Nucleic Acids Research, 1999, Vol. 27, No. 4 **1047–1055**

Sequence divergence of the RNA polymerase shared subunit ABC14.5 (Rpb8) selectively affects RNA polymerase III assembly in *Saccharomyces cerevisiae*

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Received October 9, 1998; Revised and Accepted December 18, 1998

DDBJ/EMBL/GenBank accession nos Y07643 and Y07644

ABSTRACT

ABC14.5 (Rpb8) is a eukaryotic subunit common to all three nuclear RNA polymerases. In *Saccharomyces cerevisiae*, ABC14.5 (Rpb8) is essential for cell viability, however its function remains unknown. We have cloned and characterised the *Schizosaccharomyces pombe rpb8*$^+$ cDNA. We found that *S.pombe* rpb8, unlike the similarly diverged human orthologue, cannot substitute for *S.cerevisiae* ABC14.5 *in vivo*. To obtain information on the function of this RNA polymerase shared subunit we have used *S.pombe* rpb8 as a naturally altered molecule in heterologous expression assays in *S.cerevisiae*. Amino acid residue differences within the 67 N-terminal residues contribute to the functional distinction of the two yeast orthologues in *S.cerevisiae*. Overexpression of the *S.cerevisiae* largest subunit of RNA polymerase III C160 (Rpc1) allows *S.pombe* rpb8 to functionally replace ABC14.5 in *S.cerevisiae*, suggesting a specific genetic interaction between the *S.cerevisiae* ABC14.5 (Rpb8) and C160 subunits. We provide further molecular and biochemical evidence showing that the heterologously expressed *S.pombe* rpb8 molecule selectively affects RNA polymerase III but not RNA polymerase I complex assembly. We also report the identification of a *S.cerevisiae* ABC14.5-G120D mutant which affects RNA polymerase III.

INTRODUCTION

Yeast nuclei contain three DNA-dependent RNA polymerases responsible for the synthesis of rRNAs (RNA pol I), mRNAs (RNA pol II), tRNAs (RNA pol III) and small nuclear RNAs (RNA pol II and RNA pol III). The three enzymes differ in their nuclear location, chromatographic behaviour and inhibitor sensitivity and they interact with distinct sets of transcription factors in order to locate appropriate promoters and initiate transcription (1,2). However, they have structural and functional similarities that reflect their common origin and their shared functions (3). Biochemical and genetic analyses have shown that all purified eukaryotic nuclear RNA polymerases are composed of a homologous core of four subunits, share another five subunits and are associated with several enzyme-specific subunits (4,5). The four core subunits comprise two large polypeptides that are homologous to the two largest subunits (β and β′) of the bacterial core enzyme ($\alpha_2\beta\beta'$) and form the catalytic centre of the enzyme (6–8) and two smaller polypeptides that are related to the bacterial α subunit and are required for RNA polymerase complex assembly (9–11). The five common subunits ABC27 (Rpb5), ABC23 (Rpb6), ABC14.5 (Rpb8), ABC10α (Rpb10α) and ABC10β (Rpb10β) have no bacterial counterparts while three of them (ABC27, ABC23 and ABC10β) are related to bona fide subunits of the archaeal RNA polymerase (12). (Nomenclature of RNA polymerase subunits varies; 4,5; Table 1.)

Table 1. RNA polymerase gene and subunit nomenclature

Organism	Gene	Subunit
Saccharomyces cerevisiae	*RPA190*	A190 or Rpa1
	RPB1	B220 or Rpb1
	RPC160	C160 or Rpc1
	RPB5	ABC27 or Rpb5
	RPB6	ABC23 or Rpb6
	RPC10	ABC10α or Rpb10α
	RPB10	ABC10β or Rpb10β
	RPB8	ABC14.5 or Rpb8
Schizosaccharomyces pombe	*rpb8*$^+$	rpb8
Homo sapiens	*POL2RH*	hRPB17 or hsRpb8

Nomenclature according to various biochemical (4) and genetic (5) definitions for the RNA polymerase subunits and genes mentioned in this paper.

In *Saccharomyces cerevisiae*, the five shared subunits are encoded by single copy genes that are essential for cell viability (13,14). However, so far their role in transcription remains elusive and it is not known whether they mediate similar functions in all three RNA polymerases. ABC23 is necessary for RNA polymerase I and II complex assembly, for the stability of the

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largest subunits of these enzymes (15) and for the structural and functional integrity of RNA polymerase I (16). ABC23, ABC10α and ABC10β can be replaced *in vivo* by both their *Homo sapiens* and *Schizosaccharomyces pombe* counterparts (17–19) while ABC14.5 (Rpb8) can be replaced by its *H.sapiens* orthologue at 30°C but not at 37°C (19). The ABC14.5 (Rpb8) subunit is of special interest because it is one of the two (ABC14.5 and ABC10α) common subunits that have no homologue detectable in archaeal RNA polymerases (20). Recently, it has been reported that the human orthologue of ABC14.5 interacts with the largest subunit of RNA polymerase II and with ABC23 (Rpb6) (21).

We have isolated the *S.pombe* cDNA encoding the ABC14.5 (Rpb8) orthologue protein. We found that the *S.pombe* rpb8 protein, unlike the similarly diverged human Rpb8, could not replace the *S.cerevisiae* orthologue subunit in an *rpb8*Δ strain. Considering that the *S.pombe* rpb8 sequence contains alterations significant for the function of this subunit in *S.cerevisiae*, we further investigated its specific effects by heterologous expression (in *S.cerevisiae*). Our data are the first indication that sequence alterations in a common subunit specifically affect one class of RNA polymerase *in vivo*. We additionally report the isolation of a *S.cerevisiae* ABC14.5-G120D mutant that also affects RNA polymerase III.

MATERIALS AND METHODS

Strains and media

Saccharomyces cerevisiae strains used for the plasmid shuffling complementation assays are derivatives of YSL171 [MAT**a** *his3-*Δ*200 lys2-*Δ*201 leu2-3,112 ura3-52 ade2-1 rpb8*Δ*1*::*LYS2* (pSL103: *CEN URA3 RPB8*)] (14). Rich and minimal growth media were as previously described (22). The plasmids used (Table 2) are derivatives of pBluescript, Yep351, pRS315 (Stratagene), pDB20 (23), pYeF1H (24), pNOY16 (25) and pJA483b (26).

Genetic screens

Schizosaccharomyces pombe rpb8 cDNAs were isolated in the course of a genetic screen, independent of this work, for functional complementation of the *S.cerevisiae gcn2*Δ mutation (27). The reason why rpb8 was identified in this screen is probably because it interferes with the function of RNA polymerases resulting in an overall protein synthesis decrease which consequently favours Gcn4 expression that overcomes the *gcn2* mutation. Our selection for *gcn2*Δ-complementing cDNAs relied on the inability of the *gcn2*Δ strain to grow under amino acid starvation conditions [i.e on minimal medium containing 3-aminotriazole (3-AT) that causes histidine starvation] (28). The *gcn2*Δ *leu2-2 ura3-52 GCN4-lacZ* strain was transformed with a *S.pombe* cDNA library, provided by J. D. Fikes and L. Guarente (23), carried in the pDB20 expression vector. A screen of 60 000 Ura^+ transformants yielded two plasmids, pG3 and pB3, that conferred a $3\text{-}AT^R$ phenotype on minimal medium containing 10 mM 3-amino-1,2,4-triazole (28) and blue colour on X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) indicator plates. The cDNA inserts from pG3 and pB3 were subcloned into the *Not*I site of pBluescript and sequenced (29).

pDBSc8(ABC14,5) (Table 2) DNA was *in vitro* mutagenised with hydroxylamine and used to transform the *gcn2*Δ *leu2-2 ura3-52 GCN4-lacZ* strain. Plasmids that conferred $3\text{-}AT^R$ phenotypes were selected.

