Multiprotein transcription factor UAF interacts with the upstream element of the yeast RNA polymerase I promoter and forms a stable preinitiation complex

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Like most eukaryotic rDNA promoters, the promoter for rDNA in *Saccharomyces cerevisiae* consists of two elements: a core element, which is essential, and an upstream element, which is not essential but is required for a high level of transcription. We have demonstrated that stimulation of transcription by the upstream element is mediated by a multiprotein transcription factor, UAF (upstream activation factor), which contains three proteins encoded by *RRN5*, *RRN9*, and *RRN10* genes, respectively, and probably two additional uncharacterized proteins. The three genes were originally defined by mutants that show specific reduction in the transcription of rDNA. These genes were cloned and characterized. Epitope tagging of *RRN5* (or *RRN9*), combined with immunoaffinity purification was used to purify UAF, which complemented all three (*rrn5*, *rrn9*, and *rrn10*) mutant extracts. Using *rrn10* mutant extracts, a large stimulation by UAF was demonstrated for a template containing both the core element and the upstream element but not for a template lacking the upstream element. In the absence of UAF, the mutant extracts showed the same weak transcriptional activity regardless of the presence or absence of the upstream element. We have also demonstrated that UAF alone makes a stable complex with the rDNA template, committing that template to transcription. Conversely, no such template commitment was observed with *rrn10* extracts without UAF. By using a series of deletion templates, we have found that the region necessary for the stable binding of UAF corresponds roughly to the upstream element defined previously based on its ability to stimulate rDNA transcription. Differences between the yeast UAF and the previously studied metazoan UBF are discussed.

[Key Words: *Saccharomyces cerevisiae*; *RRN5* gene; *RRN9* gene; *RRN10* gene; UAF; UBF]

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Studies on rRNA gene (rDNA) promoters in various eukaryotic systems, extending from human to yeast (*Saccharomyces cerevisiae*), have shown that despite extensive sequence diversification of the promoter regions, there is a common feature in their structural organization; the rDNA promoter consists of two elements: a core element and an upstream element (for review, see Sollner-Webb and Tower 1986; Reeder 1992; Paule 1994; Moss and Stefanovsky 1995). The core element covers ~50 nucleotides including the transcription start site and is essential (and sufficient) for specific transcription initiation. The upstream element, which is a region extending to about -150 with respect to the transcription start site, was originally identified by deletion analysis or substitution mutagenesis of promoters and is not absolutely required for specific initiation, but is required for a high level of transcription from the promoter. For example, for the human rDNA promoter, the upstream element (called upstream control element or UCE in this

system) is mapped from -156 to -107, and the core element from -45 to +18 (Haltiner et al. 1986; see also Jones et al. 1988); and for the yeast rDNA promoter, the upstream element (domain II plus III; see Discussion) is from about -146 to about -51, and the core element is from about -28 to about +8 (Musters et al. 1989; Kulkens et al. 1991; see also Choe et al. 1992). [For other systems studied, such as mouse, rat, and *Xenopus*, see Miller et al. (1985); Tower et al. (1986); Windle and Sollner-Webb (1986a,b); Henderson and Sollner-Webb (1990); Cassidy et al. (1987).] It has been suggested that the upstream element represents a site for the binding of transcription factors, which would stimulate assembly of a preinitiation complex. In some instances, including the yeast system, it has been shown that maintenance of correct spacing between these two elements is crucial in achieving a high level of transcription, suggesting that protein factors binding to the core and the upstream elements must be located at precise positions relative to each other (Windle and Sollner-Webb 1986b; Pape et al. 1990; Choe et al. 1992; Xie and Rothblum 1992).

Two transcription factors have been identified and well characterized for RNA polymerase I (Pol I) transcription in metazoan systems, UBF (upstream binding factor; Learned et al. 1986) and SL1 [promoter selectivity factor (Learned et al. 1986); also called by other names,

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e.g., TIF-IB [Schnapp and Grummt 1991]). SL1 consists of TBP (TATA-binding protein) and three other polypeptides [TBP-associated factors (TAFs)], and is essential for in vitro transcription [Comai et al. 1992; Eberhard et al. 1993; Comai et al. 1994]. In contrast to SL1, UBF, a dimer of a polypeptide containing several high mobility group (HMG) box domains [Jantzen et al. 1990; Bachvarov and Moss 1991; O'Mahoney and Rothblum 1991; McStay et al. 1991], is probably not absolutely required for Pol I transcription (e.g., see Smith et al. 1993). UBF was originally isolated as a protein that binds to the upstream element and was demonstrated to help recruit SL1 to the template [Learned et al. 1986; Bell et al. 1988; Jantzen et al. 1990, 1992]. However, UBF was also found to interact with the core element [Bell et al. 1988; Read et al. 1992]. An interaction even with a downstream region consisting of an entirely transcribed region was also observed in the *Xenopus* system [Leblanc et al. 1993]. It was suggested that such interactions are functionally important [Bell et al. 1988; Leblanc et al. 1993; Bazett-Jones et al. 1994]. In addition, mutations in the upstream element that abolish its ability to stimulate transcription did not affect the binding of UBF to the upstream element, as judged by a footprint analysis, leading to the suggestion that those mutations must affect the interaction of this element with protein factors other than UBF in this *Xenopus* system [Read et al. 1992; for similar cases in the human system, see Bell et al. 1988]. Thus, despite convincing demonstrations of the stimulatory activities of the *cis*-acting element (upstream element) and of the *trans*-acting factor (UBF), the relationship between the two has remained unclear. Efforts to identify UBF homolog genes in lower eukaryotes including *S. cerevisiae* have so far been unsuccessful (unpublished work by several investigators cited in Yang et al. 1995).

Using a genetic system developed for *S. cerevisiae*, we have isolated many yeast mutants that are defective specifically in rDNA transcription by Pol I [Nogi et al. 1991; M. Nomura et al. unpubl.]. By studying *RRN6* and *RRN7*, we have demonstrated previously that these genes encode the 102- and 60-kD subunits of a complex (Rrn6/7 complex) that also contains another protein with a molecular mass of ~66 kD [Keys et al. 1994]. The Rrn6/7 complex was shown to be essential for rDNA transcription by Pol I in vitro and to participate in the formation of a preinitiation complex, although its stable binding to the promoter depends on the initial binding of some other factors. It was argued that this Rrn6/7 complex resembles SL1 rather than UBF, even though the purified complex does not contain TBP and there are no obvious sequence similarities between Rrn6p or Rrn7p and any of the three TAFs of human SL1 [Comai et al. 1994; Keys et al. 1994]. In this paper we first describe experiments to characterize the *RRN5*, *RRN9*, and *RRN10* genes that have led to the discovery that proteins encoded by these genes are subunits of another multiprotein complex that functions as a Pol I-specific transcription factor. In contrast to the Rrn6/7 complex, this transcription factor is not absolutely required for rDNA transcription in vitro but is greatly stimulatory. We show that the complex

containing Rrn5p, Rrn9p, and Rrn10p binds specifically to the upstream element of the yeast rDNA promoter, committing the template to transcription. We then discuss similarities and differences between UBF in metazoan systems and the newly identified yeast transcription factor complex, which we call UAF (for upstream activation factor).

Results

Mutation rrn10-1 causes a preferential defect in rRNA synthesis and defines gene RRN10

Like *rrn5* and *rrn9* mutants described previously [Nogi et al. 1991], a mutant carrying *rrn10-1* (NOY696) was originally isolated from NOY418, which carries the pGAL7–35S rDNA fusion on a plasmid (pNOY103), as a mutant that grows on a galactose plate, but grows only very poorly on glucose. This mutation represents a complementation group that defines the *RRN10* gene. As was done for other *rrn* mutants, including the *rrn5* and *rrn9* mutants to be discussed in this paper, rRNA synthesis in this *rrn10* mutant was first analyzed in vivo in the presence of glucose, which represses the transcription of the pGAL7–35S rDNA fusion gene by Pol II, and was compared with rRNA synthesis in the absence of glucose, and with rRNA synthesis in the control *RRN10* strain. As can be seen in Figure 1, synthesis of large rRNAs (18S, 25S, and 5.8S rRNAs and other precursor rRNAs) in the mutant was strongly inhibited by glucose, relative to synthesis of 5S and tRNAs (lanes 5,6; see legend to Fig. 1). No such inhibition was observed for the control parent strain (lanes 3,4). We conclude that the *rrn10* mutation causes a specific defect in Pol I-mediated rDNA transcription, and rRNA synthesis in the mutant growing in galactose medium is largely achieved by transcription of the pGAL7–35S rDNA fusion gene by Pol II. (Fig. 1 also includes the results obtained with the *rrn10Δ::LEU2* deletion mutant as well as *rrn5Δ::LEU2*, *rrn9Δ::HIS3*, and *rrn7Δ::LEU2* mutant strains to be discussed below.)

