## RRN11 Encodes the Third Subunit of the Complex Containing Rrn6p and Rrn7p That Is Essential for the Initiation of rDNA Transcription by Yeast RNA Polymerase I

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A new gene, RRN11, has been defined by certain rrn mutants of Saccharomyces cerevisiae which are defective specifically in the transcription of 35S rRNA gene by RNA polymerase I (pol I). We have cloned the gene and found that it encodes a protein of 507 amino acids. We have used a strain with the chromosomal RRN11 deleted and carrying HA1 epitope-tagged RRN11 on a plasmid to isolate a protein complex containing the protein encoded by RRN11. This protein complex complemented rrn6 mutant extracts, which were previously shown to be deficient in the essential pol I transcription factor called Rrn6/7 complex or core factor (CF). The CF complex was previously shown to consist of three proteins, the 102- and 60-kDa subunits encoded by RRN6 and RRN7, respectively, and the 66-kDa subunit. The results of the above complementation experiments combined with mobility of Rrn11p in SDS-polyacrylamide gel electrophoresis analysis relative to Rrn6p and Rrn7p led to the conclusion that RRN11 encodes the 66-kDa subunit of CF. Glutathione S-transferase-Rrn11p fusion protein was found to bind strongly to 35S-labeled Rrn6p and Rrn7p but only weakly to 35S-labeled TATA-binding protein. Similarly, glutathione S-transferase-Rrn7p fusion protein bound strongly to 35S-labeled Rrn6p and Rrn11p but only weakly to 35S-labeled TATA-binding protein. These results are consistent with the fact that one can purify CF consisting of Rrn6p, Rrn7p, and Rrn11p from yeast cell extracts, but the purified complex does not contain TATA-binding protein. RRN11 was shown to be an essential gene, and [3H]uridine pulse experiments demonstrated directly that RRN11 is essential for rDNA transcription by pol I in vivo. Thus all three subunits of CF are essential for rDNA transcription. Because of the resemblance of CF to mammalian essential pol I transcription factor SL1, the amino acid sequences of Rrn11p and the other two subunits of CF were compared with those of the three TATA-binding protein-associated factors (TAFs) in the human SL1, TAF48, TAF63, and TAF110. No significant similarity was detected between two sets of the proteins. Similarity as well as differences between CF and SL1 are discussed.

Transcription of rRNA genes is central to the overall regulation of ribosome synthesis and has been studied extensively.

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1 The abbreviations used are: rDNA, genes for large rRNA; pol I, RNA polymerase I; UBF, upstream binding factor; SL1, promoter selectivity factor; TBP, TATA-binding protein; TAF, TATA-binding protein-associated factors; UAF, upstream activation factor; CF, core factor; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; kb, kilobase(s); CITE, cap-independent translational enhancer.
remained unclear. The question of how SL1, singly or together with UBF, makes pol I recruitment possible has also remained unexplored.

We have been studying transcription of rDNA by pol I in the yeast S. cerevisiae using both genetic and biochemical approaches. By using a fusion gene (pGAL7-35 S rDNA), which consists of the 35 S rRNA coding region fused to the galactose-inducible GAL7 promoter (pGAL7), we isolated mutants (rrn mutants) that showed no or only very poor growth on glucose media but could grow on galactose media because of transcription of the 35 S rDNA, which remains unclear. The question of how SL1, singly or together with UBF, makes pol I recruitment possible has also remained unexplored.

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By studying a newly defined RRN gene RRN11 we have now demonstrated that this gene encodes the previously uncharacterized subunit p66 of the Rrn6/7 complex or CF. In this paper, we first describe cloning and characterization of RRN11 and then examine interactions of the three CF subunits, Rrn6p, Rrn7p, and Rrn11p among themselves and with TBP in vitro. Independently, RRN11 was also cloned and characterized by Reeder and co-workers.

**Materials and Methods**

**Plasmids and Yeast Strains**—Yeast strains and plasmids used are listed in Table I and Fig. 1. The two plasmids pNOY345 and pNOY346 (see Fig. 1A) were isolated from the ATCC77164 yeast genomic library (CEN6 ARS4 TRP1) prepared from the strain YPH7 (provided by C. Connelly and P. Hieter). pNOY349 (CEN6 ARS4 TRP1 RRN11) was constructed by cloning the 2.6-kb Apal-NheI fragment from pNOY346 into pRS314 (19) between Apal and SpeI (see Fig. 1B). pNOY350 (CEN6 ARS4 URA3 RRN11) is a derivative of pRS316 (19) carrying the nucleotide sequence encoding three tandem copies of the SRN11 methionine codon of pNOY3165, which is a Blue-
RN11 Encodes a Subunit of rDNA Transcription Factor CF

Cloning and Sequencing of RN11 and Direct Demonstration of Its Requirement for rDNA Transcription in Vivo—Like many other rrn mutants (14), three mutants were independently isolated from NOY418, which carries the GAL7-35 S rDNA fusion on a plasmid (pNOY103) as mutants that grow on galactose but not on glucose. By standard genetic crosses with other rrn mutants and with each other, we have shown that these three mutants represent a new complementation group, which now defines the RN11 gene.

