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Mammalian Rrn3 Is Required for the Formation of a Transcription Competent Preinitiation Complex Containing RNA Polymerase I

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Mammalian Rrn3, an essential, polymerase-associated protein, is inactivated when cells are treated with cycloheximide, resulting in the inhibition of transcription by RNA polymerase I. Although Rrn3 is essential for transcription, its function in rDNA transcription has not been determined. For example, it is unclear whether Rrn3 is required for initiation or elongation by RNA polymerase I. Rrn3 has been shown to interact with the 43-kDa subunit of RNA polymerase I and with two of the subunits of SL1. In the current model for transcription, Rrn3 functions to recruit RNA polymerase I to the committed complex formed by SL1 and the rDNA promoter. To examine the question as to whether Rrn3 is required for the recruitment of RNA polymerase I to the template, we developed a novel assay similar to chromatin immunoprecipitation assays. We found that RNA polymerase I can be recruited to a template in the absence of active Rrn3. However, that complex will not initiate transcription, even after Rrn3 is added to the reaction. Interestingly, the complex that forms in the presence of active Rrn3 is biochemically distinguishable from that which forms in the absence of active Rrn3. For example, the functional complex is fivefold more resistant to heparin than that which forms in the absence of Rrn3. Our data demonstrate that Rrn3 must be present when the committed template complex is forming for transcription to occur.

Key words: Rrn3; RNA polymerase I; Transcription initiation

INTRODUCTION

Before RNA polymerase I (Pol I) can initiate specific and effective transcription, both the ribosomal DNA (rDNA) promoter and RNA Pol I have to be "transcription ready," and they must join together to form a functional initiation complex. The assembly of the intermediate and final complexes involved in this process requires a complex series of protein–DNA and protein–protein interactions. For example, the efficient and stable binding of the transcription factors to the rDNA promoter requires the coordinate binding of factors to the core and upstream promoter elements (9,36). These steps have been shown to be the targets of several different mechanisms of regulation including posttranslational modifications and the actions of antioncogenes (7,9). Thus, there is a significant body of evidence suggesting that the regulation of transcription initiation or the formation of the preinitiation complex is a major site of regulation of rDNA transcription. In addition, there is increasing evidence for postinitiation regulation of rDNA transcription (45).

Eukaryotic rDNA promoters contain a core promoter element and an upstream promoter element [for review, see (9,36)]. Two multisubunit complexes are required to commit the yeast rDNA promoter.

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Core factor (CF) and upstream activating factor (UAF), which bind to the core promoter and to the upstream element, respectively (22,23), interact specifically with TATA-binding protein (TBP) (27,46). In mammals, two known transcriptional factors are required for efficient rDNA transcription. The binding of selectivity factor (SL1) containing TBP and TBP-associated factors (TAFs) to the core promoter element is necessary and sufficient in vitro (3,11,12,26, 55). The binding of upstream binding factor (UBF) (2,20), a multiple HMG box containing architectural protein, and, possibly, a second molecule of SL1, to the upstream promoter element are required for efficient transcription in vitro. Interestingly, while the core promoter is sufficient for transcription, deletion mutants that only contain the core promoter are less than 20% as efficient as the intact promoter in the formation of a stable preinitiation complex when analyzed in competition assays against the intact promoter (5). Both SL1 and UBF are subject to regulation via phosphorylation and acetylation (14,16,17, 33,34,37,41,48) and modulatory protein–protein interactions (e.g., Rb and SV40 large T antigen). Rb, the protein product of the retinoblastoma susceptibility gene, interacts with UBF repressing Pol I transcription (7). In contrast, SV40 large T antigen activates Pol I transcription by interacting with SL1 (54).

Genetic experiments in yeast and biochemical experiments in yeast and mammalian cells demonstrate that both yeast Rrn3 and its mammalian homologue are essential for rDNA transcription [(9,31,36,51,53) and references therein]. The mammalian homologue of yeast Rrn3 has been identified as the previously described transcription initiation factor IA (TIF-IA) (4,31). The current model of transcription is based on data demonstrating that Rrn3 acts as a bridge between RNA Pol I and CF/SL1 bound to the committed rDNA promoter (8,19,30,38,47,52). A direct interaction between the 43-kDa subunit of Pol I (rpa43) and Rrn3 in the Rrn3–Pol I complex was confirmed (8, 30) as well as the direct interaction of human Rrn3 with the TAF_I110 and TAF_I68 subunits of the species-specific transcription factor SL1 (8,30,42). Despite this body of knowledge, there are both differences in the apparent mechanisms that regulate the Rrn3–Pol I interaction and significant controversies concerning the role that Rrn3 plays in transcription and the mechanism whereby RNA Pol I is recruited to the rDNA promoter.

Schnapp et al. reported that RNA Pol I complexes could be recruited to the template in the absence of active Rrn3/TIF-IA. Schnapp et al. also reported that the complexes formed in the absence of active Rrn3 were transcriptionally inactive, but that the same complexes could be activated by the addition of active Rrn3 (42). In contrast, Zomerdijk's lab (30) reported that antibodies to Rrn3 blocked recruitment of RNA Pol I to a committed template. Thus, one report would suggest that recruitment can occur independently of Rrn3/TIF-IA and the second one suggests that Rrn3/TIF-IA is required for recruitment. Interestingly, in their studies of the role of yeast Rrn3 in transcription, Aprikian et al. (1) reported results that would appear to agree, at least in part, with both reports. They demonstrated, using immobilized templates, that *S. cerevisiae* RNA Pol I could be recruited to a committed template in the absence of yRrn3. However, they also reported that the complex formed in the absence of Rrn3 was not competent and the subsequent addition of Rrn3 to this complex was insufficient to convert the incompetent complex to a competent complex.

As these differences would have significant implications with respect to the mechanism by which Rrn3 functions in transcription, we sought to determine 1) if mammalian RNA Pol I could be recruited in the absence of active Rrn3 and 2) if the complex that formed in the absence of Rrn3 could be converted to a transcriptionally active complex by the addition of active Rrn3. Our first experiments used immobilized templates. We found that complexes formed in the absence of active Rrn3 were not transcriptionally competent and could not be converted to competent complexes by the subsequent addition of Rrn3. Subsequently, we developed a modified ChiP assay to directly assess recruitment. Interestingly, in agreement with two previous reports, our results demonstrate that Pol I was recruited to a committed template in the absence of active Rrn3. However, the complexes that formed in the presence or absence of Rrn3 could be distinguished by their sensitivity to heparin and sarkosyl. In addition, the complexes that formed in the absence of active Rrn3 could not be chased from the template. These results indicate that the protein–DNA or protein–protein interactions that occur in the absence of Rrn3 are not the same as those that form in its presence. While these experiments do not preclude a role for Rrn3 in the process of initiation, they strongly suggest that Rrn3 is required for the formation of the competent preinitiation complex.

MATERIALS AND METHODS

Cell Culture

N1S1 cells or NISIC3 cells, which stably incorporate a FLAG tagged β subunit of RNA Pol I (15), were grown in RPMI supplemented with 5% horse serum and 1% fetal bovine serum. The preparation of S100 extracts from N1S1 cells has been previously

described (6). Where indicated, S100 extracts were prepared from NISIC3 cells treated with 100 μg/ml cycloheximide (CHX) (Sigma, St. Louis, MO) for 1 h. S100 extracts were dialyzed against C-20 (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA) in a Pierce (Rockford, IL) Slide-A-Lyzer mini dialysis unit overnight at 4°C before use to remove endogenous NTPs. Active, recombinant human FLAG tagged Rrn3 was expressed in Sf9 cells and purified as previously described (8).

DNA Templates

A 920-bp fragment of the rat 45S rDNA promoter was subcloned into the Bam*HI* and Eco*RI* sites of pUC 19 and used to generate rDNA templates for the immobilized template assays. Wild-type template was amplified with one of two primer pairs using a common 5′ primer (5′-GCTCACTCATTAGGCACC CCAGG-3′), based on pUC 19 sequences upstream of the rDNA insert. The reverse/downstream primers were either 5′-GGAAAACCCTTCCAGTCG-3′ or 5′-GTGCAACTCGGGAGGCACACAG-3′, which generate products of 680 or 857 bp, respectively, containing 90 bp of pUC, and fragments of the rat rDNA gene extending from −287 to either +303 or +480, respectively. pUC 19 was used as a nonspecific DNA template in some experiments. pUC template was amplified using forward 5′-CAGGGGATAACGCA GG-3′ and reverse 5′-GACGCCGGGCAAGAGCA AC-3′ primers, which generate a 1300-bp product. Primers were purchased from Integrated DNA Technologies (Coralville, IA). PCR products were purified using a Qiagen (Valencia, CA) MinElute PCR purification kit. Following elution from the Qiagen spin columns, the DNA was phenol extracted, ethanol precipitated, and resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA (TE).

Formation, Isolation, and Analysis of PIC Complexes

Five microliters of S100 (5–8 μg protein) was incubated with 60 ng PCR template (wild-type or pUC as indicated) for 20 min at room temperature in a total volume of 30 μl. Heparin or sarkosyl (Sigma) were added where indicated in a total volume of 20 μl and the incubation continued for 15 min at 30°C. Anti-FLAG M2 agarose beads (Sigma) were washed three times with C-20 and stored as a 50% slurry in the same buffer. Twenty microliters of the 50% slurry of anti-FLAG beads was added and the mixture was tumbled for 1 h at 4°C. The slurry was centrifuged at 2,000 rpm ($360 \times g$) for 20 s and the supernatant removed. The beads were washed three times with 100 μl of C-20. The bead pellet was resuspended in 50 μl of distilled water followed by the addition of 5 μl of 1 mg/ml proteinase K (Fisher). The mixture was incubated at 65°C for 15 min. The beads were centrifuged and the supernatant removed to a fresh tube. Nucleic acids were purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and the aqueous phase was then extracted with 10 μl of chloroform. The DNA from the entire reaction was amplified using 2 ng of the forward and reverse primers described above with 50 μl of Promega (Madison, WI) PCR Master mix. PCR was performed in a GeneMate Genius (ISC BioExpress, Kaysville, UT) under the following conditions: 94°C, 1 min (1 cycle); 95°C, 45 s, 55°C, 30 s, 72°C, 1 min (35 cycles); 72°C, 7 min (1 cycle). Twenty-five microliters of this reaction was further amplified in a second PCR reaction. The second reaction contained 24 ng of each primer and proceeded for 20 cycles. Control reactions demonstrated that these conditions ensure primer excess. Ten microliters from the second reaction was electrophoresed on a 1% agarose gel; DNA was visualized by ethidium bromide staining. Gels were scanned on an AlphaEaseFC Imaging System (Alpha Innotech Corp., San Leandro CA). Real-time PCR (RT-PCR) was performed with a Roche Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany). The forward primer was 5′-CCTGTCATGTTTATCCCTG-3′ and the reverse 5′-GGTGCAAGCCTCTTGGAACG-3′, which generates a 135-bp product. For RT-PCR experiments DNA and extract in a final volume of 30 μl were incubated for 10 min on ice, heparin was added at the concentrations indicated to a final volume of 50 μl, and the incubation continued for an additional 30 min at 30°C. Twenty microliters of the 50% slurry of anti-FLAG beads was added and the reaction tumbled at 4°C for 45 min. The supernatant was removed from the slurry, digested with proteinase K, and the DNA purified as described above. The first round of PCR was the same as for ethidium bromide stained gels. Two microliters of this reaction was further amplified in the second RT-PCR reaction using the Qiagen, QuantiTect SYBR Green PCR kit. PCR conditions were 95°C, 15 min (1 cycle); 95°C, 1 s, 55°C, 10 s, 72°C, 27 s (65 cycles).

