Functional analysis of the RNA polymerase II Rpb3 subunit of the fission yeast *Schizosaccharomyces pombe*

Doctor of Philosophy

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2. ABBREVIATIONS

Ade^+	adenine prototroph
bp	base pair
BSA	bovine serum albumin
C-	carboxy
CBB	Coomassie brilliant blue
CTD	carboxy terminal domain
Cs^-	cold-sensitive
DTT	dithothreitol
EDTA	ethylenediamintetraacetic acid
EGTA	ethyleneglycol bistetraacetic acid
EMM	Edinburgh minimal medium
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
LB	Luria broth
Leu^+	leucin prototroph
ME	malt extruct
N-	amino
NTA	nitlirotriacetic acid
NTD	amino terminal domain
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PMSF	phenylmethanesulfonyl fluoride
Rpb	RNA polymerase II
SDS	sodium dodecyl sulfate
TAE	Tris acetate EDTA buffer
Ts^-	temperature-sensitive
wt	wild-type
Ura^+	uracil prototroph
YE	yeast extract medium

3. SUMMARY

Three types of the nuclear RNA polymerase in eukaryotes are all multi-subunit enzymes, each consisting of more than 10 subunits. The RNA polymerase II is involved in the synthesis of mRNA and plays a key role in transcription of protein-coding genes. The RNA polymerase II from the fission yeast *Schizosaccharomyces pombe* is composed of 12 putative subunits. Sequence analysis indicated that, as in the case of other eukaryotic RNA polymerases, the largest subunit Rpb1 and the second largest subunit Rpb2 of *S. pombe* RNA polymerase II have notable homology with the β' and β subunits, respectively, of the prokaryotic RNA polymerases. The third largest subunit Rpb3 also has homology with the amino (N)-terminal domain of prokaryotic RNA polymerase α subunit (αNTD), which plays a key role in subunit assembly of this complex enzyme by providing the contact surfaces for both β and β' subunits. The *Schizosaccharomyces pombe* Rpb3 protein forms a core subassembly together with Rpb2 (the β homologue) and Rpb11 (the second α homologue) subunits as in the case of the prokaryotic $\alpha_2\beta$ complex. Several lines of evidence indicate that in addition to these core subunits, Rpb1, Rpb5, Rpb7 and Rpb8 also interact with Rpb3.

Sequence comparison between prokaryotes and eukaryotes also indicated Rpb3 has four conserved regions A to D. Among which two (A and D) are conserved among Rpb3 homologues from both prokaryotes (α subunits) and eukaryotes and two (B and C) are conserved only within eukaryotes. The regions A and D correspond to the N-terminal proximal and the C-terminal proximal regions of the αNTD, each playing critical roles in the contact with α and β subunits (motif-1) or the contact with α and β' subunits (motif-2), respectively. On the other hands, two regions in the middle part of Rpb3 protein

(Region B and C) do not exist in the prokaryotic RNA polymerases, Region B is specifically conserved in eukaryotic RNA polymerase II, and Region C is conserved among all three types of eukaryotic RNA polymerases. Therefore these two regions have been considered to be involved in eukaryote specific function(s).

In order to get insight into *in vivo* roles of Rpb3 in the assembly and function of RNA polymerase II, we have performed mutant studies for the *S. pombe rpb3* gene of RNA polymerase II. First, we carried out a systematic search for temperature-sensitive (Ts^-) or cold-sensitive (Cs^-) *S. pombe* mutants with mutations throughout the *rpb3* gene. After PCR mutagenesis of the entire *rpb3* sequence, we isolated 9 Ts^- and 3 Cs^- mutants. After the sequence analysis of mutant *rpb3* gene, each mutant was found to carry a single (or double in a few cases) mutation in one of the four regions (A to D) conserved among the eukaryotic subunit 3 homologues. The 3 Cs^- mutations were all located in the region A, in agreement with its most important role in the assembly of prokaryotic RNA polymerase, while the Ts^- mutations were scattered in all four regions. Since the metabolic stability of most Ts^- mutant Rpb3 proteins was markedly reduced at a non-permissive temperature, we predict that these mutant Rpb3 proteins are defective in the assembly or the mutant RNA polymerases containing the mutant Rpb3 are thermolabile. The assembly state of mutant RNA polymerase II was tested by treating purified mutant RNA ploymerases with low concentrations of urea. One representative Ts^- mutant complex was indeed dissociated more easily than the wild-type RNA polymerase. Moreover, the Ts^- phenotype of all the mutants were suppressed to various extents by overexpression of Rpb11, the pairing subunit in the initial stage of RNA polymerase II assembly. We conclude that the majority of *rpb3* mutations affect the subunit assembly of Rpb3, even though the extent of influence on the subunit assembly is different depending on the location of mutations.

Since the conserved regions B and C are unique for eukaryotic Rpb3 homologues, we thought that eukaryotic-specific transcription factors interact with one or both of these regions. We started the analysis of factor-dependent *in vitro* transcription activity for the mutant RNA polymerases. For this purpose, we constructed activator-dependent *in vitro* transcription system of *S. pombe* using *S.cerevisiae* GAL4-VP16. Results of the preliminary experiments indicate loss of the factor dependent transcription activity after heat treatment of the cell extracts from *rpb3* mutants carrying mutations in the B or C region.

4. INTRODUCTION

Three types of the nuclear RNA polymerase in eukaryotes are all multi-subunit enzymes, each consisting of more than 10 subunits. The RNA polymerase II is involved in the synthesis of mRNA and plays a key role in transcription of protein-coding genes. The RNA polymerase II from the budding yeast *Saccharomyces cerevisiae* consists of 12 putative subunits (Young, 1991; Sentenac *et al.*, 1992). Among 12 subunits, 5 small subunit [RPB5 (ABC27), RPB6 (ABC23), RPB8 (ABC14.5), RPB10 (ABC10β), RPB12 (ABC10α)] are shared among all three forms of *S. cerevisiae* RNA polymerase. Other 7 subunits [RPB1 (B220), RPB2 (B150), RPB3 (B44), RPB4 (B32), RPB7 (B16), RPB9 (B12.6), RPB11 (B12.5)] are specific for RNA polymerase II (Sentenac *et al.*, 1992; Thuriaux *et al.*, 1992). The RNA polymerase II from the fission yeast *Schizosaccharomyces pombe* is also composed of 12 putative subunits (Sakurai *et al.*, 1998; 1999). The isolation and sequencing of the genomic and cDNA clones have been completed for all 12 subunits (Azuma *et al.*, 1991; 1993; Kawagishi *et al.*, 1993; Miyao *et al.*, 1996; Sakurai and Ishihama, 1997; Sakurai *et al.*, 1996; 1998; Shpakovsky, 1994; Sakurai *et al.*, 1999).

Sequence analysis indicated that, as in the case of other eukaryotic RNA polymerases, Rpb1 and the Rpb2 of *S. pombe* RNA polymerase II have notable homology with the β' and β subunit, respectively, of the prokaryotic RNA polymerases (Azuma *et al.*, 1991; Kawagishi *et al.*, 1993). The fission yeast Rpb3 also has homology with the amino (N)-terminal domain of prokaryotic RNA polymerase α subunit (αNTD) (Azuma *et al.*, 1993), which plays a key role in the RNA polymerase assembly by providing the contact surfaces for both β' and β subunits (reviewed in Ishihama, 1981;1997). From the structural similarity with the prokaryotic α subunit, *S cerevisiae* homologue RPB3 subunit

had been suspected to form a dimer as an assembly core of RNA polymerase (Kolodziej *et al.*, 1989). Later, it was found that one of the small-sized RNA polymerase II specific subunits, RPB11, also has a limited homology with both eukaryotic RPB3 subunit and αNTD of prokaryotic RNA polymerases (for example see Sakurai and Ishihama, 1997). The amino acid sequences conserved among eukaryotic RPB3 and RPB11 and prokaryotic αNTD are named α motif (Dequard-Chablad *et al.*, 1991; Sentenac *et al.*, 1992). As expected from the sequence similarity, the subunits 3 and 11 were found to form a heterodimer. For instance, the *Arabidopsis thaliana* RPB3 protein can be co-immunoprecipitated with RPB11 (Ulmasov *et al.*, 1996). A recent biochemical analysis also indicated a stoichiometry of one molecule each of RPB3 and RPB11 per molecule of *S. cerevisiae* RNA polymerase II (Svetlov *et al.*, 1998).

Rpb3 lacks the sequence corresponding to the carboxyl (C)-terminal domain of α subunit (αCTD) which carries the contact sites with class-I (α-contact) transcription factors such as CRP and DNA UP elements (reviewed in Ishihama, 1993; 1997). However, recent investigation of *E. coli* RNA polymerase suggested prokaryote specific region in N-terminal domain of α subunit [α-NTD] is also involved in the transcriptional activation by interacting with another region of CRP activator protein. (Niu *et al.*, 1996). The sequence analysis indicate Rpb3 subunit has four conserved regions. The N-terminal proximal region of the Rpb3 protein (Region A) and C-terminal proximal region (Region D) has significant homology with *E. coli* αNTD (α-motif), while two regions in the middle part of Rpb3 protein (Region B and C) do not exist in the prokaryotic RNA polymerases. The region B is specifically conserved in eukaryotic RNA polymerase II and the region C is conserved among all three types of eukaryotic RNA polymerases. Therefore these two regions have been considered to be involved in eukaryote specific function(s) (Azuma *et*

al., 1993), and function of αNTD in prokaryotic α–subunit on transcription activation suggest the possible role of Rpb3 subunit in the mechanism of transcription activation.

Sequence analysis of other *S. pombe* RNA polymerase II subunits also revealed some difference from the *S. cerevisiae* RNA polymerase II subunits. For example, *S. pombe* Rpb4 subunit lacks several segments which present in the *S. cerevisiae* RPB4. Rpb4 is more smaller size like higher eukaryote human and *Arabidopsis thaliana* than that of *S. cervisiae* RPB4 subunit. Moreover, Rpb4 is essential for growth of *S. pombe* and associate with RNA polymerae II more tightly by the stoichiometric manner (Sakurai *et al.*, 1999), On the other hands *S. cervisiae* RPB4 is non-essential for growth on non-stress condition and its stoichiometry is changeable in the various growing state. In addition, *S. pombe* Rpb8 also lacks 20 amino acid residues which exist in *S ceervisae* RPB8 (Sakurai *et al.*, 1999). These difference give us the significance of studying *S. pombe* RNA polymerase II as the model organism of more higher eukaryotic transcription mechanism than that of *S. cerevisiae* RNA polymerase II.

Our study also revealed the nature of other RNA polymerase II subunit of *S. pombe*. Rpb5 was indicated to interact with Rpb1, Rpb2, Rpb3, Rpb8 and Rpb11 based on the results of far-Western analysis and GST-pulldown assay (Miyao *et al.*, 1998). Rpb6 is involved in the function for elongating step in transcription. Temperature-sensitive Rpb6 mutant showed 6-azauracil sensitivity which is characteristic to elongation defect of RNA polymerase, and this mutation was suppressed by the overexpression of TFIIS elongation factor (Ishiguro *et al.*, 1999 in press). Rpb7 forms stoichiometric complex with Rpb4 (Sakurai *et al.*, 1999). Rpb4, Rpb8, Rpb9 and Rpb11 subunits show nearly same migration rate in SDS-PAGE. This fact made it difficult to identify Rpb4 and Rpb9 by microsequencing technic for Rpb subunit from the purified RNA polymerase II complex. Up to now, Rpb4 was cloned by homology search from *S. pombe* genom database, and

Rpb9 was cloned from *S. pombe* mRNA with Rpb9 consensus sequence primer by RT-PCR method (Sakurai et al., 1998; 1999).