We used one of the mutants (NOY727, isolation number 1016) carrying rrn11-1 to detect the RN11 gene. The mutant cells were transformed with a yeast genomic library, and transformants with the growth characteristics of the wild type (RN11) were isolated on glucose plates, the plasmids were recovered, and partial sequencing of isolated yeast genomic inserts showed that the region covering the RN11 locus had already been determined as part of the yeast genome sequencing project. By constructing the plasmid pNOY349 (Fig. 1B), which complements the mutation, we were able to conclude that RN11 corresponds to an open reading frame called XM9827.09c and is located between PRP39 and CAT2 on chromosome XIII. The amino acid sequence of RN11p deduced from the nucleotide sequence is shown in Fig. 2. The protein (Rrn11p) encoded by RN11 is 507 amino acids in size with a thioredoxin-agarose beads (Sigma) as described previously (25).

[35S]Methionine-labeled TBP, Rrn6p, Rrn7p, and Rrn11p were synthesized in vitro using rabbit reticulocyte lysate systems (Promega), and pNOY3171, pNOY3173, pNOY221, and pNOY3245, respectively, were used as template (Table I; see above). These [35S]-labeled proteins were incubated with GST-Rrn11p (or GST-Rrn7p or control GST), which were attached to glutathione beads and prewashed with and suspended in buffer A (Tris-Cl, pH 7.5, 10 mM; MgCl2, 5 mM; CaCl2, 5 mM; NaCl, 200 mM; Nonidet P-40, 0.1%) containing 0.2% bovine serum albumin in a final volume of 200 μl. After 1 h at room temperature, the beads were recovered and washed extensively with buffer A (without bovine serum albumin) and proteins bound were analyzed by SDS-PAGE.

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DNA polymorphism caused by transposition of Ty1 element and is consistent with the known presence of Ty1 integration near tRNA genes (26).

We carried out standard gene disruption experiments. A diploid strain (NOY728) with one wild-type copy of RRN11 and one copy completely deleted and replaced by LEU2 (Fig. 1B) was constructed and sporulated, and tetrads were dissected. Tetrad analysis showed the expected two viable and two non-viable segregation pattern at all temperatures studied, which ranged from 20 to 37 °C, and cosegregation of spore viability and leucine auxotrophy, demonstrating that RRN11 is an essential gene (Fig. 1C).

We constructed a haploid strain with the chromosomal RRN11 deleted and carrying the pGAL7-35 S rDNA on the pNOY103 plasmid. This strain, (NOY730) was used to examine effects of the deletion of RRN11 on rDNA transcription in vivo. The strain was grown in galactose medium, shifted to glucose medium to repress rRNA synthesis from the GAL7 promoter, and then incorporation of [3H]uridine into large rRNAs was examined and compared with the control wild-type (NOY730) strain. As shown in Fig. 3, the synthesis of large rRNAs (18S, 25 S, and 5.8 S rRNAs and other precursor rRNAs) was not detected in the mutant in glucose medium, while the synthesis of 5.8 S RNA and tRNAs continued as in galactose medium (Fig. 3, lane 4 compared to lane 3). Quantitation showed that the synthesis of large rRNAs relative to 5.8 S RNA plus tRNAs in the mutant is less than 1% of that in the wild type. The results confirm the conclusion that RRN11 is an essential gene and is specifically required for transcription of rDNA.

RRN11 Encodes a Subunit of rDNA Transcription Factor CF—Based on two observations, we considered the possibility that RRN11 encodes the p66 subunit of the Rrn6/7 complex (18) (CF), the essential transcription factor containing Rrn6p and Rrn7p, and uncharacterized p66. First, the RRN11 gene is most likely identical to p66. Second, the calculated molecular mass of RRN11p is 59.2 kDa and is not very different from the apparent molecular mass (66 kDa) of p66. In order to test this possibility, we constructed a haploid strain (NOY731) with the chromosomal RRN11 deleted and carrying a triple-HA1-tagged RRN11 gene (HA1)3-RRN11 on a centromeric plasmid. This strain grew at the same growth rate as the isogenic wild-type strain, indicating that the epitope-tagged protein functions like the native protein. Extracts were prepared from this haploid strain, and protein complexes containing RRN11p were isolated by an immunoaffinity purification method using monoclonal antibodies against the HA1 epitope. If RRN11p is identical to the p66 subunit of CF, the affinity-purified preparation obtained in this way should contain CF and should be able to complement extracts prepared from an rrn6 mutant (or an rrn7 mutant) which are missing the intact CF. The results shown in Fig. 4 demonstrate that this is indeed the case. The preparation complemented the rrn6 extracts (lanes 5 and 6 compared to a positive control, lane 2, and the negative control, lane 1). The mock-purified control preparation obtained from control wild-type (RRN11) cells without HA1 epitope-tagging had no complementation activities (lanes 3 and 4). We conclude that RRN11p is complexed with RRN6p and RRN7p and is thus most likely identical to p66.