In Vitro Transcription: Immobilized Template Assays

All templates were generated by PCR and immobilized on avidin-magnetic beads (Dynal Corp, Oslo, Norway) through a biotin incorporated in a common 5′ primer. The two templates were distinguished by the 3′ PCR primer resulting in transcription of either 480- or 303-nt transcripts. Binding of the biotinylated

template to the magnetic beads was performed as recommended by the manufacturer. After 60 µl of S100 was incubated with 260 ng of immobilized template for 45 min at room temperature, the beads were collected with the magnet. Beads were washed with C-20 and the incubations continued at 30°C followed in some experiments by a second wash with C-20. Recombinant Rrn3 was added to some incubation (300 ng) at the time points indicated. Transcription was initiated by the addition of NTPs ± [α-^{32}P]UTP as indicated. The products of transcription were analyzed by denaturing urea/PAGE as previously described (44). In vitro transcription reactions using templates in solution were performed using 0.1 µg p5.1E/X linearized with Eco*RI* as template per reaction as previously described (44). The reactions were supplemented with heparin or sarkosyl as indicated.

Western Blot Analysis

SDS-PAGE and electroblot analysis were performed as described previously (15). Polyclonal rabbit antisera to mouse rpa43 were raised to recombinant rpa43 expressed in *E. coli* (Capralogics, Inc., Hardwick, MA). Monoclonal anti-FLAG antibody (M2) was purchased from Sigma Chemical Co. (St. Louis, MO) and was used as previously described (15).

RESULTS

Rrn3 Is Required for Functional Recruitment

We have previously reported that cells that have been treated with CHX are not capable of supporting in vitro transcription by RNA Pol I due to the inactivation of Rrn3 (8). We extended this observation by in vitro transcription assays using biotinylated template immobilized on avidin-magnetic beads. Results of such an experiment are presented in Figure 1. As described in Materials and Methods, these experiments utilized two different PCR templates distinguishable by size. Template A yields a transcript of 480 nt while template B yields a transcript of 303 nt. The preincubation included the S100 plus immobilized template and recombinant Rrn3 where indicated. After the preincubation, some of the complexes are washed and incubated for an additional 15 min at 30°C in the presence or absence of nucleotide triphosphates (NTPs). Following a second wash, the complexes were combined where indicated and NTPs including [^{32}P]UTP were added and the incubation continued for an additional 10 min to allow for transcription. Figure 1A is a schematic of the transcription reactions. As previously reported, extracts from CHX-treated cells cannot support transcription (Fig, 1A, lanes 1 and 2). The results in Figure 1A (lane 4) indicate that transcription only occurs when Rrn3 is included in the preincubation (compare lanes 3 and 4). Figure 1B confirms these results in that once again transcription only occurs when Rrn3 is included in the preincubation phase of transcription (Fig. 1B, lane 8). Note the absence of transcripts from template B in Figure 1B (lanes 6 and 7). In some of the reactions the capacity of preformed complexes to support a second round of transcription after a first round was investigated. Transcription was only observed for template B (Fig, 1C, lane 12) that had been incubated in the absence of NTPs prior to the addition of [^{32}P]UTP and cold NTPs. In contrast, the complex that formed on template A, which had undergone transcription, did not transcribe in the second transcription reaction (Fig, 1C, lane 12). Similarly, transcripts were not produced in the reactions depicted in Figure 1C (lanes 9–11). The presence of the transcript in Figure 1C (lane 12) also indicates that the transcriptionally competent complex dissociates after transcription (compare lanes 9–12). The results presented in Figure 1C confirm the observation that Rrn3 must be present for the preinititation complex to form correctly. The results presented in Figure 1A and B are consistent with the model that Rrn3 functions stoichiometrically in transcription, as we have previously reported (18).

RNA Polymerase I Recruited to a Template Can Be Immunoprecipitated Along With That Template

Transcription is the most direct way to determine if an extract or combinations of transcription factors and RNA polymerase have joined together to form a competent preinitiation complex. However, while transcription demonstrates that recruitment has occurred, it is not a direct measure of recruitment. Polymerase might have been recruited, but was unable to initiate or elongate. By themselves, the results presented in Figure 1 do not demonstrate that Rrn3 is required for recruitment, only that Rrn3 must be present when the preinitiation complex forms.

To further examine the nature of the initiation complex, we developed a novel assay to determine the ability of both control and CHX-treated extracts to bind to the rat ribosomal DNA promoter. The chromatin immunoprecipitation (ChIP) assay has become a commonly used tool to demonstrate that transcription factors occupy their cognate sites in vivo. We sought to modify that assay to determine if it could be used to demonstrate recruitment of RNA Pol I to a promoter. This assay combines immunoprecipitation

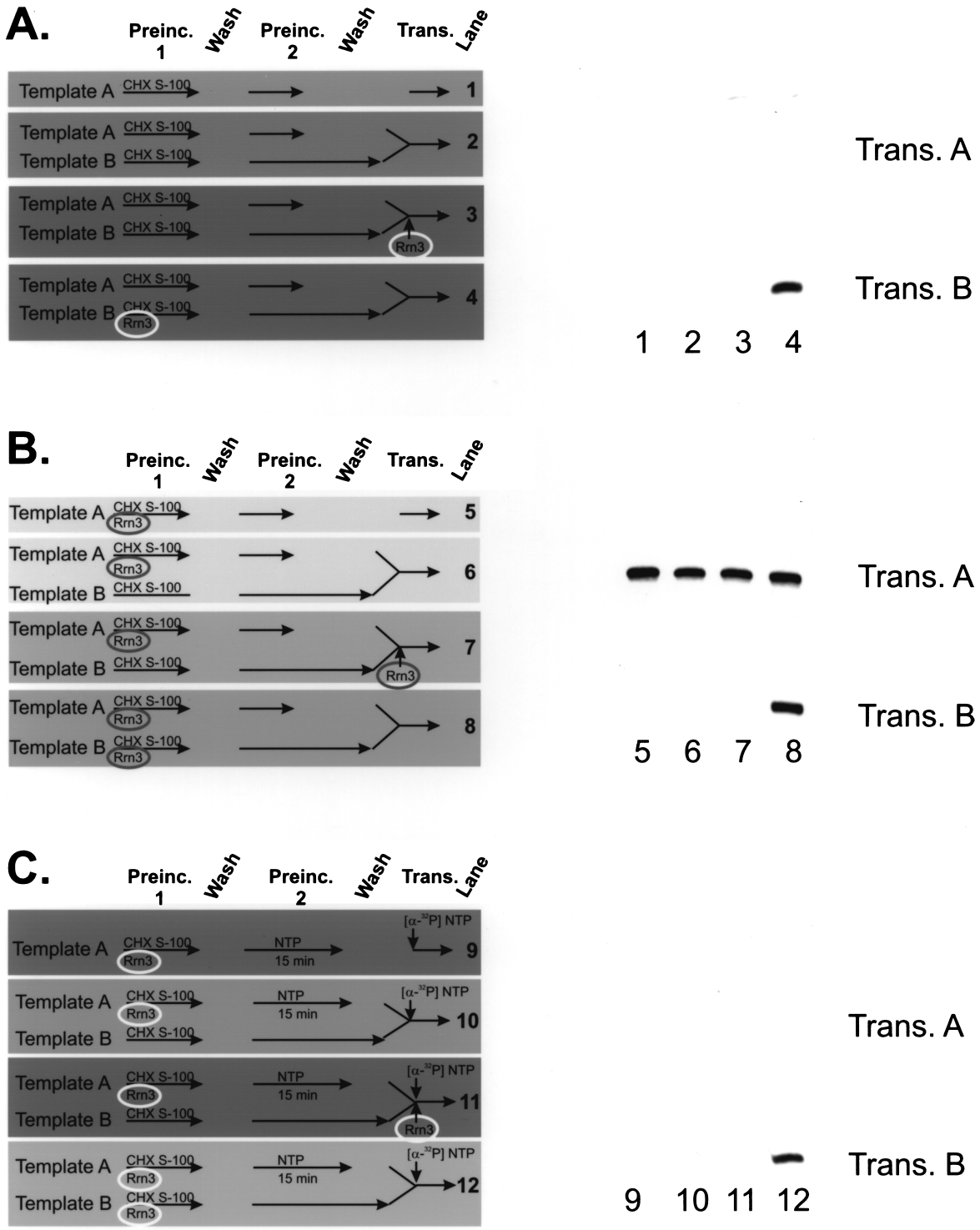

Figure 1. Rrn3 is required for "productive" recruitment. (A, B) In each reaction, the template indicated (immobilized on magnetic beads) was preincubated with an S100 extract from cells treated with cycloheximide (CHX S100) in the absence or presence of recombinant Rrn3 as indicated. Subsequently, the templates were washed in transcription buffer (gap in the horizontal line), resuspended, and incubated for an additional 15 min at 30°C (Preincubation 2). Following the second wash, the templates were again resuspended and combined as indicated. NTPs including [α-^{32}P]UTP were then added, and transcription was allowed to proceed for 10 min. Where indicated, Rrn3 was added at the beginning of the transcription reaction. The transcripts were then analyzed by denaturing urea-PAGE and autoradiography. (C) A transcription reaction was carried out after the first wash in the presence of unlabeled NTPs. The templates were then washed and pooled, and transcription was allowed to proceed in the presence of NTPs including [α-^{32}P]UTP. All templates were generated by PCR and immobilized on avidin-magnetic beads through a biotin incorporated in a common 5′ primer and were distinguished by the 3′ PCR primer, resulting in transcripts (Trans.) of either 480 (Template A) or 303 nt (Template B).

with PCR (i.e., the DNA that is bound to the protein of interest is isolated by immunoprecipitation and then detected by PCR). As we have previously reported (15), NISIC3 cells stably express the FLAG-tagged β subunit of RNA Pol I, enabling us to specifically immunoprecipate RNA Pol I with immobilized anti-FLAG antibodies. Our first experiments were designed to determine if we could demonstrate the coimmunoprecipitation of RNA Pol I with its promoter and to examine the specificity of the interaction. S100 extracts were incubated with a PCR template containing the rat ribosomal DNA promoter and immunoprecipated with anti-FLAG beads. The beads were washed and incubated with proteinase K to liberate the DNA bound to the beads. After phenol extraction, the released DNA was amplified with primers specific for the Pol I promoter. Figure 2A illustrates the steps in the assay. S100 extracts prepared from both N1S1C3 cells and N1S1 cells, the parental cell line that does not express FLAG-tagged RNA Pol I, were incubated with the DNA fragment containing the rDNA promoter. Immobilized anti-FLAG antibodies were added and the DNA bound to the immunoprecipitated RNA Pol I was purified and amplified with primers specific for the respective fragment as described in Materials and Methods. The PCR products derived from the immunoprecipitated DNA were analyzed by agarose gel electrophoresis (Fig. 2B). As shown in Figure 2B (lane 2), the rDNA promoter was found in immunoprecipitates obtained with extracts containing the FLAG-tagged Pol I, but it was not found in immunoprecipitates obtained with extracts that did not express FLAG-Pol I (lane 3).

