Rrn3 Becomes Inactivated in the Process of Ribosomal DNA Transcription*

The human homologue of yeast Rrn3, a 72-kDa protein, is essential for ribosomal DNA (rDNA) transcription. Although the importance of Rrn3 function in rDNA transcription is well established, its mechanism of action has not been determined. It has been suggested that the phosphorylation of either yeast RNA polymerase I or mammalian Rrn3 regulates the formation of RNA polymerase I-Rrn3 complexes that can interact with the committed template. These and other reported differences would have implications with respect to the mechanism by which Rrn3 functions in transcription. For example, in the yeast rDNA transcription system, Rrn3 might function catalytically, but in the mammalian system it might function stoichiometrically. Thus, we examined the question as to whether Rrn3 functions catalytically or stoichiometrically. We report that mammalian Rrn3 becomes the limiting factor as transcription reactions proceed. Moreover, we demonstrate that Rrn3 is inactivated during the transcription reactions. For example, Rrn3 isolated from a reaction that had undergone transcription cannot activate transcription in a subsequent reaction. We also show that this inactivated Rrn3 not only dissociates from RNA polymerase I, but is not capable of forming a stable complex with RNA polymerase I. Our results indicate that Rrn3 functions stoichiometrically in rDNA transcription and that its ability to associate with RNA polymerase I is lost upon transcription.

Three requirements must be met before RNA polymerase I (pol I) can initiate specific and effective transcription. Both the ribosomal DNA (rDNA) promoter and RNA polymerase I have to be “transcription ready,” and they must join together to form a functional initiation complex. The assembly of the intermediate and complex changes involved in this process requires a complex series of protein-DNA and protein-protein interactions. For example, the stable binding of the transcription factors to the rDNA promoter requires the coordinate binding of factors to the core and upstream promoter elements (1).

Eukaryotic rDNA promoters contain a core promoter element and an upstream promoter element (for review, see Ref. 1). Two multi-subunit complexes, core factor (CF) and upstream activating factor (UAF), which bind to the core promoter and to the upstream element, respectively (2, 3), are required to commit the yeast rDNA promoter. Both CF and UAF interact specifically with TATA-binding protein (TBP) (4, 5). In mammals, two known transcriptional factors are required to commit RNA polymerase. The binding of selectivity factor (SL1) (6–10), containing TBP and TBP-associated factors (TAFs) to the core promoter element is necessary and sufficient in vitro. The binding of upstream binding factor (UBF) (2, 12, 13), a multiple HMG box containing architectural protein, and possibly a second molecule of SL1 to the upstream promoter element is required for efficient transcription in vitro and template commitment. Both SL1 and UBF are subject to regulation via phosphorylation and acetylation (14–19). In addition, Rb, the protein product of the retinoblastoma susceptibility gene, interacts with UBF repressing pol I transcription (20), and SV40 large T antigen activates pol I transcription by interacting with SL1 (21).

The mechanism whereby yeast pol I is recruited to the promoter is unclear. It was established (22) that only ~2% of RNA pol I population present in an exponentially growing yeast cell is capable of promoter-specific transcription. These competent RNA pol I molecules were found to contain core RNA polymerase I subunits and Rrn3, a polymerase-associated factor.

Both genetic and biochemical experiments demonstrate that yeast Rrn3 is essential for rDNA transcription. The human homologue has been cloned and subsequently identified as the previously described transcription initiation factor IA (TIF-IA) (24). Current models suggest that Rrn3 acts as a bridge between RNA pol I and the committed RNA polymerase (25–29). A direct interaction between the 43-kDa subunit of pol I (rpa43) and Rrn3 in the Rrn3-pol I complex was confirmed (27, 28) as well as the direct interaction of human Rrn3 with the TAF110 and TAF63 subunits of species-specific transcription factor SL1 (28, 29). Despite this body of knowledge, there are still significant controversies concerning the role that Rrn3 plays in transcription.

