A Polymerase Switch in the Synthesis of rRNA in *Saccharomyces cerevisiae*

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Transcription of ribosomal DNA by RNA polymerase I is believed to be the sole source of the 25S, 18S, and 5.8S rRNAs in wild-type cells of *Saccharomyces cerevisiae*. Here we present evidence for a switch from RNA polymerase I to RNA polymerase II in the synthesis of a substantial fraction of those rRNAs in respiratory-deficient (petite) cells. The templates for the RNA polymerase II transcripts are largely, if not exclusively, episomal copies of ribosomal DNA arising from homologous recombination events within the ribosomal DNA repeat on chromosome XII. Ribosomal DNA contains a cryptic RNA polymerase II promoter that is activated in petites; it overlaps the RNA polymerase I promoter and produces a transcript equivalent to the 35S precursor rRNA made by RNA polymerase I. Yeast cells that lack RNA polymerase I activity, because of a disruption of the *RPA135* gene that encodes subunit II of the enzyme, can survive by using the RNA polymerase II promoter in ribosomal DNA to direct the synthesis of the 35S rRNA precursor. This polymerase switch could provide cells with a mechanism to synthesize rRNA independent of the controls of RNA polymerase I transcription.

It is generally accepted that rRNA in all eukaryotes is the product of transcription by RNA polymerases I and III (Pol I and Pol III), whereas mRNAs and most of the small nuclear RNAs are transcribed by RNA polymerase II (Pol II). Pol I transcribes the precursor to the 25S to 28S, 18S, and 5.8S rRNAs from ribosomal DNA (rDNA) in the nucleolus, where rRNA maturation occurs. Important *cis*-acting sequences that function in the synthesis of precursor rRNA are contained within the spacer DNA regions separating the rRNA cistrons, including those originally termed the nontranscribed spacer (NTS) (37, 45, 55). In addition to the major Pol I promoter for precursor rRNA, alternative Pol I promoter sequences, so-called spacer promoters, have been identified upstream of the major Pol I promoter in many organisms (37, 45). The intergenic spacers also contain RNA processing and termination sites, including some located close to the major initiation site for Pol I transcription (37, 45). Finally, enhancer-like elements within spacer regions of the rDNA repeat have also been described (37, 45, 55). Thus, the NTS regions of the rDNA repeat contain important *cis*-acting elements that function to regulate transcription of rRNA genes.

The yeast *Saccharomyces cerevisiae* contains multiple copies of rDNA arranged on chromosome XII as a 9.1-kb head-to-tail tandem repeat (35, 36). Each repeat unit contains the 25S, 18S, and 5.8S rRNA gene cluster transcribed by Pol I as a 35S precursor as well as the 5S rRNA gene transcribed in the opposite direction by Pol III (55). The intergenic spacer is split by the 5S rRNA gene into two regions, NTS1 and NTS2 (Fig. 1) (43). NTS1 contains a Pol I enhancer within a 190-bp *EcoRI-HindIII* fragment located 2.2 kb upstream from the transcriptional initiation site of the 35S rRNA precursor in NTS2 (9, 10). As in most organisms, rRNAs are coordinately synthesized in *S. cerevisiae*, and the steady-state levels of these

RNAs vary with growth rate and carbon, amino acid, and nitrogen sources in the medium (18, 53–55).

At least two forms of rDNA exist in *S. cerevisiae*: the tandem array on chromosome XII (35, 36), where the number of gene copies (up to 200 or so) can vary widely depending on the strain (55) (and even among clonal isolates of the same strain), and episomal copies present usually as 9.1-kb monomer circles (6, 22, 27). Those circles arise as excision products of homologous recombination between members of the tandem repeat, a process reported to be influenced by topoisomerase I and II activities (4, 21). Since each unit of rDNA contains an ARS (autonomously replicating sequence) element, these episomal copies of rDNA can be maintained as autonomous replicons (47).

In previous studies, we described an unusual and puzzling set of transcripts derived primarily from the NTS2 region of rDNA that were barely detectable in respiratory-competent (*[rho]*⁺) cells but which accumulate to high levels in certain respiratory-deficient (petite) strains (33, 34). Surprisingly, we found that the NTS RNAs, which are transcribed from the same strand as precursor rRNA (Fig. 1), are the products of Pol II rather than Pol I transcription (33). In attempting to understand the significance of these novel NTS transcripts and their relationship to rRNA synthesis in *[rho]*⁺ versus petite cells, we have made the unexpected finding that rDNA contains a cryptic Pol II promoter that is activated in petites and that this promoter contributes to the synthesis of precursor rRNA in petites, most likely from episomal copies of rDNA. Thus, even within the same cell, rRNA may be transcribed by separate RNA polymerases and presumably subject to different controls.

