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Transcription Factor UAF, Expansion and Contraction of Ribosomal DNA (rDNA) Repeats, and RNA Polymerase Switch in Transcription of Yeast rDNA

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Strains of the yeast *Saccharomyces cerevisiae* defective in transcription factor UAF give rise to variants able to grow by transcribing endogenous ribosomal DNA (rDNA) by RNA polymerase II (Pol II). We have demonstrated that the switch to growth using the Pol II system consists of two steps: a mutational alteration in UAF and an expansion of chromosomal rDNA repeats. The first step, a single mutation in UAF, is sufficient to allow Pol II transcription of rDNA. In contrast to UAF mutations, mutations in Pol I or other Pol I transcription factors can not independently lead to Pol II transcription of rDNA, suggesting a specific role of UAF in preventing polymerase switch. The second step, expansion of chromosomal rDNA repeats to levels severalfold higher than the wild type, is required for efficient cell growth. Mutations in genes that affect recombination within the rDNA repeats, *fob1* and *sir2*, decrease and increase, respectively, the frequency of switching to growth using Pol II, indicating that increased rDNA copy number is a cause rather than a consequence of the switch. Finally, we show that the switch to the Pol II system is accompanied by a striking alteration in the localization and morphology of the nucleolus. The altered state that uses Pol II for rDNA transcription is semistable and heritable through mitosis and meiosis. We discuss the significance of these observations in relation to the plasticity of rDNA tandem repeats and nucleolar structures as well as evolution of the Pol I machinery.

All eukaryotic cells have three distinct RNA polymerases that normally transcribe different sets of nuclear genes. RNA polymerase I (Pol I) is unique in that in most eukaryotic organisms, its sole function is the transcription of genes for large rRNAs (rDNA). Dedication of a separate RNA polymerase to rDNA transcription is a feature unique to eukaryotes and must have had strong selective advantages for eukaryotic organisms in evolution. However, we have recently discovered that mutants of the yeast *Saccharomyces cerevisiae* which are defective in transcription factor UAF (upstream activation factor) give rise to variants which now grow by transcribing endogenous rDNA by RNA polymerase II (Pol II) (39). (In this paper, we use the term transcription of rDNA to imply transcription of the gene encoding the 35S precursor rRNA, although the 5S RNA gene transcribed by RNA polymerase III is a part of the rDNA repeat unit in *S. cerevisiae*.) Thus, yeast cells have an inherent ability to use Pol II for rDNA transcription, but this transcription activity is apparently silenced in normal cells. Studies of the processes which enable yeast cells to grow without using the Pol I machinery may be important for understanding the normal Pol I machinery and for gaining insight into the significance of its evolution. In this paper, we describe our finding that the switch to growth using the Pol II system consists of two steps; the first step is a mutational alteration in UAF, and the second step is an expansion of chromosomal rDNA repeats. The first step, a UAF mutation, is sufficient to allow Pol II transcription of rDNA, but the overall efficiency of rRNA synthesis is apparently not sufficient for sustained cell growth. The second step, rDNA repeat expansion, represents an adaptation process, which probably involves a selection for faster-growing cells and leads eventually to a semistable state of rDNA with an increase in repeat numbers and an altered nucleolar location and morphology.

Like other eukaryotic rDNA promoters, the promoter for the gene encoding 35S precursor rRNA in *S. cerevisiae* consists of two elements, the upstream element and the core element. Basal transcription of yeast rDNA requires the core element and two transcription factors, Rrn3p and CF (core factor), in addition to Pol I. CF consists of three proteins encoded by *RRN6*, *RRN7*, and *RRN11*. For high levels of transcription, two additional factors, UAF and TBP (TATA binding protein), as well as the upstream element are required in addition to the components required for basal transcription (18, 19, 37). UAF contains three Pol I-specific protein subunits encoded by *RRN5*, *RRN9*, and *RRN10*, histones H3 and H4, and the uncharacterized protein P30 (17). In apparent agreement with the in vitro function of UAF, genes *RRN5*, *RRN9*, and *RRN10* are not absolutely required for cell growth and yeast strains with mutations in these genes can grow, albeit very slowly (19).

Slowly growing UAF mutants give rise to faster-growing variants which do not require intact Pol I and synthesize rRNA using Pol II (39). The slowly growing mutants defective in Pol I-specific UAF components are unstable because of the appearance of faster-growing variants, but they can be maintained stably by introducing a helper plasmid, e.g., pNOY103, which carries the 35S rRNA coding region fused to the galactose-inducible *GAL7* promoter. These cells can grow fairly well on galactose but extremely poorly on glucose due to repression of the fusion gene. The faster-growing variants can grow, with or without a helper plasmid, both on galactose and on glucose and were previously shown to synthesize rRNA by transcribing endogenous rDNA by Pol II (39). These cells were called PSW

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TABLE 1. Yeast strains and plasmids used

Strain or plasmid	Description
Strains	
W303-1a	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100*
NOY408-1a	*MAT*α *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 rpa135*Δ*::LEU2* pNOY102[a]
NOY408-1b	*MAT***a** *ade2-1 ura3-1 trp1-1-leu2-3,112 his3-11 can1-100* pNOY102
NOY505	*MAT*α *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100*
NOY556	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100* pNOY103
NOY566	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 rrn6*Δ*::HIS3* pNOY103
NOY703	*MAT***a** *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn9*Δ*::HIS3* pNOY103; N-PSW
NOY769	*MAT*α *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn9*Δ*::HIS3* pNOY103; N-PSW
NOY794	Same as NOY852 but *rpa135*Δ*::LEU2*
NOY852	*MAT*α *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn9*Δ*::HIS3*; PSW
NOY876	Same as NOY703 but carries pNOY199 instead of pNOY103; N-PSW
NOY877	Same as NOY852 but carries pNOY199; PSW
NOY878	Same as NOY703 but PSW
NOY896	Same as NOY703 (N-PSW) but *rpa135*Δ*::LEU2*
NOY901	Same as NOY703 (N-PSW) but *sir2*Δ*::LEU2*
NOY911	Same as NOY703 (N-PSW) but *sir3*Δ*::LEU2*
NOY912	Same as NOY703 (N-PSW) but *sir4*Δ*::LEU2*
NOY918	Same as NOY566 but *sir2*Δ*::LEU2*
NOY919	Same as NOY566 but *rrn5*Δ*::LEU2*
NOY920	Same as NOY878 but *fob1*Δ*::LEU2*
NOY921	Same as NOY703 but *fob1*Δ*::LEU2*
Plasmids	
pNOY102	High-copy-number plasmid carrying *GAL7*-35S rDNA, *URA3*, 2 μm, *amp*
pNOY103	High-copy-number plasmid carrying *GAL7*-35S rDNA, *ADE3*, *URA3*, 2 μm, *amp*
pNOY199	High-copy-number plasmid carrying *GAL7*-35S rDNA, *TRP1*, 2 μm, *amp*

[a] From Nogi et al. (26).

(polymerase switched for growth) cells, thus defining the PSW state. The original UAF mutant cells carrying the helper plasmid, which were unable to grow on glucose, were called non-PSW (referred to as N-PSW in this paper), thus defining the N-PSW state (39). The PSW state, once established, is fairly stable and can be inherited through mitosis and meiosis, but spontaneous reversion to the original N-PSW state can be easily demonstrated (39). We have now studied the reversible changes between the N-PSW and PSW states and discovered that they represent expansion and contraction of rDNA repeats.

MATERIALS AND METHODS

Strains, plasmids, and media. Yeast strains and plasmids used are listed in Table 1. Disruption of *RPA135* was done as previously described (40). For disruption of *SIR2*, a DNA fragment covering the *SIR2* chromosome region, containing a 1.8-kb deletion removing most of the *SIR2* coding region except for the first 18 amino acids and replaced by a 1.6-kb fragment containing *LEU2*, was used. For disruption of *SIR3*, a DNA fragment covering the *SIR3* chromosome region, containing a 2.5-kb *Bgl*II-*Xho*I deletion within the *SIR3* coding region replaced by the 2.5-kb *Bgl*II-*Sal*I fragment containing *LEU2*, was used. For disruption of *SIR4*, a DNA fragment covering the *SIR4* chromosome region, containing a 330-bp *Bgl*II-*Bam*HI deletion inactivating *SIR4* function (15) and replaced by the 3.1-kb *Bgl*II-*Bgl*II fragment containing *LEU2*, was used. Disruption of *SIR2*, *SIR3*, and *SIR4* genes was confirmed by the nonmating phenotype of the strains constructed. Disruption of *FOB1* was carried out by using the *Eco*RI fragment obtained from pUC-*fob1::LEU2* as previously described (20). Disruption of *RRN5* was done by using the 3.4-kb fragment carrying *rrn5*Δ*::LEU2* described previously (19).