Fath et al. (30) proposed that the phosphorylation of yeast RNA pol I regulates the formation of a functional initiation complex between RNA pol I and Rrn3. In contrast, our recently published data (28) indicate that the phosphorylation state of mammalian Rrn3 regulates this process. Aprikian et al. (31) reported that yeast RNA pol I could be recruited to the rDNA promoter in the absence of Rrn3. However, they noted that these complexes could not be converted to transcriptionally active complexes when Rrn3 was added to the reactions. In contrast Schnapp et al. (32) reported that mammalian “TIF-IA (Rrn3) is liberated from the initiation complex and facilitates transcription from templates bearing preinitiation complexes which lack TIF-IA.” This observation is significantly different from the Aprikian model. In addition, it suggests that Rrn3 functions catalytically. This is in contrast to earlier observa-

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tions that factor C*, a factor that shares many properties with Rrn3, functioned stoichiometrically (33). As these differences would have significant implications with respect to the mechanism by which Rrn3 functions in transcription, we examined the question as to whether Rrn3 functions catalytically or stoichiometrically.

A catalyst is defined as an element that modifies the rate of a chemical reaction without being consumed in the process, and without being changed by the consequences of the process. Rrn3 was shown (28) to undergo modifications that are possibly the consequence of transcription. In this study we present evidence that Rrn3 is consumed during transcription and cannot be directly "reused" in a second round of transcription. We also demonstrate that Rrn3 dissociates from pol I as a consequence of transcription. These observations provide evidence that Rrn3 serves a stoichiometric rather than catalytic function in RNA pol I transcription. This in turn supports our hypothesis that the post-translational modification of Rrn3 plays an active role in the formation of the functional Rrn3-pol I complex in mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment with Cycloheximide—**N1S1 cells were grown in RPMI1640 + 5% fetal bovine serum (34). Where indicated, cells were treated with cycloheximide, 2 μg/ml (Sigma) for one hour.

**Production of Recombinant rpa43 and Rrn3 in Sf9 Cells and Protein Purification—**S, FLAG-tagged Rrn3 and S, His rpa43 were expressed in Sf9 cells and purified using anti-FLAG agarose beads (Sigma) or nickel-nitrilotriacetic acid-agarose (Qiagen) as previously described (28). To recover recombinant FLAG-tagged Rrn3 from the *in vitro* transcription reactions, the 200-μl reactions were diluted 2.5-fold by adding 300 μl of transcription buffer containing 20 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, and 6% glycerol. The NaCl concentration was then adjusted to a final concentration of 150 mM. Both changes were necessary for the optimal binding of Rrn3 to anti-FLAG beads. 10 μl of packed anti-FLAG agarose beads were added to each reaction, and the reaction tubes were tumbled for two hours at 4 °C. After the beads were washed three times with 1 ml of wash buffer (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100), Rrn3 was eluted with 30 μl of 0.5 mg/ml FLAG peptide (Sigma). The protein was used immediately for Western blot analysis.

**Purification of UBF and SL—**UBF and SL1 were purified by DEAE Sephadex and heparin agarose chromatography as described previously (35). UBF was eluted from the heparin agarose column with 600 mM KCl, whereas SL-1 was eluted with 1000 mM KCl. At these stages of purification there is no detectable Rrn3 or pol I in either the UBF or SL preparations (data not shown).

**Co-immunoprecipitation of Rrn3/RNA Pol I—**Rrn3 that had been incubated in a transcription reaction was then incubated with anti-FLAG beads and eluted with FLAG peptide. 30 μl of eluted Rrn3 (∼7 μg) was mixed with 100 μl (∼100 μg) of recombinant rpa43 and tumbled at 4 °C overnight in a final volume of 100 μl of buffer containing 20 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, and 6% glycerol. Anti-FLAG agarose beads were added and the mixture tumbled for two hours at 4 °C. The beads were then washed as described above and Rrn3 eluted with FLAG peptide. The protein was used immediately for Western blot analysis.

**rDNA Transcription Template—**pUC5.1EX contains the rat 45S rDNA (286 to +630) promoter and pUBE/B is a PCR product of the rat 45S rDNA (+579 to +480) promoter. When truncated with EcoRI the transcription from pUC5.1EX is 632, and that from pUBE/B is 480 nts (35).