MATERIALS AND METHODS

Strains and media. The host strains of *S. cerevisiae* used in this study are two mitochondrial derivatives of strain 161U7 (*MATa ade1 lys1 ura3*): 161U7 *[rho]*⁺ and 161U7 *[rho]*⁰. Strain W3031A (*mata ade2-1 leu2-3,112 his3-11 trp1-1 ura3-1 can1-100*) (3) and a derivative of NOY4081A (32), HCW1X, were used in the plasmid shuffle experiment. To utilize the *leu2d* selectable marker on pHCW200, a *Leu2*[−] derivative of NOY4081A, HCW1X (*mata rpa135::leu2::HIS3 ade2-1 leu2-3,112 his3-11 trp1-1 ura3-1 can1-100*), was constructed. Plasmid pUCLEU

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FIG. 1. (A) The rDNA repeat of *S. cerevisiae* (top) and the rDNA minigene construct, rT7-λ (bottom). The thin arrows depict the initiation sites for the 35S and 5S rRNA transcription. The thick black arrows represent the location of NTS RNAs (33). The enhancer sequences (E; striped box) and a 3' processing site (T) for the 35S precursor rRNA are shown. The bottom diagram shows the rDNA minigene, rT7-λ, on the 2μ plasmid pSEYrT7λ or on the integrating vector YIpT7λ. The *Stu*I fragment in the 25S gene has been deleted as shown. (B) Differential expression of T7 rRNA. 161U7 [*rho*⁺] and [*rho*⁰] cells transformed with pSEYrT7λ or with YIpT7λ integrated into the rDNA repeat were grown on YNB/Craf medium. Total cellular RNA was isolated from exponentially growing cells, and Northern blot analysis was performed with labeled antisense probes for actin or T7 RNA. Levels of T7 rRNA, actin mRNA, and plasmid copy number were quantified with a Molecular Dynamics PhosphorImager. The ratio of T7 rRNA in [*rho*⁺] to that in [*rho*⁰] cells is normalized for actin mRNA levels and reporter gene copy number.

has the *leu2* gene on a *Hpa*I-*Acc*I restriction fragment in pUC19. A 600-bp *Cla*I-*Eco*RV fragment internal to the *LEU2* gene was replaced with the *HIS3* gene on an *Ssp*I-*Bst*NI fragment, forming pUC*leu2::HIS3*. The disrupted *leu2* gene was liberated from pUC*leu2::HIS3* and transformed into NOY4081A [*rho*⁰] cells, and His⁺ transformants were isolated. *Leu2*⁻ derivatives were confirmed by Southern blot analysis.

A YIp5 reporter construct (either YIpT7λ or YIp*rlacZ*) was integrated into the rDNA repeat by transforming 161U7 [*rho*⁰] cells with plasmid linearized at the *Hind*III site within NTS1. 161U7 [*rho*⁺] derivatives of the integrants were constructed by cytoduction using the *kar1-1* mutation, creating isogenic strains (30). Integration of a single copy of these reporter constructs into the rDNA repeat was confirmed by Southern blot analysis of transformants.

Cultures of yeast strains were grown at 30°C in YP medium with either 2% raffinose, 2% glucose (YPD), or 2% galactose (YPgal). Strains harboring plasmids were grown in YNB/C (6.7% yeast nitrogen base without amino acids [Difco Laboratories, Detroit, Mich.], 1% Casamino Acids, 70 mg of adenine-HCl per ml) with either 2% glucose (YNBCglu), 2% raffinose (YNBCraf), or 2% galactose (YNBCgal). For run-on transcription experiments, the Casamino Acids were omitted from the YNB/Craf medium. YNB/Craf medium containing 5-fluoroorotic acid (42) was used to select Ura⁻ isolates in the plasmid shuffle experiment.

Plasmids and constructions. Plasmids pGEMact and pGEMp19 were described previously (33), as was plasmid pSPT7 (9). The reporter construct rT7-λ was constructed by inserting a 141-bp *Pvu*II fragment from bacteriophage λ into the *Ava*II site of YcprR8 (9), and the same fragment was inserted into the *Sma*I site of pGEM3Z to form pGEM λ-14. The rDNA minigene rT7-λ was inserted into the *Bam*HI site of the 2μ plasmid pSEY8 (11) to yield plasmid pSEYrT7λ or that site in YIp5 (46) to yield the integrating vector YIpT7λ. Plasmid pGEMTY contains a 756-bp *Pst*I fragment spanning TyA and TyB (5) in

pGEM3Zf(+). Plasmid pGEMTCM contains a 1.55-kb *Pvu*II fragment containing the *TCM* gene (12) in pGEM4. Plasmid pGEMrRNA contains a 1.1-kb *Bgl*II-*Eco*RI internal fragment to the 25S rRNA gene in pGEM3Zf(+). (Promega). The β-galactosidase fusion gene constructs were derived from the CIT2-β-galactosidase fusion gene, pCIT2-605-lacZ (23). The *Sma*I-*Spe*I fragment containing the CIT2 promoter sequences was deleted from pCIT2-605-lacZ to form pCIT2Δ10. Various restriction fragments of NTS1 and NTS2 (filled in with Klenow enzyme) were ligated to pCIT2Δ10 linearized with *Eco*RI also filled in with Klenow enzyme. YEp*rlacZ*-Δ206 was constructed by inserting the 2.4-kb *Eco*RI fragment from YCprR8 into the *Eco*RI site of pCIT2-605-lacZ. The *Spe*I-*Sma*I fragment containing the CIT2 promoter sequences was then deleted. The β-galactosidase reporter construct, *rlacZ*, was subcloned into YIp5 (46) to form YIp5-*rlacZ*. Plasmid pHCW200 was made by inserting the *Sph*I-*Hind*III fragment of rDNA from 161U7 genomic DNA into those sites in the YEp*lac*181 (13) polylinker to form plasmid pHCW-S4. The 6.6-kb *Hind*III fragment from 161U7 genomic DNA was inserted into the *Hind*III site of pHCW-S4 to form pHCW100. A *leu2d* derivative, pHCW200, was created by introducing the *Hpa*I-*Nru*I fragment (filled in with Klenow enzyme) from pVT-λ (50) into the single *Bam*HI site (filled in with Klenow enzyme) of pHCW100.

