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Genetic evidence for selective degradation of RNA polymerase subunits by the 20S proteasome in *Saccharomyces cerevisiae*

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ABSTRACT

***scs32* was isolated as an extragenic suppressor of a temperature-sensitive (ts) mutation (*rpo26-31*) in the gene encoding Rpo26p, a subunit common to yeast nuclear RNA polymerases (RNAPs). *rpo26-31* also confers inositol auxotrophy, inhibits the assembly of RNAPI and RNAPII and reduces the steady-state level of Rpo26p and the largest subunit of RNAPI (Rpo11p or A190p) and RNAPII (Rpo21p). rpo26-31p accumulated to wild-type levels in the *scs32* strain; nevertheless, the amount of assembled RNAPII remained at a reduced level at high temperature. Hence, *scs32* only partially suppressed the ts phenotype and was unable to suppress the Ino$^-$ phenotype of *rpo26-31*. *SCS32* is identical to *PUP3,* which encodes a subunit of the yeast proteasome. *scs32* was able to suppress the phenotype of other ts alleles of *RPO26*, all of which reduce the steady-state level of this subunit. However, *scs32* was unable to suppress the ts phenotype of mutant alleles of *RPO21*, or result in accumulation of the unstable rpo21-4p. These observations suggest that the stability of non-functional or unassembled forms of Rpo26p and Rpo21p are regulated independently.**

INTRODUCTION

Eukaryotic nuclear transcription is carried out by three multi-subunit RNA polymerases (RNAPs), RNAPI, RNAPII and RNAPIII, whose structure and mode of function are highly conserved throughout evolution (1). All eukaryotic RNAPs have two large subunits and a number of small polypeptides, some of which are common among the three nuclear RNAPs (1). Biochemical experiments have shown that the two largest subunits of eukaryotic RNAPs associate with RNA, DNA and the nucleotide substrate, suggesting that these subunits form the catalytic center of the enzyme (2). Furthermore, analysis of extracts from cells containing mutant forms of smaller subunits in *Saccharomyces cerevisiae,* such as the 45 kDa subunit of RNAPII (Rpo23p), the 40 kDa subunit common between RNAPI and RNAPIII (AC40p), the 23 kDa subunit (Rpo26p) common among three nuclear RNAPs and the 12 kDa subunit of RNAPI (A12.2p), has shown that these subunits are required for assembly or stability of their respective RNAPs (1,3,4). The manner in which Rpo26p and A12.2p contribute to the assembly of their respective RNAPs is through stabilization of the largest subunits of these enzymes. Deletion of the gene encoding A12.2p confers a temperature-sensitive (ts) growth defect on yeast and leads to a reduction in the steady-state level of the largest subunit of RNAPI (3). A ts mutant allele of *RPO26*, *rpo26-31*, that inhibits the assembly of Rpo26p into RNAP complexes and results in decreased amount of this subunit at high temperature, also leads to reduced steady-state level of the largest subunit of RNAPI and RNAPII (4).

The observation that the steady-state level of RNAP free subunits is reduced might reflect a cellular regulatory response which serves to prevent accumulation of non-functional sub-complexes of RNAP components. Such a regulatory mechanism has been reported for mammalian cells (5–7). Heterozygous α-amanitin-resistant/α-amanitin-sensitive (AMA^r/AMA^s) cell lines express similar proportions of AMA^r and AMA^s RNAPII when grown in the absence of α-amanitin; however, when grown in the presence of this drug the inactivated AMA^s RNAPII is preferentially degraded (5–7).

We isolated spontaneous suppressors of the ts *rpo26-31* mutant allele in order to identify components of the regulatory mechanism that mediate removal of inactive RNAP subcomplexes in yeast. This report focuses on the characterization and cloning of one such suppressor mutation which lies in *PUP3*, the gene that encodes a subunit of the yeast 20S proteasome. The 20S proteasome is the catalytic component of the eukaryotic 26S proteasome, which is the major proteolytic machinery of the cell (8).

The suppressor mutation (*pup3-1*) partially suppresses the ts phenotype associated with *rpo26-31* and enables the unstable rpo26-31p to accumulate at the non-permissive temperature. However, the *pup3-1* mutation does not suppress the ts phenotype of mutations in the largest subunit of RNAPII. Nor does it enable an unstable Rpo21p mutant subunit to accumulate at the non-permissive temperature. These results suggest that the stability of non-functional forms of Rpo26p and Rpo21p are regulated independently.

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Table 1. List of strains used in this study

Strain	Genotype[a]	Reference or source
SHY101	*MATa rpo26*::pJAY97[b]	This study
SNY102	*MATα rpo26Δ:LEU2* [pSN261]	4
SNY103	*MATα rpo26Δ::LEU2* [pSN266]	4
SHY105	*MATa rpo26Δ::LEU2* [pRPO26]	This study
SHY108	*MATa rpo26Δ::LEU2* [pSN266]	This study
SHY109	*MATa rpo26Δ::LEU2* [pSN266] [pSN2]	This study
SHY110	*MATα rpo26Δ::LEU2 pup3-1* [pSN271]	This study
SHY111	*MATα rpo26Δ::LEU2 pup3-1* [pSN287]	This study
SHY112	*MATα rpo26Δ::LEU2 pup3-1* [pSN273]	This study
SHY113	*MATα rpo26Δ::LEU2 pup3-1* [pSN278]	This study
SHY158	*MATα rpo26Δ::LEU2 pup3-1* [pSN261]	
SHY201	*MATα pup3Δ2::HIS3* [pSHB16]	This study
SHY202	*MATa/α rpo21Δ::HIS3/RPO21* [pJAY101]	This study
SHY203	*MATa/α rpo21Δ::HIS3/RPO21 pup3-1/pup3-1* [pJAY101]	This study
SHY204	*MATα rpo21Δ::HIS3* [pJAY101]	This study
SHY205	*MATα rpo21Δ::HIS3 pup3-1* [pJAY101]	This study
SHY206	*MATα rpo21Δ::HIS3* [pYF1637]	This study
SHY207	*MATα rpo21Δ::HIS3* [pYF1641]	This study
SHY208	*MATα rpo21Δ::HIS3 pup3-1* [pYF1641]	This study
SHY209	*MATα rpo21Δ::HIS3 pup3-1* [pYF1637]	This study
SHY211	*MATa/α rpo26Δ::LEU2 /rpo26D::LEU2 pup3-1/PUP3 [pSN266]*	This study
SHY212	*MATα rpo26Δ::LEU2 pup3-1* [pSN266]	This study
SHY213	*MATa rpo26Δ ::LEU2 pup3-1* [pSN266]	This study
SHY216	*MATα rpo26Δ::LEU2 pup3-1* [PRPO26]	This study
SHY158	*MATα rpo26Δ::LEU2 pup3-1* [pSN261]	This study
SHY173	*MATα rpo26Δ::LEU2* [pSN261] [pEMBLyex4]	This study
SHY174	*MATα rpo26Δ::LEU2* [pSN261] [pSHB8]	This study
SHY175	*MATα rpo26Δ::LEU2* [pSN266] [pEMBLyex4]	This study
SHY176	*MATα rpo26Δ::LEU2* [pSN266] [pSHB8]	This study
SHY177	*MATα rpo26Δ::LEU2 pup3-1* [pSN266] [pEMBLyex4]	This study
SHY178	*MATα rpo26Δ::LEU2 pup3-1* [pSN266] [pSHB8]	This study
SHY179	*MATα rpo26Δ::LEU2 pup3-1* [pSN261] [pEMBLyex4]	This study
SHY180	*MATα rpo26Δ::LEU2 pup3-1* [pSN261] [pSHB8]	This study
SHY183	*MATa/α pup3-1/pup3-1*	This study
SHY188	*MATa/α pup3Δ2::HIS3/PUP3*	This study
SHY192	*MATa/α pup3Δ2::HIS3/PUP3* [pSHB11]	This study
SHY193	*MATa/α pup3Δ2::HIS3/PUP3* [pSHB16]	This study
SHY194	*MATα pup3Δ2::HIS3* [pSHB11]	This study
SHY196	*MATα pup3Δ2::HIS3* [pSHB11] [pSHB7]	This study
SHY198	*MATα pup3Δ2::HIS3* [pSHB7]	This study

