RNA polymerase I–specific subunits promote polymerase clustering to enhance the rRNA gene transcription cycle

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RNA polymerase I (Pol I) produces large ribosomal RNAs (rRNAs). In this study, we show that the Rpa49 and Rpa34 Pol I subunits, which do not have counterparts in Pol II and Pol III complexes, are functionally conserved using heterospecific complementation of the human and Schizosaccharomyces pombe orthologues in Saccharomyces cerevisiae. Deletion of RPA49 leads to the disappearance of nucleolar structure, but nucleolar assembly can be restored by decreasing ribosomal gene copy number from 190 to 25. Statistical analysis of Miller spreads in the absence of Rpa49 demonstrates a fourfold decrease in Pol I loading rate per gene and decreased contact between adjacent Pol I complexes. Therefore, the Rpa34 and Rpa49 Pol I–specific subunits are essential for nucleolar assembly and for the high polymerase loading rate associated with frequent contact between adjacent enzymes. Together our data suggest that localized rRNA production results in spatially constrained rRNA production, which is instrumental for nucleolar assembly.

Introduction

In all living organisms, multisubunit DNA-dependent RNA polymerases are built around a common set of five core subunits (α2β′βω in eubacteria; Minakhin et al., 2001) In archael and eukaryotic enzymes, five additional subunits (related through common ancestry) are required, leading to a 10-subunit extended core (Cramer et al., 2008; Kwapisz et al., 2008). In eukaryotes, three nuclear RNA polymerases are found, each transcribing a dedicated set of genes. RNA polymerase II (Pol II) transcribes all protein-coding genes and most of the small nuclear RNA genes. RNA polymerase III (Pol III) is transcribing 5S ribosomal RNA (rRNA) genes, tRNA genes, and some noncoding RNA genes. RNA polymerase I (Pol I) produces a single transcript, the large polycistronic precursor (35S pre-rRNA in yeast) processed in multiple successive steps into the mature rRNAs (in yeast, 25S, 18S, and 5.8S rRNAs). Pol II is composed of 12 subunits, including a 10-subunit extended core and a peripheral heterodimer of subunits Rpb4/Rpb7. Rpb4 and Rpb7 have counterparts in Pol I and Pol III, Rpa43/Rpa14 and Rpa17/Rpc25, respectively. Pol I and Pol III have counterparts to each of the 12 Pol II subunits and, in addition, two and five specific subunits, respectively, which may be responsible for some of the specific functions of these two enzymes. In this study, we focus on Pol I–specific subunits Rpa34 and Rpa49, which have structural homology with the Rap74/Rap30 subunits of the Pol II transcription factor TFIIF (Kuhn et al., 2007). Pol I is the most active and abundant RNA polymerase in eukaryotes. Its enormous transcriptional output can best be visualized using the DNA spread method previously developed by Miller et al. (1969), where the 35S rRNA genes (rDNA) adopt a “Christmas tree” conformation. In Saccharomyces cerevisiae,
Figure 1. **PAF53/CAST heterodimer is functionally conserved from human to budding yeast.** (A) Pol I–specific subunits in *S. cerevisiae*, *S. pombe*, *Mus musculus*, and *Homo sapiens*. (B) Nucleolar localization of CAST depends on PAF53. CAST fused with YFP (YFP-CAST) is expressed in a WT yeast producing Nop1 fused to mCherry (mCherry-Nop1) with (+) or without (−) PAF53 coexpression (see Materials and methods). (right) The overlay of both fluorescent signals (merge) is shown. (C) Immunoprecipitation of budding yeast Pol I demonstrates that association of CAST to Pol I requires human PAF53. HA-tagged CAST [HA-CAST] is detected in the supernatant (SUP) with or without coexpression of PAF53. Using IgG Sepharose to immunopurify TAP-tagged budding yeast Pol I (Rpa190-TAP), CAST copurifies with Pol I only when coexpressed with PAF53 (+). Western blot using Pap and 12CA5 revealed tagged budding yeast Pol I (Rpa190-TAP) and CAST (HA-CAST) in supernatant (SUP) and immunoprecipitated fraction (IP), respectively. (D) Nucleolar localization of PAF53 depends on CAST. PAF53 fused with YFP (YFP-PAF53) is expressed in a WT budding yeast producing Nop1 fused to mCherry (mCherry-Nop1) with (+) or without CAST (−). CAST expression is required for both a detectable YFP signal and a nucleolar localization of PAF53. (E) PAF53 expressed...
the rDNA is made of a 6.9-kb-long gene repeated ~150 times, of which about half is transcribed. Transcription of the rDNA is one to two orders of magnitude greater than the transcription of the rest of the genome. In exponentially growing cells, 0.01–10 Pol II enzymes are found per open reading frame (Bon et al., 2006), whereas >120 Pol I molecules can be detected (by Miller spread; Miller et al., 1969) at the transcribed region of each active rDNA repeat (Osheim et al., 2009).

Pol I activity is associated with the largest nucleolar body, the nucleolus, where all earlier steps of ribosome biogenesis take place. Assembly of the nucleolus is proposed to be a self-organized process, initiated by production of rRNA (Trumtel et al., 2000; Misteli, 2001; Hernandez-Verdun et al., 2002). In budding yeast, Pol I is essential, but very elegant genetic analyses have been used to show that Pol II–dependent 35S rRNA expression, albeit very inefficiently, can rescue Pol I minus mutants (Nogi et al., 1991). Importantly, in mutant cells where Pol I is inactivated and Pol II is artificially producing rRNA, nucleolar structures are still detectable, but with massive alterations (Oakes et al., 1998; Trumtel et al., 2000). Therefore, rRNA synthesis achieved from its dedicated transcriptional apparatus is important for formation of the nucleolar structure.

