyses revealed that a large fraction of cellular Rrn3p is not associated with Pol I. However, the amount of initiation-active Rrn3p which forms a stable complex with Pol I corresponds to the levels of UAF and CF which have been shown to bind the promoter. Initiation-active Rrn3p dissociates from the template during or immediately after Pol I has switched from initiation to elongation. Our data support a model in which the elongating Pol I leaves the initiation factors UAF, CF and Rrn3p close by the promoter.

Key words: Ribosome synthesis; Nucleolus; rDNA; In vitro transcription; Promoter; RNA polymerase I; Rrn3p; *Saccharomyces cerevisiae*

1. Introduction

Of all three eukaryotic RNA polymerases, RNA polymerase I (Pol I) synthesises by far the highest amount of RNA. In logarithmically growing yeast cells, Pol I generates more than 60% of the total cellular RNA. Specific mechanisms and structural arrangements of the ribosomal genes allow the high level of rRNA synthesis [1,2]. The rDNA genes are tandemly arranged in a cluster of 100–150 copies of which up to 50% can simultaneously be transcribed. In stationary yeast cells the number of the actively transcribed rDNA genes is reduced. If rDNA genes are transcribed they are covered back-to-back by many RNA-synthesising Pol I molecules. Up to 100 Pol I molecules are loaded on each rDNA gene which corresponds to one Pol I molecule every 70 nucleotides. This extremely dense packing of Pol I onto the rDNA allows the production of 40 ribosomes/min. Accordingly, many Pol I molecules have to be present in the cell. The amount of the other components of the rDNA transcription machinery in the cell depends on their role during the transcription cycle. If a transcription factor stays associated with Pol I during chain elongation its abundance should correspond to the Pol I copy numbers. If a factor is only important for promoter binding but dissociates from the elongating machinery much lower copy numbers per cell are required. In yeast, Pol I and three additional factors have been reported to be required for initiation of transcription in vitro [3]. While it was suggested that upstream activating factor UAF stays associated with the gene promoter during several rounds of transcription [4,5], two other factors, CF (core factor) [6] and Rrn3p [3,7–9], were both found to be recruited to the promoter by direct or indirect interactions with Pol I. Rrn3p dissociates from Pol I during one round of transcription, but it was not clear whether Rrn3p is released from the rRNA synthesising complex early or late during RNA chain elongation [7].

We used a recently developed method to quantify yeast proteins from crude cell extracts [10] and found high copy numbers of Pol I per cell whereas levels of UAF, CF and Pol I-bound Rrn3p were much lower. While this work was in progress a global analysis of protein expression in yeast was published [11]. However, the interpretation of the data published for the factors forming the Pol I transcription machinery is not conclusive. Many Pol I subunits and transcription factors which form complexes of stoichiometric subunit distribution either differ significantly in their cellular levels or were excluded from the analysis. In contrast, our results show that the subunits of all three initiation factors, UAF, CF and Pol I-bound Rrn3p, are present in approximately the same amounts. A logical explanation for these findings is that UAF, CF and Rrn3p are associated with the initiation complex at the rDNA promoter, but not with the elongating polymerase. Chromatin immunoprecipitation/polymerase chain reaction (ChIP/PCR) and in vitro transcription reactions support that Pol I leaves the initiation factors in proximity to the promoter. Accordingly, elongating Pol I complexes differ significantly from initiation-active complexes.

2. Materials and methods

2.1. Strains

Genomic integration of the tandem affinity purification (TAP) tag was performed as described [12,13]. The isogenic background strain was W303-1A (*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 GENE-TAP(URA3)*).

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Abbreviations: Pol, RNA polymerase; TAP tag, tandem affinity purification tag; UAF, upstream activating factor; CF, core factor

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2.2. Quantitation of TAP-tagged proteins

Cellular amounts of TAP-tagged yeast proteins were determined according to Borggrefe et al. [10].