To control for nonspecific binding, a similar experiment was carried out using either a PCR fragment of pUC 19 or the rDNA PCR fragment. The DNA bound to the immunoprecipitated RNA Pol I was purified, amplified with primers specific for the respective fragment, and analyzed as described above. The results shown in Figure 2C demonstrate the specificity of the assay (lanes 3 and 4). When extract was incubated with the PCR fragment containing the rDNA promoter, that fragment was found in the immunoprecipitate. In contrast, when the assay contained the nonspecific PCR fragment of pUC, that fragment was not found in the immunoprecipitate (Fig. 2C, lane 4). The results of an additional experiment to demonstrate the specificity of the assay are shown in Figure 2D. In this experiment both the rDNA and pUC DNA fragments were incubated with the S100 extract prepared from N1S1C3 cells. After immunoprecipatation and DNA purification, primers for both the rDNA promoter and pUC template were added to the PCR reaction and immunoprecipated DNAs were amplified simultaneously. As demonstrated by the results shown in Fig. 2D (lane 2), only the rDNA promoter was amplified, once again illustrating the specificity of the assay.

Extracts From CHX-Treated Cells Form a Complex on the rDNA Template

We next determined if extracts prepared from CHX-treated cells demonstrated the same ability to bind the rDNA promoter as control extracts. In the experiment shown in Figure 3, the rDNA fragment was incubated with either extracts from control N1S1C3 cells (lanes 1–4) or with extracts from CHX-treated N1S1C3 cells (lanes 7–10). As shown in Figure 1, the extracts from CHX-treated cells did not support specific transcription. However, the extract from CHX-treated cells was capable of forming a complex with the rDNA promoter (Fig. 3, lane 7) in the same manner as the control extract (lane 1). This binding was specific; when pUC DNA was included in the assay, it was not found in the immunoprecipitate (Fig. 3, lanes 8 and 10). Rrn3 was also added to parallel incubations (Fig 3, lanes 3, 4, 9, and 10), but it had no effect on the formation of complex. As Pol I–DNA complexes were found in the absence of added Rrn3, it was not surprising that the addition of Rrn3 had no significant effect on the DNA binding ability of the FLAG-tagged Pol I in either the control or CHX treated extracts.

Active Rrn3 Is Required for Polymerase to Dissociate From the Template Following Transcription

The above experiments indicated that transcription required that Rrn3 be added at the same time as the preinitiation complexes were formed, but that RNA Pol I could be recruited in the absence of active Rrn3. This suggested that the complexes formed in the absence of active Rrn3 would not release from the promoter and elongate. In order to examine this possibility, we next determined if Pol I would dissociate from the template following transcription.

When control extracts were incubated with the rDNA promoter, they formed a preinitiation complex that was stable following incubation at 30°C as demonstrated by the PCR product found in Figure 4A (lane 1). The DNA–Pol I complex was also detected when NTPs were added, but the reaction maintained at 4°C to prevent transcription (lane 2). However, this observation was inconclusive as a Pol I–DNA complex was also found following transcription (Fig. 4A, lane 4). This led us to consider the possibility that this same observation could result from reinitiation. As these were not immobilized templates we could not wash away excess RNA Pol I. To reduce the contri-

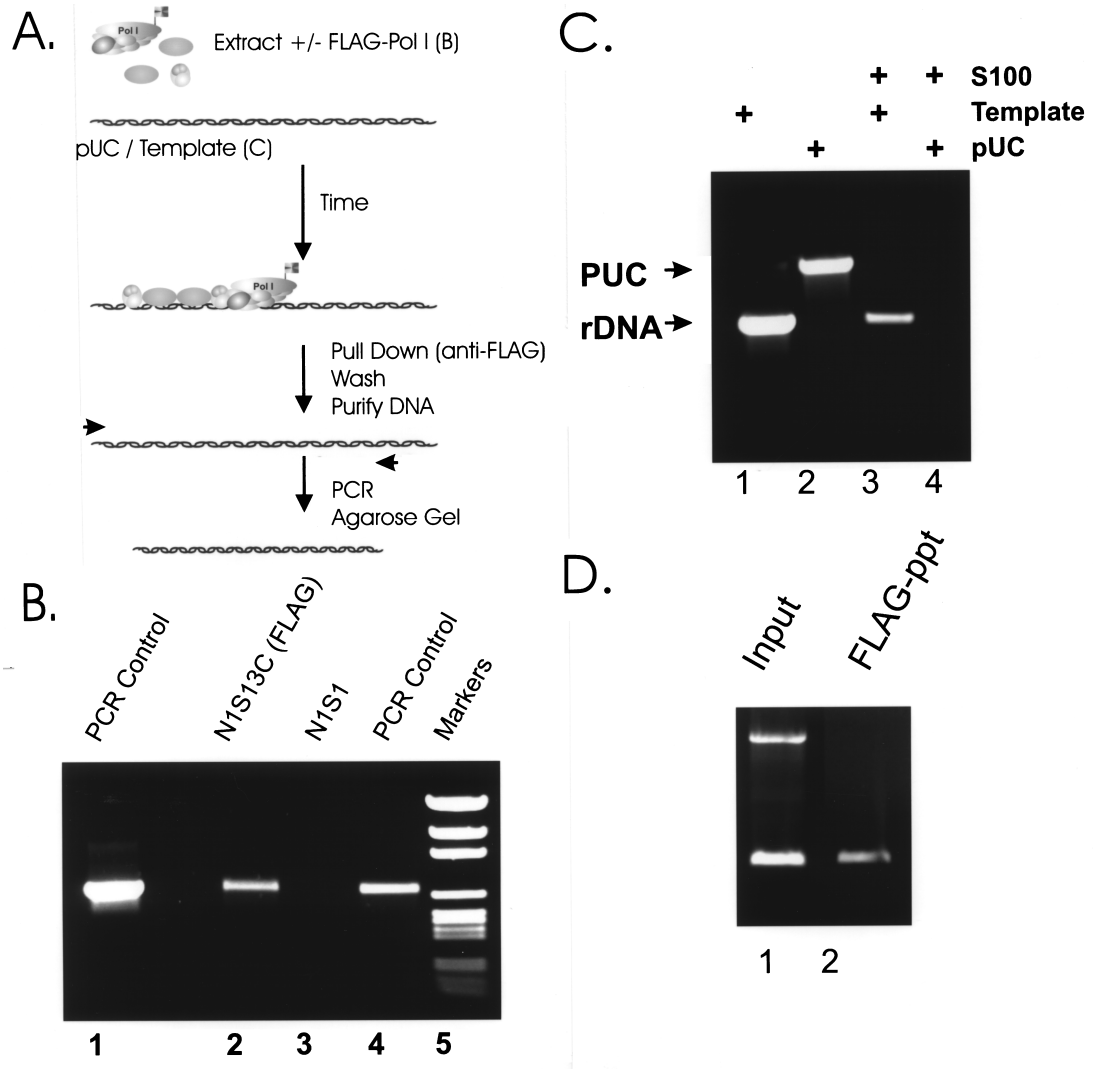

Figure 2. Coimmunoprecipation of rDNA with RNA Pol I using anti-FLAG antibody. (A) Schematic illustrating the steps in the PCR assay to detect protein–DNA interactions. (B) Five microliters of S100 from N1S1C3 (lane 2) or NISI cells (lane 3) was incubated with 60 ng of template containing the rDNA promoter. C-20 buffer was added to bring the volume to 30 µl. The incubation was carried out at room temperature for 20 min. Twenty microliters of a 50% suspension of anti-FLAG agarose beads was added and the mixture tumbled at 4°C for 1 h. The beads were washed and bound DNA isolated as described in Materials and Methods. DNA was amplified and electrophoresed on a 1% agarose gel and stained with ethidium bromide. Lane 1 depicts a positive control for the first PCR reaction, and lane 4 a positive control for the second PCR reaction. Lane 5 contains pGEM (Promega) molecular weight markers. (C) S100 extracts from N1S1C3 cells were incubated with either 60 ng of wild-type (lane 3) or pUC 19 DNA (lane 4) as described in (B). After washing the beads, DNA was isolated and amplified with primers specific for either the rDNA template or pUC 19 template. Lane 1 contains a positive control PCR reaction for the wild-type template and lane 2 contains a positive control PCR reaction for the pUC template. (D) S100 extracts from N1S1C3 cells were incubated with 60 ng of both the rDNA and pUC 19 templates as indicated in (B). The DNA immunoprecipatated from the reaction was amplified with primers for both the rDNA and pUC 19 templates. Lane 1 indicates the template input and lane 2 indicates the DNA amplified from this reaction. Primers are described in Materials and Methods. PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Wild-type template amplifies as a 690-bp fragment and pUC as a 1300-bp fragment.

bution of reinitiation to these assays, we added heparin to the assay. Heparin has been demonstrated to inhibit reinitiation, but not elongation, at low concentrations (29). [Heparin can also be used to destabilize preinitiation complexes at higher concentrations (10, 21)]. Figure 4B demonstrates that at 8 and 40 µg/ml we do in fact observe single round transcription when heparin is added at the same time as NTPs to a transcription assay (compare lanes 2 and 3 with lane 1). The addition of heparin to extract prior to the addition of template blocked the formation of the preinitiation complex (data not shown), consistent with what has been reported previously (10). However, the addition of 8 µg/ml heparin, following the preincubation of template and extract, and its presence during a subsequent incubation at either 4°C or 30°C, did