Relative plasmid copy number determination. Relative plasmid copy number was determined for all experiments which used reporter constructs. Genomic DNA was isolated as previously described (33), and standard Southern blot procedures were used. Serial dilutions of denatured genomic DNAs were applied to duplicate Nytran (Schleicher & Schuell) filters, using a dot blot apparatus (Schleicher & Schuell). As controls for cross-hybridization, 161U7 [*rho*⁺] DNA and YIpT7λ DNA were also used. Filters were hybridized with either ³²P-labeled pGEM3Zf(+) DNA or a fragment of the *LEU2* gene, using standard hybridization conditions. DNA was quantitated with a Molecular Dynamics PhosphorImager.

RNA isolation and Northern (RNA) blot analysis. Total cellular RNA was isolated from exponentially growing cells as previously described (23) or by the method of Schmitt et al. (41). An aliquot of each culture was used to prepare genomic DNA. Total RNA was fractionated over oligo(dT)-cellulose columns to obtain poly(A)⁺ RNA as described previously (40). All fractions were saved and ethanol precipitated.

Total or poly(A)⁺ RNA was fractionated by electrophoresis on 1.25% agarose-7% formaldehyde gels. RNA was transferred to a Nytran filter (Schleicher & Schuell), using a Bio-Rad Vacublotter as recommended by the manufacturer, and then cross-linked to the filter by using a UV Stratalinker 2400 (Stratagene). Hybridizations were conducted with riboprobes as previously described (33). When RNA levels were to be quantified, multiple concentrations of RNA were analyzed for each sample. Quantitation was done with a Molecular Dynamics PhosphorImager. T7 rRNA levels were then normalized for actin RNA levels and plasmid copy number.

Nuclear run-on transcription was carried out as described by Johnson and Warner (15). The ³²P-labeled RNA was hybridized to antisense RNAs immobilized on Nytran filters, using the same conditions as for the Northern blots.

Primer extension analysis. Total cellular RNA extracted from the wild-type W3031A [*rho*⁰] (30 μg) and mutant HCW1X-0 [*rho*⁰] (75 μg) strains was hybridized overnight with a 5'-end ³²P-labeled oligomer (5'-CTC TCC ACC GTT TGA CGA GGC CAT TTA C-3') in hybridization buffer (100 mM sodium citrate [pH 6.4], 300 mM sodium acetate [pH 6.4], 1 mM EDTA, 80% formamide) at 30°C. Following ethanol precipitation, the RNA was extended with avian myeloblastosis virus reverse transcriptase (Promega) at 50°C for 45 min as recommended by the manufacturer. The sample was treated with 7.5 μl of 0.5 N NaOH for 5 min at 90°C and neutralized with 7.5 ml of 0.5 N HCl, ethanol precipitated, and resuspended in loading buffer (80% formamide, 2 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). The same oligomer was used to sequence the corresponding region of pHCW200, using Sequenase 2 (United States Biochemical Corp., Cleveland, Ohio) as specified by the manufacturer.

β-Galactosidase activity. To measure expression of β-galactosidase, 10 independent transformants of each type were pooled and cultures were grown on YNB/Craf or YNB/Cglu medium. β-Galactosidase activity was measured in triplicate from whole cell extracts as previously described (42). β-Galactosidase activity was corrected for plasmid copy number.

Excision of a reporter from rDNA. Transformants of 161U7 [*rho*⁺] and 161U7 [*rho*⁰] containing the integrated copy of YIp5-*rlacZ* were grown in YNB/Craf, and an aliquot of 161U7 [*rho*⁺] was grown in the same media containing 10 μg of ethidium bromide per ml for 60 h. A dilutions of each culture was plated onto YNB/Cglu-5-bromo-4-chloro-3-β-D-galactopyranoside (X-Gal) plates, and the plates were incubated for several days at 30°C. Blue sectors, along with white colonies for controls, were picked and subcloned for further investigation.

RESULTS

Differential expression of rRNA minigenes in [*rho*⁺] and [*rho*⁰] petite cells. To examine the synthesis of rRNA in wild-type and petite cells, we have used an rDNA minigene construct containing reporter sequences for precursor rRNA (Fig. 1A). This minigene (rT7-λ), a modified form of the one de-

scribed by Elion and Warner (9), contains a 600-bp fragment of bacteriophage T7 DNA as a reporter in place of most of the transcription unit of the 35S rRNA precursor (while retaining the correct initiation and 3' processing signals). As a reporter for NTS RNAs, we have introduced a 141-bp fragment of bacteriophage λ DNA into an *Ava*II site within NTS2.

Both [*rho*⁺] and [*rho*⁰] petite derivatives (cells which lack mitochondrial DNA) of strain 161U7 were transformed with the reporter gene in two different contexts: on a 2 μ m-based plasmid, pSEYrT7 λ , and as a single copy randomly inserted in the rDNA tandem repeat by using the integrating vector construct, YIpT7 λ . The T7 rRNA content in isogenic pairs of [*rho*⁺] and [*rho*⁰] transformants was determined by Northern blot analysis of total RNA extracted from those cells. RNA loads were balanced to the levels of actin mRNA, a transcript whose abundance is the same in isochromosomal [*rho*⁺] and petite cells (34).

