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DNA Binding by the Ribosomal DNA Transcription Factor Rrn3 Is Essential for Ribosomal DNA Transcription*

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Background: Transcription initiation by RNA polymerase I requires protein-protein interactions between Rrn3, polymerase, and core factors.
Results: Mutagenesis of a putative DNA binding domain in Rrn3 had no effect on essential protein-protein interactions, but abrogated DNA binding and inactivated Rrn3 function in transcription.
Conclusion: DNA binding is essential for Rrn3 to function in transcription.
Significance: DNA binding by Rrn3 may provide an additional target to regulate rDNA transcription.

The human homologue of yeast Rrn3 is an RNA polymerase I-associated transcription factor that is essential for ribosomal DNA (rDNA) transcription. The generally accepted model is that Rrn3 functions as a bridge between RNA polymerase I and the transcription factors bound to the committed template. In this model Rrn3 would mediate an interaction between the mammalian Rrn3-polymerase I complex and SL1, the rDNA transcription factor that binds to the core promoter element of the rDNA. In the course of studying the role of Rrn3 in recruitment, we found that Rrn3 was in fact a DNA-binding protein. Analysis of the sequence of Rrn3 identified a domain with sequence similarity to the DNA binding domain of heat shock transcription factor 2. Randomization, or deletion, of the amino acids in this region in Rrn3, amino acids 382–400, abrogated its ability to bind DNA, indicating that this domain was an important contributor to DNA binding by Rrn3. Control experiments demonstrated that these mutant Rrn3 constructs were capable of interacting with both rpa43 and SL1, two other activities demonstrated to be essential for Rrn3 function. However, neither of these Rrn3 mutants was capable of functioning in transcription *in vitro*. Moreover, although wild-type human Rrn3 complemented a yeast rrn3-ts mutant, the DNA-binding site mutant did not. These results demonstrate that DNA binding by Rrn3 is essential for transcription by RNA polymerase I.

The biogenesis of the basic protein synthetic machinery is an energetically costly process that is tightly regulated (1–6). A significant fraction of these processes is involved in the synthesis and processing of the RNA backbones of the 40 S and 60 S ribosomal subunits, the 18 S and 5.8 S and 28 S rRNAs, respectively. The synthesis of rRNA is regulated at both the transcription initiation and elongation steps (1, 2, 5).

The eukaryotic ribosomal RNA genes are transcribed by RNA polymerase I (Pol I).[3] The recruitment of RNA polymerase I to the transcription start site is the result of a series of protein-protein and protein-DNA interactions between a limited number of defined transcription factors (1–4). For example, the stable binding of the transcription factors to the rDNA promoter requires the coordinate binding of factors to the core and upstream promoter elements (for review, see Ref. 1). In yeast, two multisubunit complexes, core factor and upstream activating factor, which bind to the core promoter and to the upstream element, respectively (6, 7), are required to commit the yeast rDNA promoter. Both core factor and upstream activating factor interact specifically with TATA-binding protein (8, 9). In mammals, two known transcription factor function homologously to core factor and upstream activating factor. These factors are required to efficiently commit the rDNA promoter (9–13); SL1, containing TATA-binding protein (TBP) and TBP-associated factors (TAFs), and UBF, a multiple HMG box containing architectural protein.

The committed template is a complex containing two molecules of UBF bound as a dimer to the upstream promoter element and at least one molecule of SL1 bound to the core promoter element. The cooperative interaction between these factors generates a stable committed template. The binding of SL1 to the core promoter element is necessary and sufficient for transcription *in vitro*. The binding of UBF (14–16) and possibly a second molecule of SL1 to the upstream promoter element is required for template commitment and efficient transcription *in vitro*. Both SL1 and UBF are subject to regulation via phosphorylation and acetylation (17–22). In addition, Rb, the protein product of the retinoblastoma susceptibility gene, interacts with UBF and prevents UBF-dependent activation of rDNA

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[3] The abbreviations used are: Pol I, RNA polymerase I; hRrn3, human Rrn3; CHX, cycloheximide; SL1, selectivity factor I; TAF, TBP-associated factor; TAF_I68, 68-kDa subunit of SL1; UBF, upstream binding factor; rpa43, mouse homologue of yrpa43, the 43-kDa subunit of RNA polymerase I.

transcription (23). In contrast, SV40 large T antigen activates Pol I transcription by interacting with SL1 (24).

The mechanism by which RNA polymerase I is recruited to the committed template is still not completely understood. It has been reported that RNA polymerase I can interact with both UBF and SL1 (25, 26). It was established (27) that only ~2% of the RNA polymerase I molecules in exponentially growing yeast cells are capable of promoter-specific transcription. These competent RNA polymerase I molecules, both in yeast and in mammalian cells, were found to contain core RNA polymerase I subunits and Rrn3, a polymerase-associated factor.

Both genetic and biochemical experiments have demonstrated that yeast Rrn3 is essential for rDNA transcription. The human homologue has been cloned (28) and subsequently identified as the previously described transcription initiation factor IA (TIFIA) (29). Current models suggest that Rrn3 acts as a bridge between RNA polymerase I and the committed rDNA promoter (30–34). A direct interaction between the 43-kDa subunit of RNA polymerase I (rpa43) and Rrn3 in the Rrn3-Pol I complex was confirmed (32, 33) as was the direct interaction of human Rrn3 with the TAF_I110 and TAF_I68 subunits of species-specific transcription factor SL1 (33, 34). This has led to the model that Rrn3 functions in the recruitment of RNA polymerase I to the committed template (33, 34) and that the essential role for Rrn3 is to link RNA polymerase I to SL1 (*e.g.* Ref. 34). Rrn3p associates directly with the A43 subunit of Pol I to render the polymerase competent for transcription initiation (32). The role of Rrn3p in Pol I transcription initiation may be comparable with that of prokaryotic sigma factors; however, unlike sigma factors, Rrn3p has not previously been reported to bind DNA.

In the course of studying the role of Rrn3 in the recruitment of RNA polymerase I to the committed template, we found that Rrn3 itself is a DNA-binding protein. *In silico* analysis of the sequence of Rrn3 revealed a domain, amino acids 382–400, with weak identity to the DNA binding domain of heat shock transcription factor 2 that was likely to be a helix-turn-helix. Randomization or deletion of the amino acid sequence of the putative DNA binding domain of Rrn3 abrogated its ability to bind to DNA. Additional experiments demonstrated that these mutations did not significantly affect the ability of Rrn3 to interact with either RNA polymerase I (rpa43) or TAF_I68. This in turn suggests that mutation of the DNA binding domain did not result in a grossly misfolded protein. However, these mutants were unable to activate rDNA transcription. Hence, our data indicate that Rrn3 has multiple functions in rDNA transcription and that its ability to interact with the rDNA is essential to its ability to function in transcription.

