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A34.5, a Nonessential Component of Yeast RNA Polymerase I, Cooperates with Subunit A14 and DNA Topoisomerase I To Produce a Functional rRNA Synthesis Machine†

OLIVIER GADAL,[1] SYLVIE MARIOTTE-LABARRE,[1] STÉPHANE CHEDIN,[1] ERIC QUEMENEUR,[2] CHRISTOPHE CARLES,[1] ANDRÉ SENTENAC,[1] AND PIERRE THURIAUX[1]*

Service de Biochimie & Génétique Moléculaire[1] and Département d'Ingéniérie et d'Etude des Protéines,[2] CEA-Saclay, F 91191 Gif sur Yvette cedex, France

A34.5, a phosphoprotein copurifying with RNA polymerase I (Pol I), lacks homology to any component of the Pol II or Pol III transcription complexes. Cells devoid of A34.5 hardly affect growth and rRNA synthesis and generate a catalytically active but structurally modified enzyme also lacking subunit A49 upon in vitro purification. Other Pol I-specific subunits (A49, A14, and A12.2) are nonessential for growth at 30°C but are essential (A49 and A12.2) or helpful (A14) at 25 or 37°C. Triple mutants without A34.5, A49, and A12.2 are viable, but inactivating any of these subunits together with A14 is lethal. Lethality is rescued by expressing pre-rRNA from a Pol II-specific promoter, demonstrating that these subunits are collectively essential but individually dispensable for rRNA synthesis. A14 and A34.5 single deletions affect the subunit composition of the purified enzyme in pleiotropic but nonoverlapping ways which, if accumulated in the double mutants, provide a structural explanation for their strict synthetic lethality. A34.5 (but not A14) becomes quasi-essential in strains lacking DNA topoisomerase I, suggesting a specific role of this subunit in helping Pol I to overcome the topological constraints imposed on ribosomal DNA by transcription.

In yeasts as in all other eukaryotes, the three largest species of rRNA develop from a pre-rRNA transcript that accounts for more than half of the total RNA synthesis in yeast cells (51). Pre-rRNA synthesis is catalyzed by RNA polymerase I (Pol I) in vitro (20). In vivo, the only essential function of yeast Pol I resides in the transcriptional synthesis of pre-rRNA, as the lethal phenotypes of mutants defective in Pol I are overcome by putting the pre-rRNA-encoding gene under the control of a Pol II-dependent promoter (32). The transcriptional specificity of Pol I depends on several additional Pol I-specific factors that collectively ensure the recruitment of Pol I to its promoter (26, 28). These factors presumably have some functional analogy but no detectable structural homology with animal Pol I-specific factors that determine the transcriptional specificity in Pol I (2, 17, 25, 35).

Fourteen distinct subunits were biochemically identified in yeast Pol I (9, 13, 23). Ten of them (A190, A135, AC40, ABC27, ABC23, AC19, ABC14.5, A12.2, ABC10α, and ABC10β) are conserved or even shared by the other two transcription enzymes. With the exception of A12.2, where gene inactivation leads to a temperature-sensitive lethality (31), all of the conserved or common subunits are essential for growth, as shown by the lethality of the corresponding deletions (references 13 and 46 and references therein). The two largest subunits (A190 and A135), their bacterial β′ and β counterparts, and their homologs in yeast Pol II and Pol III harbor the active site of the enzyme (19, 33, 43, 55). AC40 (a homolog of the bacterial α subunit [30]) and AC19 also belong to yeast Pol III and are related to Pol II subunits. ABC27, ABC23, ABC14.5, ABC10α, and ABC10β are common to all three enzymes. Finally, A49, A43, A34.5, and A14 have no detectable homology to the Pol II or Pol III transcription machinery. A43 is essential for growth, whereas mutants lacking A14 or A49 have limited growth defects in vivo (29, 40, 45).

We complete here the genetic characterization of Pol I subunits by isolating the *RPA34* gene, encoding A34.5, a 34.5-kDa phosphoprotein copurifying with the yeast enzyme (7, 11). *rpa34*-Δ deletions yield upon purification a structurally modified form of Pol I that also lacks A49, showing that A34.5 is a genuine Pol I subunit. However, these mutants are barely affected in their growth rates, and the mutant enzyme is catalytically active on nonspecific templates. This prompted us to investigate the properties of multiple mutants inactivated for all possible combinations of the four dispensable subunits of Pol I (A49, A34.5, A14, and A12.2) and to test their ability to support growth under conditions where ribosomal DNA (rDNA) synthesis is either naturally dependent on a functional Pol I or artificially made Pol II dependent (32). Notwithstanding the viable phenotype of individual loss-of-function mutants, our data establish that A49, A34.5, A14, and 12.2 are collectively essential for pre-rRNA synthesis. Moreover, a specific role of A34.5 in the resolution of the topological constraints inherent to rDNA transcription is indicated by the strong adverse effect of a loss-of-function mutant in cells lacking DNA topoisomerase I.

MATERIALS AND METHODS

Plasmids, strains, and in vivo techniques. The plasmids constructed in this work (Table 1) were prepared by standard in vitro recombination techniques. pA14-ADE2, pCT80TOP, and pTSV31 were respectively provided by A. Smid, R. Sternglanz, and J. R. Pringle. pTSV31 (2μm *ADE3 URA3*) was obtained by replacing the *Aat*II-*Pvu*II fragment bearing the *amp* gene and the pUC18 origin of replication of YEP352 (22) by the 2,915-bp *Aat*II-*Sty*I fragment of pBR322 (bearing the *rop* gene responsible of the low copy number, which allows propagation of *ADE3* in *Escherichia coli* [41]). *ADE3* was inserted as a 3,556-bp fragment by blunt-end cloning the 3,677-bp *Bam*HI-*Nhe*I *ADE3*+ fragment into *Hpa*I, which generated a deletion of 121 bp on the *Bam*HI side of the insert. The resulting plasmid is a 9.6-kb shuttle vector bearing *ADE3* and *URA3* in the same transcriptional orientation, with *Sac*I, *Sma*I, *Bam*HI, *Xba*I, and *Sal*I as convenient cloning sites.

Saccharomyces cerevisiae strains (Table 2) were constructed by genetic techniques based on transformation of lithium acetate-treated cells, sexual mating, and tetrad analysis, using standard media and growth conditions (45). All mu-

* Corresponding author. Mailing address: Service de Biochimie & Génétique Moléculaire, CEA-Saclay, Bât. 142, F 91191 Gif sur Yvette cedex, France. Phone: 33.1.69 08 35 86. Fax: 33.1.69 08 47 12. E-mail: thuriaux@jonas.saclay.cea.fr.

† Dedicated to the memory of Marcelle Grenson.