2.3. Preparation of the transcriptionally active fractions

Fraction PA600 (protein concentration 2.5–5 mg/ml) was generated according to [14,15].

2.4. ChIP/PCR analysis

Yeast strains that contained a chromosomally integrated TAP tag were grown at 30°C to an OD_{600} between 0.8 and 1.5. ChIP was performed as described [16]. Dilutions of 1/100 (input) or 1/2, 1/16 and 1/256 (ChIP) were analysed by PCR using the following oligonucleotides:

Promoter fragment Chip1: 5′-GTG TGA GGA AAA GTA GTT GGG AGG TA-3′; Chip promoter reverse: 5′-TAA ACG CAA AAG AAA CAC ACT CTG GG-3′;

5S RNA fragment Chip3: 5′-TGG GAT TTA GCA TAG GAA GCC AAG AA-3′; Chip 5S fragment reverse: 5′-CTG ACC GAG TAG TGT AGT GGG TGA-3′;

Termination fragment ON 10140f: 5′-CGG GGT ATT GTA AGC AGT AGA GTA-3′; Chip termination fragment: 5′-CGG GCA AAT CCT TTC ACG CTC GGG-3′.

2.5. In vitro transcription on immobilised templates

Transcription reactions on the immobilised template pcrproC− and generation of the template were performed as described previously [17]. The template contained a 34 nt long stretch downstream of the start site of RNA synthesis lacking deoxycytidine [17].

Briefly, transcription reactions contained 100 ng immobilised template, 15 μl transcription-active fraction PA600, buffer TRX (20 mM HEPES/KOH, pH 7.8), 10 mM $MgCl_2$, 5 mM EGTA, 0.1 mM EDTA, 2.5 mM dithiothreitol and 200–300 mM potassium acetate in a volume of 125 μl. After incubation at 25°C for 20 min the beads were washed twice with 200 μl ice-cold 20 mM HEPES (pH 7.8), 1 mM EDTA and 200 mM potassium acetate, resuspended in 100 μl of the same buffer supplemented with 0.2 mM of various NTP composition, and incubated for 20 min at 25°C. Supernatants were separated from the pellets and were analysed by immunoblotting.