MATERIALS AND METHODS

Cell Culture and Transfection—3T6 cells were grown in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% fetal bovine serum (Atlanta Biologicals). Mouse FM3A cells were grown in RPMI (Cellgro) supplemented with 10% fetal bovine serum (Atlanta Biologicals). To inhibit rDNA transcription, the cells were grown to a density of 2×10^6 cells/ml without changing the media. For transfection, 3T6 cells were plated at a density of 3×10^5 cells per 60-mm plate. Approximately 6 h after plating, cells were transfected with a total of 6 μg of DNA (the vector(s) expressing the required protein and pUC 19) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. When cells were cotransfected with vectors expressing Rrn3 and rpa43 or TAF_I68, they received 2 μg of Rrn3 in pCDNA3.1 and 4 μg of the vector expressing TAF_I68 or rpa43. After 40 h, the cells were scraped into lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Tween 20, containing protease inhibitors (Complete; Roche Applied Science)) and used immediately. Rat N1S1 cells were grown in RPMI1640 + 5% horse serum and 1% fetal bovine serum (35). Where indicated, cells were treated with 2 μg/ml cycloheximide (Sigma) for 1 h.

Immunopurification of Rrn3 and Coimmunoprecipitation of Rrn3 and rpa43 or TAF_I68—FLAG-tagged Rrn3 was expressed in *Sf*9 cells and purified using anti-FLAG-agarose beads (Sigma) as previously described (33). Exponentially growing 3T6 cells were cotransfected with 2 μg of FLAG-Rrn3 DNA and 4 μg of either V5-tagged rpa43 or TAF_I68 as required. Forty hours after transfection, whole cell lysates were prepared and tumbled with anti-FLAG-agarose for 2 h at 4 °C. The beads were washed 3 times with lysis buffer, and the proteins were eluted with FLAG peptide. The eluted proteins were then analyzed by SDS-PAGE and Western blotting using anti-FLAG (to detect Rrn3; Sigma) and anti-V5-HRP (to detect rpa43 or TAF_I68; Invitrogen).

Western Blot Analysis—SDS-PAGE and electroblotting were carried out as described previously (35). Monoclonal antibodies to FLAG (Sigma) and V5 peptide (Invitrogen) were used as recommended by the suppliers. The antigens were visualized by the enhanced chemiluminescent (ECL) method (Pierce).

Mutagenesis of Rrn3 and Production of Recombinant Rrn3 in Sf9 Cells and Protein Purification—Two mutations of the putative DNA binding domain of human Rrn3 were constructed. In the first, the amino acids FLEHLWKKLQDPSNPAIIR were randomized, resulting in the substitution of KIWFLLPEDNIQHRLSAKP. In the second, the same amino acids were deleted. The deletion mutant was constructed by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). The primers used were 5′-caaattgggattcgcagaggcacaggctgctggaaattatattgg and 5′-ccaatataat ttccagcagcctgtgcctctgcgaatcccaatttg. The substitution mutant of Rrn3 with random amino acids between amino acids 382 and 400 was generated by overlapping PCR. Briefly, the first fragments of Rrn3 were amplified by PCR from a template plasmid (pcDNA3-Rrn3-FLAG) using primers 5′-catcaactggctgctagaattccg (F1) and 5′-aggtttggcactcaacctatgctggatattgtcttcagggagcaaaaaccagattttgcctctgcgaatcccaatttg (R1). The second fragment of Rrn3 was amplified with primers 5′-aaaatctggtttttgctccctgaagacaatatccagcataggttgagtgccaaacctcaggctgctggaaattatattgg (F2) and 5′-gaaccgcgggccctctagactcg (R2). The primers R1 and F2 contain the sequence encoding the randomized amino acids from residue 382 to 400 of Rrn3 (underline). The primers F1 and R2 contain the recognition sites for EcoRI and XhoI (after PCR), respectively. After the resulting PCR products were purified by agarose gel electrophoresis, overlapping PCR was carried out by mixing the two fragments and the primers F1 and R1. The resultant product was purified, digested with EcoRI and XhoI,

and used to replace the wild-type sequence between the EcoRI and XhoI sites in pcDNA3-Rrn3-FLAG. All the amplification reactions were performed using Pfu DNA polymerase (Promega). All mutant constructs were confirmed by DNA sequencing. FLAG-tagged Rrn3 was expressed in *Sf*9 cells and purified using anti-FLAG agarose beads (Sigma) as previously described (33).

In Vitro Transcription—S100 extracts from control or cycloheximide-treated (100 μg/ml) N1S1 cells or from FM3A cells (grown to density of 2×10^6 cells/ml) and nuclear extracts from rat hepatoma cells were prepared essentially as described (35–37). Plasmid pU5.1E/X contains the rat 45 S rDNA (−286 to + 630) promoter. When truncated with EcoRI, the transcript from pU5.1E/X is 632 nucleotides (37). *In vitro* transcription reactions were carried as described previously (37–39).

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were carried out essentially as described previously (40) using 5% non-denaturing polyacrylamide-TAE gels (6.7 mM Tris hydrochloride (pH 7.5), 3.3 mM sodium acetate, 1 mM EDTA) equilibrated for ~2 h at 160 V with recirculating buffer. Samples were prepared during the equilibration. 25 ng of ^{32}P-labeled DNA was incubated for 10 min on ice with or without Rrn3 in a total volume of 30 μl in 20 mM HEPES (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% (v/v) glycerol. After the preincubation, an additional 20 μl of buffer was added to each sample, and the incubation was continued at 30 °C for 30 min. The competition assay was carried out with the addition of cold competitor DNA (as described below) in various molar ratios. After the 30-min incubation, 25 μl of each sample was immediately loaded onto the pre-equilibrated 5% non-denaturing gel. Samples were loaded during electrophoresis and allowed to run at constant voltage for 2 h. The gel was dried at 85 °C for 35 min and placed on a phosphor screen (GE Healthcare) overnight and analyzed with a Storm Phosphor-
Imager and ImageQuant software.