One line of studies is to determine the subunit-subunit contact network within the *S. pombe* RNA polymerase II. Various methods have been employed for this purpose, including far-Western blotting, chemical cross-linking, analysis of binary complex formation by GST-pull down assay, and yeast two-hybrid screening (Ishiguro *et al.*, 1998; Miyao *et al.*, 1996; 1998; Yasui *et al.*, 1998). Since the *in vitro* reconstitution system has not yet been established, the mechanism of subunit assembly in this laboratory has been studied by stepwise dissociation of the RNA polymerase II (Kimura *et al.*, 1996) or expressing *in vivo* Rpb proteins in various combinations (Kimura *et al.*, in press). Results altogether indicate that two large subunits, Rpb1 and Rpb2, provide platforms for the assembly of small subunits, and that Rpb3, Rpb11 and Rpb2 form the assembly core of *S. pombe* RNA polymerase II. This subunit-subunit contact network is similar to that of *E. coli* RNA polymerase, in which the α subunit contacts directly with all three core subunits, α, β and β' (Ishihama, 1981; Kimura and Ishihama, 1995a; 1995b). As in the case of $\alpha_2\beta$ complex in the formation of *E. coli* RNA polymerase, Rpb3 is involved in the formation of core subassembly Rpb2-Rpb3-Rpb11. Furthermore, Rpb3 makes direct contact *in vitro* with subunits Rpb1, Rpb2, Rpb5, Rpb7, Rpb8 and Rpb11 as analyzed by coexpression using recombinant vaculo viruses (Kimura *et al.*, in press). Coexpression study of the human RNA polymerase II subunits also indicated interaction of RPB3 with RPB1, RPB2, RPB7, RPB10, RPB11 and RPB12 (Schaller *et al.*, 1999). These observation altogether suggest that Rpb3 plays a key role in the formation of RNA polymerase II.

For identification of the *in vivo* roles of Rpb3 in the assembly and function of RNA polymerase II, we carried out mutant studies of the Rpb3 subunit in the *S. pombe* RNA

polymerase II. Previously, we isolated temperature-sensitive (Ts$^-$) *S. pombe* mutants carrying mutations in the *Rpb3* gene and carried out preliminary characterization of the RNA polymerase II isolated from the mutant strains (Azuma *et al.*, 1995). Those mutants, however, carried multiple mutations, and moreover, all the mutations were located in one of the four regions conserved among eukaryotic RNA polymerases, making it difficult to identify the crucial amino acid residues for the observed phenotypes. In this study, we carried out a more systematic search for temperature-sensitive (Ts$^-$) or cold-sensitive (Cs$^-$) *S. pombe* mutants with mutations throughout the *rpb3* gene. After PCR mutagenesis of the entire *rpb3* sequence, we isolated 9 Ts$^-$ and 3 Cs$^-$ mutants. After the sequence analysis, each mutant was found to carry a single (or double in a few cases) amino acid substitution, and most of the mutations were located within the four conserved regions A to D. Growth of the mutant *S. pombe* at non-permissive temperature was affected to various extents depending on the location of mutations. Measurement of the metabolic stability of the mutant Rpb3 proteins indicated that all the mutant *rpb3* strains showed nearly the same phenotype of probable assembly defect of the RNA polymerase II at non-permissive temperatures. The *in vitro* stability of RNA polymerase II isolated from one representative Ts$^-$ mutant strains were indeed decreased compared with the wild-type RNA polymerase. Supporting this prediction, the Ts$^-$ phenotype of all the mutants were suppressed by over-expression of Rpb11, the pairing subunit in the initial stage of RNA polymerase II assembly.

The Rpb3 mutants were also examined for specific transcription *in vitro* at permissive and non-permissive temperatures. For the characterization of these mutants, we constructed activator dependent *in vitro* transcription system of *S. pombe*. Using the *in vitro* system, we compaired activator dependent transcription activation efficiency among 6 Rpb3 Ts$^-$ mutants. The result indicated that the decrease in transcription activation level was more remarkable for the *rpb3* mutants with mutations in the regions B and C. On the

other hand, the *rpb3* mutants with mutations in the regions A and D are more thermolabile, indcating that the regions A and D play more critical roles in the subunit assembly.

5. MATERIALS AND METHODS

(a) Oligo nucleotides synthesized

3A (+297) 5'-CGCGGATCCTCTGGATAGCTCCAACATC-3' (+317)

3B (+1290) 5'-AAACTGCAGTCCGAAAGTGTTCTCTTTCACG-3' (+1269)

3C (-376) 5'-CGCGGATCCCTAAAGCTCCAGCACG-3' (-357)

3D (-10) 5'-CATTCTCATTATGTCGACAGAAACGCATATTACG-3' (+24)

3E (+983) 5'-CGCGGATCCTACCACGTGTTTTCTTCACC-3' (+963)

3I (+567) 5'-CAATGCACTGCAGAGAAATTTCCTGC-3' (+542)

NC1 (+4) 5'-GGGCCCGATTCAGAAACGCATATTAC-3' (+23)

NC2 (+486) 5'-GGTGTCCAAGAGAAGAGTTAG-3' (+466)

5' FITC labeled oligo nucleotides for sequencing

3F (-81) 5'-AGGGATCACCTAACAGTGCG-3' (-54)

3G (+242) 5'-TGTGAATACCTCAGTCATGCC-3'(+262)

3H (+602) 5'-GTCACCTACTTCAGCTGTTGC-3' (+622)

3M (+512) 5'-GCGTGATTTTGGATCGGCG-3' (+494)

3N (+1113) 5'-CATGAACGGACGCAGGCC-3' (+1096)

underlines : introduced restriction enzyme site

numbers : number of base pairs in the *rpb3* gene with respect to the translation initiation site

(b) Fission yeast strains and media

The fission yeast *Schizosaccharomyces pombe* JY741 (h^-, *ade6*-M216, *ura4*-D18, *leu1*) and JY746 (h^+, *ade6*-M210, *ura4*-D18, *leu1*) were used throughout this study. The compositions of culture media were as described in Moreno *et al.* (1994). Liquid YE medium contained 0.5% (w/v) oxoid yeast extract and 3% (w/v) glucose. Liquid EMM medium contained 3 g/liter potassium hydrogen phthalate, 2.2 g/liter Na_2HPO_4, 5 g/liter NH_4Cl, 2% (w/v) glucose, salt mixture {final concentration: 5.2 mM $MgCl_2$; 0.1 mM $CaCl_2$; 13.4 mM KCl; 0.282 mM Na_2SO_4}, vitamin mixture {final concentration: 4.2 μM pantothenic acid; 81.2 μM nicotinic acid; 55.5 μM *myo*-inositol; 40.8 nM biotin; 8.09 μM boric acid} and mineral mixture {final concentration: 2.37 μM $MnSO_4$; 1.39 μM $ZnSO_4$; 0.74 μM $FeCl_2$; 0.247 μM molybdic acid; 0.602 μM KI; 0.16 μM $CuSO_4$; 4.76 μM citric acid}. Liquid MB medium contained 0.5 g/liter KH_2PO_4, 0.1 g/liter NaCl, 5 g/liter potassium acetate, 0.5 g/liter $MgSO_4$, 0.1 g/liter $CaCl_2$, 0.5% (w/v) glucose, vitamin and mineral mixtures as described above. YE plate contained YE medium plus 2% Difco-agar. EMM plate contained EMM plus 2% Difco-agar. ME plate contained 3% Bacto-malt extract adjust to pH 5.5 with NaOH, 2% Difco-agar. Supplements added were as follows: +Ade, 120 mg/liter adenine sulfate; +Ura, 120 mg/liter uracil; +Leu, 200 mg/liter L-leucine. YE 5-FOA [5-fluoroorotic acid] plate contained 1 g/ liter 5-fluoroorotic acid, 120 mg/ liter adenine sulfate, and 25 mg/ liter uracil.

(c) *S. pombe* genetic analysis

Formation of diploid strains and random spore analysis were performed according to the published method (Moreno *et al.*, 1994). In brief, h^- mutant cells and isogenic h^+

wild-type strain JY746 were grown on YE(+Ade, +Ura) plate at 30 °C for 2 days. Then a loopful of both cells were mixed on the ME [malt extract] plate and incubated at 28 °C. For isolation of diploid strains, aliquots of cells suspension from the 1 day incubated ME plate were spread on EMM(-Ade, +Ura, +Leu) plate and incubated at 30 °C for 3 days. Diploid cells were selected as Ade^+ prototoroph by intragenic suppression of both *ade6*-M216 and *ade6*-M210 alleles in the heterozygotes. The phenotype of diploid strains were checked for growth on the YE(+Ade, +Ura) plate at 37°C for 3 days. For the random spore analysis, a loopful of cells from the 3 days incubated ME plate were suspended in 700 µl of distilled water and mixed for 5 min with vortex mixing at maximum speed. To select spores, the mixture was then added with 300 µl ethanol, incubated at room temperature for 30 min. For selection of h^+ spores, 1 µl of aliquot was plated onto EMM(15 µg/ml Ade, -Ura, +Leu) and incubated for 7 days at 30 °C. Dark red colonies (a phenotype of *ade6*-M210, tightly associated with h^+ locus) were picked up and spread on YE(+Ade, +Ura) plate for testing its Ts^- or Cs^- phenotype. The strain which confirmed linkage between *ura4* gene and mutant *rpb3* alleles were selected as Rpb3 mutant strains. This test is based on the assumption that the *ura4* gene is closely associated with the nearby located *rpb3* alleles.

(d) cDNA and genomic DNA extraction

cDNAs for Rpb3 and Rpb11 were isolated in this laboratory (Azuma *et al.*, 1993; Sakurai and Ishihama, 1997). Plasmid pSKura4, in which the *S. pombe ura4* gene is cloned into pBluescript II SK(+) at *Hin*dIII site, was provided by Dr. Y. Watanabe (Univ. Tokyo). *S. pombe* genomic DNA for PCR was extracted from JY741. In brief, about 7 x 10^8 cells were disrupted by shaking with vortex mixer in the presence of acid washed glass

beads (0.5 mm diameter), 200 µl phenol and 200 µl DNA extraction buffer {10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) SDS}, and then centrifuged at 12000 x g for 10 min. Nucleic acids in the supernatant were precipitated by adding 0.5 ml of ethanol. Pellet was washed with 70% ethanol and the dried pellet was dissolved in 200 µl of 10 mM Tris-HCl (pH 7.5), 1 mM of EDTA.

