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A specialized form of RNA polymerase I, essential for initiation and growth-dependent regulation of rRNA synthesis, is disrupted during transcription

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Only a small proportion (<2%) of RNA polymerase I (pol I) from whole-cell extracts appeared to be competent for specific initiation at the ribosomal gene promoter in a yeast reconstituted transcription system. Initiation-competent pol I molecules were found exclusively in salt-resistant complexes that contain the pol I-specific initiation factor Rrn3p. Levels of initiation-competent complexes in extracts were independent of total Rrn3p content and varied with the growth state of the cells. Although extracts from stationary phase cells contained substantial amounts of Rrn3p and pol I, they lacked the pol I–Rrn3p complex and were inactive in promoter-dependent transcription. Activity was restored by adding purified pol I–Rrn3p complex to extracts from stationary phase cells. The pol I–Rrn3p complex dissociated during transcription and lost its capacity for subsequent reinitiation *in vitro*, suggesting a stoichiometric rather than a catalytic activity in initiation. We propose that the formation and disruption of the pol I–Rrn3p complex reflects a molecular switch for regulating rRNA synthesis and its growth rate-dependent regulation.

Keywords: initiation/*in vitro* transcription/rRNA promoter/Rrn3p/*Saccharomyces cerevisiae*

Introduction

Promoter utilization by eukaryotic RNA polymerases requires accessory proteins termed general transcription factors or initiation factors. Even in the presence of saturating amounts of initiation factors, however, purified RNA polymerases typically exhibit low efficiencies of template utilization. For instance, homogenous RNA polymerase II from *Saccharomyces cerevisae* synthesized <10 specific transcripts per hundred DNA template molecules under optimal conditions with purified general factors (Sayre *et al*., 1992a,b). This matched the maximum efficiency observed in nuclear extract (Chasman *et al*., 1989), indicating that low template utilization was not due to loss of activity during purification of transcription proteins. One possible explanation for this apparent inefficiency is that only a fraction of eukaryotic RNA polymerases are active for transcription initiation at any one time. For instance, RNA polymerase II *in vivo* exists in various forms distinguished by the phosphorylation state of the C-terminal repeat domain (CTD) in the largest subunit (reviewed in Dahmus, 1994). Only the dephosphorylated form is thought to be competent for assembly into 'pre-initiation complexes' with accessory proteins on promoter DNA. Furthermore, distinct activated and non-activated RNA polymerase II complexes have been described recently in yeast (Akhtar *et al*., 1996).

Biochemical analyses of cell-free transcription systems for RNA polymerase I (pol I) from *Acanthamoeba* (Stevens and Pachler, 1973; Bateman and Paule, 1986), mouse (Tower and Sollner Webb, 1987) and yeast (Milkereit *et al*., 1997) have identified at least two different forms of polymerase, only one of which is able to initiate at the rDNA promoter. Neither the relative proportions of pol I in each form nor the underlying molecular mechanisms for establishing and/or interconverting them have been elucidated. Several reports implicate these distinct forms of pol I in growth rate-dependent regulation of rRNA synthesis. Homogeneous or enriched pol I that supports promoter-specific transcription has been isolated exclusively from logarithmically growing cells. This active form of the enzyme can complement inactive extracts from stationary phase cells in systems from *Acanthamoeba* (Paule, 1983; Bateman and Paule, 1986), mouse (Tower and Sollner Webb, 1987) and yeast (Riggs *et al*., 1995). More detailed analyses of the mouse system correlate growth-dependent initiation activity with a transcription factor that binds tightly to pol I. This factor, variously termed TIFIA (Buttgereit *et al*., 1985; Schnapp *et al*., 1990, 1993), Factor C* (Brun *et al*., 1994) and TFIC (Gokal *et al*., 1990; Mahajan and Thompson, 1990; Mahajan *et al*., 1990), can be separated from active pol I and is thought to be essential for utilization of the murine ribosomal gene promoter. Genes encoding this factor have not yet been identified.

Other pol I-specific transcription initiation factors have been described to be involved in the regulation of rRNA synthesis of higher eukaryotes. The upstream binding factor (UBF), was demonstrated to be a target molecule involved in the up- and downregulation of rRNA synthesis. The ratio of phosphorylated to non-phosphorylated UBF was suggested to define its transactivation properties (O'Mahony *et al*., 1992a,b; Voit *et al*., 1992). Furthermore, the retinoblastoma susceptibility gene product was shown to interact with UBF, resulting in the repression of pol I-dependent transcription (Cavanaugh *et al*., 1995; Voit *et al*., 1997). Recently, SV40 large T antigen, a viral protein which can stimulate cell proliferation, was shown to activate pol I-dependent transcription by its interaction with the basal pol I-specific transcription factor SL1 (Zhai *et al*., 1997).

SL1 (human) (Comai *et al*., 1992, 1994) or TIFIB (mouse or *Acanthamoeba*) (Eberhard *et al*., 1993; Radebaugh *et al*., 1994) can direct multiple rounds of pol I recruitment to the promoter (Schnapp and Grummt, 1991; Goodrich and Tjian, 1994; Beckmann *et al*., 1995;

Hempel *et al*., 1996). SL1 and TIFIB are members of a family of multisubunit general transcription factors [including TFIID (Dynlacht *et al*., 1991; Tanese *et al*., 1991; Zhou *et al*., 1992) and TFIIIB (Lobo *et al*., 1992; Taggart *et al*., 1992) containing the TATA-binding protein (TBP) in a stable complex with tightly associated proteins called TAFs.

Finally, another pol I-specific transcription factor described in mouse, TIFIC, binds directly to polymerase and appears to mediate both initiation and transcript elongation (Schnapp *et al*., 1994). Its role in growth-dependent regulation of transcription remains to be established.

Yeast pol I evidently requires two multisubunit complexes, namely CF and UAF, as well as a single-subunit transcription factor, Rrn3p, for maximal utilization of the ribosomal gene promoter *in vitro* (Keys *et al*., 1994, 1996; Yamamoto *et al*., 1996). Three essential genes (*RRN6*, *RRN7* and *RRN11*) encode the polypeptide subunits of CF, each of which is required for specific initiation *in vitro* (Keys *et al*., 1994). UAF, which is stimulatory *in vitro*, contains Rrn5p, Rrn9p, Rrn10p and the histones H3 and H4 (Keener *et al*., 1997). UAF is thought to bind upstream of the ribosomal promoter (at the upstream element) to form a stable complex which apparently helps recruit CF to the core promoter element (Keys *et al*., 1996). CF may function analogously to mammalian SL1/TIFIB, which contains TBP (Keys *et al*., 1996). Both CF and UAF interact specifically with TBP (Lin *et al*., 1996; Steffan *et al*., 1996). On a template lacking a UAF-binding site, TBP is required for stimulation of transcription mediated by UAF but not for basal transcription (Steffan *et al*., 1996). Rrn3p, which is also required for *in vitro* transcription, was suggested to interact directly with pol I since pre-incubation of Rrn3p with pol I led to a stimulation of transcription (Yamamoto *et al*., 1996).

Although all of the identified factors presumably cooperate with pol I at some point, little is known about how the polymerase itself interacts with the initiation factors. Recently, we described the resolution and characterization of distinct pol I populations from yeast whole-cell extracts using a reconstituted *in vitro* transcription system (Tschochner, 1996; Milkereit *et al*., 1997). Only a minor monomeric form of pol I was found to be active in promoter-driven transcription, whereas the bulk of pol I existed as inactive monomers or dimers. Here we show that the initiation-competent pol I population (<2% of total pol I) can be purified further as a complex in stable association with the essential initiation factor Rrn3p. We provide evidence that formation of the pol I–Rrn3p complex and its dissociation during transcription can serve as a molecular switch for transcription initiation and growth rate-dependent regulation of rRNA synthesis.