TABLE 1. Plasmids used in this study

Name	Yeast genetic markers	Origin
pA34-5	*RPA34*	Cloning of the 2.2-kb *Eco*RI fragment from λgt11-A34 (34) into the *Eco*RI site of pBluescript II(+)KS
p34ΔQ	*rpa34*-Δ (with its basic C end deleted)	Directional cloning of the 0.6-kb *Eco*RI-*Nhe*I fragment from pA34-5 between the *Eco*RI and *Xba*I sites of pUC19
pUC-his3	*HIS3*	Cloning of the 1.7-kb *HIS3*$^{+}$ *Bam*HI fragment into pUC19
pA34ΔHIS	*rpa34-Δ::HIS3*	Cloning of the 1.7-kb *Sma*I-*Xba*I fragment from pUC-HIS3 between the *Sna*BI and *Nhe*I sites of pA34-5
pBSK-D3		pBluescript II(+)KS deleted of its *Hin*dIII site by digestion with *Eco*RV and *Hin*cII followed by religation
pBSK-D3/A34	*RPA34*	Cloning of the 2.2-kb *Eco*RI fragment from pA34-5 into the *Eco*RI site of pBSK-D3
pA34ΔURA	*rpa34-Δ::URA3*	Cloning of the 1.1-kb *Hin*dIII fragment from YdpU (3) into the *Hin*dIII site of pBSK-D3/A34
pOG5-A34	*CEN6 ARSx RPA34 TRP1*	Cloning of the 1.2-kb *Eco*RI-*Nde*I fragment from pA34-5 into pFL39 (5)
pA14ADE2	*CEN6 ARSx ADE2 RPA14*	Cloning of the *RPA14 Afl*II-*Sac*I fragment from pFL44-A14 (40) between the *Sma*I and *Sac*I sites of pASZ11 (42)
pTSV31	*ORI*(2μm) *ADE3 URA3*	A yeast-*E. coli* shuttle vector with a pBR322 backbone (see Materials and Methods)
pOG1-A34	*ORI*(2μm) *ADE3 URA3 RPA34*	Directional cloning of the 2.2-kb *Bam*HI-*Sal*I fragment from pA34-5 into pTSV31

tants were constructed by mutagenesis and backcrossing in an isogenic strain set (YNN281, YNN282, YPH252, YPH499, and YPH500 [39]). MG412 and GF312-17c are unpublished strains that were given by the late Marcelle Grenson and by Gérard Faye, respectively. Zygotes and spores were isolated by using a de Fonbrunne micromanipulator. T4-1d was constructed by converting one of the *RPA34* genes of the diploid strain YNN281 × YNN282 into the *rpa34-Δ::HIS3* null allele, using a purified 3.6-kb *Eco*RI fragment from plasmid pA34ΔHIS and selecting the resulting transformants on histidine omission medium after 3 to 5 days of growth at 30°C. The transformants were checked for the expected *rpa34-Δ::HIS3/RPA34* heterozygous structure by genomic hybridization with a digitoxin-labeled sample of pA34ΔQ. One transformant was retained and subjected to tetrad analysis, yielding the *MAT*α and *MAT***a** segregants T4-1c and T4-1d (Table 2). Strain OG13-1a was constructed by replacing the *rpa34-Δ::HIS3* allele of diploid strain YNN282 × T4-1d with the 1.9-kb *Eco*RI *rpa34-Δ::URA3* fragment of pA34ΔURA, containing a *URA3* insertion flanked by six stop codons in all possible reading frames (3). Transformants were selected on uracil omission medium, checked for histidine auxotrophy by replica plating, and characterized at the molecular level by genomic hybridization with a pA34ΔQ probe. Tetrad analysis yielded viable haploid *rpa34-Δ::URA3* segregants (e.g., OG13-1a [Table 2]) in the expected 2:2 ratio. Strain OG17-1c (*top1-Δ::LEU2*) is an isogenic derivate of strain OG1-1c constructed by deleting the *TOP1* gene with the 5.1-kb *Hin*dIII fragment of plasmid pCT80Top (8).

DNA and protein sequence analysis. A34.5 was isolated on a preparative scale by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of a purified preparation of Pol I, and internal tryptic peptides were microsequenced as previously described (40). DNA sequencing was done on both strands of alkali-denatured DNA by the dideoxy method, using a modified T7 DNA polymerase (Sequenase II; U.S. Biochemical Corp.). Results were interpreted by using standard sequence analysis software.

Purification and analysis of Pol I. Pol I preparations were partially purified from haploid wild-type (YNN282) and *rpa34* (D98-4b) strains as follows. Crude extracts, prepared from 30 g of cells grown in YPD supplemented with adenine and harvested at late exponential phase (optical density of 3.5 at 600 nm), were incubated with phosphocellulose (Whatman P11) in buffer I (20 mM Tris-HCl [pH 8], 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 10% glycerol) containing 50 mM ammonium sulfate. Bound proteins were eluted with buffer I containing 400 mM ammonium sulfate. The eluate was diluted with buffer I to an ammonium sulfate concentration of 50 mM and loaded on a 1-ml Mono Q fast protein liquid chromatography column (Pharmacia) equilibrated in buffer I containing 50 mM ammonium sulfate. Pol I was recovered from this column by elution with a linear gradient of 50 to 750 mM ammonium sulfate in 43 ml of buffer I at a flow rate of 1 ml/min. The elution fraction was precipitated by addition of 1.5 volumes of saturated ammonium sulfate (pH 8.0) at 4°C. After stirring for 30 min, the solution was allowed to stand for 30 min on ice and centrifuged for 30 min at 30,000 rpm at 4°C in a 50.2 Ti rotor. The pellet was resuspended in 0.3 ml of buffer I without glycerol and clarified by centrifugation for 5 min at 15,000 rpm at 4°C, and the supernatant was layered on top of a 10 to 30% (vol/vol) glycerol gradient in buffer I with 0.3 M ammonium sulfate. After centrifugation at 40,000 rpm for 18 h at 4°C in an SW41 rotor, the protein fractions were assayed for Pol I activity in a nonspecific in vitro transcription assay on poly(dA-T)$_n$ (11, 23). The subunit composition of Pol I was determined by immunoblot analysis. Samples were run on an SDS–13% polyacrylamide gel and then transferred onto nitrocellulose membranes. Membranes were incubated with anti-Pol I antibodies (10 μg/ml). After incubation with an anti-rabbit alkaline phosphatase-conjugated secondary antibody, protein-antibody complexes were visualized by using the Promega detection system.

RESULTS

***RPA34*, a single gene located on the right arm of chromosome X, encodes the A34.5 subunit.** *RPA34* was initially isolated from a λgt11 library (prepared from yeast strain X2180C) by using an immunological screen based on anti-Pol I polyclonal antibodies (34). This clone harbors an *Eco*RI insert of 2.2 kb that was subcloned in pBluescript II(+)KS and sequenced on both strands. This 2,235-nucleotide sequence contains *RPA34* as the only complete open reading frame (Fig. 1). To prove that *RPA34* does indeed encode A34.5, this polypeptide was purified from a Pol I preparation, submitted to tryptic digestion, and microsequenced on three internal peptides. They perfectly matched the 233-amino-acid translated sequence of *RPA34* (Fig. 2). There was no significant sequence similarity between *RPA34* and any known components of the eukaryotic Pol II and Pol III transcription complexes or indeed any entry in current data banks as scanned with a truncated version of A34.5 lacking the basic C terminus, to avoid the spurious scoring of lysine-rich polypeptides. In agreement with previous immunological data (10, 24), this finding establishes that A34.5 is specifically associated with Pol I.