In this study, we focus on the role of two Pol I–specific subunits, Rpa49 and Rpa34, in Pol I transcription and nucleolar morphology. Pol I lacking Rpa34/Rpa49 has been shown to be active in transcription but impaired in elongation (Huet et al., 1975). In vivo, rpa49 and rpa34 deletion mutants are viable, but are defective in 35S rRNA production (Liljelund et al., 1992; Gadal et al., 1997). Structural insight and biochemical assays have suggested that Rpa34/Rpa49 form a heterodimer behaving like built-in elongation factors (Kuhn et al., 2007). Another clue to Rpa49 function came from the observation that in rpa49 deletion mutants, Rrn3 (TIF-IA in mouse), an essential Pol I transcription factor, is recruited to the promoter less efficiently and fails to dissociate from elongating polymerase (Beckouet et al., 2008). In this study, we demonstrate that the Rpa34/Rpa49 heterodimer is functionally conserved in evolution and required for nucleolar assembly. Using Miller spreads to analyze Pol I distribution in the absence of Rpa49 on single transcribed genes, we show that there is a decreased clustering of elongating Pol I complexes. 3D modeling of two neighboring Pol I complexes unveil how such association can regulate rRNA gene transcription cycle.

Results

The human hPAF53/CAST heterodimer is functionally conserved from human to budding yeast

We investigated the functional conservation of specific Pol I subunits from budding yeast to human (Fig. 1 A). In budding yeast, Rpa49 and Rpa34 are not essential for viability, and their amino acid sequences are poorly conserved through evolution. Orthologues of Rpa49 and Rpa34, PAF53 and PAF49 in mouse (Hanada et al., 1996; Yamamoto et al., 2004) and hPAF53 and CAST in human (also called ASE-1 or hPAF49; Panov et al., 2006), respectively, have a conserved domain organization with S. cerevisiae subunits but low sequence similarity. In budding yeast and mouse, Rpa49/Rpa34 orthologues are easily dissociated from the enzyme by high salt treatment (Huet et al., 1975; Hanada et al., 1996), and as a result, these two polypeptides have been classified either as Pol I–associated factors in mouse or as Pol I subunits in S. cerevisiae and human. A previous complementation attempt has established that Schizosaccharomyces pombe Pol I subunit Rpa51, hereafter called Sp-Rpa49, but not mouse PAF53, can complement rpa49Δ cold sensitivity (Nakagawa et al., 2003). Because Rpa49 and Rpa34 function as a heterodimer (Kuhn et al., 2007; Beckouet et al., 2008), we tested the coexpression of human CAST and hPAF53 in a complementation assay. We first investigated the localization of human CAST when expressed in wild-type (WT) budding yeast, with or without hPAF53 expression. CAST is nuclear when expressed alone but predominantly localized in the nucleolus when coexpressed with hPAF53 (Fig. 1 B). Starting from a yeast strain bearing a TAP-tagged Rpa190 (Rigaut et al., 1999), Pol I largest subunit, we purified Pol I to test the association of CAST with budding yeast Pol I. CAST is coimmunoprecipitated with budding yeast Pol I only when coexpressed with hPAF53 (Fig. 1 C). These results suggest that hPAF53/CAST heterodimer is replacing the Rpa49/Rpa34 heterodimer in budding yeast Pol I. We then investigated hPAF53 localization with or without CAST coexpression. No YFP-hPAF53 fusion protein was detected when it was expressed alone, but it was present in the nucleolus when coexpressed with CAST (Fig. 1 D). To test the idea that CAST stabilizes hPAF53 when coexpressed in budding yeast, we quantified hPAF53 by immunodetection in whole cell extracts with or without CAST (Fig. 1 E). hPAF53 is barely detectable in budding yeast extracts, even if expressed from the strong PGK1 promoter, but it accumulates in budding yeast is stabilized by CAST coexpression. PAF53 is detected in a whole cell extract from WT budding yeast expressing PAF53 (PAF53) alone or coexpressed with CAST (PAF53 CAST) using PAF53 antibody (α-PAF53). Nop1 (α-Nop1) is used as loading control. Total extract from Hela loaded in lane 1 is used as control for PAF53 detection. White lines indicate that intervening lanes have been spliced out. (F) Overexpressed PAF53 complements rpa49Δ cold-sensitive growth defect. 10-fold serial dilutions of RPA49-deleted strain expressing budding yeast RPA49 (Rpa49), empty vector (−), PAF53 expressed from a strong promoter (pRS426-PAF53), or an even stronger budding yeast promoter promoter (pRS415-PAF53) were spotted on 5-FOA-containing plates to check for complementation of the mutant cold sensitivity at 25°C (see Materials and methods for plasmid-shuffling assay). Plates without FOA (−) are used as control to confirm that the same number of cells were spotted. The strength of the complementation is evaluated by comparing plates with (+) or without (−) caffeine spotted with equivalent amount of cells.
Figure 2. \textit{Rpa49/Rpa34 heterodimer is conserved in fission yeast.} (A) In \textit{S. cerevisiae}, nucleolar localization of Sp-Rpa34 depends on Sp-Rpa49. Sp-Rpa34 fused with YFP (YFP-Sp-Rpa34) is expressed in a WT budding yeast with (Sp-Rpa49) or without (–) Sp-Rpa49 coexpression. In all cases, cells produce Nop1 fused to mCherry (mCherry-Nop1) and Nup49 fused with CFP (CFP-Nup49) to label the nucleolus and the nuclear pore complexes, respectively. (Right) Overlay of the three fluorescent signals (merge) is shown. (B) Sp-Rpa34/Sp-Rpa49 heterodimer complements budding yeast Rpa34 essential function in a Pol I mutant background \textit{(rpa135-D395N)}. 10-fold serial dilutions of \textit{rpa34Δ rpa135-D395N} double-deletion strains containing \textit{S. cerevisiae RPA34 (Rpa34)}, empty vector (–), Sp-Rpa34 (Sp-Rpa34), Sp-Rpa49 (Sp-Rpa49), or coexpressing Sp-Rpa34 and Sp-Rpa49 (Sp-Rpa34/Sp-Rpa49) were spotted on plates with (FOA) or without FOA (–), and complementation was evaluated by comparing plates with (FOA) or without FOA (–). (C) \textit{Sp-rpa34Δ} deletion barely affects growth in \textit{S. pombe}. 10-fold serial dilutions of \textit{S. pombe WT} and \textit{Sp-rpa34Δ} deletion strains in rich medium at 30°C after 48 (left) and 120 h (right). (D) \textit{Sp-rpa34Δ Sp-rpa49Δ} double deletion is viable in \textit{S. pombe}. To generate the double-mutant strain, we crossed two haploid strains bearing
when coexpressed with CAST. Interestingly, CAST depletion in mouse and human cells leads to concomitant disappearance of PAF53, whereas the level of other Pol I subunits is unaffected (unpublished data). Therefore, our results suggest that coexpression of CAST and hPAF53 is required for hPAF53 stabilization.