In previous work (18) the purified RRN6/7 complex (CF) was analyzed by SDS-PAGE and the apparent molecular masses of
the three components, Rrn6p, p66, and Rrn7p, were found to be 115, 66, and 56 kDa, respectively. Since the molecular mass of Rrn11p calculated from the DNA sequence (59.2 kDa) is near the apparent molecular mass of both Rrn7p (56 kDa) and p66, we examined the mobility of Rrn11p in SDS-PAGE relative to Rrn7p. We analyzed the same affinity-purified preparation containing triple-HA1-tagged Rrn11p ((HA1)3-Rrn11p) by SDS-PAGE followed by Western blot using anti-HA1 antibodies. For comparison, we also analyzed a sample containing (HA1)3-Rrn7p prepared from strain NOY732. The mobility of (HA1)3-Rrn11p relative to that of (HA1)3-Rrn7p (and relative to molecular weight markers) was consistent with what we expect from Rrn11p being identical to the protein observed as p66 in the previous work (data not shown). In addition, as described below, we synthesized 35S-labeled Rrn6p, Rrn7p, and Rrn11p in vitro using reticulocyte translation systems. The mobilities of these radioactive proteins in SDS-PAGE were also consistent with the results obtained in the previous work for the three components of CF, Rrn6p, Rrn7p, and p66 (Fig. 5). Thus, Rrn11p must be identical to the p66 of CF observed in the previous work.

**In Vitro Interactions among CF Subunits and TBP**—Because we have now cloned all the genes for the components of CF, one can design experiments to examine interactions of these protein components with each other and with other proteins involved in rDNA transcription such as TBP, protein components of UAF, or subunits of pol I. As a first step in this direction, we prepared GST-Rrn11p and GST-Rrn7p fusion proteins and studied their interactions with 35S-labeled Rrn6p, Rrn7p, Rrn11p, and TBP. The labeled proteins were synthesized in vitro using reticulocyte translation systems. As shown in Fig. 5A, Rrn6p and Rrn7p bound with a high efficiency to GST-Rrn11p fusion protein attached to glutathione-agarose beads in the presence of 200 mM NaCl. Similarily, Rrn6p and Rrn11p also bound with a high efficiency to GST-Rrn7p (Fig. 5B). Under the same conditions, 35S-labeled TBP bound more weakly to GST-Rrn11p and GST-Rrn7p but not to GST (Fig. 5, A and B). The amounts of 35S-labeled proteins bound to GST fusion proteins in these experiments were quantified. The following values (as percent of input) were obtained as averages from two experiments (one shown in Fig. 5): A, binding to GST-Rrn11p: TBP, 10.3 ± 3.1%; Rrn7p, 33.8 ± 6.8%; Rrn6p, 33.2 ± 7.5%. B, binding to GST-Rrn7p: TBP, 7.9 ± 1.4%; Rrn11p, 42.3 ± 8.3%; Rrn6p, 29.9 ± 15.5%. It should be noted that the amounts of GST fusion proteins used were approximately the same and that 35S-labeled Rrn6p, Rrn7p, and Rrn11p were added in approximately equal molar amounts, while the amount of 35S-labeled TBP added was about five times higher relative to these three probes. We also note that we have not succeeded in expressing a GST-Rrn6p fusion protein in E. coli and hence have not studied binding of 35S-labeled TBP to GST-Rrn6p. Although the interaction of TBP with the three individual CF subunits as well as with the assembled CF complex needs further study, we conclude that interactions among three individual CF subunits are all strong, suggesting that these three subunits also interact with each other within the assembled CF complex.

**DISCUSSION**

As stated in the introduction, two transcription initiation factors have been characterized in the yeast pol I system. One is UAF, which interacts with the upstream element of the promoter and is greatly stimulatory but is not essential. (UAF contains three proteins encoded by RRN5, RRN9, and RRN10, respectively, and probably two additional proteins.) The second is CF, which is essential for rDNA transcription and is composed of three proteins, two proteins encoded by RRN6 and RRN7, respectively, and p66. Identification of Rrn11 as the gene encoding the previously recognized p66 by the present study as well as by Reeder and co-workers6 completes donig and sequencing of all the three components of CF.