A YEP13 *S.cerevisiae* genomic DNA library, constructed by K.Nasmyth, was used for the suppression of lethality of the YSL171 strain containing only the *S.pombe* rpb8 subunit following plasmid shuffling complementation.

In vivo labelling

Cells were grown in minimal medium supplemented with 0.35 mM adenine, at 30°C, to an OD_{550} of 0.3–0.4. The *ura3* mutation was complemented by the *URA3*-expressing plasmid pDB20. An aliquot of 0.5 mCi of [5,6-^{3}H]uracil (Amersham) was added to a 20 ml culture; following labelling for 30 min, a 1000-fold excess (final concentration 200 mM) of unlabelled uracil was added and incubation continued for a further 30 min. RNA was extracted and analysed by polyacrylamide gel electrophoresis as previously described (30).

RNA polymerase I and III purification and analysis

Cells were grown in minimal medium with the required supplements, at 30°C, to an OD_{550} of 0.8–1.0. RNA polymerases III (31) and I (32) were purified as previously described from 10–20 g of cells.

Purified RNA polymerases I and III were analysed by electrophoresis in a 13% SDS–polyacrylamide gel (33) and silver staining (34). Alternatively, following electrophoresis, samples were transferred onto a PVDF membrane (Millipore) for western blot analysis, incubated with the anti-HA-epitope or anti-RNA polymerase subunits (35) and visualised by chemiluminescence (ECL; Amersham). RNA polymerase III activity was assayed in a non-specific [poly(dA-dT) template] or a specific (*U6* snRNA gene template) *in vitro* transcription assay (6).

RESULTS

The *S.pombe* rpb8 subunit is unable to functionally substitute for its *S.cerevisiae* counterpart

Two *S.pombe rpb8* cDNAs were isolated in our laboratory in a genetic screen, independent of this work (Materials and Methods). The two cDNAs, of 0.8 and 1.0 kb, respectively, correspond to two mRNAs transcribed from a single copy gene (data not shown). Nucleotide sequence analysis revealed that the two cDNAs differed in the length of their 3′-untranslated regions and contained an open reading frame (ORF) of 125 amino acid residues (EMBL accession nos Y07643 and Y07644). Sequence analysis indicated that *S.pombe* rpb8 does not contain any known consensus sequence. Multiple alignment of *S.pombe* rpb8, *S.cerevisiae* ABC14.5 and the human orthologue hRPB17 sequences showed that similarities among the three proteins are spread out over their entire sequences (Fig. 1). ABC14.5 and rpb8 display in total 44% identity and 63% similarity (BESTFIT alignment of the GCG package software) whereas ABC14.5 and its human orthologue are 38% identical and 66% similar. A region of 21 amino acids (residues 68–88) of ABC14.5 is not present in its *S.pombe* counterpart. According to our multiple alignment only six of those residues are missing from the human sequence.

Table 2. Plasmids used in this study

Plasmid	Description
pDB20+, pDB20–	Modified versions of pDB20 (*URA3*, 2μ) created by inserting a linker sequence containing the *Eco*RI, *Xho*I, *Xba*I and *Not*I sites into the *Hin*dIII site in both orientations
pB3	pDB20 (*URA3*, 2μ ori p*ADCI*) containing the ~1000 bp insert *rpb8* cDNA
pG3	pDB20 containing the ~800 bp insert *rpb8* cDNA
pDBSp8(rpb8)	pDB20+ containing an ~350 bp PCR fragment of the *S.pombe rpb8* ORF with *Eco*RI ends, ligated in the correct orientation relative to the promoter to the *Eco*RI site
pDB1+/–	pDB20+/– containing the ~1800 bp *Bam*HI fragment including the *HIS3* gene (42) filled in using Klenow DNA polymerase and blunt end ligated to the filled in *Nco*I site
pDBScp81(rpb8)	pDBSp8 containing the ~1800 bp *Bam*HI fragment including the *HIS3* gene filled in using Klenow DNA polymerase and blunt end ligated to the filled in *Nco*I site
pYeF1HA	Modified version of pYeF1H(2μ) (7). The ~800 bp *Apa*I–*Cla*I fragment, containing the promoter *GAL10-CYC1*, was replaced by the ~1500 bp *Bam*HI–*Xba*I fragment from pDB20+, containing the promoter *ADCI*. The latter fragment was filled in using Klenow DNA polymerase and blunt end ligated to the filled in *Apa*I–*Cla*I sites of pYeF1H
pYSp8(HA-rpb8)	pYeF1HA containing the N-terminus of the *S.pombe rpb8* ORF fused to the HA epitope. An ~350 bp PCR fragment containing the *rpb8* ORF of *S.pombe* with *Eco*RI ends was ligated (in-frame) to the *Eco*RI site
pDBSc8(ABC14.5)	pDB20+ containing an ~400 bp PCR fragment including the *RPB8* ORF of *S.cerevisiae*, with *Not*I ends, ligated (in the correct orientation relative to promoter DNA) to the *Not*I site
pDBSc81(ABC14.5)	pDBSc8 containing the ~1800 bp *Bam*HI fragment including the *HIS3* gene, filled in using Klenow DNA polymerase and blunt end ligated to the filled in *Nco*I site
pDBSc8Δ21(ABC14.5Δ68–88)	pDB20+ containing two PCR fragments, Sc1–67 and Sc89–146, corresponding to amino acids 1–67 and 89–146 of the *RPB8* coding region, respectively, generated from pDBS8. Sc1–67 contains an *Eco*RI site in front of the ATG codon and a blunt 3′-end. Sc89–146 contains a blunt 5′-end and a *Xba*I site following the stop codon. The two PCR fragments were simultaneously ligated to the *Eco*RI and *Xba*I sites of pDB20+
pDBSc81Δ21(ABC14.5Δ68–88)	pDBSc8Δ21 containing the ~1800 bp *Bam*HI fragment including the *HIS3* gene, filled in using Klenow DNA polymerase and blund end ligated to the filled in *Nco*I site
pDBScSp(ScSp)	pDB1– containing two PCR fragments, Sc1–88 and Sp68–125, corresponding to amino acids 1–88 of *S.cerevisiae* ABC14.5 and 68–125of *S.pombe* rpb8, were generated from pDBSc8 and pDBSp8, respectively. The Sc1–88 DNA fragment was amplified using 5′- ATAAGAAAGCGGCCGCAGCAATGTCTAACACTC-3′ and 5′-GCTCTAGATCTGTCACCAGCCTGTGG-3′ primers introducing a *Not*I site in front of the ATG codon and a *Xba*I site at the 3′-end (by replacing the S88 codon TCC by TCT). The r68–126 fragment was amplified with 5′-GCTCTAGAAAGGAAGCTGCTGATTAT-3′ and 5′-GGAATTCCCACGATCATTATTTACC-3′ primers introducing a *Xba*I site at the 5′-end (by replacing L68 by R) and an *Eco*RI site following the stop codon. The two PCR fragments were simultaneously ligated to the *Not*I and *Eco*RI sites of pDB20–
pDBSpSc(SpSc)	pDB1+ containing two PCR fragments, Sp1–67 and Sc88–146, corresponding to amino acids 1–67 of *S.pombe* rpb8 and 87–146 of *S.cervisaie* ABC14.5, generated from pDBSp8 and pDBSc8, respectively. The Sp1–67 fragment was amplified with 5′- GGAATTCCATGTCGGAATCCGTAC-3′ and 5′-GAAGATCTATCAGGGCTATTCAAATT-3′ primers introducing an *Eco*RI site in front the ATG and a *Bgl*II site following D67. Sc87–146 was amplified with 5′-GAAAGATCTCTTGCAGATGATTATGAT-3′ and 5′- ATAGTTTAGCGGCCGCGCTGCTAACGACGAATC-3′ primers introducing a *Bgl*II site at the 5′-end (by replacing S88 codon TCC by TCT) and a *Not*I site following the stop codon. The two PCR fragments were simultaneously ligated to the *Eco*RI and *Not*I sites of pDB20+
pDBSc8m(ABC14.5-G120D)	Derived from pDBP8 (*URA3*, 2μ) by hydroxylamine mutagenesis.
pDBSc81m(ABC14.5-G120D)	pDBSc8m containing the ~1800 bp *Bam*HI fragment including the *HIS3* gene, filled in using Klenow DNA polymerase and blund-end ligated to the filled in *Nco*I site
pYeC(C160)	Yep351 (*URA3*, 2μ) containing an ~5.5 kb *RPC160*-including PCR fragment with *Sma*I ends ligated to the *Sma*I site
pYeA(A190)	Yep351 (*URA3*, 2μ) containing the ~5.9 kb *Pvu*II–*Xba*I *RPA1*90-including fragment of pNOY16 filled in using Klenow DNA polymerase and blunt end ligated to the *Sma*I site
pYeB(B220)	Yep351 (*URA3*, 2μ) containing the ~5.7 kb *Eco*RI–*Hin*dIII *RPB1*-including fragment of pJA483b filled in using Klenow DNA polymerase and blunt end ligated to the *Sma*I site