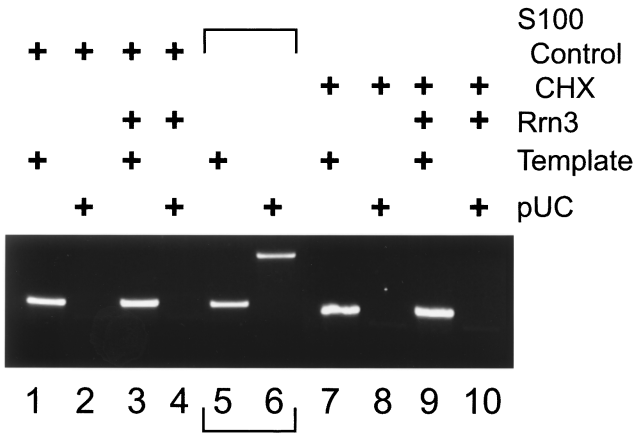

Figure 3. RNA Pol I from inactive extracts interacts with rDNA. S100 extracts from control N1S1C3 cells (lanes 1–4) or CHX-treated cells (lanes 7–10) were incubated with either wild-type template or pUC 19 template for 20 min at room temperature. Samples were processed and DNA amplified as described in Materials and Methods and the legend to Figure 2. Rrn3 (300 ng) was added as indicated (lanes 4 and 9). The products shown in lanes 5 and 6 are positive PCR controls for the wild-type and pUC templates, respectively.

not destabilize the complex (Fig. 4A, lanes 3 and 5) formed by extracts from control cells. However, if NTPs were added to initiate transcription and heparin (8 μg/ml) was added to prevent reinitiation, the template no longer coimmunoprecipitated with RNA Pol I (Fig. 4A, lane 6) (i.e., RNA Pol I had dissociated from the DNA in agreement with the model that this would result from transcription as suggested by the experiments presented in Fig. 1C).

Our results indicate that Rrn3 is required for transcription but not for recruitment. If this model is correct, then RNA Pol I from CHX-treated extracts that has been "recruited" to the template should not be "chased" from the template DNA under conditions that permit transcription. The results shown in Figure 4C confirm this model. As shown in Figure 4C (lane 6), the addition of heparin and nucleotide triphosphates to the complexes formed with a CHX-treated extract had little effect on the recovery of DNA in the anti-FLAG immunoprecipitates (compare lanes 5 and 6). This is in contrast to what we demonstrated for transcriptionally active complexes (Fig. 4A, lanes 5 and 6, Fig. 4C, lanes 2 and 3). These results indicate that although Pol I from the CHX extracts can bind to the rDNA promoter, this Pol I–DNA complex does not change under conditions that would allow transcription.

Extracts From Control and CHX-Treated Cells Differ in Their Sensitivity to Heparin

Upon examination of the data presented in Figure 4C (lanes 5 and 6), it is apparent that the addition of heparin resulted in a decreased amount of Pol I–DNA complex. This led us to examine the possibility that the preinitiation complexes formed in the absence of active Rrn3 would demonstrate a different dose–response profile than those that formed in the absence of active Rrn3. The results of such an experiment are shown in Figure 5A. Lanes 2–5 demonstrate the heparin sensitivity of complexes formed in the presence of active Rrn3 while lanes 6–9 demonstrate the sensitivity of complexes formed in the absence of active Rrn3. Comparison of the data in Figure 5A, lanes 2–5, with that of lanes 6–9 indicates that the preinitiation complexes formed in the absence of active Rrn3 are more sensitive to heparin that the preinitiation complexes formed in the presence of active Rrn3. Although the extracts from CHX-treated cells formed a complex that could be observed after 20 min in the presence of 8 μg/ml heparin (Fig. 5A, lane 8), this complex was more susceptible to disruption by heparin than were the complexes formed by control extracts (compare Fig. 5A, lane 4 with lane 8). When CHX extracts were supplemented with active Rrn3, their response to heparin more closely resembled that of the control extract (compare the signals shown in Fig. 5B, lanes 3 and 7, with those presented in Fig. 5A, lanes 4 and 5). (The second round of PCR used in the analysis of the coimmunoprecipitated DNA for the experiments presented in Fig. 5A and B was reduced from 20 cycles to 15 to increase the sensitivity of the assay.) Quantitative real-time PCR (RT-PCR) analysis was carried out in order to quantitate the differences seen in heparin sensitivity of the complexes formed by the control and CHX extracts. Fluorescence curves from both control and CHX-treated extracts incubated with and without heparin are shown in Figure 5C. In these experiments, the LightCycler assay used target-specific hybridization probes to allow flu-

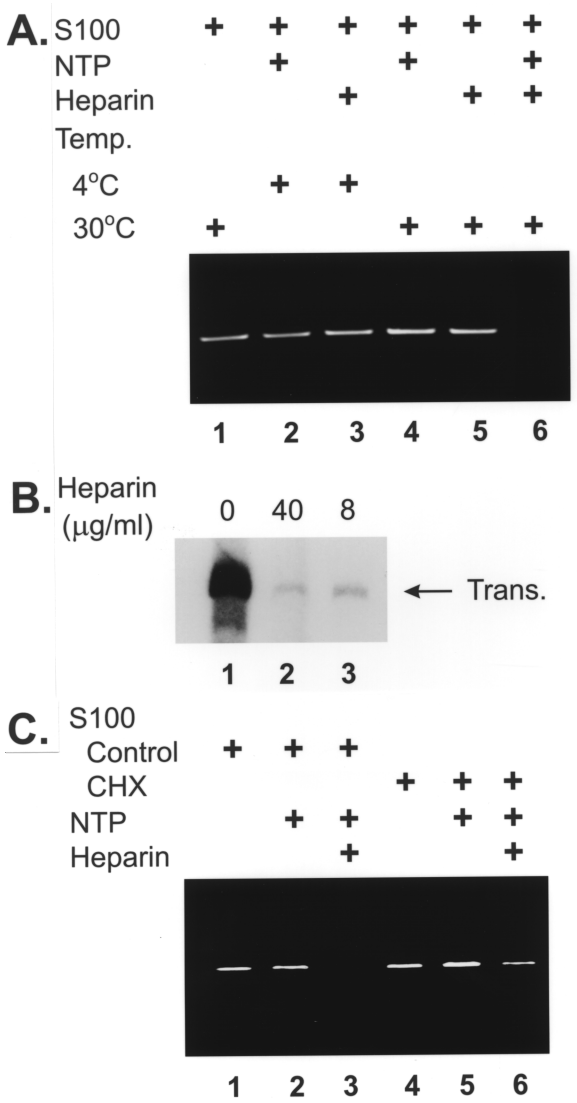

Figure 4. Transcription causes RNA Pol I to dissociate from the template. (A) S100 extracts from N1S1C3 control cells were incubated with 60 ng of wild-type template for 20 min at room temperature. NTPs (lanes 2 and 4) or NTPs plus 8 μg/ml of heparin were added (lane 6) and the reaction continued for 15 min at either 4°C ((lanes 2 and 3) or 30°C (lanes 1, 4, 5, and 6). Samples were processed and DNA amplified as described in Materials and Methods and the legend to Figure 2. (B) In vitro transcription of S100 extracts from control N1S1C3 cells in the absence (lane 1) or presence of heparin (lanes 2 and 3). Transcription conditions have been previously reported and the products analyzed by urea/PAGE and autoradiography (19). In reactions containing heparin, the NTP mixture contained unlabeled ATP, GTP, and CTP with 1 μCi of [^{32}P]UTP. (C) S100 extracts from either control N1S1C3 cells (lanes 1–3) or CHX-treated cells (lanes 4–6) were incubated with wild-type template to allow formation of the initiation complex. NTPs were added (lanes 2 and 5) or NTPs plus 8 μg/ml of heparin (lanes 3 and 6) and the reaction incubated at 30°C for 15 min. Samples were processed and the DNA amplified as described in Materials and Methods and the legend to Figure 2.

orescence resonance energy transfer-based detection of amplicons. Results, expressed as crossover points, when the log-linear part of the amplification curve crosses a fluorescence background threshold, indicate the PCR cycle number at which the amplification enters the log-linear phase (49,50). The crossover point is proportional to the initial DNA concentration of the sample and can be used as a measure of extraction efficiency (39). In addition, a melting curve for the PCR amplicon can be generated (40). Thus, the crossover value is proportional to the initial concentration of DNA in the sample (39) (i.e., the higher the crossover value the less product). A graph of the fractional change in the crossover values for control and CHX-treated extracts incubated with and without heparin, at a limited number of heparin concentrations, is presented in Figure 5D. A higher fractional change indicates less product. It should be noted that the strength of the PCR product from control extracts at 40 μg/ml heparin (Fig. 5A, lane 3) was approximately equal to that of CHX-treated extracts at 8 μg/ml heparin (Fig. 5A, lane 8.) This would suggest that the complex formed in the absence of active Rrn3 was fivefold more sensitive to the effects of heparin. Interestingly, the graph of the fractional decrease in crossover value obtained in the quantitative RT-PCR assay also demonstrated a fivefold difference (Fig. 5D). That is, the crossover value for the control extract at 8 μg/ml heparin was the same as for the CHX extract at 1.6 μg/ml heparin. A graph of the fractional change in crossover value when active Rrn3 was added to the CHX-treated extract is shown in Figure 5E. Once again, the same fivefold change in heparin sensitivity was reproduced (compare the fractional change in crossover value at 0.8 μg/ml heparin in extracts without active Rrn3 to that of 4 μg/ml heparin when these extracts are supplemented with active Rrn3). Thus, the RT-PCR experiments confirm the results observed with the ethidium bromide-stained gels. If Rrn3 was added after the complex was formed, we saw no change in the heparin sensitivity of the complex (data not shown).