RPA34 coincides with the YJL148W open reading frame (accession number Z49423) on the left arm of chromosome X of *S. cerevisiae* except for a conservative replacement (K210R) and a silent substitution (GAA→GAG) at position E202. The 2,235-nucleotide sequence determined in this work differs from the standard yeast genome sequence by 12 transitions and one transversion. These differences may admittedly be due to strain polymorphism. Prior to the complete sequencing of the yeast genome, we had assigned *RPA34* to chromosome X by chromosome hybridization and localized it on the left arm of that chromosome at about 14 centimorgans from *TIF2* by genetic crosses involving the viable *rpa34*-Δ allele. *RPA34* is also genetically close to the *RPB4* gene encoding the Pol II-specific subunit B32 (52). The physical and genetic mapping data are in full agreement (Fig. 1).

TABLE 2. Yeast mutant strains used in this study

Strain	Genotype	Origin or reference
T4-1c[a]	*MAT*α *rpa34-Δ::HIS3 ade2-1 ura3-52 lys2-801 his3-Δ200 trp1-Δ1*	In vitro deletion of *RPA34* in YNN281 × YNN282
T4-1d[a]	*MAT***a** *rpa34-Δ::HIS3 ade2-1 ura3-52 lys2-801 his3-Δ200 trp1-Δ1*	In vitro deletion of *RPA34* in YNN281 × YNN282
OG13-1a	*MAT*α *rpa34-Δ::URA3 ade2-1 ura3-52 lys2-801 his3-Δ200 trp1-Δ1*	In vitro deletion of *RPA34* in YNN281 × YNN282
D148-7d	*MAT***a** *cdc6 ura3 tif2::URA3*	32
D125-14d	*MAT*α *cdc6 ura3 ade2-1 trp1-Δ1 his3-Δ200 tif2::URA3*	Offspring of 148-7d × YNN282
MG412	*MAT*α *arg3*	X-ray mutant of 1278B
49-Δa[a]	*MAT***a** *rpa49-Δ::TRP1 ade2-1 ura3-52 lys2-801 his3-Δ200 trp1-Δ129*	
NOY504	*MAT*α *rpa12-Δ::LEU2 ura3-1 trp1-1 his3-11 leu2-3,112 ade2-1 can1-100*	31
NOY505	*MAT***a** *rpa12-Δ::LEU2 ura3-1 trp1-1 his3-11 leu2-3,112 ade2-1 can1-100*	31
SL9-6b[a]	*MAT***a** *ade2-101 lys2-801 ura3-52 trp1-Δ63 his3-Δ200 leu2-Δ1 rpa12-Δ::LEU2*	In vitro deletion of *RPA12* in YPH499 × YPH500
D98-4c[a]	*MAT***a** *rpa34-Δ::HIS3 rpa49-Δ::TRP1 ade2-1 ura3-52 lys2-801 his3-Δ200 trp1-Δ1*	Segregant of 49-Δa × T4-1c
D98-4d[a]	*MAT*α *rpa34-Δ::HIS3 ade2-1 ura3-52 lys2-801 his3-Δ200 trp1-Δ1*	Segregant of 49-Δa × T4-1c
D102-6d[a]	*MAT*α *rpa34-Δ::HIS3 rpa49-Δ::TRP1 ade2-1 ura3-52 lys2-801 his3-Δ200 trp1-Δ1 leu2-Δ1*	Offspring of D98-4c × YPH252
D102-14c[a]	*MAT***a** *rpa34-Δ::HIS3 rpa49-Δ::TRP1 ade2-1 ura3-52 lys2-801 his3-Δ200 trp1-Δ1 leu2-Δ1*	Offspring of D98-4c × YPH252
D121-9c	*MAT***a** *rpa12-Δ::LEU2 rpa34-Δ::HIS3 ade2-1 ura3-1 trp1-1 his3 leu2-3,112*	Offspring of NOY504 × D102-6d
D123-12b	*MAT***a** *rpa49-Δ::TRP1 ade2-1 ura3- his3-11 trp1*	Offspring of NOY505 × D102-6b
D123-12d	*MAT*α *rpa12-Δ::LEU2 rpa49-Δ::TRP1 ade2-1 ura3 trp1 his3 leu2-3,112*	Offspring of NOY505 × D102-6b
D124-2c	*MAT***a** *rpa12-Δ::LEU2 rpa34-Δ::HIS3 rpa49-Δ::TRP1 ade2-1 ura3 trp1 his3 leu2 lys2-801*	Offspring of D123-12d × D121-9c
D124-2a	*MAT***a** *rpa12-Δ::LEU2 rpa34-Δ::HIS3 ade2-1 ura3 trp1 his3 leu2*	Offspring of D123-12d × D121-9c
D124-2d	*MAT*α *rpa12-Δ::LEU2 rpa49-Δ::TRP1 ade2-1 ura3 trp1 his3 leu2-3,112 lys2-801*	Offspring of D123-12d × D121-9c
A14::URA3[a]	*MAT*α *rpa14-Δ::URA3 ade2-1 ura3-52 lys2-801 his3-Δ200 trp1-Δ63 leu2-Δ1*	40
OG15-22b	*MAT***a** *rpa14-Δ::URA3 rpa12-Δ::LEU2 ura3 trp1 his3 leu2 ade2-1 lys2-801* [pNOY102: *ORI*(2μm) *URA3 GAL7*-rDNA]	Offspring of D124-2c × A14::URA3
OG15-32c	*MAT***a** *rpa14-Δ::URA3 rpa34-Δ::HIS3 ura3 trp1 his3 leu2 ade2-1 lys2-801* [pNOY102: *ORI*(2μm) *URA3 GAL7*-rDNA]	Offspring of D124-2c × A14::URA3
OG15-36c	*MAT***a** *rpa14-Δ::URA3 rpa49-Δ::TRP1 ura3 trp1 his3 leu2 ade2-1 lys2-801* [pNOY102: *ORI*(2μm) *URA3 GAL7*-rDNA]	Offspring of D124-2c × A14::URA3
OG15-13c	*MAT***a** *rpa14-Δ::URA3 rpa12-Δ::LEU2 rpa34-Δ::HIS3 rpa49-Δ::TRP1 ura3 trp1 his3 leu2 ade2-1 lys2-801* [pNOY102: *ORI*(2μm) *URA3 GAL7*-rDNA]	Offspring of D124-2c × A14::URA3
GF312-17c	*MAT*α *ura3-52 leu2-3,112 trp1-289 lys2 ade2-Δ ade3-Δ*	Isogenic to GF310-131 (48)
OG1-1c	*MAT*α *rpa34-Δ::HIS3 ura3-52 ade2-Δ ade3-Δ lys2-801 his3-Δ200 trp1 leu2-3,112*	Offspring of T4-1d × GF312-17c
OG17-1c	*MAT*α *top1-Δ::LEU2 rpa34-Δ::HIS3 ura3-52 ade2 ade3-Δ lys2-801 his3-Δ200 trp1 leu2-3,112* [pOG1-A34: *ORI*(2μm) *ADE3 URA3 RPA34*]	In vitro deletion of *TOP1* in OG1-1c
D156-1c	*MAT***a** *rpa34::HIS3 ura3 ade2-Δ lys2-801 his3-Δ200 trp1 leu2*	Offspring of OG1-1c × YPH250
D156-7d	*MAT*α *rpa34::HIS3 ura3 ade2-101 lys2-801 his3-Δ200 trp1-289 leu2 top1-Δ::LEU2*	Offspring of OG1-1c × YPH250

[a] Isogenic to the YPH500 strain stock (39).