Cloning and characterization of the RRN5, RRN9, and RRN10 genes

Cloning of *RRN5*, *RRN9*, and *RRN10* genes was done using strain NOY697 carrying *rrn5-1*, strain NOY698 carrying *rrn9-1*, and strain NOY696 carrying *rrn10-1*, respectively, as was done for cloning of other *RRN* genes [Nogi et al. 1993; Keys et al. 1994]. Transformants with the growth characteristics of the wild type were isolated on glucose plates, the plasmids were recovered, and the genes were subcloned and sequenced. Physical structures of the chromosomal DNA regions containing *RRN5*, *RRN9*, and *RRN10* are shown in Figure 2, a, c, and e, and amino acid sequences of encoded proteins Rrn5p, Rrn9p, and Rrn10p, as deduced from nucleotide sequences, are shown in Figure 3.

RRN5 encodes a protein of 363 amino acids with a calculated molecular weight of 41,776 and a calculated

Figure 1. Polyacrylamide/agarose gel electrophoresis of RNA synthesized in various *rrn* mutants and the parent strain growing in galactose medium (odd-numbered lanes) and after shifting to glucose medium (even-numbered lanes). Strains NOY418 (WT), NOY699 ($\Delta 5$), NOY 696 (10-1), NOY558 ($\Delta 7$), NOY703 ($\Delta 9$), and NOY704 ($\Delta 10$) were grown at 30°C and [3 H]uridine pulse-labeling was carried out as described in Materials and methods. RNA samples containing approximately equal radioactivity were subjected to electrophoresis. Autoradiographs of gels are shown. Results from two independent experiments appear in lanes 1–6, and lanes 7–14, respectively. The positions of 25S, 18S, and 5.8S rRNAs, as well as 5S and tRNAs are indicated. Positions of three precursor rRNAs, which presumably correspond to 35S, 27S, and 20S rRNAs, respectively, are indicated by asterisks. In these experiments, radioactive large rRNAs relative to radioactive 5S plus tRNAs were quantified without correction for background radioactivity. These values obtained for mutants in glucose medium divided by the corresponding values for the wild type were 0.17 ($\Delta 5$), 0.13 (10-1), 0.022 ($\Delta 7$), 0.11 ($\Delta 9$), and 0.071 ($\Delta 10$).

isoelectric point of 6.99. The protein is hydrophilic and contains a highly acidic region near the carboxyl terminus of the molecule (position 311–346; 44% acidic amino acids). The DNA sequence analysis revealed that the *RRN5* gene is located on chromosome XII adjacent to a known gene, *PUT1*, which encodes the mitochondrial proline oxidase (Wang and Brandiss 1987) (Fig. 2a).

RRN9 encodes a protein of 365 amino acids with a calculated molecular weight of 42,786, which is slightly larger than that of Rrn5p. The calculated isoelectric point of Rrn9p is 5.11, and the protein is highly hydrophilic. From the DNA sequence upstream of *RRN9*, we found that *RRN9* is located downstream of a known

gene, *URA10*, which encodes one of the two known orotate phosphoribosyl transferase isozymes (de Montigny et al. 1990) (Fig. 2c).

The protein (Rrn10p) encoded by *RRN10* is 145 amino acids in size, its calculated molecular weight is 16,494, and the isoelectric point is 4.95. After we completed the sequencing of this gene, the DNA sequence containing this region was published as part of the yeast genome sequencing project (Van Dyck et al. 1994), enabling us to conclude that *RRN10* is on the left arm of chromosome II. *RRN10* corresponds to an open reading frame called YBL0426 in this region.

We carried out standard gene disruption experiments to confirm the expected essential requirement of the

Figure 2. Restriction enzyme maps of the *RRN5*, *RRN9*, and *RRN10* genomic regions. (a) The *RRN5* region; (b) insertion of a 2.2-kb *LEU2* fragment between the *NcoI* and *XhoI* sites to construct the *rrn5Δ::LEU2* null allele; (c) the *RRN9* region; (d) insertion of a 0.6-kb *HIS3* fragment between the *SalI* and *EcoRV* sites to construct the *rrn9Δ::HIS3* null allele; (e) the *RRN10* region; (f) insertion of a 2.2-kb *LEU2* fragment between the *PstI* and *XhoI* sites to construct the *rrn10Δ::LEU2* null allele. Protein-coding regions of *RRN5*, *RRN9*, and *RRN10* are stippled. The *LEU2* fragment is hatched; the *HIS3* fragment is cross-hatched. The 5' region of *PUT1*, located downstream of *RRN5*, and the 3' region of *URA10*, located upstream of *RRN9*, are shown by open boxes. The positions of DNA inserts encoding the triple-HA1-epitope are shown by open triangles. Arrows show the direction of transcription. Vector sequences located downstream of *RRN9* in the original library isolate (pNOY187) are shown by a solid line in c and d.

Figure 3. Predicted amino acid sequences of Rrn5p, Rrn9p, and Rrn10p. The nucleotide sequences of *RRN5*, *RRN9*, and *RRN10* have been submitted to the GenBank data base, under accession numbers U43680, U43681, and U43682, respectively.

three cloned genes for growth and to construct strains that would be useful for purification of proteins encoded by the genes. DNA fragments carrying disrupted genes described in Figure 2, b, d, and f, *rrn5Δ::LEU2*, *rrn9Δ::HIS3*, or *rrn10Δ::LEU2*, were used to construct diploid strains that carry one chromosome with the wild-type *RRN* allele and another chromosome with one of the corresponding genes replaced by the disrupted allele (NOY679, NOY678, and NOY677, respectively; Table 1). These diploid strains were sporulated, tetrads were dissected on YEP–glucose medium, and their segregation patterns were monitored at 25°C or 30°C. To our initial surprise, we found that these genes were not absolutely required for spore germination or cell growth. As can be seen in Figure 4, a–c, tetrads from three diploid strains, *RRN5/rrn5Δ::LEU2*, *RRN9/rrn9Δ::HIS3*, and *RRN10/rrn10Δ::LEU2*, all gave a 2:2 segregation pattern with two large colonies and two (or occasionally one or zero) very small colonies. The very small colonies carried the *LEU2* or *HIS3* genes used for disruption and represent haploid deletion segregants. In contrast, paral-

lel tetrad dissection of a diploid strain, *RRN7/rrn7Δ::LEU2* (NOY548), showed the segregation pattern of two viable and two nonviable spores (Fig. 4d), as reported previously (Keys et al. 1994). Thus, it appears that in contrast to *RRN6* and *RRN7*, none of the three genes studied here, *RRN5*, *RRN9*, and *RRN10*, is absolutely essential. It should be noted that the original mutant strains, including those used for gene cloning, were able to grow very slowly on glucose media, but their growth was sufficiently weak so that the strains could be used to clone the genes by complementation.

Using the diploid strains carrying the disrupted *rrn5* (or *rrn9* or *rrn10*) described above, we have constructed haploid strains with the chromosomal *RRN5* (or *RRN9* or *RRN10*) disrupted and carrying the pGAL7–35S rDNA fusion on the pNOY103 plasmid (NOY699, NOY703, and NOY704; see Fig. 2 and Table 1). These strains can grow on galactose but very poorly on glucose. Some of these strains were used to make extracts described below. In addition, strains with the chromosomal *RRN5* (or *RRN9*) disrupted and carrying a triple-HA1-tagged *RRN5* (or *RRN9*) gene on a centromeric plasmid were also constructed (NOY662 and NOY674) and utilized to detect and affinity purify Rrn5p (or Rrn9p) with monoclonal antibodies against the HA1 epitope, as described below.

Complementation of *rrn5*, *rrn9*, and *rrn10* mutant extracts in vitro and purification of a multiprotein complex containing Rrn5p, Rrn9p, and Rrn10p

Preliminary experiments showed that extracts (PC-300 plus D-300 fractions; see Materials and methods) prepared from *rrn5*, *rrn9*, or *rrn10* mutants showed very weak activities in transcription of rDNA templates, relative to extracts from the wild-type strain (data not shown; for the *rrn10* extract, see Fig. 8, lane 1, discussed below). Therefore, we attempted to purify Rrn5p, Rrn9p, and Rrn10p, or protein complexes containing these proteins to see whether they complement respective mutant extracts.

We started with extracts from NOY674, which expresses triple-HA1-tagged Rrn9p [(HA1)₃–Rrn9p], and ex-

Figure 4. Tetrad analysis of diploid strains, NOY679 (*RRN5/rrn5Δ::LEU2*) (a), NOY678 (*RRN9/rrn9Δ::HIS3*) (b), NOY677 (*RRN10/rrn10Δ::LEU2*) (c), and NOY548 (*RRN7/rrn7Δ::LEU2*) (d). Diploid cells were sporulated, tetrad spores were dissected, and haploid segregants were grown on YEP–glucose plates for 10 days at 30°C.

