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RRN3 gene of *Saccharomyces cerevisiae* encodes an essential RNA polymerase I transcription factor which interacts with the polymerase independently of DNA template

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***RRN3* is one of the *RRN* genes specifically required for the transcription of rDNA by RNA polymerase I (Pol I) in *Saccharomyces cerevisiae*. We have cloned the gene, determined the nucleotide sequence, and found that it is an essential gene which encodes a protein of calculated molecular weight of 72 369. Extracts prepared from *rrn3* mutants were defective in *in vitro* transcription of rDNA templates. We used extracts from a strain containing an epitope-tagged Rrn3 protein to purify a factor that could complement the mutant extracts. Using immunoaffinity purification combined with Mono Q chromatography, we obtained an essentially pure preparation of Rrn3p which complements the mutant extracts. By carrying out template commitment experiments, we found that Rrn3p is not part of the pre-initiation complex that is stable through multiple rounds of transcription. We also found that pre-incubation of Rrn3p with purified Pol I leads to stimulation of transcription upon subsequent mixing with DNA template and other transcription reaction components. Single-round transcription experiments using the detergent Sarkosyl showed that this stimulation is due to increased efficiency of formation of a Sarkosyl-resistant pre-initiation complex. Thus, Rrn3p appears to interact directly with Pol I, apparently stimulating Pol I recruitment to the promoter, and is distinct from two other Pol I-specific transcription factors, the Rrn6/7 complex and the Rrn5/9/10 complex (UAF), characterized previously.**

Keywords: rDNA transcription/RNA polymerase I/*RRN3* gene/transcription factor/yeast

Introduction

RNA polymerase I (Pol I) is one of the three nuclear RNA polymerases in eukaryotes and is dedicated to transcription of rRNA genes (rDNA) in the nucleolus. In contrast to the complexity of the Pol II initiation pathway that involves an array of basal transcription initiation factors, the initiation pathway for Pol I has generally been thought to be simpler, utilizing perhaps only two factors in several metazoan *in vitro* systems, UBF (upstream binding factor) and SL1 (promoter selectivity factor; also called TIF-IB) (e.g. see a review on eukaryotic transcription complexes by Zawel and Reinberg, 1995; for more specialized reviews on transcription by Pol I, see Reeder, 1992; Paule, 1994; Moss and Stefanovsky, 1995). SL1 consists of TBP (TATA binding protein) and three TAFs (TBP-associated factors) (Comai *et al.*, 1992; Eberhard *et al.*, 1993), and is itself, or together with UBF, able to form a stable initiation complex which is capable of recruiting Pol I in the absence of any other factors, and of committing the template to multiple cycles of transcription (Comai *et al.*, 1992; see also Schnapp and Grummt, 1991). The gene for UBF was cloned from a variety of organisms and characterized (reviewed in Moss and Stefanovsky, 1995). Although UBF is a DNA binding protein with low sequence specificity, and the basis of its positioning at the exact sites in the promoter region is unclear, it has the ability to bend and wrap DNA, and the resulting structure is thought to help bind SL1 (Bazett-Jones *et al.*, 1994; Copenhaver *et al.*, 1994; Hu *et al.*, 1994; Putnam *et al.*, 1994). Most recently, Tjian and co-workers succeeded in cloning the genes for the three TAFs of human SL1, and reconstituting active SL1 from the four recombinant protein molecules (Comai *et al.*, 1994; Zomerdijk *et al.*, 1994). Thus, at least in the human cell system, correct initiation of rDNA transcription can take place *in vitro* using two well purified factors, UBF and SL1, and purified Pol I together with a suitable DNA template. Nevertheless, the possibile existence of some other transcription factors is not really excluded. Thus, Grummt and co-workers have reported a requirement for two additional factors, TIF-IA and TIF-IC, in addition to UBF and SL1, for initiation in a mouse *in vitro* system (e.g. see Schnapp and Grummt, 1991). However, neither of these factors has been purified completely, and their genes have not been cloned. Because purified Pol I preparations used for *in vitro* experiments in these mammalian systems were not well characterized, the lack of the requirement for additional factors in the human system might be explained by the presence of small amounts of transcription factors in the purified Pol I preparations. Alternatively, the possibility has been suggested that the additional factors described by Grummt and co-workers might have been derived from Pol I because of harsh conditions used for purification of these factors (e.g. see discussion in Paule, 1994).

RNA polymerases from *Saccharomyces cerevisiae* are probably the best characterized among eukaryotic nuclear RNA polymerases from various sources. *S.cerevisiae* Pol I (yeast Pol I) consists of 14 subunits, and genes for all of these subunits have been cloned and characterized (Thuriaux and Sentenac, 1992, and references therein; Nogi *et al.*, 1993; Smid *et al.*, 1995; Thuriaux *et al.*, 1995), thus allowing unequivocal identification of other factors required for transcription. We have used a genetic approach to identify transcription factors specifically required for Pol I transcription in *S.cerevisiae*. Starting

from strains carrying the 35S rRNA gene fused to the *GAL7* promoter (p*GAL7*–35SrDNA) on a plasmid, mutants were isolated which are specifically defective in transcription of chromosomal rDNA by Pol I, but whose growth can be rescued by rRNA synthesis from the plasmid-encoded hybrid gene by Pol II. The mutants have been classified in at least a dozen complementation groups and the genes (*RRN* genes) that complement the mutational defects have been cloned and characterized. Five of these genes were found to encode subunits unique to Pol I (*RRN1*, *RRN2*, *RRN13*, *RRN12* and *RRN4*, encoding the A190, A135, A49, A43 and A12 subunits, respectively) (Nogi *et al.*, 1991, 1993; Thuriaux *et al.*, 1995; our unpublished experiments). Seven other genes (*RRN3*, *RRN5*, *RRN6*, *RRN7*, *RRN9*, *RRN10* and *RRN11*) were found to encode proteins which do not correspond to any of the 14 subunits of Pol I. In addition, extracts from strains with mutations in these seven genes showed no or greatly reduced Pol I transcription activities *in vitro* and were complemented by protein fractions from the wild-type strain, i.e. these seven genes appear to encode proteins that function as Pol I-specific transcription factors. By studying *RRN6* and *RRN7*, we have demonstrated previously that the encoded proteins, Rrn6p and Rrn7p, together with another protein of 66 kDa (p66) form an essential transcription factor complex called the Rrn6/7 complex, which lacks TBP, but resembles SL1 in metazoan systems (Keys *et al.*, 1994). More recently, we have demonstrated that three proteins, Rrn5p, Rrn9p and Rrn10p, encoded by *RRN5*, *RRN9* and *RRN10*, respectively, form yet another multiprotein transcription factor which contains two more uncharacterized protein components. This latter complex (called the Rrn5/9/10 complex or UAF for upstream activation factor) was demonstrated to bind directly to the upstream element of the yeast rDNA promoter in the absence of any other factor, forming a stable complex that is able to recruit the Rrn6/7 complex (possibly together with other factors) and committing the template to transcription (Keys *et al.*, 1996). In this respect, UAF appears to carry out the function originally proposed for UBF in metazoan systems, although there are some significant differences between the two (Keys *et al.*, 1996). Here we describe cloning and characterization of gene *RRN3* and show that it encodes an essential transcription factor, which is distinct from the above two transcription factor complexes, the Rrn6/7 complex and UAF.