These experiments show that T7 rRNA transcripts derived from the reporter construct integrated into the rDNA repeat are about eightfold more abundant in [*rho*⁺] cells than in the [*rho*⁰] petite cells (Fig. 1B). We have also tested several other strains for this effect and obtain the same results (data not shown). This difference in T7 rRNA abundance is consistent with the finding that [*rho*⁺] strains contain three- to fourfold more rRNA than do petites (8a), as large a difference as has been observed in rRNA content among yeast strains (54). That petites should contain less rRNA than their isochromosomal [*rho*⁺] counterparts is the expected result, since under identical growth conditions, petites have slower growth rates than [*rho*⁺] cells, and growth rate is known to be an important determinant of rRNA content in yeast cells (19, 53, 54).

In sharp contrast to the foregoing results, we have consistently observed that when the same minigene construct is expressed from the multicopy plasmid pSEYrT7 λ , the T7 rRNA content is three- to fivefold less per gene copy in [*rho*⁺] cells than in petites (Fig. 1B). This anomalous behavior of the T7 rRNA reporter gene is not restricted to this 2 μ m-based construct, as we have also observed a similar decrease of T7 rRNA content in [*rho*⁺] versus [*rho*⁰] cells with the reporter gene on a *TRP1-ARS1* high-copy-number plasmid (data not shown). Thus, the levels of T7 rRNA expressed from multicopy plasmids are opposite what would be expected for Pol I synthesis of rRNA, suggesting that localization of the reporter construct may influence its expression.

T7 rRNA synthesis from episomal templates in petites has characteristics of Pol II transcription. The expression of T7 reporter sequences from the 2 μ m plasmid mimics the behavior of the Pol II-derived endogenous NTS RNAs, which we previously showed are greatly elevated in [*rho*⁰] compared with [*rho*⁺] cells (33, 34). This finding raised the interesting possibility that some fraction of rRNA synthesis in petites might also be the product of Pol II transcription and perhaps selectively from extrachromosomal copies of rDNA. A general property of most Pol II transcripts is that they are polyadenylated at their 3' ends. Since the 35S rRNA precursor is rapidly processed to produce the 18S, 5.8S, and 25S rRNAs, we have used the rDNA minigene to first test whether any reporter rRNA transcripts derived from this construct might be polyadenylated. The minigene is convenient for these experiments, since it lacks the internal posttranscriptional signals of the 35S rRNA but still contains a 3' processing site and putative polyadenylation sites as well. Using the [*rho*⁺] and [*rho*⁰] transformants of strain 161U7 described in Fig. 1, total RNA was isolated from these cells and fractionated on oligo(dT)-cellulose columns to separate poly(A)⁺ from poly(A)⁻ RNAs; the various fractions were then analyzed by Northern blot hybrid-

FIG. 2. (A) T7 rRNA expressed from a 2 μ m vector fractionates as poly(A)⁺ RNA in [*rho*⁰] cells. Total RNA from [*rho*⁺] and [*rho*⁰] 161U7 cells transformed with pSEYrT7 λ or YIpT7 λ (Fig. 1) was fractionated on oligo(dT)-cellulose columns to obtain poly(A)⁺ RNA. Equal units of optical density at 260 nm of each fraction were subjected to Northern blot analysis using antisense T7, 25S rRNA, and actin riboprobes. (B) α -Amanitin-sensitive transcripts from rDNA sequences. 161U7 [*rho*⁺] and [*rho*⁰] cells transformed with pSEYrT7 λ , grown on YNBraff, were subjected to run-on transcription in the presence and absence of 200 μ g of α -amanitin per ml. The ³²P-labeled RNA was hybridized to antisense RNAs immobilized on Nytran filters.

ization with probes for T7 rRNA, 25S rRNA, and actin mRNA sequences (Fig. 2A). In all cases, the majority of actin mRNA fractionates as poly(A)⁺ RNA (fraction d), while most of the mature 25S rRNA is unbound and appears in the flowthrough and wash fractions (fractions b and c). T7 rRNA, when expressed from the rDNA repeat in both [*rho*⁺] and [*rho*⁰] cells, follows the fractionation of the 25S rRNA. However, when the minigene is expressed from pSEYrT7 λ , there is a striking shift in the distribution of transcripts between [*rho*⁺] and [*rho*⁰] cells, in which a portion of the T7 rRNA in [*rho*⁰] cells now fractionates as poly(A)⁺ RNA. It is highly likely that competition between cleavage and polyadenylation occurs in these RNAs. Perhaps the chromosomal versus episomal location of transcription influences this competition. However, the presence of substantial poly(A)⁺ T7 rRNA expressed from pSEYrT7 λ suggests that the episomal location of rDNA sequences is important for this pattern of Pol II expression in [*rho*⁰] cells.