The region immediately upstream of *RPA34* contains a divergently transcribed sequence potentially encoding two small nucleolar RNAs involved in rRNA maturation (54). This is reminiscent of the divergent *RPA190-RPA43* gene cluster encoding the A190 and A43 subunits of Pol I (45) and of the adjacent genes encoding the Pol III-specific transcription factor TFIIIA and the ABC23 subunit common to Pol I, II, and III (1, 53). This organization may help in coordinating the synthesis of functionally related gene products via shared promoter elements. However, all of the genes encoding other Pol I subunits are dispersed on the yeast genome, and their immediate neighbor genes are unrelated to rRNA metabolism.

Properties of the amino acid sequence of A34.5. A34.5 is a hydrophilic protein of 233 amino acids, with an average hydrophilicity index of 0.78 and a large proportion of charged residues (98 of 233). The predicted isoelectric point (8.7) is more basic than the experimentally determined value of 7.1 (9), as expected for a phosphoprotein. The discrepancy between the calculated mass (26,847 Da) and apparent mass of 34.5 kDa determined by electrophoretic migration (11) presumably reflects aberrant electrophoretic properties related to this abundance of charged residues.

The most striking feature of A34.5 is its highly charged and predominantly basic C-terminal domain, with the last 44 amino acids including 21 lysines, 3 arginines, 3 histidines, and 11 glutamates. The abundance of lysines generates several potential nuclear localization signals (6), but their biological relevance to nuclear targeting in vivo is doubtful, since a β-galac-

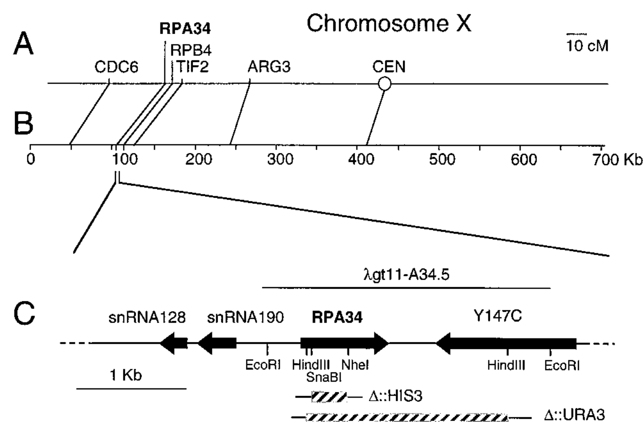

FIG. 1. Organization of the *RPA34* region. (A) Genetic localization of *RPA34* on the left arm of chromosome X, based on the updated version of the MIPS server. The genetic position of *RPA34* was deduced from tetrad analysis using a cross between strains D120-10b (*arg3 ura3 his3 rpa34-Δ::HIS3*) and D125-14d (*ura3 his3 cdc6 tif2::URA3*). A sample of 21 tetrads revealed a loose linkage to *ARG3* (11 parental ditypes [PDT]:9 tetratypes [TT]:2 recombinant ditypes [RDT]) and a close linkage to *TIF2* (15 PDT:6 TT:60 RDT) and *CDC6* (7 PDT:14 TT:0 RDT). CM, centimorgans. (B) Physical map of the left arm of chromosome X. (C) Close-up of the *RPA34* region, showing the genes immediately surrounding *RPA34*, the extent of the initial 112λgt11 clone (isolate 2 of Riva et al. [34]), and the positions of the restriction sites used in this study. The two striped boxes denote the chromosomal fragments deleted in *rpa34-Δ::HIS3* and *rpa34-Δ::URA3*.

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1
MSKLSKDYVS DSDSDDEVIS NEFSIPDGFK KCKHLKNFPL NGDNKKKAKQ
51
QQVWLIKFPS NVDISKLKSL PVDFESSTTM TIDKHDYKIM DDTDIESSLT
101
QDNLSNMTLL VPSESKESLK IASTAKDNAP LQFDKVFSVS ETAKIPAIDY
151
SKVRVPRKDV PKVEGLKLEH FATGYDAEDF HVAEEVKENK KEPKKRSHHD
201
DEEESSEKKK KKKEKKEKRE KKDKKDKKKK HRD
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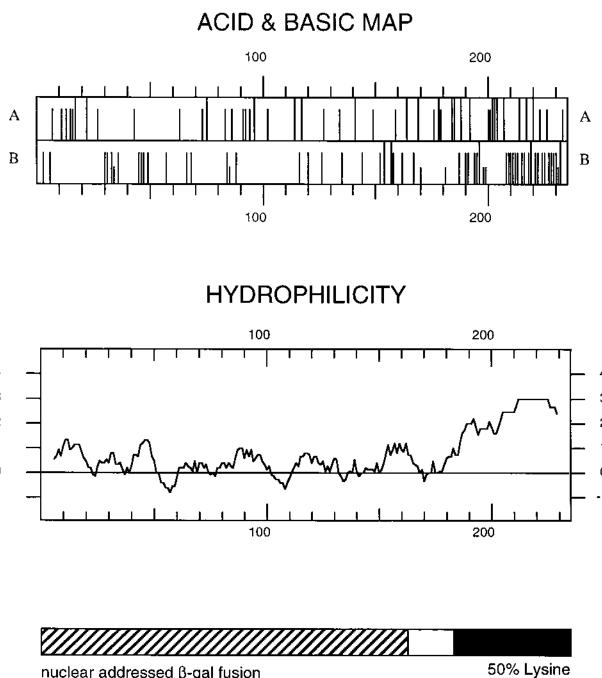

FIG. 2. Characteristics of the amino acid sequence of A34.5. The amino acid sequence indicated is deduced from the nucleotide sequence of the *RPA34* open reading frame. The three microsequenced tryptic peptides are underlined. The diagram immediately below the charge and hydrophilicity profiles identifies two distinct regions corresponding to the positively charged, lysine-rich C terminus (black box) and to the β-galactosidase fusion (striped box) resulting in a nucleus-localized chimeric protein (12).

tosidase fusion to the first two-thirds of A34.5 and thus lacking the basic C terminus, is nevertheless targeted to the nucleus (12) (Fig. 2). Glu, Lys, and Arg are strong α-helix formers (Pα values of 1.59, 1.23, and 1.21), and software-assisted modeling of the last 23 amino acids (using the Sybil software from Tripos, St. Louis, Mo.) showed that this region could be organized as a right-handed α-helix stabilized by ionic pairing.

***RPA34* deletions have little effect on growth.** *rpa34-Δ::HIS3* (hereafter called *rpa34-Δ*) has a deletion of the first half of *RPA34* and insertion of a 1.8-kb fragment containing the *HIS3* gene. *rpa34-Δ::URA3* is a full deletion extending into the C-terminal half of the Y147C open reading frame downstream of *RPA34*, with a *URA3* insertion flanked by stop codons in all six reading frames (Fig. 1). Both deletion mutants are indistinguishable from their isogenic wild-type parents on YPD plates at temperatures ranging from 16 to 37°C. Moreover, there was no detectable effect of *rpa34-Δ* on Pol I activity in vivo, as measured by pulse-labeling in the presence of tritiated uracil (data not shown). Thus, *RPA34* is dispensable under standard laboratory conditions. However, careful growth measurements in liquid cultures revealed a slightly reduced growth rate compared to an isogenic wild-type strain (Fig. 3). We also observed the progressive displacement of *rpa34-Δ* mutant cells in a mixed culture with the wild-type parent (from 45 to 30% of the cell population after six doubling times in liquid YPD), which confirmed the slight competitive disadvantage of the mutant under these growth conditions.