Results

Cloning and characterization of the RRN3 gene

Of the mutants defective in transcription by Pol I which we have isolated, six belong to the complementation group which we call *RRN3*. All the *rrn3* mutants were isolated from the strain carrying the p*GAL7*–35SrDNA fusion gene on a plasmid (pNOY103) at 36°C as mutants which can grow on galactose but not on glucose. Synthesis of RNA in an *rrn3-1* mutant strain before and after shift from galactose to glucose medium was analyzed previously, and a very severe reduction in the synthesis of large rRNAs relative to 5S and tRNA synthesis was demonstrated for the mutant strain after the transfer from galactose to glucose medium (Nogi *et al.*, 1991). Using this mutant

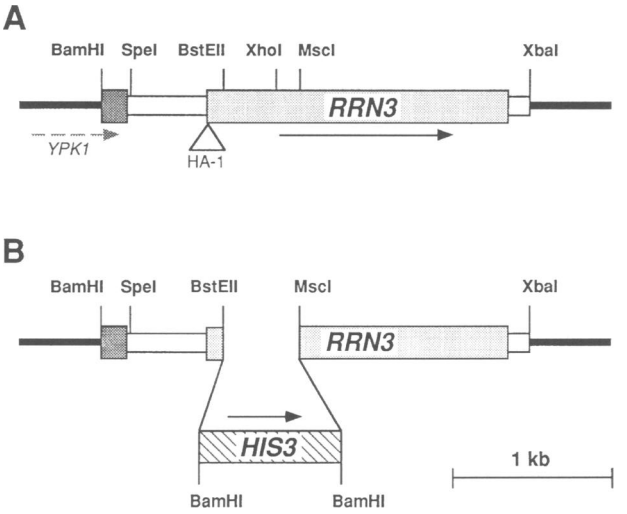

Fig. 1. Restriction enzyme map of *RRN3*. (**A**) The *Bam*HI–*Xba*I fragment present in pNOY202; (**B**) the DNA fragment in pNOY3228 that was used to construct the *rrn3Δ::HIS3* null allele. The protein coding region of *RRN3* is stippled as is the distal part of *YPK1*. The position of the oligonucleotide encoding the HA1 epitope (in pNOY333) is indicated by an open triangle. Restriction enzyme sites relevant to plasmid and strain constructions are shown. Arrows indicate direction of transcription.

strain (NOY675), we cloned the *RRN3* gene by complementation. The 2.7 kb *Bam*HI–*Xba*I fragment which carried the gene responsible for complementation was sequenced and its physical structure is shown in Figure 1A. The amino acid sequence of the encoded protein, Rrn3p, deduced from the nucleotide sequence is shown in Figure 2A, and the distribution of acidic and basic amino acid residues in the protein is shown in Figure 2B.

RRN3 encodes a protein of 627 amino acids with a calculated molecular weight of 72 369 and a calculated isoelectric point of 4.4. One unique feature of the protein is a highly acidic region in the middle of the molecule. This region (33 amino acids from position 249 to 281; shown in bold in Figure 2A) contains 25 glutamic plus aspartic acids, but its significance is unknown. By comparing the nucleotide sequence we obtained for the *RRN3* DNA fragment with sequences in the data bank, we found that *RRN3* is located on the left arm of chromosome XI, 499 nucleotides downstream of *YPK1* (a protein kinase gene; see Chen *et al.*, 1993; Figure 1A). It is now evident that *RRN3* corresponds to an open reading frame called YKL125w found as a result of the yeast sequencing project (Dujon *et al.*, 1994). We have also shown, by appropriate genetic experiments, that the cloned gene corresponds to the gene originally defined by *rrn3* mutations (our unpublished experiments).

Gene disruption experiments confirmed the expectation from the phenotype of *rrn3* mutants that *RRN3* is an essential gene. A diploid strain (a derivative of NOY397) carrying a disrupted *rrn3* gene (*rrn3Δ::HIS3*, see Figure 1B), together with the wild-type gene (*RRN3*) on the other chromosome, produced, upon sporulation, tetrads with two viable and two non-viable spores, and the wild-type *HIS3* gene used for disruption was not recovered in the viable spores.

We have constructed a haploid strain with the chromosomal *RRN3* disrupted by *HIS3* and carrying the p*GAL7*–

A

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  1 MMAFENTSKR PPQDFVAPID QKKRKVQFSD STGLVTLQPE  EIKDEVFSAA MYSRFVKSAL DDLDKNDSTQ IGIIANQVAL
 81 PSKNPERIND KNLNILLDIL SSNINRIESS RGTFLIQSII  NFEKWWELPP HTLSKYIYFI KILCSSIPKW WQDVSMILVS
161 CFILPIKQTV CHHDMLKYFL RMIPSSMGFI DTYLAKFFPN  KNDTRRKLVN YTSNLLKLRG YCSELGFQIW SLLIEKIISI
241 DVELQNELDE LDDDVDDDDL EEVDLEDDDD LDDDSGDDDD  ENCGNSNEEL RSGAADGSQS DSEDMDIIEG MDGTEEYNVE
321 LTQGIKELST KLDSILTLVS THVEEQVTPE SLESGEGVGV  FNTLTTLFKT HVLPTYYTRS IQYIMFHVSQ QQLELMDSFL
401 VTLIDISFAV NEAAEKKIKS LQYLGSYIAR AKKLSRTQII  FVASYLTSWL NRYVIEREEE VDQRGGMERF KHFYAAFQAL
481 CYIFCFRHNI FRDTDGNWEC ELDKFFQRMV ISKFNPLKFC  NENVMLMFAR IAQQESVAYC FSIIENNNNE RLRGIIGKAD
561 SDKKENSAQA NTTSSSWSLA TRQQFIDLQS YFPYDPLFLK  NYKILMKEYY IEWSEASGEY ESDGSDD
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B

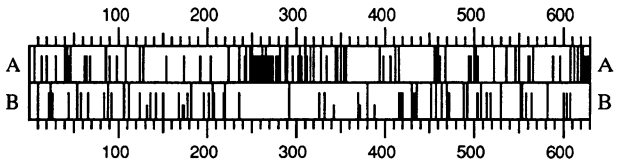

Fig. 2. (**A**) Predicted amino acid sequence of Rrn3p based upon the nucleotide sequence. An aspartic acid-rich region is shown in bold. (**B**) Acid/base map of Rrn3p. Acid map: half bar, Asp; full bar, Glu. Base map: small bar, His; intermediate bar, Lys; full bar, Arg.