Generation of Labeled DNA for EMSA—The 5′ primer (5′-cctgtcatgtttatccc-3′) of the 135-bp DNA fragment was labeled using [γ-^{32}P]ATP. The kinase reaction was performed with 40 pmol of primer and T4 polynucleotide kinase (Promega) in a total volume of 20 μl. The reaction was incubated at 37° for 30 min, after which the volume was adjusted to 40 μl with water. The labeled primer was then purified with Chroma Spin +TE-10 columns (Clontech) as described by the manufacturer. The purified primer was added to a PCR reaction containing 40 pmol of the 3′ primer (3′-caaccttctccgaacgtgg-5′) and PCR Master Mix (Promega) in a total volume of 100 μl. PCR was performed in the Bio-Rad MyCycler™ Thermal Cycler (95 °C for 1 min, 1 cycle; 95 °C for 45 s, 46 °C for 30 s, 72 °C for 1 min, 35 cycles; 72 °C for 7 min, 1 cycle; 4 °C hold). The PCR product was purified with the Qiagen MinElute PCR purification kit.

Generation of Cold DNAs for EMSA Competition Assays—The 135-bp DNA was used as a specific competitor and referred to as DNA #1. PCR was done as described above. A 120-bp DNA fragment was chosen upstream of the area of interest and referred to as DNA #2. The forward primer used for DNA #2 was 5′-GGATCCTCCCCGGTC-3′, and the reverse primer was 5′-GGTCGACCTTAGAACC-3′. An 84-bp DNA fragment was chosen downstream from the area of interest and referred to as DNA #3. The forward primer for DNA #3 was 5′-GGAAGAGGCTTGCACC-3′, and the reverse primer was 5′-AAGCTTCAAGCATCGAAGAGGC-3′. PCR for DNAs #2 and #3 were performed in the GeneMate Genius as follows: 95 °C for 1 min, 1 cycle; 95 °C for 45 s, 55 °C for 30 s, 72 °C for 1 min, 35 cycles; 72 °C for 7 min, 1 cycle; 4 °C hold. PCR products were purified using the Qiagen MinElute PCR Purification kit.

In Vivo Complementation in Yeast—Wild-type and random forms of human Rrn3 as well as wild-type *Saccharomyces cerevisiae* Rrn3 were cloned into pRS426TEF so that Rrn3 would be expressed from the constitutive *TEF1* promoter. A strain of *S. cerevisiae* expressing a temperature-sensitive allele of *RRN3* (NOY1075; Ref. 41) was transformed with plasmid expressing WT hRrn3, plasmid expressing the randomized form of hRrn3, plasmid expressing *S. cerevisiae* Rrn3, or an empty vector. Each plasmid carried a *URA3* marker, rendering cells URA+. Liquid cultures were grown to stationary phase in SD−Ura medium at 23 ° with aeration. Ten microliters and then 10-fold serial dilutions of these starter cultures were spotted on SD−Ura agar. Images shown are duplicate plates that were incubated at the permissive (30 °C) or non-permissive temperatures (37 °C) for 5 days before imaging.

RESULTS

Identification of the DNA-binding Site for Rrn3 in the rDNA Repeat—In the course of investigating the role of Rrn3 in the formation of a functional, transcription initiation complex, we found that Rrn3 bound to the rDNA, specifically to a 411-bp fragment spanning the transcription initiation site (data not shown). Those experiments used a modified ChIP assay to detect the interaction of FLAG-Rrn3 with the rDNA promoter. To analyze this result in greater detail, we developed an EMSA assay for the interaction of Rrn3 with the rDNA. Our initial experiments used that same 411-bp fragment containing the transcription initiation site (−286 to + 124). To more narrowly define the Rrn3 DNA-binding site, we split that fragment into three smaller pieces and used the fragments in a competition EMSA. As shown in Fig. 1, when the fragment spanning the transcription initiation site was labeled, the other two fragments were much less effective as competitors. These results demonstrated that Rrn3 bound to the 135-bp fragment spanning the transcription initiation site (Fig. 1).

Identification of the DNA Binding Domain of Rrn3—Having found that Rrn3 was a DNA-binding protein, we hypothesized that its ability to bind DNA might play a role in its function in transcription. To examine this question, we sought to identify the DNA binding domain of Rrn3. Prosite analysis of the primary sequence of Rrn3 demonstrated a region (amino acids 379–400) with weak identity to the consensus sequence of the DNA binding domain of the heat shock transcription factor 2 (Fig. 2, *panels A* and *B*). To determine if this region plays a role in transcription, we constructed both a randomization mutant of that domain and deleted that domain from Rrn3. As shown in Fig. 3, either randomization (*Sub. mut.*, *panels A* and B) or deletion of amino acids 382–400 (deletion or Δ mutant; *panels C* and *D*) resulted in forms of Rrn3 that did not bind to the rDNA

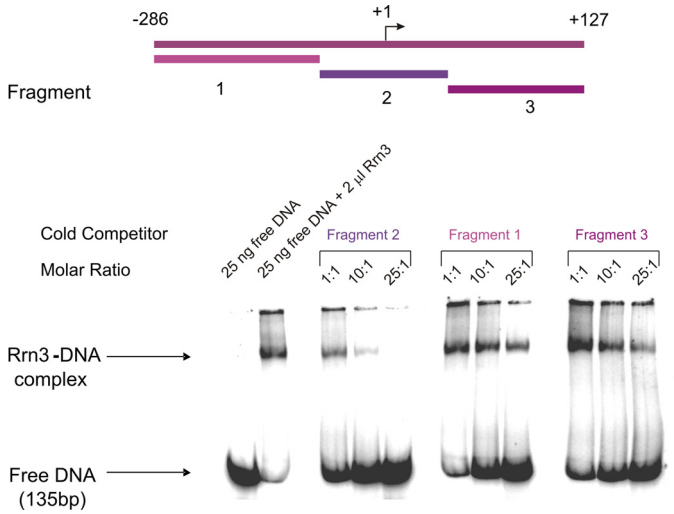

FIGURE 1. **Rrn3 is a DNA-binding protein.** *A*, competition EMSA demonstrates that Rrn3 binds to a 134-bp fragment spanning the transcription initiation site of the rat rDNA promoter. The *upper panel* indicates the region from −286 through +130 (extending from the BamH1 site in the original gene to the HindIII site) of the rat rDNA promoter, and the three PCR fragments that are generated using the appropriate primers and contain the indicated regions. Fragment 1 was found to bind specifically to Rrn3 in preliminary experiments (not shown). The competition EMSA used labeled Fragment 1, purified recombinant Rrn3 (~0.3 μg), and increasing amounts of the competing fragments (molar ratios). Fragment 1 is 135 bp and extends from −73 to +61. Fragment 2 is 121 bp and extends from −196 to −76. Fragment 3 is 85 bp and extends from +46 to +130. *B*, 40 ng of the purified Rrn3 (two different preparations) used in the EMSA were fractionated by SDS-PAGE, and the gel was stained using the SilverQuest Silver stain kit from Invitrogen.