3. Results and discussion

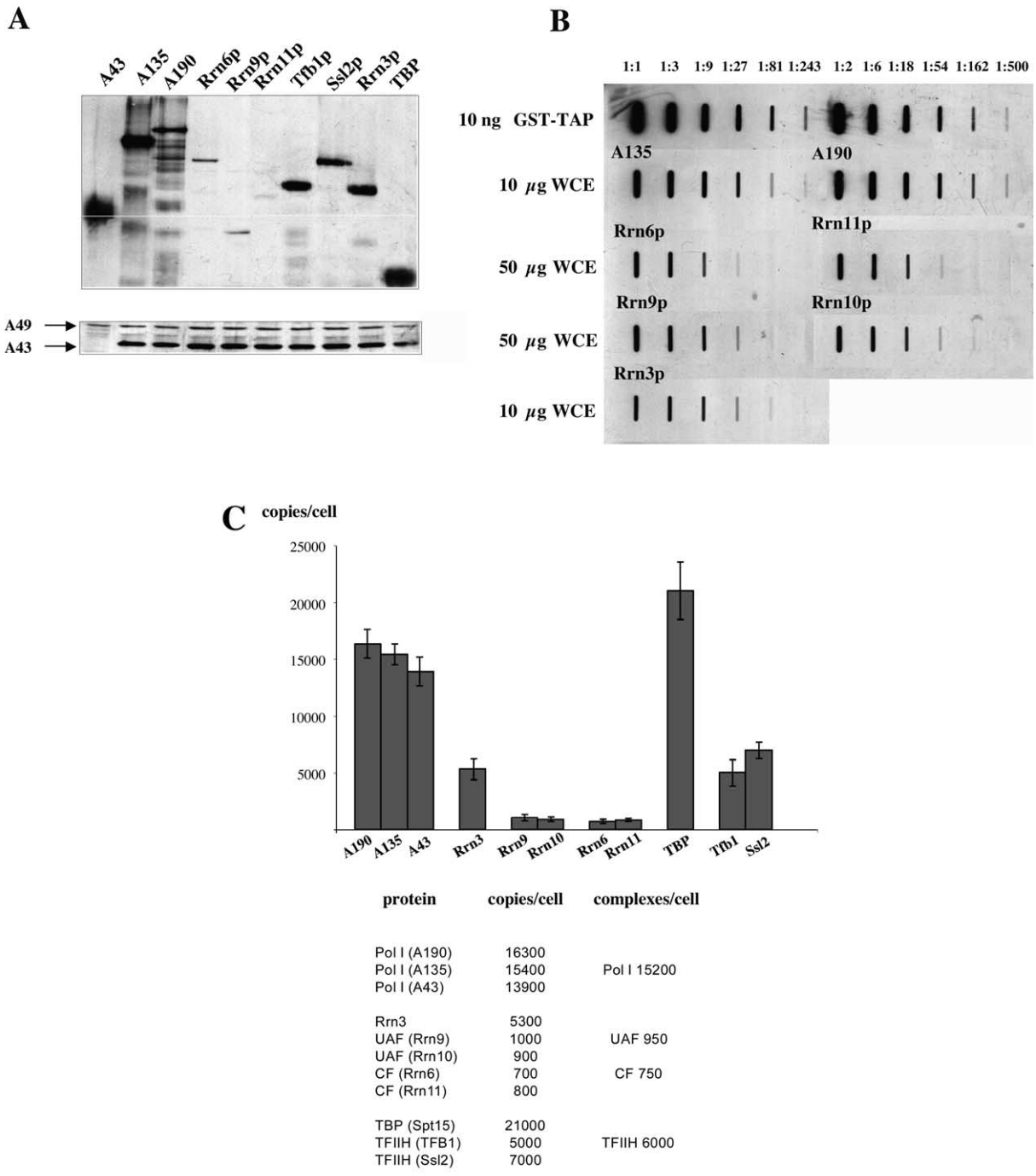
3.1. Quantitation of Pol I and Pol I-dependent transcription factors in crude cell extracts

We employed a recently published method to quantify yeast proteins bearing a chromosomally integrated TAP tag. Pol I subunit Rrn3p and components of UAF and CF were TAP-tagged at the C-termini of their genomic loci. As controls, components of the Pol II machinery were tagged of which cellular copy numbers had previously been determined. Cells were grown to mid-log phase, lysed and the tagged proteins were analysed by Western blot analysis using anti-peroxidase IgG (Fig. 1A). No background IgG binding was observed. All fusion proteins migrated predominantly as single bands except for the Pol I subunit A190. The amounts of tagged proteins were determined by dot blot analysis using homogeneous glutathione *S*-transferase (GST)-TAP fusion protein as external standard. Increasing dilutions (1:3 to 1:500) of the various cell extracts and of the pure GST-TAP fusion protein were spotted onto polyvinylidene difluoride (PVDF) membrane and subjected to a one step immunodetection assay [10] (Fig. 1A). The signals were quantified using a Chemiluminescence imaging system (LAS3000, Fuji; AIDA software). All analyses were performed in triplicate. In control experiments we found no evidence that the various TAP tags change the expression levels of the corresponding hybrid proteins: (i) there was no difference in growth rate between wild type yeast and the tagged strains; (ii) quantitation of two or more different subunits of one multiprotein complex led to similar copy numbers of the respective complex (Fig. 1C). Strikingly, quantitation of the tagged TBP and TFIIH subunits (Ssl2 and TFB3) revealed similar copy numbers as published by Kornberg and colleagues [10], underlining the reproducibility of the method. In contrast, in the genome-wide analysis of protein expression in yeast, the levels of TBP, TFIIH and of most Pol I subunits differ from our results [11]. The inconsistency is perhaps due to methodical uncertainties using such large approaches or to differences in the methods of extract preparation or quantitation. For instance, it is unlikely that the cellular amounts of the single Pol I subunits differ more than 10-fold as was estimated by the genome-wide analysis [11]. Moreover, the basal transcription factor TBP, which is required for all three eukaryotic transcription systems, could be hardly detected in the genome-wide analysis although its high cellular abundance is well documented [10,18]. Our results are, however, entirely consistent with other information about the mechanism of Pol I-dependent transcription and the roles of Pol I- and Pol II-dependent transcription factors.