YEP-galactose, YEP-glucose (YEPD), synthetic galactose, and glucose media were described previously (26). The following supplements were added to the synthetic media as appropriate to satisfy nutritional requirements: Casamino Acids (5 mg/ml), tryptophan (20 μg/ml), adenine (20 μg/ml), and uracil (20 μg/ml).

Spot test for PSW and N-PSW phenotype. Individual colonies formed on YEP-galactose media were picked and suspended in 100 μl of H_2O, and 5-μl aliquots of 10-fold serial dilutions were spotted on YEP-galactose and YEPD plates. They were usually incubated at 30°C for 7 days.

Analysis of chromosome XII by contour-clamped homogeneous electric field (CHEF) electrophoresis. Chromosomal DNA from yeast strains was isolated as previously described (35) and electrophoresed in 0.8% agarose (SeaKem LE; FMC BioProducts, Rockland, Maine)–0.5× Tris-borate-EDTA buffer by using a CHEF Mapper (Bio-Rad, Richmond, Calif.), programmed for a switch time of 300 to 900 s for 68 h at 14°C and with an included angle of 120° (21). After electrophoresis, the gel was stained with 0.5 mg of ethidium bromide per ml for 30 min at room temperature, destained in water for 1 to 2 h, and then photographed. The gel was transferred to a nylon membrane (Zeta-Probe GT; Bio-Rad) and then analyzed by Southern hybridization with ^{32}P-labeled rDNA and *SIR3* probes (24). The rDNA probe used was the 613-bp *Sma*I-*Eco*RV fragment spanning positions −210 to +403 (+1 is the Pol I transcription start site). The *SIR3* probe used was the 754-bp *Pst*I-*Hin*dIII fragment within the *SIR3* coding region.

EM and FISH. Yeast strains were grown at 25°C, and electron microscopy (EM) analysis was carried out as previously described, using a JEOL 100CX electron microscope (28). Fluorescence in situ hybridization (FISH) analysis was also carried out as described previously (28) except that the rDNA probe used was a 6.9-kb DNA carrying the 35S rRNA coding region (+1 to +6922) with extra 15 nucleotides derived from a multicloning site of a plasmid vector.

Other methods. Isolation of DNA and Southern hybridization analysis were carried out as described by Maniatis et al. (24). Analysis of 5′ ends of precursor rRNA by primer extension was carried out as described previously (18, 39), using the primer 5′-ACACGCTGTATAGAGACTAGGC-3′, which hybridizes to 35S precursor rRNA 130 nucleotides downstream of the Pol I start site. Quantification in both analyses was done with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

RESULTS

Transition from the N-PSW state to the fully established PSW state is an adaptation process. Yeast strains carrying a deletion of *RRN9* were analyzed for growth by spot testing serial dilutions on galactose and glucose plates. N-PSW strain NOY703 is able to grow well on galactose due to the presence of a helper plasmid carrying *GAL7*-35S rDNA (Fig. 1A Gal, rows a and b). Conversely, NOY703 grows poorly on glucose with colonies appearing after a long incubation (7 to 10 days at 30°C) at a frequency of approximately 10^{-3} to 10^{-4} (Fig. 1A Glu, rows a and b). When colonies were picked from the glucose plate, resuspended in water, and spot tested again on glucose and galactose plates, growth on galactose was still

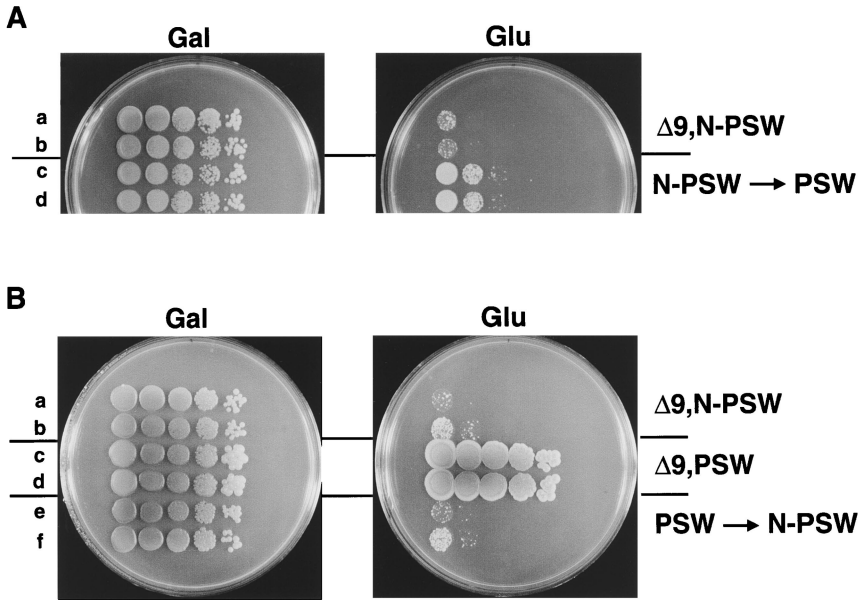

FIG. 1. Spot test of *rrn9*Δ N-PSW strain NOY703 and PSW variants derived from it. (A) Two independent colonies of NOY703 formed on a galactose plate were analyzed by spotting aliquots of 10-fold serial dilutions of suspension of colonies on YEP-galactose (Gal) and YEPD (Glu) (rows a and b). Several discrete colonies, which had just formed on YEPD by plating large numbers of NOY703 cells on glucose (similar to colonies shown in rows a and b), were combined and similarly analyzed (rows c and d). (B) Two independent colonies of NOY703 (rows a and b), NOY878 (rows c and d), and an N-PSW revertant derived from NOY878 (rows e and f) formed on galactose plates were analyzed by the spot test as for panel A. Plates were incubated at 30°C for 10 days.

better than that on glucose as indicated by colony size, and only a small fraction (ca. 1% in the example in Fig. 1A) of the cells retained the ability to grow on glucose (Fig. 1A, rows c and d). However, after repeated streaking on glucose plates, larger colonies were easily obtained, and these clones showed better growth on glucose than on galactose. Spot test analysis of one such established PSW strain, NOY878, which was derived from NOY703 and was used in the present and previous work (39), is shown in Fig. 1B (rows c and d). These observations demonstrate that between the N-PSW and PSW states are intermediates which are able to grow weakly on glucose and moreover are unstable such that they tend to lose the ability to form colonies on glucose. Thus, switching from N-PSW to PSW states is an adaptation process that occurs through selection for better-growing variants on glucose plates.

Correlation of expansion of rDNA repeats with the switch from N-PSW to PSW states. In the course of analysis of rDNA in PSW strains, we discovered that the amount of rDNA in these strains is much higher than that in N-PSW strains. The results of a typical Southern analysis of rDNA in the wild-type (WT; NOY556), *rrn9*Δ N-PSW (NOY703), and *rrn9*Δ PSW (NOY878) strains are shown in Fig. 2 (lanes 1 to 3). *SIR3*, a single-copy gene on chromosome XII, the same chromosome that carries the rDNA repeats, was used for normalization. It was found that the *rrn9*Δ N-PSW strain showed about a 2-fold reduction and the *rrn9*Δ PSW strain showed about a 2.6-fold increase in rDNA copy number relative to the WT strain. The reduction in the copy number of rDNA repeats in the *rrn9*Δ N-PSW strain was not unexpected, since a twofold reduction in rDNA copy number was previously observed for a strain (NOY408-1a) carrying a deletion in an essential Pol I subunit gene (*RPA135*) and growing on galactose using the *GAL7*-35S rDNA fusion gene on a helper plasmid (21). This strain was also analyzed, together with its control *RPA135* strain (NOY408-1b), and the previous finding, a twofold reduction in rDNA copy number, was confirmed (Fig. 2, lanes 4 and 5). The absolute copy number of rDNA in this control strain was previously estimated to be approximately 150 per genome. Using this number for the current control strain (NOY556), one can calculate that the *rrn9*Δ N-PSW strain has approximately 80 copies and the *rrn9*Δ PSW strain has approximately 400 copies. Thus, the switch from the N-PSW to the PSW state is accom-