[a]All strains in this study are derived from *Saccharomyces cerevisiae* strain W303 (*can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*) obtained from R.Rothstein.
[b]In this strain the expression of *RPO26* is driven by the inducible *GAL1* promoter. For a detailed description of the *RPO26* locus refer to Archambault *et al*. (9).

MATERIALS AND METHODS

Yeast strains and growth media

A list of strains used in this study is given in Table 1. Strain SHY103 was used for isolation of spontaneous suppressors of *rpo26-31* ts phenotype. Strains SHY101 and SHY105 are identical to JAY476 (*pGAL-RPO26*) (9) and JAY444 [*RPO26Δ::LEU2* (pRPO260)] (9), respectively, except they have the opposite mating type. Strains SHY212 and SHY213 were isolated as follows: first, the *scs32* suppressor strain was mated with SHY108 to create the diploid strain SHY211 (Table 1), which then was sporulated and used for tetrad dissection. SHY212 and SHY213 represent Leu$^+$, *MAT*α and *MATa*, respectively, haploid progenies that showed suppression of the *rpo26-31* ts phenotype (*pup3-1*). SHY183 was constructed as follows: strain SHY212 was mated with W303-1A, the resultant diploid was sporulated and tetrads were dissected on YPD solid medium. Since the *pup3-1* allele did not confer a discernible

phenotype on yeast in the presence of *RPO26*, the ability of *rpo26-31* to confer a growth defect at 35°C in the absence of *pup3-1* was used to decipher the allele present at the *PUP3* locus. Tetrads in which the ts phenotype of *rpo26-31* was no longer suppressed were judged to have segregation of *pup3-1* with *RPO26*; haploids with *RPO26 pup3-1* combination were chosen and the identity of their *PUP3* allele was confirmed by PCR amplification of this locus from genomic DNA, followed by sequence analysis. SHY183 was constructed by mating two of the haploids isolated in this way.

Cells were grown in rich medium or in defined medium supplemented with required amino acids as described (10). Minimal medium lacking inositol was prepared according to Culbertson and Henry (11). Cells were grown in low sulfate medium (LSM) as described (4) for the purpose of metabolic labeling with [^{35}S]methionine.

Plasmids

Plasmids pSN261 and pSN266 have been described previously (4); they contain *RPO26* and *rpo26-31*, respectively, in pFL39 (*TRP1 CEN ARS*) (12). Plasmid pSN2 is a derivative of pUN80 (*URA3 CEN4 ARS1*) (13), which lacks the sequences between *Xba*I–*Eco*RI in the polylinker. pSHB1 contains an ~7.0 kb fragment of chromosome V (Fig. 2), which was isolated from a YCp50-based yeast genomic library (see below) based on the ability to complement the suppression of *rpo26-31* ts phenotype by *pup3-1*. pSHB2 contains an ~3.0 kb fragment, *Hin*dIII (position 32 in YCp50 upstream of insert)–*Eco*RI (in the insert; Fig. 2), from pSHB1 cloned into pRS316 (14). pSHB3 contains an ~4.0 kb fragment, *Eco*RI (in the insert)–*Sal*I (position 654 downstream of insert in Ycp50; Fig. 2) from pSHB1 cloned into pRS316. pSHB4 was derived from pSHB3 by digestion of the latter with *Bam*HI and re-ligation of the plasmid (Fig. 2). pSHB5 was constructed by cloning a 1.2 kb *Bam*HI–*Eco*RI fragment from pSHB3 into pRS316 (Fig. 2). pSHB7 contains a 2.7 kb *Ssp*I (28 bp upstream of *PUP3* translation initiation codon)–*Sal*I fragment from pSHB3 cloned into pYGAL (a gift from Frank Jones). The same fragment has also been cloned into *Sma*I–*Sal*I sites of pEMBELyex4 (*pGAL1 URA3* 2 µm) to construct pSHB8 (Fig. 2). The expression of *PUP3* is driven by the repressible *GAL10* and *GAL1* promoters in pSHB7 and pSHB8, respectively. pYGAL contains the *PGK* transcription–termination sequence on a *Bgl*II–*Hin*dIII fragment cloned into the *Sph*I–*Hin*dIII sites of pJAY99 (constructed by J.Archambault) polylinker. pJAY99 contains the *pGAL10* promoter on an *Eco*RI–*Sma*I fragment cloned into the *Eco*RI–*Sma*I sites of pFL39. To construct pSHB9, the 4.0 kb *Eco*RI–*Sal*I insert of pSHB3 was cloned into a derivative of pRS316 in which the *Spe*I site in the polylinker has been destroyed by digestion and end-filling. pSHB11 was derived from pSHB3 by removing the polylinker sequences between *Eco*RI and *Sst*II. Plasmid pSHB16 is identical to pSHB11, except it contains the *pup3-1* mutant allele. It was constructed as follows: *pup3-1* was rescued from the chromosome (see below) and a *Bam*HI–*Xba*I fragment (Fig. 2) containing the *pup3-1* mutation was used to replace the analogous fragment in pSHB11. pJA452 (constructed by J.Archambault), pJA457 (15) and pYF1641 contain a 5.7 kb *Eco*RI–*Hin*dIII fragment containing *RPO21*, *rpo21-23* and *rpo21-4*, respectively, cloned into pFL39. pDJ40 (constructed by D.Jansma) contains a 7.0 kb *Hin*dIII–*Hin*dIII fragment carrying *rpo21-1* on pFL39.