Another important criterion for Pol II transcription is its unique sensitivity to low concentrations of α -amanitin. We have compared the α -amanitin sensitivities of run-on transcription of endogenous rDNA sequences and T7 rRNA expressed from pSEYrT7 λ in [*rho*⁺] and [*rho*⁰] cells, using the permeabilized yeast cell system described by Elion and Warner (10). In addition to a probe for endogenous and reporter (λ) NTS transcripts, we included probes for actin (31), TCM (12), and Ty1 elements (5), all of which are transcribed by Pol II. The results of this analysis (Fig. 2B) show that 200 μ g of α -amanitin per ml inhibits almost completely run-on transcription from the Pol II genes in both [*rho*⁺] and [*rho*⁰] cells. In [*rho*⁺] cells,

FIG. 3. (A) RNA Pol II promoters in rDNA sequences. A series of promoter fusion constructs was made, fusing NTS sequences to a *CIT2*- β -galactosidase fusion gene such that all *CIT2* regulatory sequences are removed. The enhancer sequences (E), the core Pol I promoter (hatched box), and the Pol I initiation site (arrow) are also shown. The promoter fusion constructs (*rlacZ* [a], *rlacZ-2226* [b], *rlacZ-1208* [c], *rlacZ-206* [d], and *rlacZΔ206* [e]) on 2μ m plasmids were transformed into 161U7 [ρ^+] and [ρ^0] cells. The β -galactosidase activity for each construct was corrected for plasmid copy number, and the standard deviation is shown. (B) Diagram of the Pol II promoter region of rDNA. The Pol I core promoter is shown in the hatched box. The Pol II promoter region lies within the stippled box as defined by the β -galactosidase fusion constructs. The three consensus ARS elements (■) and a putative TATA sequence (●) are shown. Putative polyadenylation sites (triangles) are located near the major 3' ends of the 35S rRNA (indicated as T_0 , T_1 , and T_2). R, *EcoRI*; H, *HindIII*; A, *AvaII*; S, *SmaI*; B, *BamHI*.

α -amanitin has no effect on either rRNA or T7 rRNA run-on transcripts as would be expected for Pol I transcription. However, we have consistently observed in these experiments that roughly 50% of the endogenous rRNA run-on transcription in [ρ^0] cells is sensitive to α -amanitin. Moreover, in [ρ^0] cells, synthesis of a large fraction of T7 rRNA is sensitive to inhibition by α -amanitin to about the same extent as the control Pol II genes, suggesting that the majority of T7 rRNA derived from pSEYrT7 λ in [ρ^0] cells is the product of Pol II transcription.

rDNA sequences can direct the synthesis of high levels of β -galactosidase activity in gene fusions. The data presented thus far suggest that rDNA contains a cryptic Pol II promoter which is activated in petites. To localize the putative Pol II promoter element(s), a 2.4-kb segment of yeast rDNA including the enhancer, NTS1, NTS2, and the Pol I transcriptional start site for the 35S rRNA precursor was fused to a portion of the *Escherichia coli lacZ* gene containing the first 24 amino acids of the yeast *CIT2* gene (38). The fusion places the NTS2 sequences 15 bp upstream of the *CIT2* ATG codon such that all *CIT2* upstream regulatory sequences including the TATA box have been removed. This construct, *rlacZ* (Fig. 3A, construct a), on a 2μ m vector (YEprlacZ) was transformed into 161U7 [ρ^+] and [ρ^0] cells, and β -galactosidase activity was measured in whole cell extracts. As shown in Fig. 3A, not only does the entire NTS region including the enhancer element give rise to robust β -galactosidase activity in extracts of the transformants, but that activity is about sixfold higher in the [ρ^0] petite cells than in [ρ^+] cells.

To further define the Pol II promoter regions in rDNA, we analyzed a series of deletion constructs of YEprlacZ (Fig. 3A, constructs b to e). Analysis of β -galactosidase activity in [ρ^+] and [ρ^0] cells transformed with these constructs show that the region of rDNA encompassing the Pol I promoter, together with NTS2, is required to support β -galactosidase synthesis. The data also show that the Pol I enhancer element, which has been reported to augment Pol I transcription of rDNA reporter gene constructs by ~ 15 -fold (9), has no effect on β -galactosidase expression (Fig. 3A, constructs b and c). In addition, the Pol I promoter region, as defined by the minimal sequences necessary for Pol I transcription (29), is necessary but not sufficient for β -galactosidase expression (Fig. 3A, constructs d and e). This result confirms the observation that a Pol I promoter alone cannot produce translatable mRNA (26, 44). Thus, a Pol II promoter functioning in the same direction as the Pol I promoter can be defined within rDNA extending from the *AvaII* site in NTS2 to the *EcoRI* site downstream of the Pol I transcriptional start site (Fig. 3B).

Preferential activation of Pol II transcription from episomal rDNA. Earlier experiments suggested that the location of the rDNA influenced the expression of the Pol II rDNA promoter. We have used construct *rlacZ* (Fig. 3A) to test whether the Pol II rDNA promoter is inactive in the chromosomal repeat and primarily active in episomal copies of rDNA. The experimental prediction is that β -galactosidase expression from the *rlacZ* construct would be low when the construct present in the rDNA repeat and high when it was present episomally as a