We next tested the ability of the human subunits to complement the rpa49Δ and/or rpa34Δ phenotypes by plasmid-shuffling assays (see Materials and methods). hPAF53 on its own is unstable; therefore, we expressed hPAF53 under the control of the strong PGK1 or the very strong TEF1 promoter. rpa49Δ cold sensitivity was corrected by hPAF53 only when the pTEF1 promoter was used (Fig. 1 F). rpa4Δ has little growth defect, but it is hypersensitive to caffeine (1,3,7-trimethylxanthine; Beckouet et al., 2008). We assayed the ability of CAST, hPAF53, or both to suppress caffeine sensitivity. CAST and hPAF53 complemented the rpa34Δ mutant phenotype only when coexpressed (Fig. 1 G). Therefore, our results suggest that the CAST/hPAF53 heterodimer can substitute for the endogenous budding yeast Rpa49/Rpa34 within Pol I and can perform the function of the S. cerevisiae heterodimer in vivo.

The Rpa49/Rpa34 heterodimer is also conserved in fission yeast
Fission yeast Sp-Rpa49 can substitute for budding yeast Rpa49 (Nakagawa et al., 2003). A recent database search revealed that the S. pombe SPBC11G11.05 open reading frame (hereafter named Sp-Rpa34) has weak but significant sequence homology with Rpa34 (Beckouet et al., 2008). We cloned and expressed the fission yeast counterpart of budding yeast Rpa34. We next demonstrated that double-deletion mutants Sp-rpa34 and Sp-rpa49) were spotted on rich media (left; –), 10-fold serial dilutions of Sp-rpa34Δ Sp-rpa49Δ stb5 (middle; +6-AZA), or 40 µg/ml mycophenolic acid (right; +MPA) and grown for 4 d at 25°C.

The C-terminal domains of budding yeast Rpa34 contain a nucleolar localization signal
We showed that Rpa49 overexpression could suppress all known phenotypes of rpa34Δ, suggesting that Rpa34 function is restricted to stabilization of Rpa49 (Beckouet et al., 2008), but in higher eukaryotes, the orthologue of Rpa34 has several specific functions of its own (Yamazaki et al., 1999; Yamamoto et al., 2004; Panov et al., 2006). Our sequence analysis reveals that these polypeptides share a lysine-rich C-terminal domain. This domain is not required for interaction with Rpa49 (Beckouet et al., 2008), and we hypothesized that this feature could be responsible for a function unrelated to Rpa49. To verify this hypothesis, we decided to perform a search for all possible genetic backgrounds in which rpa34Δ is lethal. We performed a global genetic interaction mapping (GIM; Decourty et al., 2008) using rpa34Δ as bait. We confirmed two previously identified synthetic lethal interactions, top1Δ and rpa14Δ (Gadal et al., 1997), and identified two novel genetic backgrounds, gcr2Δ and stb5Δ, in which rpa34Δ became essential (Fig. 3 A). However, Rpa49 overexpression could suppress the rpa34Δ phenotype in all tested backgrounds. Moreover, the truncated version of Rpa34 lacking the C-terminal domain was functional, revealing no growth phenotype associated with the absence of this particular domain.

We next tested whether the nucleolar localization of Rpa34 also depends on Rpa49 in budding yeast. In contrast to CAST and Sp-Rpa34, Rpa34 remains in the nucleolus even in the absence of Rpa49 (Fig. 3 B). We next tried to identify which domain of budding yeast Rpa34 could behave as a nucleolar localization signal functioning independently from Rpa49. The nucleolar localization of Rpa34 in Rpa49Δ background is lost upon truncation of the C-terminal domain (Fig. 3 C). The nucleolar targeting of Rpa34 C domain in S. cerevisiae appears to be species specific. Despite the apparent similarity between the C-terminal domains of Rpa34, CAST, and Sp-Rpa49, the nucleolar targeting of CAST and Sp-Rpa49 are strictly dependent on the coexpression of their respective binding partner, PAF53 or Sp-Rpa49. In contrast, Rpa34 in S. cerevisiae has two nucleolar targeting mechanisms. One depends on Rpa49 association, the other on the Rpa34 C-terminal domain.

The single Sp-rpa34 or Sp-rpa49 deletion. After meiosis, we analyzed the growth of single- and double-mutant offspring. Viability of the double mutant was confirmed in 20 tetrads. A representative tetratype tetrade is shown, with WT, single Sp-rpa34, or Sp-rpa49 deletion and double Sp-rpa34Δ Sp-rpa49Δ (ΔΔ) genotype. (E) The sensitivity of the Sp-rpa34Δ deletion strain to mycophenolic acid and 6-azauracil is suppressed by Sp-rpa49Δ overexpression. 10-fold serial dilutions of Sp-rpa34Δ Sp-rpa49Δ strain on rich media [left; –], 75 µg/ml 6-azauracil (middle; +6-AZA), or 40 µg/ml mycophenolic acid (right; +MPA) and grown for 4 d at 25°C.
Figure 3.  The C-terminal domain of Rpa34 is a nucleolar localization signal.  (A) Rpa34 C-terminal domain is fully dispensable in vivo. 10-fold serial dilutions of four double-mutant budding yeast strains, rpa34Δ top1Δ (top left), rpa34Δ stb5Δ (top right), rpa34Δ rpa14Δ (bottom left), or rpa34Δ gcr2Δ (bottom right), are shown. Double mutants containing RPA34 [Rpa34], empty vector (−), Rpa34-ΔC driven from a Ppgk1 promoter [Rpa34-ΔC], or overexpressing Rpa49 [2µ-RPA49] were spotted on 5-FOA–containing plates to check for complementation of the lethality of the double mutant. Plates were incubated for 3 d at 30°C. Strength of the complementation was evaluated by comparing plates with (FOA) or without FOA (−). (B) Nucleolar localization of Rpa34 is independent of Rpa49. Rpa34 fused with YFP [YFP-Rpa34] is expressed in a WT budding yeast or in a mutant lacking Rpa49 (rpa49Δ). Both strains produce Nop1 fused to mCherry [mCherry-Nop1]. (right) Overlay of both fluorescent signals (merge) is shown. (C) Nucleolar localization of Rpa34-ΔC is dependent on Rpa49. Rpa34-ΔC fused with YFP [YFP-Rpa34ΔC] is expressed in WT or in a mutant lacking Rpa49 (rpa49Δ). Both strains produce Nop1 fused to mCherry [mCherry-Nop1]. (right) Overlay of both fluorescent signals (merge) is shown.
Rpa34 and Rpa49 influence nucleolar morphology