Our analysis revealed a 17-fold excess of Pol I molecules compared to UAF and CF. In contrast, only a three-fold excess of Pol I with regard to Rrn3p was observed (Fig. 1A–C). Rrn3p is able to form a stable complex with Pol I which resists stringent buffer conditions like 1.5 M potassium acetate and 0.5% NP-40. This complex represents the initiation-active form of Pol I [7,15]. To find out how much Rrn3p is stably associated within the initiation-active Pol I–Rrn3p complex, we gel-filtrated whole cell extracts in the presence of 1.5 M potassium acetate and 0.5% NP-40 on a Superose 6 column (Fig. 2). About 70% of the Rrn3p applied to the column could be detected in three separated peaks. 21% of the recovered Rrn3p eluted with the void volume of the column, about 24% co-migrated with Pol I, whereas the largest fraction containing 55% of Rrn3p migrated with the molecular mass of monomeric Rrn3p. If only the Pol I-bound Rrn3p is taken into account which eluted between fraction 14 and 18 from the column (and which is transcriptionally active) [15], about 24%, corresponding to 1200 copies of Rrn3p per cell, are incorporated in transcriptionally active complexes. This copy number resembles the cellular copies of CF and UAF, suggesting that about 1000 molecules of each are sufficient to form the pre-initiation complex. In contrast, many more Pol I molecules are required for rRNA synthesis. Assuming that most Pol I molecules are engaged in RNA chain elongation but not in transcription initiation, this result means that a transition between the initiation- and elongation-active Pol I takes place close by the promoter which finally results in promoter clearance and dissociation of Pol I from the initiation factors.

However, from these results it is not clear why we observed a five-fold amount of cellular Rrn3p versus UAF and CF. Possible explanations for this finding are: (i) a major fraction of Rrn3p accompanies Pol I on at least a short stretch of the rDNA, however, this form of Rrn3p is dissociated from Pol I under the stringent conditions used for the gel filtration experiment; (ii) the excess of unbound Rrn3p is required to capture released Pol I molecules which might result in a complex that can be disrupted using high salt conditions before its incorporation into the pre-initiation complex; (iii) Rrn3p not bound to Pol I might be involved in other cellular mechanisms rather than rDNA transcription.

The nature of the Rrn3p fraction that co-eluted with Pol I



protein	copies/cell	complexes/cell
Pol I (A190)	16300	
Pol I (A135)	15400	Pol I 15200
Pol I (A43)	13900	
Rrn3	5300	
UAF (Rrn9)	1000	UAF 950
UAF (Rrn10)	900	
CF (Rrn6)	700	CF 750
CF (Rrn11)	800	
TBP (Spt15)	21000	
TFIIH (TFB1)	5000	TFIIH 6000
TFIIH (Ssl2)	7000	

Fig. 1. Pol I and Pol I-specific transcription factors differ in their cellular amounts. A: Whole cell extracts (50 μg) of TAP-tagged yeast strains were immunoblotted and probed with rabbit anti-peroxidase IgG. As additional loading control the blot was redeveloped with antibodies directed against Pol I subunits A43 and A49 (lower panel). B: Immuno-dot blot probed with rabbit anti-peroxidase IgG. Dilution series (1:3) of 10 ng and 5 ng of GST-TAP (left and right panel) and 10 μg or 50 μg of crude extracts from the TAP-tagged strains indicated were transferred to a PVDF membrane. C: Cellular amounts of Pol I subunits and transcription factors.