The Preinitiation Complex Formed by Extracts From Control Cells Is Sensitive to Low Concentrations of Sarkosyl

Several laboratories have used sarkosyl to distinguish between the various stages/complexes associated with transcription initiation (10,21). It has been noted that the formation of preinitiation complexes by RNA Pol I is inhibited by a lower concentration of sarkosyl than that which will inhibit elongation (13). For example, Kato et al. reported that preinitiation complex formation was sensitive to 0.015–0.02%

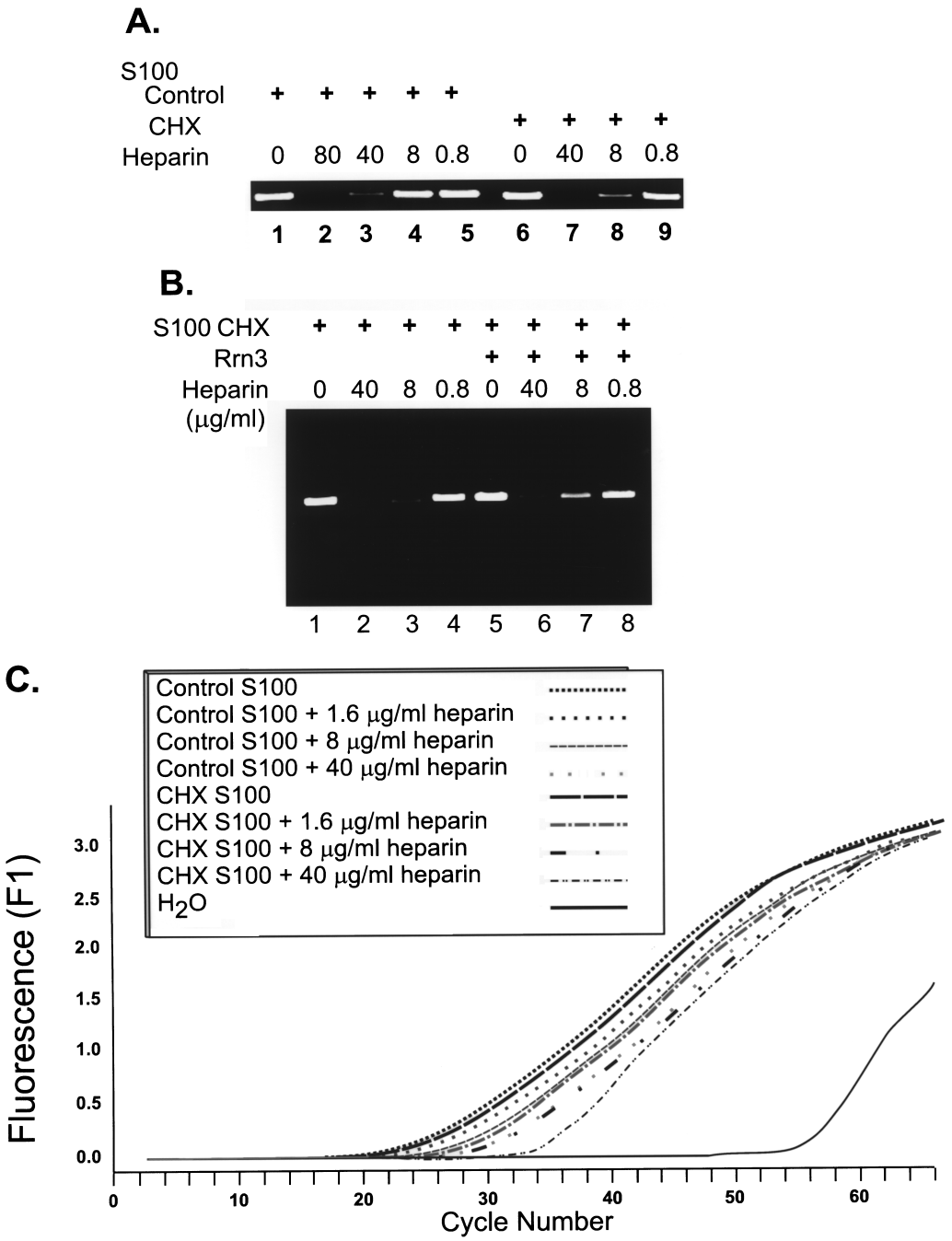

Figure 5. Preinitiation complexes formed in the presence or absence of Rrn3 differ in their sensitivity to heparin. (A) S100 extracts from either control (lanes 1–5) or CHX-treated (lanes 6–9) N1S1C3 cells were incubated with 60 ng of wild-type template for 15 min at room temperature. Heparin was added at the concentration indicated in the total volume of 20 μl and the incubation continued for an additional 15 min at 30°C. Samples were processed and DNA amplified as described in Materials and Methods and the legend to Figure 2. (B) S100 extracts from CHX-treated N1S1C3 cells, some of which had been preincubated with 300 ng Rrn3 for 20 min (lanes 5–8), were incubated with wild-type template (lanes 1–8) for 20 min at room temperature. Heparin or buffer was then added to the final concentrations indicated in a final volume of 20 μl and the incubation continued for 15 min at 30°C. Samples were processed and DNA amplified as described in Materials and Methods and the legend to Figure 2. (C) RT-PCR fluorescence curves for S100 extracts from control or CHX-treated N1S1C3 cells incubated in the presence or absence of varying amounts of heparin. The experiment was carried out as indicated in (A). Conditions for RT-PCR are as stated in Materials and Methods. The figure shows a representative experiment. Crossover values for a typical experiment were control, 27.68; control plus 1.6 μg/ml heparin, 29.14; CHX plus 1.6 μg/ml heparin, 30.46; control plus 8 μl/ml heparin, 30.75. (D) Graphic representation of fractional change in crossover value of S100 extracts from control and CHX-treated N1S1C3 cells incubated with or without heparin as indicated. The fractional change in crossover values was calculated relative to control S100 value. Values represent the average of three independent experiments ± SD. (E) S100 extracts from CHX-treated N1S1C3 were incubated with heparin or heparin and Rrn3 as described in (B). The fractional change in crossover values was calculated relative to the value with no heparin. Values represent the average of four independent experiments ± SD.

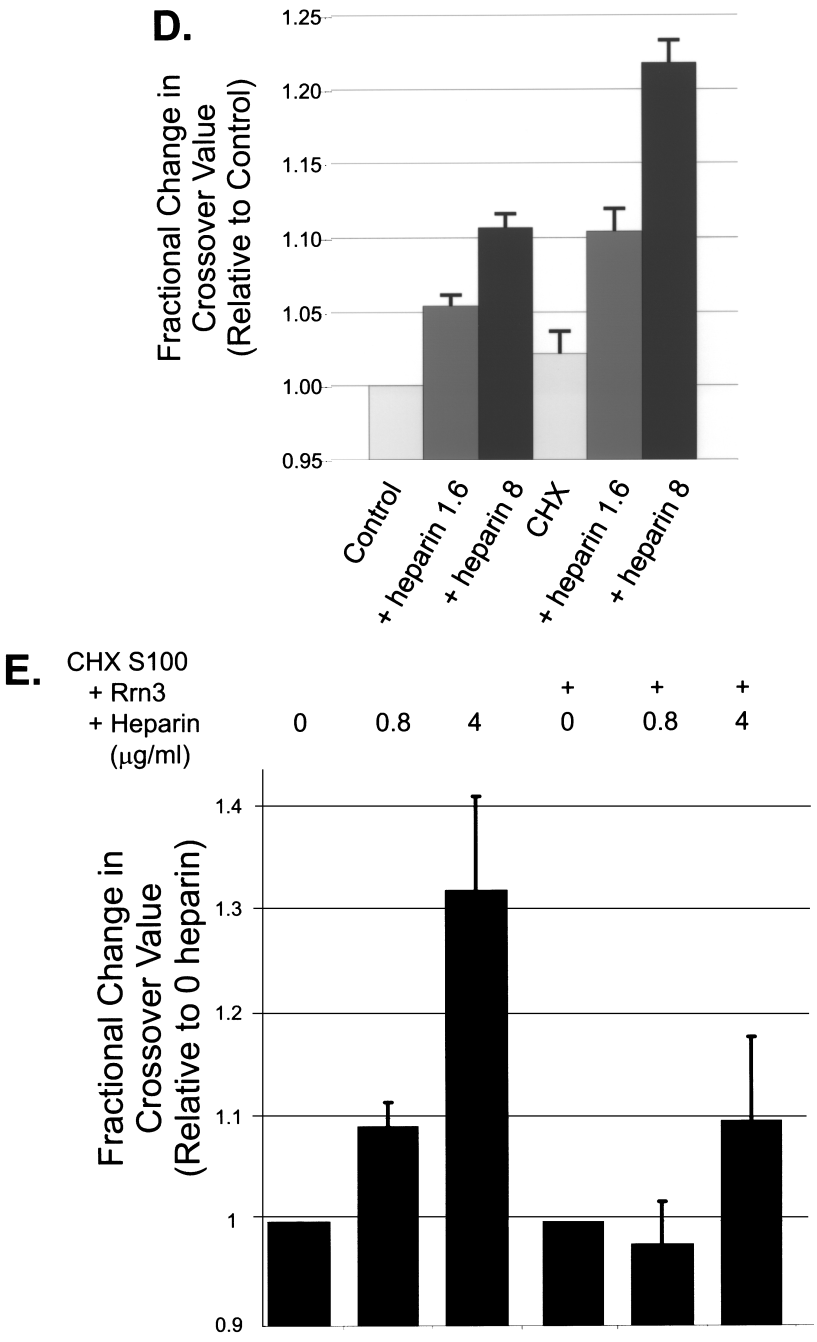

Figure 5. Continued.

sarkosyl (21). As predicted by their observations, we found that if sarkosyl (0.025%) is added to an S100 from control extracts before the addition of template, no initiation complex is formed (Fig. 6, lane 1). However, when we carried out the same experiment using extracts from CHX-treated cells, we found that the extract (or at least the RNA Pol I in that extract) formed a sarkosyl-resistant complex with the DNA (Fig. 6, lanes 3 and 5). Interestingly, the sarkosyl-resistant extracts from CHX-treated cells became sensitive to sarkosyl if active Rrn3 was added to the extracts (lanes 4, 5, and 6). The results presented in Figure 6 (lanes 5 and 6) indicate that this conversion occurred in a dose-dependent manner.