35S rDNA fusion gene on a plasmid. This strain (NOY604; called '*Δrrn3*' strain below) can grow on galactose, but not on glucose, and was used to make extracts which do not contain Rrn3p. We have also constructed a haploid *rrn3Δ::HIS3* strain carrying a HA1 epitope-tagged *RRN3* gene on a centromere plasmid (pNOY333). This strain (NOY657; called 'HA1–*RRN3*' strain below; see Materials and methods) grew at the same rate as the control strain carrying the wild-type *RRN3*, indicating that HA1-tagged Rrn3p (HA1–Rrn3p) can functionally replace Rrn3p *in vivo*. Extracts from this strain were used to detect and affinity purify HA1–Rrn3p with monoclonal antibodies against the HA1 epitope.

Purification of HA1–Rrn3p and demonstration of its complementation activity using rrn3 mutant extracts

Extracts were prepared from the HA1–*RRN3* and *rrn3* mutant (*Δrrn3* or *rrn3-1*) strains and fractionated first over phosphocellulose and then DEAE columns, yielding two protein fractions, a PC-300 (eluting from the phosphocellulose column at 300 mM KCl) and a D-300 fraction (eluting from the DEAE column at 300 mM KCl). As described previously (Keys *et al.*, 1994), accurate initiation of transcription of rDNA template can be achieved by a combination of PC-300 and D-300 fractions obtained from wild-type strains. Wild-type PC-300 contains Pol I and another unidentified factor(s) (our unpublished experiments), whereas D-300 contains the remaining factors required for our *in vitro* rDNA transcription assay including the Rrn6/7p complex (Keys *et al.*, 1994), UAF (Keys *et al.*, 1996; see Introduction) and Rrn3p (detected by SDS–PAGE followed by Western blot analysis; data not shown). Extracts from the *rrn3* mutant strains are inactive for specific transcription by Pol I (Figure 3, lane 1). To see if Rrn3p is in fact the factor deficient in the *rrn3* mutant extracts, HA1–Rrn3p was immunoaffinity purified using HA1-specific antibodies and added to the *rrn3* mutant extracts, and *in vitro* transcription assays were carried out. A control preparation was obtained from an *RRN3* strain (NOY388), which does not carry the HA1-tagged *RRN3*, by following the same procedure. As shown in Figure 3, addition of the affinity-purified HA1–Rrn3p preparation to *rrn3* mutant extracts showed active rDNA

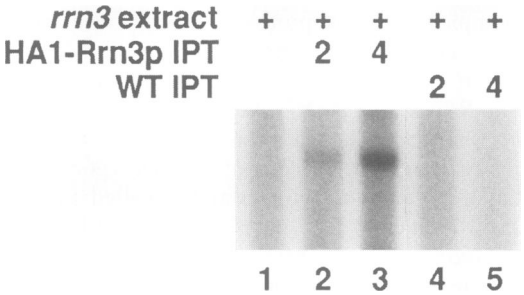

Fig. 3. Complementation of *rrn3* extracts by an immunoaffinity-purified HA1–Rrn3p preparation. Transcription was carried out using an *rrn3-1* mutant extract and in the presence of 2 and 4 μl of HA1–Rrn3p preparation obtained from NOY657 ('HA1–Rrn3p IPT'; lanes 2 and 3), 2 and 4 μl of control preparation obtained from NOY388 without HA1 tagging ('WT IPT'; lanes 4 and 5) or without any addition (lane 1). An autoradiogram revealing RNA transcripts is shown.

transcription (lanes 2 and 3), while the control preparation did not (lanes 4 and 5), indicating that the factor complementing *rrn3* mutant extracts is Rrn3p itself or a complex containing Rrn3p.

The active factor was purified further by subjecting the affinity-purified preparation to Mono Q column chromatography. Fractions obtained after salt gradient elution were analyzed for activity to complement *Δrrn3* extracts (Figure 4A), for proteins by SDS–PAGE and silver staining (Figure 4C) and specifically for Rrn3p by Western blot analysis (Figure 4B). As expected, peak fractions containing HA1–Rrn3p complemented *Δrrn3* extracts; furthermore, these fractions showed only one major protein component as a silver-stained protein band which corresponded to HA1–Rrn3p revealed by Western blot. The molecular mass calculated from the mobility of this band was also consistent with that calculated from its amino acid sequence (~73 kDa). Thus, Rrn3p does not form a stable multiprotein complex and appears to function singly as a transcription factor in our *in vitro* system.

In other experiments, we examined the native molecular mass of Rrn3p as a transcription factor by superose 6 gel filtration chromatography, and obtained results indicating that at least a major fraction of Rrn3p in D-300 fraction exists as a monomer (data not shown).

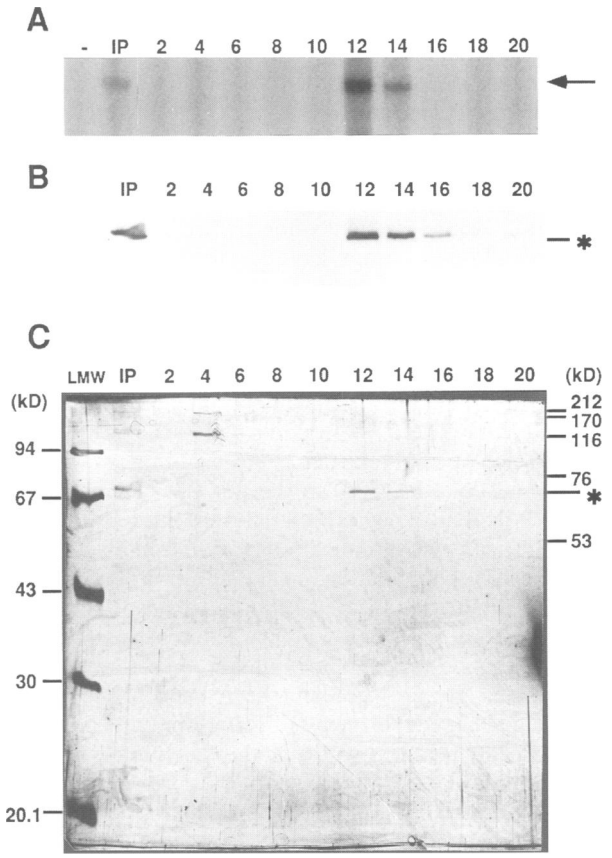

Fig. 4. Purification of Rrn3p by a Mono Q column chromatography. The preparation obtained after immunoaffinity purification was applied to a Mono Q column, and even numbered fractions obtained after elution were analyzed for their activity to complement *rrn3* mutant extract (**A**), for HA1–Rrn3p by SDS–PAGE followed by Western immunoblot analysis with HA1–antibody (**B**), and for proteins by SDS–PAGE followed by silver staining (**C**). Fraction numbers are given, and IP is the preparation before the Mono Q column step. In (A), a control tube without any addition is also shown ('–'), and in (C) the positions of molecular weight size markers are indicated on the left (lane LMW) and on the right (lane not shown). The position of the RNA transcript is shown by an arrow (in A) and that of HA1–Rrn3p is shown by an asterisk (in B and C).