in the void volume of the gel filtration remains obscure. It could either be due to an artificial aggregation using the high salt conditions applied, or Rrn3p could co-migrate with the large nucleolar substructure that has previously been described [19]. This macromolecular assembly contains Pol I, and many other nucleolar factors involved in rRNA synthesis and processing. It could also be isolated from transcriptionally inactive cells suggesting that an organised structure independent of active rRNA synthesis provides a scaffold for the regular formation of the nucleolus. This substructure is not reminiscent of a Pol I holoenzyme, however, it supports the existence of a structural organisation which drives the assembly and formation of the nucleolus [19].

3.2. *Initiation factors are dissociated from elongation-active transcription complexes*

To rule out that the Pol I-specific transcription initiation factors accompany Pol I during RNA chain elongation we

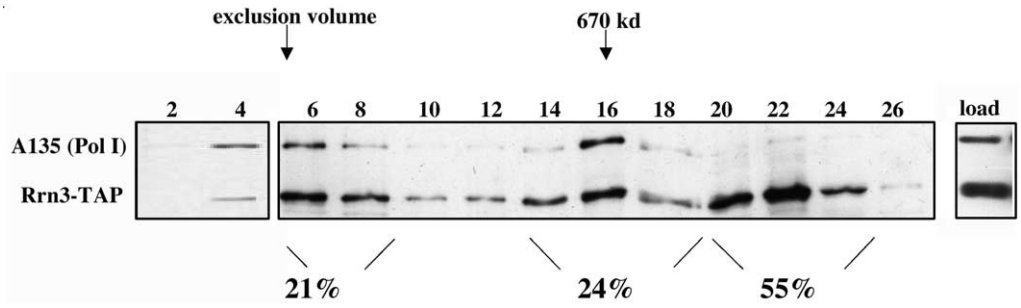


Fig. 2. Distribution of Rrn3p after gel filtration using high salt conditions. 50 μg of yeast whole cell extracts derived from strain Rrn3p-TAP were gel-filtrated on Superose 6 using a buffer containing 0.5% NP-40 and 1.5 M potassium acetate. Using these conditions the initiation-active Pol I–Rrn3p complex can be separated from bulk Rrn3p. Every second fraction (25 μl; 50%) of the gel filtration was analysed by Western blotting. 50% of each fraction was independently quantified by immuno-dot blot analysis (not shown). The distribution of Rrn3p recovered after gel filtration is indicated.

performed ChIP/PCR experiments using primers specific for the rDNA promoter region and the termination site. In control reactions a DNA fragment of the 5S rDNA was PCR-amplified which is located between the rDNA repeats but which is transcribed by Pol III. We found accumulation of UAF exclusively at the promoter fragment whereas Pol I was detected in association with both the initial and terminal rDNA fragment (Fig. 3A). These findings are consistent with previous results which suggested that UAF remains bound at the rDNA promoter during the transcription cycle [4]. In contrast, it was reported that CF can dissociate from the pre-initiation complex in vitro after Pol I has cleared the promoter and it was suggested that CF is recruited to the promoter for each round of initiation [6]. This conclusion was drawn although only a minor amount of template-bound CF was released under the conditions used. Therefore, we asked whether the major fraction of CF – that stays associated with the template – accompanies Pol I during RNA chain elongation. Our ChIP/PCR experiments underline that most CF does not move along the template together with Pol I in a holoenzyme-like fashion since accumulation of CF is only found at the promoter fragment (Fig. 3A).

Transcription initiation factor Rrn3p was also found to be associated in proximity to the promoter, but not to the terminator sequence (Fig. 3A). While this work was in preparation a manuscript was published by Nomura and colleagues which fully supports our data that UAF and Rrn3p are associated with the promoter while only Pol I is found in association with the entire rRNA coding region [20].