result of its excision from the rDNA repeat. Accordingly, we first constructed an isogenic pair of 161U7 [*rho*⁺] and [*rho*⁰] cells containing *lacZ* (Fig. 3A) randomly integrated into the chromosomal rDNA repeat, using the integrating plasmid YIprlacZ. All of the initial [*rho*⁺] and [*rho*⁰] transformants containing the YIprlacZ integration were white on X-Gal plates (data not shown), suggesting that the Pol II rDNA promoter is not active in the tandem repeat regardless of the random site of integration. To confirm the activation of this Pol II promoter upon excision from the repeat, we took advantage of an observation that petite cells can have as much as ~30-fold more episomal rDNA than [*rho*⁺] cells (data not shown). We reasoned that conversion of the [*rho*⁺] cells to petites would result in an increase in the amount of episomal rDNA, including the *lacZ* reporter sequences, and thus lead to an increased expression of β -galactosidase activity. Therefore, [*rho*⁺] cells containing the *lacZ* reporter integrated into the rDNA repeat were grown for ~30 generations on YNB-Craff (see the legend to Fig. 1) in the presence of 10 μ g of ethidium bromide per ml in order to induce petites; as controls, isogenic [*rho*⁺] and [*rho*⁰] *lacZ* integrants were also grown in parallel on the same medium without ethidium bromide. Samples of the [*rho*⁺] and [*rho*⁰] transformants and the ethidium bromide-induced petites were screened on X-Gal plates. Blue colonies and sectors were observed only with the ethidium bromide-induced petites (data not shown). Four blue subclones from the ethidium bromide-induced petites and four white isolates from the primary [*rho*⁺] and [*rho*⁰] transformants were analyzed for the abundance of the excised plasmid by Southern blot analysis of an *Nco*I digest of total genomic DNA (Fig. 4) and for β -galactosidase activity in crude extracts.

In both the [*rho*⁺] and [*rho*⁰] primary transformants, episomal copies of the fusion gene, which are evident as a 10.7-kb *Nco*I fragment, are barely detectable, and the β -galactosidase activity in these cells, normalized to the YIprlacZ copy number, is low (2.3 and 7.0 U per plasmid copy, respectively) (Fig. 4B). However, in the ethidium bromide-induced petites, the β -galactosidase activity is more than 11-fold higher (27.6 U per plasmid copy) than in the [*rho*⁺] cells, and there is a comparable increase in the amount of episomal reporter sequences. These results show that the rDNA Pol II promoter is active primarily on episomal copies.

Yeast cells can survive in the absence of Pol I. Nogi et al. (32) showed previously that Pol II transcription of rRNA can, in principle, provide functional rRNA. In those experiments, cells of strain NOY4081A that lack Pol I activity because of a disruption of the *RPA135* gene, which encodes subunit 2 of the enzyme, are viable when they harbor a plasmid (pNOY102; Fig. 5A) in which the 35S rRNA transcription unit is under the control of the inducible *GAL7* promoter. These cells can grow only on galactose medium, showing that a strong Pol II promoter can drive the synthesis of functional rRNAs and that Pol I activity per se is not required for cell viability. To determine whether the cryptic Pol II promoter that we have found in yeast rDNA can produce functional rRNA, we introduced plasmid pHCW200 (Fig. 5A), a 2- μ m-based plasmid containing NTS2 and the entire 35S precursor rRNA transcription unit into cells of strain NOY4081A in exchange for plasmid pNOY102. A *Leu2*⁻ [*rho*⁰] derivative of strain NOY4081A, designated HCW1X [*rho*⁰], was transformed with pHCW200. Transformants were grown on YNBraff medium containing uracil to select for cells containing pHCW200 and subsequently placed on YNBraff medium containing 5-fluoro-orotic acid to select for cells that had lost pNOY102. Figure 5B shows that HCW1X [*rho*⁰] cells containing just plasmid pNOY102 grow on medium containing galactose but not on medium contain-

FIG. 4. In petites, the rDNA Pol II promoters are activated upon excision from the rDNA repeat. (A) The rDNA-*lacZ* fusion gene, YIprlacZ, integrated into the rDNA repeat is shown. Homologous recombination between flanking rDNA sequences will result in the excision of the plasmid sequences, and the monomer excision product is shown below. (B) Southern blot analysis of white and blue colonies. Upon Southern blot analysis of *Nco*I-digested DNA with pGEM3Zf(+) as a probe, excised copies of the plasmid are present as a 10.7-kb band, while the integrated plasmid yields a 7.1-kb band. The probe also hybridizes with an adjacent 9.3-kb *Nco*I fragment that contains the integrated *lacZ* gene and flanking rDNA sequences. Regions that hybridize to the probe are represented in the construct as a heavy black line and Amp (ampicillin gene). Aliquots of the cultures were also analyzed for β -galactosidase activity. The average β -galactosidase (β -gal) activity (nanomoles per minute per milligram of protein) in all four subclones normalized for copy number of all plasmid sequences is shown for each cell type. The ratio of the 10.7-kb band to the 7.1-kb band, indicating the fraction of that construct that is episomal, is also shown. EB, ethidium bromide-induced.

ing glucose. However, all of the Ura⁻ isolates of HCW1X [*rho*⁰] cells transformed with pHCW200 grew well on both carbon sources, although more slowly than cells containing pNOY102 on YPgal medium.