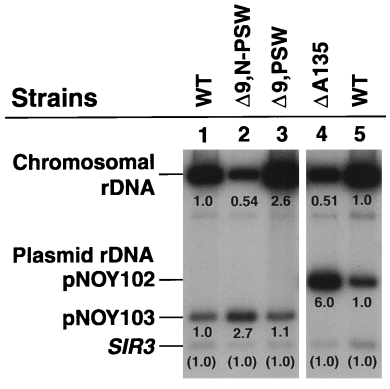

FIG. 2. Comparison of chromosomal rDNA copy numbers of NOY556 (WT), NOY703 (Δ9,N-PSW), NOY878 (Δ9,PSW), NOY408-1a (ΔA135), and NOY408-1b (WT). DNA from these five strains were digested with *Hin*dIII and *Pst*I, and the digests were subjected to agarose gel electrophoresis, followed by transfer to a nylon membrane and hybridization with a mixture of ^{32}P-labeled rDNA probe and *SIR3* probe. An autoradiogram is shown with an inserted gap between lanes 3 and 4. Radioactivity in each DNA band was quantified with a PhosphorImager. The values for chromosomal rDNA (and plasmid rDNA) were first normalized to the values for reference *SIR3* DNA. These values obtained for NOY703 (lane 2) and NOY878 (lane 3) were then divided by corresponding values for control strain NOY556 (lane 1), and the ratios calculated are shown below the pertinent bands. Similarly, the normalized values obtained for NOY408-1a (lane 4) were divided by corresponding values for control strain NOY408-1b (lane 5), and the ratios calculated are shown below the pertinent bands. It should be noted that growth of both NOY703 (lane 2) and NOY408-1a (lane 4) is achieved by transcription of *GAL7*-35S rDNA on helper plasmids (NOY103 and NOY102, respectively), and copy numbers of the helper plasmids were higher than those carried by the control strains due almost certainly to selection.

panied by an approximately fivefold increase in rDNA copy number.

To examine whether the increase in rDNA copy number is due to the formation of extrachromosomal rDNA circles or an increase in rDNA copies associated with chromosome XII (i.e., repeat expansion), we separated chromosomes of these strains by CHEF electrophoresis and analyzed chromosome XII by Southern hybridization using a rDNA probe and a control probe for a single-copy gene on the same chromosome, *SIR3* (Fig. 3A). The Pol I deletion strain previously studied was again analyzed in parallel. As was found previously (21), chromosome XII of the wild-type strain showed a broad band indicating a heterogeneous population with an average estimated size of 2.8 Mb (Fig. 3A, lane 1). This size roughly corresponds to the sum of the calculated size of non-rDNA sequence, 1.1 Mb, plus 150 copies of the 9.1-kb rDNA unit. The Pol I deletion strain showed a heterogeneous size distribution ranging from 1.4 to 1.9 Mb, with an average estimated size of 1.8 Mb (Fig. 3, lane 5), which corresponds to the sum of 1.1-Mb non-rDNA plus 80 copies of the 9.1-kb rDNA unit. The Δ9 N-PSW strain showed one or sometimes two heterogeneous chromosome XII bands (Fig. 3A, lane 2; Fig. 3B, lanes 2 and 9) which showed a mobility similar to bands seen for the Pol I deletion strain (Fig. 3A, lane 5).

The PSW strain contained a very large chromosome XII which entered into the gel but showed a very slow mobility, as judged by hybridization with rDNA and *SIR3* probes as well as by ethidium bromide staining of gels (Fig. 3A, lane 3; Fig. 3B, lanes 3 and 10). Repeating many CHEF analyses of chromosomes as well as standard agarose gel electrophoretic analysis of DNA, we found that most rDNA in *rrn9*Δ PSW strains is associated with chromosome XII and that the fraction present as extrachromosomal circles is small and is not different from that in the control wild-type strain (data not shown). It should be noted that intensities of the signals seen with the rDNA probe relative to the signals seen with the *SIR3* probe are clearly different among these strains and support the conclusion that rDNA repeat numbers decrease in the *rrn9*Δ N-PSW strain and increase in the *rrn9*Δ PSW strain.

The increase in rDNA repeat numbers seen for PSW strains is a reversible change correlated with their ability to grow without Pol I. A N-PSW clone obtained from the *rrn9*Δ PSW strain (as described above and shown in Fig. 1B, rows e and f) was analyzed for the size of chromosome XII. As shown in Fig. 3A, the size(s) of chromosome XII was decreased and the mobility of the heterogeneous band was similar to that seen before switch to the PSW state (and comparable to the ΔA135 strain) (Fig. 3A; compare lane 4 with lanes 2 and 5).

Two rDNA states with different rDNA repeat numbers are inherited through meiosis. We have previously shown that the PSW state can be inherited not only through mitosis but also through meiosis (39). In crosses between PSW and N-PSW strains, both PSW and N-PSW haploid segregant clones were obtained after growth as diploids, followed by sporulation and tetrad dissection. Although an exact 2:2 segregation pattern was not always observed because of an apparently increased frequency of switching in the PSW/N-PSW diploid state and perhaps during meiosis, a clear segregation of the phenotypes indicated their association with a chromosome, presumably chromosome XII (39). We have now examined the state of chromosome XII in such diploids as well as haploid segregants in such a cross. As shown in Fig. 3B, independent diploid clones obtained from the cross showed a copy of chromosome XII with expanded rDNA repeats and a copy with decreased rDNA repeats (lanes 5 to 7). Such diploid clones, analyzed after the cross without extensive colony purification, almost