To obtain a higher resolution at which point Rrn3p is released from Pol I during RNA chain elongation we performed promoter-dependent transcription reactions on immobilised templates in which transcription was stalled after 35 nucleotides. We have previously described that Rrn3p, which is tightly bound to Pol I, is released from Pol I during one round of transcription in vitro [7]. This was confirmed by Aprikian et al. [6] using immobilised templates. However, it was not clear whether Rrn3p dissociation occurs at the promoter, during RNA chain elongation or when transcription is terminated. From our ChIP/PCR data we conclude that Rrn3p leaves the rDNA before Pol I reaches the termination site, but it is possible that Rrn3p travels along the rDNA for a certain distance before the transcription machinery enters the termination region. Therefore, we investigated whether Rrn3p is still associated with the elongating Pol I after the promoter has been cleared which could explain the higher amount of cellular Rrn3p compared to the promoter-bound factors. Using an immobilised template which lacks cytidines for the first 35 nucleotides after the transcription start site the movement of Pol I can be stalled at position 35 if CTP was omitted from the transcription reaction [17]. Rrn3p was released into the supernatant no matter whether all four nucleotides or only the three nucleotides ATP, UTP and GTP were included in the transcription reaction (Fig. 3B). In contrast, when transcription cannot take place in the absence of nucleotides or if only ATP was present, no Rrn3p was detected in the supernatant indicating that Rrn3p is still bound to the template. In control reactions using templates that are lacking the rDNA promoter no Rrn3p was detected in the supernatant after the immobilised template had been washed with buffer (Fig. 3B).

Once Rrn3p is dissociated from Pol I in vitro, the Pol I–Rrn3p complex cannot be re-formed [7]. Whether either yeast Rrn3p, like its human homologue hRrn3p [21], or yeast Pol I is 'consumed' during one round of transcription remains to be determined. It is possible that the phosphorylation state of Pol I and/or Rrn3p is involved in the regulation of this reversible interaction [22].

From our data we conclude that none of the known yeast transcription factors stays associated with Pol I during RNA chain elongation and that Rrn3p leaves both Pol I and the promoter shortly after or during promoter clearance. In summary, these findings do not support the idea that Pol I and the transcription factors are recruited to the promoter in a single holoenzyme-like complex.

Although it is not clear from our quantitation data how many Pol I molecules are actually involved in the transcription process, it is obvious that at least a major fraction of Pol I is engaged in RNA chain elongation. It was estimated by electron microscopic analysis that the density of Pol I on the rDNA of higher eukaryotes is one molecule every 100 bp [23]. If a similar density is true for yeast, about 5000 Pol I molecules are involved in rRNA chain elongation provided approximately 50% of the 150 tandemly repeated rDNA units are simultaneously transcribed in yeast [24]. The cellular excess of Pol I molecules compared to initiation factors underlines that elongating Pol I complexes differ significantly from initiation complexes. It is possible that this transition represents a rate-limiting step for the transcription process, which reflects an additional possibility to regulate the efficiency of transcription. This idea is supported by our observation that