Plasmid pSHB15 was constructed as follows for the purpose of obtaining a chromosomal deletion of the *PUP3* gene: a 601 bp (fragment 1) and an ~2.2 kb (fragment 2) fragment were PCR amplified from pSHB1. Primers used to amplify fragment 1 were K/O1 (5′-AATAGAACTTGGATCCGAC-3′), which contains a *Bam*HI site (underlined) and hybridizes to the coding strand of *PUP3* encompassing 21 bp downstream of *PUP3* translation–initiation codon and K/O2 (5′-CCCGAATTCC**CGCTACACTC**-3′), which contains an *Eco*RI site (underlined) and hybridizes to the non-coding strand (bold) located at 580 bp upstream of *PUP3* ORF. Primers used to amplify fragment 2 were K/O3 (5′-CGCGGATCC**TCGGTTCATGG**-3′), which contains a *Bam*HI site (underlined) and hybridizes to sequences in the non-coding strand located immediately downstream of *PUP3* ORF (bold), and the reverse primer that hybridize to the *LACZ* sequences in the plasmid. Fragment 1 was digested with *Bam*HI–*Eco*RI and cloned into pBluescript to obtain plasmid pSHB12. Fragment 2 was digested with *Bam*HI and *Not*I (397 bp downstream of *PUP3* ORF), and this 397 bp fragment was cloned in *Bam*HI–*Not*I sites of pSHB12 to obtain pSHB13. A 1.8 kb *Bam*HI fragment containing *HIS3* from pJJ215 (16) was cloned into the *Bam*HI site of pSHB13 to obtain plasmid pSHB15 with *HIS3* inserted in the same orientation as *PUP3*.

Isolation of suppressors of rpo26-31

Forty independent colonies of strain SNY103 9 [*rpo26*Δ::*LEU2* (pSN266)] were grown exponentially in liquid culture for 2 days at 23°C. An equivalent of 10^6 cells from each culture was spread on 40 Glucose (–Trp and –Leu) solid medium; the Petri plates were incubated at 37°C for 2 days. Each plate contained an average of nine colonies growing at the non-permissive temperature. To avoid true revertants, 40 colonies that grew at 37°C but at a considerably slower rate than wild-type (one representative from each plate) were chosen for further consideration.

Characterization of the suppressors

The following experiments were performed in order to determine whether the suppression phenotype was due to extragenic mutations rather than second-site mutations in *rpo26-31*. First, the pSN266 plasmid carrying the *rpo26-31* allele was purified from the suppressor strains, and after passage through *Escherichia coli*, was introduced into strain JAY444 [*rpo26*Δ*::LEU2* (pRPO26)]. Trp$^+$ transformants were relieved of pRPO26 by plasmid shuffling, and the growth phenotype of cells was compared with the rpo26-31 strain (SNY103) at 35°C. Second, the plasmid carrying *rpo26-31* was replaced with pRPO26 (*RPO26 URA3 CEN ARS)* in the suppressor strains. An independent preparation of plasmid carrying *rpo26-31* (pSN266) was used to replace wild-type *RPO26* by plasmid shuffling (17), and the growth phenotype of cells was tested at 35°C.

In order to determine whether the suppressor mutations were dominant or recessive, the suppressor strains were mated with strain SHY101 in which the expression of *RPO26* is under the control of the repressible *GAL1* promoter. The ability of the resultant diploid strains to grow at 35°C was tested in the presence of glucose (expression of chromosomal *RPO26* is repressed).

Strain SHY213 was mated to the 20 recessive suppressor strains, as well as to SHY103 as control. The growth phenotype of the diploid strains was tested at 35°C in order to test if any of the recessive suppressors are allelic to *scs32*.

Cloning of *SCS32*

Since the *scs32* suppressor strain did not exhibit a phenotype in the presence of *RPO26*, the *SCS32* gene was cloned by complementation of the suppression of *rpo26-31* ts phenotype by *scs32*. Prior to the cloning of *SCS32*, experiments were performed to ascertain that the suppression by *scs32* was due to mutation of a single gene. Strain SHY212 (*scs32*) was mated with SHY109 (*SCS32*), the diploid was sporulated and used for tetrad dissection. Thirteen tetrads were dissected, all of which showed a 2:2 segregation of the suppression phenotype, indicating that the suppressor mutation resides in a single gene. Strain SHY212 was transformed with a plasmid library (18) containing 10–15 kb *Sua*3AI partial digests of yeast genomic DNA cloned in the *Bam*HI site of YCp50 (*URA3 CEN4 ARS1*). A total of 10 087 Ura^+ transformants were patched on glucose (–Trp, –Ura and –Leu) solid medium at 35 and 30°C. Through this primary screen, 23 colonies were identified that no longer were able to grow at 35°C. In a secondary screen, the 23 putative positive transformants were relieved of the plasmid library using a plasmid-shuffling assay (17), and the ability of 5-FOA resistant cells to grow at 35°C was determined. Only three of the 23 putative positive colonies were able to grow at 35°C (suppress the ts phenotype of *rpo26-31*) in the absence of the library plasmid. Plasmid DNA was isolated from these three, passed through *E.coli*, re-introduced into yeast strain SHY212 and the ability of Ura^+ transformants to grow at 35°C was determined. Only one plasmid (pSHB1) was able to complement the suppression phenotype of *scs32* following a second transformation.

Rescue of the *pup3-1* mutation from the chromosome

Plasmid pSHB9 was digested with *Spe*I (581 bp upstream of *PUP3* ORF) and *Bgl*II (132 bp downstream of *PUP3* ORF), the plasmid was gel-purified and it was introduced into strain SHY212 (*rpo26-31 pup30-1*). Plasmid DNA was prepared from Ura^+ transformants that were able to grow at 35°C (did not show complementation of suppression by *pup3-1*). Following passage through *E.coli*, plasmids were used for sequencing of *PUP3* ORF.

Chromosomal deletion of *PUP3*

The entire insert of plasmid pSHB15 was released on a *Sal*I–*Sst*I 2.9 kb fragment and was introduced into the diploid yeast strain LP112 (W303-1A/B). His^+ transformants were sporulated and used for tetrad dissection.

Test of allele specificity of suppression by *scs32*

Plasmids pSN271, 273, 278 and 287 containing *rpo26-32*, *-30*, *-33* and *-34* ts alleles, respectively, were used to replace pRPO26 (*RPO26 URA3*) in strains SHY216 and JAY444 (as control), by plasmid shuffling. The ability of *scs32* to suppress the ts growth defect of these mutant alleles of *RPO26* was analyzed at 37°C.

Construction of an *RPO21* deletion in *scs32* and *SCS32* background

Plasmid pRP196 (a gift from R.Young) contains a deletion allele of *RPO21* in which a *Bgl*II fragment containing a portion of *RPO21* open reading frame (ORF) has been replaced with *HIS3* (19). pRP196 was digested with *Eco*RI (to release the insert) and was used to transform diploid yeast strains SHY183 (*pup3-1/pup3-1*) and LP112 (*PUP3/PUP3*). His^+ transformants were sporulated and were used for tetrad dissection. At least 10 tetrads were dissected for each strain, all of which showed co-segregation of the His^+ phenotype with lethality. Plasmid pJAY101 (*RPO21 URA3 CEN ARS*) (15) was introduced into the His^+ diploids described above, and Ura^+ His^+ transformants were used for tetrad dissection. pJAY101 was able to rescue the lethality of His^- haploid progeny and allowed growth of complete tetrads, confirming that the lethality of His^+ transformants was due to deletion of *RPO21* sequences in the chromosome. His^+ Ura^+ haploids isolated from the above mentioned tetrads (strains SHY204 and SHY205) were used to test the ability of *scs32* to suppress ts phenotype of mutations in *RPO21*.