In spite of the sequence similarities between the two yeast orthologues and in spite of the fact that the slightly more divergent human sequence can functionally complement an *rpb8*Δ strain, we found that the *S.pombe* rpb8 cannot substitute for the *S.cerevisiae* ABC14.5 subunit. An *rpb8*Δ strain expressing both the *S.cerevisiae* and the *S.pombe* subunit proteins from two different plasmids was tested for viability by a plasmid shuffling complementation assay (22). To express the *S.pombe* protein in a similar context to the endogenous ABC14.5, the *S.pombe rpb8* coding region was fused downstream of the *S.cerevisiae RPB8* gene promoter and 5′-untranslated region and the *ADC1* terminator was added. A similar construct (to ensure comparable levels of expressed protein) containing the *S.cerevisiae RPB8* coding region was used as a positive control. In that assay, only cells expressing the *S.cerevisiae* ABC14.5 protein were able to grow (data not shown), suggesting that the *S.pombe* protein could not functionally substitute for its *S.cerevisiae* counterpart *in vivo*. The same result was observed when *S.pombe rpb8* was expressed

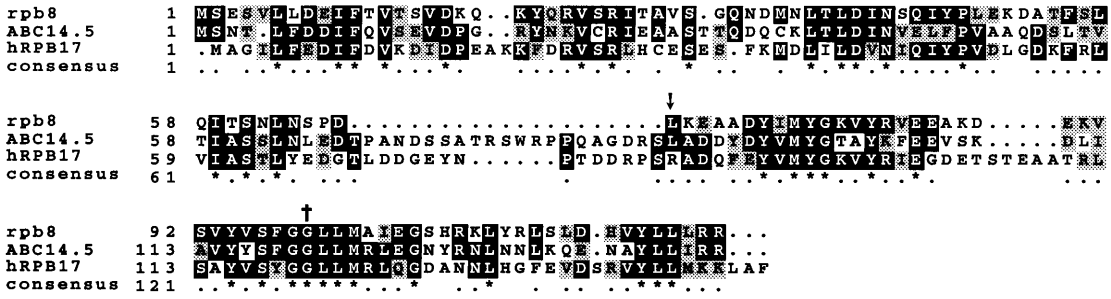

Figure 1. Amino acid sequence comparison of Rpb8 orthologues. Multiple alignment of the *S.pombe* (rpb8) (EMBL accession nos Y07643 and Y07644), *S.cerevisiae* (ABC14.5) (EMBL accession no. X53289) and *H.sapiens* (hRPB17) (GenBank accession no. U37689) amino acid sequences using PILEUP of the GCG program and BOXSHADE 3.21 (K.Hofmann and M.Baron, http://ulrec3.unil.ch/software/BOX_form.html). Identical residues are indicated by black shading. Conservative amino acid substitutions are indicated by grey shading. Asterisks in the consensus line indicate identical residues in all three sequences and dots indicate residue similarities. The arrow indicates the replacement of L68 by R in the ScSp hybrid protein (Table 2) which had no phenotypic effect. The cross indicates the lethal mutation G120D in ABC14.5.

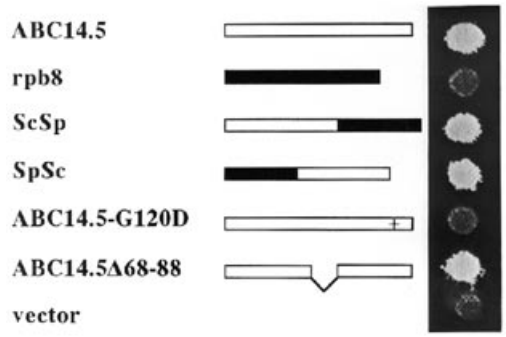

Figure 2. Ability of Rpb8 orthologues and derivative proteins to complement *S.cerevisiae rpb8*Δ. Patches of transformed cells grown to confluence on minimal medium plates, replica plated on minimal medium supplemented with 5-FOA and incubated at 30°C for 7 days. *S.cerevisiae rpb8*Δ containing a *CEN URA3 RPB8* plasmid (strain YSL171; Materials and Methods) was transformed with *HIS3*, 2μ plasmids (Table 2) overexpressing *S.pombe* rpb8 (pDBSp81), *S.cerevisiae* ABC14.5 (pDBSc81), its derivatives ABC14.5Δ68–88 (pDBSc81Δ21) and ABC14.5-G120D (pDBSc81m) and cross-species hybrid proteins (pDBScSp and pDBSpSc) or transformed with control vector (pDB1). (The *CEN URA3 RPB8* plasmid was lost by growth in 5-FOA.) The corresponding inserts are shown schematically: *S.cerevisiae*, empty bars; *S.pombe*, black bars; amino acid substitution, cross; nucleotide deletion, gap.

from the *ADC1* promoter on a high copy number plasmid (Fig. 2). Thus, we conclude that the heterologously expressed *S.pombe* rpb8 subunit contains residue differences that render it unable either to be assembled into the RNA polymerase complexes or to carry out all the essential functions of the endogenous *S.cerevisiae* ABC14.5 subunit.