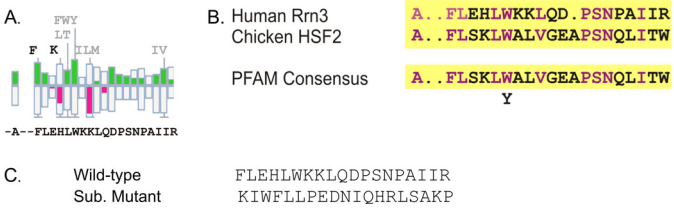

FIGURE 2. **Identification of the DNA binding domain of Rrn3.** *A*, shown is alignment of the sequence of Rrn3 (*bottom sequence*) with the consensus DNA binding domain of heat shock transcription factor 2 (*top sequence*, XP_419760.2) obtained using MotifScan (53). The relevant sequence from human Rrn3 is presented using the *black residues at the bottom of the histogram*. The *underlying gray rectangles* represent the maximal score possible at every position of the query. The areas of the rectangles located *below the axis* are negative. The amino acids of the profile consensus that might contribute the most to the profile score are represented in *gray* at the *top of the background histogram*. *Green* indicates identity. Please refer to Falquet *et al.* (53) for additional details. *B*, shown is alignment of the sequences of human Rrn3 and chicken heat shock transcription factor 2. *C*, the sequence of the substitution (*Sub.*) mutant of the putative DNA binding domain of Rrn3 is shown.

promoter. In our first experiment designed to determine if this region played a role in the function of Rrn3, we either deleted amino acids 382–400 of Rrn3 or randomized the sequence (FLEHLWKKLQDPSNPAIIR was changed to KIWFLLPEDNIQHRLSAKP) by PCR mutagenesis. Isolated FLAG-tagged constructs of the mutants were expressed and purified by anti-FLAG affinity chromatography. The immunoaffinity-purified proteins were used in EMSA assays to determine if they bound to DNA (Fig. 3). As shown in Fig. 3*A*, in contrast to the wild-type protein (*lanes 2* and *3*), the substitution mutant failed to generate a shift (*lanes 4* and *5*). Similar results were obtained with the deletion mutant. When added to an EMSA assay in equal amounts to wild-type Rrn3, the deletion mutant failed to

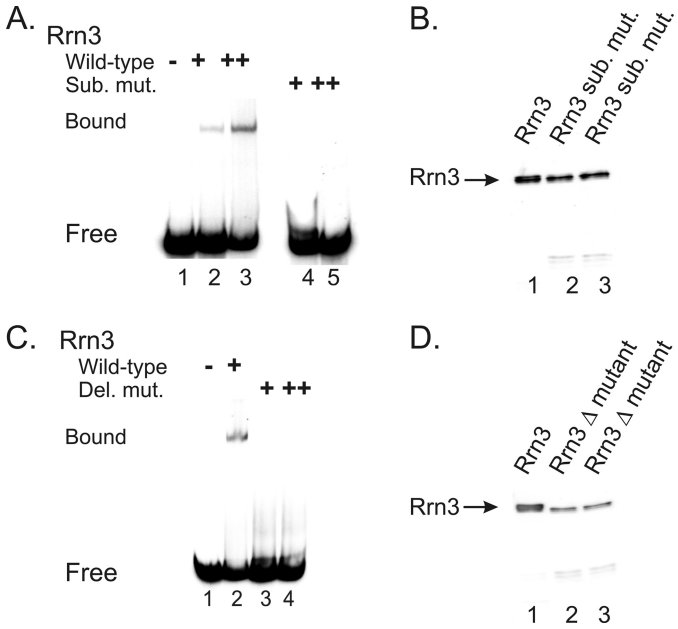

FIGURE 3. **Amino acids 382–400 of Rrn3 are required for DNA binding.** Randomization or deletion of amino acids 382–400 eliminates DNA binding by Rrn3. In *panel A*, EMSA assays, described in the legend to Fig. 1, were carried out with either increasing amounts of affinity-purified wild-type Rrn3 (*lanes 2* and *3*) or substitution mutant of Rrn3 (*sub. mutant*, *lanes 4* and *5*). In *panel C*, the EMSA assays were carried out with either affinity-purified wild-type Rrn3 (*lane 2*) or two separate isolates of the deletion mutant (*lanes 3* and *4*). Fragment 1, the 135-bp fragment described in the legend to Fig. 1 was used in these EMSAs. The amounts of the various recombinant proteins used in *panels A* and *C* were adjusted after Western analyses of equal volumes of the purified proteins. *Panels B* and *D* present the results of Western blots of the different recombinant proteins using anti-FLAG antibody. The results of these blots were also used to adjust the amount of protein added to the transcription assays presented in Fig. 4. In this case, 2.5× the volume of the deletion mutant was added to the DNA binding assays (this figure) and transcription assays (Fig. 4) as compared with wild-type protein.

demonstrate a shift (Fig. 3*C*, compare the results portrayed in *lane 2* with those in *lanes 3* and *4*). Quantitation of Western blots of the wild-type and mutant protein isolates (Fig. 3, *panels B* and *D*) were used to assure that equal protein amounts were used in the binding assays. These data are consistent with the model in which we identified a domain in Rrn3 that is essential for binding to DNA.

The DNA Binding Domain of Rrn3 Is Required for Rrn3 to Function in Transcription—Current models for the function of mammalian Rrn3 focus upon the ability of the protein to simultaneously interact with SL1 and RNA polymerase I and do not include a DNA binding function. Thus, we sought to determine if either the deletion or substitution mutants of the DNA binding domain of Rrn3 affected the protein ability to function in rDNA transcription.

These experiments were carried out using two different assay systems. In the first of these assay systems, Rrn3 has been inhibited by treatment of the cells with cycloheximide (33). The second assay is based upon the observation that Rrn3 is inactivated when FM3A cells are nutrient-deprived (42) after growth to a high density in suspension. In two experiments (Fig. 4) we used S100 extracts prepared from cells treated with cycloheximide (*S100 CHX*). We previously demonstrated (33) that 1) S100 extracts prepared from cells treated with CHX cannot support rDNA transcription *in vitro* (*lane 1*), 2) treatment with CHX