RNA Pol I purified from an *rpa34-Δ* strain lacks the A34.5 and A49 subunits. Pol I was partially purified from isogenic wild-type and *rpa34-Δ* mutant strains, using a nonspecific transcriptional assay on poly(dA-T) to monitor enzyme activity. The active enzyme recovered from the mutant strain (Fig. 4) corresponded to the Pol I* form, which lacks A34.5 and A49 (23). In assays using similar quantities of the purified mutant and wild-type enzymes as determined by Western blotting, the specific activity of the mutant enzyme was reduced by a factor of about 2 compared to the wild-type control, whereas Huet et al. (23) found the activities of the native and Pol I* forms to be similar on poly(dA-T) at low salt concentrations. Thus, A34.5 and A49 do not directly contribute to the catalytic activity of the enzyme.

rpa49-Δ mutants inactivated for A49 grow slowly at 30°C (29) and not at all at 25°C (Fig. 3), in sharp contrast to the fast growth of *rpa34-Δ*. This finding implies that A49 is incorporated into the *rpa34-Δ* mutant Pol I in vivo and that the production of the Pol I* form upon purification of the *rpa34-Δ* cell extracts reflects the instability of the mutant enzyme in vitro. Thus, A34.5 stabilizes the association of A49 with Pol I.

Multiple inactivation of the dispensable subunits A12.2, A14, A34.5, and A49. Figure 3 summarizes the growth defect associated with single deletions of the nonessential subunits of yeast Pol I. Deleting A14 leads to a distinct growth defect at 37°C but is otherwise phenotypically silent (40). Deleting A49 generates a viable but slow-growing mutant at 30°C (29) and completely prevents growth at 25°C. Finally, the *rpa12-Δ* deletion leads to a temperature-sensitive defect with a severe growth defect at 30°C when constructed in a YPH500 strain as in this work (Fig. 3), whereas the *rpa12-Δ* mutant strain NOY504, constructed in the W303 background (31), grows

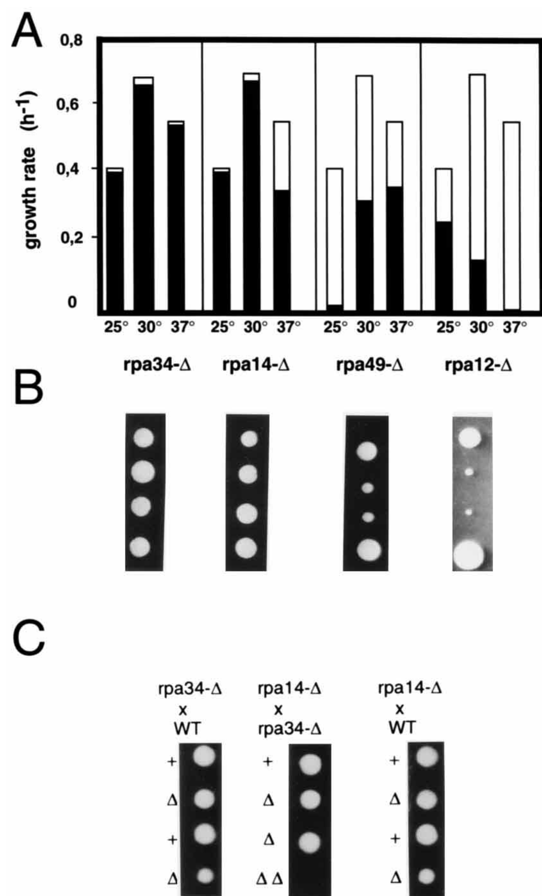

FIG. 3. Growth properties of strains with the dispensable Pol I subunits deleted. (A) Diagram summarizing the growth rates of the mutant strains T4-1c (*rpa34*-Δ), A14::URA3 (*rpa14*-Δ), 49-Δa (*rpa49*-Δ), and SL9-6b (*rpa12*-Δ) compared to their isogenic wild-type parent YPH500 (denoted by white boxes). Table 2 provides complete strain genotypes. Cells were grown in liquid YPD medium at the temperatures indicated, and growth was monitored on a Hach turbidimeter (one turbidimetric unit corresponds to an optical density of 0.008 at 600 nm and to about 10^5 haploid *S. cerevisiae* cells/ml). (B) Individual tetrads from crosses between the same *rpa*-Δ mutants and their isogenic parents YPH500 or YPH499. Cells were grown on YPD at 30°C for 3 to 4 days. (C) Growth properties of single tetrads originating from crosses T4-1d (*rpa34*-Δ) × YNN282 (wild type [WT]), T4-1c (*rpa34*-Δ) × A14::URA3 (*rpa14*-Δ), and A14::URA3 (*rpa14*-Δ) × YNN282 (wild type). The complete strain genotypes are given in Table 2. The genotype of each segregant is summarized on the left side (Δ denotes the *rpa34*-Δ or *rpa14*-Δ allele; + symbolizes the corresponding wild-type alleles). Note the absence of the *rpa14*-Δ *rpa34*-Δ double mutant (ΔΔ) in the case of the T4-1c × A14::URA3 cross. These data were extended to at least 15 tetrads for each cross.

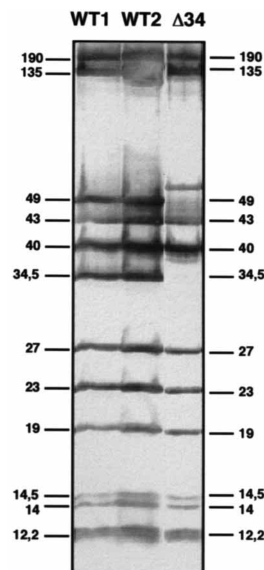

FIG. 4. Subunit composition of Pol I from wild-type and *rpa34*-Δ*::HIS3* (D98-4b) strains. Lane WT1, highly purified wild-type Pol I prepared as described in references 9 and 13; lanes WT2 and Δ34, partially purified Pol I prepared from isogenic wild-type (YNN282 [lane WT2]) and *rpa34*-Δ*::HIS3* (D98-4b [lane Δ34]) strains. See Materials and Methods for the purification procedure. A sample corresponding to 30 μl of the most concentrated glycerol gradient fraction was separated by SDS-polyacrylamide gel electrophoresis, and the subunit composition was determined by Western blot analysis using anti-yeast Pol I antibodies. Note the absence of the A49 and A34.5 subunits in the mutant polymerase compared to the two wild-type controls. A minor band of about 55 kDa present in the mutant preparation corresponds to an unidentified polypeptide that occasionally copurifies with Pol I but is eliminated upon further purification without any detectable effect on enzymatic activity. Sizes are indicated in kilodaltons.

substantially better at 30°C. Tetrad analysis established that this is due to an extragenic suppressor of *rpa12*-Δ that partly restores growth at 30°C.