Further Evidence That Different RNA–Polymerase I Complexes Form in the Presence and Absence of Active Rrn3

The above experiments demonstrated that although RNA Pol I could be recruited to the DNA in the ab-

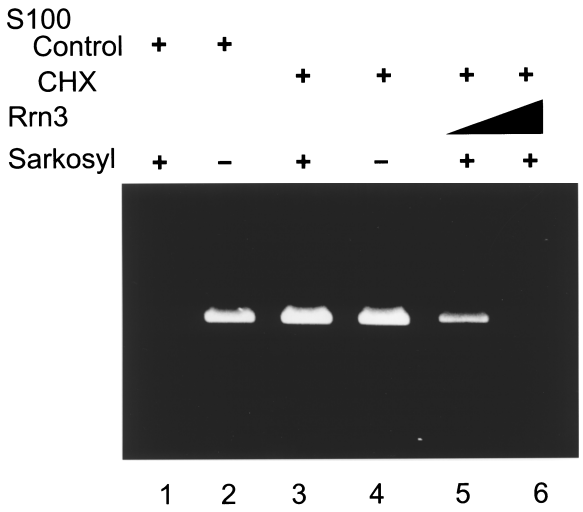

Figure 6. Preinitiation complexes, formed in the presence or absence of Rrn3, differ in their sensitivity to sarkosyl. Reactions containing S100 extracts from control (lanes 1 and 2) or CHX-treated cells (lanes 3–6) were supplemented with sarkosyl (as indicated) to a final concentration of 0.025%. As indicated, the S100 extracts from CHX-treated N1S1C3 cells were preincubated with either 150 ng (lane 5) or 300 ng of Rrn3 (lane 6) at room temperature for 20 min. Sixty nanograms of wild-type template was then added to the reaction and the incubation continued for an additional 20 min at room temperature. Samples were processed and DNA amplified as described in Materials and Methods and the legend to Figure 2.

sence of active Rrn3, the Pol I–DNA complex that formed was different than that formed in the presence of active Rrn3. We, and others, have reported that Rrn3 can interact with RNA Pol I and SL1 independently (8,30). This raised the interesting possibility that Rrn3 might be recruited to the template, via its interaction with SL1, independent of its association with RNA Pol I. To address this question, extracts from CHX- treated cells were incubated with purified, recombinant FLAG-Rrn3 and/or template in different temporal sequences as illustrated in the schematic in Figure 7A. The resulting complexes were then immunoprecipitated with anti-FLAG antibodies and analyzed for the presence of RNA Pol I by Western blots for rpa43 (Fig. 7B), a subunit of RNA Pol I, and a separate PCR assay for the presence of template (Fig. 7C; in these experiments Rrn3 is FLAG-tagged, not RNA Pol I). The immunoprecipitates were also probed with anti-FLAG antibody to control for the efficiency of Rrn3 immunoprecipitation (data not shown.). As Rrn3 can assemble with RNA Pol I, one would expect that formation of a polymerase–Rrn3 complex would be independent of the presence of template. This is, in fact, demonstrated by the signal in lane 2 (Fig. 7B). In agreement with the above-described transcription and PCR analyses, we also found evidence for a Pol I–Rrn3 complex when DNA was added to the incubation (Fig 7B, lane 3). Interestingly, the amount of immunoprecipitable Pol I–Rrn3 complex was significantly reduced when the polymerase was first incubated with template (Fig. 7B, lane 5). This suggests that polymerase that has been recruited to the template in the absence of Rrn3 can no longer interact with Rrn3. If RNA Pol I is omitted from the reaction, no polymerase is found in the immunoprecipitate (lane 6).

We next analyzed these complexes for the presence of DNA (Fig. 7C). As expected, template was found in the immunoprecipitates formed when the extracts from the CHX-treated cells were supplemented with Rrn3 prior to the addition of DNA (Fig. 7C, lane 3). Surprisingly, we did find DNA in the immunoprecipitate formed when Rrn3 was added to the committed template (Fig. 7C, lane 5). Our experiments would indicate that this does not reflect the formation of a competent preinitiation complex (Fig. 1) or the formation of a complex between Rrn3 and RNA Pol I as there is only a trace amount of polymerase in this immunoprecipitate (Fig. 7B, lane 5). As Rrn3 can interact with SL1 (8,30,42) in the absence of template, we believe that the DNA that is being immunoprecipitated is the result of the recruitment of Rrn3 to SL1 bound to the template in the absence of Pol I. As our previous assays indicate that Pol I has in fact been "recruited" to the template, the relative paucity of RNA Pol I (rpa43) in the immunoprecipitate indicates that Rrn3 is in fact being excluded from those nonfunctional complexes that formed in its absence. Whether this does in fact represent an interaction between Rrn3 and SL1 will require additional experiments. When Rrn3 is incubated with the DNA, an insignificant amount of DNA is coimmunoprecipitated in comparison with the amount that coimmunoprecipitates with Rrn3 in a functional complex (Fig. 7C, compare lanes 2 and 6).

DISCUSSION

The synthesis of a transcript represents the endpoint of a process that includes the formation of the competent initiation complex and elongation. While previous experiments from several laboratories demonstrated that Rrn3 was required for transcription, there was no consensus as to the step in transcription that was dependent upon Rrn3. As Rrn3 has been shown to interact with components of mammalian SL1 or yeast core factor, as well as RNA Pol I, it has been presumed that it would at least function in recruitment. However, at least two manuscripts have reported that Rrn3 may not be required for recruitment (1,42). Thus, it was, and is, necessary to derive assays

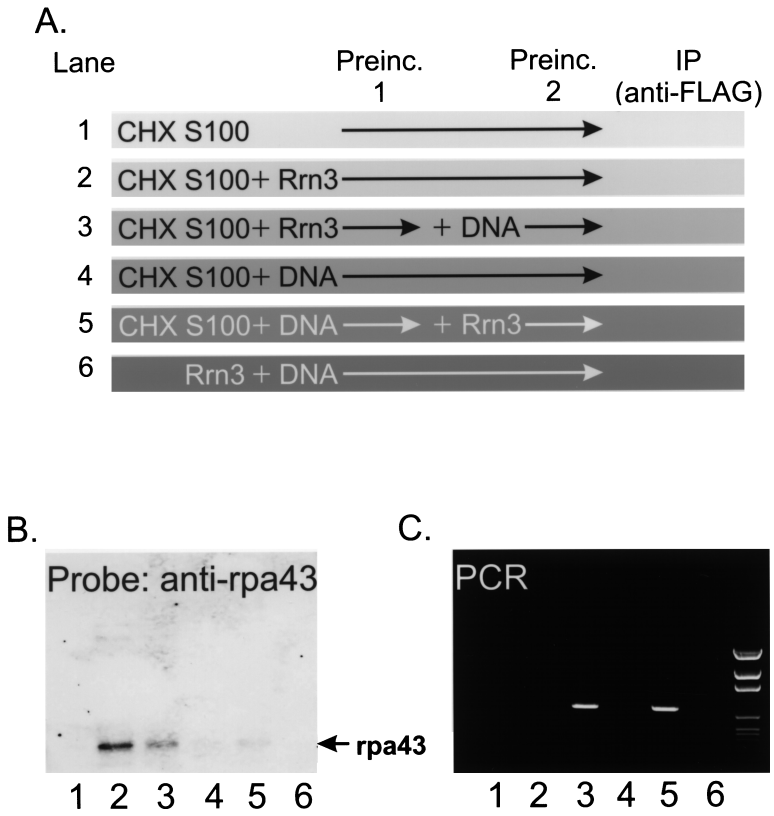

Figure 7. Rrn3 does not bind to RNA Pol I that has been recruited in the absence of active Rrn3. (A) Schematic of the reactions shown in (B) and (C). Each preincubation was done for 20 min at RT followed by immunoprecipitation with anti-FLAG agarose beads. (B) S100 (120 µl) from CHX-treated NISI cells was incubated with either 1.5 µg FLAG-tagged Rrn3 (lanes 2 and 3) or 500 ng of DNA (lanes 4 and 5) for 20 min at RT (preincubation 1). DNA (lane 3) or FLAG-tagged Rrn3 (lane 5) was then added to the reactions, and the incubation continued for another 20 min (preincubation 2). Twenty-five microliters of packed anti-FLAG agarose beads was added and the incubation continued for 1 h at 4°C. The beads were eluted with 50 µl of FLAG peptide (500 µg/ml) and the entire eluate fractionated by SDS-PAGE, transferred to PVDP, and probed with an antibody to rpa43. (C) S100 (5 µl) from CHX-treated NISI cells was incubated with either 0.3 µg FLAG-tagged Rrn3 (lanes 2 and 3) or 50 ng of template DNA (lanes 4 and 5) as indicated (preincubation 1). After a second incubation supplemented with either DNA (lane 3) or Rrn3 (lane 5) as indicated in the schematic, 10 µl of packed anti-FLAG agarose beads was added, and the incubation continued for 1 h at 4°C. Following that incubation, the DNA was isolated and amplified as described in Materials and Methods and the legend to Figure 2.

that demonstrate a functional role for Rrn3 in the various steps of transcription. The immobilized template assays presented in this report indicate that the formation of a competent preinitiation complex requires the presence of active Rrn3. However, by itself transcription does not demonstrate whether a factor is required for one or more steps in initiation and/or elongation or whether it has even been assembled into a complex. Although others (1) have combined the immobilized template assay with Western blots to demonstrate the recruitment of specific factors, we were unable to demonstrate specific binding/recruitment using this assay.

Hence, the modified ChIP assay described in this article was derived in order to determine if the recruitment of RNA Pol I required active Rrn3. Our results clearly indicate that Pol I can be recruited to the template in the absence of active Rrn3. However, using this assay, we were able to demonstrate that the preinitiation complex that forms in the presence of active Rrn3 will proceed to elongation, while that which forms in its absence will not. Further, our data demonstrate distinct differences between the complexes that form in the presence of active Rrn3 and those that form in its absence. For example, the binding of Pol I recruited in the absence of active Rrn3 is fivefold more sensitive to heparin than the complex formed in the presence of active Rrn3. Moreover, the transcription assays and the complex assays demonstrate that the "preinitiation" complex that forms in the absence of active Rrn3 cannot be converted to an active complex by the addition of Rrn3.

The DNA binding assay described in this report is very similar to the standard ChiP assay with the exceptions that 1) we do not cross-link the protein to DNA and 2) a PCR fragment of DNA is the target

of the DNA binding protein rather than chromatin. Although we have increased its sensitivity by the inclusion of two rounds of PCR amplification, our controls indicate that the assay is specific. Moreover, the results of the assays using ethidium bromide-stained gels agree with the results obtained with quantitative RT/PCR. However, it should be pointed out that the assay performed employing whole cell extracts (S100 preparations) is not capable of demonstrating a direct interaction with the DNA. This can only be accomplished by using purified proteins.