Table 1. Yeast strains and plasmids used

Strain or plasmid	Description
Strain	
NOY397	<i>MATa/MATα 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</i>
NOY418	<i>MATa ade2 ade3 leu2 ura3 lys2 can1</i> pNOY103 (Nogi et al. 1991)
NOY423	<i>MATα ade2 ade3 leu2 ura3 trp1 can1</i> [a meiotic segregant of the cross between strains CH1305 (Kranz and Holm 1990) and NOY396 (Nogi et al. 1991)]
NOY424	NOY423 carrying pNOY103
MOY610	<i>MATa/MATα ade2/ade2 ade3/ade3 leu2/leu2 ura3/ura3 trp1/trp1 can1/can1</i>
NOY697	<i>MATa ade2 ade3 leu2 ura3 lys2 can1 rrn5-1</i> pNOY103
NOY705	<i>MATa ade2 ade3 leu2 ura3 lys2 can1 rrn5-2</i> pNOY103
NOY698	<i>MATα ade2 ade3 leu2 ura3 trp1 his can1 rrn9-1</i> pNOY103
NOY696	<i>MATα ade2 ade3 leu2 ura3 lys2 his can1 rrn10-1</i> pNOY103
NOY679	<i>MATa/MATα 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 RRN5/rrn5Δ::LEU2</i>
NOY548	<i>MATa/MATα 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 RRN7/rrn7Δ::LEU2</i> (Keys et al. 1994)
NOY678	<i>MATa/MATα 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 RRN9/rrn9Δ::HIS3</i>
NOY677	<i>MATa/MATα ade2/ade2 ade3/ade3 leu2/leu2 ura3/ura3 trp1/trp1 can1/can1 RRN10/rrn10Δ::LEU2</i>
NOY577	<i>MATα ade2 ade3 leu2 ura3 trp1 his can1 rrn5::TRP1</i> (contains <i>TRP1</i> insertion at the <i>Clal</i> site, which is within the codon Ile 161 of <i>RRN5</i>), pNOY103
NOY662	NOY577 carrying pNOY330 instead of pNOY103
NOY699	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn5Δ::LEU2</i> pNOY103
NOY558	<i>MATα ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11 can1-100 rrn7Δ::LEU2</i> pNOY103 (Keys et al. 1994)
NOY703	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn9Δ::HIS3</i> pNOY103
NOY674	NOY703 carrying pNOY332 instead of pNOY103
NOY690	<i>MATα ade2 ade3 leu2 ura3 trp1 can1 rrn10Δ::LEU2</i>
NOY704	<i>MATa ade2 ade3 leu2 ura3 trp1 can1 rrn10Δ::LEU2</i> pNOY103
Plasmid	
pRS315	yeast- <i>E. coli</i> shuttle vector (Skierski and Hieter 1989); <i>LEU2</i> , <i>CEN6</i> , <i>ARSH4</i>
GTEP1	derivative of pBluescript IISK(-); contains a <i>NotI</i> fragment that encodes three copies of the HA1 epitope sequence YPYDVPDYA (Roof et al. 1992)
pSIRT	a derivative of pEMBL Yc30-Δ6 that carries the mini rDNA gene called SIRT (Musters et al. 1989)
pNOY103	high-copy-number plasmid carrying <i>GAL7-35S</i> rDNA, <i>ADE3</i> , <i>2μ</i> , <i>amp</i> (Nogi et al. 1991)
pNOY230	the original plasmid carrying <i>RRN5</i> recovered from a YCpN1 (<i>CEN3 ARS TRP1</i>) bank
pNOY187	the original plasmid carrying <i>RRN9</i> on a ~5-kb DNA fragment, recovered from a YCpN1 bank
pNOY337	the original plasmid carrying <i>RRN10</i> on a ~3.6-kb DNA fragment recovered from a YEp13 (<i>LEU2</i> , <i>2μ</i> , <i>amp</i>) bank
pNOY328	derivative of pPC97 (<i>LEU2</i> , <i>CEN6</i> , <i>amp</i> ; a gift from D. Nathans; similar to pPC62 described by Chevray and Nathans 1992); the 1.6-kb <i>XhoI</i> - <i>XhoI</i> fragment carrying the <i>ADC1</i> promoter was removed; retains multicloning sites present upstream of the <i>ADC1</i> terminator
pNOY330	carries ~1.7-kb DNA fragment encoding triple-HA1-tagged Rrn5p inserted between <i>Sall</i> and <i>SacI</i> of pNOY328 (see text)
pNOY335	carries ~2.1-kb <i>BamHI</i> - <i>NaeI</i> fragment containing <i>RRN9</i> inserted between <i>BamHI</i> and <i>NaeI</i> of pRS315
pNOY332	carries ~2.2-kb DNA fragment encoding triple HA1-tagged Rrn9p between <i>BamHI</i> and <i>NaeI</i> of pRS315
pNOY3114	2.7-kb <i>BglII</i> fragment carrying <i>LEU2</i> (from YEp13) inserted into the <i>BamHI</i> site of pUC19
pNOY3220	derivative of pBluescript KS(+); carries a <i>NotI</i> - <i>SacI</i> fragment encoding 37 amino acids, which contain three copies of the HA1 peptide sequence, inserted between the <i>NotI</i> and <i>SacI</i> sites
pNOY3124	(= pATH23) TrpE fusion/expression vector (Koerner et al. 1991)

tracts from NOY662, which expresses triple-HA1-tagged Rrn5p [(HA1)₃-Rrn5p], and used immunoaffinity purification as an initial purification step. By testing fractions after this step for the presence of proteins encoded by several *RRN* genes using polyclonal antibodies in addition to testing (HA1)₃-Rrn9p [or (HA1)₃-Rrn5p] using anti-HA1 monoclonal antibody, it soon became clear that Rrn5p, Rrn9p, and Rrn10p copurify, and the preparations that were active in complementing *rrn9* (or *rrn5*)

extracts were also active in complementing *rrn5* (or *rrn9*) and *rrn10* extracts. Figure 5 summarizes the purification scheme used for extracts containing (HA1)₃-Rrn5p as well as for those containing (HA1)₃-Rrn9p.

The results of analysis of fractions obtained from the final Superose 12 gel filtration column are shown in Figure 6 for a purification starting from an extract containing (HA1)₃-Rrn5p. It can be seen that both Rrn9p and Rrn10p copurified with (HA1)₃-Rrn5p as assayed by

Figure 5. Scheme for purification of Rrn5/9/10 complex (UAF).

SDS-PAGE followed by immunoblot analysis of column fractions (Fig. 6b). The column fractions were also analyzed for proteins by SDS-PAGE followed by silver staining (Fig. 6a). Five bands were consistently observed in the fractions containing Rrn5p, Rrn9p, and Rrn10p (see legend to Fig. 6). The apparent molecular masses of these five proteins were 58, 50, 30, 18, and 17 kD, as estimated from positions of molecular mass markers; the 58-kD component corresponded to (HA1)₃-Rrn5p and the 50-kD component corresponded to Rrn9p for the preparation obtained from the (HA1)₃-RRN5 strain, and the 17-kD component corresponded to Rrn10p, as judged by comparison with immunoblot analysis. The sum of the apparent molecular mass of these five proteins is 173 kD, and this is roughly consistent with the peak position of the active complex in this sizing column; the peak position was slightly behind the size marker (β-amylase (206 kD) but several fractions ahead of another size marker, BSA (66 kD) (data not shown). Thus, Rrn5p, Rrn9p, and Rrn10p appear to be components of a multiprotein complex (Rrn5/9/10 complex). This complex appears to contain two additional proteins with sizes of ~30 kD (p30) and ~18 kD (p18), respectively (see legend to Fig. 6 for other protein bands seen in Fig. 6a). It should be noted that p30 is not TBP (27 kD) and the purified preparation does not contain TBP, as analyzed by Western blot with polyclonal antibodies against yeast TBP (data not shown). Column fractions were also analyzed for their ability to complement *rrn5*, *rrn9*, and *rrn10* mutant extracts (Fig. 6c–e). The three complementation activities were coeluted from the column, and their peak fractions (Fig. 6, fractions 35–39) corresponded to the peak fractions revealed by protein gels. We conclude that a multiprotein complex containing Rrn5p, Rrn9p, and Rrn10p (and probably two additional proteins, p30 and p18) is the transcription factor missing in extracts prepared from *rrn5*, *rrn9*, or *rrn10* mutants. [It should be noted that a close examination of the data shown in Fig. 6 suggests that the protein composition of the complex

may not be homogeneous; notably the ratio of p30 to p18 changes from earlier fractions to later fractions. Thus, establishing p30 and p18 as genuine components of the Rrn5/9/10 complex must await cloning of their genes and appropriate genetic and biochemical analyses.]

We note that the molecular mass of (HA1)₃-Rrn5p calculated from the amino acid sequence is ~46 kD [41.8 kD for Rrn5p plus 4.3 kD for the (HA1)₃ epitope] and is considerably smaller than 58 kD estimated from its mobility in SDS-PAGE. Similarly, the molecular mass of Rrn9p calculated from the amino acid sequence is ~42.8 kD, which is smaller than 50 kD estimated from the mobility in SDS-PAGE. We do not know the reason for these discrepancies.

To confirm the presence of Rrn5p, Rrn9p, and Rrn10p (and p30 and p18) in a single transcription factor complex, we repeated purification of the active complex starting from NOY674, which carries the (HA1)₃-RRN9 gene, instead of the (HA1)₃-RRN5 strain. The same purification method was used with an immunoaffinity purification as the first step. Fractions from the final Superose 12 gel filtration column were analyzed for protein components using SDS-PAGE followed by silver staining and Western immunoblot, and for *in vitro* complementation activities. The results confirmed the conclusion obtained with the preparations purified from the (HA1)₃-RRN5 strain. Activities that could complement *rrn5*, *rrn9*, and *rrn10* mutant extracts were copurified as a complex containing Rrn5p, (HA1)₃-Rrn9p, and Rrn10p. The presence of the two other proteins, p30 and p18, in the complex was also demonstrated (data not shown).