**Protein Extracts—**S-100, whole cell extracts from control or cycloheximide-treated cells were prepared essentially as described (34, 36).

**In Vitro Transcription—**In *in vitro* transcription reactions were carried out as described previously (37, 38).

**Western Blot Analysis—**SDS-PAGE and electroblot analysis were performed as described previously (28). Polyclonal rabbit antiserum to the 194-kDa (β') and 127-kDa (β) subunits of pol I have been described previously (34). Polyclonal, rabbit antiserum to Rrn3 and rpa43 were raised to purified, recombinant Rrn3 or rpa43, respectively. S peptide conjugated to horseradish peroxidase (S-HRP conjugate, Novagen) was used as recommended by the suppliers. Monoclonal anti-FLAG antibodies were used as recommended by the supplier (Sigma).

**RESULTS**

**Cycloheximide Inactivates Rrn3—**We have previously reported that whole cell extracts prepared from CHX-treated cells cannot support transcription *in vitro* and that transcription activity can be restored by addition of Rrn3 (28). Thus, our evidence is consistent with the model that treatment with cycloheximide results in the inactivation of mammalian Rrn3. However, it is possible that cycloheximide inactivates rDNA transcription through another pathway, and the rescue of rDNA transcription by exogenous Rrn3 is due to a redundancy in the mechanism of rDNA transcription, i.e. Rrn3 can replace another component. To test this model we isolated Rrn3 from cells treated with cycloheximide and from control cells (Fig. 1A). 3T3 cells were transfected with pCDNA3.1FLAG-Rrn3 as described previously (28). Twenty-four hours later, one half of the cells were treated with cycloheximide for one hour. Rrn3 was purified from whole cell extracts of both groups of cells by immuno-affinity purification using immobilized anti-FLAG antibodies (28). Equal amounts of the two forms of Rrn3 were added to extracts from cycloheximide-treated cells to determine whether they were equally active in transcription (Fig. 1B). The observation that Rrn3 purified from cells treated with cycloheximide (lane 3) was <5% as active as the Rrn3 purified from untreated cells (lane 4) provided additional evidence that treatment with cycloheximide resulted in the inhibition of Rrn3 activity. Moreover, when this result is considered with other observations, it is consistent with the model that the only component of the rDNA transcription apparatus that is inactivated in extracts from cycloheximide-treated cells is Rrn3.

**Rrn3 Is the Limiting Factor in *in Vitro* Transcription Assays—**The question as to whether Rrn3 functions catalytically or stoichiometrically has been examined indirectly (32), and those authors reported data consistent with a model in which Rrn3 functions catalytically. As this question has a significant effect on our understanding of the mechanisms involved in transcription, we examined this question using three different approaches.

Our first series of experiments were based on the premise that if Rrn3 undergoes some inactivation as the result of transcription, it should become limiting in the transcription reac-
and 60 min. Lanes 3
imide-treated S-100 N1S1 protein extract, 1.5 ng of recombinant Rrn3
by 30 min (Fig. 2
the accumulation of transcription products reached a plateau
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A
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Experimental Procedures,’’ using 5 μl of control N1S1 S-100 extract, to which recombinant Rrn3 was added as indicated. Lanes 1–5 show a time course without addition of Rrn3. Reaction time was 5, 30, 60, 90, and 120 min. Lanes 6–8 show the reactions that were run for additional 15, 30, or 60 min after adding 100 ng of Rrn3 at 60 min. C, transcription reactions were performed using 5 μl of cycloheximide-treated S-100 N1S1 protein extract, 1.5 ng of recombinant Rrn3 and 0.1 μg template pU5.1EX. Lanes 1 and 2 show reactions run for 30 and 60 min. Lanes 3 and 4 show reactions that were run for 60 min without isotope. Isotope was added at 60 min along with 0.5 ng Rrn3 (lane 4) or without Rrn3 (lane 3), and transcription was allowed to proceed for another 15 min. Transcription reactions, purification of the in vitro synthesized RNA, and PAGE analysis of the transcription reactions were carried out as described under “Experimental Procedures.” Trans., the 632-nt transcript that results from correct initiation. Int. Std., internal standard added for the recovery of nucleic acids.