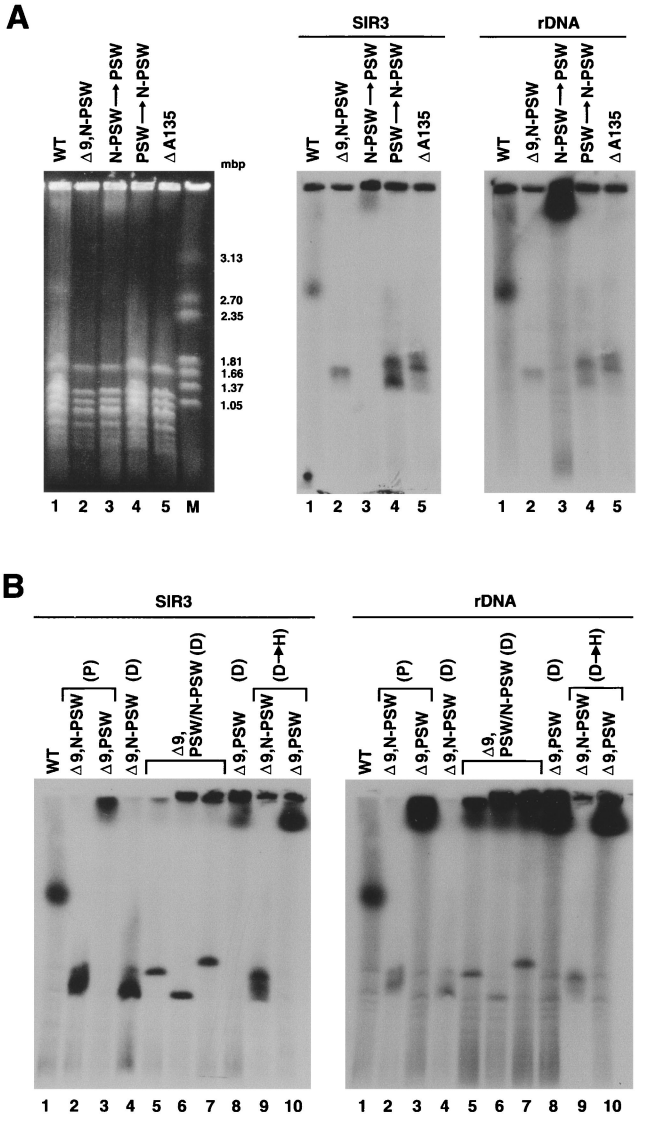

FIG. 3. Correlation between the sizes of chromosome XII as analyzed by CHEF electrophoresis and PSW/N-PSW phenotypes. (A) Chromosomal DNA was isolated from strains NOY505 (lane 1) and NOY703 (lane 2), PSW strain NOY878 (lane 3), an N-PSW strain derived spontaneously from NOY878 (lane 4), and strain NOY408-1a (lane 5). The size of chromosome XII was then analyzed by CHEF electrophoresis. Size markers (lane M) are *Hansenula wingei* chromosomes, and their sizes are indicated in megabase pairs. Left, chromosome patterns revealed by staining with ethidium bromide; middle and right, autoradiograms obtained after hybridization with a *SIR3* probe and an rDNA probe, respectively. (B) Chromosomal DNA was isolated from the following strains and analyzed by CHEF electrophoresis as for panel A: NOY505 (lane 1), NOY703 (lane 2), NOY877 (lane 3) (NOY703 and NOY877 are parents [P] of diploids [D] shown in lanes 5 to 7), a diploid obtained after the cross of N-PSW strain NOY876 and N-PSW strain NOY769 (lane 4), three independent diploid clones obtained after the cross of N-PSW strain NOY703 and PSW strain NOY877 (lanes 5 to 7), a diploid strain obtained after the cross of two PSW strains NOY877 and NOY878 (lane 8), and two haploid segregants (D→H) from the cross of N-PSW strain NOY703 and PSW strain NOY877, one showing the N-PSW phenotype (lane 9) and the other showing the PSW phenotype (lane 10). Autoradiograms obtained after hybridization with a *SIR3* probe and an rDNA probe, respectively, are shown. It should be noted that in panel B, a portion of the gel containing the initial sample plugs corresponding to lanes 1 to 5 was inadvertently lost before the gel was subjected to autoradiography. Therefore, radioactive signals in sample wells representing chromosome XII, which was present in incompletely digested cells or spheroplasts and failed to enter the gel, are not seen in these lanes.

always showed the PSW phenotype (39). Thus, the copy of chromosome XII with the expanded rDNA repeats derived from the PSW parent strain apparently functions as a dominant allele. The two chromosome XII species with different rDNA repeats can coexist within the same diploid nucleus and can be segregated into individual haploid spores through meiosis, as confirmed by CHEF analysis of clones of haploid segregants; segregant clones with the PSW phenotype showed a chromosome with expanded rDNA repeats and those with the N-PSW phenotype showed a chromosome with reduced rDNA repeats (Fig. 3B, lanes 9 and 10). Thus, the expanded state of rDNA on chromosome XII is stable enough to be maintained through meiosis. Figure 3B also includes the analysis of two control diploids, one obtained by a cross between two N-PSW strains and the other obtained by a cross between two PSW strains. The former showed the chromosome XII with decreased rDNA repeats (lane 4), and the latter showed the chromosome with expanded rDNA repeats (lane 8). These results give further support to the correlation between the expanded rDNA state on chromosome XII and the PSW phenotype. Interestingly, the shorter chromosome XII derived from the *rrn9*Δ N-PSW parent in the PSW/N-PSW diploids showed a band with a size distribution more homogeneous than that of the N-PSW haploid parent or the N-PSW diploid (Fig. 3B; compare lanes 5 to 7 with lanes 2 and 4) or the N-PSW haploid segregants (lane 9), and their sizes varied depending on the diploid clone examined (lanes 5 to 7). A possible reason for this observation is discussed below.

Mutation of *FOB1* decreases the frequency of switching from the N-PSW state to the PSW state. The observed correlation of expansion of rDNA repeats with the PSW phenotype suggests that rDNA repeat expansion is necessary to attain the PSW state. Alternatively, the expansion of rDNA could simply be a consequence of PSW. To distinguish between these two alternatives, we examined the effects of deletion of the *FOB1* gene on the switching from N-PSW to PSW states. The *FOB1* gene was previously demonstrated to be required for expansion/contraction of rDNA repeats (21). It was found that strain NOY408-1a, which carries the *rpa135*Δ mutation (and helper plasmid pNOY102), showed a reduction in the number of rDNA repeats to about 80 repeats on average (Fig. 3A, lane 5) and that the introduction of the missing *RPA135* gene induced a gradual increase in repeat number back to the normal level, about 150. Derivatives of the *rpa135*Δ strain carrying a *fob1* deletion were constructed; these showed a reduced rDNA repeat number with more homogeneous repeat number distribution. These *fob1*Δ *rpa135*Δ strains did not show a significant increase in repeat numbers upon reintroduction of the *RPA135* gene, demonstrating a requirement of *FOB1* for rDNA expansion (21). It should be noted that DNA replication fork blocking aided by Fob1 protein at the site distal to the 35S rRNA coding region (20) appears to stimulate recombination, leading to a stimulation of rDNA expansion/contraction. Thus, rDNA expansion and contraction take place almost certainly during DNA replication and may require many generations of cell growth to attain large changes in repeat numbers.

We constructed *fob1* deletion derivatives of *rrn9*Δ N-PSW strain NOY703 and compared the frequency of switching of these strains to the PSW state with that of the control N-PSW strain NOY703. Many independent colonies which formed after streaking these strains on galactose plates were analyzed by a spot test. Examples of the results are shown in Fig. 4B. It was found that the control N-PSW *FOB1* strain formed PSW variants with a frequency ranging from 10^{-4} to 10^{-3}. In contrast, the N-PSW *fob1*Δ strain showed a frequency less than 10^{-5} in all the colonies analyzed, i.e., a large reduction, by a factor of

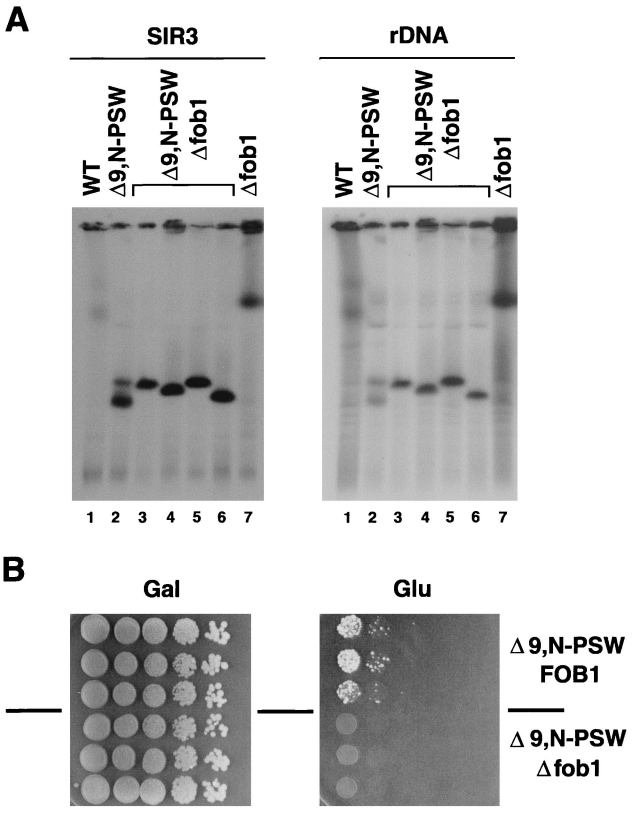

FIG. 4. Effect of a *fob1* mutation on the frequency of switch from the N-PSW to PSW states. (A) Chromosomal DNA was isolated from the following strains and analyzed by CHEF electrophoresis: NOY505 (lane 1), NOY703 (lane 2), four independent *fob1* deletion isolates obtained from NOY703 by disruption of *FOB1* (lanes 3 to 6), and a *fob1* deletion isolate obtained from the control strain NOY505 (lane 7). The left and right panels show autoradiograms obtained after hybridization with a *SIR3* probe and a rDNA probe, respectively. (B) Three independent colonies of NOY703 (Δ9, N-PSW) and those of a *fob1*Δ mutant derived from NOY703 were analyzed by spot test on YEP-galactose (Gal) and YEPD (Glu).

10 to 100 or higher, in the frequency of the switch. Thus, the *fob1*Δ mutation inhibits switching from the N-PSW to the PSW state. We conclude that the expansion of rDNA repeats is the cause of the switch to the PSW state and not a consequence of the switch.

It should be noted that the *rrn9*Δ *fob1*Δ N-PSW strains used in this experiment showed a more homogeneous chromosome XII band than the band for the parent *FOB1* strain and that mobility differed depending on the *fob1*Δ clones obtained after the transformation used for their construction (Fig. 4A, lanes 3 to 6 compared with lane 2 or with lanes 2 of Fig. 3A and B). The rDNA repeats appear to continue to expand and contract in *rrn9*Δ strains as well as in *rpa135*Δ and WT strains, showing a heterogeneity in the size of chromosome XII, but such expansion and contraction are greatly inhibited by deletion of *FOB1* (21). The *rrn9*Δ *fob1*Δ N-PSW strain was constructed from the *rrn9*Δ *FOB1* N-PSW strain by a standard gene disruption method. Individual *fob1*Δ transformants may have carried different rDNA repeat numbers at the time of *FOB1* deletion; upon depletion of the Fob1 protein during growth on selective plates, rDNA expansion and contraction ceased, and individual clones may have been left carrying different, yet relatively homogeneous, repeat numbers.