The upstream element is required for stimulatory activity of UAF

As described in the introductory section, Pol I promoters consist of two domains: the core element, which is essential, and the upstream element, which is not essential but stimulatory. As already noted above, extracts prepared from *rrn5*, *rrn9*, or *rrn10* mutants showed great reduction in Pol I transcription activity; nevertheless, we observed weak but definite activity in these extracts. Therefore, we thought that the complex might be involved in the stimulation mediated by the upstream element of the Pol I promoter. Transcription experiments using *rrn10* extracts were carried out in which two DNA templates were compared: one with the wild-type promoter (called pSIRT; see Fig. 7a), and the other with a mutant promoter carrying a deletion from -208 to -91 (i.e., deletion of a region abolishing most of the stimulation by the upstream element; see Musters et al. 1989; Kulkens et al. 1991; Fig. 7b). With the mutant promoter, no significant stimulation by the Rrn5/9/10 complex was observed (Fig. 8, cf. lane 4 and 2), whereas a large stimulation was observed with the wild-type promoter (Fig. 8, cf. lane 3 and 1; see the legend). In the absence of the complex, no significant difference was observed in transcription between the wild-type and the mutant promoter (Fig. 8, cf. lane 1 and 2; see the legend), indicating that stimulation by the upstream element is mediated by

Figure 6. Purification of the Rrn5/9/10 complex (UAF) from NOY662 expressing $(HA1)_3$ -Rrn5p. The complex was purified as diagrammed in Fig. 5 and described in Materials and methods. (a) Individual fractions eluted from Superose 12 column analyzed by SDS-PAGE followed by silver staining of protein [120 μ l of Superose fraction per lane; concentrated by Microcon 10 (Amicon); fractions 33–42, as indicated]. Twenty microliters of the column-load fraction (Mono S peak fraction concentrated by ultrafiltration) was applied to lane L. The positions of molecular weight markers (LM) are as indicated. (b) Western

blot analysis of Superose 12 fractions. Forty microliters of Superose fraction was applied per lane. After transferring onto a PVDF (Immobilon-P, Millipore) filter, the filter was cut in half. The top portion was first analyzed using anti-Rrn9p polyclonal antisera to detect Rrn9p. After washing, the same filter was subjected to analysis with anti-HA1 monoclonal antibody to detect $(HA1)_3$ -Rrn5p. The bottom half of the filter was analyzed using anti-Rrn10p polyclonal antisera. Ten microliters of column-load fraction was applied to lane L. (SDS-PAGE for *a* did not contain dithiothreitol; SDS-PAGE for *b* contained it and was run for a longer time than that for *a*.) (c–e) Activities of the Superose 12 fractions to complement *rrn5*, *rrn9*, and *rrn10* mutant extracts were analyzed by in vitro transcription. Transcription was carried out with D-300 and PC-300 fractions prepared from *rrn5* (NOY705) (c), *rrn9* (NOY703) (d), and *rrn10* (NOY696) (e) mutant strains, supplemented with 1.5 μ l of the indicated Superose fraction or with 0.5 μ l of column-load fraction (lane L). In lane C, control reactions were carried out using mutant D-300 and PC-300 fractions with no other addition. We note that the SDS-PAGE gel stained with silver showed a clear band just below the Rrn9p band (a). In other experiments with different preparations, such a band was not seen. It is not known whether this band represents a genuine component distinct from Rrn9p. Similarly, the p30 band appears as a doublet in this gel, but this was not seen in other experiments. We also note that there are other faint bands [e.g., one at ~80-kD position and another at ~39-kD position] in the peak fractions showing stimulatory activity. The significance of these bands is not known.

this transcription factor complex. For this reason, we call the Rrn5/9/10 complex UAF.

UAF is necessary and sufficient for template commitment

It was demonstrated previously that when an rDNA template was incubated with extracts or crude transcription factor fractions in the absence of nucleoside triphosphates, a transcription factor(s) forms a stable complex with the template and fails to participate in the transcription of a subsequently added second template. This was shown for several Pol I transcription systems (Cize-

wski and Sollner-Webb 1983; Wandelt and Grummt 1983; Kownin et al. 1985; Clos et al. 1986; Kato et al. 1986), including yeast systems using crude extracts (Kulkens et al. 1991), *rrn6* mutant extracts supplemented with the Rrn6/7 complex (Keys et al. 1994), or partially purified chromatographic fractions (Riggs et al. 1995). We carried out similar template commitment experiments using *rrn10* extracts supplemented with UAF and examined a possible role of UAF in the formation of stable preinitiation complex.

As shown in Figure 9, when *rrn10* extracts supplemented with UAF were preincubated with a template DNA, the transcription of a subsequently added second

Figure 7. rDNA template, pSIRT, and deletion mutant templates. (a) The minigene SIRT (contained in plasmid pSIRT) aligned with a rDNA unit. Solid bars represent the coding regions of the rRNA genes. Transcribed and nontranscribed spacers are represented by stippled and open bars, respectively. Boxes, designated P and E, are the Pol I promoter and the enhancer/terminator regions, respectively. (b) The promoter region and various deletion constructs. The names of deletion constructs indicate the first and the last base pair deleted. Locations of the upstream element (UE) and the core element within the promoter are indicated.

template was greatly reduced, as expected, indicating the formation of a stable complex of some transcription factors with the first template (lanes 2 and 3, cf. controls, lanes 1, 4, and 5). When UAF was omitted from the preincubation step and added immediately following the second template, both templates were transcribed (Fig. 9a, lanes 7 and 8; cf. control, lane 6), that is, the commitment to the first template during preincubation required UAF. The converse experiment, preincubation of the first template with UAF followed by the addition of the second template and the *rrn10* extracts, showed transcription of the first template and little or no transcription of the second template (Fig. 9, lanes 10 and 11; cf. controls, lanes 9, 12, and 13). These results demonstrate that UAF is apparently bound stably to a template and that UAF is necessary and sufficient for template commitment.

Template competition analysis of promoter deletion mutants

To define the promoter region responsible for the binding of UAF, we carried out template competition experiments using various 5'- and 3'-deletion promoters (see Fig. 7b) as the first template for preincubation with UAF. As shown in Figure 10a, a deletion from -208 to -155 partially impaired the ability of the first template to sequester UAF from transcription of the second template (lane 3; in some other experiments, no impairment or

only a very weak impairment was observed). Deletions extending to -119 and beyond abolished this ability; that is, transcription of the second template took place without any significant decrease relative to control (Fig. 10a, cf. lanes 4-6 and 7). [It should be noted that 5' deletion of the first template to -155 decreased the template activity itself only slightly, if any, and deletion to -119 and to -91 caused a very large decrease in the template activity of the first template but did not abolish it; deletion to -26 abolished transcription of the first template completely in this system. Thus, the 5' boundary of the promoter, as judged by template activity (not by competition activity), is between -155 and -119, and the boundary between the upstream and core elements is somewhere between -91 and -26. These results are very similar to the results of previous analyses (Musters et al. 1989; Kulkens et al. 1991; Choe et al. 1992; see legend to Fig. 10).]

Similar template competition experiments were also carried out by a series of 3'-deletion mutants of the first template. The ability to bind UAF, and thus prevent transcription of the second template, was not significantly affected when the promoter was deleted to -41 from downstream, even though the template activity was lost when the deletion extended to -2 and beyond into the core region (Fig. 10b, lanes 4-6; see legend). When the deletion extended to -70 and beyond, the ability of the first template to bind UAF was lost, and transcription of the second template took place to ap-

Figure 8. Effects of UAF on transcription of rDNA using template with or without the upstream element. In vitro transcription reactions were carried out using *rrn10* mutant extract with or without addition of purified UAF (0.6 μ l of Superose peak fraction). pSIRT is the template used in lanes 1 and 3 (wild-type promoter). pSIRT Δ -208/-91, which contains an internal deletion removing an upstream promoter sequence, is the template used in lanes 2 and 4. Relative amounts of 32 P-labeled nucleotide incorporated into specific transcripts were quantitated using a PhosphorImager. The values obtained were 1.0, 0.74, 10.1, and 0.50 for lanes 1–4. In other experiments, relative levels of specific transcripts were quantitated by densitometric analysis of autoradiographs. Similar results were obtained, and the degree of stimulation by UAF for the wild-type promoter ranged from $\sim$ 10- to $\sim$ 25-fold. We note that the two templates used contain the 5S RNA gene (see Fig. 7a), and that the amounts of 5S RNA transcripts produced in the same reactions were not significantly different among the four reactions (not shown here), thus serving as an internal control in these experiments.

proximately the same degree as the control (Fig. 10b, cf. lanes 7–9 and 10). These experiments define the region (5' border near -155; 3' border between -70 and -41) that is required for the stable binding of UAF leading to template commitment.