Fig. 2. Rrn3 becomes a limiting factor during transcription in vitro. A, schematic depicting the theoretical result of adding recombinant Rrn3 to a transcription reaction after 60 min, assuming that Rrn3 has become rate-limiting. B, transcription reactions, prepared as described under “Experimental Procedures,” using 5 μl of control N1S1 S-100 extract, to which recombinant Rrn3 was added as indicated. Lanes 1–5 show a time course without addition of Rrn3. Reaction time was 5, 30, 60, 90, and 120 min. Lanes 6–8 show the reactions that were run for additional 15, 30, or 60 min after adding 100 ng of Rrn3 at 60 min. C, transcription reactions were performed using 5 μl of cycloheximide-treated S-100 N1S1 protein extract, 1.5 ng of recombinant Rrn3 and 0.1 μg template pU5.1EX. Lanes 1 and 2 show reactions run for 30 and 60 min. Lanes 3 and 4 show reactions that were run for 60 min without isotope. Isotope was added at 60 min along with 0.5 ng Rrn3 (lane 4) or without Rrn3 (lane 3), and transcription was allowed to proceed for another 15 min. Transcription reactions, purification of the in vitro synthesized RNA, and PAGE analysis of the transcription reactions were carried out as described under “Experimental Procedures.” Trans., the 632-nt transcript that results from correct initiation. Int. Std., internal standard added for the recovery of nucleic acids.

allowed to proceed for 60 min in the absence of radioactive probe (lanes 3 and 4). After 60 min of transcription, [α32P]-UTP was added to both reactions, and transcription was allowed to proceed for an additional 30 min. Rrn3 was added to only one reaction (lane 4). When the transcription reactions were complete, the in vitro synthesized RNA was isolated, fractionated by urea-PAGE, and detected by autoradiography. The absence of transcript in lane 3 suggests that an essential component(s) was consumed during the first 60 min of reaction. The observation that transcripts accumulated when the reaction was supplemented with Rrn3 at the same time as isotope was added (lane 4), suggests that Rrn3 was the “consumed” component. Taken together the results shown in Fig. 2 demonstrate that Rrn3 is inactivated or consumed as a result of transcription.

Template Commitment Assay—Our second approach was to use a modified template commitment assay (Fig. 3). Two templates, distinguished by the sizes of the transcripts they support, were used in these assays (template 1, p5.1E/X, 632 nt transcript; and template 2, pUE/B, 480 nt transcript). The first experiments used extracts from cycloheximide-treated cells as the use of unfractionated extracts avoids artifacts associated with purification and fractionation. As shown in Fig. 3, if both templates are added at the same time, both are transcribed (lane 1). However, if the reaction is incubated with only one template followed by the addition of the second template after 60 min (followed in turn by an additional 30 min of transcription), transcription is only observed from the template that was incubated with Rrn3 first (template 1, lanes 2 and 3). When the second template 2 was added together with Rrn3, again both templates are transcribed (lane 4). These data suggest that, under these conditions, Rrn3 mediates an essential transcription step and is the limiting component in the transcription reaction.

To ensure that Rrn3 was the limiting component in the transcription reactions, a similar experiment was carried out,
the first transcription reaction, the recovery of Rrn3 was determined by SDS-PAGE and Western blot analysis of the transcription reactions were carried out as described under “Experimental Procedures.” Trans. 1, the 480-nt transcript that results from the correct initiation on template 1. Trans. 2, the 632-nt transcript from template 2. Int. Std., internal standard added for the recovery of nucleic acids.

and the reactions were supplemented with purified UBF and SL1, so that those factors would not be limiting (Fig. 4). When purified, Rrn3, SL1, and UBF were added to an in vitro reaction mixture containing S-100 from CHX-treated N1S1 (as a source of RNA pol I) and either template 1 or 2, each template was transcribed (lanes 2 and 3 and 5 and 6). If both templates (Temp 1 and 2) were added at time 0, both templates are transcribed (lane 7). However, if the second template mixture containing SL1 and UBF is added after 20, 40, or 60 min, only the first (Temp 1) template is transcribed (lanes 8–10). As SL1 and UBF were added along with the second template, they are not limiting factors in these reactions. This suggests that once Rrn3 commits to the initiation complex, it cannot contribute to transcription from a second template.