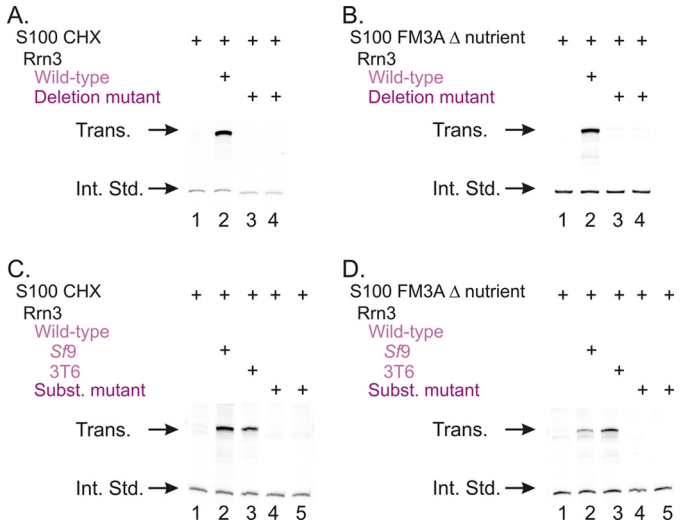

FIGURE 4. **Amino acids 382–400 of Rrn3 are required for Rrn3 to function in transcription.** Substitution of amino acids 382–400 abrogates the ability of Rrn3 to activate rDNA transcription. The addition of wild-type Rrn3 (expressed in 3T6 cells and purified by anti-FLAG antibody affinity purification) to an S100 from cells treated with cycloheximide (*A*) or to an S100 from nutrient deprived FM3A cells (*B*) results in an extract capable of carrying out transcription (*lane 2*). However, the addition of the DNA binding domain deletion mutant of Rrn3 (expressed in 3T6 cells and purified by anti-FLAG antibody affinity purification) does not result in an extract capable of rescuing transcription in either assay (*panels A* and *B*, *lanes 3* and *4*; two different preparations of the deletion mutant). The addition of purified, ectopically expressed, wild-type Rrn3 to an S100 from cells treated with cycloheximide (*C*) or to an S100 from nutrient deprived FM3A cells (*D*) results in an extract capable of carrying out transcription (*lanes 2* and *3*). However, the addition of the purified, ectopically expressed DNA binding domain substitution mutant (*Subst. mutant*) of Rrn3 does not result in an extract capable of rescuing transcription in either assay (*panels C* and *D*, *lanes 4* and 5; two different preparations of the substitution mutant). The Rrn3 added in *lanes 2* and *3* was purified by anti-FLAG affinity purification from either baculovirus-infected *Sf*9 cells or transiently transfected 3T6 cells, respectively. The mutant Rrn3 was isolated from transiently transfected 3T6 cells. Western blots using anti-FLAG antibody were used to adjust the amount of protein added to the transcription assays as in Fig. 3.

results in the inactivation of Rrn3, and 3) S100 extracts from CHX-treated cells will support transcription when supplemented with active Rrn3 (Fig. 4*A*, *lane 2*). As shown in Fig. 4*A*, the addition of wild-type Rrn3 (isolated from 3T6 cells transfected with a vector expressing FLAG-tagged Rrn3) to inactive S100 extracts prepared from cells treated with cycloheximide (*lane 1*) results in extracts capable of carrying out transcription (*lane 2*). However, when we added either of two separate isolates of the deletion mutant of the DNA binding domain of Rrn3 (prepared in the same way) (*lanes 3* and *4*), that protein was incapable of rescuing transcription. The second assay system used Rrn3-inactive extracts prepared from nutrient-deprived FM3A cells (42). As shown in Fig. 4, *panel B*, S100 extracts derived from nutrient-deprived FM3A (S100 FM3A Δ Nutrient) are inactive (*lane 1*) unless supplemented with Rrn3 (*lane 2*). However, the addition of either of two separate isolates of the deletion mutant of Rrn3 failed to rescue transcription (*lanes 3* and *4*). These results would indicate that amino acids 382–400 of Rrn3 were required for Rrn3 to function in transcription. However, there is the possibility that they themselves do not serve a function but serve as a required spacer between adjacent functional domains.

To control for that possibility, we determined if the substitution mutant could function in transcription using the two assay systems described above. In one experiment (Fig. 4*C*), we used S100 extracts prepared from cells treated with CHX, and in the second (Fig. 4*D*) we used S100 extracts from nutrient-deprived FM3A cells. As shown in Fig. 4*C*, the addition of wild-type Rrn3, expressed in either *Sf*9 or 3T6 cells, to inactive S100 extracts (*lane 1*) prepared from cells treated with cycloheximide results in extracts capable of carrying out transcription (*lanes 2* and *3*). However, when we added either of two separate isolates of the DNA binding domain substitution mutant of Rrn3 (*lanes 4* and *5*), that protein was incapable of rescuing transcription in either assay. As shown in Fig. 4*D*, *lanes 1–3*, S100 extracts derived from nutrient-deprived FM3A are inactive unless supplemented with Rrn3. However, when these extracts are supplemented with either of two separate isolates of the substitution mutant of Rrn3 (*lanes 4* and *5*), they are still inactive.

This series of experiments is consistent with the model in which we defined the DNA binding domain of Rrn3, and this binding domain is required for Rrn3 to function in transcription. However, similar results could be obtained if either the deletion of amino acids 382–400 or the randomization of those amino acids had general effects on the structure of Rrn3, *i.e.* the Rrn3 used in these experiments could be misfolded. We and others have demonstrated that Rrn3 can interact both with the 43-kDA subunit of RNA polymerase I (rpa43) and the 68-kDa subunit of SL1 (TAF_I68) both *in vitro* or in cotransfection/co-immunoprecipitation experiments (33, 34). We hypothesized that at least one if not both of the domains required for the protein-protein interactions that have previously been described as being essential for Rrn3 function should provide an independent assay for the structure of the Rrn3 mutants. Thus, we examined the possibility that the substitution or deletion mutant of Rrn3 would still interact with either rpa43 or TAF_I68.

DNA Binding by Rrn3 Is Independent of Its Ability to Interact with RNA Polymerase I or SL1—The possibility that mutant forms of Rrn3 would interact with one or both of these two proteins would provide independent means for assaying the functionality of Rrn3 and its mutants. As such, they provide an indirect reflection of the state of the folding of the protein. For example, if the deletion of the DNA binding domain only affects that function, one would expect rpa43 would coimmunoprecipitate with either the substitution or deletion form of Rrn3 essentially as it does with the wild type.

As shown in Fig. 5, *panel A*, when 3T6 cells were cotransfected with vectors that support the expression of FLAG-Rrn3 and V5-rpa43 (rpa43 tagged with the V5 epitope), rpa43 coimmunopurified with FLAG-Rrn3 over immobilized anti-FLAG antibodies (*lanes 2* and *5*). Similarly, when the 3T6 cells were cotransfected with vectors that support the expression of the substitution mutant of Rrn3 (FLAG-SMRrn3) and V5-rpa43, rpa43 coimmunopurified with the FLAG-tagged substitution mutant of Rrn3 (*lanes 3* and *6*). It is interesting to note that although the substitution mutant was not expressed to the same level as wild-type Rrn3 in these experiments, the amount of rpa43 in the immunoprecipitate was the same. Additional experiments will be required to determine if this represents an increased affinity of the substitution mutant and rpa43.