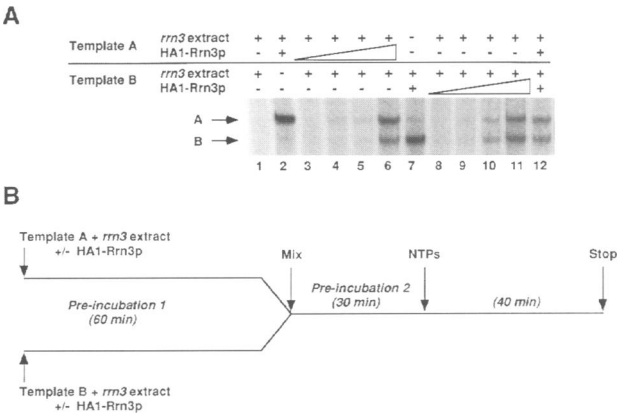

Fig. 5. Rrn3p is not part of a committed template–factor complex that is stable through multiple rounds of transcription. Two different rDNA templates, A and B, were incubated separately with buffer (template B in lane 2, and template A in lane 7), or with equal amounts of Δ*rrn3* extract with or without supplement of increasing amounts of affinity-purified HA1–Rrn3p to one of the templates, as indicated. After a 60 min pre-incubation, the two extract–template mixtures were combined in a new tube and incubated for an additional 30 min (pre-incubation 2). Transcription was initiated by the addition of nucleoside triphosphates (NTPs). RNA products were analyzed by urea–PAGE. An autoradiogram is shown in (**A**) and an outline of the experiment is shown in (**B**). The reaction mixtures contained 0 (lane 1), 0.5, 1, 2, and 4 μl each (lanes 3–6 and 8–11) of affinity-purified HA1–Rrn3p. Lanes 2 and 7 contained 4 μl each, and each pre-incubation in lane 12 contained 2 μl of HA1–Rrn3p. Lane 1 shows that, in the absence of Rrn3p, there is no transcription from either template. Lanes 2 and 7 show that the template that was pre-incubated (pre-incubation 1) with the Δ*rrn3* extract (and HA1–Rrn3p) was transcribed preferentially and the other template that was not pre-incubated with the Δ*rrn3* extract (and HA1–Rrn3p) was transcribed only slightly, indicating that pre-incubation of a template with extracts allows formation of a stable pre-initiation complex. Lane 12 is a positive control, showing that both templates, when pre-incubated with the Δ*rrn3* extract (and HA1–Rrn3p), were able to be transcribed equally.

Rrn3p is not part of a committed template–factor complex that is stable through multiple rounds of transcription

As shown in previous studies with crude yeast extracts or partially purified fractions (Kulkens *et al.*, 1991; Riggs *et al.*, 1995) and with *rrn6* mutant extracts supplemented with the Rrn6/7 complex (Keys *et al.*, 1994), the first step of transcription of rDNA *in vitro* is formation of a stable pre-initiation complex. Protein factors bind stably to the rDNA promoter prior to the formation of the first phosphodiester bond by Pol I. Once bound to the promoter, these factors remain bound throughout multiple rounds of transcription, i.e. polymerase recruitment, initiation, elongation, termination and subsequent re-initiation. Thus, when an excess of template is incubated with the protein factors to allow formation of a stable initiation complex, a second template subsequently added fails to be transcribed, i.e. the first template is committed to transcription. We have found recently that incubation of purified UAF with a template forms a stable complex, and that extracts which are specifically deficient in UAF fail to do so, i.e. UAF is necessary and sufficient for template commitment (Keys *et al.*, 1996). We have also shown previously that the Rrn6/7 complex itself fails to form a stable complex with template DNA, but joins the stable pre-initiation complex with the help of other factor(s) [UAF and possibly other factor(s)] (Keys *et al.*, 1994). Using purified Rrn3p, we first carried out template commitment experiments and found that Rrn3p alone does not form a stable complex with template DNA (data not shown), consistent with the conclusion that UAF is necessary and sufficient for template commitment. The following experiments were then carried out to see whether Rrn3p, like the Rrn6/7 complex, joins the stable pre-initiation complex and becomes unavailable to subsequently added template, according to the scheme shown in Figure 5B.

Two different templates were pre-incubated separately and simultaneously with Δ*rrn3* extract to allow initiation factors from the extract to bind stably to both templates. These two templates can be distinguished by the sizes of their transcripts. To one template, increasing (and subsaturating) amounts of HA1–Rrn3p were added. After 1 h, the two pre-incubating mixtures were combined, incubated together for 30 min, and the transcription reaction was started by the addition of nucleoside triphosphates. If Rrn3p is part of the stable pre-initiation complex, then only the template pre-incubated with Rrn3p will be transcribed. If Rrn3p does not bind stably to the complex,

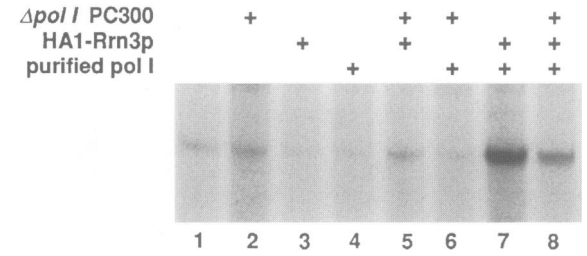

Fig. 6. Pre-incubation of Rrn3p with purified Pol I leads to stimulation of transcription. Three components, PC-300 fraction from NOY446 (ΔPol I PC-300), affinity-purified HA1–Rrn3p and purified Pol I (~3 μg) were pre-incubated in 17.5 μl of buffer, individually or in combination as indicated, for 60 min, then supplemented with template, Δ*rrn3* D-300 fraction, and the components omitted in the first incubation. After an additional 30 min incubation, the transcription assay was carried out (in 40 μl reaction mixtures). Because of the presence of ammonium sulfate in the purified Pol I preparation used, the final reaction mixtures contained 15 mM ammonium sulfate in addition to salts in the standard assay mixture. RNA products were analyzed by urea–PAGE. An autoradiogram is shown.

then Rrn3p will be free to act on both templates and both will be transcribed. The latter result was obtained (Figure 5A, lanes 3–6, 8–11; see the figure legend for various control lanes). Thus, Rrn3p is not part of the pre-initiation complex that is stable through multiple rounds of transcription.