Genomic DNA for Southern blot analysis was extracted as described in Moreno *et al.* (1994). Cells were collected by centrifugation from 15 ml culture (5 x 10^7 cells/ml), and suspended in 600 µl of 1.2 M sobrbitol, 40 mM EDTA and 2 mg/ml Zymolyase-100T (Seikagaku Kogyo). After incubation at 37 °C for 30 min, collected by centrifugation, resuspended in 700 µl 50 mM Tris-HCl (pH 7.5), 50 mM EDTA, and lysed by addition of 70 µl 10% (w/v) SDS. To remove cell debris and insolble materials, 250 µl 5 M sodium acetate was added to lysate, and incubated for 30 min at 4 °C, centrifuged at 12000 x g for 10 min. Then genomic DNA was precipitated from the supernatant by additon of 1 ml isopropanol. The pellet was resuspended in 200 µl 50 mM Tris-HCl (pH 7.5), 50 mM EDTA, 20 µl g/ml RNase A and incubated at 37°C for 60 min. Then DNA was phenol extracted and ethanol precipitated again. The pellet of genomic DNA was dissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.

For isolation of integration vector, genomic DNA was digested by *Bgl*II in 300 µl reaction at 37 °C for 2 hrs, and the DNA fragments were purified using QIA prep Spin plasmid miniprep kit (QIAGEN), and eluted with 100 µl H_2O. The DNA fragments were ligated by adding 5 U of T4 DNA ligase (Takara) in a total volume of 200 µl at 16 °C for 12 hrs. DNA pellet was ethanol precipitated and dissolved in 10 µl of H_2O. *E. coli* DH5 was transformed with this DNA solution. Integration vectors were recovered from *E. coli* transformant and checked for the restriction map by cutting with *Bam*HI and *Pst*I. Mutations in the *rpb3* were also checked by DNA sequencing.

(e) Transformation of *S. pombe*

Integration vectors and plasmids for *S. pombe* were used for transformation of JY741 strain by lithium acetate method (Okazaki *et al.*, 1990). About 1 x 10^8 cells were inoculated in 50 ml of liquid MB(+Ade, +Ura, +Leu) medium, and incubated 6 hrs at 30 °C. Cells were collected by centrifugation at 5000 x g for 5 min, washed with 10 ml of distilled water, and then washed with 5 ml of 0.1 M lithium acetate. Cells were suspended in 500 µl of 0.1 M lithium acetate, and 50 µl of aliquot was mixed with 1 µg of integration vectors or plasmids, 10 µl of 2 mg/ml heat denatured salmon testis DNA and 290 µl of 0.1 M lithium acetate containing 50% (w/v) polyethlenglycol (MW 3350 Sigma). The mixture was incubated at 30 °C for 30 min and then at 43 °C for 15 min. The cells were collected by brief centrifugation and suspended in 1000 µl of distilled water. A 100 µl aliquot of the cell suspension was plated onto EMM plate. For transformation of pSK-ura4-rpb3, EMM(+Ade, -Ura, +Leu) plate was used. EMM(+Ade, +Ura, -Leu) plate was used for pREP vectors which carrying *LEU2* gene as a selectable marker.

(f) Mutagenesis of the *rpb3* gene

Mutagenesis of the *rpb3* gene was carried out by PCR. To avoid production of multiple mutation, PCR was carried out without presence of Mn^{2+} ion as described (Mitsuzawa *et al.*, 1995). 5'-Proximal portion between -376 and +560 with respect to the translation initiation site (N, amino terminal proximal) and 3'-proximal portion between +297 and +1290 (C, carboxyl terminal proximal) were PCR-amplified separately, by set of primers 3C and 3I or 3A and 3B respectively (Fig. 1). The condition of PCR reaction was 2.5 U of Ampli-taq DNA polymerase (Perkin Elmer), 1 µM of each primer, 100 mM

each dNTP, 4 µl of JY741 genomic DNA, 1 x reaction buffer (Perkin Elmer) total volume 100 µl. The condition of incubation was 94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min for 20 cycles by Gene-Amp 9600 thermal cycler (Perkin Elmer). The PCR products were purified by Wizard PCR preps (Promega) and digested by *Bam*HI and *Pst*I. Digested fragments were purified by ethanol precipitation and then ligated into *Bam*HI, *Pst*I digested pSKura4 vector by T4 DNA ligase (ligation kit version II, Takara) to generate pSK-ura4-rpb3(N) or pSK-ura4-rpb3(C) libraries, respectively. *E. coli* DH5 was transformed (Competent High, Toyobo) with this mixture. Total 16 independent libraries were constructed for pSK-ura4-rpb3(N) and 15 independent libraries for pSK-ura4-rpb3(C).

(g) Construction of the *rpb3* mutants

Two-step replacement method of Kaiser *et al.* (1994) was employed for the construction of *rpb3* mutants. First, 1 µg of both pSK-ura4-rpb3(N) and pSK-ura4-rpb3(C) plasmids were linealized by cutting at a unique *Bgl*II site located at +450 of the *rpb3* gene and transformed into the *S. pombe* JY741 strain using the lithium acetate method (Okazaki *et al.*, 1990). Transformants were plated on to EMM(+Ade, -Ura, +Leu). Approximately 1000 Ura^+ transformants were grown at 30 °C for 5 days on 9 cm diameter EMM plates and then replica-plated onto YE(+Ade, -Ura) plates. After 3 days incubation at 37 °C or 7 days at 20 °C, candidate colonies of temperature-sensitive (Ts^-) or cold-sensitive (Cs^-) mutants were isolated. Total screen number was 1 x 10^5. For construction of the *S. pombe* strains carrying only the mutant *rpb3* gene without the second incomplete *rpb3* gene (see Fig. 1), we carried out the second-step screening for isolation of Ura^- Ts^- strains on 5-fluoroorotic acid (5-FOA) plate. (Kaiser *et al.*, 1994). The representative 7 Ts^- strains (Ts3-30, Ts3-53, Ts3-84, Ts3-154, Ts3-176, Ts3-229, Ts3-

231) were cultured at 30 °C in 5 ml of liquid YE(+Ade, +Ura), 100 µl of saturated culture was plated onto YE 5-FOA plate. After incubation at 30 °C for 2 days, 5-FOA resistant colonies were replica plated onto YE(+Ade, +Ura) plates and Ts^- selection was performed as described above. The isolated Ura^- Ts^- strain was confirmed its gene organization by Southern blot analysis.

(h) Mutation site determination by sequencing of *rpb3* alleles

The sites of mutations in the isolated Ts^-, Cs^- strains were analyzed by cycle sequence with fluorescent primers. At first, the mutant *rpb3* alleles were amplified by PCR using set of primers 3C and 3B (see synthesized oligo nucleotide). Total 100 µl of mixture containing 0.5 µM of each primer, 200 µM each dNTP, 2U of Vent DNA polymerase (NEB), 1 x reaction buffer (NEB), 4 µl gemomic DNA was incubated 30 cycles of 95 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min by Gene-Amp 9600 thermal cycler (Perkin Elmer). PCR products were purified by Wizard PCR preps (Promega), 0.1 pmol of *rpb3* PCR products and 2 pmol of fluorescent primers [For the transformant of pSK-ura4-rpb3(N), 3F; 3G; 3N. For the pSK-ura4-rpb3(C), 3H; 3M] were subject to Thermo Sequenase™ cycle sequencing kit (Amersham-Pharmacia) and analyzed DNA sequencer DSQ-1500L (Simadzu).

(i) Southern blot analysis

Gene organizations of mutagenized strains were confirmed by Southern blot analysis. The genomic DNA (0.1 µg each) was digested with *Bgl*II or *Xba*I at 37 °C for 12 hrs. After electrophoretic separation of the digested products in the 1% TAE agarose

gel, The gel was soaked in the 0.25 M HCl for 10 min, 0.5 M NaOH, 0.5 M NaCl for 25 min and 0.5 M Tris-HCl (pH 7.5), 0.5 M NaCl for 30 min. DNA fragments were blotted onto nitrocellulose membranes with 10 X SSC buffer {0.15 M sodium citrate, 1.5 M NaCl} 9 hrs by capillary method (Southern, 1975). Digested DNA were UV-crosslinked on the membrane and probed with 20 ng/ml of random labeling florescent probe which produced from the primer 3A-3B PCR product or the pSKura4 plasmid as a template. After hybridization at 60 °C 12 hrs, membrane was washed with 1 X SSC {15 mM sodium citrate, 150 mM NaCl}, 0.1% SDS at 60 °C for 10 min and then 0.5 X SSC {7.5 mM sodium citrate, 75 mM NaCl} at 60 °C for 10 min and hybridized bands were visualized with the Gene Image kit (Amersham-Pharmacia).

(j) Preparation of cell lysates

Each mutant strain was cultured at 30 °C in 150 ml liquid YE(+Ade +Ura) medium. At a cell density of 1 x 10^7 cells/ml, an aliquot of 100 ml was centrifuged to harvest the control cells. The rest of culture was diluted with prewarmed same YE medium to make the final concentration of 1 x 10^6 cells/ml and the incubation was continued at 38 °C. At 6 hr after the temperature shift, an aliquot of 100 ml was removed and cells were harvested by centrifugation. The rest of culture was incubated until 12 hr after the temperature shift. Preparation of cell lysates was carried out essentially according to Kaiser *et al.* (1994). Cells were harvested by centrifugation, washed with 50 mM Tris-HCl (pH 7.5) containing 10 mM sodium azide, treated at 100 °C for 3 min and stored at -20 °C until use. Frozen cells (about 1 x 10^8) were suspended in 30 µl of 80 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol and 1.5% (w/v) DTT, and disrupted with a Beat-Beater (Biospec Products) in the presence of acid washed glass beads (0.5 mm diameter), for 6

times each for 30 sec at the maximum speed. The efficiency of cell disruption was routinely more then 95% as checked by microscopic observation.

(k) Polyacrylamide gel electrophoresis and Western blot analysis.

Cell lysates were treated for 1 min at 100 °C in the presence of 2% (w/v) SDS and after addition of 0.1 mg/ml Xylene cyanol and subjected to 10% SDS-PAGE. For Western blot analysis, proteins in gels were electro-blotted onto nitrocellulose membranes using a semi-dry transfer unit in a transfer buffer {20 mM Tris-HCl (pH 7.5), 150 mM glycine, 0.1% SDS and 20% methanol} at 1 mA/cm^2 for 1 hr. After blocking with 5% (w/v) skim milk in phosphate buffered saline [PBS(-)] the membrane was incubated for 1 hr with antibodies raised in rabbits against RNA polymerase II subunits (Ishiguro *et al.*, 1998) which was diluted 1000 fold with 1% skim milk PBS(-). Monoclonal antibody against α-tubulin DM1A was purchased from Sigma (Ding *et al.*, 1998 Sigma) which was diluted 500 fold with 1% (w/v) skim milk PBS(-). The membrane was then washed by 0.5% (w/v) Tween-20 PBS(-) and incubated at room temperature for 1 hr with 2000-fold diluted horseradish peroxidase-conjugated goat anti-rabbit IgG serum or anti-mouse IgG (Cappel). Antibodies bound on the membranes were visualized using the chemiluminescence (ECL) kit (Amasham-Pharmacia). The intensity of stained protein bands was measured with a densitometer (Quantity One, PDI inc.). For quantitative measurement of Rpb3 proteins, the Western blot analysis was repeated at least three times. The level of Rpb3 was determined after correction for the level of α-tubulin.