Results

Initiation-competent monomeric pol I is stably associated with Rrn3p

Our aim was to isolate yeast RNA polymerase I in an active form for promoter-dependent transcription. For the fractionation scheme diagrammed in Figure 1, pol I purification was monitored with three different assays: (i) quantitative immunoblotting with antibodies directed against the pol I-specific subunit A_{49} or A_{190} (kindly provided by A.Sentenac and colleagues); (ii) non-specific RNA chain elongation with single-stranded or nicked DNA templates (Roeder, 1974); and (iii) a promoter-dependent run-off transcription assay performed in the presence of two other essential fractions. One of the two accessory fractions (designated TBP-cpl in Figure 1) contains a 240 kDa protein complex that includes TBP; the other (fraction B600) is a crude fraction that lacks pol I activity. As shown previously, the polymerase-containing fraction (B2000; Figure 1) resolved into three fractions by size-exclusion chromatography. Two of these were inactive for promoter-dependent transcription: one contained predominantly dimers while the other contained the majority of monomeric pol I. A third fraction contained monomeric pol I that was active for promoter-dependent transcription (Milkereit *et al*., 1997). On this and other sizing columns, initiation-competent pol I migrated with a slightly higher molecular mass than did the bulk of (inactive) monomeric pol I, providing the first indication that additional subunit(s) might be associated with the active form of the enzyme (Milkereit *et al*., 1997).

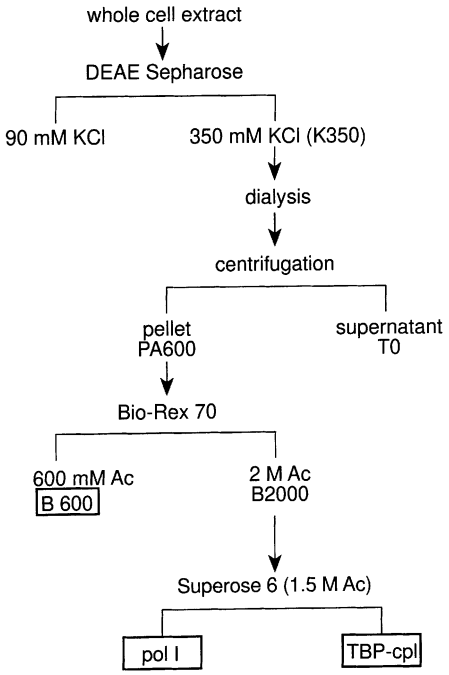

Fig. 1. Purification scheme of pol I and fractions used for the reconstituted *in vitro* transcription from yeast whole-cell extracts.

Rrn3p is known to be essential for promoter-directed yeast pol I transcription (Yamamoto *et al*., 1996). To determine the fate of Rrn3p in our fractionation scheme, fractions were analysed by immunoblotting with affinity-purified antibodies specific for Rrn3p. Both Rrn3p and pol I were detected in the B2000 fraction (data not shown). Proteins in this fraction were resolved further on a Superose-6 gel filtration column in the presence of 1.5 M potassium acetate. Even under these stringent conditions, Rrn3p and monomeric pol I co-eluted from the column (Figure 2A, upper and lower panels). No Rrn3p was detected in fractions eluting at the volume expected for monomeric Rrn3p (with a predicted molecular mass of 72 kDa; data not shown). We performed two

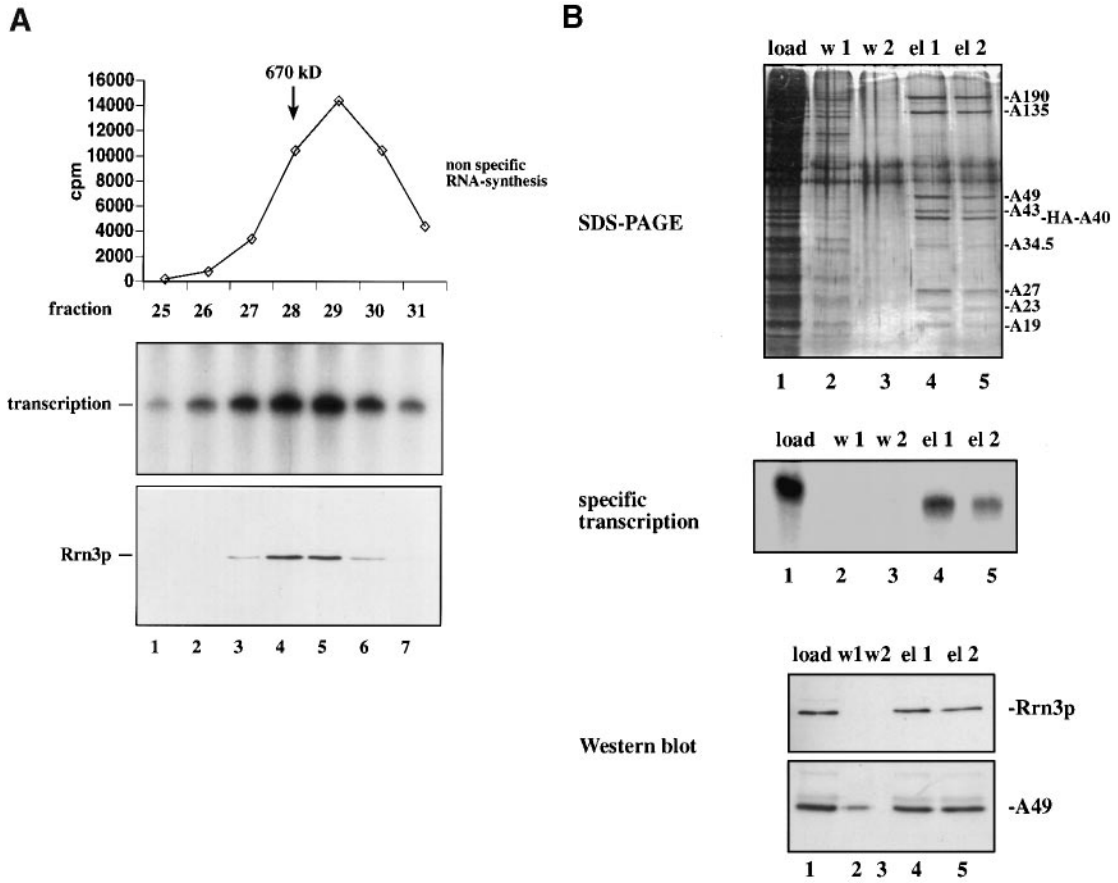

Fig. 2. Rrn3p is stably associated with monomeric initiation-competent pol I. (**A**) Gel filtration of initiation-competent pol I (50 µl of fraction B2000) with a Superose-6 column in the presence of 1.5 M potassium acetate. A 1.5 µl aliquot of the 240 kDa, TBP-containing protein complex and 1 µl of fraction B600 were added to 1.5 µl of each fraction from the column for the reconstituted transcription assay (middle panel). Each 40 µl and 4 µl were tested for Western blot analysis (lower panel) and non-specific RNA synthesis (upper panel), respectively. (**B**) Co-immunopurification of Rrn3p with pol I. Fraction B2000 was incubated with HA-specific antibodies attached to Sepharose beads (BAbCO). After washing with buffers containing 600 mM potassium acetate (see Materials and methods) (lanes 2 and 3), the column was eluted with 1 mg/ml HA-peptide dissolved in the same acetate buffer (lanes 4 and 5). Then 1% of the load and 10% of each fraction were separated on 10% SDS–PAGE and silver stained (upper panel) or blotted onto a PVDF membrane (Millipore) and developed with anti-Rrn3p antibodies (lower panel). Specific transcriptional activity of 1% of the load and 5% of each fraction is depicted in the middle panel.

additional experiments to confirm that promoter-dependent initiation activity co-purified with Rrn3p in association with pol I. First, pol I, Rrn3p and initiation activity were co-purified by immunoaffinity chromatography exploiting a haemagglutinin (HA)-tagged AC40 pol I subunit (Figure 2B). In addition, pol I and Rrn3p also were co-purified by metal chelate affinity-chromatography using a histidine-tagged ABC23 pol I subunit (data not shown). Taken together, these data strongly suggest that Rrn3p is a component of a stable pol I enzyme complex that supports accurate transcription initiation *in vitro*.