Differences in the 67 N-terminal residues of the two orthologues contribute to the functional distinction of the *S.pombe* rpb8 expressed in *S.cerevisiae*

In order to test whether the 21 residue region present in ABC14.5 and absent in rpb8 was responsible for the non-hetero complementation of ABC14.5, we constructed a deletion expressing ABC14.5Δ68-88 protein and examined the functionality of this mutant by plasmid shuffling complementation assay. High copy expression of ABC14.5Δ68-88 in cells lacking the endogenous ABC14.5 subunit supported normal growth at 30 (Fig. 2), 16 and 37°C (data not shown). Therefore, residues 68–88 are not essential for ABC14.5 protein function and consequently their absence from the *S.pombe* rpb8 subunit does not account for the functional distinction between the two orthologues. To roughly map the domain on the *S.pombe* protein responsible for the observed functional difference in *S.cerevisiae*, hybrid proteins generated by interchanging the N- and C-termini of the *S.cerevisiae* and *S.pombe* subunits were examined by plasmid shuffling complementation assay. High copy expression of the hybrid ScSp bearing the N-terminus of *S.cerevisiae* ABC14.5 (amino acids 1–88) and the C-terminus of *S.pombe* rpb8 (amino acids 68–125) rescued the lethal phenotype of *S.cerevisiae rpb8*Δ (Fig. 2), suggesting that the C-terminal regions of the two proteins are functionally equivalent. A similar result was obtained with the hybrid SpSc bearing the N-terminus of *S.pombe* rpb8 (amino acids 1–67) and the C-terminus of *S.cerevisiae* ABC14.5 (amino acids 89–146) (Fig. 2) but, in that case, a slightly slow growth phenotype at 30°C (data not shown) and a more severe defect at 37°C (lanes vector and row SpSc in Fig. 3B) were observed. We conclude that the functional distinction between the two yeast subunits was partly due to their N-terminal divergent regions (residues 1–67 in *S.cerevisiae* ABC14.5). However, since both hybrid proteins were functional in *S.cerevisiae* at 30°C, a cumulative effect of several amino acid substitutions throughout the entire length of *S.pombe* rpb8 must be responsible for its inability to substitute for the *S.cerevisiae* ABC14.5 subunit. Since *H.sapiens* hRPB17 is able to substitute for the *S.cerevisiae* ABC14.5 subunit (17,19), we suggest that important functional differences of *S.pombe* rpb8 reside within residues 1–67 at positions where the *S.cerevisiae* and *H.sapiens* sequences are identical and distinct from the *S.pombe* sequence.

Overexpression of the *S.cerevisiae* C160 subunit protein allows the *S.pombe* rpb8 subunit to functionally replace ABC14.5 in *S.cerevisiae*

The results obtained from the N- and C-terminal exchange experiments between ABC14.5 and rpb8 suggested that *S.pombe* rpb8 might be competent in carrying out some but not all of the functions of ABC14.5. To further analyse these defects, we used a plasmid shuffle complementation assay to select plasmids from a high copy *S.cerevisiae* genomic library that could rescue the lethal phenotype of *S.pombe* rpb8-containing *S.cerevisiae rpb8*Δ. Two

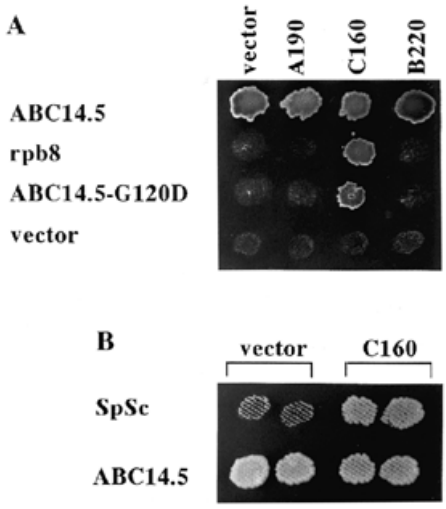

Figure 3. Ability of Rpb8 orthologues and derivative proteins to complement *S.cerevisiae rpb8*Δ in the presence of high copy C160 protein. Patches of transformed cells grown to confluence on minimal medium, replica plated on minimal medium supplemented with 5-FOA and incubated at 30°C for 7 days (**A**) and at 37°C for 4 days (**B**). *Saccharomyces cerevisiae rpb8*Δ containing a *CEN URA3 RPB8* plasmid (strain YSL171) co-transformed with one of the *HIS3*, 2μ plasmids (Table 2) expressing either *S.pombe* rpb8 (pDBSp81) or *S.cerevisiae* ABC14.5 (pDBSc81) or ABC14.5-G120D (pDBSc81m) or the SpSc hybrid subunit proteins and one of the *LEU2*, 2μ plasmids expressing either *S.cerevisiae* A190, B220 or C160 subunit proteins, as indicated. pDB1 and Yep351 were used as control vectors, respectively. (The *CEN URA3 RPB8* plasmid was lost by growth in 5-FOA.)

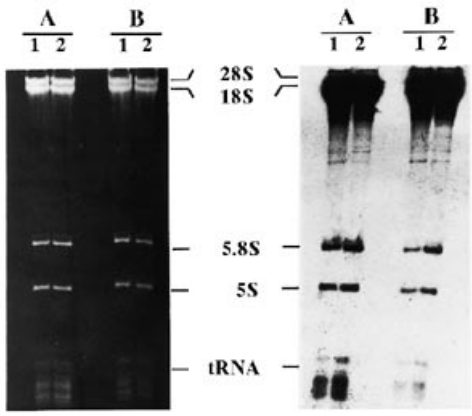

Figure 4. Substitution of the endogenous *S.cerevisiae* ABC14.5 subunit by *S.pombe* rpb8 (in the presence of high copy C160) affects tRNA synthesis. An autoradiogram of radiolabelled RNAs is shown on the right side. Pulse-chase *in vivo* radiolabelling of RNAs in the *S.cerevisiae rpb8*Δ strain containing a complementing allele of *S.cerevisiae RPB8* on a single copy plasmid (strain YSL171) additionally transformed with two high copy plasmids expressing either (**A**) the endogenous *S.cerevisiae* ABC14.5 (pDBSc81) and C160 subunits (pYeC) (used as wild-type control) or (**B**) the *S.pombe* rpb8 (pDBSp81) and *S.cerevisiae* C160 subunits (pYeC) (The *CEN URA3 RPB8* plasmid was lost following growth in 5-FOA.) RNAs were labelled as described in Materials and Methods by a 30 min pulse (1) followed by a 30 min chase (2). Each lane contained ~60 000 c.p.m. of total RNA sample. Ethidium bromide staining of the same RNA samples is shown on the left side. Each lane contained ~3 μg of total RNA.

groups of clones were isolated from this screen. As expected, fast growing transformants harboured plasmids with *RPB8*-containing inserts. A second category of transformants had a slow growth rate at 30°C (doubling time 8 h) and harboured plasmids with overlapping insert sequences. Restricted nucleotide sequencing analysis of these inserts identified the *RPC160* gene, which encodes the largest subunit of RNA polymerase III, C160 (36). High copy expression of *RPC160* rescued the lethal phenotype of *S.pombe* rpb8-containing *S.cerevisiae rpb8*Δ (Fig. 3A). Additionally, it suppressed the temperature-sensitive phenotype of the SpSc hybrid (Fig. 3B). The *RPA190* (37) and *RPB1* (38) genes, encoding the largest subunits of RNA polymerase I (A190) and II (B220), respectively, were not identified in the above screen and did not rescue the lethal phenotype of the *S.pombe* rpb8 subunit when tested individually (Fig. 3A). These findings indicate an essential genetic interaction between the *S.cerevisiae* C160 and ABC14.5 subunits and point to a defect of the heterologously expressed *S.pombe* rpb8 subunit specifically in RNA polymerase III complex assembly.