The level of Rpb3 in 125 μg each of cell extracts used for *in vitro* transcription assays was determined by quantitative Western blotting using the authentic purified *S.*

pombe RNA polymerase II sample as standard (Azuma *et al.*, 1993, Kimura, M., Sakurai, H. and Ishihama, A., submitted for publication).

(l) Construction of *S. pombe* mutants carrying the His-tagged mutant Rpb3 genes

A sequence coding for hexahistidine (H_6) tag was added to the N-termini of two mutant *rpb3* genes, *rpb3-30* and *rpb3-231*. The construction of the strains, Ts3-30H and Ts3-231H, carrying these recombinant *rpb3* genes were performed essentially according to Kimura *et al.* (1997). In brief, the mutant *rpb3* genes were digested with pairs of restriction enzyme and the *rpb3* fragments carrying the mutation were replaced by the corresponding fragments of the wild-type *rpb3* gene into pUC-ura4-rpb3H vector (Kimura *et al.*, 1997). The mutant *rpb3H* gene was transformed into the mutant strain which carried the same mutation with the mutant *rpb3H* gene.

For construction of the Ts3-30H strain, the fragment between nucleotide +4 and +486 of the mutant *rpb3-30* gene was PCR-amplified using a 5' primer attached with the *Apa*I site sequence at the 5' end [NC1], and 3' primer with the complementary sequence of nucleotide positions between +466 and +486 [NC2]. The PCR fragment was digested with *Apa*I and *Bgl*II, and cloned into pUC-ura4-rpb3H between the *Apa*I and *Bgl*II sites to construct pUC-ura4-rpb3-30H. For construction of the Ts3-231H strain, a *Bgl*II (+450) - *Sna*BI (+1177) fragment was isolated from plasmid pSK-ura4-rpb3-231 and cloned into pUC-ura4-rpb3H between the *Bgl*II and *Sna*BI sites to construct pUC-ura4-rpb3-231H. The *rpb3* gene sequence was checked for all these recombinant plasmids. The plasmids pUC-ura4-rpb3-30H and pUC-ura4-rpb3-231H were digested with *Kpn*I and the linearized DNAs were introduced into the original Ts^- strain Ts3-30 and Ts3-231, respectively. The resulting Ura^+ transformants were plated on 5-FOA [5-fluoroorotic acid] plates, and the

presence of His-tag sequence in the respective *rpb3* gene was examined for the 5-FOA resistant colonies after PCR amplification.

(m) Purification of His-tagged RNA polymerases

The *S. pombe* strains carrying the wild-type (Kimura *et al.*, 1997) or mutant *rpb3* genes (see above), each being attached with the His-tag sequence, were grown at 30 °C in liquid YE(+Ade, +Ura) medium to 5 x 10^7 cells/ml and harvested by centrifugation. The cells were suspended in 2 x cell volume of 1.5 x lysis buffer {50 mM Tris-HCl (pH 8.0), 2% (v/v) glycerol, 0.1 mM EDTA, 0.1 M $(NH_4)SO_4$ and 1 mM dithiothreitol} containing a mixture of protease inhibitors {1 mM PMSF [phenylmethylsulfonyl fluoride], 100 μg/ml benzamidine HCl, 10 μg/ml leupeptine, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml *N*-tosyl-L-lysine chloromethyl ketone, 10 μg/ml *N*-tosyl-L-phneylalanine chloromethyl ketone, 5 μg/ml chymostatine, and 2.5 μg/ml antipain} and disrupted with a Mini-Lab high pressure homogenizer (Rannie Inc.) at a pressure below 12,000 psi and keeping the temperature under 15 °C. The cell lysate was centrifuged at 15,000 x g for 30 min at 4 °C, and 0.01 volume of 10% (w/v) polyethyleneimine was added to the supernatant. The precipitates formed after 1 hr storage at 4 °C were recovered by centrifugation at 15,000 x g for 20 min at 4 °C, and the bound proteins were extracted with an extraction buffer {50 mM Tris-HCl (pH 7.6), 0.1 mM EDTA and 0.2 M $(NH_4)SO_4$}. After centrifugation at 15,000 x g for 30 min at 4 °C, the supernatant was pooled and dialyzed over night against TGM buffer {50 mM Tris-HCl (pH 8.0), 20% (v/v) glycerol, 10 mM 2-mercaptoethanol and 0.5 mM PMSF} containing 0.3 M $(NH_4)SO_4$. After centrifugation at 15,000 x g for 30 min at 4 °C, the supernatant was loaded onto Ni^{2+}-charged nitrilotriacetic acid (NTA) agarose column (QIAGEN) equilibrated with the same

buffer. The column was washed with 20 x column volume of TGM buffer containing 0.3 M $(NH_4)SO_4$ and 20 mM imidazole, and successively with 4 x column volume of TGM buffer containing 0.3 M $(NH_4)SO_4$ and 50 mM imidazole. The proteins were then eluted with a linear gradient of imidazole from 50 to 200 mM in the TGM buffer containing 0.3 M $(NH_4)SO_4$. Fractions containing the RNA polymerase II were pooled and stored at -80 °C.

(n) Partial dissociation of RNA polymerase II

Step-wise dissociation of the RNA polymerase II was carried out essentially according to Kimura *et al.* (1997). 3.36 pmoles of wild-type or mutant RNA polymerase II, as determined by measuring the amount of Rpb3 protein by Western blotting, were dialyzed against the TGM buffer containing 150 mM $(NH_4)SO_4$ and 20 mM imidazole, and then mixed gently with 10 µl of Ni^{2+}-nitrilotriacetic acid agarose (QIAGEN) for 2 hrs at 4 °C. Then resin was collected by centrifugation at 5000 x g for 1 min. Three resin-bound RNA polymerase II samples were incubated 2 hr at 4 °C with TGM buffer containing 0, 2, or 4 M urea, 150 mM $(NH_4)SO_4$ and 20 mM imidazole, respectively. The resin was suspended in the respective buffer containing same concentration of denaturant as used for washing and resin-bound proteins were eluted with 10 µl of TGM buffer containing 100 mM $(NH_4)SO_4$ and 150 mM imidazole, and analyzed by SDS-PAGE.

(o) Overexpression of Rpb11 in the *rpb3* Ts^- strain

For regulated expression of *rpb11*, the *rpb11* gene was cloned into pREP1, pREB41 and pREP81 (Maundrell, 1993), yielding pREP1(rpb11), pREP41(rpb11) and

pREP81(rpb11) (H. Mitsuzawa, unpublished). Using these plasmids, the expression of *rpb11* could be achieved at three different levels in the absence of thiamine. All Ts$^-$ strains and wild-type strain were transformed with pREP1(rpb11), pREP41(rpb11), pREP81(rpb11) or control pREP41. The transformants were grown on EMM(+Ade, +Ura, -Leu) plate containing 2 μg/ml thiamine chloride at 30 °C for 3 days, and the cells were suspended into distilled water. After adjustment of the cell density, 5 μl aliquot of each cell suspension was spotted on EMM(+Ade, +Ura, -Leu, -Thi) plate. The cell growth was monitored at 37 °C.

(p) Preparation of GAL4-VP16 fusion protein

GAL4-VP16 fusion protein was purified as described in Chasman *et al.* (1989). The expression plasmid of GAL4-VP16 and transcription template pGAL4CG$^-$ were kindly gifted from Dr. H. Sakurai, (Kanazawa Univ.). GAL4-VP16 was expressed in *E. coli*, JM109 under control of the *tac* promoter. One liter of *E. coli* culture in LB medium was grown at 37 °C to 0.7 A_{600} and expression of the fusion proteins was induced by adding isopropylthiogalactoside [to 1 mM], After 2 hrs incubation, cells were harvested, washed with 20 mM HEPES-NaOH (pH 7.5) [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 0.2 M NaCl, suspended in 25 ml of buffer A {20 mM HEPES-NaOH (pH 7.5), 20 mM 2-mercaptoethanol, 10 μM Zinc acetate, 2 μg/ml of each leupeptin, pepstatin A, and *N*-tosyl-L-phneylalanine chloromethyl ketone, 20 μg/ml benzamidine HCl, and 200 μg/ml PMSF} containing 0.2 M NaCl and lysed by sonication until A_{600} was reduced by 90%. All subsequent operation was performed at 4 °C. A cured extract was derived by centrifugation of the lysate for 20 min at 10,000 x g. Proteins were precipitated with polyethylenimine at a final concentration of 0.25% (w/v), and the pellet

was washed with buffer A containing 0.4 M NaCl. After centrifugation at 30,000 x g for 30 min at 4 °C, proteins were eluted form PEI pellet with 20 ml of buffer A containing 0.75 M NaCl, After centrifugation at 30,000 x g for 10 min at 4°C, the supernatants were combined, and proteins were precipitated by adding solid $(NH_4)SO_4$ to 35% saturation. The precipitated proteins were collected by centrifugation at 30,000 x g for 30 min at 4°C, $(NH_4)SO_4$ pellet was suspended in buffer A (without NaCl) to give a conductivity equal to that of buffer A with 0.1 M NaCl (5.39 mS/m at 20 °C), loaded onto DE-52 column (2 ml bed volume, Whatman) which was pre-equilibulatd with buffer A containing 0.1 M NaCl, and eluted with a gradient of NaCl from 0.1 to 0.4 M. Peak fractions were collected and loaded onto heparin-Sepharose CL-6B column (2 ml bed volume, Amersham-Pharmacia) pre-equilibrated in buffer A with 0.2 M NaCl. The protein was eluted with buffer A containing 0.6 M NaCl. The purified fraction contained two proteins, which migrated to 33 kDa and 40 kDa positions by 10 % SDS-PAGE. Since the N-terminal sequences of 15 amino acids were identical between two proteins and the same with GAL4-VP16 as determined by N-terminal amino acid sequencing (Procise, Applied BioSystem). The protein concentration of the top fraction was 5.6 mg/ml as measured by the method of Bradford (protein assay kit, Bio Rad) using BSA as standard. The purified GAL4-VP16 protein was diluted to 335 μg/ml with a dialysis buffer (see transcription extract preparation) and stored at -80 °C until use.