Only a minor proportion of yeast RNA polymerase I is competent for initiation

Initiation-competent pol I from the sizing column (Milkereit *et al*., 1997) was applied to a MonoQ column and eluted with a salt gradient (Figure 3A). More than 75% of the specific transcription activity loaded onto the column was recovered. The peak fractions of pol I protein, as determined by immunoblotting (fractions 20 and 21), did not coincide with promoter-dependent transcription activity. The peak of specific activity eluted in fraction 22 (Figure 3A, lower panel), which contained <15% of the polymerase, as determined by Western blot analysis. Evidently, monomeric pol I was resolved into two populations on the MonoQ column, only one of which was active in promoter-driven transcription. We tested this fraction for Rrn3p content; as in the gel filtration experiments, initiation activity coincided with the appearance of Rrn3p (Figure 3A, middle and lower panels). Titrating initiation-incompetent pol I (MonoQ fraction 20) into Rrn3p-containing fractions (e.g. MonoQ fraction 23) neither stimulated nor inhibited specific initiation (Figure 3A, lane 8). This result showed that the weak promoter-dependent activity of fractions containing the highest concentrations of pol I (fractions 20 and 21) was not due to the presence of an inhibitor, and that the total amount of pol I was not limiting in the strongly active Rrn3p-containing fractions. SDS–PAGE analysis of MonoQ fractions (Figure 3B) showed comparable degrees of purity in peak fractions for non-specific (Figure 3B, lane 1) and promoter-specific pol I activity (Figure 3B, lane 2). However, in addition to the typical pattern of pol I subunits, a few other polypeptide bands were unique to fraction 22 (Figure 3B). One of these corresponded to a polypeptide with an apparent molecular mass of 72 kDa,

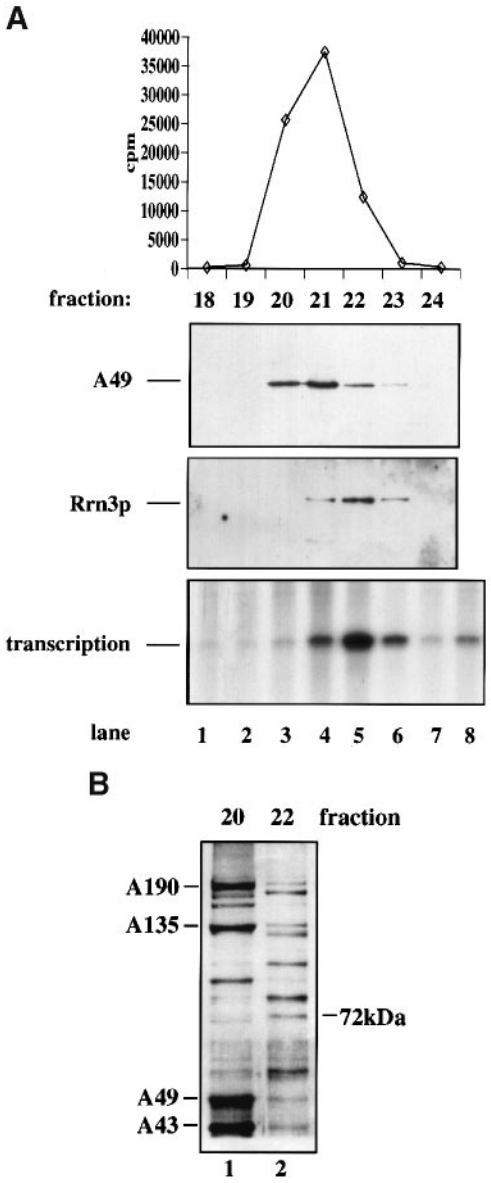

Fig. 3. A small proportion of pol I is associated with Rrn3p. Co-purification of Rrn3p and transcriptional activity on Mono Q. Monomeric pol I which had been separated from pol I dimers on a Sephacryl S-300 column was loaded onto a MonoQ column and eluted at ~1.1 M potassium acetate applying a linear gradient from 600 to 1300 mM potassium acetate. (**A**) Two µl of each fraction were tested in non-specific RNA synthesis (upper panel) and promoter-dependent transcription (lower panel) in the presence of 1.5 µl of TBP-cpl and 1 µl of fraction B600, respectively. In the assay illustrated in lane 8, 1 µl of fraction 20 was mixed with 1 µl of fraction 23 before starting the transcription reaction. Five µl of each fraction were analysed by Western blotting with antibodies against the pol I-specific subunit A49 and against Rrn3p (middle panels). (**B**) Eighty percent each of the peak fractions from a MonoQ column in non-specific activity of RNA synthesis (lane 1) and promoter-dependent activity (lane 2) were separated on an 8% SDS–polyacrylamide gel and silver stained. The positions of pol I subunits and the size of Rrn3p (72 kDa) are indicated.

consistent with the predicted mass of Rrn3p (Figure 3B, lane 2), which was recognized by the anti-Rrn3p antibodies. We conclude that only a small proportion of yeast pol I can be isolated from whole-cell extract in an initiation-active form, and that this initiation-competent enzyme fraction contains Rrn3p.

A pol I–Rrn3p complex represents a subform of pol I highly active in initiation

Immunoaffinity purification of the pol I–Rrn3p complex from the B2000 fraction using antibodies directed against the N-terminal peptide of Rrn3p allowed a more detailed analysis of initiation-competent pol I. After elution of the co-immunoprecipitated complexes with an excess of the N-terminal Rrn3p peptide, SDS–PAGE analysis revealed a seemingly stoichiometric relationship between pol I subunits and a polypeptide of the apparent molecular mass of 72 kDa, which obviously resembled Rrn3p (Figure 4A; the stoichiometry is inferred from silver staining intensity, which may not reflect accurately the relative abundance of these particular proteins). No other proteins in the 65–200 kDa mass range were visible, indicating that additional polypeptides in this range present in the initiation-active MonoQ fraction 22 (Figure 3B) were not required for promoter-dependent initiation. More importantly, the immunopurified pol I–Rrn3p complex possessed a very high specific activity, with 3–4 ng of the purified complex being sufficient to saturate the reconstituted transcription assay (Figure 4A, lower panel). This corresponded to a specific activity of 15 pmol transcripts per mg of pol I. Good recovery of specific activity through all purification steps indicated that the putative pol I–Rrn3p complex identified by these experiments is highly stable (Table I). The large increase in specific activity of the pol I–Rrn3p complex during purification evidently was not due to the loss of inhibitory activities during the purification procedure. Mixing crude pol I-containing fractions (K350, T0, B2000) with initiation-competent pol I did not reduce the yield of transcripts in the reconstituted assay (data not shown). Quantitative immunoblotting and transcription assays revealed that $<2\%$ of the pol I present in the B2000 fraction resided in the pol I–Rrn3p complex.

Nomura and colleagues reported that the majority of Rrn3p in crude extracts is monomeric (Yamamoto *et al*., 1996). Immunoprecipitation with antibodies directed against the N-terminus of Rrn3p confirmed that the majority of Rrn3p is not associated with the initiation-competent pol I complex: a large proportion of Rrn3p was immunoprecipitated from fractions that were inactive in pol I-dependent transcription (such as T0) with no co-precipitation of pol I (Figure 4B, left panel, lane 2). However, when Rrn3p was immunoprecipitated from fractions containing initiation-competent pol I, such as K350 and PA600 (data not shown) or B2000 (Figure 4B, left panel, lane 1), a significant proportion of pol I was co-precipitated. After extensive washing, immunoprecipitated pol I–Rrn3p complexes were assayed for promoter-dependent transcription by adding template, fractions B600, the TBP complex and nucleotide substrates to the beads (Figure 4B, right panel, lane 1). Although a similar amount of Rrn3p was precipitated from fractions B2000 and T0, efficient initiation of rRNA synthesis was restricted to immunoprecipitated pol I–Rrn3p complexes from fraction B2000. The slightly elevated transcriptional activity visible in Figure 4B, lane 2 (right panel) is probably due to some pol I–Rrn3p complexes still present in fraction T0. Indeed, long exposures of the Western blot depicted in Figure 4B (left panel) also showed trace amounts of co-precipitated pol I in lane 2. No initiation activity co-precipitated with empty beads (Figure 4B, lane 3).