Given the viability of individual deletions inactivated for either A12.2, A14, A34.5, or A49, we constructed all possible combinations between these deletions and tested them for growth under conditions where rDNA synthesis was either naturally dependent on a functional Pol I or made Pol II dependent by an appropriate genetic construction (32). Viable double or triple mutants inactivated for either A12.2, A34.5, or A49 could be recovered, indicating that the corresponding deletions are epistatic with respect to the capacity to support growth. However, the viable *rpa12*-Δ *rpa49*-Δ double mutants accumulated the temperature-sensitive and cold-sensitive defects of *rpa12*-Δ or *rpa49*-Δ alone and grew less well at 30°C than the individual *rpa12*-Δ and *rpa49*-Δ parents (Table 3). Moreover, they could be recovered only when starting from the *rpa12*-Δ mutant strain constructed in a W303 background (31), not when the same allele was introduced in the YPH500 strain used in this work, an effect which is presumably due to the extragenic suppressor of *rpa12*-Δ described above.

Crosses between *rpa14*-Δ and any of the *rpa12*-Δ, *rpa34*-Δ, or *rpa49*-Δ parents systematically failed to segregate the corresponding double mutants irrespectively of the genetic context used to construct the *rpa12*-Δ allele. Viable *rpa14*-Δ *rpa34*-Δ double mutants could be recovered in the presence of a plasmid-borne wild-type *RPA34* allele. The plasmid could not be lost from the double mutant, although it was readily lost in a wild-type strain under similar conditions, showing that viable double mutants indeed depend on the presence of the complementing plasmid. Similarly, *rpa12*-Δ *rpa14*-Δ and *rpa14*-Δ *rpa49*-Δ double mutants were viable only when bearing the wild-type *RPA14* gene on a replicative plasmid. Finally, all of the double, triple, or quadruple mutants simultaneously lacking A14 and/or A12.2, A34.5, or A49 subunits could be recovered in the presence of pNOY102 (32), a plasmid harboring the 35S rRNA gene under the transcriptional control of the Pol II-dependent promoter of *GAL7* (Fig. 5). These mutant strains were strictly dependent on the presence of pNOY102 and could grow only in the presence of galactose, which demonstrates that the colethality between *rpa14*-Δ and *rpa12*-Δ, *rpa34*-Δ, or *rpa49*-Δ is entirely due to a defect in rDNA transcription.

In summary, a coherent pattern of genetic interactions discriminates between A14 (which becomes essential upon inac-

TABLE 3. Growth pattern of multimutant strains defective in the Pol I-specific subunit A12.2, A34.5 or A49

Strain	Genotype (Pol I subunits)[a]	Doubling time[b]	Growth pattern[c]		
			25°C	30°C	37°C
YPH500	Wild type	1 h 40 min	++	+++	+++
NOY504	*rpa12*-Δ::*URA3*	2 h 30 min	++	++	−
D98-4d	*rpa34*-Δ::*HIS3*	1 h 45 min	++	+++	+++
D123-12b	*rpa49*-Δ::*TRP1*	2 h 30 min	−	++	++
D124-2a	*rpa12*-Δ::*URA3 rpa34*-Δ::*HIS3*	2 h 40 min	++	++	−
D124-2d	*rpa12*-Δ::*URA3 rpa49*-Δ::*TRP1*	4 h 40 min	(−)	+	−
D102-14c	*rpa34*-Δ::*HIS3 rpa49*-Δ::*TRP1*	2 h 10 min	−	++	++
D124-2c	*rpa12*-Δ::*URA3 rpa34*-Δ::*HIS3 rpa49*-Δ::*TRP1*	4 h 20 min	−	+	−

[a] Complete genotypes are in Table 2.
[b] In liquid YPD at 30°C.
[c] Growth was scored on solid medium (YPD plates) according to a decreasing scale: +++, ++, +, (−), −.

tivation of any of the other three subunits) and a group defined by subunits A12.2, A34.5, and A49, which can be simultaneously inactivated in viable triple mutants. The fact that synthetic-lethal effects can be rescued by expressing pre-rRNA from a Pol II-specific promoter demonstrates that these subunits are exclusively involved in rRNA synthesis. Finally, the genetic inactivation of A12.2 and A49 has cumulative effects in vivo.

Synergy between A34.5 and DNA topoisomerase I. There are numerous indications that DNA topoisomerase I plays a critical role in the transcription of yeast rDNA and yet *top1*-Δ deletions inactivated for DNA topoisomerase I have little or no effect on growth, which is at least partly due to a functional redundancy with the other two yeast DNA topoisomerases, topoisomerases II and III (see reference 49 for a review). Remarkably, *top1*-Δ *rpa34*-Δ double mutants simultaneously inactivated for DNA topoisomerase I and subunit A34.5 have a very slow growth rate, as shown by the genetic recombination and complementation tests illustrated in Fig. 6. Tetrad analysis data show that a cross between an *rpa34*-Δ and a *top1*-Δ parent generates slow-growing segregants which are invariably double mutant (Fig. 6B). To confirm that this slow growth was indeed due to the *rpa34*-Δ deletion itself (rather than to a spurious mutation genetically linked to *RPA34*), we carried out a genetic complementation test in which the *rpa34*-Δ × *top1*-Δ cross was done in the presence of pOG1-A34, a *URA3*-bearing plasmid with a wild-type copy of *RPA34*. This time, the *top1*-Δ *rpa34*-Δ segregants had a wild-type-like growth rate (Fig. 6A). As expected, chasing the *URA3* plasmid pOG1-A34 in the presence of fluoro-orotic acid (4) restored the slow-growth-rate pattern.

The synthetic lethality observed between the genetic inactivation of DNA topoisomerase I and A34.5 is specific to the latter subunit, since crosses between *top1*-Δ and any one of the *rpa12*-Δ, *rpa14*-Δ, and *rpa49*-Δ mutations inactivated for the other three dispensable subunits yielded a normal 2:2 segregation of viable *top1*-Δ segregants (Fig. 5 and data not shown). The Pol II enzyme also contains an enzyme-specific component, subunit B32, that is dispensable in vivo since the corresponding deletion (*rpb4*-Δ) mutants are slow growing but viable (52). In this case too, tetrad analysis showed no synthetic lethality between *rpb4*-Δ and *top1*-Δ.

DISCUSSION

Fourteen polypeptides are biochemically associated with purified yeast Pol I. Their genetic characterization, started 10 years ago by Riva et al. (34), had so far identified 13 of the corresponding genes (references 13 and 46 and references therein). We extend this study to the *RPA34* gene, encoding subunit A34.5. A34.5 is a 34.5-kDa phosphoprotein copurifying with yeast Pol I (7, 11), but a biologically irrelevant copurification could not be ruled out since a simplified Pol I* form lacking polypeptides A34.5 and A49 is catalytically active in