Rrn3p interacts with a purified RNA polymerase I preparation

In the course of template commitment experiments (data not shown; see above), we observed that longer pre-incubation of Δ*rrn3* extracts and template in the presence of HA1–Rrn3p gave greater transcriptional activity than when Rrn3p was first omitted and added subsequently in the second short pre-incubation. We studied this phenomenon in more detail. First, we found that pre-incubation of Δ*rrn3* extracts and HA1–Rrn3p without the DNA template also stimulates transcription (data not shown). Second, we found that HA1–Rrn3p incubated alone or with bovine serum albumin has no stimulatory effect (data not shown), suggesting that the stimulation is a result of an interaction of Rrn3p with some component(s) in the extract. Since Δ*rrn3* extract is made up of two components, PC-300 and D-300 fractions, we examined which of these fractions is responsible for this pre-incubation phenomenon, and found that pre-incubation of Rrn3p with *rrn3* PC-300, but not with *rrn3* D-300, showed the stimulation (data not shown).

As stated earlier in this paper, *rrn3* PC-300 contains Pol I and another factor(s) required for rDNA transcription (our unpublished experiments). To see if Rrn3p was interacting with Pol I itself, the experiment was repeated using three components for pre-incubation, a purified Pol I preparation ('Pol I'), PC-300 from a Pol I-deficient mutant strain (NOY446) carrying a disrupted *rpa135* gene ('ΔPol I PC-300') and HA1–Rrn3p. Various combinations of the three components were pre-incubated for 1 h, after which *rrn3* D-300, template and the omitted components were added, and the mixtures were incubated for another 30 min before nucleoside triphosphates were added to start the transcription reaction. Activity was greatly stimulated by pre-incubation of the purified Pol I and HA1–Rrn3p (Figure 6, lane 7 compared with lane 1 without pre-incubation, and with other lanes). We found that the degree of stimulation ranged from 15- to 20-fold when quantitated in several experiments. It should be noted that the purified Pol I preparation may not be completely pure (see Materials and methods), but pre-incubation of Rrn3p with PC-300 from the Pol I-deficient mutant (ΔPol I PC-300) did not give stimulation (Figure 6, lane 5 compared with lane 2), while pre-incubation of Rrn3p with PC-300 from the wild-type strain gave a large stimulation, as mentioned above, indicating that the factor interacting with Rrn3p is absent as a consequence of the deletion of the A135 subunit of Pol I. [ΔPol I PC-300 actually showed partial inhibition of the activation caused by mixed incubation of Rrn3p with purified Pol I (lane 8 compared with lane 7). We think that this inhibition is probably non-specific, because the addition of D-300 (instead of ΔPol I PC-300) to the mixture of Rrn3p and purified Pol I also caused partial inhibition (data not shown).] These results indicate that Rrn3p interacts with the purified Pol I preparation, presumably with Pol I itself, leading to stimulation of transcription. However, attempts to demonstrate a direct physical interaction between Rrn3p and Pol I by co-immunoprecipitation or sedimentation analysis on a glycerol gradient have been unsuccessful (our unpublished experiments). Therefore, the physical interaction between the two, which has been inferred from their functional interaction, appears to be weak.

We have compared transcription of a non-specific DNA template, calf thymus DNA, using Pol I with and without pre-incubation with HA1–Rrn3p. No significant stimulation (or inhibition) of the rate of non-specific transcription was observed after pre-incubation of Pol I with Rrn3p, relative to a control without pre-incubation (data not shown). Therefore, stimulation of rDNA transcription by pre-incubation of Pol I with Rrn3p is specific, suggesting that the stimulation is perhaps at the initiation step. Single-round transcription experiments described in the next section confirm this suggestion. We also note that, in similar non-specific transcription experiments using calf thymus DNA as template, Killeen and Greenblatt (1992) showed that RAP30–glutathione-*S*-transferase fusion protein, but not glutathione-*S*-transferase, inhibited non-specific transcription by Pol II. Thus, Rrn3p appears to be different from the RAP30 subunit of TFIIF in this respect (see Discussion).

Rrn3p is required for efficient transcription initiation by RNA polymerase I

The experimental data described above indicate that Rrn3p is a transcription factor for Pol I and interacts with Pol I prior to transcription initiation, but is not stably bound to a committed pre-initiation complex at the promoter through multiple rounds of transcription. However, the data do not distinguish whether the function of Rrn3p is required for transcription initiation or for efficient elongation (e.g. 'anti-pausing' function). To study this question, we examined whether a large stimulation of transcription observed with the pre-incubation of Rrn3p with the (Pol I-containing) Δ*rrn3* PC-300 is a result of stimulation of initiation of transcription. Single-round transcription experiments using the detergent Sarkosyl were carried out for this purpose. Previous work has shown that a suitable concentration of Sarkosyl, when added after the formation of an initiated

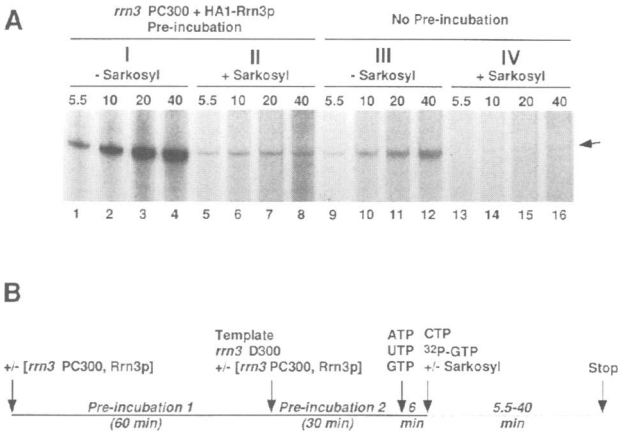

Fig. 7. Pre-incubation of Rrn3p with Δ*rrn3* PC-300 increases the *in vitro* transcriptional efficiency assayed in a single-round transcription in the presence of Sarkosyl. Affinity-purified HA1–Rrn3p (20 μl) and Δ*rrn3* PC-300 (~30 μg protein) were mixed in 87.5 μl of buffer and, either directly or after pre-incubation for 60 min, added to buffer containing template (1 μg) and Δ*rrn3* D-300 (~90 μg protein). After 30 min of incubation (pre-incubation 2), transcription was initiated by addition of the first three templated nucleotides, ATP, UTP and GTP. After 6 min, the fourth templated nucleotide, CTP, and [^{32}P]GTP were added together with Sarkosyl (0.025%) and incubated. Total reaction volume was 200 μl. Equal volumes (40 μl) were removed from the reaction and stopped with EDTA and phenol at 5.5, 10, 20 and 40 min. RNA products were analyzed by urea–PAGE. An autoradiogram is shown in (**A**) and an outline of the experiment is shown in (**B**). In the original autoradiogram, lanes 13–16 showed faint but clear transcripts (positions indicated by an arrow), which became clearer upon longer exposure. The intensity of the bands showed that the transcript increased slightly between 5.5 and 10 min, and then remained about the same thereafter. It should be noted that the sequence of correct transcripts starts with AUGC, and hence the first three nucleotide substrates were added before Sarkosyl (and [^{32}P]GTP) addition in this experiment. In other experiments, all the four nucleoside triphosphates were added simultaneously with Sarkosyl and the same results were obtained. In other experiments, template and Δ*rrn3* D-300 were pre-incubated separately for 30 min to allow formation of a stable, committed pre-initiation complex before mixing with a pre-incubated mixture of Δ*rrn3* PC-300 plus Rrn3p. Similar results were obtained. As stated in the text, addition of the same concentration of Sarkosyl at the time of mixing of the two pre-incubated mixtures, Δ*rrn3* PC-300 plus Rrn3p and template plus Δ*rrn3* D-300, abolished transcription completely, indicating that the concentration of Sarkosyl used was sufficient to inhibit recruitment of 'activated' Pol I (or Pol I plus Rrn3p) to a committed pre-initiation complex consisting of DNA template and other components in D-300 (including UAF).