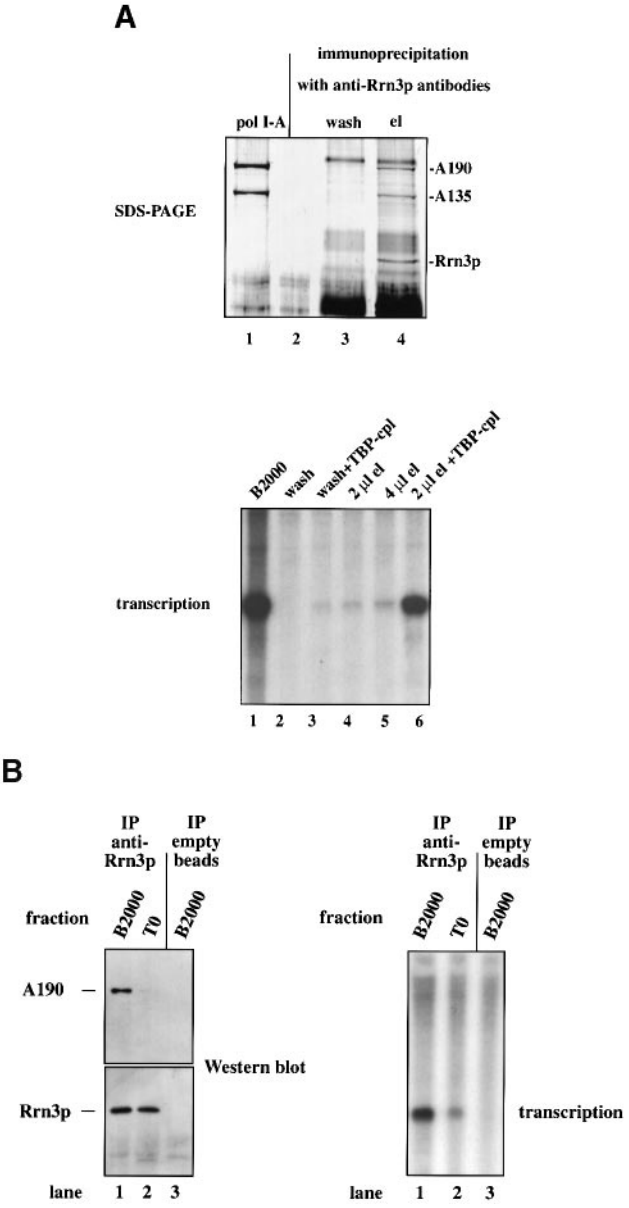

Fig. 4. Immunoprecipitated Rrn3p is highly active in transcription initiation if it is incorporated in a pol I–Rrn3p complex.
(**A**) Immunoprecipitation was performed with anti-Rrn3p antibodies as described in Materials and methods. After elution of the precipitated complex with an excess of the N-terminal Rrn3p peptide, half of the last wash step (lane 3) and half of the eluate (lane 4) were separated on a 10% SDS–polyacrylamide gel and stained with silver (upper panel). The purified pol I-A (200 ng) kindly provided by A.Sentenac and colleagues is depicted in lane 1. The sizes of the two largest pol I subunits and of Rrn3p are indicated. Lower panel: transcription initiation assay with the eluted pol I–Rrn3p complex. One µl of fraction B600 was added to 1 µl of fraction B2000 (containing 200 ng of pol I) or to 2 and 4 µl of the eluate (containing 2 and 4 ng of pol I, respectively) in either the presence (lane 6) or absence (lanes 4 and 5) of 1.5 µl of TBP-cpl and assayed for transcription initiation. Control reactions with the same amounts of fractions B600 and/or TBP-cpl and 4 µl of the wash step are shown in lanes 2 and 3. Note the reduction of non-specific radioactivity in lane 6 in comparison with lane 1.
(**B**) Eighty µl of fraction B2000 (lanes 1 and 3) and 200 µl of fraction T0 were incubated with 40 µl of protein A–Sepharose beads covered with anti-Rrn3p antibodies (lanes 1 and 2) or empty beads (lanes 3). Half of each washed complex was subjected to Western blot analysis (left) or to transcription reactions in the presence of 1 µl of fraction B600 and 1.5 µl TBP-cpl (right). Western blots were developed with anti-pol I antibodies (directed against subunit A190) (upper panel) and anti-Rrn3p antibodies (lower panel).

Consistent with published results (Yamamoto *et al*., 1996), initiation activity could be detected when immunoprecipitated Rrn3p from the transcriptionally inactive fraction T0 (which was not complexed with pol I) was supplemented with ~3 µg of initiation-inactive pol I, which did not contain Rrn3p (fraction 20 of the MonoQ column) (data not shown). However, transcription efficiency was insignificant compared with that of the pol I–Rrn3p complex isolated from fraction B2000, even with a 1000-fold greater amount of pol I and a large excess of Rrn3p.

The observation that the majority of Rrn3p is not associated within the initiation-competent pol I complex suggests that either pol I or Rrn3p has to be modified to enable an interaction between the two partners. Indeed, analysis of Rrn3p-containing yeast fractions on two-dimensional gels revealed more than two different charged populations of Rrn3p (data not shown). However, a possible correlation with their activities could not been deduced thus far, since the pol I-associated Rrn3p failed to migrate into the first dimension of the gel.

Taken together, these data strongly suggest that a distinct pol I complex, consisting of pol I core enzyme, Rrn3p and possibly another associated factor(s), is formed either prior to or simultaneously with the start of promoter-dependent rRNA synthesis. Genetic and biochemical analyses have shown that Rrn3p activity is necessary for transcription initiation (Yamamoto *et al*., 1996). We propose that additional factors and/or modifications of Rrn3p or pol I are required to form a functional pol I–Rrn3p complex. While the conditions required for formation of the pol I–Rrn3p complex remain unknown, our results clearly show that only a pre-formed complex is able to initiate transcription efficiently *in vitro*.

During transcription the pol I–Rrn3p complex is disrupted and its capacity to initiate rRNA synthesis is exhausted

Although the pol I–Rrn3p complex was stable during purification and extended incubation in buffers used for *in vitro* transcription, the complex appeared to disintegrate during transcription. pol I could be still co-immunoprecipitated with Rrn3p after a 1 h incubation with all transcription components except the template (Figure 5A, lane 2) or nucleotide substrates (Figure 5A, lane 3). In contrast, when transcription was allowed to proceed for 1 h, pol I no longer co-precipitated with Rrn3p (Figure 5A, lane 4). Gel filtration experiments previously had demonstrated that no free Rrn3p was present in the fractions used for the reconstituted assay before *in vitro* transcription (data not shown), indicating that all Rrn3p that could be immunoprecipitated after the transcription reaction without pol I was indeed released from the pol I–Rrn3p complex.

If co-immunoprecipitation experiments before and after transcription were performed using the HA-tagged pol I, an analogous result was obtained (data not shown): co-immunoprecipitation of pol I and Rrn3p was observed exclusively before, but never after the transcription reaction.

The effect of transcription on the stability of the pol I–Rrn3p complex was tested in an order-of-addition experiment (Figure 5B). After pre-incubation of template E with pol I–Rrn3p complex and all necessary transcription factors, transcription was started by the addition of nucleo-

Table I. Purification of initiation-competent pol I from yeast whole-cell extracts

Fraction/step	Protein (mg)	Volume (ml)	Activity (U)	Specific activity (U/mg)	Relative specific activity
Whole-cell extract	2100	450			
K350	900	200	12 800	14.2	1
PA600	60	5	17 500	292	21
B2000	8.25	2.3	9800	1190	84
Sephacryl S-300	0.6	6	2570	4283	302
MonoQ PC 1.6/5	0.042	0.5	1950	46 428	3270

One unit is defined as the amount required to produce 0.1 fmol of accurately initiated transcripts in the reconstituted initiation assay. Initiation activity could not be detected in whole-cell extracts and was apparently diminished in fraction K350 due to the presence of inhibitors. The protein concentration after the MonoQ column was estimated by silver-stained SDS gels and by Western blotting.

tide substrates; a second template (template B) that had been pre-incubated with fractions B600 and TBP-cpl was then added along with fresh nucleotides at various time points. When transcription of template E was allowed to proceed for >40 min (Figure 5B, lane 3–5), no transcripts were generated from the second template (template B), indicating depletion or sequestration of pol I–Rrn3p activity. In control reactions in which transcription was prohibited by omitting either template (Figure 5B, lane 7) or nucleotides (Figure 5B, lane 8), pol I–Rrn3p initiation activity remained stable and apportioned equally to both templates when transcription was allowed. The diminished capacity to transcribe template B after transcription had ensued on template E was not explained by sequestration of the pol I–Rrn3p complex in a stable initiation complex on template E. In the absence of transcription, pol I–Rrn3p activity was distributed equally to templates B and E, even after prolonged pre-incubation exclusively with template E and all remaining transcription factors (data not shown).