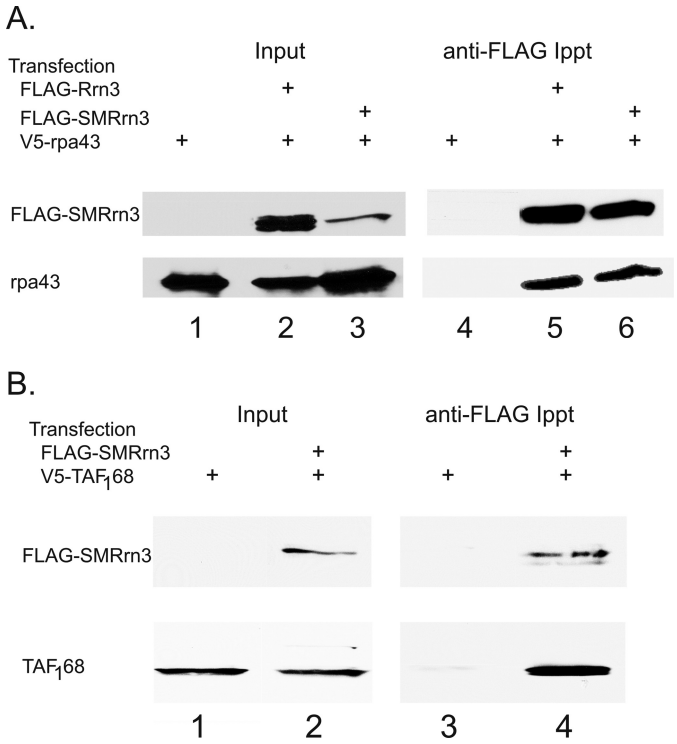

FIGURE 5. **Substitution of amino acids 382–400 of Rrn3 does not affect the ability of Rrn3 to interact with the 68-kDa subunit of SL1 (TAF$_I$68) or rpa43.** *A*, 3T6 cells were co-transfected with vectors supporting the expression of either FLAG-tagged wild-type or substitution mutant (SMRrn3) of Rrn3 and V5-tagged rpa43. Forty-eight hours post-transfection, the cells were harvested, and the FLAG-Rrn3 was purified using immobilized anti-FLAG antibodies. The immunopurified and coimmunopurified proteins were fractionated by SDS-PAGE and analyzed with either anti-FLAG antibodies (*FLAG-Rrn3*) or with anti-V5 antibodies (*V5- rpa43*). TAF$_I$68 coimmunoprecipitated both with the wild-type (*lane 5*) and mutant (*lane 6*) form of Rrn3 but not with anti-FLAG beads alone (4). *B*, 3T6 cells were co-transfected with vectors supporting the expression of FLAG-tagged substitution mutant (*SMRrn3*) of Rrn3 and V5-tagged TAF$_I$68. Forty-eight hours post-transfection, the cells were harvested, and the FLAG-SMRrn3 was purified using immobilized anti-FLAG antibodies. The immunopurified and coimmunopurified proteins were fractionated by SDS-PAGE and analyzed with either anti-FLAG antibodies (*FLAG-SMRrn3*) or with anti-V5 antibodies (*V5-TAF$_I$68*). TAF$_I$68 coimmunoprecipitated with the mutant (*lane 4*) form of Rrn3 but not with anti-FLAG beads alone (25). *Ippt.*, immunoprecipitate.

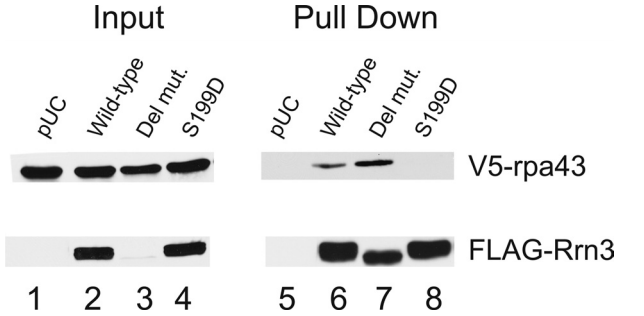

FIGURE 6. **Deletion of amino acids 382–400 of Rrn3 does not affect the ability of Rrn3 to interact with rpa43.** 3T6 cells were co-transfected with vectors supporting the expression of either FLAG-tagged wild-type or deletion mutant (Δ382–400) of Rrn3 and V5-tagged rpa43. Forty-eight hours post-transfection, the cells were harvested, and the FLAG-Rrn3 was purified using immobilized anti-FLAG antibodies. The immunopurified and coimmunopurified proteins were fractionated by SDS-PAGE and analyzed with either anti-FLAG antibodies (*FLAG-Rrn3*) or with anti-V5 antibodies (*V5-rpa43*). As shown in *lanes 2* and *3* (*bottom panel*), the deletion mutant was poorly expressed in comparison to the wild type. However, rpa43 coimmunoprecipitated with both the wild-type (*lane 6*) and mutant (*lane 7*) forms of Rrn3.

We next sought to determine if the substitution of amino acids 382–400 of Rrn3 had an effect on the ability of Rrn3 to interact with TAF$_I$68 (Fig. 5, *panel B*). When a vector coding for V5-TAF$_I$68 was cotransfected with a vector coding for the FLAG-tagged substitution mutant of Rrn3 (SMRrn3), V5-TAF$_I$68 coimmunoprecipitated with the mutant (*lanes 2* and *4*). The specificity of the coimmunoprecipitation is confirmed by the lack of signal in the immunoprecipitate obtained in the absence of expression of FLAG-SMRrn3 (*lanes 1* and *3*).