We have shown that the three components of CF interact strongly with each other in vitro. Under the same conditions, Rrn11p and Rrn7p interact with TBP more weakly; the interaction of the third CF subunit, Rrn6p, with TBP has not been studied in the same way. These observations are consistent with the fact that one can purify the complex consisting of Rrn6p, Rrn7p, and Rrn11p from yeast cell extracts, but the purified complex (CF) does not contain TBP (18). In addition, we have found that a temperature-sensitive rrn7 mutation can be suppressed by the presence of a multicopy plasmid carrying RRN6,6 suggesting that the direct interaction between Rrn6p and Rrn7p observed in vitro also takes place in vivo. Regarding the weak interactions observed between the TBP and Rrn7p (or

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6 D. Lalo and M. Nomura, unpublished data.
Rrn11p), although they appear to be specific in vitro, their significance in vivo has to be examined by other means. Using a yeast two-hybrid system, we have observed an interaction between Rrn6p and TBP within yeast cells, but we have not demonstrated a similar interaction between TBP and Rrn7p (or Rrn11p) with this method. It should be noted that apparently specific physical interactions observed in vitro do not necessarily prove functional significance of such interactions in vivo. For example, it has been demonstrated that the ability of TBP to interact with transcriptional activators in vitro is not directly relevant to its ability to support activated transcription in vivo (27). Purified CF, consisting of Rrn6p, Rrn7p, and Rrn11p, binds to GST-TBP only very weakly in vitro. The interaction was observed in the presence of 200 mM potassium glutamate but not under conditions similar to those used in the present study, that is, in the presence of 200 mM KCl. Thus, it is possible that some direct interactions between TBP and Rrn11p (or Rrn7p) observed in this study, although they are apparently specific, might not take place in the mixture of TBP and assembled CF complex even under the same in vitro conditions, perhaps because of masking of the TBP-interacting sites in uncomplexed Rrn11p (or Rrn7p). This and related questions are under current study.

As mentioned above, the Rrn6/7/11 complex, CF, appears to interact with TBP specifically, although weakly, and thus resembles SL1 of metazoan systems, which consists of TBP and three TBP-associated proteins (TAFs). Because of the resemblance of CF to mammalian SL1, the sequences of Rrn6p and Rrn7p were previously compared with those of the three TAFs in the human SL1, TAF4, TAF63, and TAF110. No sequence similarity was detected between two sets of the proteins (10, 18). With the sequence of Rrn11p now available, we compared it with the sequences of the three TAFs in the human SL1. No significant similarity was detected. In view of the well recognized evolutionary divergence of components in the pol I transcription system, the absence of primary sequence similarity between human SL1 and yeast CF components may not be surprising. Nevertheless, it is noteworthy that some unique sequence features are not shared. For example, TAF63 in the human SL1 contains two putative zinc fingers, and it was suggested that they might be involved in binding of TAF63 to promoter DNA as observed in ultraviolet cross-linking experiments (10). No such zinc finger motif is present in Rrn11p (or Rrn6p or Rrn7p). Similarly, as noted previously (18), the leucine zipper-like motif found in Rrn6p, which might be involved in an interaction with some other protein, is not present in human TAF proteins. Thus, it is still premature to conclude that CF and SL1 are really functionally homologous. It should also be noted that although CF appears to interact with TBP specifically, UAF also shows a specific interaction with TBP, and this interaction is in fact stronger than that between CF and TBP in vitro. Elucidation of the exact functional roles of TBP in both the yeast and the metazoan pol I transcription systems is an important subject of future study and may also help to settle the question of whether CF and SL1 are functional homologues.

As stated above, previous studies have demonstrated that UAF is greatly stimulatory but is not essential for rDNA transcription, whereas CF is essential for rDNA transcription (16, 18). This conclusion was obtained first from in vitro experiments and then confirmed by in vivo analyses using a set of yeast mutants which carry a deletion in one of the three genes, RRN5, RRN9, and RRN10, which encode subunits of UAF, or a deletion in RRN6 or RRN7, which encodes a subunit of CF. Strains carrying rrn5, rrn9, or rrn10 deletions are viable, and in vivo $[^3H]$uridine pulse-labeling experiments also indicated the presence of weak but detectable rDNA transcription by pol I in these deletion strains (16). In contrast, strains carrying rrn6 or rrn7 deletions are nonviable (16, 18), and no residual rDNA transcription by pol I was detected in $[^3H]$uridine incorporation experiments (16). Experiments described in this paper have demonstrated that a strain with a complete RRN11 deletion is nonviable, and no pol I-dependent rDNA transcription was detected in similar $[^3H]$uridine pulse-labeling experiments in vivo. Thus, all three subunits of CF, Rrn6p, Rrn7p, and Rrn11p, appear to be essential for the function of CF, the essential transcription factor of the yeast pol I. Now that the genes for all three subunits are available, we should be able to study in more detail functional roles of this essential transcription factor in rDNA transcription in relation to other molecular components participating in this important process.

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