Disruption of the pol I–Rrn3p complex obviously occurred during or after one single round of transcription, since no multiple rounds of transcription could be observed with our purified fractions in the promoter-dependent run-off assay (Figure 5C). This was measured by chasing a paused ternary transcription complex in the absence or presence of Sarkosyl or heparin to resume RNA chain elongation and possibly reinitiation of transcription: stable paused ternary complexes were formed by transcribing a template which lacked deoxycytidine within the first 34 nucleotides after the start site of the promoter-dependent RNA synthesis using a nucleotide triphosphate mixture (NTPs) without CTP (Tschochner and Milkereit, 1997). Transcripts were stalled after the synthesis of 34 nucleotides, but could be re-extended quantitatively after the addition of CTP (Tschochner and Milkereit, 1997). The presence of 0.025% Sarkosyl or 0.5 M heparin before addition of the nucleotides completely abolished transcription initiation (Yamamoto *et al*., 1996) (Figure 5C, lanes 2 and 3) and also disrupted pre-initiation complexes formed at the promoter in the absence of NTPs (data not shown). However, the same amounts of heparin and Sarkosyl were not able to dissociate a halted ternary pol I–DNA–RNA complex, since paused complexes could resume RNA chain elongation in the presence of both reagents (Figure 5C, lanes 4–7). A time course experiment in the presence of heparin or Sarkosyl revealed about the same efficiency of transcription as if the experiment was performed without heparin or Sarkosyl (Figure 5C, compare lanes 4 and 6 with lane 9, and lanes 5 and 7 with lane 12), indicating that all elongating pol Is are incapable of resuming reinitiation. (To ensure that only DNA-bound pol I was analysed during the elongation reactions, pre-initiation complexes formed at the immobilized template were washed before addition of the NTPs. An excess of fractions B600 and TBP-cpl could be added after washing the DNA-bound pre-initiation complex without any change in the efficiency of transcription.)

Our findings indicate an irreversible physical disruption of the pol I–Rrn3p initiation complex during or after one round of transcription, suggesting that pol I or Rrn3p acquire a different state during transcription in which one or both of them are no longer able to interact with each other and thus are no longer able to initiate transcription.

Growth-dependent regulation of transcription is dependent on the presence of the pol I–Rrn3p complex

We used our purification scheme (Figure 1) to purify pol I and pol I-specific initiation factors from stationary phase yeast cultures. Although the enrichment of pol I in terms of non-specific activity in fraction PA600s was comparable with fraction PA600g derived from growing cells, fraction PA600s was not active in promoter-driven transcription (Figure 6A, lane 11). The component(s) that regulates pol I transcription in response to growth rate is thought to be closely associated with the enzyme (Buttgereit *et al*., 1985; Cavanaugh and Thompson, 1985; Bateman and Paule, 1986; Tower and Sollner Webb, 1987; Schnapp *et al*., 1990; Riggs *et al*., 1995). Our observations suggest that the pol I–Rrn3p complex might be involved in this regulation process in yeast. Indeed, addition of the purified pol I–Rrn3p complex from the MonoQ column (fraction 22) to fraction PA600s isolated from stationary phase yeast cells restored an ability to utilize the ribosomal gene promoter (Figure 6A, lanes 8 and 10). In contrast, substitution of fraction PA600s with either initiation-inactive pol I (fraction 20 of the MonoQ column) (Figure 6A, lanes 7 and 9), TBP-cpl (lanes 6 and 9) or recombinant Rrn3p (data not shown) failed to restore accurate transcription.

Exchange experiments between the corresponding fractions derived from cells of the two different growth states confirmed this result: with the exception of the polymerase-containing fraction itself, all fractions necessary for transcription initiation could be exchanged without a significant loss in transcriptional activity (Figure 6B, lanes