transcription complex, has no effect on elongation, but prevents re-initiation of the terminated polymerase, thus allowing only a single round of transcription (Hawley and Roeder, 1987; Schnapp and Grummt, 1991).

As described schematically in Figure 7B, a mixture of HA1–Rrn3p and Δ*rrn3* PC-300 (containing Pol I) were added to Δ*rrn3* D-300 and template either after 1 h of pre-incubation (reactions I and II) or without pre-incubation (reactions III and IV). The mixtures were incubated for 30 min (second pre-incubation), followed by the addition of ATP, UTP and GTP to allow initiation of transcription, but not extension of transcripts beyond the trinucleotide stage (the correct transcripts start with AUGC; see legend to Figure 7). After 6 min, CTP and [^{32}P]GTP were added simultaneously with Sarkosyl (final concentration, 0.025%; reactions II and IV) or H_2O (reactions I and III). [This concentration of Sarkosyl, if added before the start of the second pre-incubation, was sufficient to inhibit transcription completely (data not shown; see also figure legend).] The transcription reaction was allowed to proceed for 5.5, 10, 20 and 40 min. In the absence of Sarkosyl, formation of the transcript increased steadily from 5.5 to 40 min, the activity being consistently greater with pre-incubation (Figure 7A, lanes 1–4 compared with 9–12). With pre-incubation, and in the presence of Sarkosyl, transcript formation leveled off at some time between 5.5 and 10 min (Figure 7A, lanes 5–8). This is consistent with what we expect from a single round of transcription. Without pre-incubation of Rrn3p with Δ*rrn3* PC-300, a transcript was seen only faintly in the presence of Sarkosyl (Figure 7A, lanes 13–16; see the legend). These results strongly suggest that pre-incubation of HA1–Rrn3p with Pol I in Δ*rrn3* PC-300 increased the number of transcription-competent pre-initiation complexes relative to the reaction mixtures without such pre-incubation, resulting in a large difference in the number of complete transcripts produced in a single round of transcription. If Rrn3p does not play any role in initiation of transcription, i.e. all the reaction mixtures had the same number of initiation complexes at the time of [^{32}P]GTP (and Sarkosyl) addition, one would expect a slow but eventual appearance of the transcript even in the absence of re-initiation in the reaction without pre-incubation (reaction IV), which was not the case (lanes 13–16; see the legend). This observed failure of appearance of a significant amount of the transcript in reaction IV cannot be explained by an inadequate amount of 'elongation-competent' Pol I due to the absence of pre-incubation, because a significant amount of transcript was produced even at 5.5 min in the absence of pre-incubation of Rrn3p with Pol I, when re-initiation of transcription was allowed by the absence of Sarkosyl (reaction III; Figure 7A, lane 9). We also note that no accumulation of radioactive RNA bands or smears with smaller sizes were observed in autoradiograms for samples without pre-incubation (reactions III and IV) relative to samples from reaction mixtures with pre-incubation (reactions I and II), that might be expected if stimulation obtained with pre-incubation is due to increased efficiency of the elongation step (data not shown).

Discussion

We have purified the protein encoded by *RRN3*, Rrn3p, as an epitope-tagged protein, HA1–Rrn3p, and demonstrated that it is an essential transcription factor required for rDNA transcription by Pol I *in vitro*. This conclusion fully confirms the previous results obtained from *in vivo* experiments that the *RRN3* gene is an essential gene, but becomes dispensable if rDNA transcription is achieved by Pol II by genetic manipulations, and that *rrn3* mutants are specifically defective in Pol I transcription under restrictive conditions (Nogi *et al.*, 1991).

Our experiments strongly suggest that Rrn3p is not part of the stable pre-initiation complex and, therefore, must play a role(s) distinct from the Rrn5/9/10 complex (UAF) or the Rrn6/7 complex. It was found that Rrn3p interacts with Pol I in the absence of DNA template and stimulates transcription upon subsequent mixing with rDNA transcription mixtures. We do not know how this stimulation takes place. We have found that the degree of stimulation of transcription observed by pre-incubation of Rrn3p with

purified Pol I increases with the time of pre-incubation, reaching a maximum after 2–3 h incubation at 22°C (data not shown). Thus, the reaction, which takes place during pre-incubation and is responsible for stimulation of transcription, is rather slow under the conditions used. One possibility is that the interaction causes a conformational change of Pol I, increasing the efficiency of its loading to the template–factor complex at the core promoter. However, it is also possible that the 'activated' Pol I (or Pol I–Rrn3p complex) has a higher efficiency in melting the DNA template to form an 'open complex,' or in catalyzing the first phosphodiester bond or in stimulating 'promoter clearance.' In addition, there is a possibility that the activated Pol I has a higher efficiency in chain elongation. Our single-round transcription experiments using the detergent Sarkosyl have shown that pre-incubation of Pol I with Rrn3p apparently increased the amount of a transcription-competent pre-initiation complex, thus favoring the model of stimulation of an initiation step(s). It should be noted that Rrn3p stimulates formation of a pre-initiation complex and yet, as mentioned above, does not remain as part of a stable pre-initiation complex. It is possible that Rrn3p might be part of a pre-initiation complex, but is released very soon after the start of transcription (or is released together with Pol I upon transcription termination) and is utilized by different (competing) templates, i.e. Rrn3p is not part of a complex that is stable through multiple rounds of transcription. This and related questions are under current study.