We have shown that the Pol I–specific Rpa49/Rpa34 heterodimer is functionally conserved from yeast to humans. We hypothesized that Pol I–specific subunits might be responsible for the two most prominent features of Pol I transcription: nucleolar assembly and high RNA polymerase loading rate per gene. We first focused on nucleolar alterations caused by the absence of Pol I–specific subunits. We labeled the nuclear periphery using GFP-Nup49, a nuclear pore protein, and the nucleolus using mCherry-Nop1, an abundant nucleolar protein, in WT, rpa34Δ, and rpa49Δ cells (Fig. 4 A). Nucleolar alterations in rpa34Δ and rpa49Δ cells were determined by thresholding the fluorescent nucleolar signal detected using confocal microscopy in a large number of cells (Berger et al., 2008). Because the cumulative distribution function accurately describes volume distribution in large samples, we used this representation to evaluate the nucleolar size for each mutant (Fig. 4 B). The nucleolar size increases considerably in rpa49Δ cells, suggesting that the integrity of nucleolar structure is compromised (Fig. 4 A). We next performed an ultrastructural study of the nucleus in rpa49Δ and rpa34Δ mutants. For optimal preservation of the nuclear structure, chemical fixation was avoided, and the cells were cryoimmobilized before low temperature substitution. The nuclear structure, chemical fixation was avoided, and the cells were determined by thresholding the fluorescent nucleolar signal detected using confocal microscopy in a large number of cells (Berger et al., 2008). Because the cumulative distribution function accurately describes volume distribution in large samples, we used this representation to evaluate the nucleolar size for each mutant (Fig. 4 B). The nucleolar size increases considerably in rpa49Δ cells, suggesting that the integrity of nucleolar structure is compromised (Fig. 4 A). We next performed an ultrastructural study of the nucleus in rpa49Δ and rpa34Δ mutants. For optimal preservation of the nuclear structure, chemical fixation was avoided, and the cells were cryoimmobilized before low temperature substitution. The nuclear ultrastructure is altered in both mutants (Fig. 4, C and D). In rpa34Δ cells, the nucleolus is decondensed with areas of low electron density. In rpa49Δ cells, the nucleolar morphology is dramatically altered: its characteristic crescent shape disappeared, and the dense material redistributed in the full volume of the nucleus.

Cryofixation also allows the direct observation of individualized cytoplasmic ribosomes. The number of ribosomes in rpa34Δ cells is similar with WT, but it is significantly reduced in rpa49Δ cells (Fig. 4 E). To investigate a defect in translation, we performed a polysome profile in WT and the rpa49Δ mutant. Interestingly, we detected polysomes in both backgrounds, but with the appearance of half-mers in rpa49Δ cells, reminiscent of a 60S biogenesis defect (Fig. 4 F). Such a defect was previously observed in a Pol I mutant strain defective for rRNA elongation (Schneider et al., 2007). Therefore, the morphological analysis demonstrates that Rpa49 is required for normal nucleolar morphology and efficient ribosome biogenesis. The rpa49Δ strain contains fewer 60S subunits relative to 40S, which is reminiscent of a Pol I elongation defect.

Reduction in the number of rDNA repeats can partially compensate for the alterations in nucleolar morphology caused by Rpa49 deletion

In the rpa49Δ mutant, rRNA production still sustains growth, but the nucleolus appears fully decondensed. To spatially constrain rRNA production, we decided to use mutant cells with a decreased number of rDNA repeats (Cioci et al., 2003). rDNA copy number can be stabilized at 25 copies by Fob1 deletion without significant growth defect (Cioci et al., 2003). To support normal rRNA production from such a low number of rDNA genes, the number of Pol I complexes per gene is significantly increased (French et al., 2003). We first demonstrated that double-mutant rpa49Δ fob1Δ strains are viable at 30°C, having either 190 or 25 rDNA copies (Fig. 5 B). To confirm this surprising result, we establish that rDNA copy number in both strains is unaffected by rpa49 deletion (Fig. 5 A). Growth of the rpa49Δ strain is severely impaired at 25°C (Liljendul et al., 1992). Comparing growth of the rpa49Δ fob1Δ strains containing 25 versus 190 rDNA copies, we noted that the cold sensitivity is alleviated upon reduction in the number of rDNA repeats (Fig. 5 B).

Quantitative fluorescence analysis revealed that in presence of Rpa49, the reduction from 190 to 25 rDNA copies resulted in a nucleolus of normal size (Fig. 5 C). Electron microscopy observations showed that the nucleolus in the 25 rDNA copies background is characterized by an identifiable crescent-shaped area of fibrillo-granular material adjacent to the nuclear envelope (Fig. 5 D). Interestingly, in the Rpa49 deletion strain with 25 rDNA copies, but not with 190 copies, a crescent-shaped nucleolus is detectable in most cells (60%), as evaluated by fluorescence microscopy (Fig. 5 C). Similarly, the nucleolar morphology is partially restored at the ultrastructural level because a dense structure occupying one third of the nuclear surface and in contact with the nuclear envelope can be detected (Fig. 5 D). These data suggest that a decrease in the rDNA copy number is enough to establish a partial nucleolar organization in the absence of Rpa49.