(q) Preparation of cell extract for transcription assay

S. pombe strains Ts3-30, Ts3-53, Ts3-84, Ts3-154, Ts3-176, Ts3-231 and wild-type JY741 were grown on liquid YE (+Adenine, +Uracil) at 30 ^{o}C. Cells were harvested at 5 x 10^7 cells/ml, and stored in -80 ^{o}C freezer until use. Cells (7 g) in liquid nitrogen were

disrupted with Cryo-press™ cell disrupter CP-100W (Microtec Nition Inc., Tokyo). After 1 min disruption, the cells were removed from the chamber surface, and the disruption was repeated for another 1 min. All subsequent operations were performed at 4 °C, essentially according to the published protocols by Woontner *et al.* (1993). In brief, the frozen disrupted cells were suspended in 20 ml of 200 mM Tris-acetate buffer (pH 7.9) containing 0.39 M $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 20% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT) and a protease inhibitor mixture (see Results for the composition). The suspension was centrifuged at 10,000 x g for 10 min, and the supernatant was centrifuged again at 60,000 x g for 75 min with a Ti60 rotor (Beckman). To the final supernatant, 337 mg/ml of solid $(NH_4)SO_4$, was added to make a final concentration of 2.94 M, followed by the addition of 10 µl of 1 M KOH per 1 g of solid $(NH_4)_2SO_4$ for neutralization. After stirring at 4 ^oC for 30 min, precipitated proteins were collected by centrifugation at 30,000 x g for 30 min. The pellet was dissolved in 800 µl of 20 mM HEPES-KOH [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] buffer (pH 7.5) cotaining 10 mM $MgSO_4$, 20% (v/v) glycerol, 1 mM EDTA, 10 mM EGTA, 5 mM DTT, 1 mM PMSF, 2 mM benzamidine HCl, 0.5 mg/ml leupeptin, 0.35 µg/ml bestatin, 0.4 µg/ml pepstatin A, 10 mM sodium fluoride {for only NaF (+) samples}. The samples were dialyzed against the same buffer containing 1 mM PMSF but lacking other protease inhibitors [0.5 mM sodium fluoride for only NaF (+) samples] until the conductance of a 1 : 201 dilution reached between 50 to 75 µS/cm. The dialyzate was then centrifuged at 10,000 x g for 10 min to prepare the "cell extract" for *in vitro* transcription assay. The protein concentration was in the range from 25 to 40 mg/ml. Aliquots of 100 µl were stored at -80 °C until use.

(r) *in vitro* transcription reactions

The reaction mixture contained the followings in 30 μl volume: 50 mM HEPES-KOH (pH 7.5), 10% (v/v) glycerol, 90 mM potassium glutamate, 0.75% (w/v) polyethylenglycol (PEG, Mr 3350, Sigma), 10 mM magnesium acetate, 5 mM EGTA, 2.5 mM DTT, 30 mM phosphocreatine, 0.4 mM ATP, 0.4 mM CTP, 10 μM [α-^{32}P]UTP (3 x 10^4 cpm/pmol), 20 μg/ml pGAL4CG$^-$ plasmid, 150 U human placental ribonuclease inhibitor (Takara, Shiga), 1.4 U/ml creatine phosphokinase (type I, Sigma) with or without 335 μg Gal4-VP16 activator protein, and 1 μg α-amanitin. Transcription was initiated by adding 20 μl reaction mixture into 10 μl aliquot of the cell extracts containing 125 μg proteins. The reaction was carried out for 30 min at 24 °C, and terminated by adding 20 μl of 200 mM NaCl, 20 mM EDTA containing 40 U of RNase T1. After incubation at 24 °C for 10 min for digestion of background non-specific RNA (specific transcripts do not contain G residues), a 150 μl mixture of 200 mM NaCl, 20 mM EDTA, 0.3% (w/v) SDS and 10 μl of 10 mg/ml Proteinase K was added, and incubated for 20 min at 30 °C. After phenol extraction and ethanol precipitation, the transcription products were suspended in 12 μl of 80% formamide containing 1 mM EDTA, 1 mg/ml each of xylencyanol and bromophenol blue. The samples were heat denatured at 98 °C for 3 min, and separated by electrophoresis on 5% polyacrylamide-7 M urea gel. The gel was fixed for 15 min in 10% methanol-10% acetate, and washed for 15 min in distilled water. Dried gel was subjected to autoradiography on Fuji RXU film for 1.5 hrs with intensifying screen, and the radioactivity of individual transcripts were measured with phosphoimager (Instant Imager, Packard). To test the thermostability of transcription apparatus, the cell extracts were incubated at 41 °C for 1 min, and then transcription reaction was carried out at 37 °C for 30 min by mixing the prewarmed reaction mixture. Gels were exposed to imaging plates for 15 min, and the intensities of radioactive gel bands were measured with BAS station (Fuji Film, Tokyo).

6. RESULTS

(a) Isolation of temperature-sensitive or cold-sensitive *S. pombe* mutants

Isolation of the *rpb3* mutants of *S. pombe* was performed by PCR mutagenesis *in vitro* of the *rpb3* gene. In order to avoid the multiple mutations, we carried out PCR in the absence of Mn^{2+} addition. PCR was carried out independently for the N-terminal and C-terminal proximal halves of the *rpb3* gene (Fig. 1). After screening of 1 x 10^5 transformants, we isolated 25 candidates of temperature-sensitive (Ts^-) mutants and 18 candidates of cold-sensitive (Cs^-) mutants.

In order to confirm that the Ts^- and Cs^- phenotypes are conferred by mutations in the *rpb3* gene, Ts^- or Cs^- phenotype were tested again for the Ura^+ progenies after a random spore analysis. A total of 9 Ts^- and 3 Cs^- were isolated after this secondary screening (Fig. 2, Table 1). All the Cs^- mutants, Cs3-39, Cs3-41 and Cs3-59, also showed Ts^- phenotype. For further confirmation of the Ts^- phenotype, we recovered the integration vectors from the genomic DNA of representative 7 Ts^- strains (see below). After transformation of all the recovered *rpb3* genes into the wild-type *S. pombe*, we obtained transformants showing the same Ts^- phenotype as the original Ts^- strains. Fig. 3 shows the results of Ts3-154, Ts3-176 and Ts3-231. However, not all the transformants showed Ts^- phenotype, persumably because the mutant *rpb3* genes on plasmids might be incorrectly inserted into the chromosome. We confirmed that at least 6 Ts^- mutations were recessive, because the heterozygotes between Ts^- strains and wild-type JY746 were no more temperature sensitive (Fig. 4).

(b) Identification of mutations in the *rpb3* gene

The sites of mutation were determined by direct sequencing of the *rpb3* genes amplified by PCR from genomic DNA of Ts^- or Cs^- mutants. The amino acid substitutions deduced from the respective DNA sequences are shown in Fig. 5 and Table 1. All the mutants were found to carry a single or double point mutations within the *rpb3* gene, each leading to one or two amino acid substitutions within the Rpb3 protein. All these mutations are located within the four regions, A to D, of Rpb3 protein conserved among the Rpb3 homologues from eukaryotes (Fig. 5).

(c) Isolation of Ura^- Ts^- strains

For detailed analysis of the phenotypes of the *rpb3* mutants, we focused on 7 representative Ts^- strains, *i.e.*, two (Ts3-30 and Ts3-53) carrying a single amino acid substitution in the region A, one (Ts3-84) carrying a point mutation in the region B, two (Ts3-154 and Ts3-176) carrying a single mutation in the region C, and two (Ts3-229 and Ts3-231) carrying a single mutation in the region D (Fig. 5). Since the Ura^+ Rpb3 mutant strains harbored another incomplete *rpb3* sequence and an integration vector sequence on the chromosome (for the gene organization see Fig. 1) and were genetically unstable, we selected the stable Ura^- Ts^- strains on 5-FOA [5-fluoroorotic acid] plate according to the published method (Kaiser *et al.*, 1994). For confirmation of the gene organization, the stable Ura^- colonies were subjected to Southern blot analysis. Genomic DNA from these strains were digested with *Bgl*II and *Xba*I, and subject to Southern blot analysis using both

rpb3 coding sequence and integration vector pSKura4 as probes. All 7 Ura$^-$ Ts$^-$ strains retained the single *rpb3* gene, but lacked the plasmid sequence (Fig. 6).

(d) Growth characteristics of the *rpb3* mutants

For characterization of the phenotypes of the Ts$^-$ *rpb3* mutants, we first analyzed the growth rates at both permissive and non-permissive temperatures. For some mutants such as Ts3-53 (95% of the rate of wild-type), Ts3-84 (75%), Ts3-176 (83%), Ts3-229 (76%) and Ts3-231 (93%) showed significantly reduced rates of the cell growth even at a permissive temperature at 30 °C (Table 1).

The growth rate was also measured at a non-permissive temperature. Upon transfer of the 30 °C culture to 38 °C, all the Rpb3 mutants stopped the cell growth even though the time of growth cessation was different depending on the site of mutations (Fig. 7). Based on the growth characteristics, the mutants could be classified into three groups. The group I mutants including Ts3-53, Ts3-176 and Ts3-229 grew slowly even at a permissive temperature, continued to grow at slightly reduced rates immediately after the temperature up-shift, but finally stopped to grow after prolonged incubation. The group II mutants including Ts3-30 and Ts3-84 grew faster than other Rpb3 mutants in the early phase after the temperature up-shift, but stopped to grow after about 10 h. Afterward the cell density stayed at plateau levels. The group III mutants including Ts3-154 and Ts3-231 grew at the highest rates among the Rpb3 mutants examined and reached the maximum level of cell density after the temperature up-shift. However, there is apparently no significant correlation between the grouping of Ts$^-$ mutants and the location of *rpb3* mutations.

Overall growth characteristics in the liquid medium is in good agreement with the growth on YE plate. Although the temperature limits that allowed the growth of Ts mutants on agar plates are lower than those observed on the liquid medium, most of the Rpb3 mutant strains except for the group III mutants completely stopped cell growth at 37 °C. The group III mutants, Ts3-154 and Ts3-231, stopped growth at 38 °C (Fig 2).

(e) Metabolic stability of the mutant Rpb3 proteins

The intracellular concentrations of Rpb3 protein in each of the *rpb3* mutant strains was measured at various time points after the temperature up shift to a non-permissive temperature. All the mutant strains were transferred from 30 °C to 38 °C, and the cells were harvested at 0, 6 and 12 h after the temperature up-shift. The relative amount of Rpb3 protein in a fixed amount of total proteins of cell lysates was measured by a quantitative Western blotting using the antibodies against the Rpb3 protein (Fig. 8). As experimental controls, the levels of Rpb2 and α-tubulin were measured.

The intracellular levels of Rpb3 in cells grown at the permissive temperature at 30 °C are significantly different among the mutant strains. The level was the highest for the wild-type strain, in good agreement with the concept that the rate of cell growth correlates with the level of RNA polymerase (Ishihama, 1981). The levels were the lowest for all the group I mutants, again supporting the interpretation that the slow growth rates of Ts3-53, Ts-176 and Ts3-229 are attributed to the low levels of RNA polymerase II in these strains. The relative amount of Rpb3 in all Ts$^-$ mutants decreased concomitantly with the increase in culture time at 37 °C, because the amount of total proteins per cell continued to increase at least up to the time point measured (12 h after the temperature shift). The level of Rpb2

in the mutants also decreased, but the rate in decrease of Rpb2 level was slower than that of Rpb3. Severe reduction of Rpb2 was observed in Ts3-53, Ts3-84 and Ts3-154 (Fig. 8).

(f) Sensitivity of mutant RNA polymerase II against protein denaturant

For detailed analysis of the mutant RNA polymerases carrying the mutant Rpb3 subunits, we purified the RNA polymerase II from two representative mutant strains, Ts3-30 and Ts3-231, each producing the mutant Rpb3 with Asn30Tyr (region A) and Met231Thr (region D) substitution, respectively. For quick purification of the mutant RNA polymerases, a nucleotide sequence encoding hexahistidine (His_6) tag was inserted into the 5'-termini of the mutant *rpb3* genes in these mutant *S. pombe* strains according to Kimura *et al.* (1997). The mutant RNA polymerase II was isolated from cells grown at a permissive temperature of 30 °C by Ni^{2+} affinity chromatography.