We also note that the homogeneous and variously sized

shorter chromosome XII bands seen in PSW/N-PSW diploid clones (Fig. 3B, lanes 5 to 7) could be explained in a similar way. Fob1p was shown to be present in the nucleolus (9). Perhaps it may be bound mostly to rDNA repeats on chromosome XII. Fob1p that had allowed expansion/contraction of the chromosome XII in the N-PSW nuclei may have been sequestered by competition to the expanded chromosome XII from the PSW strain after nuclear fusion in the cross, leading to cessation of expansion and contraction of the N-PSW chromosome XIIs.

Effects of mutations in *SIR* genes and genes for Pol I and CF subunits. We have previously shown that in addition to UAF, both Pol I and transcription factor CF are important in the maintenance of normal yeast nucleolar structures (27–29). In addition, there are some proteins known to be involved in the maintenance of rDNA chromatin structure. For example, Sir2 protein is present in the yeast nucleolus in addition to being present at telomere regions (11). Sir2p is known to be required for silencing of some Pol II reporter genes inserted into rDNA (1, 10, 36) and for decreasing the rate of recombination within the rDNA repeats (12). Therefore, we examined mutations in some of these genes with respect to switching to the PSW state.

PSW strains can grow in the absence of Pol I (39) or CF (see below). Yet analysis of individual colonies of NOY408-1a (which is *rpa135*Δ and carries a helper plasmid) by the galactose-glucose spot test did not reveal the appearance of cells able to form colonies on glucose, i.e., no switching, while a control *rrn9*Δ strain showed switching to PSW (Fig. 5A). By spreading more cells on glucose plates, we failed to detect appearance of any PSW colonies from NOY408-1a cultures grown in galactose medium (less than 1 in 10^7 cells [data not shown]). However, strains carrying both the UAF mutation and the Pol I mutation and growing on galactose with a helper plasmid (e.g., NOY896, which is *rrn9*Δ *rpa135*Δ pNOY103) can switch to the PSW state (Fig. 5A). Analysis of many independent colonies by the spot test indicated that the *rrn9*Δ *rpa135*Δ strain showed a switching frequency similar to that for the control *rrn9*Δ strain. Thus, the *rpa135*Δ mutation does not inhibit or significantly stimulate the switch from the N-PSW to PSW states and is unable to cause the switch by itself.

Strain NOY566 carrying a deletion in one of the CF subunit genes (*rrn6*Δ*::LEU2*) and growing on galactose with the helper plasmid pNOY103 also failed to produce cells able to grow on glucose (Fig. 5B, upper samples). When this mutation was combined with a UAF mutation (*rrn5*Δ), switching to the PSW state was observed (Fig. 5B, lower samples), and its efficiency was similar to that observed for the control *rrn5*Δ N-PSW strain carrying a helper plasmid (data not shown). Thus, the presence of intact UAF, but not Pol I or CF, appears to be important for preventing switching to the PSW state.

SIR3 and *SIR4* are required for silencing at the telomeres and silent mating loci as is *SIR2* but are not required for silencing of a Pol II reporter gene inserted into rDNA (23, 36). We examined the effects of individual deletion of *SIR2*, *SIR3*, and *SIR4* genes on the switch from N-PSW to PSW in the *rrn9*Δ background. N-PSW *rrn9*Δ strain NOY703 and its *sir2*, *sir3*, and *sir4* deletion derivatives were grown on YEP-galactose plates. All had similar growth rates. Twelve single colonies from each strain were analyzed for the frequency of PSW variants by the spot test. The frequency for the *rrn9*Δ *sir2*Δ colonies in this and other similar experiments ranged from $\sim 10^{-4}$ to 10^{-1}, whereas that for the control NOY703 strain ranged from $\sim 10^{-4}$ to 10^{-3} (Fig. 5C and other data not shown). The median value for the former was clearly much (at least 10-fold) higher than for the latter. In contrast, *sir3*Δ and *sir4*Δ did not show such an increase (Fig. 5C; apparent negative effects observed with these mutations were not studied further).

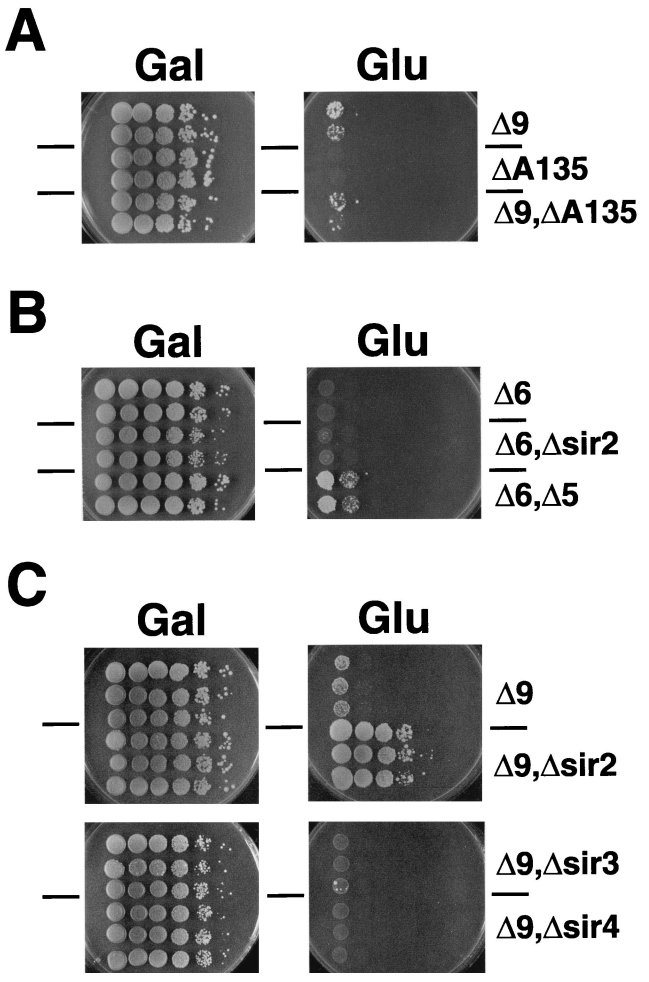

FIG. 5. Efficiency of switching from the N-PSW to PSW states. (A) Two independent colonies of strains carrying *rrn9*Δ (NOY703; N-PSW), *rpa135*Δ (NOY408-1a), and *rrn9*Δ *rap135*Δ (NOY896) were analyzed by spot test on YEP-galactose and YEPD. (B) Two independent colonies of strains carrying *rrn6*Δ (NOY566), *rrn6*Δ *sir2*Δ (NOY918), and *rrn6*Δ *rrn5*Δ (NOY919) were analyzed by spot test on YEP-galactose and YEPD. (C) N-PSW strains carrying *rrn9*Δ (NOY703), *rrn9*Δ *sir2*Δ (NOY901), *rrn9*Δ *sir3*Δ (NOY911), and *rrn9*Δ *sir4*Δ (NOY912) were grown on a YEP-galactose plate. Three single colonies from each strain were analyzed by spot test.

Although the *sir2*Δ mutation increased the frequency of switching to the PSW state when combined with a UAF mutation, the *sir2*Δ mutation itself was unable to allow switching to the PSW state. This conclusion can be drawn from the fact that the introduction of *sir2*Δ into NOY566 (*rrn6*Δ pNOY103) did not allow the strain to form PSW variants, whereas introduction of a UAF mutation (*rrn5*Δ) was able to do so (Fig. 5B, middle compared to lower samples). Thus, disruption of *SIR2* stimulates switching to the PSW state, presumably by stimulating the rate of expansion and contraction of rDNA repeats, but does not by itself cause the switching.