Immunoprecipitation of RNAPII from [^{35}S]methionine-labeled cell extracts

Strains SNY102, SNY103, SHY158 and SHY212 (Table 1) were metabolically labeled with [^{35}S]methionine at 37°C, and RNAPII complexes were immunoprecipitated from crude extracts essentially as described (4). The monoclonal antibody 8WG16 (20), which recognizes a C-terminal domain (CTD) unique to the largest subunit of RNAPII (Rpo21p), was used for these experiments. An independent SNY102 extract was used for immunoprecipitation with a monoclonal antibody (Pharmingen) to human retinoblastoma protein (Rbp) as negative control. Immunoprecipitated complexes were separated on an SDS–PAGE gel (10% acrylamide) along with a purified preparation of RNAPII. The gel was stained for protein and analyzed by autoradiography.

RESULTS

Suppressors of a ts mutation in *RPO26*

Spontaneous suppressors that suppress a ts allele of *RPO26* (*rpo26-31*), the gene encoding a subunit common to yeast RNAPs, were isolated. Cells containing the *rpo26-31* allele are auxotrophic for inositol, grow slowly at 30°C and are unable to grow at or above 35°C (4). Suppressors were obtained by spreading 40 independent cultures of an *rpo26-31* strain at 37°C and isolating one colony from each plate. On average, nine in every 10^6 colonies were able to grow at 37°C for each plate. The suppressor strains were called *scs1* through to *scs40* (for suppressor of mutation in common subunit). Except for scs5, all of the suppressors supported similar growth rates at the non-permissive temperature; the growth rate of cells in the presence of *scs5* was considerably lower under these conditions. All suppressors provided better suppression of the ts phenotype at 35°C than at 37°C; hence, the growth rate of cells was monitored only at 35°C for the remainder of this study.

Characterization of suppressor strains

Two observations indicated that the suppressor strains contained extragenic mutations, rather than second-site mutations in *rpo26-31*. First, *rpo26-31* containing plasmids purified from the suppressor strains continued to confer a ts phenotype, indistinguishable from *rpo26-31*, when they were introduced into an isogenic strain that lacked a suppressor mutation. Second, when the plasmid carrying *rpo26-31* in the suppressor strains was replaced with an independent preparation of the same plasmid (see Materials and Methods), the growth rate of these newly

transformed strains was identical to the original suppressor strains, indicating that the latter strains contained an extragenic suppressor mutation.

The growth phenotype of suppressor strains in the presence of wild-type *RPO26* was tested at various temperatures (15, 23, 30 and 37°C) in order to determine whether the suppressor mutations generated a secondary phenotype. The growth rate of *RPO26* cells in the presence of the suppressor mutations was indistinguishable from wild-type under these conditions (not shown). Since the suppressor mutations themselves did not confer a discernible growth-defect, suppression of the ts phenotype of *rpo26-31* was used for further characterization of the suppressor strains.

Suppression of the *rpo26-31* ts phenotype was tested in diploid strains heterozygous for the suppressor mutations in order to identify recessive suppressors (see Materials and Methods). Of the 40 diploids tested, 20 failed to grow at 35°C (failed to suppress the *rpo26-31* ts phenotype), thus identifying recessive suppressors. The remainder of the diploid strains showed an intermediate growth phenotype at 35°C, suggesting that the suppressors in this group are due to semi-dominant mutations. The recessive suppressors were used for further study, since they provided a more easily scorable growth phenotype.

rpo26-31 mutants require inositol for growth (4), a phenotype which often is associated with mutations in genes that encode components of RNAPII (9,15,21–25) and which stems from poor induction of the *INO1* gene in the absence of inositol (21,22,24). Of the 20 recessive suppressors, only *scs32* and *scs5* were unable to support growth of the *rpo26-31* strain in the absence of inositol (Fig. 1A). When tested in the presence of wild-type *RPO26*, neither *scs32* nor *scs5* conferred an Ino⁻ phenotype (not shown). The failure of *scs32* and *scs5* to suppress the inositol auxotrophy, which is an RNAPII-specific defect, suggested that they might contain compensatory mutations in cellular components that specifically rescue the assembly defect of RNAPI (and perhaps RNAPIII). Under these circumstances, the functional defect imposed on RNAPII by *rpo26-31* may not be corrected and the cells would remain auxotrophic for inositol. In order to explore this possibility, *scs32* and *scs5* were characterized further.

In order to identify suppressor mutations that are allelic to *scs32*, the mutant strain was crossed to the panel of 20 recessive suppressor strains, as well as to the original *rpo26-31* strain as control, and the growth phenotype of the diploids at 35°C (suppression of *rpo26-31* ts phenotype) was tested. The *scs32* suppressor was chosen for this test because it provided better suppression than *scs5* of the ts phenotype conferred by *rpo26-31*. None of the diploid strains (except for homozygous *scs32*) was able to grow at 35°C, indicating that the *scs32* mutation is not allelic to any of the recessive suppressors in our collection.

Cloning of *SCS32*

The *SCS32* gene was cloned by complementation of the suppression of the *rpo26-31* ts phenotype by *scs32* (see Materials and Methods). A library of yeast genomic DNA was used to transform the *scs32* suppressor strain. Among 10 087 transformants, one contained the plasmid pSHB1 with a 7.0 kb insert (Fig. 2), which prevented the suppression of *rpo26-31* ts phenotype by *scs32* (prevented growth at 35°C). Restriction digestion and subcloning experiments were used to locate the complementing region on a 4.0 kb fragment (Fig. 2). Sequencing analysis of the