In order to test the integrity of RNA polymerase complexes, we then treated the purified RNA polymerases fixed to Ni^{2+}-nitrilotriacetic acid-agarose beads, with increasing concentrations of urea added as a protein denaturant according to the published procedure (Kimura *et al.*, 1997). After soaking with the buffer without urea the resin was treated with the dissociation buffer containing increasing concentration of urea. The proteins retained on the resin were eluted with imidazole and subject to SDS-PAGE. Up to 2 M urea buffer, all RNA polymerase II subunits remained bound to the resin (Fig. 9). Upon treatment with 4 M urea, Rpb5, Rpb6 and Rpb7 subunits were dissociated from the wild type RNA polymerase II. After treatment with 4 M urea the significant difference was observed between the wild-type and Ts3-30 mutant RNA polymerases. In the case of Ts3-30 RNA polymerase, not only Rpb5, Rpb6, Rpb7 but also Rpb2 and Rpb11 were released from the resin. On the other hand, the urea induced dissociation pattern of subunits from

Ni^{2+}-resin bound Ts3-231 mutant RNA polymerase was essentially the same with that of wild-type RNA polymerase. Previously, Kimura *et al.* (1997) observed that Rpb2 and Rpb11 were still retained bound with the Rpb3 even after treatment with 6 M urea. The reduction of Rpb2 and Rpb11 association with the mutant Rpb3 indicates that the integrity of the Rpb2-Rpb3-Rpb11 core subassembly is reduced for the Ts3-30 mutant RNA polymerase II.

(g) Suppression of *rpb3* mutations by overexpression of Rpb11

In the case of *E. coli* RNA polymerase, the initial step of subunit assembly is the formation of α dimer (Ishihama, 1981). The corresponding step in *S. pombe* is the formation of Rpb3-Rpb11 heterodimer. If the defect of RNA polymerase assembly in the Rpb3 mutants is attributed to the decrease in the association with Rpb11, the defect may be overcome by overexpression of the pairing subunit Rpb11. We tried to suppress the assembly defect of mutant Rpb3 proteins by overexpression of Rpb11.

The *rpb11* cDNA was cloned into pREP series vectors. By depletion of thiamine from the culture media, three different levels of the expression can be induced using three different vectors, pREP1 > pREP41 > pREP81. Approximately 100 fold difference was observed in the expression level between pREP1 and pREP41 and between pREP41 and pREP81 (Maundrell, 1993). All the Ts^- strains and the wild-type *S. pombe* were transformed with the three expression vectors, pREP1(rpb11), pREP41(rpb11), pREP81(rpb11), and a control vector pREP41 without any insertion. After plating the same number of transformant cells on EMM(+Ade, +Ura, -Leu, -Thi) plate, we found that all the Ts^- strains were suppressed by these Rpb11 expression vectors, allowing growth at 37 °C, while the transformation of the control plasmid did not affect the Ts^- phenotype.

The level of the suppression depended on the expression level (Fig. 10). The suppression efficiency was much higher for the two group III mutants, Ts3-154 and Ts3-231, compared with other Ts$^-$ mutants. In contrast, the efficiency of suppression was the lowest for the strain Ts3-30 carrying the *rpb3-30* mutation in the region A, indicating the assembly defect, in particular at the step of Rpb3-Rpb11 heterodimer formation, is the most severe for this region A mutant. For other group I and group II mutants, the suppression was observed by achieving the intermediate expression of Rpb11, suggesting that these mutants also carry the intermediate level of assembly defect of Rpb3.

(h) Establishment of an improved system of activator-dependent *in vitro* transcription

As an initial attempt for the functional assay of RNA polymerases from *S. pombe rpb3* mutants, we tried to set up a cell-free system of activator-dependent specific transcription using *S. pombe* extracts. The composition of transcription reaction mixture was essentially as described by Woontner *et al.* (1991), because it was reported that cell extracts from various yeast species including *S. pombe* possess the ability, albeit at low level, of activated transcription directed by the *S. cerevisiae CYC1* promoter in the presence of GAL4-VP16 fusion activator protein. The template plasmid pGAL4CG$^-$ used contained 377 bp G-less cassette sequence (Swadogo and Roeder, 1987) attached to the GAL4 binding site sequence and the *S. cerevisiae CYC1* promoter (Lue *et al.*, 1989). Using the template without G residues, transcription takes place without GTP, and moreover, background non-specific transcripts, if any, can be digested by RNase T1 treatment. Transcription products were purified essentially according to the methods of Liao *et al.* (1991). Transcription product synthesized in this system gave a single band on polyacrylamide gel electrophoresis in the presence of urea. However, three different bands were observed for transcripts by the *S. cerevisiae* cell extract (Woontner *et al.*, 1990).

Probably the selection of transcription start site by the *S. pombe* transcription apparatus may be more accurate than the *S. ceveisiae* extract (Russell, 1983).

The activity of *in vitro* transcription with use of the promoter and the activator protein from *S. cerevisiae* was, however, not so high for the *S. pombe* extract as compared with the homologous *S. cerevisiae* extract (Woontner *et al.*, 1993). For instance, the basal transcription in the absence of activator addition was not detected for the *S. pombe* extract. An *in vitro* transcription system with a high sensitivity enough to quantitate even the basal transcription is necessary for comparison of the activation efficiency of RNA polymerase among various *rpb3* mutant strains. Our effort has been focussed on the modification of the procedure for cell extract preparation. After several lines of attempt to improve the *in vitro* transcription system, we modified the procedure of cell extract preparation in the followings: (1) use of Cryo-press cell disrupter instead of commonly used disruption procedure with glass beads; (2) cell disruption in liquid nitrogen; (3) addition of high concentrations of protease inhibitors; and (4) addition of sodium fluoride as a phosphatase inhibitor (Hames and Higgins, 1993).

For purification of native forms of the RNA polymerase II from *S. pombe,* we used a cocktail of protease inhibitors, which contains 1 mM (PMSF), 2 mM benzamidine-HCl, 0.5 μg/ml leupeptine, 0.35 μg/ml bestatin, and 0.4 μg/ml pepstatin (Woontner *et al.*, 1993). In addition, we prepared a modified version of the cocktail which contains 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.64 mM benzamidine-HCl, 10 μg/ml leupeptine, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml *N*-tosyl-L-lysine chloromethyl ketone, 10 μg/ml *N*-tosyl-L-phneylalanine chloromethyl ketone, 5 μg/ml chymostatine, and 2.5 μg/ml antipain (Azuma *et al.*, 1993). Influence of these two protease inhibitors cocktails on the yield of the transcription activity was tested by adding them in the cell extraction buffer. Fig. 11 (upper panel) shows specific transcripts synthesized by the different cell extracts in the prerence and absence of GAL4-VP16. The levels of both basal

and activated transcription were not so different with or without the addition of protease inhibitors. Apparently the effect of high concentration of protease inhibitors was not obvious. In the case of RNA polymerase II purification, the recovery of enzyme activity was significantly increased by the addition of protease inhibitors (Azuma *et al.*, 1993). The major factor for the observed improvement might be due to cell disruption in liquid nitrogen, but not to addition of the protease inhibitors. When the levels of RNA polymerase II subunits were analyzed by Western blotting, however, the concentration of RNA polymerase II proteins was found to be decreased (Fig. 11, lower panel). In particular, the marked reduction was observed for the largest subunit, Rpb1. The failure of concomitant reduction in the transcription activity implies that the level of RNA polymerase II in the cell extracts is not limiting under the assay conditions employed. We can not exclude another possiblity that the cell extracts contain unused or unassembled Rpb suubnits which are sensitive to protease digestion. To avoid unexpected protein degradation, we then decided to prepare the cell extracts in the presence of high concentration of the protease inhibitors.

Possible effect of the addition of sodium fluoride, (Hames and Higgins, 1993), was also examined. As shown in Fig. 11, the addition of sodium fluoride obviously improved activated transcription by the GAL4-VP16 protein to the level approximately 2-fold higher than that observed with the extract prepared in its absence. Thus, we decided to routinely include NaF during the preparation of cell extracts. No significant difference was, however, observed in the basal transcription (without GAL4-VP16) between the extracts prepared in the presence or absence of sodium fluoride.

The GAL4-VP16 fusion protein used was expressed in *E. coli* and purified by two step chromatography on DEAE cellulose and heparin-sepharose columns essentially according to Chasman *et al.* (1989). The GAL4-VP16 thus purified contained two protein

bands on SDS-PAGE, which migrated to the positions of 33 and 40 kDa on 10% gel (Fig. 12). The peak fraction (fraction No. 15) was used for the transcription assay. After amino (N)-terminal sequencing, however, both bands had the same sequence with the authentic GAL4 protein (data not shown), indicating that the fast migrating band is a carboxy (C)-terminal truncated fragment of GAL4-VP16. In the transcription assays herewith described, this mixture of native and truncated GAL4-VP16 was used without further purification.

The optimum concentration of GAL4-VP16 to give the maximum activation of transcription by the *S. pombe* extract was then examined. For the titration, the GAL4-VP16 was diluted in a stepwise manner with the dialysis buffer containing 5 mM DTT and protease inhibitors, and the results are shown in Fig. 13. The level of specific transcript increased concomitantly with the incease in amount of the activator addition, and the maximum activation was achieved with 335 ng of GAL4-VP16 per reaction. For comparative studies of the activities of *rpb3* mutant RNA polymerases, we analyzed the optimal amount of GAL4-VP16 for each *rpb3* mutant cell extract, but It turned out that the amount of 335 ng GAL4-VP16 is always the optimal amount if the same amounts of cell extract s were used (data not shown).

(i) Comparison of the Rpb3 content in the *rpb3* mutant extracts

Using the *in vitro* transcription assay system thus established, we examined the activity of cell extracts from six different Rpb3 Ts mutants. First we prepared cell extracts from strains grown at 30 ^{o}C. Since the amount of Rpb3 in the whole cell lysates is significantly different among the mutant strains (see Fig. 8; Yasui *et al.*, 1995), we measured, prior to the transcription assay, the concentration of Rpb3 protein in each cell etract by quantitative Western blotting. Fig. 14 shows the Western blot of 125 μg each of the extracts. Using the known amount of Rpb3 in the reference RNA polymerase as

standard for caliculation, the extracts used were found to contain 3.28 - 6.36 x 10^{10} molecules of Rpb3. The level was the highest amount for the wild-type JY741 cell extract, while the Rpb3 levels in the mutant cell extracts were lower than the maximum level in the wild-type extract, in agreement with the observations in Fig. 8 that the assemly of Rpb3 into the RNA polymerase II is defective, to various extents for all the *rpb3* mutants. The level of Rpb3 in the Ts3-53 extract was the lowest and about half of the maximum level in the wild-type extract. Again this agrees with the finding that, in the case of *E. coli* α subunit, the sequence including Asn53 within the region A plays an essential role in the initial stage of assembly, being involved in both α dimerization and binding of the β subunit (Kimura and Ishihama, 1995a; 1995b; 1996). Strong correlation was not observed between the growth rate and the content of Rpb3.