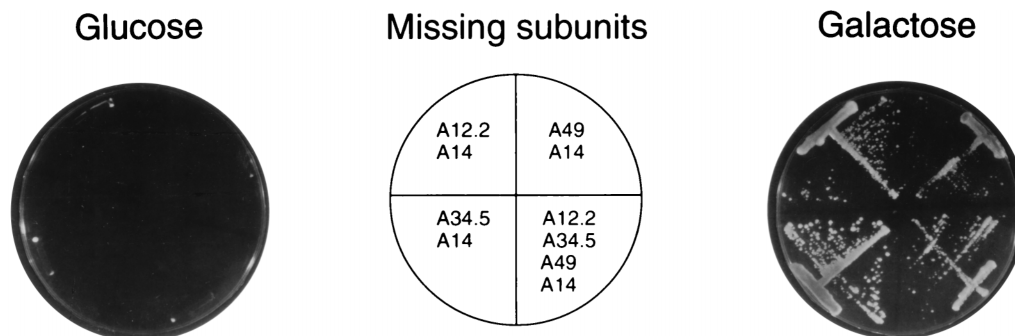

FIG. 5. Growth properties of mutant strains inactivated for subunit A14 and for one or all three of the A12.2, A34.5, and A49 subunits: rescue by Pol II-dependent transcription of the rDNA gene. Double mutants combining the *rpa14*-Δ::*URA3* deletion with *rpa34*-Δ::*HIS3* (strain OG15-32c), *rpa12*-Δ::*LEU2* (strain OG11-22b), or *rpa49*-Δ::*TRP1* (strain OG15-36c) and one quadruple mutant inactivated for all four subunits (strain OG15-13c) were complemented by the 2μm *URA3 GAL7*-rDNA plasmid pNOY102, which allows Pol II-dependent synthesis of the 35S pre-rRNA precursor using the galactose-inducible promoter of *GAL7* (32). The genetic pedigrees and complete genotypes of these strains are given in Table 2. Yeast cells were streaked on glucose-containing medium (left; repression of the *GAL7* promoter) and galactose-containing medium (right; induction of the *GAL7* promoter). Growth was monitored after 5 days at 30°C. Note that growth is supported only on the galactose medium, indicating a strict dependency on the transcriptional synthesis of the 35S rRNA precursor. The missing subunits are denoted by their conventional symbols A12.2, A14, A34.5, and A49.

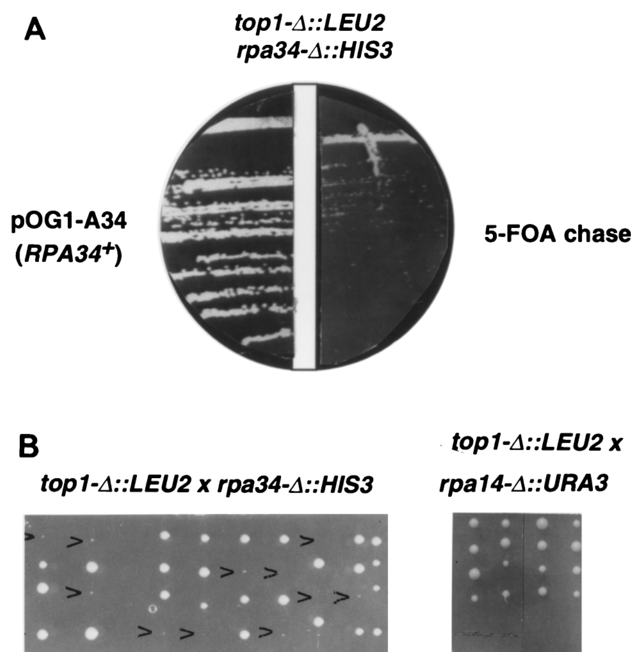

FIG. 6. Synergy between the *top1-Δ::LEU2* and *rpa34-Δ::HIS3* alleles. (A) Slow growth of the *top1-Δ::LEU2 rpa34-Δ::HIS3* strain OG17-1c bearing the *RPA34*$^+$ plasmid pOG1-A34 (see Table 2 for complete genotypes) after 4 days on YPD at 30°C (right sector) compared to an isogenic strain bearing the *RPA34*$^+$ plasmid. (B) Tetrad analysis from a cross between strains D156-7d (*top1-Δ::LEU2*) and D156-1c (*rpa34-Δ::HIS3*). Meiotic asci yielded very slowly growing segregants that are barely detectable after 3 days at 30°C (the corresponding microcolonies are denoted by arrows). The frequency of these segregants (about 25%) was typical of a two-gene segregation. Also shown are the normal (*rpa34-Δ*) and almost normal (*top1-Δ*) growth patterns of the single-mutant parents. The first tetrad corresponds, from top to bottom, to the following segregants: *rpa34-Δ top1-Δ*, *top1-Δ* (note the slightly reduced growth), + +, and *rpa34-Δ*. Upon further growth for 8 days at 30°C, the double-mutant segregants eventually formed medium-sized colonies that were reisolated and tested for prototrophy on leucine and histidine omission media. They were all prototrophic for both characters, indicating that they are *top1-Δ rpa34-Δ* recombinants. The right panel illustrates the viable segregation in a control cross between D156-7d (*top1-Δ::LEU2*) and A14::URA3 (*rpa14-Δ::URA3*), documenting a lack of synthetic lethality between *rpa14-Δ* and *top1-Δ*.

vitro when tested on nonspecific DNA templates (23). Indeed, our genetic and physiological data establish that A34.5 functionally belongs to the Pol I transcription complex but also show that this functional association is of a subtle nature, since loss-of-function mutants have essentially no growth phenotype in an otherwise wild-type background.

The amino acid sequence of A34.5 suggests no obvious function in terms of conserved amino acid motifs. Nevertheless, its strongly hydrophilic nature and numerous polar and charged residues point to a peripheral location on Pol I, in keeping with its easy dissociation from the enzyme during purification (23). In particular, a positively charged C-terminal domain, possibly structured in an α-helix, may protrude from A34.5 and from the whole Pol I protein. In agreement with previous immunological data (10, 24), there is no significant sequence homology to components of the other two eukaryotic transcription machines or, for that matter, to any amino acid sequence predictable from the yeast DNA genome. A34.5 therefore has no structural counterpart in the other two transcription complexes. In fact, it bears no resemblance to any other known or predicted protein in current data banks and might conceivably be lacking in other eukaryotic phyla.

The Pol I-specific subunits A49, A14, and A12.2 are dispensable for growth at 30°C (29, 31, 40) but are essential (A12.2 and A49) or important (A14) at 25 or 37°C. The genetic inactivation of A34.5 has instead hardly any effect on growth and rRNA synthesis in vivo. Minor growth defects could be detected only by careful growth rate measurements and by competition experiments with isogenic wild-type strains. Moreover, the Pol I* form lacking A34.5 and A49 is catalytically active in vitro when tested on nonspecific DNA templates, which rules out a direct role in the DNA-dependent RNA polymerization reaction. The multiple-mutant combinations inactivated for two, three, or four of the dispensable subunits were all tested for the ability to grow on rich medium at 30°C, i.e., under conditions where the single mutants are all viable. This finding showed that yeast cells simultaneously inactivated for A49, A34.5, and A12.2 are still viable (at least in some laboratory strain backgrounds) but that their inactivation is invariably lethal in a *rpa14-Δ* strain lacking A14. These subunits therefore fall into two classes (A14 and A49/A34.5/A12.2) that cannot be simultaneously inactivated.