RNA Pol I distribution on single genes is dependent on Rpa49

Having shown that nucleolar assembly is dependent on Rpa49, we next evaluated whether a high RNA Pol I loading rate per gene requires Rpa49. Miller spreads are the only technique allowing counting of individual Pol I on a single gene (Miller et al., 1969). However, the ability to quantify and accurately localize polymerase on a single gene relies on obtaining high contrast chromatin spreads from mutant yeast cells. The chromatin spreading technique was set up in our laboratory using strain NOY886, which contains 42 actively transcribed rDNA copies (Fig. 6 A; French et al., 2003). Because of the very high loading rate in mutant backgrounds with reduced rDNA copy number, polymerases often appear as a continuous track, making accurate counting of single polymerases difficult. We took advantage of negatively stained Miller spreads obtained with low frequency (see Materials and methods) to demonstrate that continuous tracks are best analyzed by assuming the presence of a polymerase every 12 nm along such track (Fig. 6 C). We next analyzed Pol I density and distribution on the rRNA genes in 25 or 190 rDNA copy strains with or without Rpa49 using Miller spreads (Fig. 7 A). In the presence of Rpa49, we observed 62 ± 11 Pol I complexes per single rRNA gene in the 190 rDNA copy strain. However, in this strain, we were unable to identify rDNA in chromatin spreads in the absence of Rpa49, probably because of a very low Pol I occupancy of the rDNA. We next analyzed chromatin spreads in strains with 25 rDNA copies and identified single transcription units both in the presence and absence of Rpa49 (Fig. 7 A). We detected 122 ± 17 Pol I complexes per gene in the presence of Rpa49 and 36 ± 5 Pol I

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Figure 4. **Rpa34 and Rpa49 are required for nucleolar integrity.** (A) Nucleolar structure of rpa49Δ and rpa34Δ cells assessed by fluorescent microscopy. Representative confocal sections of strains TMS1-1a (WT), BEN18-1a (rpa34Δ), and BEN19-1a [rpa49Δ] expressing TetR-GFP staining the nucleus, GFP-Nup49 staining the NPC (NPC/nucleus), and mCherry-Nop1 staining the nucleolus (mCherry-Nop1) are shown. [right] Overlay of both fluorescent signals (merge) is shown. (B) Cumulative distribution function of the nucleolar volume in Pol I mutants. The nucleolar volume was estimated by thresholding using the intensity of the mCherry fluorescent signal in WT, rpa49Δ, and rpa34Δ mutant cells (nWT = 907; nrpa49Δ = 620; nrpa34Δ = 928). (C) Ultrastructural study of cryofixed, cryosubstituted WT, rpa34Δ, and rpa49Δ budding yeast cells. Representative sections of the nuclei are depicted, with manual segmentation of nucleolus bounded by red lines. The cytoplasmic ribosomal contents in the three budding yeast strains are depicted in a selected frame. (D) Quantification of ultrastructural morphological alterations in Pol I mutants. Nucleolar area normalized by nuclear surface is estimated by manual segmentation of electron microscopy sections (n = 20). (E) Quantification of the ribosomal cytoplasmic content in Pol I mutants. Ribosomal content of representative sections (n = 20) was manually counted and expressed as ribosomes per micrometer cubed. (F) Polysomal profiles (OD260 nm) after sucrose density gradient centrifugation derived from the rpa49Δ mutant and WT strain. The positions of 40S ribosomal subunits, 60S ribosomal subunits, polysomes, and half-mer polysomes (H) are indicated.
Figure 5. **rpa49Δ phenotype in strains with 190 or 25 copies of rDNA.** (A) rDNA copy number is unaffected by rpa49 deletion in a fab1Δ deletion background. PFGE of chromosomes from rpa49Δ fab1Δ strains resulting from invalidation of the rpa49Δ gene in Fab1Δ strains containing either 25 or 190 copies of rDNA or from original fab1Δ strains. Separated chromosomes were stained with ethidium bromide (right). Hybridization of rDNA probes was performed to estimate chromosome XII size (left). (B) Growth phenotype of the fab1Δ rpa49Δ double-mutant strains bearing 190 or 25 copies of rDNA. 10-fold serial dilutions of strains BEN20-1a (25 rDNA copies rpa49Δ fab1Δ) and BEN21-1a (190 rDNA copies rpa49Δ fab1Δ) deleted for rpa49 and containing 25 or 190 copies, respectively, of rDNA and an empty vector (−) or bearing Rpa49 (Rpa49) were spotted on plates with [FOA] or without FOA (−) and grown at 25°C for 7 d. (C) Nucleolar structure of Pol I mutants assessed by fluorescent microscopy. Representative confocal sections of strains NOY1071 (25 rDNA copies fab1Δ), NOY1064 (190 rDNA copies rpa49Δ), and BEN20-1a (25 rDNA copies rpa49Δ) containing GFP-Nup49 staining the NPC (GFP-Nup49) and mCherry-Nop1 staining the nucleolus (mCherry-Nop1). (right) Overlay of both fluorescent signals (merge) is shown. (D) Ultrastructural study of cryofixed, cryosubstituted strains with 25 rDNA copies with [WT] or without Rpa49 (Rpa49Δ).
Figure 6. Quantification of polymerases along transcribed rDNA genes. (A) Visualization of active genes in rDNA. Using a mutant strain with a reduced number of rDNA copies (strain NOY886; 42 rDNA copies), we obtained high quality spreads from total nucleolar DNA. (B) Quantification of actively transcribed rDNA genes. The quantification of active genes in mutant strain (42 active gene in NOY886) is compatible with previous results (French et al., 2003). (C) Visualization of individual polymerases on negatively stained Miller spread. In mutant strains with 25 rDNA copies, Pol I density on each gene is very high. Using higher magnification (bottom left), we can still detect individual polymerases, which can be quantified (right; red circles). Note that a nascent rRNA can be visualized from each detected polymerase.