Schizosaccharomyces pombe rpb8 causes an RNA polymerase III deficiency in *S.cerevisiae*

Since overexpression of C160 rescued the lethal phenotype of *S.pombe* rpb8-containing *S.cerevisiae rpb8*Δ, we were able to investigate the effect on RNA polymerase III function of substituting the *S.pombe* rpb8 subunit for its endogenous *S.cerevisiae* (ABC14.5) counterpart. For this, we have examined the *de novo* synthesis of both tRNAs and rRNAs in an *rpb8*Δ strain overexpressing *S.cerevisiae* C160 and *S.pombe* rpb8 subunits, as well as in an isogenic control strain overexpressing C160 and ABC14.5 protein, by *in vivo* labelling with [^{3}H]uracil (30 min pulse, 30 min chase). While both cultures, for the same amount of cells, yielded similar quantities of RNA, the incorporation of [^{3}H]uracil was 3-fold less in the RNA isolated from the chimeric strain in agreement with its slow growth rate (doubling time 8 h). 5S rRNA accumulated at equimolar ratios and it was synthesised at similar rates, relative to the 5.8S rRNA, in both strains, whereas the accumulation and the rate of synthesis of tRNAs was reduced by 2-fold (estimated by the NIH Image 1.60/68K program following scanning of the autoradiogram) in the chimeric strain (Fig. 4). These results indicate that RNA polymerase III transcription is impaired when *S.pombe* rpb8 is substituted for the endogenous ABC14.5 subunit. However, it was not possible to determine whether the cross-species subunit substitution resulted in lower amounts of assembled RNA polymerase III or whether it affected RNA polymerase III activity. Additionally, since the [^{3}H]RNA in a 30 min pulse essentially reflected the synthesis of rRNAs, the reduced incorporation of [^{3}H]uracil into RNA in the chimeric strain suggested that *S.pombe* rpb8 might also have affected RNA polymerase I transcription, possibly via its action on the RNA polymerase III complex (30).

The *S.pombe* rpb8 subunit is poorly assembled into the RNA polymerase III complex in *S.cerevisiae* and overexpression of the C160 subunit protein favours its incorporation even in the presence of the endogenous ABC14.5 subunit

To investigate to what extent the *S.pombe* rpb8 subunit was incorporated in the RNA polymerase III complex and the activity

of the chimeric enzyme, we purified and analysed RNA polymerase III from the same *S.cerevisiae rpb8*Δ strains that we used for the genetic and *in vivo* labelling analyses: (i) expressing ABC14.5 from a single copy plasmid and *S.pombe* rpb8 from a high copy plasmid; (ii) expressing both *S.pombe* rpb8 and *S.cerevisiae* C160 from high copy plasmids; and (iii) expressing only ABC14.5 from a single copy plasmid as a wild-type control. The RNA polymerase III purified from all three strains exhibited similar chromatographic behaviour, although we consistently obtained 10-fold less enzyme from the strain containing only the *S.pombe* rpb8 protein (ii). The subunit composition of the purified enzymes was analysed by SDS–PAGE and the *S.pombe* rpb8 subunit was identified by parallel electrophoretic analysis of the *S.pombe* RNA polymerases I and II (32) that were available (Fig. 5). (The *S.pombe* rpb8 subunit had an apparent molecular mass of 12.8 kDa whereas the *S.cerevisiae* ABC14.5 subunit had an apparent molecular mass of 14.5 kDa.)

While in RNA polymerase III purified from the strain lacking the endogenous ABC14.5 subunit (ii) (Fig. 5, lane 3) *S.pombe* rpb8 was detectable, in the enzyme purified from the strain containing both subunit counterparts (i) (Fig. 5, lane 2) the endogenous ABC14.5 subunit was preferentially incorporated (~95%) and *S.pombe* rpb8 (although overexpressed) represented <5% of the incorporated ABC14.5 protein. This result indicated that the *S.pombe* rpb8 subunit was not efficiently assembled in RNA polymerase III and accounted for the low yields of the enzyme recovered from the strain expressing only *S.pombe* rpb8. However, the possibility that the low yield of rpb8-containing enzyme was due to enzyme instability during purification cannot be excluded. Western blot analysis of the chimeric and wild-type enzymes eluted from the first chromatography column (heparin-hyper D), using antibodies directed against entire RNA polymerase III (*S.pombe* rpb8 was not detected), showed a similar subunit composition but again a lower amount of the chimeric enzyme (data not shown).

We have also compared the activity of the two *S.pombe* rpb8-containing enzymes with that of wild-type RNA polymerase III in both specific and non-specific *in vitro* transcription assays. The three purified enzymes (i, ii and iii) had equivalent specific activities in non-specific and specific transcription assays (6) using poly(dA-dT) or the gene encoding *U6* snRNA as template (data not shown). These findings, in combination with those obtained from the *in vivo* labelling experiment, suggest that substitution of *S.pombe* rpb8 for the endogenous ABC14.5 subunit resulted in a lower amount of RNA polymerase III assembled *in vivo*, while it did not affect the transcription properties of the chimeric RNA polymerase III.

We further examined the incorporation of *S.pombe* rpb8 into the RNA polymerase III complex in the presence of both the high copy *S.cerevisiae* C160 protein and the endogenous *S.cerevisiae* ABC14.5 subunit. We analysed the subunit composition of the enzyme purified from: (i) the *rpb8*Δ strain expressing ABC14.5 from a single copy plasmid and overexpressing the *S.pombe* HA-rpb8 protein (the substitution of HA-rpb8 for rpb8 did not alter the growth rate; data not shown); and (ii) the above strain additionally overexpressing *S.cerevisiae* C160 protein. The two enzyme preparations exhibited the same chromatographic behaviour and had the same *in vitro* specific activity on poly(dA-dT) as wild-type enzyme (data not shown). The western blot analysis shown in Figure 6 provides biochemical evidence that overexpression of the C160 protein favours the incorporation of the *S.pombe* rpb8 subunit even in the presence of endogenous