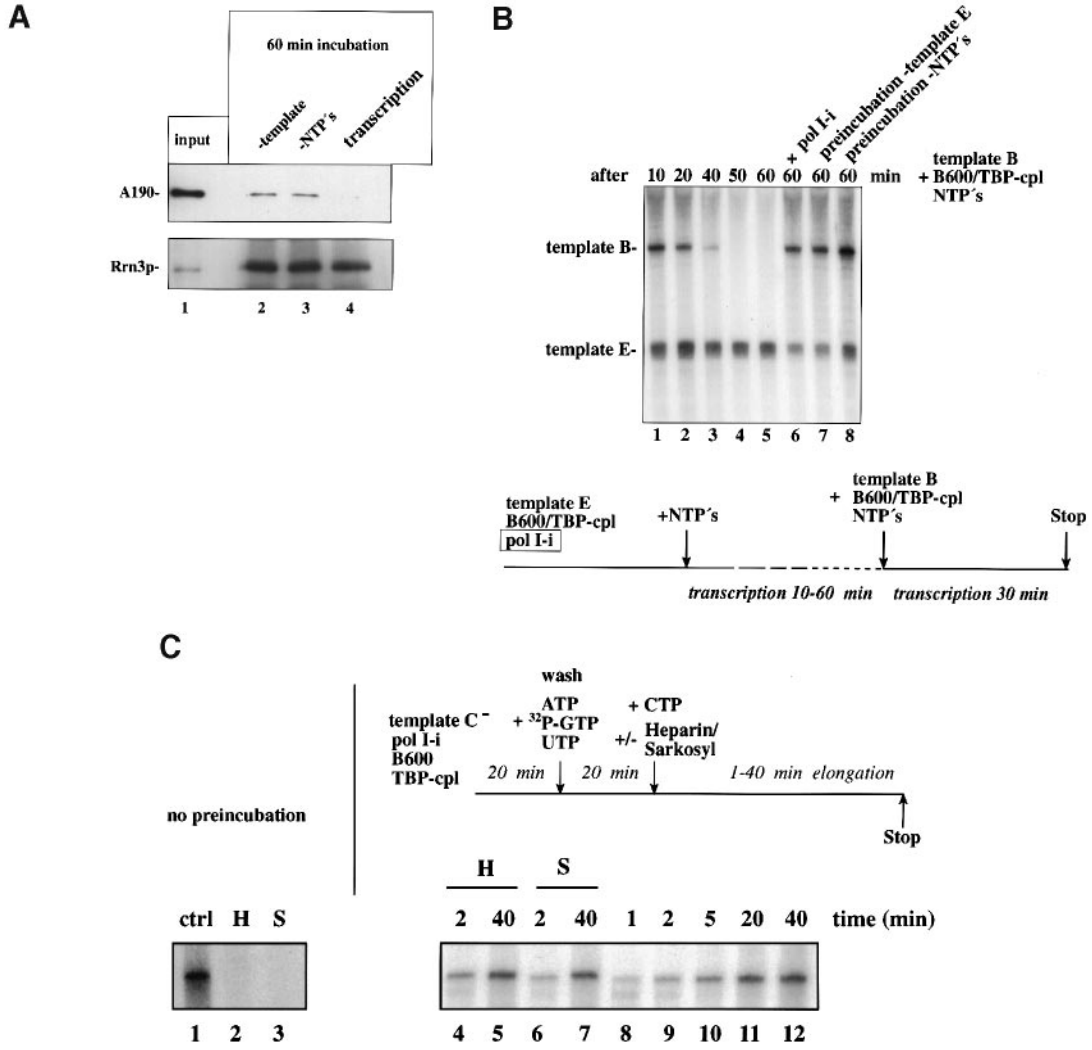

Fig. 5. Dissociation of pol I–Rrn3p complex during one round of transcription and loss of its transcriptional activity. (**A**) Co-immunoprecipitation of Rrn3p and pol I before and after transcription. Transcription assays containing 40-fold amounts of all ingredients (40 µl of fraction B600, 60 µg of fraction B2000) were performed in the absence of template (lane 2) or nucleotides (lane 3), or in the presence of 4 µg of template and 0.2 mM NTPs (lane 4). After 60 min incubation at 25°C, immunoprecipitation was carried out with anti-Rrn3p antibodies. Precipitated proteins were separated on a 10% SDS–polyacrylamide gel, blotted onto PVDF membranes and screened with antibodies directed against the largest subunit of pol I, A190 (upper panel), and Rrn3p (lower panel). Five µg of fraction B2000 was blotted on lane 1. (**B**) The pol I–Rrn3p complex loses its ability to start rRNA synthesis during ongoing transcription. As indicated in the scheme at the bottom, template E (pSES5 linearized with *Eco*RV) was pre-incubated with fraction B600, TBP-cpl and the pol I–Rrn3p complex (pol I-i) (MonoQ fraction 22) and transcription was started with the addition of nucleotides. At the indicated time points (lanes 1–6), template B (pSES5 linearized with *Bam*HI) was added together with fraction B600, TBP-cpl and fresh NTPs and incubated for a further 30 min. In lane 6, pol I-i was added together with the second template, fraction B600 and TBP-cpl after transcription of template E had proceeded for 60 min. Lanes 7 and 8 show control reactions lacking either template E (lane 7) or nucleotides (lane 8) during the first 60 min of incubation. (**C**) *In vitro* generated run-off transcripts are synthesized in one single round of transcription. Transcription was performed on immobilized templates which contained the pol I promoter, but lacked cytidine within the first 34 nucleotides form the start site. Lanes 1–3: *in vitro* reconstituted transcription in the absence (ctrl, control, lane 1) or in the presence of 0.5 mg/ml heparin or 0.025% Sarkosyl (lanes 1 and 2, respectively). Heparin and Sarkosyl were added to the template prior to the protein fractions. Lanes 4–12: a pre-initiation complex was formed with fractions B600, pol I-i and TBP-cpl on the immobilized template for 20 min. The pre-initiation complexes were washed to remove unbound pol I and transcription factors. ATP, [^{32}P]GTP and UTP were added to generate a ternary elongation complex (Tschochner and Milkereit, 1997). After a further 20 min of incubation, CTP and either heparin or Sarkosyl were added to give final concentrations of 0.5 mg/ml and 0.025%, respectively (lanes 4–7). Control reactions were performed without heparin or Sarkosyl (lanes 8–12). Transcription elongation was stopped after the indicated times.

1–6). Only pol I purified from growing cells supported promoter-driven transcription in the reconstituted system.

Western blot analysis demonstrated depletion of Rrn3p in the pol I-containing B2000 fraction derived from stationary phase yeast relative to the B2000 fraction from growing cells (Figure 6B, compare lanes 7–10). By contrast, equal amounts of pol I, TBP and Rrn10p, a component of the UAF complex, could be detected in both fractions. Rrn3p did not co-purify with pol I when fractionation was performed according to our purification scheme starting with cell extracts from non-growing cells, suggesting that Rrn3p is either completely missing in stationary phase cells or that it fails to form an active pol I–Rrn3p complex. Western blot analysis of total cell extracts from growing and stationary phase yeast revealed that similar amounts of Rrn3p are present in both extracts (Figure 7A, lanes 1 and 2), as well as in the K350 fraction (Figure 7A, lanes 4–7). Although the total yield of pol I

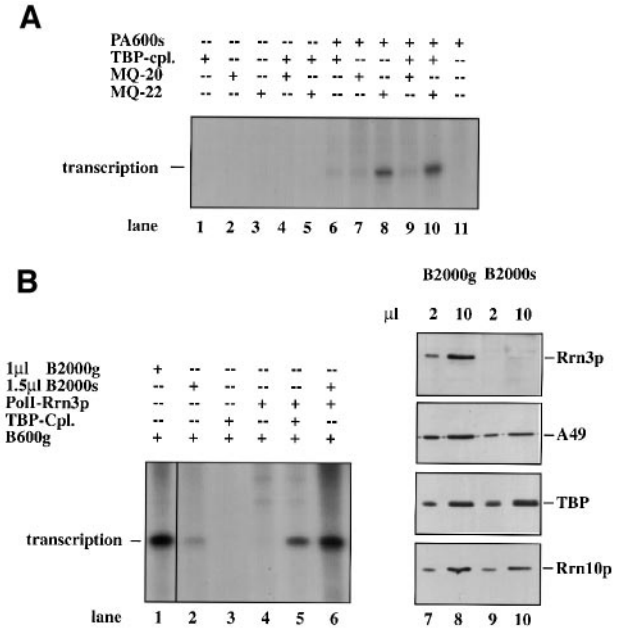

Fig. 6. Extracts from stationary phase cells require pol I–Rrn3p complex to initiate transcription. (**A**) Addition of initiation-competent pol I (pol I–Rrn3p complex) to the transcriptional inactive fraction PA600 derived from stationary phase cells restores transcription initiation. Reconstitution experiments were performed as indicated with 1 µl of PA600s (fraction PA600 isolated from stationary phase cells), 1.5 µl of TBP-cpl and 1.5 µl of the MonoQ fractions 20 and 22 (see Figure 3A). (**B**) Pol I-containing fraction B2000s isolated from stationary phase cells lacks both transcriptional activity and Rrn3p, but regains activity if pol I–Rrn3p is added. Left panel: substitution experiments were performed as indicated with 1 µl of fraction B2000g (isolated from growing cells), 1.5 µl of B2000s (isolated from stationary phase cells), 1.5 µl of pol I-i (pol I–Rrn3p complex), 1.5 µl of TBP-cpl and 1 µl of fraction B600g (isolated from growing cells, respectively). Right panel: Western blot analysis of fraction B2000 derived from growing cells (B2000g) and stationary phase cells (B2000s). Blots were developed with the indicated antibodies.

was slightly reduced in cell extracts of quiescent cells when compared with extracts of growing cells (Figure 7A, compare lane 1 with 2), it was possible to obtain a similar enrichment of pol I from stationary phase cells with our purification procedure. However, Rrn3p did not co-precipitate with pol I during dialysis of fraction K350 against buffers of low salt concentrations, indicating that a tight association of Rrn3p and pol I is lacking in stationary phase cells. This assumption was verified by immunoprecipitation experiments with whole-cell extracts derived from growing and quiescent cells of yeast strain LS149, which contains a HA-tagged AC40 subunit (Figure 7B). When immunoprecipitation was performed with anti-HA antibodies, a comparable amount of pol I was precipitated in both cell extracts (Figure 7B, lanes 3 and 4). In contrast, co-immunoprecipitation of Rrn3p with pol I was achieved exclusively in cell extracts derived from growing yeast (Figure 7B, lane 1). These data indicate that the pol I–Rrn3p complex is absent in stationary phase cells and that this deficit can account for the failure of stationary phase cell extracts to generate rRNA.

Discussion

We have shown that yeast pol I can be purified in an active monomeric form associated with Rrn3p. Only a small percentage of pol I was found to be in this initiation-competent form. The remainder of the polymerase failed to stimulate transcription efficiency. The basis for the large pool of inactive pol I is unclear, but there are two possible explanations. First, the initiation-competent form may be unable to withstand the purification procedure. However, this hypothesis is inconsistent with our observations that purification of initiation-competent pol I complexes through several chromatographic steps yielded a good recovery of transcriptional activity, and that the complexes are resistant to high salt concentrations. The second explanation is that only a small percentage of intracellular pol I molecules are primed for initiation. Perhaps the inactive pol I represents a separate subpopulation(s) that reflects different functional states not tested here, such as RNA chain elongation and/or reinitiation of transcription. Indeed, most of the pol I in mitotic cells might be present in an elongation-specific form, since pol I has to transcribe long stretches of DNA containing clusters of several hundred tandemly repeated rDNA genes. Thus, only a small percentage of pol I might be involved in promoter recognition *de novo*. Once the promoter has been cleared, the enzyme might dissociate from initiation factors in a transition to a form required for efficient RNA chain elongation.