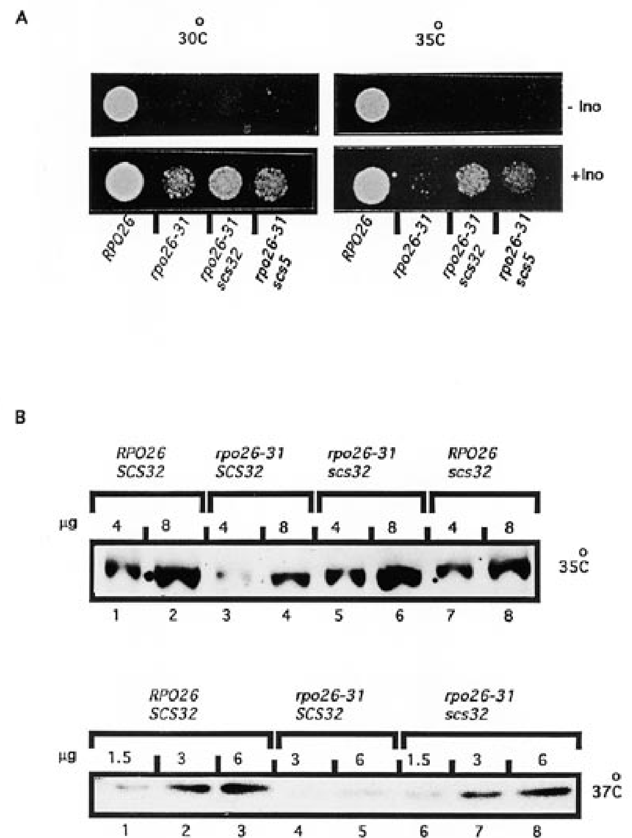

Figure 1. Suppression of the growth phenotype and reduction in the amount of Rpo26p imposed by *rpo26-31*. (**A**) *scs32* suppresses the ts phenotype of *rpo26-31* but not the Ino⁻ phenotype imposed by this mutant allele. Similar numbers of cells were spotted on solid growth medium in the absence (top) or presence (bottom) of inositol, and incubated at the indicated temperatures. (**B**) The steady-state amount of rpo26-31p is returned to wild-type levels in the presence of *scs32*. Strains SNY102 (*RPO26 SCS32*), SNY103 (*rpo26-31 SCS32*), SHY212 (*rpo26-31 scs32*) and SHY158 (*RPO26 scs32*), were grown exponentially at 23°C, shifted to 35 and 37°C for 6 h and cell extracts were prepared. Indicated amounts of protein from each extract were used for protein-blot analysis using a polyclonal antibody to Rpo26p.

ends of the insert followed by a search in the DNA database showed that it contains a portion of *RAD51* and upstream sequences of this gene located on chromosome V. Analysis of the sequence upstream of *RAD51* identified two divergently transcribed overlapping open reading frames (Fig. 2, ORF1 and ORF2). Further subcloning of the insert showed that at least one of these ORFs is required for complementation of the suppression phenotype (Fig. 2). A search on the database for homologous protein sequences identified the ORF transcribed divergently from *RAD51* (ORF2) as *PUP3* (putative proteasomal subunit 3) which has been identified previously (26) based on homology with the rat (27) and bovine (28) proteasomal subunits RC10-IIp and θ, respectively. ORF1 did not show a significant homology to any protein in the database.

Several lines of evidence indicate that *SCS32* is *PUP3*. First, when expression of *PUP3* was placed under the control of *GAL1* promoter (plasmid pSHB8) and introduced into the *scs32* strain (SHY212), the transformants were able to grow at 35°C only when the expression of *PUP3* was repressed (in the presence of

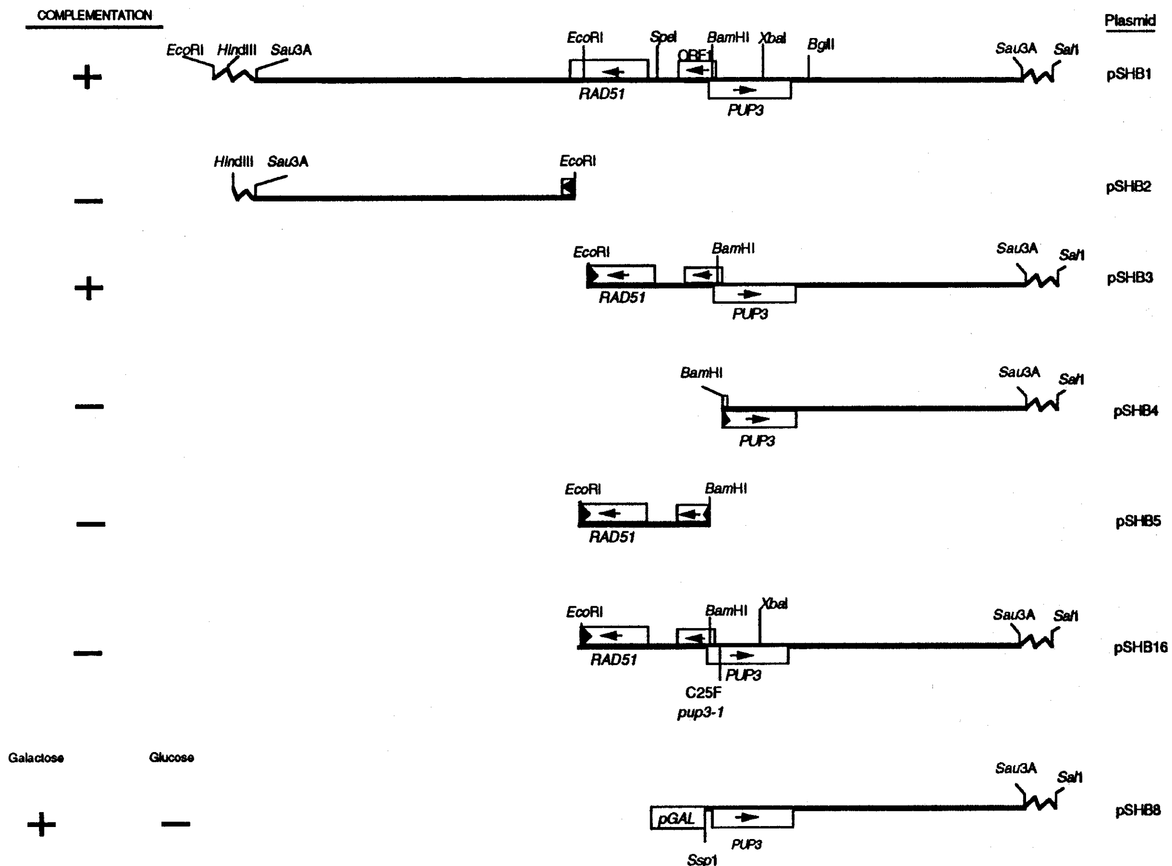

Figure 2. Molecular cloning of *PUP3* (*SCS32*) and identification of the *pup3-1* (*scs32*) mutation. *PUP3* was cloned by complementation of suppression of *rpo26-31*. A YCp50-based genomic library, made from *Sau*3A-digested yeast DNA, was introduced into strain SHY212 (*rpo26-31 scs32*) and transformants were tested for growth at 35°C. Plasmid pSHB1, which was able to inhibit cell growth at 35°C (complemented the suppression by *scs32*), contained a 7.0 kb insert from chromosome V. In order to identify the complementing region of the insert, pSHB1 was digested with various restriction enzymes as indicated, the shown fragments were subcloned into pRS316 and tested for the complementation of the suppression phenotype. The solid line represents the yeast genomic insert and the wavy lines indicate YCp50 sequences. Plasmid pSHB16 contains the *pup3-1* (*scs32*) suppressor allele of *PUP3* (*SCS32*) which was rescued from strain SHY212 as outlined in Materials and Methods. In plasmid pSHB8 expression of *PUP3* is under the control of the inducible *GAL1* promoter. This plasmid was used to show that the expression of *PUP3* (in the presence of galactose) is necessary and sufficient for the complementation of the suppression of *rpo26-31* ts phenotype by *scs32*.

glucose). This indicated that the expression of *PUP3*, but not *ORF1*, was necessary and sufficient to complement the suppression of *rpo26-31* by *scs32* (Fig. 2). Second, rescue of chromosomal *PUP3* from the *scs32* strain followed by sequence analysis identified a mutation (C25F) in the *PUP3* ORF. When tested in the scs32 strain, the mutant form of *PUP3* (*pup3-1*) was not able to complement suppression of the *rpo26-31* ts phenotype by *scs32*. Third, it has been shown previously that the steady-state level of rpo26-31p is reduced significantly at 37°C (4). Analysis of the steady-state level of rpo26-31p showed that this subunit accumulates to wild-type levels in the presence of *scs32* both at 35 and 37°C (Fig. 1B), consistent with the observation that the *scs32* strain has a mutation in a putative catalytic subunit of the cellular proteasome.