To demonstrate that the Ura⁻ isolates still contain the disrupted copy of *RPA135* and lacked plasmid pNOY102, two independent Ura⁻ colonies, designated HCW1X-0 [*rho*⁰] and HCW1X-P [*rho*⁰], were chosen for further analysis. An *EcoRV*-*Xho*I digest of genomic DNA was hybridized with a probe for *RPA135* sequences. Both of the Ura⁻ isolates still contain a 4.5-kb band representing the disrupted allele (Fig. 5C, lanes 2 to 5), while the wild-type control (lane 1) shows the expected 1.6-kb band. To confirm the loss of pNOY102 in the

FIG. 5. (A) Wild-type (WT) and disrupted alleles of *RPA135* and plasmids pNOY102 and pHCW200. Relevant restriction sites: *Xho*I (X), *Hind*III (H), *Pst*I (P), *Eco*RV (RV), *Nru*I (N), *Sph*I (S), and *Sma*I (S). pHCW200 contains rDNA sequences from the *Sph*I site in NTS2 through the *Hind*III site in the enhancer element. pNOY102 contains rDNA sequences from the first nucleotide of the 35S rRNA through the same *Hind*III site in the enhancer (32). (B) Yeast cells can survive without Pol I. Serial dilutions of wild-type W3031A [*rho*⁰] cells and HCW1X [*rho*⁰] cells or two Ura⁻ isolates (HCW1X-O [*rho*⁰] and HCW1X-P [*rho*⁰]) were plated on YPD or YPgals medium. The plasmids harbored by the *rpa135::leu2::HIS* disruption strain are shown on the left. (C to E) Southern blot analysis of Ura⁻ isolates from the plasmid shuffle. Genomic DNA was digested with *Xho*I and *Eco*RV (C) or *Hind*III (D and E) and subjected to Southern blot analysis using the a 456-bp PCR fragment from the 5' end of *RPA135*. The *GAL7* probe was a 262-bp PCR fragment from the *GAL7* promoter. The pGEM3Zf(+) probe was the entire plasmid. Lane 1, wild-type strain W3031A [*rho*⁰]; lane 2, strain HCW1X [*rho*⁰]; lane 3, strain HCW1X [*rho*⁰] transformed with pHCW200; lanes 4 and 5, two Ura⁻ isolates of strain HCW1X [*rho*⁰], HCW1X-O [*rho*⁰] and HCW1X-P [*rho*⁰], respectively.

Ura⁻ isolates, a 262-bp fragment of the *GAL7* promoter was hybridized to a *Hind*III digest of genomic DNA. Lanes 4 and 5 in Fig. 5D show that the 1.1-kb band containing *GAL7* sequences derived from pNOY102 is absent in the Ura⁻ isolates. All strains show a 2.4-kb band corresponding to genomic *GAL7* sequences. To ensure the integrity of pHCW200 and to verify the complete loss of pNOY102 sequences, the previous blot was stripped and reprobed with pGEM3Zf(+) DNA. These data (Fig. 5E) show that plasmid pNOY102 is present only in the control strains (lanes 2 and 3) as a 5.3-kb fragment, while the Ura⁻ isolates (lanes 4 and 5) contain intact pHCW200 sequences present as an 8.1-kb fragment. Thus, in

cells containing just pHCW200, all of the rRNA must be provided by Pol II transcription of rDNA.

The cryptic Pol II promoter in rDNA utilizes the same initiation site for 35S rRNA synthesis as the Pol I promoter. To determine the initiation site for the rDNA Pol II promoter, we mapped the 5' ends of the 35S rRNA precursor RNAs by primer extension in HCW1X-O [*rho*⁰] cells lacking Pol I activity. Total RNA from wild-type and *RPA135*-disrupted cells containing only plasmid pHCW200 was hybridized to a 5'-end ³²P-labeled antisense 28-mer corresponding to a region of the 35S precursor rRNA located 105 nucleotides downstream of the wild-type (Pol I) initiation site, and the primer was ex-

FIG. 6. Primer extension of precursor rRNA from cells lacking Pol I. The 5' end of the 35S rRNA precursor was mapped by primer extension using RNA isolated from wild-type (WT) W3031A [*rho*⁰] and mutant HCW1X-O [*rho*⁰] (Δ I) cells and a 5'-end ³²P-labeled oligomer (5'-CTC TCC ACC GTT TGA CGA GGC CAT TTA C-3'). The same oligomer was used to sequence the corresponding region of pHCW200. The sequencing reactions and primer extensions were analyzed on an 8.0% acrylamide-6 M urea sequencing gel.

tended with avian myeloblastosis virus reverse transcriptase. The same oligonucleotide was used to generate a sequencing ladder from plasmid pHCW200. Figure 6 shows that the primer extension products are the same for the Pol I- and the Pol II-derived 35S precursor rRNA, which corresponds to the previously mapped location for the 5' end of the 35S rRNA precursor (1). This finding is highly suggestive that this Pol II rDNA promoter is physiologically significant, since it is unlikely that a fortuitous Pol II promoter would use the identical initiation site for synthesis of the rRNA precursor as Pol I.

DISCUSSION

Our results provide evidence for a transcriptional switch from Pol I to Pol II in the synthesis of rRNA in certain petite strains of *S. cerevisiae*. This switch appears to involve the selective activation of cryptic Pol II promoters from episomal rDNA elements rather than from an rDNA repeat on chromosome XII. The following lines of evidence support the conclusion of such a Pol I-to-Pol II switch. First, a large fraction of the reporter rRNA transcripts in petites derived from a multicopy rRNA minigene are polyadenylated, and their synthesis is sensitive to inhibition by concentrations of α -amanitin that inhibit Pol II but not Pol I transcription. Second, fusions of rDNA sequences to the *E. coli lacZ* gene have localized the Pol II rDNA promoter within a region of NTS II overlapping the Pol I promoter. The Pol II rDNA promoter is inactive in the chromosomal repeat and activated upon excision from the repeat. Finally, cells which lack RNA polymerase I can survive by using the endogenous Pol II rDNA promoter to direct synthesis of the 35S rRNA precursor.