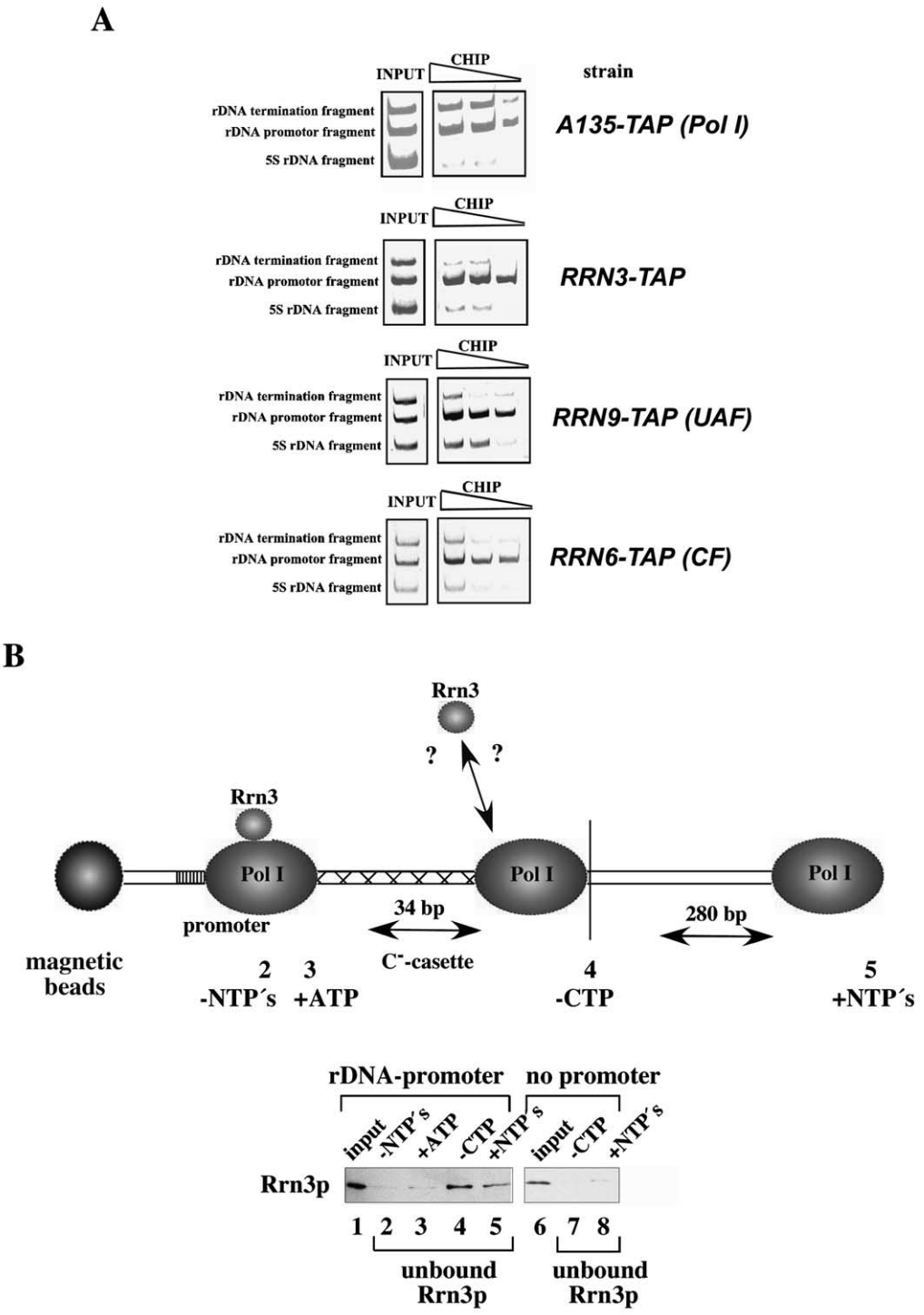


Fig. 3. UAF, CF and Rrn3p are not part of the elongating Pol I complex. A: ChIP/PCR with rabbit anti-peroxidase IgG was carried out using strains containing chromosomally TAP-tagged A135, Rrn3p, Rrn9p (UAF) and Rrn6p (CF). Increasing dilutions of the immunoprecipitated samples were analysed by PCR using primers specific for the rDNA promoter and the termination region. The 5S rDNA fragment served as an internal control that is not transcribed by Pol I. B: Rrn3p is released from the template immediately after transcription initiation. Transcription reactions on immobilised template were performed with fraction PA600 which contains all factors required for transcription initiation including Pol I-bound Rrn3p. After 10 min preincubation with the transcription-active fraction PA600 the beads were washed with transcription buffer lacking nucleotides and subsequently supplemented with buffer containing either no (−NTPs), ATP (+ATP), GTP, UTP, ATP (−CTP) or all nucleotides (+NTPs). In the presence of all four nucleotides (+NTPs), an approximately 280 nt long run-off transcript is generated. In the absence of CTP (−CTP) transcription paused at the first CTP (position 34) [17]. Purified and washed ternary complexes were allowed to resume elongation for 20 min. The supernatants were analysed for the presence of Rrn3p by Western blotting.