Analysis of the assembly of RNAPII

As described above, rpo26-31p accumulated to wild-type levels at 37°C in the presence of *pup3-1*, yet the ts growth defect was only partially suppressed and the cells remained auxotrophic for inositol (Fig. 1). It has been shown previously that the amount of assembled RNAPII is notably reduced at 37°C in the *rpo26-31* mutant strain (4). Furthermore, although over-expression of this mutant allele from a high-copy plasmid partially suppresses the Ino$^-$ and ts phenotypes, it cannot rescue completely the RNAPII assembly defect (4). The amount of assembled RNAPII in the *scs32* strain was monitored at 37°C in order to investigate whether partial suppression of the ts phenotype and the lack of suppression of the inositol auxotrophy was due to the inability of this enzyme to assemble to normal levels. Yeast strains containing all four combinations of wild-type and mutant alleles of *RPO26* and *SCS32* were metabolically labeled with [^{35}S]methionine at 37°C. RNAPII complexes were immunoprecipitated from crude extracts, using a monoclonal antibody to the CTD (20), and were separated on an SDS–PAGE gel. As shown in Figure 3, the amount of newly assembled RNAPII was reduced in the presence of *rpo26-31* (lane 3) compared to wild-type (lane 2, compare intensity of bands corresponding to RNAP subunits). Furthermore, this assembly defect was not rescued in the presence of *scs32* (Fig. 3, lane 4), although rpo26-31p accumulated to approximately wild-type levels under these conditions (Fig. 1B).

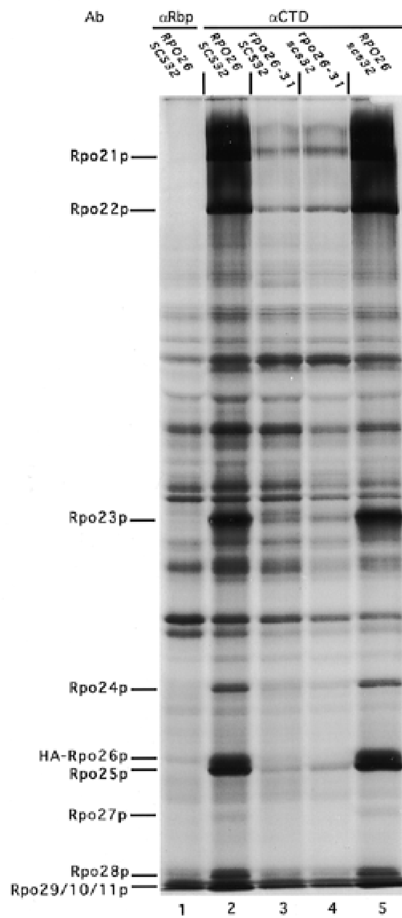

Figure 3. Assembly of RNAPII in the presence and absence of *pup3-1* (*scs32*). Strains SNY102 (lane 2), SNY103 (lane 3), SHY212 (lane 4) and SHY158 (lane 5) were metabolically labeled with [^{35}S]methionine at 37°C for 2 h, and RNAPII complexes were immunoprecipitated from crude extracts using a monoclonal antibody (8WG16) to the CTD of Rpo21p. A separate immunoprecipitation was done with an SNY102 extract using a monoclonal antibody to human Rb protein (lane 1), as a negative control. The immunoprecipitated complexes were subjected to electrophoresis in a 10% polyacrylamide–SDS gel. The position of migration of the RNAPII subunits is shown on the left.

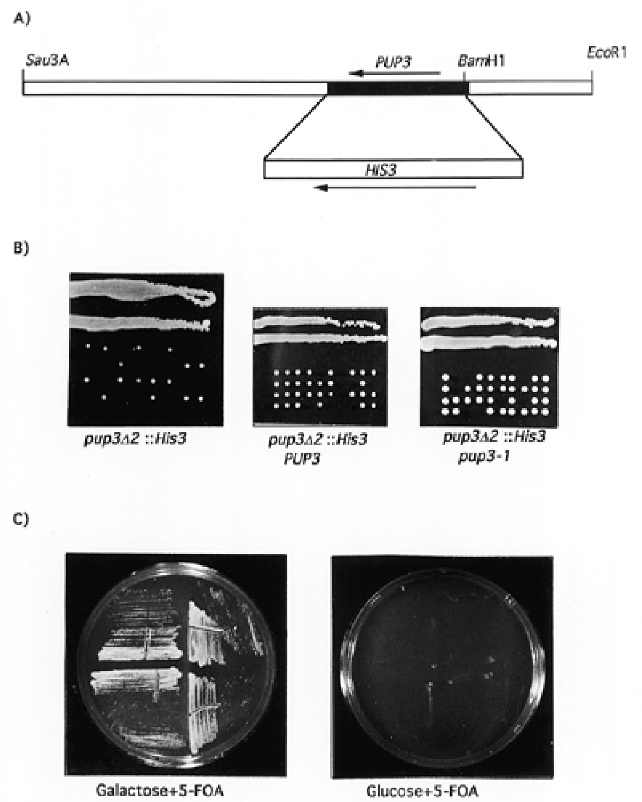

Figure 4. Construction of a *PUP3* deletion strain. (**A**) Schematic representation of the *PUP3* deletion allele indicating the region of the ORF that was replaced by *HIS3*. (**B**) Phenotype of *PUP3* deletion. A yeast strain (SHY188) heterozygous for the *PUP3* deletion allele was sporulated (left column); this gave rise to only two viable tetrads, both of which were His$^-$ (wild-type *PUP3*). The lethal phenotype of the *PUP3* deletion strain was rescued by plasmids pSHB11 (*PUP3*; middle) and pSHB16 (*pup3-1*; right) as described in Materials and Methods. (**C**) Expression of *PUP3* is necessary and sufficient for the viability of *PUP3* deletion strains. Plasmid pSHB7 (*pGAL10–PUP3*) was introduced into strain SHY194 [*pup3Δ::HIS3* (*pSHB11*)] and the ability of cells to lose pSHB11 (become resistant to 5-FOA) was tested when the expression of *PUP3* was repressed in the presence of glucose or induced in the presence of galactose. The result of this experiment is shown with four independent His$^+$ spores from the sporulation shown in (B) (middle column).