Rrn3p is a stable component of the initiation-competent pol I complex

Rrn3p was described previously as a protein essential for efficient transcription initiation by yeast pol I (Yamamoto *et al*., 1996). Although they could not observe a direct physical interaction between pol I and Rrn3p, Nomura and co-workers postulated a weak interaction between these components because pre-incubation of affinity-purified, HA-tagged Rrn3p with purified pol I led to a stimulation of transcription *in vitro*. Isolated HA-tagged Rrn3p used for the reported reconstituted transcription assays apparently was not involved in a stable multi-protein complex since only one major protein component (Rrn3p) could be detected after affinity-purification of Rrn3p, and gel filtration experiments indicated that Rrn3p from whole-cell extracts exists predominantly as a monomer (Yamamoto *et al*., 1996). In contrast, we found transcriptionally active pol I complexes only when Rrn3p was physically associated with pol I: specific transcriptional activity co-purified and co-precipitated together with the assembled pol I–Rrn3p complex, but was never found in fractions (such as fraction T0) which were depleted of the pol I–Rrn3p complex, despite the fact that these fractions contained both free pol I and Rrn3p. After extended pre-incubation of purified Rrn3p lacking pol I and free pol I, we observed that at least a small proportion of free Rrn3p can bind to pol I and support transcription initiation. However, building up a functional complex in this way appeared to be much less favourable because the resulting transcription efficiency was dramatically lower than that of the pre-assembled pol I–Rrn3p complex. Furthermore, recombinant Rrn3p failed to support transcription initiation together with purified pol I in the reconstituted assay (data not shown). It is possible that a distinct activity absent from the purified pol I and Rrn3p fractions is required to bring one or both partners into the appropriate state to interact with each other. Alternatively,

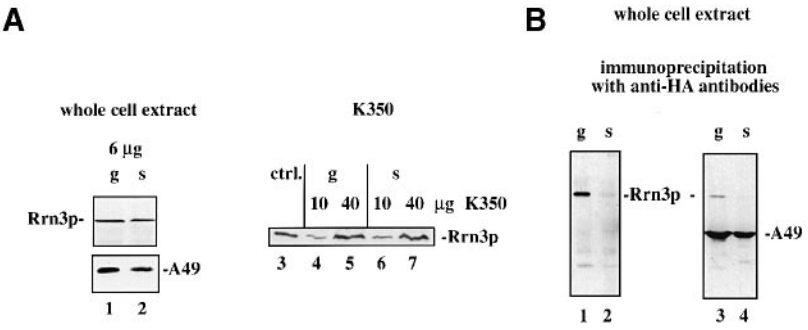

Fig. 7. Pol I–Rrn3p complex is not detected in whole-cell extracts of stationary phase cells. (**A**) Western blot analysis of whole-cell extracts (left panel) and of fraction K350 (right panel) derived from growing (g) and stationary phase (s) yeast. Blots were developed with antibodies against the pol I-specific subunit A_{49} and against Rrn3p as indicated. (**B**) Immunoprecipitation of pol I from whole-cell extracts of growing (g) and stationary phase (s) cells. Reactions were performed as described in Materials and methods, but using 60 µl of immobilized anti-HA antibodies (BAbCO) and 3.9 mg of protein of whole-cell extracts in a total volume of 0.65 ml. Whole-cell extracts were prepared from yeast strain LS149 which contained a HA-tagged AC40 subunit. Lanes 3 and 4 represent the same blot as shown on the left hand side (lanes 1 and 2), but redeveloped with pol I-specific anti-A49 antibodies.

an additional component required for transcription initiation might be present in the pol I–Rrn3p complex but lacking in the fractions containing the free components (Figure 3B). On the other hand, the existence of a whole set of additional components associated within the initiation-active complex, as is the case for the pol I holoenzymes reported in mammals and plants (Saez-Vasquez and Pikaard, 1997; Seither *et al*., 1998), can be excluded for several reasons. (i) Recently published results showed that initiation-competent pol I migrated on gel filtration columns with a slightly increased molecular mass if compared with the initiation-inactive monomeric pol I core enzyme (Milkereit *et al*., 1997). (ii) Comparison of the protein pattern of the fraction containing monomeric initiation-competent pol I after gel filtration and homogenous inactive pol I core enzyme (pol I-A) revealed differences in the polypeptide composition only in the mol. wt range 49–200 kDa (Milkereit *et al*., 1997). The resolution of these additional proteins could be improved using the PAGE system shown in Figure 3B. However, after immunoprecipitation with anti-Rrn3p antibodies, only the band corresponding to the molecular weight of Rrn3p remained associated with initiation-active pol I (Figure 4A), which suggests that this is the only component tightly attached to the core enzyme. (iii) Electron microscopic inspection of the pol I–Rrn3p complex led to the localization of Rrn3p on the core enzyme without any evidence for a dramatic change in core enzyme structure or for any further associated factor(s) (P.Schultz, P.Milkereit and H.Tschochner, in preparation).

Future experiments with recombinant Rrn3p and purified pol I will focus on the question of whether a modifying activity and/or a missing factor is required to form an initiation-active enzyme.

Involvement of pol I–Rrn3p complex in initiation of rRNA synthesis and growth-dependent regulation

Cellular rDNA transcription is closely regulated according to the growth state of the cell. Investigations with reconstituted *in vitro* transcription systems from mouse, *Acanthamoeba* and yeast have demonstrated that the regulated activity is closely associated with the pol I fraction (Buttgereit *et al*., 1985; Cavanaugh and Thompson, 1985; Riggs *et al*., 1995; Bateman and Paule, 1986; Tower and Sollner Webb, 1987; Schnapp *et al*., 1990). Promoter-dependent pol I activity is isolated exclusively from growing cells (Bateman and Paule, 1986; Tower and Sollner Webb, 1987; Schnapp *et al*., 1990, 1993) and can be separated from the initiation-inactive enzyme. In the mouse system, the regulated component (TIFIA/TFIC/Factor C*) could be isolated from pol I during the purification procedure and appeared to comprise sufficient activity by itself to restore transcription initiation both with purified pol I in a reconstituted system and with inactive cell extracts from stationary phase cells (Mahajan and Thompson, 1990; Schnapp *et al*., 1990, 1993; Brun *et al*., 1994). In contrast, our results indicate that in yeast it is the formation of a pol I–Rrn3p complex that mediates initiation of rRNA synthesis and growth-dependent regulation of transcription. The presence of non-associated cellular Rrn3p and free pol I in extracts from stationary phase cells or in our reconstituted transcription assay is not sufficient for *de novo* rDNA transcription. Furthermore, neither combinations of free pol I and free Rrn3p purified from cells in exponentional and stationary phases nor the addition of recombinant Rrn3p to extracts derived from quiescent cells were capable of stimulating transcription initiation (data not shown). Several explanations for these different results are conceivable. First, yeast and mammals might regulate initiation of rDNA transcription differently. Secondly, the mouse factors described may represent an activity different from Rrn3p which is involved in the pathway to form an initiation-competent pol I complex. Thirdly, unlike the mammalian transcription systems, the fractions used in our system might lack the activity which is required to form the complex. Fourthly, since a functional pol I–Rrn3p complex has a strikingly high specific transcriptional activity and the vast majority of these two components in the cell are not involved in this particular complex, a minor amount of murine pol I embedded in such a complex may have escaped detection in fractions required for proper regulation of murine rRNA synthesis. Since none of the genes coding for TIFIA, TFIC and Factor C* have been identified, it remains an open question as to whether one of these represents the mammalian counterpart of Rrn3p.

Other potential mechanisms for regulating rDNA transcription have been described. As mentioned in the Introduction, both UBF and SL1 were suggested as target molecules of the initiation complex which are involved in the up- and downregulation of rRNA synthesis. Thus, it seems very probable that multiple control points exist in the pathway to activate or repress rDNA transcription in the cell. In this respect, it is worth noting that transcription efficiency in our *in vitro* system is slightly reduced when the pol I–Rrn3p complex is assayed with the B600s fraction derived from stationary phase cells instead of fraction B600g from growing cells (data not shown). A second independent growth-dependent mechanism might also function in yeast and might affect transcription factor(s) present in fraction B600. Alternatively, it is possible that formation of the polI–Rrn3p complex is mediated by an active upstream regulatory factor such as UBF.