A similar series of experiments was carried out to determine if the deletion of amino acids 382–400 Rrn3 had an effect on the ability of Rrn3 to interact with either rpa43 or TAF$_I$68 (Fig. 6). 3T6 cells were cotransfected with a vector coding for V5-rpa43 and an empty vector (pUC) or vectors coding for various FLAG-tagged variants of Rrn3: wild-type Rrn3, Δ382–400 Rrn3 (*Del. Mut.*), or a substitution mutant (S199D). The substitution of an acidic residue for the serine at 199 has been reported previously to inhibit the ability of Rrn3 to interact with both SL1and RNA polymerase I (43). The data presented in *lanes 6* and *7* of Fig. 6 demonstrate that rpa43 coimmunoprecipitated with the deletion mutant of Rrn3. Furthermore, the experiment confirms the previous observation (43) that substitution of a glutamate for serine 199 results in a form of Rrn3 that does not bind rpa43 (compare the results presented in *lanes 4* and *8* with those presented in *lanes 2* and *6*). Inspection of the input side of the experiments demonstrates that the levels of the deletion mutant in the starting extracts were very low. Thus, we used, in a typical experiment, three times as many cells for those experiments as we used for the experiments studying either the wild-type or the substitution mutant. It is interesting to note that the deletion mutant of Rrn3 appears to bind better to rpa43 than does the wild type. In a parallel series of experiments, we determined that the deletion of amino acids 382–400 of Rrn3 had no affect on its ability to interact with TAF$_I$68 (data not shown). These data indicate that the deletion of the putative DNA binding domain of Rrn3 does not affect its ability to interact with either RNA polymerase I (rpa43) or SL1 (TAF$_I$68). These data strongly suggest that the deletion construct has a significant degree of the secondary structure of the wild-type protein.

The observations that both the deletion mutants and substitution mutants of Rrn3 could interact with both TAF$_I$68 and rpa43 are consistent with the model that the mutations have not had global affects on the tertiary structure of Rrn3. Although it is formally possible that the mutations have had significant local effects on the structure of Rrn3, in particular on the DNA binding domain, these mutations have not inhibited the ability of Rrn3 to function as a bridge between SL1 and RNA polymerase I.

The DNA Binding Domain of Human Rrn3 Is Required for Complementation of the Lethal Phenotype of an RRN3-ts Mutant by Human Rrn3—To determine whether the DNA binding domain human RRN3 was required for function *in vivo*, we assayed its ability to rescue a yeast strain in which the genomic RRN3 allele was rendered nonfunctional by a temperature-sensitive mutation (S213P, NOY1075; Ref. 41). Rrn3 is essential for rRNA gene transcription; hence, this strain is inviable at the nonpermissive temperature due to the absence of Rrn3 activity. NOY1075 was transformed with either wild-type

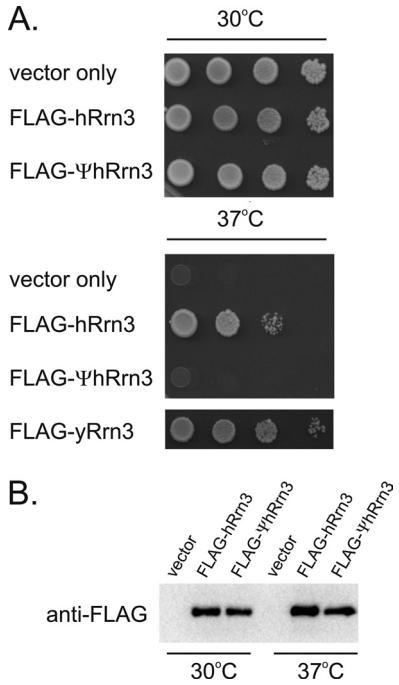

FIGURE 7. **Randomization of amino acids 382–400 inactivates Rrn3 *in vivo*.** *A*, the random mutant of human Rrn3 fails to complement the lethal phenotype of an rrn3-ts mutant. rrn3-ts strain NOY1075 was transformed with high copy number plasmid expressing wild-type hRrn3 from the constitutive TEF1 promoter (*hRrn3*), a derivative of that plasmid expressing the randomized form of hRRN3 (Ψ*hRrn3*), an empty vector (*Vector*), or wild-type yeast Rrn3. Each plasmid carried a URA3 marker, rendering cells URA+. Liquid cultures were grown to stationary phase in SD−Ura medium at 23 °C with aeration. Ten microliters of the liquid cultures, and then 10-fold serial dilutions of these starter cultures were spotted on SD−Ura agar. Plates were incubated at 30 °C for 3 days or 37 °C for 5 days before imaging. FLAG-tagged Rrn3 from *S. cerevisiae* (yRrn3) was expressed as an additional positive control, and those cells were imaged after only 3 days, as the yeast protein supports more efficient growth than the human homologue. *B*, cells expressing FLAG-tagged hRrn3 and ΨhRrn3 were grown to early log phase (A_{600} = 0.2) at 30 °C in SD−Ura medium. The cultures were split and incubated an additional 3 h at 30 or 37 °C for 3 h. Cells were harvested and lysed, and 4 μg of total protein from each crude extract was analyzed by SDS-PAGE and Western blot, probing for the FLAG epitope.

human cDNA, the mutant in which amino acids 382–400 were randomized (ΨhRrn3) or the empty expression vector and grown under appropriate selection on SD−Ura media as described under "Materials and Methods." After equal volumes (10 μl) of cultures grown at the permissive temperature were serially diluted (10×) onto SD−Ura plates to monitor RRN3 function, the plates were then incubated at 30 or 37 °C (Fig. 7.). Cells expressing human Rrn3 (hRrn3 or ΨhRrn3) grew at the permissive temperature, 30 °C. When the same cells were grown at 37 °C on SD−Ura, only the colonies that expressed wild-type hRrn3 grew. Those cells transformed with ΨhRrn3 or the empty expression construct were inviable. To control for the possibility that the ΨhRrn3 was not expressed or was significantly less stable than the wild-type hRrn3, Western blots were carried out. As shown in Fig. 7, *panel B*, both the wild-type and Ψ forms of hRrn3 were expressed at essentially equal levels. The ability of the human gene to complement the RRN3ts mutation demonstrates that its function in Pol I transcription is conserved between yeast and humans as previously demonstrated by Moorefield *et al.* (28). The observation that ΨhRrn3 failed to complement the rrn3-ts mutation demonstrates that the identified DNA binding domain is important for Rrn3 function *in vivo* as well as *in vitro*.

DISCUSSION

It has generally been considered that the Rrn3 functions in rDNA transcription by facilitating the recruitment of transcription competent RNA polymerase I to the committed template (*e.g.* 34). Clearly, the ability of Rrn3 to interact with both the 110- and 68-kDa subunits of SL1 and as well as the 43-kDa subunit of RNA polymerase I, rpa43, demonstrates a mechanism through which the polymerase-associated factor could accomplish this function. Furthermore, the observation that treatment of cells with cycloheximide results in the inactivation (dephosphorylation) of Rrn3 and the inhibition of its ability to interact with RNA polymerase I (33) provides further evidence in support of this model. On the other hand, our finding that DNA binding by Rrn3 is essential for rDNA transcription could be seen as a complement to that model and to other models of how RNA polymerase I recognizes the committed template.