complexes per gene in its absence. We then measured the distance between adjacent polymerases to evaluate whether the Pol I molecules were randomly distributed on the rRNA genes (Fig. 7 B). The distances between polymerases depend intrinsically on the Pol I density on each rRNA gene, which varies between strains. Therefore, for each polymerase load, we first compared the empirical measurements of distance between polymerases with the corresponding random distribution predicted (Fig. 7 B). In all cases, the observed distance between polymerases was shorter than that predicted for the random distribution. These results suggest clustering of polymerases. We have introduced a new parameter in our simulations to take this effect into account. When 60% of polymerases are modeled as being in direct contact with each other and 40% are randomly...
Figure 7. **Miller spread of 190 and 25 rDNA copy strains with or without Rpa49.** (A) Representative Miller spread from NOY1071 bearing 25 rDNA copies (25 rDNA copies [WT]), BEN20-1a bearing 25 rDNA copies and rpa49Δ (25 rDNA copies [rpa49Δ]), and NOY1064 bearing 190 rDNA copies (190 rDNA copies [WT]). (right) Interpretive tracing of the genes is shown. Polymerases that appear on the gene are shown on the tracing by black dots. (B and C) Cumulative distribution function of the distances between adjacent polymerases in strains NOY1071 (red; 25 rDNA copies [WT]), BEN20-1a (blue; 25 rDNA copies [rpa49Δ]), and NOY1064 (green; 190 rDNA copies [WT]) is shown. Solid lines, empirical measurement; dashed lines, random (B) or simulated (C).
Figure 8. Two successive elongating Pol I complexes allow a contact between Rpa49 and Rpa43 subunit of adjacent molecules. (A) Frequent association of adjacent Pol I depends on Rpa49. We generated diploid cells expressing two versions of Pol I containing an Rpa190 subunit fused to either the Myc or HA tag. These tagged proteins are efficiently detected in cell extract supernatants (SUP). After formaldehyde cross-linking and extensive DNase treatment, we immunoprecipitated Pol I using anti-HA antibodies (IP). We evaluated the amount of polymerase dimers/multimers by assessing the extent of Myc-tagged Rpa190 coprecipitation. We use a strain bearing only Myc-Tag Pol I as negative control (no HA tag; lanes 1 and 4). The presence of dimers/multimers is dependent on Rpa49, as shown by lack of Myc-tagged Rpa190 coimmunoprecipitation in rpa49 deletion background (rpa49Δ; compare lanes 5 and 6). (B) 3D model of Pol I as determined by cryo–electron microscopy. The positions of the Pol I–specific Rpa43/Rpa14 and Rpa49/Rpa34 heterodimers are shown in purple and in green, respectively. (C) Fitting of the atomic structure of the highly homologous Pol II elongation complex (Protein Data Bank accession no. 2YU9) into the cryo–electron microscopy structure of RNA Pol I. The transcribed DNA is shown in red, and the 10-subunit core Pol II is shown in blue.
distributed, the simulated data are in good agreement with our experimental measurements in the case of the 190 and 25 rDNA copy strains in the presence of Rpa49 (Fig. 7 C). However, in the 25 rDNA copy/rpa49Δ strain, we measured larger distances between polymerases compared with the predictions of the model. Therefore, we propose that the interactions between successive Pol I complexes are strongly reduced in the absence of Rpa49, accounting for the reduction in the number of adjacent Pol I complexes. Therefore, Rpa49 is required to ensure high density of Pol I per rDNA gene. Furthermore, our data suggest that Rpa49 is involved in establishing contact between adjacent Pol I complexes.

**Molecular modeling of contacts between adjacent polymerases on rDNA genes**

Rpa49 seems to be required for interaction between adjacent transcribing polymerases. To confirm the requirement on Rpa49 for contact between adjacent polymerases, we coexpressed in a single diploid cell two of Pol I complex’s largest subunits tagged with different epitopes, Myc and HA. By purifying HA-tagged Pol I from the chromatin-bound fraction, we could evaluate the amount of polymerase dimers or multimers by assessing the extent of copurification of Myc-tagged Pol I. In WT cells, after formaldehyde cross-linking and with extensive DNase treatment, we could detect frequent Pol I dimers (Fig. 8 A). In absence of Rpa49, the proportion of Pol I dimers or multimers is dramatically reduced (Fig. 8 A, compare lane 5 with lane 6).

Using Miller spreads and communoprecipitations, we show that elongating Pol I enzymes are in frequent contact in an Rpa49-dependent manner. To characterize potential molecular interactions occurring between successive polymerases, we modeled the structure of a Pol I dimer in an elongation state on DNA (Fig. 8 D). We used a cryo–electron microscopy density map of Pol I obtained as described previously (De Carlo et al., 2003) onto which the Rpa43 and Rpa49/Rpa34 heterodimer are localized far away from each other in different parts of the Pol I complex (Fig. 8 B; Bischofer et al., 2002; Kuhn et al., 2007).

To model the DNA upstream and downstream of the Pol I transcription bubble, we used the atomic structure of the corresponding RNA Pol II elongation complex (Protein Data Bank accession no. 2YU9; Fig. 8 C; Wang et al., 2006). A second elongation complex was then positioned according to the following constraints: (a) enzymes were placed in contact, (b) the intervening DNA connecting the two polymerases was set as linear, and (c) this linker DNA was modeled as B-DNA to find the rotational register between the two elongating polymerases (Fig. 8 D). The minimal distance between two successive active sites was found to be of 34 bp, which is in good agreement with ExoIII footprinting experiments on RNA Pol II elongation complexes (Samkurashvili and Luse, 1998). Interestingly, in this model, the Rpa49/Rpa34 subunits of one polymerase are brought in close proximity to the Rpa43/Rpa14 heterodimer of the following Pol I. Genetic evidence previously suggested that Rpa49 and Rpa43 are functionally linked (Beckouet et al., 2008). To test whether Rpa49 directly associates with Rpa43, we coexpressed GST-tagged Rpa49 and HA-tagged Rpa43 in *Escherichia coli*. Because Rpa49 and Rpa34 form heterodimers, we used GST-tagged Rpa49 and HA-tagged Rpa34 as positive controls. Upon purification on glutathione Sepharose beads, both Rpa34 and Rpa43 copurify with GST-Rpa49 (Fig. 8 E, lanes 2 and 6). In contrast, HA-tagged Rpa43 or HA-tagged Rpa34 are not copurified with GST alone (Fig. 8 E, lane 4 and 8). Therefore, genetic and biochemical assays suggest a direct interaction between Rpa43 and Rpa49.