Rrn3p interacts with Pol I in the absence of template DNA and stimulates recruitment of Pol I, but does not remain as part of stable pre-initiation complex. In these respects, Rrn3p appears to resemble TFIIF, specifically the Rap30 subunit, studied in metazoan Pol II transcription systems. It is known that TFIIF, which consists of two subunits, Rap30 and Rap74, has an affinity for Pol II and stimulates loading of Pol II onto promoter–factor complexes [e.g. a promoter that has bound TFIID (or TBP) and TFIIB], and that it is released from the template soon after transcription initiation, although it has the ability to reassociate with Pol II in an elongation complex stalled at pausing sites, presumably carrying out the second function, i.e. stimulation of passage through the pause sites (Buratowski, 1994; Zawel and Reinberg, 1995; Zawel *et al.*, 1995). This latter property of TFIIF, the role in elongation, appears to be absent for Rrn3p; although our experiments do not rigorously exclude the possibility of such a role for Rrn3p, we have so far failed to observe any indication of stimulation of elongation, as described in the Results section. In addition, as also mentioned in the Results section, we carried out experiments similar to those done by Killeen and Greenblatt (1992) and examined the question of whether stimulation of specific transcription initiation caused by pre-incubation of Rrn3p with Pol I is accompanied by suppression of non-specific initiation, but no evidence for such suppression was obtained. Thus, while Rrn3p resembles TFIIF, there are also differences between the two.

Using a mouse Pol I transcription system, Grummt and co-workers have shown that rDNA transcription requires two factors, TIF-IA and TIF-IC, in addition to UBF, TIF-IB (SL1) and Pol I (Schnapp and Grummit, 1991). Although TIF-IC has not been purified, its properties resemble Rrn3p. TIF-IC, as judged by its activity, was shown to interact with Pol I in the absence of template (Schnapp *et al.*, 1994) and apparently joins the pre-initiation complex only after prior binding of TIF-IB (SL1), UBF and Pol I (Schnapp and Grummt, 1991). They have also shown that TIF-IC is not only required for the formation of Sarkosyl-resistant initiation complexes, but also plays a role in elongation by suppressing pausing of Pol I, thus resembling Pol II-specific factor TFIIF (Schnapp *et al.*, 1994). As mentioned above, we have not observed any evidence for suppression of pausing by Rrn3p in our preliminary studies. TIF-IA was identified as a protein that complements transcriptionally inactive extracts obtained from quiescent mouse cells (Schnapp *et al.*, 1993), and may correspond to factor C* studied by Sollner-Webb and co-workers (Brun *et al.*, 1994). We have not studied the question of whether Rrn3p plays any role in growth-dependent regulation of rDNA transcription in yeast cells.

Since the genes for TIF-IC and TIF-IA (or C*) have not been cloned, we cannot compare the sequence of Rrn3p with these Pol I transcription factors from mouse cells. We have compared the Rrn3p sequence with sequences of subunits of TFIIF, Rap30 in particular, both from mammalian cells (Aso *et al.*, 1992; Finkelstein *et al.*, 1992; Sopta *et al.*, 1989; Horikoshi *et al.*, 1991) and yeast (Henry *et al.*, 1994). No significant similarity that would suggest homologous functions was observed in any of these sequence comparisons. Thus, apparent functional similarity, e.g. between Rrn3p and the Rap30 subunit of mammalian TFIIF, is not apparent in their primary sequences. Rap30 is the smaller subunit of TFIIF that is responsible for interaction with Pol II, and its functional resemblance to bacterial σ factors has been emphasized both in relation to their interactions with core polymerases and their interaction with template DNA (Sopta *et al.*, 1989; McCracken and Greenblatt, 1991; Tan *et al.*, 1994). In fact, the presence of limited sequence homology between the two has been reported both in their polymerase binding domains (Sopta *et al.*, 1989; McCracken and Greenblatt, 1991) and DNA binding domains (Tan *et al.*, 1994). In this connection, we note that Rrn3p interacts *in vitro* with the A49 subunit strongly and with the A34.5 subunit weakly, but not with other yeast Pol I subunits tested (D.Lalo, J.S.Steffan and M.Nomura, unpublished experiments). Both the A49 and A34.5 subunits are unique to Pol I and not shared by Pol II or Pol III. If these *in vitro* interactions are relevant to the functional interaction of Rrn3p with Pol I revealed by the large stimulation of transcription activity observed after pre-incubation of the two, such interaction might involve mostly a structure unique to Pol I. Thus, we might not expect similarity in primary sequence between Rrn3p and its possible functional homologs in the Pol II systems.

In summary, we have demonstrated that Rrn3p is a Pol I-specific transcription factor; it interacts with Pol I, causing an apparent stimulation of formation of a pre-initiation complex, and plays an essential role(s) in rDNA transcription. This transcription factor is distinct from the 14 subunits of Pol I as well as two other yeast transcription initiation factors, UAF and the Rrn6/7 complex. Rrn3p has now been purified and its gene is available. Thus, future study should be able to answer questions regarding

Table I. Yeast strains and plasmids used

Strain or plasmid	Description
Strains	
NOY388	*MAT***a** *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,11 can1-100*
NOY397	*MAT***a**/α *ade2-1/ade2-1 ura3-1/ura3-1 his3-11/his3-11 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/can1-100*
NOY446	*MAT*α *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rpa135Δ::LEU2* pNOY102 (Nogi *et al.*, 1991)
NOY604	*MAT*α *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn3Δ::HIS3* pNOY103
NOY657	*MAT*α *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn3Δ::HIS3* pNOY333
NOY675	*MAT***a** *ade2 ade3 leu2 ura3 trp1 his can1 rrn3-1* pNOY103
Plasmids	
pRS316	*E.coli*–yeast shuttle vector carrying *CEN6 ARSH4 URA3* (Sikorski and Hieter, 1989)
pNOY102	high copy number plasmid carrying *GAL7*–35S rDNA, *URA3*, 2 μ, *amp* (Nogi *et al.*, 1991)
pNOY103	high copy number plasmid carrying *GAL7*–35S rDNA, *ADE3*, *URA3*, 2 μ, *amp* (Nogi *et al.*, 1991)
pNOY202	YEp351 (Hill *et al.*, 1986) derivative with 2.7 kb *Bam*HI–*Xba*I fragment carrying *RRN3*
pNOY333	a derivative of pRS315 (*CEN6 ARSH4 LEU2*; Sikorski and Hieter, 1989) with a fragment carrying HA1 epitope-tagged *RRN3* inserted between the *Bam*HI and *Xba*I sites (the first two amino acids in the *RRN3* coding region, MM, is mutated to AG)
pNOY3228	pUC19 derivative with a fragment carrying *rrn3Δ::HIS3* between the *Bam*HI and *Xba*I sites
pSIRT	a derivative of pEMBL Ye30-Δ6 that carries the mini rDNA gene called SIRT (Musters *et al.*, 1989)
pSIRTΔ+39/+128	a derivative of pSIRT (Musters *et al.*, 1989; Kulkens *et al.*, 1991; see Materials and methods)

the exact function(s) of Rrn3p in rDNA transcription by Pol I in yeast cells.