Therefore, the failure of RNAPII to assemble to normal levels in the presence of *scs32* provides an explanation for partial suppression of the ts phenotype and lack of suppression of the Ino$^-$ phenotype associated with *rpo26-31*.

Chromosomal deletion of *PUP3*

Chromosomal *PUP3* (along with the overlapping ORF, see above) was replaced with *HIS3* (Fig. 4A) in the diploid strain LP112 in order to determine whether the product of *PUP3* is essential for growth (see Materials and Methods). His$^+$ diploids were sporulated and used for tetrad dissection. All diploids analyzed (20 in total), showed only two viable spores (Fig. 4B), all of which were His$^-$. The lethality of His$^+$ spores was rescued by plasmid pSHB3 (Fig. 4B), which contains the 4.0 kb *Eco*RI–*Sal*I complementing fragment (Fig. 2), suggesting that *PUP3*, the overlapping ORF, or both are required for viability. His$^+$ haploids containing pSHB3 were transformed with plasmid pSHB7, which contains *PUP3* expressed conditionally from the *GAL10* promoter. The ability of these cells to lose pSHB3 was tested by plasmid shuffling (ability to grow on 5-FOA), in the presence (on galactose) or absence (on glucose) of *PUP3* expression. As shown in Figure 4C, cells were able to lose pSHB3 only when *PUP3* was expressed (in the presence of galactose), indicating that *PUP3*, and not the overlapping ORF, is essential for viability.

A plasmid carrying *pup3-1* was able to support growth of the *PUP3* deletion strain (Fig. 4B). This indicated that the *pup3-1* mutation did not completely abolish the activity of Pup3 proteasomal subunit and is consistent with the observation that *scs32* does not confer a growth defect in the presence of *RPO26*.

Specificity of suppression by *scs32*

The ability of *scs32* to suppress the phenotype of other ts mutations in *RPO26* was tested in order to determine if the

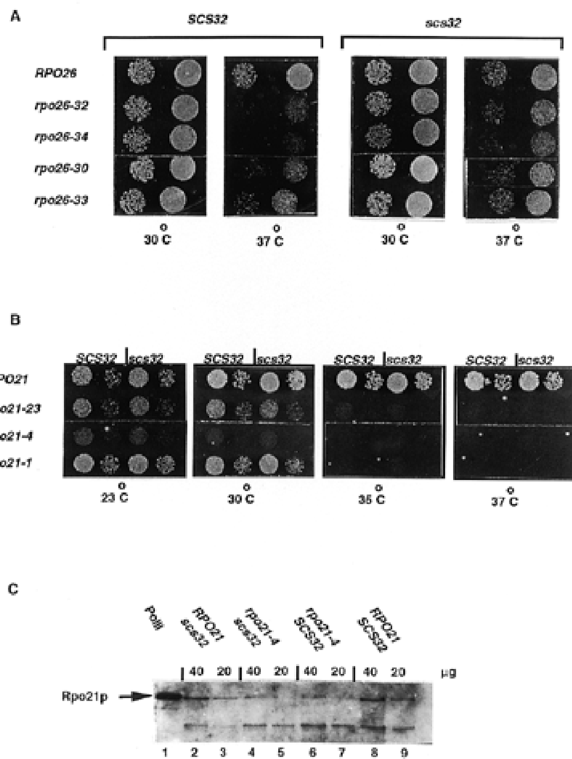

Figure 5. Specificity of suppression by *scs32*. Growth phenotype conferred by various ts mutant alleles of *RPO26* (**A**) or *RPO21* (**B**) in the absence or presence of the *pup3-1* (*scs32*) mutation. A suspension of cells in the form of a drop was applied on solid medium and the Petri plates were incubated at the indicated temperatures. For each strain, 10 µl of a cell suspension at 2×10^5 or 2×10^4 cells/ml was used for the drop tests. (**C**) Steady-state level of rpo21-4p in the presence and absence of *pup3-1*. Strains SHY206 (*RPO21 PUP3*), SHY207 (*rpo21-4 PUP3*), SHY208 (*rpo21-4 pup3-1*) and SHY209 (*RPO21 pup3-1*) were grown exponentially at 23°C and shifted to 37°C for 6 h. Cell extracts were prepared and the indicated amounts of protein were monitored for the amount of Rpo21p using the polyclonal antibody (α-B185) that recognizes this subunit. The band below Rpo21p represents cross-reactivity of α-B185 with an unknown protein which was used as an internal loading control.

suppression was allele-specific. *scs32* was able to partially suppress the ts phenotype of *rpo26-30*, *-32*, *-33* and *-34* at 37°C (Fig. 5A); this is consistent with the fact that these mutant alleles also reduce the steady-state level of Rpo26p (4). Next, the ability of *scs32* to suppress ts mutations in another polymerase subunit was tested. The gene encoding the largest subunit of RNAPII was replaced with *HIS3* (see Materials and Methods) in two isogenic strains carrying either the *SCS32* or *scs32* allele. The growth of these strains was supported by the plasmid-encoded *RPO21*, which then was replaced with plasmids carrying various ts alleles of *RPO21* (*rpo21-1*, *-4* and *-23*) by plasmid shuffling (17).

These *RPO21* ts alleles were chosen for two reasons; first, they show differing degrees of growth defect at the non-permissive temperature; *rpo21-4* has the most-severe growth defect and *rpo21-23* the least (Fig. 5B). Second, *rpo21-1* and *rpo21-4* are known to be defective in the assembly/stability of RNAPII since the growth defect of *rpo21-1* can be suppressed by over-expression of the mutant subunit (Jansma and Friesen, unpublished data), and the ts phenotype of *rpo21-4* is associated with reduced steady-state of Rpo21p (Fig. 5C) and can be suppressed by an increase in the gene dosage of *RPO26* (9). *rpo21-23* is defective in elongation of transcription due to reduced binding-affinity for the transcription elongation factor SII (29,30). *scs32* was unable to suppress the ts phenotype of any of these mutant alleles at all temperatures tested (23, 30, 33 and 37°C) (Fig. 5B). Cells harboring *rpo21-4* are auxotrophic for inositol, have a slow growth phenotype at 30°C and are unable to grow at or above 35°C (15), a growth characteristic similar to cells containing *rpo26-31*. Protein-blot analysis showed that, similar to rpo26-31p, the steady-state level of rpo21-4p was reduced at 37°C (Fig. 5C, compare lanes 6 and 7 with lanes 8 and 9), but unlike rpo26-31p, rpo21-4p did not accumulate to wild-type levels in the presence of *scs32* (Fig. 5C, compare lanes 4 and 5 with lanes 6 and 7). Thus, suppression by *scs32* does not extend to ts mutations in another polymerase subunit.