Discussion

UAF is a Pol I transcription factor that is not essential but stimulatory

Experiments described in this paper demonstrate that the three proteins, Rrn5p, Rrn9p, and Rrn10p, encoded by *RRN5*, *RRN9*, and *RRN10*, respectively, are components of a multiprotein complex that functions as a transcription factor in rDNA transcription by Pol I. First, the complex has been purified both from a strain expressing HA1-tagged *RRN5* and from a strain expressing HA1-tagged *RRN9*, by procedures that include a specific immunoaffinity purification step followed by three column chromatography steps, and the presence of Rrn5p, Rrn9p, and Rrn10p (together with two additional proteins) was demonstrated in both instances. In addition, the purified complex had the ability to stimulate transcription by three different extracts prepared from *rrn5*, *rrn9*, and *rrn10* mutants, respectively. Therefore, it is clear that these three different mutant extracts are deficient in Pol I transcription activity because they lack this transcrip-

tion factor complex. As mentioned above, we call this complex UAF.

In contrast to extracts prepared from *rrn6* and *rrn7* disruption mutants [Keys et al. 1994], where no rDNA transcription activity was detectable, extracts prepared from *rrn5*, *rrn9*, and *rrn10* disruption mutants all showed very weak, but detectable activity to transcribe rDNA specifically. Thus, the UAF complex is not absolutely required for rDNA transcription in vitro, but serves as a stimulatory factor. This conclusion also appears to apply to rDNA transcription in vivo. As shown in this paper, the *RRN5*, *RRN9*, and *RRN10* genes are not absolutely required for cell growth, whereas the *RRN7* and *RRN6* genes are essential [Keys et al. 1994]. The difference between the two groups of Pol I transcription factor genes is almost certainly attributable to the presumed difference in the requirements of their gene products for rDNA transcription in vivo. To confirm this presumed difference, we carried out in vivo pulse-labeling experiments. Disruption mutant strains Δ rrn5, Δ rrn9, or Δ rrn10, which carry the pGAL7-35S rDNA fusion gene on plasmid pNOY103, were grown in galactose media, shifted to glucose media, and then incorporation of [3 H]uridine into large rRNAs was examined and compared with the Δ rrn7 strain carrying the same pGAL7-35S rDNA fusion gene on pNOY103. We observed very weak but definite synthesis of 3 H-labeled 18S and 25S rRNAs in glucose for the former three disruption (Δ rrn5, Δ rrn9, and Δ rrn10) strains, while the synthesis of these rRNAs was undetectable for the Δ rrn7 strain [M. Nomura et al. unpubl.; see also Fig. 1 and the legend]. It should be noted that in previous experiments using an *rrn7* mutant (as well as an *rrn6* mutant), weak but definite synthesis of 18S and 25S rRNAs was observed [Nogi et al. 1991]. The *rrn7* mutant (*rrn7-1*) used in the previous work carries an amber mutation changing a glutamine codon at 250 to a stop codon [D.A. Keys et al., unpubl.], whereas the *rrn7* mutant used in the new experiments carries a deletion (*rrn7* Δ ::*LEU2*). Perhaps more importantly, the repression of the GAL7-35S rDNA fusion gene in the previous experiments was done by the addition of glucose to galactose medium without removing galactose, whereas in the new experiments repression was achieved with glucose in the absence of galactose, leading to presumably much stronger repression than in the previous experiments. These differences may explain the difference in (the degree of) residual rRNA synthesis in *rrn7* mutants between the previous and the new experiments. Because of uncertainty in the degree of the residual transcription of rDNA by Pol II in this system combined with very weak rRNA synthesis in these deletion mutants in glucose media, it is difficult to definitively prove the above-mentioned proposition, namely that Rrn6p and Rrn7p are absolutely required for Pol I transcription, whereas the requirement of Rrn5p, Rrn9p or Rrn10p for rDNA transcription by Pol I is more relaxed in vivo. Nevertheless, [3 H] uridine pulse-labeling experiments like that shown in Figure 1 tend to support this proposition.

Even though *RRN5*, *RRN9*, and *RRN10* are not abso-

Figure 9. Formation of a committed UAF–template complex. Template commitment experiments were performed using *rrn10* mutant extract and purified UAF (a Superose peak fraction) as outlined in *b*. The mutant extract and purified UAF together (lanes 1–5) or individual fractions (mutant extract; lanes 6–8, or purified UAF; lanes 9–13) were preincubated with the first template (400 ng) for 60 min. The second template (400 ng) was then added, together with *rrn10* extract or UAF that was omitted during the first preincubation, as indicated. After a second preincubation of 30 min nucleoside triphosphates (NTPs) were added, and transcription was allowed to proceed for 40 min. RNA was analyzed by urea–PAGE followed by autoradiography (*a*). Template A is pSIRT giving a specific transcript of 765 nucleotides; template B is pSIRTΔ + 39/ + 128, giving a specific transcript of 675 nucleotides. The positions of these two transcripts are indicated as A and B, respectively. In lanes 4 and 5 and 12 and 13, preincubation with *rrn10* extract and/or UAF was done using nonspecific DNA (pBluescript vector DNA; indicated as V) followed by the addition of rDNA template to show that the absence of transcription of the second added template (in lanes 2 and 3 and 10 and 11) is attributable to specific and stable binding of UAF to the first rDNA template.

lutely essential genes and the UAF complex containing the encoded proteins is not absolutely required for in vitro rDNA transcription, the degree of stimulation by UAF observed in vitro is large, ranging from ~10- to ~25-fold stimulation. The stimulation provided by UAF in vivo might even exceed this increase. In the experiments mentioned above (see legend to Fig. 1), one can calculate reduction in large rRNA synthesis in $\Delta rrn10$ (or $\Delta rrn5$ or $\Delta rrn9$) strains. Disregarding the contribution of residual rDNA transcription by Pol II in glucose media and normalizing to the sum of 5S RNA and tRNA synthesized by Pol III, we can conclude, for example, that the reduction of rRNA transcription in the $\Delta rrn10$ mutant is ~93%. Thus, UAF, though not essential, appears to play an important function in rDNA transcription in vivo.

UAF mediates stimulation by the upstream element

We have demonstrated that the large stimulation by the upstream element is mediated by UAF; with a template

lacking the upstream element, UAF does not give any significant stimulation of transcription by Pol I, and conversely, in the absence of UAF, the upstream element does not confer any significant stimulation. It appears that UAF is the factor essential for stimulation by the upstream element, and no other component (in our extracts) can replace UAF to mediate the stimulation.

We have also demonstrated that UAF binds to the upstream element and commits that template to transcription. Our experiments show that UAF is necessary and sufficient for template commitment and therefore defines the basis of stimulation of transcription by the upstream element mediated by UAF in our in vitro transcription system. Of course, our in vitro system uses arbitrarily defined “crude extracts” (D-300 plus PC-300 fractions) and may be missing some important factors that might also be capable of stimulation by (and/or binding to) the upstream element. However, we think that such factors, even if present, might carry out perhaps a secondary role and will not be able to substitute for the role of UAF. This argument is based on the fol-

Figure 10. Template competition analysis of promoter mutants to define the region required for the stable association of UAF with the rDNA template. Template competition experiments were performed as diagramed in *c*. Purified UAF [0.6 μ l of Superose peak fraction] was preincubated with the first DNA template (400 ng). Then a second wild-type DNA template (200 ng) was added together with mutant *rrn10* extract, and after a second preincubation, nucleoside triphosphates were added to allow transcription to begin. A series of 5'-promoter deletions were used as first template in *a*; a series of 3'-promoter deletions were used in *b*. The second template in *a* is pSIRT Δ +39/+128, containing a wild-type promoter and generating a specific transcript of 675 nucleotides, 90 nucleotides shorter than the transcript generated from the 5'-promoter deletion mutants, when they are active. The second template in *b* is pSIRT (wild type), which generates a transcript of 765 nucleotides that is longer than the transcripts produced from transcriptionally active 3'-deletion mutants used as the first template. It should be noted that longer 3' deletions are expected to produce correspondingly shorter transcripts when they are active. Thus, the transcripts from the first templates seen in lanes 2 and 3 in *b* are expected to be 90 nucleotides and 102 nucleotides, respectively, shorter than the transcript from the second template. Note that templates used for lanes 4–6 had the ability to sequester UAF, yet failed to produce any transcripts. The latter result is consistent with the results of previous analyses to define the 3' boundary of the promoter (see text). Nonspecific DNA (400 ng pBluescript vector DNA) was used in the first preincubation in lanes 1 and 7 in *a*, and lanes 1 and 10 in *b*. UAF was omitted from the reaction in lanes 1 of *a* and *b*.

lowing observations: First, the degree of stimulation by the upstream element observed in vivo (by at least a factor 5; done by the use of plasmids carrying a minigene and its deletion mutants; see Musters et al. 1989) can be accounted for by the stimulation observed by the present in vitro system (by a factor of 10–25); second, in vivo [³H]uridine incorporation experiments using *rrn5*, *rrn9*, and *rrn10* mutants directly demonstrate a major and specific role of these gene products in rDNA transcription by Pol I, and calculation shows that UAF stimulates rDNA transcription in vivo by at least a factor of ~15 (see the preceding section); and third, *rrn5*, *rrn9*, and *rrn10* disruption strains show a severe reduction in their growth rates, although they are still able to form colonies, and their growth defects can be substantially overcome by a helper plasmid carrying the pGAL7–35S rDNA fusion gene, indicating again the importance of these genes in Pol I function in vivo.