Materials and methods

Strains, plasmids, gene cloning and genetic methods

Yeast strains and plasmids used in this study are listed in Table I. Standard media and genetic methods were used as described previously (Keys *et al.*, 1994). Strain NOY675 carrying the *rrn3-1* mutation (isolation number *416*) was used to clone the *RRN3* gene by complementation. The yeast genomic library based on the YCpNI vector (Nakayama *et al.*, 1985) was used for this purpose. DNA sequence was determined by the dideoxy method with the Tag Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Strains carrying disrupted *rrn3* were constructed as follows (see Figure 1). The 2.7 kb *Bam*HI–*Xba*I fragment present in pNOY202, which carries *RRN3*, was subcloned into pUC19 between *Bam*HI and *Xba*I. DNA of the resultant plasmid was digested with *Bst*EII and *Msc*I, and 479 bp of the *RRN3* coding region were replaced by a 0.9 kb *Bam*HI fragment carrying the *HIS3* gene. Ligation was carried out after filling DNA ends with Klenow DNA polymerase. The resultant plasmid (pNOY3228) was digested with *Spe*I and *Xba*I; the fragment containing the *rrn3Δ::HIS3* was transformed into a diploid strain (NOY397) and His^+ transformants were selected. One such transformant (NOY597) was confirmed by tetrad analysis to carry the expected *rrn3Δ::HIS3* on one chromosome and was used to construct a haploid strain (NOY604) carrying *rrn3Δ::HIS3* on the chromosome and the p*GAL7–35S* rDNA on a plasmid (pNOY103).

NOY657 carrying the gene encoding HA1 epitope-tagged Rrn3p on a plasmid (pNOY333) was constructed in the following way. First, mutagenesis was carried out to attach the HA1 epitope from influenza virus (Wilson *et al.*, 1984), YPYDVPDYA, to the amino-terminus of Rrn3p. The 40 bp oligonucleotide, G CCT CAT *ATG* TAC CCA TAC GAC GTC CCA GAC TAC GCT AGC (*ATG* is the new start codon), which encodes MYPYDVPDYAS, was introduced immediately in front of the ATG start codon of *RRN3* by oligonucleotide-directed mutagenesis (Kontis and Goldin, 1993). A derivative of M13mp18 carrying *RRN3* in the entire 2672 bp *Bam*HI–*Xba*I fragment was used as template, and a 71 base oligonucleotide containing the above 40 nucleotide sequence flanked by *RRN3* sequences was used as a mutagenesis primer. The new *Bam*HI–*Xba*I fragment (2712 bp) was subcloned into the *Bam*HI–*Xba*I sites of pRS316, leading to pNOY218. pNOY333 was then constructed by mutating the first two amino acids of the original Rrn3p, Met Met, to Ala Gly using a similar mutagenesis method. A derivative of M13mp18 carrying the template strand of the HA1-tagged *RRN3* gene constructed above was used as template, and the 35 base oligonucleotide [GT ATT CTC AAA AGC *GCC GGC* GCT AGC GTA GTC TGG (the opposite strand of *GCC GGC* corresponding to the sequence coding for Ala Gly)] as a mutagenesis primer. A derivative of pRS316 carrying this substitution mutation was then constructed as above, leading to pNOY333. Thus, the mutant protein encoded by this plasmid is different from Rrn3p in two respects: the first two amino acids MM are altered to AG, and 11 amino acids, MYPYDVPDYAS, are added in front of these two amino acids, AG. For convenience sake, herein we call the mutant hybrid gene 'HA1–*RRN3*' and the encoded mutant protein 'HA1–Rrn3p'.

Transcription assays

In vitro reactions were performed as described previously (Keys *et al.*, 1994). Two circular supercoiled DNA templates, pSIRT and pSIRTΔ+39/+128, were used (Musters *et al.*, 1989; Kulkens *et al.*, 1991). pSIRT carried a mini-rRNA gene called SIRT, which has both the promoter and the transcription terminator regions. This template gives a specific transcript of ~765 nucleotides (Keys *et al.*, 1994), and was used as template A in the experiments described in Figure 5. pSIRTΔ+39/+128 carries a 90 bp deletion (and an addition of 15 bp derived from the *Bal*31 cassette; Musters *et al.*, 1989; Kulkens *et al.*, 1991) downstream from the promoter, making the size of the specific transcript ~690 nucleotides, and was used as template B in Figure 5, and as template in all other experiments. 'Crude extracts' used for transcription assay consisted of PC-300 fraction (4–8 μg) and D-300 fraction (15–20 μg) and, together with template (200 ng) and other additions, the final reaction volume was 40 μl. Reactions were carried out for 40 min at room temperature (~22°C). RNA was recovered and analyzed by 5% urea–PAGE followed by autoradiography (Keys *et al.*, 1994). For *rrn3* extracts, D-300 from either NOY675 (*rrn3-1*) (for the experiment shown in Figure 3) or D-300 from NOY604 (*rrn3Δ::HIS3*) (for all other experiments) was used in combination with PC-300 from NOY604. Pol I used in the experiments shown in Figure 6 was purified according to a method described previously (Riva *et al.*, 1982). Peak fractions obtained after the final glycerol gradient steps were pooled and stored at −70°C before experimental use.

Purification of HA1–Rrn3p

Immunoaffinity purification of HA1–Rrn3p from NOY657 was carried out essentially as previously described for Rrn6/7p complex (Keys *et al.*, 1994). Extract (~200 ml from 120 g of cells) was mixed with ~5 ml of protein G–Sepharose carrying anti-HA1 antibodies and gently rolled on a roller drum at 4°C for 2 h. Antibody beads were washed twice with 100 ml of TA300/10 buffer [TA buffer (Tris–acetate/EDTA/Mg^{2+} buffer containing protease inhibitors; see Keys *et al.*, 1994) supplemented with 300 mM KCl, 10% glycerol, and 0.1% Tween 20], once with 30 ml of TA300/10 buffer, and twice with 30 ml TA200/10 buffer (TA buffer containing 200 mM KCl, 10% glycerol and 0.1% Tween 20). Antibody beads were then mixed with 5 ml of TA200/10 buffer containing 1 mg/ml HA1 peptide and left at room temperature for 30 min to elute HA1–Rrn3p. Antibody beads were recovered, and the elution step was repeated. Eluates were combined and loaded on an 8 ml Mono Q column (Pharmacia) at 1 ml/min. Proteins were eluted by a linear gradient from TA200/10 buffer (without Tween 20) to TA buffer containing 500 mM KCl and 10% glycerol in 15 min at 1 ml/min. One ml fractions were collected. To each fraction, proteinase inhibitors were added at the final

concentrations listed: 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml each of pepstatin, bestatin, aprotinin, chymostatin, leupeptin and antipain. Fractions were stored at −70°C. For experiments shown in Figures 3, 5, 6 and 7, immunoaffinity purification was done on a small scale using an elution buffer which is TA buffer containing 200 mM potassium glutamate, 10% glycerol and 1 mg/ml HA1 peptide, and preparations without Mono Q column chromatography were used.

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