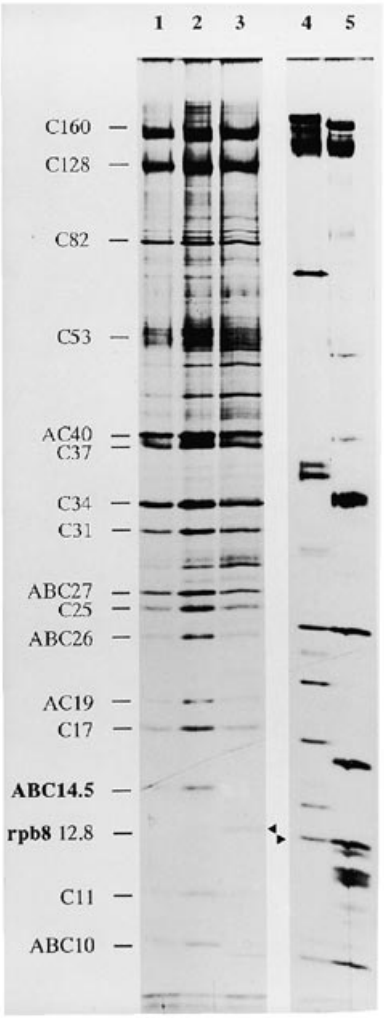

Figure 5. Incorporation of the *S.pombe* rpb8 subunit in RNA polymerase III of *S.cerevisiae*. Silver staining pattern of RNA polymerase subunits analysed by SDS–PAGE. RNA polymerase III was purified from (1) the *S.cerevisiae rpb8*Δ strain containing a complementing allele of *S.cerevisiae RPB8* on the single copy plasmid *CEN URA3 RPB8* (strain YSL171) or (2) the same strain additionally transformed with a high copy plasmid expressing the *S.pombe* rpb8 protein (pDBSp81) or (3) co-transformed with two high copy plasmids expressing the *S.pombe* rpb8 (pDBSP81) and the *S.cerevisiae* C160 (pYeC) subunit proteins, respectively, after growth in 5-FOA (to lose the *CEN URA3 RPB8* plasmid). RNA polymerases I (4) and II (5) were purified from wild-type *S.pombe*. The positions of different RNA polymerase III subunits are indicated on the left side. The *S.pombe* rpb8 subunit is indicated by arrowheads.

ABC14.5. The strain overexpressing C160 yielded 10-fold less enzyme and contained mainly the *S.pombe* HA-rpb8 subunit (RNA pol III, lane 4). In contrast, in the absence of overexpressed C160, the RNA polymerase III contained mainly endogenous ABC14.5 (RNA pol III, lane 3).

The *S.pombe* rpb8 subunit is efficiently assembled into the RNA polymerase I complex in *S.cerevisiae*

The data presented above led us to the conclusion that the inability of *S.pombe* rpb8 to substitute for the endogenous *S.cerevisiae* subunit was essentially due to its defective assembly into RNA

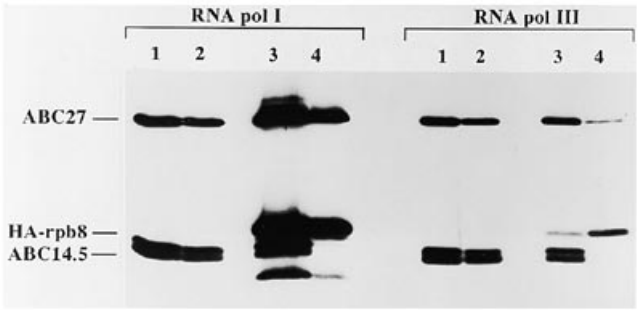

Figure 6. Comparative incorporation of the *S.pombe* rpb8 subunit in RNA polymerases I and III of *S.cerevisiae*. Western blot analysis of RNA polymerase I and III subunits. Aliquots of 1.0 (1) and 0.5 µg (2) of enzymes purified from wild-type *S.cerevisiae* are shown as controls of enzyme quantitation. Enzymes were purified from the *S.cerevisiae rpb8*Δ strain containing a complementing allele of *S.cerevisiae RPB8* on a single copy plasmid (*CEN URA3 RPB8* plasmid) (strain YSL171) and transformed with a high copy plasmid expressing the *S.pombe* rpb8 protein (pDBSp81) (3) or co-transformed with two high copy plasmids expressing the *S.pombe* rpb8 (pDBSp81) and the *S.cerevisiae* C160 (pYeC) subunit proteins, respectively (4). Specific anti-HA and anti-ABC14.5 antibodies were used for immunodetection of *S.pombe* rpb8 and *S.cerevisiae* ABC14.5, respectively. Anti-ABC27(Rpb5) antibody was used as an internal quantitative control.

polymerase III and suggested that *S.pombe* rpb8 was efficiently assembled into RNA polymerase I. To test this hypothesis, we performed subunit composition analysis (as described for RNA polymerase III) of RNA polymerase I purified from two strains: (i) the *rpb8*Δ strain expressing ABC14.5 from a single copy plasmid and overexpressing the *S.pombe* HA-rpb8 subunit; and (ii) the same strain as in (i) additionally overexpressing the C160 subunit protein. The amounts and activities of the RNA polymerase I enzyme purified from each strain assayed on poly(dA-dT) were found to be comparable with those of a wild-type control strain. Western blot analysis showed that ABC14.5 and HA-rpb8 were similarly represented in the RNA polymerase I of strain (i) (Fig. 6, RNA pol I, lane 3). Moreover, as revealed by silver staining of the RNA polymerase I subunits, the amount of overexpressed *S.pombe* rpb8 was even higher than the amount of the endogenous ABC14.5 subunit (data not shown). Curiously enough, over-expression of C160 promoted the assembly of *S.pombe* rpb8 into RNA polymerase I, as in the RNA polymerase III complex (Fig. 6, RNA pol I, lane 4).

A *S.cerevisiae* ABC14.5-G120D lethal mutant is rescued by overexpression of the *S.cerevisiae* C160 subunit protein

Our results that sequence alterations in *S.pombe* rpb8 cause RNA polymerase III-specific defects when expressed in *S.cerevisiae* led us to look for ABC14.5 mutants that would show similar effects. A single point mutant of *S.cerevisiae* ABC14.5 unable to substitute for the wild-type ABC14.5 subunit was isolated by the same genetic screen used for the isolation of the *S.pombe rpb8* cDNAs (Materials and Methods; Fig. 3). The single nucleotide mutation in the ORF at position 419 resulted in a G120D substitution. ABC14.5-G120D did not substitute for the wild-type ABC14.5 either when expressed in a similar context to the endogenous ABC14.5 subunit or when expressed on a high copy number plasmid (Fig. 2). G120 is present in a seven amino acid stretch [YXS(F/Y)GGLL] conserved among all known Rpb8 protein sequences (19; also Fig. 1). Therefore, the G120D mutation disrupted an essential highly conserved function of ABC14.5.

This highly conserved region is also probably involved in the interaction of ABC14.5 with C160 since we found that the lethality of the ABC14.5-G120D mutant was rescued by high copy co-expression of the *RPC160* gene but not the *RPA190* or *RPB1* genes (Fig. 3A). Examination of the patterns of *in vivo* labelled tRNAs and rRNAs obtained from the *rpb8*Δ strain co-overexpressing the ABC14.5-G120D and C160 subunits showed specifically reduced accumulation of tRNAs (data not shown), indicating that RNA polymerase III transcription was impaired by the ABC14.5-G120D mutation.