We have previously reported that Rrn3 is a phosphoprotein and that treatment of cells with CHX causes a rapid dephosphorylation of Rrn3 as well as its dissociation from Pol I (8). The data presented in Figure 3 indicate that although extracts from CHX-treated cells do not have functional Rrn3, the Pol I in these extracts is capable of binding, or being recruited, to the rDNA promoter. However, this binding is unproductive; we can neither observe transcription nor do we observe nucleotide-dependent release of the polymerase from the template. Thus, our results agree with the observations reported by Aprikian et al. (1) and Schnapp et al. (42). They both concluded that Rrn3 is not required for the recruitment of Pol I to the rDNA promoter. However, the data from the immobilized template assays and the DNA immunoprecipitation assays indicate that only preinitiation complexes formed in the presence of active Rrn3 will support transcription. This conclusion agrees with that reached by Aprikian et al. (1) in their study on the formation of the preinitiation complex by the *S. cerevisiae* rDNA transcription apparatus, and disagrees with that reported by Schnapp et al. (42). Thus, despite differences in yeast and mammals in the regulation of Rrn3 activity and/or the mechanisms that regulate the association of Rrn3 with RNA Pol I, there is a consensus as to the requirement for Rrn3 for the formation of a functional preinitiation complex.

We have attempted to demonstrate biochemical differences between the complexes formed by RNA Pol I bound to the rDNA promoter in the presence and absence of functional Rrn3 with two known inhibitors of initiation complex formation, namely heparin and sarkosyl. The inclusion of heparin in the assay allowed us to demonstrate that RNA Pol I dissociated from the template under conditions that allowed transcription. The observation that Pol I that has been "recruited" in the absence of active Rrn3 does not become displaced from the template following the addition of NTP and heparin again illustrates that even though the polymerase binds to the DNA it cannot carry out transcription.

Additional differences in the protein–DNA complexes formed by RNA Pol I in the presence or absence of active Rrn3 are evidenced by the differences in their sensitivity to heparin (Fig. 5A). The effect of heparin upon transcription is demonstrated by the very low level of transcription (single round) supported by control extracts at this concentration (8 μg/ml) (Fig. 4B, lane 3). This is consistent with the finding that at a high concentration of heparin (40 μg/ml) most of the Pol I that has been recruited is displaced from the preinitiation complex as indicated by the lack of coimmunoprecipitated DNA (Fig. 5A, lane 3). On the other hand, the polymerase recruited in the absence of active Rrn3 is fivefold more sensitive to the effect of heparin. When the concentration of heparin is reduced to 8 μg/ml, the preinitiation complex formed in the presence of active Rrn3 is relatively stable. However, the "preinitiation" complex, formed by RNA Pol I in the absence of active Rrn3, is still sensitive. The addition of Rrn3 reverses this affect (Fig. 5B, lane 7). It is interesting to note that the reconstitution appears to result in the formation of a DNA–protein complex that once again is five times more stable than that formed by extracts that do not contain active Rrn3. Although there are significant differences in the results overall, they are quite consistent. It should be kept in mind that these experiment only examined the effect of a single concentration (300 ng) of Rrn3 and a relatively short preincubation. It is possible that higher concentrations or a longer preincubation will result in a stronger reconstitution. It should also be pointed out that we have used different preparations of recombinant Rrn3 throughout these experiments without having an assay to compare their relative activities.

It is interesting to note that sarkosyl has opposite effects on the stability/formation of the two DNA–protein complexes than that observed with heparin (i.e., the complex that forms in the presence of functional Rrn3 is more sensitive to sarkosyl than the complex formed in its absence). At a single concentration of detergent (0.025%) the control extracts do not form any detectable complexes, while the CHX-treated extracts support the formation of a sarkosyl-resistant complex. Interestingly, the preincubation of extracts from CHX-treated cells with Rrn3 reduced the resistance of the recruited polymerase to sarkosyl. The results, presented in Figure 6 (lanes 5 and 6), indicate that this conversion occurred in a dose-dependent manner. It should be pointed out that attempts to show a dose-dependent effect of sarkosyl on the formation/resistance of the preinitiation complex did not demonstrate a clear pattern, and that it was only at 0.025% sarkosyl that we observed a reproducible difference between the complexes that formed in the presence or absence of functional Rrn3. In addition,

as stated in the legend to Figure 6, this difference was only seen if Rrn3 was incubated with the S100 at room temperature before the addition of template. Clearly the ability of heparin and sarkosyl to inhibit rDNA initiation occurs by different mechanisms.

The data presented in this article indicate that the DNA–protein (RNA Pol I) interactions supported by extracts from control and CHX-treated cells are very different. Rrn3 does not appear to be required for the recruitment of RNA Pol I to the rDNA promoter. However, its presence is required prior to recruitment of RNA Pol I to the committed template for transcription to proceed, and our data clearly demonstrate differences in the preinitiation complexes that form in its absence or presence. Thus, Rrn3 is essential for at least one step prior to transcription initiation, recruitment.

Transcription initiation includes at least four events: 1) the binding of polymerase/holoenzyme to a promoter; 2) the isomerization of the resulting complex accompanied by strand opening (open complex); 3) the iterative synthesis and release of abortive transcripts; and 4) the achievement of continuous elongation accompanied by the escape of the enzyme from the promoter. This mechanism for initiation can be considered to be a sequential pathway. However, evidence from studies on transcription initiation at the several bacterial promoters [e.g., the LacUV5, λP_RAL, and T7A1 promoters (24,25,32)] supports the possibility that there may in fact be a branched pathway that includes the option for the formation of a "moribund" complex at the same level as the formation of the productive open complex. In this model, the moribund complex, which is defined as the ability to synthesize only abortive transcripts (24), does not proceed through abortive synthesis to elongation, but results in the formation of a terminal complex, with a greater half-life than that of the abortive and escaping complexes. In this context it is worthwhile to note that deletion or modification of the CTD of the large subunit of eukaryotic RNA Pol II may result in an analogous inhibition of transcription in vivo. For example, Lux et al. (28) have shown that recognition of the c-myc promoter by Pol II with a CTD truncated to five repeats is not affected in the context of chromatin, while subsequent steps like stable PIC formation and isomerization to an elongation competent complex are severely impaired.

An argument by analogy would be that that in the absence of active Rrn3, RNA Pol I may be recruited to the template resulting in a complex that continues abortive transcription but cannot make the transition to an elongating complex. It is also interesting to note that the transition from initiating to elongating Pol II is both associated with and dependent upon changes in the phosphorylation state of the CTD (35,43). This may also be analogous (albeit inversely) to the situation with regard to the phosphorylation state of Rrn3. We have shown that dephosphorylated Rrn3 cannot interact with RNA Pol I, and that treatment with cycloheximide inactivates Rrn3 by causing its dephosphorylation (8). Further, we have demonstrated that Rrn3 is inactivated upon transcription (18). Based on this series of observations we have proposed a model for the cyclical association of Rrn3 with RNA Pol I that is dependent upon the phosphorylation state of Rrn3; Rrn3 must be phosphorylated to interact with RNA Pol I and function in recruitment/initiation and Rrn3 is inactivated by dephosphorylation upon transcription (8,18).

The present results demonstrate that in the absence of active Rrn3, RNA Pol I can still make sufficient contacts with the committed template to be recruited. However, the conformation of the Pol I–template complex is constrained in a way that prevents promoter escape/elongation. Whether this demonstrates a requirement for Rrn3 in recruitment would appear to be a semantic argument at this stage. Clearly, polymerase that has been "recruited" in the absence of active Rrn3 cannot transcribe. Subsequent experiments will have to determine if Rrn3 functions in both recruitment and a subsequent step in initiation.

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REFERENCES

1. Aprikian, P.; Moorefield, B.; Reeder, R. H. TATA binding protein can stimulate core-directed transcription by yeast RNA polymerase I. Mol. Cell. Biol. 21: 4847–4855; 2001.
2. Bazett-Jones, D. P.; Leblanc, B.; Herfort, M.; Moss, T. Short-range DNA looping by the *Xenopus* HMG-box transcription factor, xUBF. Science 264:1134–1137; 1994.
3. Bell, S. P.; Learned, R. M.; Jantzen, H. M.; Tjian, R. Functional cooperativity between transcription factors UBF1 and SL1 mediates human ribosomal RNA synthesis. Science 241:1192–1197; 1988.
4. Bodem, J.; Dobreva, G.; Hoffmann-Rohrer, U.; Iben, S.; Zentgraf, H.; Delius, H.; Vingron, M.; Grummt, I. TIF-IA, the factor mediating growth dependent control of ribosomal RNA synthesis, is the mammalian