The upstream region required for stable binding of UAF

Experiments using various deletion mutant templates have clearly defined the upstream region of the promoter required for the stable binding of UAF. The 5' boundary of this region is near –155, and the 3' boundary is between –41 and –70. Previously we carried out similar template commitment experiments using crude extracts and obtained the evidence for the presence of a factor (which is now identified as UAF) interacting with the upstream region (Kulkens et al. 1991). The results were generally similar to the present one. However, there are some discrepancies in defining the 5' and the 3' boundaries of the region responsible for template commitment. In the previous study, a deletion extending to –119 showed a partial (not a full) reduction in the ability to bind the factors, and a more extensive deletion was re-

quired to achieve full reduction. Similarly, the 3' deletion extending to -70 caused a full reduction in the ability to bind UAF in the present study, whereas the same deletion did not cause such a reduction and deletion had to go further to achieve a full reduction. The differences are probably derived from the use of highly purified UAF in the present experiment for preincubation with the first template, whereas crude extracts were used in the previous studies. Thus, in the absence of other components, UAF may interact only weakly with the 3'-4-70/+128 template. Other factors whose binding depends on the binding of UAF may join and stabilize formation of the preinitiation complex containing UAF and the DNA template, leading to the removal of UAF (and other factors) from the second template in experiments using crude extracts but not in the present experiments. A similar explanation can also be made for the results obtained for the 5'-end deletion mutations in the previous experiments which used crude extracts. In this regard, it should be noted that the Rrn6/7 complex was shown previously not to bind stably to the rDNA template by itself but to join a stable preinitiation complex in the presence of other factors present in the extracts made from *rrn6* (or *rrn7*) deletion mutants (Keys et al. 1994). Thus, the Rrn6/7 complex, alone or together with other factors, might strengthen the initial interaction of UAF with DNA template.

In the previous mutational analyses of the promoter both in vivo and in vitro, the upstream region important for transcription of the yeast Pol I promoter was divided into two regions, domain II and domain III, and the core element was called domain I (Musters et al. 1989; Kulkens et al. 1991). The basis for defining separate domains in the upstream region was provided by linker scanning mutations between domains II and III, which had no significant effects on transcription activity or on competition ability in template competition experiments (Musters et al. 1989; Kulkens et al. 1991). However, there was no clear functional basis to separate these two domains, and UAF appears to interact with the region covering both domains. Thus, in agreement with Choe et al. (1992), we will now call the region covering both domain II and III simply the upstream element. Thus, defined in this way, the region necessary for the stable binding of UAF appears to roughly correspond to the upstream element.

Comparison of UAF with UBF in other eukaryotic systems

As stated above, UBF isolated from other eukaryotic systems interacts with the upstream element and has been considered to be the mediator of the stimulatory activity of this *cis*-acting element. Like the upstream element, both protein factors UAF and UBF are only stimulatory and are not absolutely required. Thus UAF and UBF are similar in these respects. However, there are several significant differences between the two. First, the interaction between UAF and the upstream element appears to be strong enough to be able to commit the template for

transcription. Our experiments have demonstrated not only that UAF alone is sufficient but also that UAF is necessary for template commitment. In the case of other systems, UBF alone does not appear to be sufficient for template commitment. In the mouse system, TIF-IB (SL1) alone, but not mUBF (mouse homolog of UBF) alone, is able to form a template-committed complex, whereas mUBF acts to stimulate the formation of such committed complexes (Schnapp and Grummt, 1991). In the *Xenopus* system, McStay et al. (1991) have concluded that xUBF (*Xenopus* homolog of UBF) and Rib1 (*Xenopus* homolog of SL1) are both required for template commitment. In the rat system, the presence of rUBF (rat homolog of UBF) appears to stimulate the formation of a template-committed complex (Smith et al. 1990), but the published data do not allow an answer to the question of whether rUBF alone is sufficient for template commitment, nor the question of whether it is necessary for template commitment.

The second difference between UBF and UAF is that UAF does not appear to make a significant interaction with the core element region, whereas UBF appears to interact with the core in addition to the upstream element. This was demonstrated for human (e.g., Bell et al. 1988), rat (Pikaard et al. 1990), and *Xenopus* (Bell et al. 1989; Pikaard et al. 1989, 1990; Read et al. 1992; Leblanc et al. 1993). It was shown that UBF significantly stimulates transcription of rDNA even using a mutant template missing the upstream element entirely, although to a lesser extent as compared with the wild-type template (Bell et al. 1988). In contrast, no significant stimulation of rDNA transcription by UAF was observed with a template missing the upstream element in the yeast system nor could such a deleted template sequester UAF in template competition experiments.

The third and perhaps a most obvious difference is their structures. Whereas UBF is a homodimer, UAF consists of at least three and possibly five different proteins, three of which, Rrn5p, Rrn9p, and Rrn10p, have no sequence resemblance to UBF. The UBF polypeptide consists of five to six copies of the HMG box with the amino-terminal domain required for dimerization and a highly acidic carboxy-terminal domain. These structural features, including the primary sequences, are highly conserved from *Xenopus* to human (Jantzen et al. 1990, 1992; Bachvarov and Moss, 1991; McStay et al. 1991; O'Mahony et al. 1991). No such sequence feature is present in Rrn5p, Rrn9p, or Rrn10p. In addition, these three proteins as well as two other proteins in the purified UAF preparation did not cross-react with antibodies raised to *Xenopus* UBF or antibodies to a yeast HMG box protein, NHP6A (D.A. Keys et al., unpubl.; for NHP6A, see Kolodrubetz and Burgum 1990). UBF is a DNA-binding protein with low sequence specificity (Copenhaver et al. 1994; Hu et al. 1994), and its activity in rDNA transcription almost certainly depends on its ability to bend and wrap DNA carried out by the dimerization domain and one or more of the HMG box domains (Bazett-Jones et al. 1994; Putnam et al. 1994). Thus, the absence of an HMG box in any of the three characterized subunits of

UAF is significant. Of course, it is still possible that the other uncharacterized components might contain the HMG box, and the exact comparison of structures of these two transcription factors, UBF and UAF, must await cloning and sequencing of the genes for p30 and p18. Nevertheless, it should be noted that the three proteins, Rrn5p, Rrn9p, and Rrn10p, which are all essential for the function of UAF as a Pol I transcription factor, together comprise most of the mass of UAF. UBF does not have any domain resembling these proteins. Thus, it would be very remarkable if UAF is a functional yeast homolog of UBF.

As discussed earlier, UAF appears to initiate assembly of the transcription-competent preinitiation complex by recruiting other factors including the Rrn6/7 complex, which together with TBP may represent a functional yeast homolog of SL1 (Keys et al. 1994). UAF does not resemble UBF; rather its complexity resembles TFIIC in Pol III transcription systems, which recruits TFIIB, the essential Pol III transcription factor containing TBP, and which contains multiple protein subunits (six in the yeast TFIIC, and five in the human TFIIC2; for review, see Willis, 1993; Geiduschek and Kassavetis 1995).

As emphasized by Choe et al. (1992) and also discussed in this paper, general structural features of rDNA promoters are well conserved between the yeast promoter and the metazoan promoters from frog to human, suggesting a conservation of mechanisms of initiation used by Pol I transcriptional machineries. Thus, the question of whether UAF is really a functional homolog of UBF is important and deserves further investigation. In this connection, it should be noted that UBF was reported to be complexed with TBP in TFIID fractions and to play a role in Pol II transcription (Kwon and Green 1994). It should also be noted that UBF is an abundant nuclear protein found mostly in the nucleolus (Chan et al. 1991; Roussel et al. 1993; Zatssepina et al. 1993), and its amount was reported to be ~50,000 copies per cell; these levels are in great excess of the ~200 copies of the rRNA gene and 100–400 copies of the SL1 molecules per cell (Bell et al. 1988; see also McStay et al. 1991). In our yeast system, the amount of UAF is comparable to that of the Rrn6/7 complex, which is approximately a few hundred per cell (D.A. Keys et al., unpubl.), compared with ~120 copies of the rRNA gene per cell. Thus, it is possible that UBF might have some other functions besides recruitment of SL1 at the rRNA gene promoter (and enhancer). It has been suggested (Putnam et al. 1994) that UBF might play a structural role by binding to DNA in the nucleolus and displacing nucleosomes or preventing nucleosome assembly, thereby maintaining rDNA free from repression mediated by histones and some other repressors (Kuhn and Grummt 1992; Kuhn et al. 1993).