Pol II synthesis of an equivalent 35S rRNA precursor. These studies have identified a functional Pol II promoter in NTS2 that overlaps the core Pol I promoter. In NTS2-*lacZ* fusion genes, the Pol I promoter sequences is necessary but not sufficient for β -galactosidase activity. This region contains a variation of a TATA box element, 5'-AATAAAA-3', which in other contexts is functional for Pol II transcription. In addition, primer extension studies of the Pol II-derived 35S rRNA demonstrate that both Pol I and Pol II initiate transcription at the same nucleotide 79 nucleotides downstream from this putative TATA element. It is notable that construct e (Fig. 3A), which has no detectable β -galactosidase activity, destroys a high-affinity binding site for REB1p, a protein known to bind to

FIG. 7. Model for a Pol I-to-Pol II switch in rRNA synthesis. Pol I transcription of the 35S precursor rRNA (template is indicated by the large black box) occurs from the ribosomal repeat on chromosome XII. The 5S rRNA gene is shown by the small black box. Excision events lead to episomal copies of rDNA. In petites, the Pol II cryptic promoters on these episomal copies are activated, leading to Pol II synthesis of 35S precursor rRNA.

both Pol I and Pol II promoters (17, 28). In addition to the Pol II promoter elements, there are typical signals for Pol II polyadenylation and termination at the 3' end of the rRNA gene cluster. For example, within the Pol I enhancer and overlapping the 3' end of the 25S rRNA are T-rich repeats, TTTTAT (Fig. 3B), which, on the basis of studies with well-defined Pol II genes in *S. cerevisiae* (14, 39), should work as efficient polyadenylation signals. It is noteworthy that for Pol I transcription of the 35S precursor rRNA, there are multiple processing and termination sites downstream of the 25S rRNA gene, including one or more within the enhancer element (16, 49). Thus, not only are the Pol II regulatory signals intermingled with the Pol I signals, but the Pol II signals function to produce an equivalent 35S rRNA in the absence of RNA Pol I.

A polymerase switch model. We envisage at least two processes that could be involved in the polymerase switch from Pol I to Pol II (Fig. 7). First, in response to petite formation, there is a shift in the equilibrium between episomal and chromosomal copies of rDNA, yielding an increase in the steady-state amount of episomal rDNA. This could occur by an increase in recombination within the rDNA repeat or, alternatively, by some selection in petites for cells containing higher amounts of episomal rDNA. In either case, the presence of an ARS element on rDNA would enable the episomal copies to replicate and probably to be amplified as well. It is known that the amount of episomal rDNA, which would be a function of both excision and reintegration events, is influenced by the activity of topoisomerases I and II (4, 21). Further, topoisomerase activity is required for transcription of rRNA genes in the tandem repeat but not for extrachromosomal rDNA (2, 3). rDNA contains a hot spot of recombination, *HOT1*, that includes the enhancer and Pol I promoter regions (20, 51, 52); thus, there is the possibility that some form of dedicated control of rDNA recombination exists which may be related to the Pol I-to-Pol II switch. Regulation of any one of these potential control points could influence the distribution of chromosomal versus episomal rDNA, which in turn could affect the template availability for Pol II transcription of rDNA.

The second process in the polymerase switch would be the selective activation in petites of the episomal rDNA Pol II promoters, similar to what has been observed for petite-specific increases in transcription of a number of nuclear genes (23). However, the specific signals involved in the response of nuclear genes to the mitochondrial state are not known. It is also unclear why Pol II activation does not occur from rDNA in the tandem repeat. Pol II is not completely excluded from rDNA sequences in the nucleolus, since Pol II genes inserted into the rDNA repeat can be functionally expressed. Perhaps specific factors required for Pol I transcription (for a review, see reference 48), which would be locally concentrated in the nucleolus, are inhibitory to Pol II transcription of rDNA in the tandem repeat. In this connection, Dhar et al. (7, 8) found that human rDNA sequences amplified in Chinese hamster cells are transcribed only by Pol II. Those authors suggested that the absence of human-specific Pol I transcription factors in those cells favors a default Pol II transcription of rDNA. It is intriguing that cryptic Pol II promoters have been identified in the Pol I promoter vicinity in other systems (26, 44). Similarly, it has been argued that the polymerase switching observed between Pol II and Pol III in the transcription of some snRNA genes may depend on the binding of transcription factors, such as TFIID, which could dictate the particular RNA polymerase complex that engages the template (24, 25, 48).

It has recently been established that Pol I activity is dispensable in *S. cerevisiae* if rRNA synthesis is put entirely under the control of a Pol II promoter, such as *GAL7* (32). Our findings show that polymerase switching of rRNA synthesis occurs through the activation of a cryptic rDNA promoter and is a physiological response to at least one kind of cellular perturbation, the loss of a functional mitochondrial genome. One plausible view, therefore, is that Pol II transcription of rDNA provides a backup, which under certain conditions could contribute to the synthesis of total rRNA required to maintain cell viability. Such a backup would likely not be subject to the same controls as Pol I transcription of rDNA and could have evolved if distinct RNA polymerases were derived from a common ancestor as is suggested by the existence of common subunits among all three eukaryotic RNA polymerases (48, 56).

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