homolog of yeast Rrn3p. EMBO Rep. 1:171–175; 2000.
5. Cassidy, B.; Haglund, R.; Rothblum, L. Regions upstream from the core promoter of the rat ribosomal gene are required for the formation of a stable transcription initiation complex by RNA polymerase l in vitro. Biochim. Biophys. Acta 909:133–144; 1987.
6. Cavanaugh, A. H.; Gokal, P. K.; Lawther, R. P.; Thompson, E. A. Glucocorticoid inhibition of initiation of transcription of the DNA encoding rRNA (rDNA) in lymphosarcoma P1798 cells. Proc. Natl. Acad. Sci. USA 81:718–721; 1984.
7. Cavanaugh, A. H.; Hempel, W. M.; Taylor, L. J.; Rogalsky, V.; Todorov, G.; Rothblum, L. I. Activity of RNA polymerase I transcription factor UBF blocked by Rb gene product. Nature 374:177–180; 1995.
8. Cavanaugh, A. H.; Hirschler-Laszkiewicz, I.; Hu, Q.; Dundr, M.; Smink, T.; Misteli, T.; Rothblum, L. I. Rrn3 phosphorylation is a regulatory checkpoint for ribosome biogenesis. J. Biol. Chem. 277:27423–27432; 2002.
9. Cavanaugh, A.; Hirschler-Laszkiewicz, I.; Rothblum, L. I. Ribosomal DNA transcription in mammals. In: Olson, M. O. J., ed. The nucleolus. Kluwer Academic/Plenum Publishers; 2003:89–129.
10. Cavanaugh, A. H.; Thompson, E. A. Hormonal regulation of transcription of rDNA. Initiation of transcription by RNA polymerase I *in vitro*. J. Biol. Chem. 261: 12738–12744; 1986.
11. Comai, L.; Tanese, N.; Tjian, R. The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. Cell 68:965–976; 1992.
12. Comai, L.; Zomerdijk, J. C.; Beckmann, H.; Zhou, S.; Admon, A.; Tjian, R. Reconstitution of transcription factor SL1: exclusive binding of TBP by SL1 or TFIID subunits. Science 266:1966–1972; 1994.
13. Hanada, K.; Song, C. Z.; Yamamoto, K.; Yano, K.; Maeda, Y.; Yamaguchi, K.; Muramatsu, M. RNA polymerase I associated factor 53 binds to the nucleolar transcription factor UBF and functions in specific rDNA transcription. EMBO J. 15:2217–2226; 1996.
14. Hannan, K. M.; Brandenburger, Y.; Jenkins, A.; Sharkey, K.; Cavanaugh, A.; Rothblum, L.; Moss, T.; Poortinga, G.; McArthur, G. A.; Pearson, R. B.; Hannan, R. D. mTor-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. Mol. Cell. Biol. 23:8862–8877; 2003.
15. Hannan, R. D.; Hempel, W. M.; Cavanaugh, A.; Arino, T.; Dimitrov, S. I.; Moss, T.; Rothblum, L. Affinity purification of mammalian RNA polymerase I. Identification of an associated kinase. J. Biol. Chem. 273: 1257–1267; 1998.
16. Heix, J.; Vente, A.; Voit, R.; Budde, A.; Michaelidis, T. M.; Grummt, I. Mitotic silencing of human rRNA synthesis: Inactivation of the promoter selectivity factor SL1 by cdc2/cyclin B-mediated phosphorylation. EMBO J. 17:7373–7381; 1998.
17. Hirschler-Laszkiewicz, I.; Cavanaugh, A.; Hu, Q.; Catania, J.; Avantaggiati, M. L.; Rothblum, L. I. The role of acetylation in rDNA transcription. Nucleic Acids Res. 29:4114–4124; 2001.
18. Hirschler-Laszkiewicz, I.; Cavanaugh, A. H.; Mirza, A.; Lun, M.; Hu, Q.; Smink, T.; Rothblum, L. I. Rrn3 becomes inactivated in the process of ribosomal DNA transcription. J. Biol. Chem. 278:18953–18959; 2003.
19. Imazawa, Y.; Hisatake, K.; Nakagawa, K.; Muramatsu, M.; Nogi, Y. The fission yeast RPA21 subunit of RNA polymerase I: An evolutionarily conserved subunit interacting with ribosomal DNA (rDNA) transcription factor Rrn3p for recruitment to rDNA promoter. Genes Genet. Syst. 77:147–157; 2002.
20. Jantzen, H-M.; Admon, A.; Bell, S. P.; Tjian, R. Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. Nature 344:830–836; 1990.
21. Kato, H.; Nagamine, M.; Kominami, R.; Muramatsu, M. Formation of the transcription initiation complex on mammalian rDNA. Mol. Cell. Biol. 6:3418–3427; 1986.
22. Keys, D. A.; Vu, L.; Steffan, J. S.; Dodd, J. A.; Yamamoto, R. T.; Nogi, Y.; Nomura, M. RRN6 and RRN7 encode subunits of a multiprotein complex essential for the initiation of rDNA transcription by RNA polymerase I in *Saccharomyces cerevisiae*. Genes Dev. 8: 2349–2362; 1994.
23. Keys, D. A.; Lee, B. S.; Dodd, J. A.; Nguyen, T. T.; Vu, L.; Fantino, E.; Burson, L. M.; Nogi, Y.; Nomura, M. Multiprotein transcription factor UAF interacts with the upstream element of the yeast RNA polymerase I promoter and forms a stable preinitiation complex. Genes Dev. 10:887–903; 1996.
24. Kubori, T.; Shimamoto, N. Physical interference between *Escherichia coli* RNA polymerase molecules transcribing in tandem enhances abortive synthesis and misincorporation. Nucleic Acids Res. 25:2640–2647; 1997.
25. Kubori, T.; Shimamoto, N. A branched pathway in the early stage of transcription by Escherichia coli RNA polymerase. J. Mol. Biol. 256:449–457; 1996.
26. Learned, R. M.; Cordes, S.; Tjian, R. Purification and characterization of a transcription factor that confers promoter specificity to human RNA polymerase I. Mol. Cell. Biol. 5:1358–1369; 1985.
27. Lin, C. W.; Moorefield, B.; Payne, J.; Aprikian, P.; Mitomo, K.; Reeder, R. H. A novel 66-kilodalton protein complexes with Rrn6, Rrn7, and TATA-binding protein to promote polymerase I transcription initiation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 16: 6436–6443; 1996.
28. Lux, C.; Albiez, H.; Chapman, R. D.; Heidinger, M.; Meininghaus, M.; Brack-Werner, R.; Lang, A.; Ziegler, M.; Cremer, T.; Eick, D. Transition from initiation to promoter proximal pausing requires the CTD of RNA polymerase II. Nucleic Acids Res. 33:5139–5144; 2005.
29. Milkereit, P.; Tschochner, H. A specialized form of RNA polymerase I, essential for initiation and growth-dependent regulation of rRNA synthesis, is disrup-

ted during transcription. EMBO J. 17:3692–3703; 1998.
30. Miller, G.; Panov, K. I.; Friedrich, J. K.; Trinkle-Mulcahy, L.; Lamond, A. I.; Zomerdijk, J. C. hRRN3 is essential in the SL1-mediated recruitment of RNA Polymerase I to rRNA gene promoters. EMBO J. 20: 1373–1382; 2001.
31. Moorefield, B.; Greene, E. A.; Reeder, R. H. RNA polymerase I transcription factor Rrn3 is functionally conserved between yeast and human. Proc. Natl. Acad. Sci. USA 97:4724–4729; 2000.
32. Motoki, S.; Ranjan, S.; Nobuo, S. Generality of the branched pathway in transcription initiation by *Escherichia coli* RNA polymerase. J. Biol. Chem. 277:15407–15412; 2002.
33. Muth, V.; Nadaud, S.; Grummt, I.; Voit, R. Acetylation of TAFI68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. EMBO J. 20:1353–1362; 2001.
34. O'Mahony, D. J.; Smith, S. D.; Xie, W.; Rothblum, L. I. Analysis of the phosphorylation, DNA-binding and dimerization properties of the RNA polymerase I transcription factors UBF1 and UBF2. Nucleic Acids Res. 20:1301–1308; 1992.
35. Palancade, B.; Bensaude, O. Investigating RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation. Eur. J. Biochem. 270:3859–3870; 2003.
36. Paule, M. Transcription of ribosomal RNA by eukaryotic RNA polymerase I. In: Conaway, R.; Conaway, J., eds. Transcription: Mechanisms and regulation. New York: Raven Press; 1994:83–105.
37. Pelletier, G.; Stefanovsky, V. Y.; Faubladier, M.; Hirschler-Laszkiewicz, I.; Savard, J.; Rothblum, L. I.; Cote, J.; Moss, T. Competitive recruitment of CBP and Rb-HDAC regulates UBF acetylation and ribosomal transcription. Mol. Cell 6:1059–1066; 2000.
38. Peyroche, G.; Milkereit, P.; Bischler, N.; Tschochner, H.; Schultz, P.; Sentenac, A.; Carles, C.; Riva, M. The recruitment of RNA polymerase I on rDNA is mediated by the interaction of the A43 subunit with Rrn3. EMBO J. 19:5473–5482; 2000.
39. Read, S. J. Recovery efficiences on nucleic acid extraction kits as measured by quantitative LightCycler PCR. Mol. Pathol. 54:86–90; 2001.
40. Ririe, K. M.; Rasmussen, R. P.; Wittwer, C. T. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. 245:154–160; 1997.
41. Russell, J.; Zomerdijk, J. C. RNA-polymerase-I-directed rDNA transcription, life and works. Trends Biochem. Sci. 30:87–96; 2005.
42. Schnapp, A.; Schnapp, G.; Erny, B.; Grummt, I. Function of the growth-regulated transcription initiation factor TIF-IA in initiation complex formation at the murine ribosomal gene promoter. Mol. Cell. Biol. 13: 6723–6732; 1993.
43. Sims, R. J.; Belotserkovskaya, R.; Reinberg, D. Elongation by RNA polymerase II: the short and long of it. Genes Dev. 18:2437–2468; 2004.
44. Smith, S.; Oriahi, E.; Lowe, D.; Yang-Yen, H. F.; O'Mahony, D.; Rose, K.; Chen, C.; Rothblum, L. I. Characterization of factors that direct transcription of rat ribosomal DNA. Mol. Cell. Biol. 10:3105–3116; 1990.
45. Stefanovsky, V.; Langlois, F.; Gagnon-Kugler, T.; Rothblum, L. I.; Moss, T. Growth factor signaling regulates elongation of RNA polymerase I transcription in mammals via UBF phosphorylation and r-chromatin remodeling. Mol. Cell 21:629–639; 2006.
46. Steffan, J.; Keys, D.; Dodd, J.; Nomura, M. The role of TBP in rDNA transcription by RNA polymerase I in *Saccharomyces cerevisiae*: TBP is required for upstream activation factor-dependent recruitment of core factor. Genes Dev. 10:2551–2563; 1996.
47. Thuriaux, P.; Mariotte, S.; Buhler, J. M.; Sentenac, A.; Vu, L.; Lee, B. S.; Nomura, M. RPA43 in *Saccharomyces cerevisiae* encodes an essential subunit of RNA polymerase I. J. Biol. Chem. 270:24252–24257; 1995.
48. Voit, R.; Grummt, I. Phosphorylation of UBF at serine 388 is required for interaction with RNA polymerase I and activation of rDNA transcription. Proc. Natl. Acad. Sci. USA 98:13631–13636; 2001.
49. Wittwer, C. T.; Herrmann, M. G.; Moss, A. A.; Rasmussen, R. P. Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques 22: 130–131, 134–138; 1997.
50. Wittwer, C. T.; Ririe, K. M.; Andrew, R. V.; David, D. A.; Gundry, R. A.; Balis, U. J. The LightCycler: A microvolume multisample fluorimeter with rapid temperature control. BioTechniques 22:176–181; 1997.
51. Yamamoto, R. T.; Nogi, Y.; Dodd, J. A.; Normura, M. RRN3 gene of *Saccharomyces cerevisiae* encodes an essential RNA polymerase I transcription factor which interacts with the polymerase independently of DNA template. EMBO J. 15:3964–3973; 1996.
52. Yuan, X.; Zhao, J.; Zentgraf, H.; Hoffmann-Rohrer, U.; Grummt, I. Multiple interactions between RNA polymerase I, TIF-IA and TAF(I) subunits regulate preinitiation complex assembly at the ribosomal gene promoter. EMBO Rep. 3:1082–1087; 2002.
53. Yuan, X.; Zhou, Y.; Casanova, E.; Chai, M.; Kiss, E.; Grone, H. J.; Schutz, G.; Grummt, I. Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis. Mol. Cell 19:77–87; 2005.
54. Zhai, W. G.; Tuan, J. A.; Comai, L. SV40 large T antigen binds to the TBP-TAF(I) complex SL1 and coactivates ribosomal RNA transcription. Genes Dev. 11: 1605–1617; 1997.
55. Zomerdijk, J. C.; Beckmann, H.; Comai, L.; Tjian, R. Assembly of transcriptionally active RNA polymerase I initiation factor SL1 from recombinant subunits. Science 266:2015–2018; 1994.