**Discussion**

In this study, we addressed the function of two Pol I–specific subunits, Rpa49 and Rpa34. We showed that heterodimers formed by human or fission yeast orthologues are functional in budding yeast, demonstrating a functional conservation of these two Pol I–specific subunits in the course of evolution. We also showed that these subunits are playing an important role in the formation of the nucleolus and are required to produce a high density of elongating Pol I on rDNA genes.

**RNA Pol I evolutionary conservation**

All eukaryotic cells exploit three distinct RNA polymerases to transcribe their nuclear genome. Of these, Pol I is the most divergent enzyme, probably because of its highly specialized function: it transcribes only a single gene encoding large rRNAs. Our data demonstrate that despite considerable variation in sequence, two of the Pol I–specific subunits forming a heterodimer, Rpa49/Rpa34, have orthologues throughout the eukaryotic domain, suggesting that functions of this dimer are evolutionally conserved. Our results show that overexpression of Rpa49 can rescue the growth defects in genetic backgrounds in which *rpa34Δ* is lethal. The C-terminal domain of Rpa34 is responsible for nucleolar localization, and it functions independently from Rpa49. Nucleolar localization sequences are known to interact with various nucleolar factors (Lechertier et al., 2007), suggesting the possibility of a direct interaction between the C-terminal domain of Rpa34 and *S. cerevisiae* nucleolar proteins.

Indeed, several potential partners of Rpa34 have been identified in an unbiased two-hybrid interaction screen between Rpa34 and the complete genome. In this screen, three nucleolar...
functions, Gro1, a cofactor of the RNA helicase Pbp43, and two components of the C/D snORNP complexes Nop56 and Nop58 were shown to interact with Rpa49 (Beckouet et al., 2008). Therefore, the C-terminal domain is involved in the recruitment of species-specific binding partners, which are responsible for nucleolar targeting.

Pol I transcription influences nucleolar morphology

The nucleolus is the largest nuclear domain, the integrity of which depends directly on rRNA production (Trumtel et al., 2000; Hernandez-Verdun et al., 2002). This structure is conserved in all eukaryotes, and most nuclear steps of ribosome biogenesis take place within the nucleolus. In the absence of Rpa34 and especially Rpa49, nucleolar assembly is severely compromised. Importantly, the altered nucleolar morphology in rpa49-null strains correlates with a significant reduction in the number of ribosomes per cell. When we artificially decreased the number of rDNA copies from 190 to 25, in the absence of Rpa49, we observed a partial nucleolar reassembly. Therefore, we propose that the initial event required for self-organization of the nucleolus is a spatially constrained rRNA synthesis, which initiates the chain of events leading to formation of nucleolar structures.

Function of the Rpa49/Rpa34 heterodimer in Pol I transcription

A previous study revealed a close relationship between Rrn3, the two heterodimers Rpa49/Rpa34, and Rpa43/Rpa14 (Beckouet et al., 2008). The release of the Pol I- specific initiation factor Rrn3 during elongation requires Rpa49, despite diametrically opposite locations within a single Pol I complex (Beckouet et al., 2008). While our work was in progress, an elegant structural approach to understanding how contacts between adjacent Pol I, during elongation, affect 3D organization of the nucleolus was presented (Beckouet et al., 2008). We now show that Rpa49 is required to allow contact between adjacent polymerases. 3D modeling of adjacent contiguous polymerases gives a hint as to how Rrn3 could be released. Rrn3 and its interacting partner Rpa43 behave as extragenic suppressors of the rpa49 phenotype, and Rpa49 and Rpa43 when co-expressed in E. coli are interacting, arguing for a physical contact between both subunits (Beckouet et al., 2008).

From our model, we propose that adjacent polymerases form a head to tail “camel caravan” in which Rpa49 from one Pol I molecule is in direct contact with Rpa43 from the neighboring molecule. These interactions determine the distance between successive Pol I complexes. In the absence of Rpa49, adjacent complexes are unable to interact, and this could lead to the lack of Rrn3 release (Beckouet et al., 2008) and to the increased distance between polymerases (this study). All of these data together strongly suggest that the two Pol I-specific subunits Rpa49 and Rpa34 are essential for nucleolar assembly and formation of “Pol I caravans,” two unique Pol I features. We now need to understand how contacts between adjacent Pol I, mediated by Rpa49, could affect yeast nucleolar integrity, impacting on rDNA organization in the nuclear space.

Materials and methods

Yeast strains, plasmid construction, and plasmid-shuffling assays

Yeast strains used in this study are listed in Table S1, and oligonucleotides are listed in Table S2. Yeast strains were constructed by meiotic crossing and plasmid DNA transformation. S. cerevisiae-null alleles with KanMX4 insertions were obtained from EUROCARG and were checked using ad hoc PCR amplifications. The Sp-rpa34 gene deletion in S. pombe strain BENSP1-1a was performed by homologous recombination using PCR-amplified fragments with oligos 746 and 747 with plasmid p29802 as template. Sp-rpa49 (rpa51A) was obtained as previously described using plasmid pYN1003uds (Nakagawa et al., 2003), pDONR201 (Invitrogen), pREP41 (Baso et al., 1993), pRS413, pSR434, pSR452 (Sikorski and Hieter, 1989), pV2000, pV2008 (Van Mullem et al., 2003), p29802, pCNOD5-Pep43 (Cherry-NOP1) (Beckouet et al., 2007), pCFP-NUP49 (CFP-NUP49: Berger et al., 2008), pFLS-HCSS, pGID-CII (Beckouet et al., 2007), pOGS-RPA34 (Gadal et al., 1997), pENTR-PAF53, pENTR-CAST, pENTR-HA-CAST (Panov et al., 2006), pOG1-A34 (Beckouet et al., 2008), pENTR-RPA49 (Beckouet et al., 2008), pYN1003uds (Nakagawa et al., 2003), and pNOY373 (Wai et al., 2000) were previously described. pE5 and pENTR-Rpa34DC58 were provided by E. Bertrand and F. Beckouet, respectively.