Altered localization of the nucleolus in polymerase switched strains. We examined the structure of the nucleolus in PSW strains by using EM and immunofluorescence microscopy (IFM). Since the presence of helper plasmids such as pNOY103, which allows transcription of the *GAL7*-35S rDNA fusion gene by Pol II, may lead to formation of several

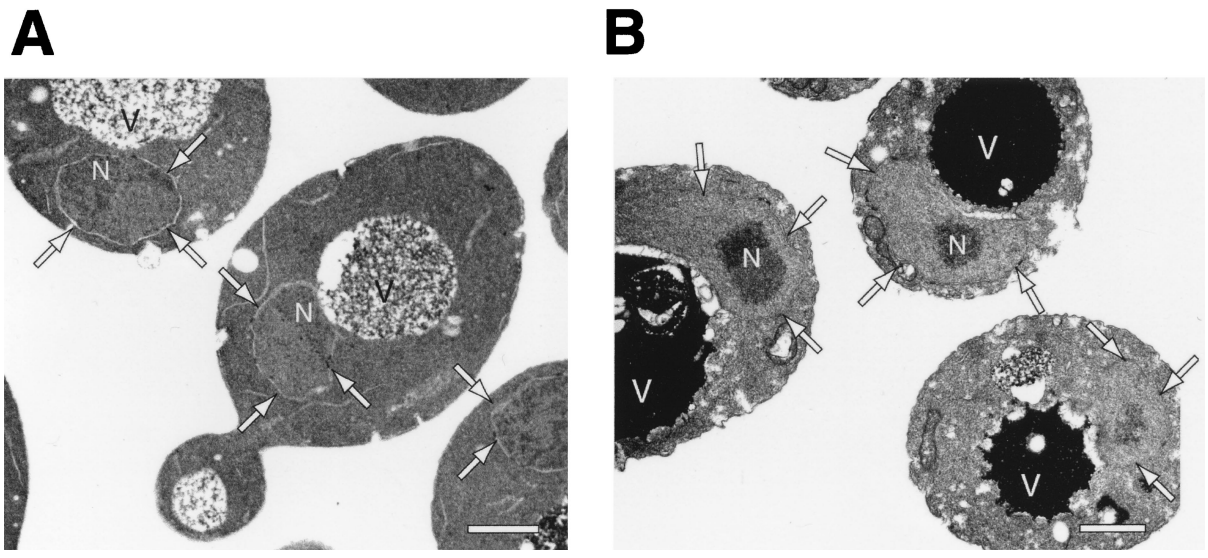

FIG. 6. EM analysis of the nucleolus in control strain NOY505 (A) and PSW strain NOY794 (B). The strains were grown in YEPD at 25°C to an A_{600} of about 0.5, and samples were prepared for EM analysis. The nuclear envelope is marked with arrows to serve as a point of reference, and the nucleolus (electron-dense areas within the nucleus) is indicated as N. The vacuole is indicated as V. Bars, 1 μm.

mininucleoli (27, 28), thus complicating the analysis, we examined PSW strains without such helper plasmids. Figure 6 shows electron micrographs of thin sections of cells of a *rrn9*Δ *rpa135*Δ PSW strain (NOY794) and cells of a wild-type strain (NOY505). The control cells showed electron-dense nucleolar materials at or near the nuclear periphery, forming the normal crescent-shaped nucleolus (Fig. 6A). In contrast, the nucleolus of the *rrn9*Δ *rpa135*Δ PSW strain (without any helper plasmid) showed a round nucleolus, which is distant from the nuclear periphery. Quite often, the nucleolus in the PSW strain revealed two different parts, one with higher and the other with lower electron density. Although we have not studied the basis of the presence of two subnucleolar regions, a similar feature as well as the interior localization of the nucleolus was observed for strains with chromosomal rDNA deletions complemented by the *GAL7*-35S rDNA fusion gene on a multicopy plasmid (28). IFM using antibodies against nucleolar protein Ssb1p (3) also supported the interior localization of the nucleolus in PSW strains, which was clearly different from the crescent structure along the nuclear periphery seen in the control strains (data not shown).

Localization of expanded rDNA repeats in a *rrn9*Δ PSW strain was then examined by FISH, and the results were consistent with the interior localization of the nucleolus in the PSW strain revealed by EM and IFM described above. As shown in Fig. 7, 4′,6-diamidino-2-phenylindole (DAPI) staining of PSW cell nuclei carried out under the conditions of FISH revealed a "hole" with much reduced DNA staining, and rDNA was seen surrounding this hole. For the control cells, this rDNA arrangement was not observed and rDNA was seen as either a cap, a bar, or a collection of dots mostly located at the nuclear periphery as reported previously (13, 28). Although the conditions for sample preparation are different for EM, IFM, and FISH, the holes surrounded by rDNA seen in PSW cells by FISH may correspond to round nucleoli seen by EM and IFM. We conclude that the sites of rDNA transcription as well as ribosome assembly in PSW cells are different from those in normal yeast cells and are at more interior locations compared with the normal site at the nuclear periphery.

Transcription of rDNA by Pol II in N-PSW *rrn9*Δ strains. Cells which can grow reasonably well on glucose (i.e., are able to form colonies on glucose) are defined to be in the PSW state, whereas cells that cannot grow or can grow only extremely slowly on glucose (no visible colonies after 7 to 10 days of incubation) are defined to be in the N-PSW state (39). By defining the N-PSW state in this way, we originally assumed that transcription of rDNA by Pol II takes place only in the PSW state and not in the N-PSW state. Weak residual transcription observed in the UAF mutants under conditions of repression of the *GAL7* promoter was interpreted to be due to the basal transcription of rDNA by Pol I, as was observed in in vitro experiments (19, 39). We have now examined this previous assumption and found that it is incorrect; transcription of rDNA by Pol II actually takes place in *rrn9*Δ strains even in the N-PSW state, though very weakly.

Using a primer extension analysis, we have previously shown that transcription of rDNA by Pol II in established PSW strains starts at several positions upstream from the start site (+1) seen for transcription by Pol I, ranging from −9 to −95 and with a major site at −29 (39). We used the same method and examined the question of whether Pol II transcription of rDNA takes place in N-PSW strains. In experiments shown in Fig. 8A, *fob1*Δ derivatives of *rrn9*Δ N-PSW and *rrn9*Δ PSW strains (NOY921 and NOY920, respectively) were used to minimize the occurrence of switching to PSW in N-PSW cultures. Control strains W303-1a and NOY408-1a were also analyzed. Cells were grown in galactose medium and divided into two parts; one part was shifted to glucose medium, and the other was kept in galactose medium. One hour after the shift, cells were harvested and RNA was prepared. Primer extension was then carried out to examine 5′ ends of rRNA precursors from these strains.

We found, contrary to the original assumption, that N-PSW strains showed the presence of transcripts, which corresponded to the transcripts made by Pol II in the control PSW strain under the condition of repression of *GAL7*-35S rDNA on the helper plasmid (Fig. 8, lanes 8′ and 6).The amounts of those Pol II-specific precursor rRNAs were simply much lower than