(j) Comparison of the transcription activities among the Rpb3 mutants

For quantitative assay of the transcription activities for all the mutant cell extracts, we next measured the RNA synthesis acitivity. Fig. 15 shows one example of transcription assay using the same amount of cell extracts from all the *rpb3* mutants. In the presence of α-amanitin, the activated transcription was completely inhibited, indicating that the observed transcription was RNA polymerase II-dependent. However, the concentration of α-amanitin required for complete inhibition of *S. pombe* transcription was about three times higher than that of *S. cerevisiae* system. For comparative studies of the *rpb3* mutants, we adjusted all the cell extracts to the same protein concentration as to include 125 μg in a volume of 10 μl added to the transcription assay. For this amount of proteins, a linear relationship was observed between the extract volume and the transcription activity.

Under the optimum conditions of GAL4-VP16-dependent *in vitro* transcription thus established, the activities of all the mutant extracts as well as wild-type extract were examined in parallel. Results are shown in Fig. 15. The level of basal transcription in the

absence of GAL4-VP16 was not so different between the strains, but the level of activated transcription was variable. The level of activated tarnscription by the wild-type cell extract was 5.5 fold higher than that of basal transcription. The 6.52-fold activation level by the Ts3-231 was further higher than the wild-type. Activation of 5.38 fold by Ts3-53 is as much as that with the wild-type cell extract, but the activation of 4.03, 3.81, 3.77 and 4.30 fold by the Ts3-30, Ts3-84, Ts3-154 and Ts3-176 cell extracts, respectively, was slightly lower than that of wild-type. The activation levels of Ts3-84, Ts3-154 and Ts3-176 were nearly 60% of the level of wild-type. Ts3-30 and Ts3-53 showed intermediate levels of the activity, with approximately 85% of the wild-type level. The activity of Ts3-231 was higher than the wild-type level.

(k) Heat inactivation of transcription activity of the Rpb3 mutants

The thermostability of mutant RNA polymerases was examined by measuring the remaining activity after preincubation of the cell extracts at high temperatures. Preliminary experiments indicated that preincubation of the mutant cell extracts for short periods at 37 ^{o}C resulted in marked reduction in the transcription activities (data not shown). In the experiment shown in Fig. 16, the cell extracts were incubated at 41 ^{o}C for 1 min, and then transcription reaction was carried out at 37 ^{o}C by mixing the prewarmed reaction mixture. Under such a strict condition, the transcription activity was decreased approximately hundred folds. For this reason, the intensities of radioactive gel bands were measured with a BAS station (Fuji Film, Tokyo). The activities are shown as the relative values to the level of basal transcription by the wild-type cell extract. The level of activated transcription by the mutant Ts3-30 and Ts3-154 extracts remained almost the same level as the wild-type JY741 extract, but the transcription levels by other mutant cell extracts were reduced to various extents. The reduction of transcription activity was the largest for Ts3-176. Since the activities of both basal and activated transcription were decreased, the observed decrease in transcription activities reflects inactivation of the basal transcription apparatus

containing Ts3-176 Rpb3 rather than inactivation in the response to the activator. Likewise, both basic and activated transcription by the cell extracts from three mutant strains, Ts3-53, Ts3-84 and Ts3-231, decreased in parallel, even though the inactivation levels were lower than that of Ts3-176.

7. DISCUSSION

(a) Amino acid substitutions in temperature sensitive mutant Rpb3

To get insight into structure-function relationship of Rpb3, we tried to isolate as many temperature-sensitive or cold-sensitive mutants as possible, each carrying a single mutation in the *rpb3* gene. We isolated 9 Ts^- mutants and 3 Cs^- mutants, all containing a single mutation (or two in one case) in the *rpb3* gene (see Table 1). The *rpb3* mutations in the 9 Ts^- mutants are clustered in all the four conserved regions, among which two (A and D) are conserved among Rpb3 homologues from both prokaryotes (α subunits) and eukaryotes and two (B and C) are conserved only within eukaryotes (see Fig. 5). The regions A and D, each carrying the well-conserved α motif-1 and -2, respectively (Dequard-Chablad *et al.*, 1991; Sentenac *et al.*, 1992; Ulmasov *et al.*, 1996), and correspond to the N-terminal proximal and the C-terminal proximal regions of the αNTD, each playing critical roles in the contact with α and β subunits (motif-1) or the contact with α and β' subunits (motif-2), respectively (Heyduk *et al.*, 1996; Kimura and Ishihama, 1995a; 1995b; Zhang and Darst, 1998).

Mutations in the region A: The region A is conserved in not only eukaryotic RPB3 polypeptides but also prokaryotic α subunits. In the case of *E. coli* RNA polymerase, this region plays the most critical role in the assembly of RNA polymerase by providing the contact surface for the α (dimerization) and β subunits, thereby forming the $\alpha_2\beta$ assembly intermediate (Kimura and Ishihama, 1995a; 1995b).

Among total of 9 Ts$^-$ and 3 Cs$^-$ mutants analyzed, 4 Ts$^-$ (Ts3-24, Ts3-30, Ts3-31 and Ts3-53) and 3 Cs$^-$ (Cs-39, Cs3-41 and Cs3-59) mutants carried a single (or double in the case of Cs3-59) amino acid substitution in the region A (Fig. 5). Ts3-24 carries a single base substitution of A70C which leads to Thr24Pro change near the N-terminal end of the region A. Ts3-30 strain carries A131T mutation, resulting in Asn30Tyr substitution. The Asn30 is located on the putative helix 1 of the N-terminal half of region A (Zhang and Darst, 1998), and is conserved among wide varieties of the subunit 3 homologue including the *E. coli* RNA polymerase α subunit (see Fig. 5). In the case of *E. coli* α subunit, both the sites for α dimerization and β-subunit binding are located on this helix 1 (Kimura and Ishihama, 1995a; 1995b; Zhang and Darst, 1998), and one of the dimerization-defective mutations is located at Gly40 next to this conserved Asn residue (Kimura and Ishihama, 1995b). The Gly40 of *E. coli* α subunit also corresponds to the site of one of the double-mutant allele *RPB3-1* in the temperature-sensitive *S. cerevisiae* strain (Kolodziej and Young, 1991).

The 3 Cs$^-$ mutants isolated in this screening all carried mutations in a narrow region between residues 38 and 59 in the central part of region A (see Fig. 5). Cs3-59 carried two mutations, C156T and A265T, which lead to amino acid substitutions, Ala38Val and Asp59Val, while Cs3-39 and Cs3-41 carried a single amino acid substitution, Glu39Lys and Pro41Leu, respectively. The sites corresponding to two β-subunit contact sites, Arg45 and Leu48, in *E. coli* αNTD are both located between the 3 Ts$^-$ mutations and the 3 Cs$^-$ mutations (see Fig. 5). The *rpb3-53* mutation in Ts3-53 leads to change of the conserved Asn53 to Asp in the C-terminal part of region A. The corresponding Tyr68 on *E. coli* αNTD can be protected from hydroxide radical cleavage after binding of the β subunit (Heyduk *et al.*, 1996; Zhang and Darst, 1998).

A mutation in the region B: The region B includes the putative metal binding sequence, CXCX3CX2C, from positions 90 to 99 in the *S. pombe* Rpb3 sequence. Since the resgion B is conserved only among the eukaryotic RNA polymerase II but not in the corresponding subunits of RNA polymerase I and III, this region has been considered to be involved in functions related to the RNA polymerase II such as interaction with either transcription factors for protein-coding genes or RNA polymerase II-specific subunits. In our mutant collection, only one Ts3-84 mutant carried a point mutation T340C in this region, which leads to substitution of Ser for the conserved Leu84 near the N-terminal edge of region B (Fig. 5).

Mutations on the region C: The region C of Rpb3 is conserved only among three forms of the eukaryotic RNA polymerase but is absent in the prokaryotic homologues. Therefore, this region is considered to be related to a eukaryotic-specific function(s). We isolated one Ts$^-$ mutant, Ts3-154, carrying a point mutation T550C which leads to Ile154Thr change in the Rpb3 protein near the N-terminal half of region C.

Mutations on the region D: The region D is conserved among wide varieties of Rpb3 homologues from eukaryotes to prokaryotes. Ts3-176 and Ts3-229 mutants carry a single mutation, Ala176Pro and Phe229Ser, respectively, on the putative β-sheet structure formed by the C-terminal half of region C (Zhang and Darst, 1998), while Ts3-231 carries a mutation, Met231Thr, on the β-sheet structure in the C-terminal half of region D. The α-motif 2 exists in the C-terminal portion of the region D. In the corresponding region of *E. coli* αNTD, small insertions at positions 180 and 200 cause defects in β' binding without affecting the assembly of $\alpha_2\beta$ (Kimura and Ishihama, 1995a). The residues 180 and 200 of αNTD are protected from the hydroxide radical cleavage after binding of β' subunit (Heyduk *et al.*, 1996). Thus, the genetic and biochemical studies indicate that the β-sheet structures including these residues are considered to form the contact surface with the β'

subunit (Heyduk *et al.*, 1996; Kimura and Ishihama, 1995a; 1995b; Zhang and Darst, 1998). The *rpb3-176* corresponds to the same residue as position 180 in *E. coli* αNTD while the *rpb3-229* is situated at the C-terminal-proximal side of the residue 200 (Fig. 5). The *rpb3-176* mutation also corresponds to the same position with one of the double mutations of *S. cerevisiae RPB3-1* allele (Kolodziej and Young, 1989).

(b) Growth characteristics of the *rpb3* mutants

Mutations affecting the molecular assembly of macromolecular complexes often lead to cold-sensitive phenotypes because of the involvement of hydrophobic interactions for protein-protein contacts. Thus, the finding that the 3 *rpb3* mutations conferring the Cs^- phenotype are all located in the region A of Rpb3 is itself in good agreement with the concept that the region A plays the most important role in the assembly of RNA polymerase II. However, we focused our detailed analysis on the set of 7 Ts^- mutants because these Ts^- mutations are distributed along all four conserved regions of the Rpb3 protein.

From the growth characteristics of temperature-sensitive Rpb3 mutants (Fig. 7) and the metabolic stability of mutant Rpb3 proteins (Fig. 8), we classified the Ts^- mutants into three groups: The group I mutants including Ts3-53, Ts3-176 and Ts3-229; the group II mutants including Ts3-30 and Ts3-84; and the group III mutants including Ts3-154 and Ts3-231. The difference in the time of growth cessation after the temperature up-shift may be attributed to the difference in intracellular levels of the functional RNA polymerase II (see below). The Ts^- characteristics of group I mutants seems to be due to the decrease in the level of RNA polymerase II at not only non-permissive but also permissive temperatures; and both group II and III mutants appear to carry defect of the Rpb3

assembly at non-permissive temperatures, but the defect is more severe for the group II mutants. However, Ts3-84 (group II) grew faster than other Rpb3 mutants in the early phase after the temperature up-shift, then stopped to grow after about 10 h and the amount of mutant Rpb3 decreased stepwise manner (Fig 8).