**Rrn3 Isolated From a Transcription Reaction Does Not Function in a Second Reaction**—The above experiments provide strong evidence that once Rrn3 has been utilized in a transcription reaction, it cannot function in a second reaction. To directly test this model, we designed an experiment that consisted of two consecutive, Rrn3-dependent transcription reactions (Fig. 5a). FLAG-Rrn3 was added to an initial, Rrn3-dependent transcription reaction and then purified from that reaction and tested in a second Rrn3-dependent transcription reaction. If Rrn3 were to be inactivated as a result of transcription then it would not function in the second transcription reaction. NTPs and/or template were omitted from some reactions to control for the specificity of the inactivation. In addition, the recovery of Rrn3 was determined by SDS-PAGE and Western blotting (Fig. 5b, lower panel). As shown in Fig. 5b (upper panel), when Rrn3 was isolated from a complete transcription reaction, we did not observe transcription from the second Rrn3-dependent reaction (lane 3). In contrast, if NTPs (lane 4), DNA (lane 5), or both NTPs and DNA (lane 6) were omitted from the first incubation, we observed transcription from the second transcription reaction. These results demonstrate that Rrn3 becomes inactivated in a transcription reaction, and that the inactivation is NTP and template-dependent. Thus, Rrn3 becomes inactivated as the result of transcription.

**Transcription Results in the Dissociation of Rrn3 from Core RNA Polymerase I**—Milkereit and Tschochner (22) have shown that active RNA polymerase I (capable of promoter-specific transcription) is associated with Rrn3 and inactive polymerase I does not contain Rrn3. These authors and Aprikian et al. (31) also demonstrated that Rrn3 dissociated from pol I as a consequence of transcription. Subsequent studies by Fath et al. (30)
suggest that the dissociation of the Rn3-pol I complex is the result of a change in the phosphorylation state of RNA polymerase I and does not reflect a change in Rrn3. We have reported previously (28) that mammalian Rrn3 is inactivated by dephosphorylation and have observed that Rrn3 is inactivated as a result of transcription. Thus, if the mammalian rDNA transcription system is homologous to the yeast system, we would expect to see dissociation of the Rrn3-pol I complex, but the dissociation would be due to changes in Rrn3 and not RNA pol I.

As a first step in assessing this model, we determined if Rrn3 dissociates from RNA polymerase I as a consequence of transcription (Fig. 6, upper panel). In vitro transcription reactions were carried out ± NTPs as described above. Rrn3 was purified from the transcription reactions that had been supplemented with FLAG-Rrn3 by anti-FLAG affinity purification. The immunoprecipitates were fractionated by SDS-PAGE, transferred to Immobilon P, and the blots were then stripped and reprobed with polyclonal antibodies to the β′ subunit of RNA polymerase I (A194), and with anti-Rrn3 antibodies (Rrn3).

Our first series of experiments provided evidence that Rrn3 functions stoichiometrically and the above experiments demonstrated that Rrn3 dissociates from core RNA polymerase I as a consequence of transcription. This suggests that the dissociation of the Rrn3-pol I complex might result from a change in Rrn3 that both inactivates the polymerase-assoc-

FIG. 6. Rrn3 dissociates from RNA polymerase I as a result of transcription. A schematic depicting the experimental protocol. B, FLAG-tagged Rrn3 was affinity purified from either a complete transcription reaction (lane 2), or from reactions where either NTPs (lanes 3 and 5) and/or template (lanes 4 and 5) were omitted. The affinity purified Rrn3-RNA polymerase I complexes were fractionated by SDS-PAGE and blotted to Immobilon P. The blots were probed with polyclonal antibodies to the β′ subunit of RNA polymerase I (A194), stripped, and reprobed with polyclonal anti-Rrn3 antibodies (Rrn3).