Pol I is more concentrated at the promoter than at the termination segment in the ChIP/PCR experiments (see Fig. 3A). The underlying mechanism which triggers the transition from initiation to elongation remains to be determined. It is likely that (de-)phosphorylation of Pol I subunits is involved in this step, since we have recently found that Rrn3p-bound Pol I exhibits a different phosphorylation pattern than the bulk of Pol I [22].

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References

[1] Grummt, I. (2003) Genes Dev. 17, 1691–1702.
[2] Moss, T. and Stefanovsky, V.Y. (2002) Cell 109, 545–548.
[3] Keener, J., Josaitis, C., Dodd, J. and Nomura, M. (1998) J. Biol. Chem. 273, 33795–33802.
[4] Keys, D.A. et al. (1996) Genes Dev. 10, 887–903.
[5] Steffan, J.S., Keys, D.A., Dodd, J.A. and Nomura, M. (1996) Genes Dev. 10, 2551–2563.
[6] Aprikian, P., Moorefield, B. and Reeder, R.H. (2001) Mol. Cell. Biol. 21, 4847–4855.
[7] Milkereit, P. and Tschochner, H. (1998) EMBO J. 17, 3692–3703.
[8] Peyroche, G., Milkereit, P., Bischler, N., Tschochner, H., Schultz, P., Sentenac, A., Carles, C. and Riva, M. (2000) EMBO J. 19, 5473–5482.
[9] Yamamoto, R.T., Nogi, Y., Dodd, J.A. and Nomura, M. (1996) EMBO J. 15, 3964–3973.
[10] Borggrefe, T., Davis, R., Bareket-Samish, A. and Kornberg, R.D. (2001) J. Biol. Chem. 276, 47150–47153.
[11] Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K. and Weissman, J.S. (2003) Nature 425, 737–741.
[12] Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Seraphin, B. (1999) Nat. Biotechnol. 17, 1030–1032.
[13] Puig, O., Rutz, B., Luukkonen, B.G., Kandels-Lewis, S., Bragado-Nilsson, E. and Seraphin, B. (1998) Yeast 14, 1139–1146.
[14] Tschochner, H. (1996) Proc. Natl. Acad. Sci. USA 93, 12914–12919.
[15] Milkereit, P., Schultz, P. and Tschochner, H. (1997) Biol. Chem. 378, 1433–1443.
[16] Hecht, A. and Grunstein, M. (1999) Methods Enzymol. 304, 399–414.
[17] Tschochner, H. and Milkereit, P. (1997) FEBS Lett. 410, 461–466.
[18] Lee, T.I. and Young, R.A. (1998) Genes Dev. 12, 1398–1408.
[19] Fath, S., Milkereit, P., Podtelejnikov, A.V., Bischler, N., Schultz, P., Bier, M., Mann, M. and Tschochner, H. (2000) J. Cell Biol. 149, 575–590.
[20] Claypool, J.A., French, S.L., Johzuka, K., Eliason, K., Vu, L., Dodd, J.A., Beyer, A.L. and Nomura, M. (2004) Mol. Biol. Cell 15, 946–956.
[21] Hirschler-Laszkiewicz, I., Cavanaugh, A., Mizra, A., Lun, M., Hu, Q., Smink, T. and Rothblum, L.I. (2003) J. Biol. Chem. 278, 18953–18959.
[22] Fath, S., Milkereit, P., Peyroche, G., Riva, M., Carles, C. and Tschochner, H. (2001) Proc. Natl. Acad. Sci. USA 98, 14334–14339.
[23] Foe, V.E. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 723–740.
[24] Dammann, R., Lucchini, R., Koller, T. and Sogo, J.M. (1993) Nucleic Acids Res. 21, 2331–2338.