Dissociation of the pol I–Rrn3p complex and its correlation with transcriptional activity

Although a pre-assembled pol I–Rrn3p complex appeared to be very stable in our purification procedure, its disruption was accomplished rapidly by ongoing transcription. Disruption of the complex was accompanied by a diminished capacity to initiate transcription at the promoter (Figure 5). This result is in agreement with a previous observation that mouse Factor C* activity is exhausted early in the transcription process (Brun *et al*., 1994). A post-translational modification of Factor C* was postulated to prevent the factor from regaining transcriptional activity. Our results support a modified version of this suggestion and provide a molecular basis for the following model. (i) Accurately initiated transcripts depend on the presence of a functional pol I–Rrn3p complex. (ii) Since this initiation-competent pol I complex apparently represents only a minority of total pol I in the cell, the majority of pol I might be involved in other functions such as RNA chain elongation, reinitiation, etc. (iii) Once the initiation-competent pol I complex is disrupted during or after transcription, the single components are no longer able to form a complex and thus fail to support accurate transcription initiation. Most of the pol I and Rrn3p present in whole-cell extracts do not interact with each other and, therefore, appear to be 'silent' for initiation of rDNA transcription. (iv) To regain their competence to reassemble within an initiation-active complex, free Rrn3p and/or pol I must be (re)activated in an unknown way restricted to growing cells. Future experiments should test whether and how the non-associated components are modified to restore the capability to assemble, and how this activation is accomplished in a growth rate-dependent manner.

Materials and methods

Strains and templates

Yeast wild-type strain BJ926, strain YF2089, which contained a His_6-tagged ABC_{23} pol I subunit (kind gift of Drs S.Nouraini and J.D.Friesen, Toronto), and strain LS149, which contained a His_6- and HA-tagged AC_{40} pol I subunit (kind gift of Dr Sentenac and colleagues), were used for preparation of the extracts and subsequent fractionation. Plasmid pSES5 (Stewart and Roeder, 1989) was used as template for the initiation assay and was linearized either with *Eco*RV or with *Bam*HI, which resulted in specific initiated transcripts of 244 and 432 nucleotides, respectively.

In vitro transcription

Transcription reactions were performed as described elsewhere (Milkereit *et al*., 1997). Conditions for *in vitro* transcription reactions at immobilized templates and purification of the ternary complex have been published previously (Tschochner and Milkereit, 1997). Radiolabelled transcripts in dried gels were quantitated if necessary on a PhosphorImager.

Preparation of whole-cell extracts and fractions used for the reconstituted transcription assay

Fraction B600, TBP-cpl and fraction B2000 were generated on a large scale according to Milkereit *et al*. (1997). Preparations of whole-cell extracts on a small scale were performed as described (Grandi *et al*., 1993) with the exception that 2× 20 min of bead beating were appended after Zymolyase treatment. To achieve a better breakage of stationary phase cells, four rounds of bead beating were performed. [Note that the protein concentration of lysates derived from growing cells was still ~3-fold compared with stationary phase cells, which is due to the increased cell wall stability of stationary phase yeast (Werner-Washburne *et al*., 1993).] After the cells were broken, the lysate was centrifuged for 15 min at 4°C at 14 000 *g* and the supernatant was used for immunoprecipitation. For one preparation of whole-cell extracts on a small scale, 50 ml of a yeast culture at OD_{600} = 6 or its equivalent at higher or lower cell densities was used.

Purification of initiation-competent pol I

Thirty litres of yeast were grown in YPD to an A_{600} of 2–3, harvested by centrifugation, and fraction B2000 was prepared as previously described (Milkereit *et al*., 1997). After dialysis against buffer BU300 [buffer BU contained 20% glycerol, 20 mM HEPES pH 7.8, 2 mM $MgCl_2$, 0.02 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine and was supplemented with potassium acetate to give 300 mM potassium acetate], 750 µl of fraction B2000 containing 4 mg/ml protein were loaded onto a Sephacryl S-300 column (120 ml) (Pharmacia). The column was developed with buffer BU300 at a flow rate of 0.4 ml/min, and 0.75 ml fractions were collected. Inspection by electron microscopy revealed that dimers eluted from fractions 28–32 and monomers from fractions 34–38. Monomeric initiation-competent pol I was separated from the bulk pol I on a MonoQ column (0.1 ml) (SMART, Pharmacia) with a linear gradient from 600 to 1300 mM potassium acetate in buffer BU in a total volume of 2 ml. The flow rate was 0.05 ml/min, up to 2 ml of the monomeric pol I peak fractions from the Sephacryl-S-300 column were loaded, and 0.05 ml fractions were collected. Pol I eluted at ~1.1 M acetate from the column. To show the stable association of Rrn3p with pol I during gel filtration, 50 µl of fraction B2000 were loaded on a Superose 6-column (SMART, Pharmacia) and proceeded at a flow rate of 15 µl/min of buffer BU1500 (buffer BU containing 1500 mM potassium acetate). Fractions of 50 µl were collected.

Large-scale preparation of whole-cell extracts from stationary phase cells

Generation of whole-cell extracts and the fractionation procedure were basically the same as from growing cells, with the exception that yeast cells were grown until no increase in the optical density could be detected (OD_{600} 10–11). To ensure that rRNA synthesis was completely downregulated (Riggs *et al*., 1995), the cells were harvested after a further cultivation for 12 h.

Immunoaffinity purification of the pol I–Rrn3p complex

A 0.3 ml aliquot of fraction B2000 (1.8 mg/ml) derived from strain LS149 containing a HA-tagged AC40 subunit was adjusted to 1 ml with buffer BU600 (buffer BU supplemented with 600 mM potasium acetate; 2.5 mM mercaptoethanol was used instead of 1 mM DTT). After incubation with 50 µl of anti-HA antibodies attached to Sepharose beads (BAbCO) for 2 h at 4°C, the beads were washed with 4× 1 ml of buffer BU600. Bound pol I was eluted with 1 mg/ml HA-peptide in buffer BU600.

Antibodies

Antibodies against Rrn3p were generated in rabbits against a synthetic peptide corresponding to the N-terminal sequence of Rrn3p (MMAFENTSKR) that was coupled to a branched polylysine core (Posnett and Tam, 1989). Antibodies against the C-terminal peptide of Rrn3p (SEASGEYESDGSDD) were produced as described (Stenbeck *et al*., 1993). The same procedure was performed to generate antibodies against the N-terminus of Rrn10 p (MDRNVYEACSN). All antibodies

were affinity-purified with the peptide coupled to epoxy-activated Sepharose 6B (Pharmacia).

Immunoprecipitation of the pol I–Rrn3p complex with anti-Rrn3p antibodies

Approximately 1 µg of affinity-purified anti-Rrn3p antibodies was coupled to 20 µl of protein A–Sepharose (Pharmacia) for 2 h at 4°C, washed twice with 20 mM HEPES pH 7.8 and twice with buffer IP (10 mg/ml milk powder, 20 mM HEPES pH 7.8, 600–900 mM potassium acetate, 0.5% NP-40) and used for one immunoprecipitation experiment. Either 60 µg of fraction B2000 or 200 µl of fraction T0 were incubated in the presence of buffer IP for 2 h at 4°C, washed with 3× 0.5 ml of buffer IP without milk powder and 1× 0.5 ml with 20 mM HEPES pH 7.8. Washed beads were either used for *in vitro* transcription or were resuspended in SDS sample buffer and applied to SDS–PAGE. If the complex should be eluted from the beads, 2 mg of B2000 were incubated with 0.15 ml of protein A–Sepharose which had been attached with ~8 µg of affinity-purified anti-Rrn3p antibodies. Incubation and wash steps were as described above and elution was performed with 1 mg/ml of the N-terminal peptide of Rrn3p in buffer BU600.

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