DISCUSSION

One of the open questions concerning the function of the RNA polymerase shared subunits is whether they have similar and/or distinct functions in each RNA polymerase class. In this paper we present the first genetic and biochemical evidence showing that sequence alterations in a shared subunit (Rpb8) primarily affect RNA polymerase III. We have identified the *S.pombe* rpb8 protein, homologous to the *S.cerevisiae* ABC14.5 (Rpb8) subunit, which in contrast to the human orthologue cannot functionally replace ABC14.5 in *S.cerevisiae*. We found this intriguing and assumed that the *S.pombe* subunit contains divergent residues, adapted to the structure and function of the *S.pombe* RNA polymerases, in regions of unique importance for its heterologous function in *S.cerevisiae*. To delimit these important regions, we examined molecularly and biochemically the effects of heterologous expression of *S.pombe* rpb8 in *S.cerevisiae*. We found that a region of 21 amino acids (68–88) of ABC14.5 which is absent in *S.pombe* rpb8 does not account for the functional distinction between the two homologues. In fact, a recently published structural description of ABC14.5 revealed that this 21 amino acid sequence is included in a large 24 amino acid unstructured ω-loop (39). We have shown by domain exchange experiments that differences within the N-terminal 1–67 residues of *S.pombe* rpb8 contribute to its functional distinction in *S.cerevisiae*, while the C-terminal regions of the two counterparts are functionally equivalent. Regional comparison of the two yeast amino acid sequences showed that the N-terminal halves are somewhat less similar (40% identity) than the C-terminal portions (48% identity). In fact we can see some important residue differences in the N-terminal half of *S.pombe* rpb8 that are conserved in both the *S.cerevisiae* ABC14.5 and human hRPB17 sequences (Fig. 1). For example, significant structural consequences might result from the ABC14.5-P17, HRPB17-P17 to rpb8-K18 (at the end of the β-strand I; 39) and the ABC14.5-E66, HRPB17-D67 to rpb8-P66 (at the end of the α-helix B; 39) changes. In agreement with these observations, the C-terminal half of ABC14.5 appears significantly more structured (and therefore less prone to residue changes) than the N-terminal half (39). In fact, the only lethal single point mutant of *S.cerevisiae* ABC14.5 that we isolated is G120D, contained in a seven residue sequence of the C-terminal half [YXS(F/Y)GGLL], conserved in all known Rpb8 sequences (19).

Considering that the N-terminal half of *S.pombe* rpb8 contains important residue alterations affecting its function in *S.cerevisiae*, we further investigated its defect in complementing the lethality of *S.cerevisiae rpb8*Δ. We have genetically identified the largest subunit (C160) of RNA polymerase III as a high copy suppressor

of the lethal phenotype. Overexpression of the largest subunits of RNA polymerases I and II had no effect. These data suggest an interaction between the C160 and ABC14.5 subunits and the involvement of the largest subunit in the assembly of ABC14.5 (or rpb8) in the RNA polymerase III complex. They additionally indicate that when *S.pombe* rpb8 is heterologously expressed in *S.cerevisiae*, it causes a specific defect in RNA polymerase III. This conclusion was verified by the demonstration of a relative decrease in tRNA synthesis, similar to that seen in several RNA polymerase III mutants (30). Biochemical analysis of RNA polymerases I and III purified from *S.cerevisiae* strains overexpressing *S.pombe* rpb8, in the presence of endogenous ABC14.5, showed directly that *S.pombe* rpb8 was poorly incorporated into RNA polymerase III, whereas it was incorporated at a much higher frequency into the RNA polymerase I complex. The concomitant overexpression of C160 promoted preferential assembly of the *S.pombe* subunit into RNA polymerase III even in the presence of the endogenous ABC14.5 subunit. Since we found that the chimeric RNA polymerase III (containing *S.pombe* rpb8) was similarly active to the wild-type enzyme *in vitro*, we conclude that the inability of the *S.pombe* subunit to functionally replace the endogenous *S.cerevisiae* subunit was due to its defective assembly in RNA polymerase III and that overexpression of C160 rescued the lethal phenotype because it facilitated its incorporation.

Our data argue that sequence alterations in a common subunit, such as sequence divergence in *S.pombe* rpb8, result in a specific RNA polymerase III defect. RNA polymerase I was not defective in our assays and we have preliminary evidence that RNA polymerase II function was also not affected (by examination of the levels of several RNA polymerase II transcripts in the same strains that we have tested for RNA polymerase I and II deficiencies; A.Voutsina and D.Alexandraki, unpublished observations). Why is only RNA polymerase III affected by sequence alterations in a shared subunit? We could assume that the *S.pombe* rpb8 subunit contains sequences particularly diverged in RNA polymerase III interacting regions. This hypothesis is corroborated by other reports pointing to a certain degree of species specificity of RNA polymerase III transcription (40,41). Alternatively, we could hypothesise that the divergent *S.pombe* rpb8 sequences are not specific for interaction with one class of RNA polymerase, rather one class of polymerase (RNA polymerase III) is less tolerant of mutations than the other two RNA polymerase complexes. In fact, it has been shown that mutations in the conserved regions of the largest subunits of RNA polymerases, although tolerated in A190 and B220, are lethal in C160 (5). The latter explanation probably applies best to the lethal phenotype obtained with the *S.cerevisiae* mutant subunit ABC14.5-G120D, which was altered in a cross-species conserved region and was also rescued only by overexpression of the polymerase III C160 subunit. (Biochemical proof that this mutant affects only RNA polymerase III awaits efficient purification of the mutant enzyme.) Finally, an increased sensitivity of RNA polymerase III might also be assumed from the fact that all the RNA polymerase III subunits are essential whereas certain RNA polymerase I- and II-specific subunits are not strictly required for viability (5). Therefore, it is possible that ABC14.5 (Rpb8) is essential for cell viability only in RNA polymerase III.

One unexpected finding in our results is that overexpression of the C160 subunit promoted the assembly of the overexpressed *S.pombe rpb8* subunit also in the RNA polymerase I complex. One explanation, based on relative amounts and binding constants of the various subunits, would be that, assuming that the *S.cerevisiae* C160 subunit interacts more tightly with *S.cerevisiae* ABC14.5 (Rpb8) than with *S.pombe* rpb8 (and presumably ABC14.5 interacts more tightly with C160 than with A190), excess C160 protein would sequester the pool of ABC14.5 leaving rpb8 (also in excess) in polymerase I. However, if this explanation was true, in a wild-type strain overexpressing only the C160 subunit, sequestration of the ABC14.5 subunit would also occur and that would result in polymerase I and/or III defects. This has not been observed in our strains. An alternative explanation is that the overexpressed C160 subunit interacts reversibly with *S.pombe* rpb8 and is able to deliver it to an RNA polymerase I subcomplex. This interpretation (totally hypothetical) raises questions as to the mode of assembly and nuclear addressing of multisubunit RNA polymerases, which are entirely unaswered.

In conclusion, the RNA polymerase common subunits may have a very basic role in the assembly or in the catalytic function of all RNA polymerase classes and/or they may have distinct functions in each class providing structural platforms for other interacting molecules. The data presented in this paper show that sequence alterations of an RNA polymerase shared subunit affect one class of enzyme. This differential behaviour may be related to slightly different interaction interfaces and/or to a different environment of these subunits in the three RNA polymerases. Consistently, limited proteolysis of ABC23 (Rpb6) in RNA polymerases I, II and III indicates a very different accessibility of this subunit in the three complexes (M.Riva and C.Carles, personal communication). Identification of mutants in common subunits specifically affecting each form of enzyme will facilitate the investigation of the function of these subunits and their assembly in RNA polymerases I, II and III.