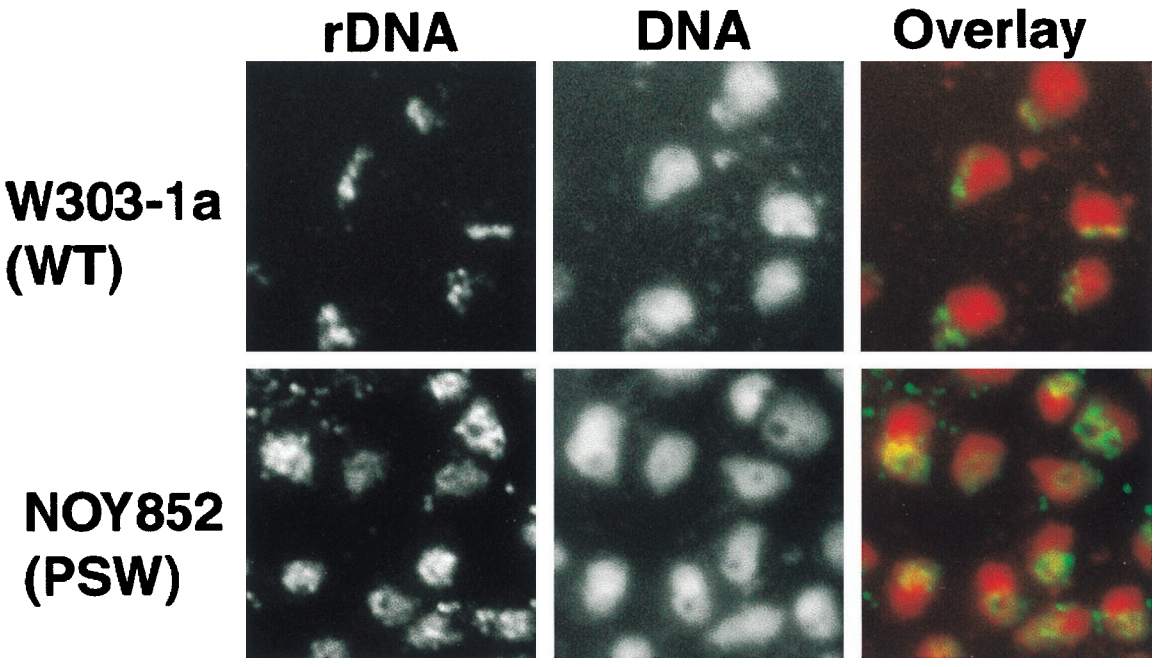

FIG. 7. FISH analysis of rDNA in PSW strain NOY852 and control strain W303-1a. Yeast strains were analyzed for rDNA and DNA as described in Materials and Methods. Images of rDNA and DNA were pseudocolored green and red, respectively, giving overlapped regions yellow in overlay. Individual images are shown in black and white. Note that in the PSW strain, many of the DAPI-stained nuclei have a hole with decreased DAPI staining and that rDNA appears to surround these holes. In the control strain, such a hole surrounded by rDNA was rarely seen.

that found in the control PSW strain (approximately 15% of the control PSW strain level; see the legend to Fig. 8). No transcript with the Pol I start site (+1) was detected. It should be noted that Pol I deletion (*rpa135*Δ) and CF deletion (*rrn6*Δ) strains did not show any transcripts unique to the PSW strains; only faint transcripts which corresponded to the one with the *GAL7* promoter start site as the 5′ end were observed (Fig. 8; compare lanes 4 and 12 with lanes 3 and 11, respectively). Figure 8 also includes the results obtained for the *rrn6*Δ *sir2*Δ strain and the *rrn6*Δ *rrn5*Δ N-PSW strain used in the experiments shown in Fig. 5. The former did not show any PSW-specific transcripts (lane 14), as was the case with the *rrn6*Δ strain, whereas the latter showed PSW-specific transcripts (lane 16), as did the *rrn9*Δ N-PSW strain. The difference between the two strains is correlated with the difference in their abilities to switch to the PSW state shown in Fig. 5B. It appears that inactivation of UAF by the *rrn9*Δ deletion (or the *rrn5*Δ deletion) is sufficient to allow Pol II to transcribe chromosomal rDNA, even though this transcription is apparently not enough to allow cells to grow without helper plasmid, and the expansion of rDNA repeats is required for improved cell growth.

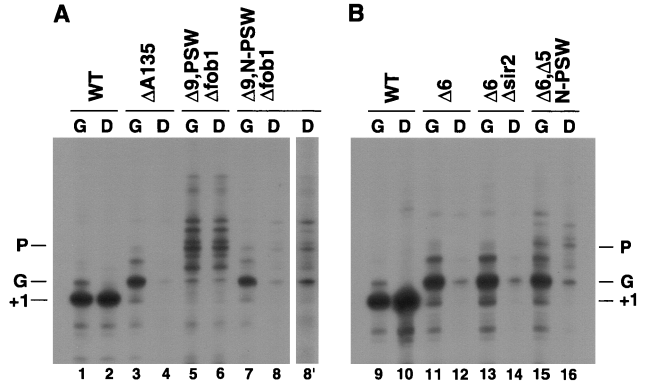

FIG. 8. Primer extension analysis of primary transcripts for detection of rDNA transcription by Pol II. RNA was prepared from the following strains growing on synthetic galactose medium supplemented with Casamino Acids, tryptophan, and adenosine (lanes G) and 1 h after shift from galactose to glucose synthetic medium with the same supplements (lanes D): W303-1a (lanes 1, 2, 9, and 10), NOY408-1a (lanes 3 and 4), NOY920 (lanes 5 and 6), NOY921 (lanes 7 and 8), NOY566 (lanes 11 and 12), NOY918 (lanes 13 and 14), and NOY919 (lanes 15 and 16). Primer extension reactions were done in parallel, but gel electrophoresis and autoradiography were done in two separate groups (shown in A and B) with the same WT samples included. Autoradiograms (exposure times, 12 h [A] and 18 h [B]) are shown. Lane 8′ is the same as lane 8 after a longer exposure, which was equivalent to ~30 h. Positions indicated as +1, G, and P correspond to the start site for the Pol I rDNA promoter, that for the *GAL7* promoter, and a major site (−29) among the 5′ ends identified for rDNA transcripts in PSW strains, respectively. Three independent experiments were carried out, and the amounts of Pol II-specific precursor rRNAs found in NOY921 in glucose (lane 8 or 8′) were 15.4% ± 2.0% of those in control PSW strain NOY920 (lane 6).

DISCUSSION

Model for RNA polymerase switch. The results presented in this report demonstrate that switching to the PSW state, i.e., the state that allows growth using the Pol II system, involves two steps: a mutation in UAF and an expansion of rDNA repeats. Table 2 summarizes information obtained on the WT, N-PSW, and PSW states. We found that some mutational alterations of UAF are sufficient to allow Pol II transcription of rDNA, although transcription is weak (~15% of the control PSW strain) and is apparently not sufficient to allow cellular growth. After repression of the *GAL7* promoter by glucose, N-PSW UAF mutants can continue to grow and divide at least for several generations by using preexistent ribosomes, and possibly also some new ribosomes, that would continue to be synthesized by the weak rDNA transcription by Pol II. This residual growth may be sufficient to allow UAF mutants to

TABLE 2. Summary of information on *rrn9*Δ N-PSW, *rrn9*Δ PSW, and control *RRN9* (WT) strains

Strain	Growth without Pol I	No. of rDNA repeats	Nucleolus	rDNA transcription by Pol II
WT	−	~150	Crescent, periphery	−
*rrn9*Δ N-PSW	−	~80	Not studied	+
*rrn9*Δ PSW	+	~400	Round, interior	+++

continue *FOB1*-dependent expansion and contraction of rDNA repeats at least for a while so that a small fraction of cells (10^{-3} to 10^{-4}) can form colonies, enabling some of them to establish a fully competent PSW state.

UAF is unique in playing an essential role in silencing Pol II transcription of rDNA. Mutations in other genes, those for subunits of CF or Pol I or Sir2p, a known component of rDNA chromatin, do not allow Pol II transcription. The simplest possible mechanism for silencing is that UAF binds to the upstream element of the rDNA promoter (19) and by itself or in combination with other interacting proteins forms a structure that inhibits Pol II transcription. According to this model, UAF may be a crucial component of rDNA-specific chromatin and is probably bound to the promoter region regardless of the state of Pol I activity. The second step, a *FOB1*-dependent expansion of rDNA repeats, is slow and involves intermediate states, presumably reflecting states of chromosome XII with somewhat increased, but not yet fully expanded, rDNA repeats. Cells in such intermediate states may be able to grow somewhat more efficiently than the original N-PSW cells, leading to formation of tiny colonies, but may lose the increased rDNA repeats due to an instability of the expanded states, returning frequently back to the N-PSW state, as the experiments shown in Fig. 1 indicate. We suggest that only fully or nearly fully expanded rDNA repeats (about 400) can establish a relatively stable structure(s), i.e., a Pol II-specific nucleolar structure, thus preventing further increase in rDNA repeats or frequent loss of the increased repeats.

It should be noted that switch from the *rrn9*Δ N-PSW to *rrn9*Δ PSW states is clearly an adaptation process which takes place on glucose plates. Fully established PSW cells do not exist in N-PSW cultures grown in galactose by transcribing the artificial fusion gene on a helper plasmid.

Deletion of *SIR2* was shown to increase the efficiency of switching, though it does not cause switching to the PSW state by itself; i.e., it stimulates the second step without causing any change equivalent to the first step. Since *sir2* mutations are known to increase the frequency of recombination within rDNA repeats (12), stimulation of *FOB1*-dependent repeat expansion can be explained on this basis; *sir2* mutations are expected to increase the rate of both expansion and contraction and consequently increase the frequency of rDNA repeats with sufficiently high numbers (about 400) to form a reasonably stable Pol II-specific nucleolar structure and establish the PSW state.