FIG. 7. After transcription, Rrn3 has lost its ability to associate with RNA polymerase I. Upper panel, a schematic depicting the experimental protocol. Lower panel, transcription reactions, complete (lane 4) or incomplete (lane 5, lacking NTPs), were supplemented with S, FLAG-Rrn3. The Rrn3 was then purified by immunopurification with immobilized anti-FLAG antibodies and used in co-immunoprecipitation reactions with 100 μg of purified, recombinant Rpa43. The products of the co-immunoprecipitation reactions were fractionated by SDS-PAGE and blotted to Immobilon P. The blots were probed with S peptide conjugated to horseradish peroxidase (S-HRP conjugate, Novagen) as recommended by the suppliers to detect Rrn3 (Rrn3). The blots were then stripped and reprobed with polyclonal anti-rpa43 antibodies (rpa43).

To test this hypothesis we determined if Rrn3 isolated from a transcription reaction could bind to recombinant rpa43 (Fig. 7, upper panel). Transcription reactions were supplemented with FLAG- and S-tagged Rrn3. After transcription, the Rrn3 was isolated on immobilized anti-FLAG beads. The immobilized Rrn3 was then incubated with purified recombinant rpa43 tagged with S peptide. After the beads were washed, the bound proteins were fractionated by SDS-PAGE and analyzed by Western blotting with antibodies to rpa43 and S peptide (to detect rpa43 and Rrn3). Rrn3 isolated from complete transcription reactions did not bind rpa43 (Fig. 7, lower panel, lane 4). The loss of ability to interact with rpa43 was not due to nonspecific effects of the incubation at 30 °C, as the Rrn3 isolated from reactions lacking NTPs and template retained its ability to interact with rpa43 (lane 5). This confirms that the dissociation of the Rrn3-pol I complex is due to a change in Rrn3.

DISCUSSION

Cavanaugh et al. (28) and Bodem et al. (24) have reported that Rrn3 is inactivated when cells are treated with cycloheximide. Those reports were based on the observations that the addition of Rrn3 to in vitro transcription reactions containing inactive extracts from cycloheximide-treated cells would reconstitute transcription. However, those experiments did not directly demonstrate that Rrn3 was inactivated in the extracts. The possibility existed that the added Rrn3 was compensating for the lack of another factor. The observation that Rrn3 isolated from cycloheximide-treated cells was inactive established that Rrn3 activity was the “target” of cycloheximide. Also, this observation validated the use of cycloheximide-treated extracts as the source of RNA polymerase I, SLI, and UBF for some of the in vitro transcription reactions reported herein.
Three independent assays were used to examine the hypothesis that mammalian Rrn3 functions stoichiometrically rather than catalytically, i.e. Rrn3 becomes inactivated as a consequence of rDNA transcription. In our first experiments we found that Rrn3 becomes rate-limiting as the transcription proceeds. In our second set of experiments we found that once committed to a template that is transcribed, Rrn3 cannot subsequently become engaged on a second template. This result is in direct contrast with the results of Schnapp et al., (32) who reported “after initiation, TIF-IA is liberated from the initiation complex and facilitates transcription from templates bearing preinitiation complexes which lack TIF-IA.” This discrepancy could be explained if due to technical differences, Schnapp et al. (32) were observing the reactivation, and hence recycling, of Rrn3. It is also formally possible that the inactivation we observed was due to a nonspecific reaction or to an artifact of the reaction conditions.

Thus, we examined this problem using a third approach that would allow us to directly assess the possibility that Rrn3 was becoming inactivated. Rrn3 was repurified from transcription reactions, and its ability to support transcription in a subsequent reaction was assessed. These assays demonstrated that Rrn3 was inactivated as a consequence of transcription. Controls such as the omission of NTPs and/or template from the first incubation, as well as Western blots of the purified Rrn3, demonstrated that the loss of Rrn3 activity was not due to the incubation alone or to nonspecific degradation but was a consequence of rDNA transcription.