ACKNOWLEDGEMENTS

We thank G. Thireos and A. Sentenac for critical comments and advice, D. Tzamarias, E. Georgatsou, Y. Papanikolau and A. Smid for helpful discussions, J. D. Fikes and L. Guarente for providing the *S.pombe* cDNA library, N. A. Woychik and R. Young for providing the YSL171 strain, P. Thuriaux for providing the *RPA190* and *RPB1* clones, P. Benos for providing the pDB20+/– plasmids and Lila Kalogeraki for photographic assistance. We thank S. Conlan who kindly improved our English. This work was supported by the Greek Ministry of Industry, Energy and Technology and by a short-term EMBO fellowship awarded to A.V. M.R. and C.C. thank the Human Frontier Science Program Organization for supporting the work.

REFERENCES

1 Roeder,R.G. (1996) *Methods Enzymol.*, **273**, 4737–4743.
2 Thuriaux,P. and Sentenac,A. (1992) In Broach,J., Pringle,J.R. and Jones,E.W. (eds), *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 2, pp. 1–48.
3 Zawel,L. and Reinberg,D. (1995) *Annu. Rev. Biochem.*, **64**, 533–561.
4 Sentenac,A. (1985) *CRC Crit. Rev. Biochem.*, **18**, 31–90.

5 Sentenac,A., Riva,M., Thuriaux,P., Buhler,J.M., Treich,I., Carles,C., Werner,M., Ruet,A., Huet,J., Mann,C., Chiannikulchai,N., Stettler,S. and Mariotte-Labarre,S. (1992) In McKnight,S.L. and Yamamoto,K.R. (eds), *Transcriptional Regulation* (Monograph 22). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
6 Dieci,G., Hermann-Le Denmat,S., Lukhtanov,E., Thuriaux,P., Werner,M. and Sentenac,A. (1995) *EMBO J.*, **14**, 3766–3776.
7 Thuillier,V., Brun,I., Sentenac,A. and Werner,M. (1996) *EMBO J.*, **15**, 618–629.
8 Treich,I., Carles,C., Sentenac,A. and Riva,M. (1992) *Nucleic Acids Res.*, **20**, 4721–4725.
9 Lalo,D., Carles,C., Sentenac,A. and Thuriaux,P. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 5524–5528.
10 Mann,C., Buhler,J.M., Treich,I. and Sentenac,A. (1987) *Cell*, **48**, 627–637.
11 Pati,U. and Weissman,S.M. (1990) *J. Biol. Chem.*, **265**, 8400–8403.
12 Langer,D., Hain,J., Thuriaux,P. and Zillig,W. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 5768–5772.
13 Carles,C., Treich,I., Bouet,F., Riva,M. and Sentenac,A. (1991) *J. Biol. Chem.*, **266**, 24092–24096.
14 Woychik,N.A., Liao,S.-M., Kolodziej,P.A. and Young,R.A. (1990) *Genes Dev.*, **4**, 313–323.
15 Nouraini,S., Archambault,J. and Friesen,J.D. (1996) *Mol. Cell. Biol.*, **16**, 5985–5996.
16 Lanzendorfer,M., Smid,A., Klinger,C., Schultz,P., Sentenac,A., Carles,C. and Riva,M. (1997) *Genes Dev.*, **11**, 1037–1047.
17 McKune,K., Moore,P.A., Hull,M.W. and Woychik,N.A. (1995) *Mol. Cell. Biol.*, **15**, 6895–6900.
18 Shpakovski,G.V. (1994) *Gene*, **147**, 63–69.
19 Shpakovski,G.V., Acker,J., Wintzerith,M., Lacroix,J.F., Thuriaux,P. and Vigneron,M. (1995) *Mol. Cell. Biol.*, **15**, 4702–4710.
20 Klenk,H.P., Clayton,R.A., Tomb,J.F., White,O., Nelson,K.E., Ketchum,K.A., Dodson,R.J., Gwinn,M., Hickey,E.K., Peterson,J.D., Richardson,D.L., Kerlavage,A.R., Graham,D.E., Kyrpides,N.C., Fleischmann,R.D., Quackenbush,J., Lee,N.H., Sutton,G.G., Gill,S., Kirkness,E.F., Dougherty,B.A., McKenney,K., Adams,M.D., Loftus,B., Venter,J.C. *et al.* (1997) *Nature*, **390**, 364–370.
21 Acker,J., de Graaff,M., Cheynel,I., Khazak,V., Kedinger,C. and Vigneron,M. (1997) *J. Biol. Chem.*, **272**, 16815–16821.
22 Guthrie,C. and Fink,G.C. (1991) *Methods Enzymol.*, **194**, 302–305.
23 Fikes,J.D., Becker,D.M., Winston,F. and Guarente,L. (1990) *Nature*, **346**, 291–294.
24 Cullin,C. and Minvielle-Sebastia,L. (1994) *Yeast*, **10**, 105–112.
25 Wittekind,M., Kolb,J.M., Dodd,J., Yamagishi,M., Memet,S., Buhler,J.M. and Nomura,M. (1990) *Mol. Cell. Biol.*, **10**, 2049–2059.
26 Archambault,J., Schappert,K.T. and Friesen,J.D. (1990) *Mol. Cell. Biol.*, **10**, 6123–6131.
27 Roussou,I., Thireos,G. and Hauge,B.M. (1988) *Mol. Cell. Biol.*, **8**, 2132–2139.
28 Kanazawa,S., Driscoll,M. and Struhl,K. (1988) *Mol. Cell. Biol.*, **8**, 664–673.
29 Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1987–1998**)** *Current Protocols in Molecular Biology.* Greene Publishing Associates and Wiley-Interscience, New York, NY.
30 Hermann-Le Denmat,S., Werner,M., Sentenac,A. and Thuriaux,P. (1994) *Mol. Cell. Biol.*, **14**, 2905–2913.
31 Huet,J., Manaud,N., Dieci,G. Peyroche,G., Conesa,C., Lefebvre,O., Ruet,A., Riva,M. and Sentenac,A. (1996) *Methods Enzymol.*, **273**, 249–267.
32 Riva,M., Buhler,J.M., Sentenac,A., Fromageot,P. and Hawthorne,D. (1982) *J. Biol. Chem.*, **257**, 4570–4577.
33 Laemmli,U.K. (1970) *Nature*, **259**, 680–685.
34 Morrissey,J.H. (1981) *Anal. Biochem.*, **117**, 307–310.
35 Buhler,J.M., Huet,J., Davies,K.E., Sentenac,A. and Fromageot,P. (1980) *J. Biol. Chem.*, **255**, 9949–9954.
36 Moyle,M., Hofmann,T. and Ingles,C.J. (1986) *Biochem. Cell. Biol.*, **64**, 717–721.
37 Memet,S., Gouy,M., Marck,C., Sentenac,A. and Buhler,J.M. (1988) *J. Biol. Chem.*, **263**, 2830–2839.
38 Allison,L.A., Moyle,M., Shales,M. and Ingles,C.J. (1985) *Cell*, **42**, 599–610.
39 Krapp,S., Kelly,G., Reischl,J., Weinzierl,R.O. and Matthews,S. (1998) *Nature Struct. Biol.*, **5**, 110–114.
40 Lagna,G., Kovelman,R., Sukegawa,J. and Roeder,R.G. (1994) *Mol. Cell. Biol.*, **14**, 3053–3064.
41 Wang,Z. and Roeder,R.G. (1997) *Genes Dev.*, **11**, 1315–1326.
42 Struhl,K. (1985) *Nucleic Acids Res.*, **13**, 8587–8601.