The double, triple, and even quadruple mutants devoid of all the dispensable subunits are rescued under conditions where the rDNA genes are artificially made Pol II dependent (32) by expressing a plasmid-borne rDNA under the control of a Pol II-specific promoter. Thus, these dispensable subunits functionally belong to Pol I, and this is their only essential function, as was already known to be the case for A12.2 (31). What is, therefore, their role in Pol I-dependent transcription? As discussed below, three possibilities come to the mind on the basis of currently available data on A34.5. A structural role in maintaining the quaternary structure of Pol I is indicated by the properties of the *rpa34-Δ* mutant enzyme and also by its synthetic lethality with *rpa14-Δ*. A more questionable role in the specific recognition of a hypothetical UBF-like preinitiation factor is indirectly suggested by the curious observation that A34.5 binds the murine UBF factor in vitro (36). Finally, a role in resolving the topological constraints raised during rDNA transcription is suggested by the very poor growth of *rpa34-Δ* mutants lacking type I DNA topoisomerase I.

In vitro, the purified *rpa34-Δ* mutant enzyme is entirely of the Pol I* type (23), which means that it retains all Pol I subunits except A34.5 itself and the semidispensable subunit A49. A49 is presumably retained in vivo, since strains inactivated for A49 (*rpa49-Δ*) or for both A34.5 and A49 (*rpa34-Δ rpa49-Δ*) have a clear-cut growth defect, in contrast to the fast-growing *rpa34-Δ* cells. Thus, one of the main effects of A34.5 could well be to stabilize the binding of A49 to the core Pol I. This raises the intriguing possibility that the Pol I* form, which is also detected upon purification of a wild-type strain (23), is physiologically relevant and coexists in vivo with the complete enzyme. A parallel characterization of the *rpa14-Δ* mutant yielded a catalytically inactive enzyme that lacks the essential subunits ABC23 and A43 but retains all other subunits, including A12.2, A34.5, and A49 (40). Mutants lacking either A14 or A34.5 therefore have nonoverlapping effects on the quaternary structure of Pol I. We propose that these two subunits cooperate in vivo to anchor a subset of three essential (A43 and ABC23) and semidispensable (A49) subunits on the heteromultimeric structure of Pol I, which would provide a simple explanation of the synthetic lethality of the corresponding mutants (Fig. 7).

Studies on yeast Pol III suggest that three of its specific subunits (C31, C34, and C82) allow the specific recruitment of Pol III through a direct interaction between C34 and the $TFIIIB_{70}$ component of the preinitiation complex (44, 50). In the case of yeast Pol I, preinitiation depends on the TATA-box-binding protein (18, 38), on a strictly essential initiation

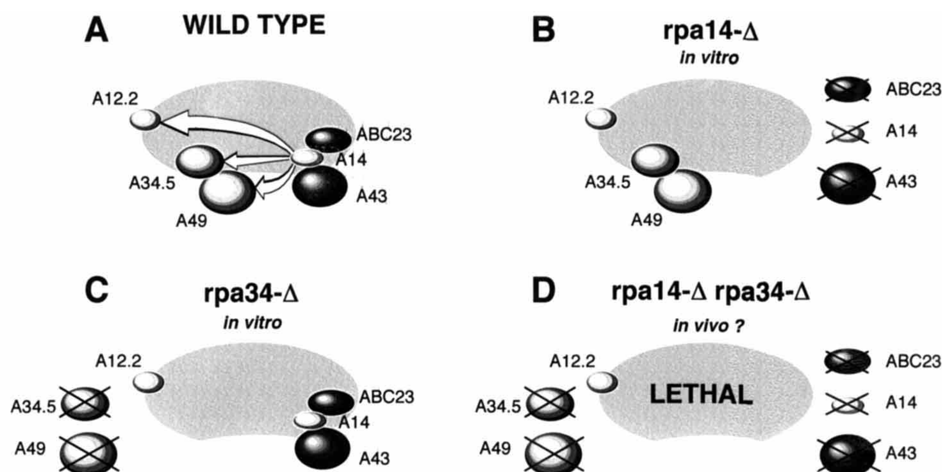

FIG. 7. A tentative model explaining the synthetic lethality of A14 (*rpa14*-Δ) and A34.5 (*rpa34*-Δ). (A) Possible spatial relationships between Pol I subunits in the wild-type enzyme, based on previous biochemical and genetic data (23, 40) and on the present work. Two groups of subunits (A14/A43/ABC23 and A34.5/A49) that are lost in vitro upon inactivation of either A14 or A34.5 are represented by two sets of ovals. Overlapping ovals correspond to subunits believed to be in topological contact on the basis of the purification properties of mutant forms lacking A14 (40) or A34.5 (this work), but their relative orientation is arbitrary. Essential subunits (ABC23, present in all three polymerases, and the Pol I-specific subunit A43) are dark shaded, whereas light-shaded ovals define the dispensable polypeptides A14, A34.5, and A49. A fourth nonessential subunit, A12.2, is arbitrarily located outside the two groups' interacting subunits. Arrows denote synthetic-lethality effects between dispensable subunits. The large grey-shaded form symbolizes the remaining core of Pol I subunits. (B and C) In vitro subunit composition in the viable single mutants inactivated for A14 (B) or A34.5 (C). (D) Probable consequences on the double mutant in vivo, assuming that it cumulates the effects observed in vitro on the single mutants.

factor formed of three distinct subunits (27, 28), and on a somewhat dispensable heteromultimeric factor, UAF, that binds to the upstream promoter element of the rDNA gene (26). In mammals, Pol I initiation depends on several transcription factors, including UBF, a monomeric protein that carries five high-mobility-group boxes and a strongly acidic C tail and that binds to the upstream region of the mammalian rDNA locus (25). In vitro, UBF directly binds to murine Pol I, and this binding appears to be mediated by a 53-kDa component of Pol I that is a homolog of the yeast A49 subunit (21). However, there is no obvious equivalent of UBF, as judged from the yeast genomic sequence. The mammalian UBF was also found to bind A34.5 in a far-Western blotting assay (36). This is not due to a trivial electrostatic contact between the basic C terminus of A34.5 and the acidic C terminus of UBF, since a UBF form devoid of its acidic tail still binds A34.5. Nevertheless, the interaction is probably nonspecific, as A34.5 also binds several yeast proteins unrelated to Pol I, such as the τ138, τ131, and τ95 subunits of the Pol III transcription factor TFIIIC or the TFIIIA and TFIIIB70 factors also participating in the Pol III transcription system (17a).

Our observation that mutants lacking DNA topoisomerase I are slow growing when they also lack A34.5 suggests that A34.5 specifically helps the Pol I machine to overcome the topological constraints imposed on the rDNA during transcription. *top1*-Δ mutants lacking DNA topoisomerase I have little or no effect on growth, but this is due to the presence of other topoisomerases since, in combination with a temperature-sensitive *top2* allele, *top1*-Δ mutants are defective in pre-rRNA elongation with little or no adverse effect on mRNA synthesis (8, 37, 47). *top1*-Δ mutants also have fairly dramatic effects on the electrophoretic mobility of rDNA (16) and on its accessibility to psoralen (14), and they enhance mitotic recombination between rDNA repeats (15). These specific effects on the rDNA locus presumably reflect its particularly heavy transcription and suggest that supercoil removal is a major rate-limiting factor in Pol I-mediated transcription. In addition to the direct removal of DNA supercoil by DNA topoisomerases, there might therefore be a need for other mechanisms carried out by Pol I itself through its A34.5 subunit and allowing the topological constraints inherent to rDNA transcription to be overcome.

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