Significance of the requirement of rDNA repeat expansion for establishment of the PSW state. There are two different models to explain the requirement of rDNA repeat expansion for establishing the PSW state. The first model assumes that in *rrn9*Δ N-PSW strains, all of the rDNA repeats are accessible to Pol II and transcribed in a productive way leading to ribosome formation and that a higher gene dosage is required simply to increase the overall rate of rDNA transcription to meet the need for growth. In this case, one has to explain the formation of a distinct nucleolar structure in PSW cells, which is localized at an interior site rather than at the original site at the nuclear periphery. Perhaps UAF is a key element for retaining rDNA at the nuclear periphery and *rrn9*Δ cells in the N-PSW state have rDNA (and the Pol II-specific nucleolus) at an interior site. Expansion of rDNA repeats may simply increase the rRNA synthesis rate without changing the location of the nucleolus. Unfortunately, it has been difficult, for technical reasons, to study the nucleolus in *rrn9*Δ N-PSW cells which do not carry a helper plasmid, and thus, we have no information on this question (Table 2).

The second model assumes that only a fraction of the rDNA repeats is accessible to the Pol II machinery or is transcribed productively to lead to ribosome formation. Perhaps the Pol II machinery is not freely diffusible in the yeast nucleus, as suggested by previous studies for higher eukaryotic cells (4, 14). In addition, mobility of rDNA repeats on chromosome XII in interphase may also be limited within certain "chromosome territories" (25), thus making formation of the nucleolus at an interior site(s) difficult. According to this model, DNA repeat expansion is required to form a nucleolar structure at a suitable site in addition to (or rather than) a simple increase of repeat numbers to increase transcription rate by a high gene dosage. Such a Pol II-specific nucleolar structure may be required for more efficient rDNA transcription by Pol II and/or subsequent steps, rRNA processing, rRNA modification, and ribosome assembly. In connection with the second model, it should be noted that transcription of some Pol II reporter genes integrated into rDNA repeats is known to take place. However, it has not been demonstrated that these reporter genes integrated into any of the repeats can be transcribed by Pol II. Clearly, further experiments are required to settle these issues and distinguish between the two (and other possible) models.

The presence of a round nucleolus localized at an interior site(s) in PSW strains is consistent with the results of our previous studies using yeast mutants with chromosomal rDNA completely deleted (28). Such a mutant, when complemented by a plasmid carrying a single rDNA repeat transcribed by Pol I, contained many mininucleoli preferentially localized at the nuclear periphery. In contrast, the same mutant, when complemented by a plasmid carrying the *GAL7*-35S rDNA fusion gene transcribed by Pol II and growing on galactose, contained a rounded nucleolus that lacked extensive contact with the nuclear envelope and resembled that observed in the PSW strains studied in this work. These observations indicate the presence of separate nuclear subregions, one favorable for rDNA transcription by Pol I followed by ribosome assembly and the other favorable for rRNA synthesis by Pol II (using the fusion gene) followed by ribosome assembly.

Relation to rDNA silencing of Pol II reporter genes. Silencing of certain Pol II reporter genes inserted into rDNA has been reported (1, 10, 36). One feature of this silencing system is the requirement of *SIR2* but not *SIR3* and *SIR4*. Similarly, *SIR2*, but not *SIR3* or *SIR4*, was shown to play a role in decreasing the rate of recombination within rDNA repeats (12). In the present system, the second step in switching to the PSW state is stimulated by a *sir2* mutation but not by a *sir3* or *sir4* mutation. Thus, a rDNA chromatin structure containing Sir2p postulated to be important for rDNA silencing of reporter genes or inhibition of recombination appears to play a role in decreasing switching to the PSW state by decreasing the rate of expansion and contraction of rDNA.

It is clear that the roles of *SIR2* and UAF in silencing of Pol II transcription of rDNA are fundamentally different. In contrast to UAF mutations, *sir2* deletion itself does not allow rDNA expansion to the PSW state (or rDNA transcription by

Pol II without expansion). It appears that UAF, presumably together with other unidentified components, prevents Pol II transcription of rDNA directly, and Sir2p is not involved in this repressive chromatin structure, which is almost certainly located at rDNA promoter regions. In this connection, it should be noted that in in vitro experiments both Pol I and CF, together with TBP and Rrn3p, join the UAF-promoter complex to form a preinitiation complex (18, 19). In addition, both Pol I and CF are known to be important, like UAF, for the maintenance of intact nucleolar structures (28), yet neither Pol I subunit deletion nor CF deletion allows rDNA expansion to the PSW state. Perhaps the weak rDNA transcription by Pol II, which was observed in UAF deletion but not Pol I subunit deletion or CF subunit deletion mutants, is important, allowing residual growth and selective pressure to continue, leading to an eventual establishment of the PSW state.

Significance of large tandem repeat numbers of rDNA genes. In most eukaryotes, rDNA genes are tandemly repeated at one or a few chromosomal loci, the nucleolar organizers. The repeat number at a locus is generally large but is highly variable among organisms, ranging from less than 100 to over 10,000 per haploid genome. The repeat numbers often vary significantly not only among related species but also among different strains of the same species (reviewed in reference 22; for variations within *S. cerevisiae*, see references 2 and 30). It was often assumed that the presence of large numbers of rDNA genes reflects a demand for high rates of rRNA synthesis to meet cellular growth needs. However, the large variations of gene number among closely related organisms are difficult to explain on this basis. In addition, a given organism does not appear to require the presence of all rDNA gene copies for normal growth rates. We have previously described the construction of a yeast strain which carries only 25% (i.e., ca. 40 repeats) of the normal rDNA repeats (21). This strain was constructed by first deleting *RPA135* in the presence of a helper plasmid, which caused reduction of the rDNA repeat numbers followed by deletion of *FOB1*, preventing expansion and contraction of the repeats, and finally by reintroducing the missing *RPA135* gene. This yeast strain and a control yeast strain with ~150 rDNA repeats showed identical growth rates (21) and rRNA synthesis rates (16). Thus, it is clear that normal yeast cells use only a fraction of the ~150 rDNA repeats to synthesize rRNA by Pol I, even under conditions of near maximum growth rates. Previous analyses of rDNA chromatin structure using psoralen cross-linking also showed that only a fraction of the rDNA copies is transcribed in actively growing cells in a variety of systems including yeast (5, 8). As we discussed above in connection with the requirement of rDNA repeat expansion for the formation of a new Pol II-specific nucleolar structure, extra rDNA repeats might be present simply to form suitable nucleolar structures rather than to function as template for rRNA synthesis. Perhaps the number of rDNA repeats unique to each organism reflects the presence of particular nucleolar structures unique to these organisms (and environmental or developmental conditions).

We consider rDNA expansion and the alteration of nucleolar structures in PSW strains as an extreme example of a general plasticity of the nucleolar structure. The *FOB1*-dependent expansion and contraction of rDNA repeats might be used for changing nucleolar structure in response to environmental changes, in addition to the well-discussed role in the maintenance of sequence homogeneity throughout many rDNA repeats. In this regard, we note that there have been studies reporting heritable changes in rDNA copy numbers in flux induced by specific environmental changes (6, 7). For *S. cerevisiae*, it was reported that cells grown at the optimal temperature of 30°C led to an increase in rDNA repeat numbers relative to cells grown at 22°C (32). The plasticity of rDNA repeat numbers as well as nucleolar structures may be advantageous to organisms. However, nucleolar structures are complex. In addition, the nucleolus may play functional roles other than synthesizing ribosomes (31, 33, 34, 38). Thus, clear understanding of this subject must await further studies of nucleolar structure and functions.

There are three features of rDNA transcription in most eukaryotes that distinguish it from rRNA synthesis in eubacteria or archaea: (i) the use of a distinct RNA polymerase, Pol I, (ii) the presence of tandemly repeated rRNA genes (with exceptions of some lower eukaryotes which carry many copies of rDNA plasmids or minichromosomes), and (iii) the presence of the nucleolus as the site of transcription, rRNA processing, and ribosome assembly. Separation of the site of rDNA transcription, using a unique polymerase presumably for the purpose of efficiency and regulation, may have had selective advantages for eukaryotic organisms, and the evolution of the three features of rDNA transcription might have been interrelated. The RNA polymerase switch induced by mutations in UAF affects all of these three features. Thus, further studies on this system may provide some insight into not only the functional significance of these features in general cell biology but also the question of their evolution in eukaryotes.

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M.O. and I.S. contributed equally to this work.

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