DISCUSSION

Previous studies have shown that the ts *rpo26-31* mutation reduces the steady-state level of Rpo26p at a high temperature (4). This reduction is due to degradation of Rpo26p since, as reported in this communication, an extragenic suppressor (*scs32*) of *rpo26-31* ts phenotype that allows accumulation of this subunit to normal levels contains a mutation in a catalytic subunit (Pup3p) of the yeast 20S proteasome. The 20S proteasome is the catalytic core of the 26S proteasome, which is the major proteolytic machine of eukaryotes, both in the nucleus and cytoplasm (8). This complex enzyme is involved in a variety of cellular events (reviewed in 31) and is responsible for degradation of proteins that are misfolded due to heat stress or incorporation of the arginine analog canavanine (32–34). The crystal structure of archaebacterial and yeast 20S proteasome has been determined (35,36). Both the archaebacterial and eukaryotic 20S complexes are composed of a stack of four rings each containing seven subunits (31); catalytic or β subunits form the two inner rings whereas regulatory or α subunits form the two outer rings of the proteasome complex (36). Although the archaebacterial complex is formed by seven identical α and β subunits (36), eukaryotic 20S complexes contain 14 distinct subunits (35).

β-type subunits are synthesized as inactive precursors which are activated during the assembly of the 20S proteasome upon removal of the N-terminal pro-sequence and exposure of an N-terminal catalytic Thr residue (reviewed in 37). Structural analysis of the yeast 20S complex has shown that only three out of seven β-type subunits are processed into active catalytic subunits (Pup1, Pre3 and Pre2; 35). The remainder of the β-type subunits are predicted to contribute amino acid side chains to the catalytic pocket of the active subunits (35).

The following observations indicate that Pup3p interacts with and contributes to the trypsin-like activity of the Pup1 subunit. (i) Determination of the crystal structure of the yeast proteasome has revealed that Pup3p and Pup1p form neighboring subunits and are involved in extensive protein–protein interactions (35). (ii) Residues K58 in Pup1p and E151 in Pup3p (double-underlined in Fig. 6) are predicted to form a salt bridge; destabilization of Pup3p caused by the E151K substitution causes lethality, which can be suppressed by a compensatory K58E mutation in Pup1p (38). (iii) Asp114, Asp120 and Cys118 in Pup3p (underlined in Fig. 6) are predicted to contribute to the trypsin-like activity of Pup1p (35). The homologous Cys residue

Figure 6. Partial sequence alignment of α- and β-type proteasomal subunits. The sequence alignment was performed using the PileUp programme. The amino acid mutated in *pup3-1* (*scs32*) and the equivalent residue in other proteasomal subunits are enclosed in a rectangle. S1–S4 show regions of α and β subunits from *Thermoplasma acidophilum* that have been shown by X-ray crystallography to form four strands of a β-pleated sheet that is a conserved secondary structure of these subunits. The underlined residues in the Pup3p sequence are predicted from the crystal structure of yeast 20S proteasome to contribute to the trypsin-like activity of the Pup1p subunit. The double-underlined residue in Pup3p forms a salt bridge with K58 in Pup1p. The following are the abbreviations used: Sc, *S.cerevisiae*; Rn, *Rattus norvegicus*; Hs, *Homo sapiens*; Dm, *Drosophila melangaster*; At, *Arabidopsis thalania*; Dd, *Dictyostelium discoideum*; Xl, *Xenopus laevis*; Bt, *Bos taurus*; Ta, *T.acidophilum*.

in the bovine Pup3p (subunit θ) cross-links to *N*-ethylmaleimide, which is a specific inhibitor of the trypsin-like activity of the bovine 20S complex (28). These observations suggest that mutations which alter the function of Pup3p would reduce the trypsin-like activity of the yeast 20S proteasome.

Sequences flanking the mutation in *pup3-1* (C26F) are highly conserved among all available proteasomal subunits (Fig. 6). These sequences participate in the formation of one strand (strand S2, underlined in Fig. 6) of a β-pleated sheet that is observed in all proteasome subunits (35,36). The cysteinyl residue which is mutated in pup3-1p is not predicted to participate in the catalytic activity of the proteasome and is not a conserved amino acid. These observations, in combination with the fact that C26 is never substituted with an amino acid containing a bulky side chain (Fig. 6), suggest that the presence of phenylalanine at the position of C26 is not favorable and might generate a minor structural change, which as a consequence may lead to reduced amount of the trypsin-like activity. The *pup3-1* strain has no detectable growth and sporulation defect, suggesting that the presence of the trypsin-like activity of the proteasome is not essential for yeast function. This is in agreement with the recent observation that inactivating the trypsin-like activity of the yeast proteasome by mutagenizing the catalytic Thr in Pup1 does not lead to a detectable growth defect (38).

Reductions in the steady-state level of the largest subunit of RNAPII (Rpo21p) (Fig. 3; 4) and of RNAPI (Rpo11p) (4) are also associated with the ts *rpo26-31* allele. Restoration of the steady-state level of rpo26-31p to wild-type, either by mutating *PUP3* or by over-expression of rpo26-31p, does not rescue the reduction in the amount of Rpo21p (Fig. 3) or Rpo11p (4). This observation suggests that the mechanism by which the steady-state level of Rpo26p is maintained is probably independent from that responsible for Rpo21p and Rpo11p. This conjecture is supported by the observation that, although *scs32* can partially suppress the ts phenotype of *RPO26* mutant alleles (perhaps by allowing the accumulation of the mutant subunit), it fails to suppress the ts phenotype of *RPO21* mutant alleles or allow rpo21-4p to accumulate in a wild-type *RPO26* background. This apparent subunit-specificity of *scs32* action can be interpreted in at least two ways. First, it is possible that the first cleavage event for the rpo26-31 protein (but not for rpo21-4p) is mediated by the tryptic activity, and that further cleavages cannot occur otherwise. In the presence of pup3-1p the tryptic activity of the 20S proteasome would be reduced which can potentially block further degradation of the rpo26-31p. Alternatively, the apparent specificity of *scs32* to Rpo26p might simply be due to the ability of unassembled or mutant Rpo21p to be recognized more efficiently by the proteasome than mutant or unassembled Rpo26p. Under

these circumstances, a marginal decrease in the activity of the proteasome would be sufficient for Rpo26p but not for Rpo21p (or Rpo11p) to accumulate to wild-type levels.

One mechanism by which Rpo21p could be recognized more efficiently by the proteasome is by rapid ubiquitin-modification. Except for isolated examples where ubiquitination serves a signaling role (39–41), ubiquitin-modification of cellular proteins marks them for rapid degradation by the 26S proteasome (31). Indeed, it is known that the largest subunit of RNAPII (Rpo21p) is ubiquitinated in yeast by the ubiquitin ligase Rsp5p whose interaction with Rpo21p is mediated by the CTD (42). Knowledge that CTD is a domain unique to Rpo21p suggests that Rsp5p specifically targets Rpo21p (among the subunits of RNAPII) that is free of association with the other polymerase subunits, thus mediating selective removal of the unwanted Rpo21p. It is also possible that there are Rpo11p- or Rpo26p-specific ubiquitin ligases whose identification awaits further investigation.

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