The finding that Rrn3 is a DNA-binding protein raises the formal possibility that Rrn3 is the same as the 70-kDa DNA-binding protein reported by Yamamoto *et al.* (44). In their manuscript, those authors reported the finding of an essential rDNA transcription factor that bound to the rDNA promoter in the presence of SL1. However, they concluded that their factor and Rrn3/TIF-IA were not the same. They observed that the 70-kDa protein was easily separated from RNA polymerase I in their initial chromatography step (43), whereas Rrn3 is tightly associated with the polymerase (43). Subsequently, that same laboratory reported the species-specific interaction of transcription factor p70 with the ribosomal DNA promoter (45). Thus, our finding that the human form of Rrn3 binds to the rat rDNA promoter and activates transcription from that promoter, both, would appear to obviate the possibility of identity.

Our EMSA experiments clearly demonstrate that Rrn3 binds within 70 bp of the rDNA promoter. Fragment 1 extends from −73 to +61 (+1 being the transcription initiation start site), and fragments that flank fragment 1 do not compete for binding with the same efficiency. We have so far been unable to obtain definitive footprinting results needed to identify the DNA-binding site. However, it may be that the DNA binding properties of Rrn3 are similar to those of SL1 and UBF.

The original experiments on the binding of UBF to the rDNA promoter identified a large region from circa −50 to −125 (*e.g.* Refs. 14 and 46) that was protected in DNase footprinting experiments. More strikingly, the various vertebrate forms of UBF are interchangeable, *i.e. Xenopus* UBF yields a footprint over the rat rDNA promoter that is virtually identical to that generated by rat UBF (46). However, subsequent experiments have failed to identify a "consensus" DNA binding element in any of the vertebrate rDNA promoters. This has led some to refer to UBF as a nonspecific or "sequence-tolerant" DNA-binding protein (47). A similar situation exists for SL1; there is no defined DNA recognition element. In fact, it was originally reported that human SL1 did not function independently as a DNA-binding protein but cooperated with UBF (48) and that "UBF1 recruits SL1 to the template and directs binding to an extended region encompassing sequences in the UCE."

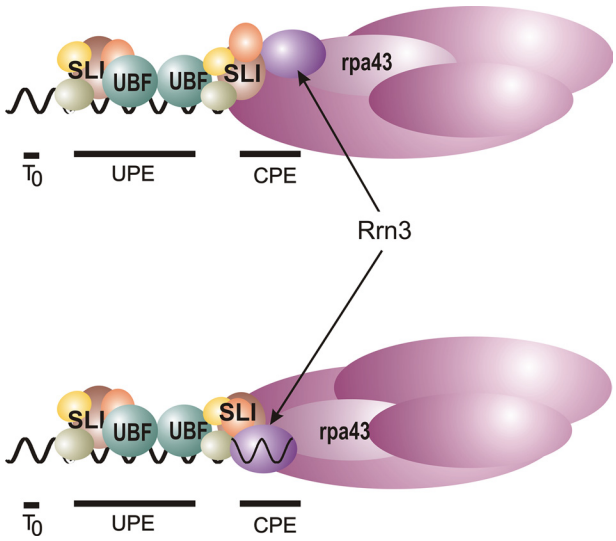

FIGURE 8. **Schematic comparing two models for the role of Rrn3 in transcription by RNA polymerase I.** In the *top scheme*, Rrn3 functions as a bridge between RNA polymerase I and SL1. In the *bottom scheme*, Rrn3 binds to the rDNA promoter and also functions as a bridge between RNA polymerase I and SL1. *UPE*, upstream promoter element; *CPE*, core promoter element.

Although subsequent experiments (37, 49) have demonstrated that SL1 is a DNA-binding protein, the recognition element has not been identified.

With these caveats in mind, we are presently carrying out additional footprinting, competition, and mutagenesis experiments designed to identify the Rrn3 DNA-binding site. In this context, two observations appear to be relevant. First, both Muramatsu *et al.* (50) and Sollner-Webb *et al.* (51) reported that the 3′ boundary of essential elements of the rDNA promoter extended to at least +9. Second, we reported (52) that the sequences from +2 to +16 of the rat, mouse, and human rDNA promoters were identical. This suggests that this region may have functional significance, and although we are doing the footprinting assays, we will mutate this region and determine if it is essential for Rrn3 binding and rDNA transcription. Finally, it is possible that DNA binding by RNA polymerase I and SL1 occurs simultaneously in the form of the binding of a holoenzyme (25). In this model, both Rrn3 and SL1, acting in concert, would contribute to the recognition of the promoter (Fig. 8).

Our computer analysis indicated that the region from amino acid 379 to 400 of Rrn3 might function in DNA binding. Thus, we constructed both the deletion mutant as well as the substitution (randomization) mutant of that site to examine the possibility that the putative DNA binding domain was in fact required for DNA binding by Rrn3 and might also play a role in the ability of Rrn3 to function in transcription. In the event that the deletion mutant affected Rrn3 activity, we considered the possibility that the substitution (randomization) mutant, which has the same amino acid composition as the putative DNA binding domain of wild-type Rrn3, would be less likely to be grossly misfolded. However, the mutation of even a single amino acid can have deleterious effects on the overall folding of a protein. Hence, it is necessary to have positive controls for the biochemistry of the mutant before it is possible to draw a conclusion with regard to the function of the mutated domain.

We and others (33, 34) have shown that Rrn3 interacts with both rpa43 and TAF_I68. When we examined the ability of the substitution mutant of Rrn3 to interact with either rpa43 or TAF_I68, we found that its activity was essentially identical to that of wild-type Rrn3. Similar results were obtained when we examined the ability of the deletion mutant to interact with rpa43 or TAF_I68. To ensure the quality of these assays, we determined if the mutagenesis of serine 199 to alanine would inhibit the ability of Rrn3 to interact with rpa43 as has been reported (43). This was confirmed. Thus, the demonstration that the substitution and deletion mutants can bind to both rpa43 and TAF_I68 strongly indicates that neither of these mutant forms of the protein is significantly misfolded. Hence, the inability of these mutants to function in transcription is most likely due to their inability to bind to DNA and not to the loss of other functions. In the future it will be informative to determine if the region between amino acids 382 and 400 is both necessary and sufficient for binding to the rDNA promoter, *i.e.* will it drive the binding of another protein to the rDNA?

In summary, our data are consistent with a new model for the mechanism of action of Rrn3. In this model Rrn3 is a DNA-binding protein that binds within 60 bp of +1, and its ability to bind to DNA as well as its interactions with SL1 and RNA polymerase I are required for function.

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