In the case of UAF, the functions of the three genetically characterized subunits, Rrn5p, Rrn9p, and Rrn10p, are specifically in rDNA transcription, as judged by the *in vivo* genetic experiments; a severe growth defect caused by disruption of any of the genes for these subunits can be remedied (though partially) by transcription of the pGAL7–35S rDNA fusion gene by Pol II. [³H]uri-

dine pulse-labeling experiments using *rrn5*, *rrn9*, and *rrn10* mutants have also directly demonstrated a specific *in vivo* function of these proteins in rDNA transcription by Pol I. These *in vivo* experiments, combined with the *in vitro* experiments presented in this paper, provide unequivocal evidence that UAF is a Pol I-specific transcription factor interacting with the upstream element of the yeast Pol I promoter. So, in view of many clear differences between UAF and UBF discussed above, it appears that there are two alternative possibilities. One is that yeast and higher eukaryotes use very different transcription factors to mediate the stimulatory activity of the upstream element. The other possibility is that a multi-protein transcription factor similar to the yeast UAF is also present in higher eukaryotes and has not been discovered so far. This second possibility may deserve serious experimental investigation.

Materials and methods

Media, buffers, strains, and plasmids

YEP–glucose medium, YEP–galactose medium, synthetic galactose medium, and synthetic glucose medium were described previously (Nogi et al. 1991; Keys et al. 1994).

The strains and plasmids used in this study are listed in Table 1. To construct the diploid strain NOY610, the haploid strain NOY423 was first transformed with plasmid p43A, carrying the *HO* gene (Harashima et al. 1984). One of the transformants was subsequently cured of the resident plasmid, assayed for its ability to sporulate, and named NOY610. Other procedures used for HA1 epitope tagging and gene disruption are described below.

Cloning and sequencing of *RRN5*, *RRN9*, and *RRN10*

Strains NOY697, NOY698, and NOY696 were used to clone *RRN5*, *RRN9*, and *RRN10* genes, respectively. For cloning of *RRN5* and *RRN9*, the yeast genomic library (Nakayama et al. 1985) carrying chromosomal DNA inserted at the *Bam*HI site of YCpN1 vector (*CEN3 ARS1 TRP1*) was used, and for cloning of *RRN10*, the YEp213-based yeast genomic library (Hasegawa et al. 1989) was used. DNA sequences of pertinent fragments were determined by the dideoxy method with the Tag DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Construction of *rrn5*, *rrn9*, and *rrn10* deletion mutants

Two disruption mutants were constructed: one is an insertion of an 850-bp DNA fragment carrying *TRP1* at the *Clal* site within *RRN5* (*rrn5::TRP1*; see Fig. 2a for the *Clal* site), and the other is a deletion replacing the region between *Nco*I and *Xho*I within *RRN5* by *LEU2* (*rrn5Δ::LEU2*; see Fig. 2b). To construct the *rrn5::TRP1* allele, the 3.3-kb *Hind*III fragment carrying *RRN5* obtained from pNOY230 was inserted into the *Hind*III site of pUC19. This plasmid was digested with *Clal*, and the 850-bp *Clal*–*Bgl*III fragment carrying *TRP1* was inserted at the *Clal* site by a blunt-end ligation. The resultant plasmid was digested with *Kpn*I and *Nae*I and the 1.5-kb fragment containing *rrn5::TRP1* was transformed into NOY424, which is *trp1* and carries pNOY103, selecting *Trp*⁺ transformants on a galactose plate. One of the transformants, NOY577, can grow on galactose without losing pNOY103 but grows very poorly on glucose. To construct the *rrn5Δ::LEU2* allele, a plasmid (pNOY3231)

carrying a 1.9-kb *HindIII*–*Bam*HI fragment containing the *RRN5* gene in pUC19 was digested with *Nco*I and *Xho*I, which resulted in an internal deletion of *RRN5* (see Fig. 2b). The deleted region of the *RRN5* gene in pNOY3231 was replaced with a 2.2 kb *Xho*I–*Sal*I fragment containing the *LEU2* gene (which was obtained from pNOY3114) by a blunt-end ligation. The resulting plasmid was digested with *Bam*HI and *Nde*I, which released the 3.4-kb fragment carrying *rrn5Δ::LEU2*. This fragment was transformed into diploid strain NOY397. *Leu*⁺ transformants were selected and NOY679, which is one of the transformants, was confirmed by tetrad analysis to contain one copy of the *RRN5* gene replaced by the *LEU2* gene.

A 2.2-kb *Bam*HI–*Sph*I fragment containing *RRN9* (see Fig. 2c) derived from pNOY187 was ligated between the *Bam*HI and *Sph*I sites of pUC19. The resulting plasmid was cut with *Sal*I and *Eco*RV to remove 612 bp of the *RRN9*-coding region, blunted with T4 DNA polymerase, and then ligated with a blunted 600-bp *Bam*HI fragment containing *HIS3* (see Fig. 2d). The resulting plasmid containing the *HIS3* fragment replacing the *RRN9* *Sal*I–*Eco*RV fragment was digested with *Ssp*I and the 1.5-kb fragment containing *rrn9Δ::HIS3* was transformed into diploid strain NOY397, selecting for *His*⁺ transformants. One of the transformants, NOY678, was confirmed by tetrad analysis to carry *rrn9Δ::HIS3* on one chromosome.

A 3.3-kb *Bam*HI–*Hind*III fragment containing *RRN10* was isolated from pNOY337 and ligated between the *Bam*HI and *Hind*III sites of pUC19. The resulting plasmid was digested with *Pst*I and partially digested with *Xho*I to remove a 478-bp *Pst*I–*Xho*I fragment, and then blunted using *Escherichia coli* DNA polymerase (Klenow fragment). A blunted 2.2-kb *Sal*I–*Xho*I fragment containing *LEU2* was inserted between the original *Pst*I and *Xho*I sites of *RRN10* (see Fig. 2f). The resultant plasmid was digested with *Bam*HI–*Hind*III, and the fragment carrying *rrn10Δ::LEU2* was transformed into diploid strain NOY610, selecting for *Leu*⁺ transformants. One of the transformants, NOY677, was confirmed by tetrad analysis to carry the expected *rrn10Δ::LEU2* on one chromosome.

Construction of pNOY330, encoding (HA1)₃-tagged *Rrn5p*

A 1.6-kb *RRN5* fragment was synthesized by PCR amplification using an oligonucleotide, which hybridizes at a position ~470 nucleotides upstream of the initiator methionine codon and introduces a unique *Sal*I site at one end of the PCR product, and another oligonucleotide, which hybridizes at a position near the stop codon and introduces a sequence (GCGGCCGC) containing a unique *Not*I site (underlined) following the last amino acid (lysine) codon (AAA) of *RRN5* at the other end. pNOY230 was used as template for the PCR reaction. After cutting with *Sal*I and *Not*I, the PCR product was ligated between the *Sal*I and *Not*I sites of pNOY328, a *LEU2*-marked *CEN* plasmid, to create pNOY329, which contains a full-length *RRN5* including its native promoter with a unique *Not*I site at the 3' end of the *RRN5* coding region. A 0.12-kb *Not*I–*Sac*I fragment encoding three tandem copies of the 9-amino-acid HA1 epitope (followed by Ala-Gln-Cys-Stop) was isolated from pNOY3220 (see below) and ligated between the *Not*I and *Sac*I sites of the above plasmid (pNOY329) to generate pNOY330, fusing the (HA1)₃-epitope coding sequence in-frame to the sequence encoding the carboxyl terminus of *Rrn5p*. The plasmid, pNOY330, constructed in this way encodes (HA1)₃–*Rrn5p*, which has 38 additional amino acids [GGRI(HA1)G(HA1)GS (HA1) AQC; HA1 is a 9-amino-acid HA1-epitope sequence, YPYDVPDYA] at the end of *Rrn5p*.

Plasmid pNOY3220 is a convenient source for the *Not*I–*Sac*I fragment encoding three tandem copies of the HA1 epitope used

above and was constructed by PCR using GTEP1 (Roof et al. 1992) as template, modifying the *Not*I–(HA1)₃–*Not*I structure present in GTEP1 to the *Not*I–(HA1)₃–*Sac*I structure; the distal end is now GCTCAGTGCTAGAGCTC (encoding AQC and the stop codon TAG overlapped with the *Sac*I site which is underlined) rather than GCTCAGTGCGGCCGC (encoding the AQC followed by the *Not*I site underlined). The PCR product was digested with *Not*I and *Sac*I, and the fragment was cloned between the *Not*I and *Sac*I sites of pBluescript KS(+), yielding pNOY3220.

Construction of pNOY332, which encodes (HA1)₃-tagged *Rrn9p*

The 837-bp *Eco*RI–*Sal*I DNA fragment containing the 3' end of the *RRN9* coding region and the downstream untranslated region (—AACATATGAATGAGA—; the stop codon underlined) was first subcloned into a pUC19 plasmid. The oligonucleotide GCGGCCGC (the *Not*I site underlined) was introduced between the codon for the last amino acid (ATA for Ile) and the stop codon (TGA) of *RRN9* by PCR reaction. A *Bsm*I–*Nae*I fragment, which now carries the 3' end of *RRN9* containing the *Not*I site, was then used to replace the corresponding *Bsm*I–*Nae*I fragment in the wild-type *RRN9* gene carried on plasmid pNOY335, resulting in pNOY336. Finally, the 114-bp *Not*I fragment containing (HA1)₃ prepared from GTEP1 was inserted into pNOY336. The resultant plasmid, pNOY332, encodes (HA1)₃–*Rrn9p*, which has 40 amino acids [GGRIF(HA1)G(HA1)GS(HA1)AQCGR] at the end of *Rrn9p*.