These observations were consistent with our hypothesis that Rrn3 and not RNA polymerase I was inactivated during mammalian rDNA transcription. However, this did not preclude the possibility that the mammalian Rrn3-pol I complex would dissociate as a consequence of transcription as has been reported for yeast Rrn3-RNA polymerase I. Rather, our model suggests that if the complex were to dissociate it would be due to a change in the activity of Rrn3.

Subsequently, we found that Rrn3 did dissociate from RNA polymerase I as a consequence of transcription, as predicted by the studies of Milkereit and Teschchner (22) and Aprikian et al. (31) on yeast rDNA transcription. However, we found that the ability of Rrn3 to interact with RNA polymerase I was significantly reduced as a consequence of transcription. Because our analyses demonstrated that the dissociation of the Rrn3-pol I complex and the loss of the ability of Rrn3 to interact with rpa43 was not due to a nonspecific activity or degradation, it is likely that the loss of Rrn3 activity is the result of a transcription-dependent, post-translational modification.

This hypothesis agrees with our model (28) in which following transcription initiation, Rrn3 becomes dephosphorylated (deactivated) and loses its ability to bind to RNA pol I. In this model, the modification of Rrn3 would be a major regulatory pathway in rRNA synthesis. The question about the presence and role of modification of pol I in the mammalian rDNA transcription system remains open. Milkereit and Teschchner (22), who proposed 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,” also observed that in yeast, in contrast to mammalian systems, the presence of non-associated cellular Rrn3 and free pol I is not sufficient for de novo rDNA transcription. The authors suggested several possible explanations for these apparently different mechanisms of regulation in yeast and mammals. 1) They proposed that the yeast and mammalian Rrn3 homologues might play different roles in transcription. 2) They suggested that the yeast in vitro transcription system might lack an activity necessary to form the Rrn3-pol I complex. 3) They suggested that the fractions used in the reconstituted mammalian transcription systems might be cross-contaminated.

It would be beyond the scope of this manuscript to draw any conclusions on the presence or absence of an activity in the yeast extracts. However, with the exception of the possibility that Rrn3 is regulated differently in yeast and mammals, there do not seem to be other possible explanations for the differences between the results of the studies on Rrn3 in the yeast mammalian and yeast rDNA transcription systems. It is unlikely that the yeast and mammalian Rrn3 homologues play different roles in transcription. Miller et al. (29) and vanavaugh et al. (28) have demonstrated that mammalian Rrn3 interacts with SL1, the mammalian homologue of yeast core factor. Moreover, the use of recombinant Rrn3 by Bodem et al. (24), and in these experiments, significantly reduces the possibility of cross-contamination.

Our data indicate that yeast and mammals regulate the initiation of rDNA transcription differently. Fath et al. (30) demonstrated that non-phosphorylated Rrn3 was able to form a transcriptionally competent RNA polymerase I-Rrn3 complex in yeast. This contrasts with our previous report (28) that dephosphorylated Rrn3 cannot neither activate transcription nor bind to RNA polymerase I. On the other hand, they demonstrated that the phosphorylation state of yeast RNA polymerase I is the determining factor in the formation of the RNA pol I-Rrn3. Although our experiments are not consistent with a controlling role for RNA polymerase I, they do not eliminate the possibility that the phosphorylation status of RNA polymerase I contributes to the formation of the Rrn3-RNA polymerase I complex.

Our data also fit a model presented recently by Dundr et al. (11). The authors analyzed the kinetics of assembly and elongation of the mammalian RNA polymerase I using in vivo microscopy. Their observations suggest that the RNA pol I initiation complex is assembled on the rDNA promoter from individual components via metastable intermediates. In addition, those authors observed a rapid exchange of each protein between the nucleoplasm and the nucleolus, suggesting that components of the RNA polymerase initiation complex are not necessarily recycled from transcription reaction to transcription reaction. Although the experiments presented in this manuscript do not address some of the questions raised by Dundr et al. (11), their observations are consistent with a model in which, some, if not all, of the components of the rDNA transcription apparatus can act stoichiometrically.

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