pSR425-PgK was constructed by ligating the Ndel fragment from pVV2000 in the Smal site of pRS425. pCNOD08, a gateway vector that allows strong transcription from the TEF1 promoter, was constructed in three steps. First, the 1,281-bp Xhol-Ncol fragment of pSR425-PgK containing promoter PgfK and antf lambda recombinase site was inserted into XhoI-Ncol-digested vector pSKB3X (GTP technology) to generate plasmid pCNOD02. Second, promoter PgfK was amplified by PCR using oligonucleotides 439 and 440 and yeast OD11-1a genomic DNA as template. The Xbal-Xhol-digested PCR product was cloned into the same site of pCNOD02 to generate plasmid pCNOD05. The 1,281-bp Ncol-Xhol fragment of pCNOD05 was inserted into pSR425-PgK digested with Ncol and Xhol generating pCNOD08. pSR423-Pet was constructed by subcloning the Drol–Saci fragment from pSR425-PgK in the same site of pSR423. pCFP-Nup49-His was constructed by ligating the fragment from HindIII (filled in with Klenow) to SacI in the Smal–Saci of pSR413. pREP41-gat was made a gateway destination vector by ligating the PCR-generated fragment using oligos 740 and 741 and pSR425-PgK as the template, cut VspI–Xhol inserted in Ndel–SalI of pREP41. The following plasmids were constructed using Gateway technology (Invitrogen): (a) pENTR-Rpa34, by a BP reaction with pDONR201 and PCR-generated fragment amplified using oligonucleotides 463 and 440 and genomic DNA from S. cerevisiae strain OD11-1a as a template, and (b) pENTR-Sp-Rpa34 and pENTR- Sp-Rpa49, by a BP reaction with pDONR201 and PCR fragment generated using oligonucleotides 742–743 or 744–745, respectively, and genomic DNA from S. cerevisiae strain TG11 as template. pRS425-PgK and pSR425-PgK-HA were constructed by LR reaction between destination vectors and entry vector and were used for in vivo selection in E. coli.

Complementation was tested using plasmid-shuffling assays. A null allele of haploid tester strain was complemented by the corresponding WT gene borne on URA3-containing plasmids. Fluoroorotate (FOA) is toxic for URA3− strains (Boeke et al., 1984). FOA is used to apply a strong positive selection on cells without plasmids containing WT genes. Complementation was tested by examining whether plasmids expressing a given cDNA could bypass the lethal phenotype, monitored by FOA colonies.

GIM

GIM analysis of pRA34 deletion mutant was performed as described previously (Decourty et al., 2008). Microarray data were normalized using MATLAB (MathWorks, Inc.), and relative enrichments are described in Table S3. Genetic interactions of interest were individually confirmed by plasmid-shuffling experiments. Deletion mutants of interest from the EUROCARG collection were mated individually with the query strain carrying RPA34 deletion tag with NAT marker under the control of the
Expression of GST-Rpa49 and Rpa43 and Rpa34 in 

After centrifugation, yeast cells were resuspended in synthetic complete medium (DIFCO), mounted on a slide, and observed in the fluorescence microscope. Wide-field fluorescent images were captured with a microscope (IX81; Olympus) equipped with a polychrome V monochromator and a camera (CoolSNAP HQ; Roper Industries) controlled with Meta- Morph acquisition software (version 6; Universal Imaging Corp.). Confocal microscopy was performed using a Nikon dish confocal system (Revolution; Andor) installed on a microscope (IX-81), featuring a confocal spinning disk unit (CSU22; Yokogawa) and a cooled electron multiplying charge-coupled device camera (DU 888; Andor). The system was controlled using the FAST mode of Revolution IQ software (Andor). Images were acquired using a 100X Plan Apo 1.4 NA oil immersion objective and a twofold lens in the optical path. Single laser lines used for excitation were diode-pumped solid-state lasers exciting GFP fluorescence at 488 nm (50 mW; Coherent) and mCherry fluorescence at 561 nm (50 mW; Cobolt, jive), and a bandpass emission filter (Em01-R488/568-15; Semrock) allowed collection of the green and red fluorescence. Pixel size was 65 nm. For quantification of nucleolar volume, z stacks of 41 images with a 250-nm z step were used. Exposure time was 200 ms. Digital pictures were processed using Photoshop software (version CS3; Adobe).

Electron microscopy and quantitative analysis of Miller spreads

Simulated distances and 3D modeling

We estimated experimental measurement errors of 2 pixels of systematic bias and 2 pixels of random error, with a pixel size of 3 nm. Random and simulated distances between adjacent polymers were determined assuming 185 possible Pol I positions on a single rDNA gene (12 nm for each polymerase distributed along a 2.22-µm-long gene). Measurement error was added to each distance, random noise being approximated using the normal law of 5 nm of variance. Each random and simulated data set corresponds to 100 genes with 142, 62, or 36 polymers per gene for 25 copy, 190 copy WT, and 25 copy strains bearing rpa49Δ, respectively. Distances were generated using MATLAB. Fitting of atomic coordinates into electron microscopy density maps, modeling of two successive elongating Pol I molecules, and image creation were performed using University of California San Francisco chimera (Pettersen et al., 2004).

Online supplemental material

Table S1 lists yeast strains used in this study. Table S2 lists oligonucleotides used in this study. GIM analysis of RPA34 deletion mutant was performed as described previously (Decourty et al., 2008). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201006040/DC1.

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