It should be noted that pNOY330 and pNOY332 complement *rrn5Δ::LEU2* and *rrn9Δ::HIS3*, respectively, indicating that triple-HA1 tagging of *Rrn5p* or *Rrn9p* at the end of the carboxyl terminus does not apparently affect the functions of these proteins.

Polyclonal antibodies against *Rrn5p*, *Rrn9p*, and *Rrn10p*

Rrn5p, *Rrn9p*, and *Rrn10p* were isolated as fusion proteins to prepare polyclonal antibodies against these proteins. An *RRN5* gene with a unique *Bgl*II site upstream of the start codon and a unique *Bam*HI site downstream of the stop codon was synthesized by PCR amplification and was inserted into the *Bam*HI site of the expression vector pNO3124 [pATH23; see Koerner et al. 1991], fusing full-length *RRN5* coding sequence in-frame with the carboxy-terminal coding region of *trpE*. *TrpE*–*Rrn5p* fusion protein was expressed in *E. coli* DH5α cells containing this plasmid, and isolated by extraction of insoluble membrane/wall pellets with SDS followed by preparative SDS-PAGE.

A 0.77-kb *Hinc*II fragment encoding amino acids 54–310 of *RRN9* was inserted into the *Sma*I site of the expression vector pGEX-3X (Pharmacia), fusing the *RRN9* fragment in-frame with the carboxy-terminal coding region of glutathione *S*-transferase (GST). The GST–*Rrn9p* fusion protein was isolated as an insoluble precipitate from *E. coli* cells and purified by a preparative SDS-PAGE.

A 2.6-kb *Pst*I–*Sal*I *RRN10* fragment was isolated from pNOY337 and inserted between the *Pst*I and *Sal*I sites of pRS315, placing *Bam*HI and *Sma*I sites immediately upstream of the *Pst*I site. A 2.0-kb *Bam*HI–*Hind*III *RRN10* fragment was then isolated from this plasmid and inserted between the *Bam*HI and *Hind*III sites of the expression vector pET15b (Novagen), fusing the His₆-tagged coding sequence [MGSSHHHH-HHSSGLVPRGSHMLEDPGP] in-frame to codon 9 of *RRN10*, generating plasmid pNOY3233. His-tagged *Rrn10p* was affinity purified from an *E. coli* strain containing pNOY3233 using a Ni(II)SO₄ column, followed by preparative SDS-PAGE. Using

these fusion protein preparations, rabbit polyclonal antibodies were produced by Bethyl Laboratories (Texas).

Purification of (HA1)₃-Rrn5p and (HA1)₃-Rrn9p complexes

Monoclonal antibody 12CA5, specific to the HA1 epitope, and the epitope peptide (YPYDVDPYA) were obtained from Berkeley Antibody Co. (Richmond, CA). The 12CA5 antibody was cross-linked to protein G-Sepharose (Pharmacia) using dimethyl pimelimidate according to the protocol provided by the supplier of the reagent. Extracts were prepared from 50–100 grams of cells from strains expressing (HA1)₃-tagged Rrn5p (NOY662) or (HA1)₃-tagged Rrn9p (NOY674). Cells were homogenized in 350 ml of buffer containing 400 mM ammonium sulfate, 200 mM Tris-acetate (pH 8.0), 10% glycerol, 10 mM Mg acetate, 10 mM β-mercaptoethanol, 1 mM PMSF, 0.1% Tween 20, and 5.5 mM benzamidinium-HCl, plus 150 ml of acid-washed glass beads (0.4–0.5 mm diam.) using a 400 ml Bead-beater apparatus (Biospec Products, Bartlesville, OK), with 30 pulses of 30 sec with a 90 sec pause between each pulse at a reduced voltage of 95 V. After centrifugation at 30,000 rpm for 30 min in a Beckman type Ti-35 rotor, the supernatant was added to 5 ml of protein G-Sepharose beads carrying cross-linked 12CA5 antibodies (~20 mg of IgG/ml), together with 10 μg/ml of the following protease inhibitors: aprotinin, chymostatin, leupeptin, antipain, bestatin, and pepstatin. The antibody beads and supernatant were rolled gently in a bottle on a roller drum for 4 hr at 4°C. The supernatant was then gently pulled off, and the antibody beads were washed batchwise five times with 50 ml of TA buffer [20 mM Tris-acetate (pH 8.0), 0.2 mM EDTA, 10 mM Mg acetate, 10 mM β-mercaptoethanol, 1 mM PMSF, 20% glycerol] containing 450 mM KCl (TA/450). Bound complexes were continuously and simultaneously eluted from the antibody column with HA1 peptide (1 mg/ml in TA/450) and loaded directly onto a 5-ml heparin-Sepharose cartridge (HiTrap heparin, Pharmacia) equilibrated in TA/450. This was accomplished by pouring the washed antibody beads into a column, which was in turn connected in series to the heparin column. The flowthrough from the heparin column (containing HA1 peptide, which does not bind to heparin) was then recirculated by peristaltic pump back onto the antibody column. The HA1-peptide-containing buffer was circulated through the two columns overnight at 4°C at a flow rate of ~1 ml/min. The two columns were then disconnected, the heparin column was washed with several volumes of TA/450, and bound proteins were eluted with a salt gradient of 450–1000 mM KCl in TA buffer. Peak fractions containing HA1-tagged proteins (~720 to ~770 mM KCl) were identified by immunoblot analysis using anti-HA1 antibody, pooled, diluted to ~375 mM KCl in TA buffer, and applied to a 1-ml Mono S column equilibrated in TA buffer containing 375 mM KCl (TA/375). After washing with TA/375, bound proteins were eluted with a salt gradient of 375–1000 mM KCl in TA buffer. Peak fractions containing HA1-tagged proteins (~550 to ~600 mM KCl) were pooled, concentrated by Centricon 10 (Amicon) ultrafiltration, and applied to a 25-ml Superose 12 gel filtration column equilibrated in TA buffer containing 10% glycerol, 450 mM KCl. The Superose column was run at 0.15 ml/min, and 0.3-ml fractions were collected and stored at –70°C.

In vitro transcription reactions

Transcription extracts [PC-300 (eluting from the phosphocellulose column at 300 mM KCl) and D-300 (eluting from the DEAE column at 300 mM KCl) fractions] were prepared as described previously (Keys et al. 1994) from *rrn5-2* mutant strain NOY705, from *rrn9* deletion-mutant strain NOY703, and from

rrn10-1 mutant strain NOY696. In vitro transcription reactions were performed as described previously (Keys et al. 1994). Typical in vitro reactions contained, in the 40-μl reaction volume, 3–6 μl of D-300 fraction (~20 μg of protein) plus 3–9 μl of PC-300 fraction (~2–4 μg of protein) and 200 ng (5 μg/ml) of circular supercoiled DNA template in addition to other standard components. The DNA template used for most assays is pSIRT containing a yeast rDNA minigene that generates a specific transcript of ~765 nucleotides that is apparently unprocessed and correctly terminated (Keys et al. 1994; for the structure of pSIRT, see Fig. 7a and Musters et al. 1989). For template competition experiments, derivatives of pSIRT containing internal deletions starting at position –208 and extending downstream toward the promoter (5' deletion series) or starting at position +128 and extending upstream toward the promoter (3' deletion series) were used, as described previously (see Fig. 7b; Musters et al. 1989; Kulkens et al. 1991). Preincubation of DNA template with purified UAF (Figs. 10 and 9, lanes 9–13) was done in reaction mixture (10 μl) supplemented with 0.4% BSA. Typical in vitro reactions contained 175 mM potassium glutamate (no KCl); in vitro reactions using purified (HA1)₃-Rrn5p or (HA1)₃-Rrn9p complexes (Superose 12 fractions; i.e., purified UAF) were performed with 100 mM potassium glutamate and 40 mM KCl, as Superose 12 fractions were not dialyzed to remove the KCl. Reactions were carried out for 40 min at room temperature, and radioactive RNA synthesized was analyzed by 5% urea-PAGE followed by autoradiography, as described previously (Keys et al. 1994).

Analysis of RNA labeled in vivo

Cells were grown in synthetic galactose medium supplemented with casamino acids, tryptophan, and adenosine at 30°C to a cell density (*A*₆₀₀) of ~0.2. Each culture was divided into two parts. To one culture, [³H]uridine (174 mCi/mmol; 100 μCi/ml) was added, and after a 60-min incubation the labeling was stopped by immersing culture flasks into a dry ice-ethanol bath. The other culture was centrifuged, and cells were washed with water and resuspended in synthetic glucose medium with the same supplementation. After incubation at 30°C for 1 hr, [³H]uridine-pulse-labeling was carried out in the same way. RNA was isolated and analyzed by electrophoresis on a 2% polyacrylamide, 0.5% agarose composite gel followed by autoradiography as described previously (Nogi et al. 1991).

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