(c) Metabolic stability of the mutant Rpb3 proteins

In *E. coli*, the metabolic stability of subunits is recognized as a marker for monitoring the assembly state of RNA polymerase, because the assembled RNA polymerase is conserved for generations without detectable degradation, but unassembled free subunits are degraded (Ishihama, 1981). Likewise, assembly-defective mutant RNA polymerase proteins of *S. cerevisiae* are metabolically unstable, but become stable once they are assembled into the core enzyme (Kolodziej and Young, 1989; 1991; Mann *et al.*, 1987). We then examined the intracellular level and metabolic stability of Rpb3 as indications for monitoring its assembly state.

If the metabolic stability of Rpb3 correlates with the level of its assembly, the faster decrease in Rpb3 for the group-II and group-III mutant strains at non-permissive temperature may represent that the defect in subunit assembly at the non-permissive temperature is more severe for these group mutants. Since these mutants continued to grow for certain periods after the temperature up-shift, the mutant Rpb3 proteins already assembled into the RNA polymerase prior to the temperature shift must be stable and functional even at the non-permissive temperature. From these results, we concluded that the reduction in Rpb3 protein level in the group-II and group-III mutants after the temperature up-shift was attributed to the degradation of unassembled mutant Rpb3 proteins, leading to reduction in the functional RNA polymerase II and cessation of the cell

growth. The intracellular level of Rpb3 is lower for some Ts⁻ mutants even at the permissive temperature of 30 °C (see Fig. 8). The low level of Rpb3 might be attributed to the decreased rate of Rpb3 synthesis in these mutants, but this possibility is unlikely because the mutations are located within the Rpb3 coding sequence. Instead the assembly of mutant Rpb3 proteins into RNA polymerase may be inefficient in these *rpb3* mutants or the metabolic stability of mutant Rpb3 may be decreased. If this is the case, the time of growth retardation after the temperature up-shift may be correlated with the pre-shift level of assembled RNA polymerase.

The site of amino acid substitution of these mutant are nearly located on the reported *E. coli* assemble defective mutations. This support the notion that reduction of mutant Rpb3 protein is due to assembly defects. However, stability of assembled Rpb3 remains to be elucidated.

(d) Sensitivity of mutant RNA polymerase II against protein denaturant

Previously, we established an *in vitro* test system for checking the integrity of RNA polymerase II assembly (Kimura *et al.*, 1997), in which the resin-bound RNA polymerase II via H_6-tag is treated with increasing concentrations of protein denaturants to identify the subunits remained in the polymerase complexes. Using this system, the integrity of RNA polymerase II was tested for two representative mutant strains, Ts3-30 and Ts3-231. In the case of one Ts⁻ mutant RNA polymerase (Ts3-30), the purified RNA polymerase is dissociated *in vitro* with urea easier than the wild-type RNA polymerase II (see Fig. 9), indicating that the association of Rpb3-30 mutant protein to the core subassemblies is weaker than that of wild-type Rpb3. The Ts3-30 mutant carries a single amino acid substitution Asn30Tyr in the conserved region A of Rpb3, which corresponds to the site of

E. coli α subunit for α dimerization and β subunit binding (Kimura and Ishihama, 1995a; 1995b). Since the reduction of Rpb11 is much faster than that of Rpb2 upon treatment with increasing concentrations of urea, one possibility is that the region including Asn30 plays a critical role in binding of Rpb11. In the case of wild-type of *S. pombe* RNA polymerase II, both Rpb2 and Rpb11 are still retained bound with the Rpb3 even after treatment with 6 M urea (Kimura *et al.*, 1997). The reduction of Rpb2 and Rpb11 association with the mutant Rpb3 indicates that the integrity of the Rpb2-Rpb3-Rpb11 core subassembly, which is equivalent with the assembly intermediate $\alpha_2\beta$ of *E. coli* RNA polymerase, is reduced for the Ts3-30 mutant RNA polymerase II. Thus, the assembly defect is much severe for the region A mutant than the Ts3-231 carrying the mutation Met231Thr within the region D. The results are consistent with the prediction that the region A plays a critical role in the early steps of RNA polymerase assembly.

(e) Suppression of *rpb3* mutations by overexpression of Rpb11

The formation of α dimer is the initiation step of RNA polymerase assembly in *E. coli* (Ishihama, 1981). Rpb11 is the second homologue of prokaryotic α subunit in the case of eukaryotes, Rpb3 forms the heterodimer with Rpb11 instead of Rpb3 homodimer (Ulmasov *et al.*, 1996; Svetlov *et al.*, 1998). As an attempt to test the association step of mutant Rpb3 with Rpb11, we then tested possible suppression of the assembly-defective mutations of Rpb3 by overexpression of Rpb11. The overexpressed Rpb11 suppressed the Ts^- phenotype of all the Rpb3 mutants but to various extents (Fig. 10). The level of Rpb11 expression needed for effective suppression correlates with the level of assembly defect of mutant Rpb3. For instance, the expression of low levels of Rpb11 effectively suppressed the growth of group III mutants, Ts3-154 and Ts3-231, while high levels of

Rpb11 expression were needed to observe significant levels of the suppression of Ts3-30 carrying a mutation in the region A and with a severe defect in RNA polymerase assembly (see Fig. 9). In the case of Ts$^-$ mutant RNA polymerases I and III carrying the mutant subunit AC40 (the third largest and a common subunit of *S. cerevisiae* RNA polymerases I and III), multicopy suppressors have been isolated, which were mapped at either the AC19 gene (another α homologue in *S. cerevisiae* RNA polymerases I and III) or ABC10β (or RPB10) gene (Lalo *et al.*, 1993). In the case of *S. pombe* RNA polymerase II, both subunits (Rpb10 and Rpb11) are involved in the formation of subassembly core, though the affinity to the assembly core is higher for Rpb11 (Kimura *et al.*, 1997). The specificity of suppression by Rpb11 needs to be confirmed by overexpression of subunits other than Rpb11.

(f) Activator-dependent *in vitro* transcriptio system of *S. pombe*

The activator-dependent *in vitro* transcription system has been established for the budding yeast *S. cerevisiae*. Weentner *et al.* (1993) tried to establish a similar system using *S. cerevisiae* GAL4-VP16 as activator and cell extracts from four different yeast strains. The results indicated that the activity of *S. pombe* cell extract was not strong enough to detect the basal transcription in the absence of GAL4-VP16 addition. To improve the *in vitro* transcription by *S. pombe* extracts, we modified the procedure of extract preparation in various points. The most effective modification was the change in disruption method of *S. pombe* cells. Previously we noticed marked inactivation of the RNA polymerase II during disruption of *S. pombe*, in particular due to heat inactivation (Azuma *et al.*, 1991). The heat-induced inactivation of transcription activity was significantly reduced by disrupting cells in liguid nitrogen using Cryo-press cell

disrupter™. We also added high concentrations of various protease inhibitors to the extraction buffer. The addition of the cocktail of protease inhibitors, however, did not give significant apparent increase in the transcription activity, but the levels of Rpb proteins were reduced in the absence of high concentrated protease inhibitors as measured by Western blotting. Since the amount of cell extracts used in this study were at the range in which a linear relationship exists between the amount of proteins and the transcription activity, it appears that the cell extracts used in this study contain some non-functional Rpb proteins which are sensitive to protease digestion. In fact, we found that the cell extract contains considerable amounts of unassembled Rpb proteins (Kimura, M., Sakurai, H. and Ishihama, A., submitted for publication). The effective improvement of activated transcription was also achieved by the addition of sodium fluoride as a phosphatase inhibitor (Hames and Higgins, 1993). However, it remains unsolved whether the level of phosphorylation of Rpb1 CTD is affected by the addition of sodium fluoride.

The optimal concentration of the synthetic activator, GAL4-VP16, to give the maximum transcription activity by the *S. pombe* extracts was 335 ng per 30 µl of the transcription mixture. When the *S. cerevisiae* extract was used otherwise under the same conditions, the requirement for GAL4-VP16 was as low as 80 ng (Woontner *et al.*, 1990), indicating that the affinity of GAL4-VP16 to *S. pombe* transcription apparatus is 4 times weaker than that of *S. cerevisiae*. In the *S. cerevisiae* system, several candidates of the VP16 receiver molecule have been proposed, including mediator complex (Hengartner *et al.*, 1995) and general transcription factors TFIIB (Lin *et al.*, 1991) and TFIIH (Xiao *et al.*, 1994). However, no suggestion of direct contact between GAL4-VP16 and Rpb3 has been provided. Since the decrease in transcription activity coupled with the deletion of CTD repetition in RPB1 was observed in VP16-dependent transcription system (Liao *et al.*, 1991), the role of GAL4-VP16 on *in vitro* transcription system is considered to be recruitment of the transcription apparatus to DNA promoter.

(g) Levels of RNA polymerase proteins in *S. pombe* extracts

The intracellular concentrations of all twelve subunits of the RNA polymerase II has been determined using a quantitative immunoblotting method (Kimura, M., Sakurai, H. and Ishihama, A., submitted for publication). The content of Rpb3 is the least among the twelve suubnits, indicating that the intracellular concentration of RNA polymerase II is determined by the level of Rpb3. Since Rpb3 plays a key role in the RNA polymerase II assembly, it is natural to consider that the level of RNA polymerase II decreases in the *rpb3* mutants. The content of Rpb3 subunit in the mutant cell extracts was measured by the quantitative Western blotting (Fig. 14). The amount of Rpb3 was the highest in the wild-type cell extract, while the Rpb3 level in the mutant cell extracts were reduced to various extent. However, the basal transcription by the mutant cell extracts remained nearly at the same level, indicating that the level of functional RNA polymerase is maintained as a fixed level even though the efficiency of subunit assembly is different between the *rpb3* mutants. In the case of *E. coli*, the assembled RNA polymerase is metabolically stable but unassembled subunits are subject to degradation by proteases (Ishihama, 1981). Likewise, unassembled Rpb proteins appear to be degraded rapidly, even though the level of functional RNA polymerase stays constant (see Fig. 11).

(h) Difference in transcription activities among Rpb3 mutants

The above results indicate that all the Ts mutant Rpb3 are, more or less, defective in the assembly function of RNA polymerase at various steps of subunit-subunit association depending on the site of mutation. In order to examine the possible influence of these mutations on the activation function, we tested GAL4-VP16-dependent transcription for some representative Rpb3 mutants (Fig. 15). The level of activated transcription was lower, albeit at different levels, for the Rpb3 mutant cell extracts than that of wild-type. The

decreased level of activation transcription was more remarkable for the *rpb3* mutants with mutations in the regions B and C, which are present only in eukaryotic Rpb3 homologues. This finding itself suggests the involvement of regions B and C in the activated transcripton. The internal part of Rpb3 may be the targets for interaction with eithter eukaryotic RNA polymerase II-specific subunits or certain transcription factors. A mutant *S. cerevisiae* carrying double mutation Cys92Arg and Ala159Gly in the *RPB3* gene is temperature-sensitive as detected by reduction in the activation of transcription *in vitro* and *in vivo* (Tan *et al.*, 2000). The mutation sites of Ts3-84 and Ts3-154 are close to the positions of Cys92Arg and Ala159Gly mutations in *S. cerevisiae*. In the case of *E. coli* α subunit, the αCTD plays a major role in recognition and dinteraction with class-I activators and DNA UP elements (Ishihama, 1993; 1997). However, an αNTD mutation has been identified, which interacts cAMP-CRP (Niu *et al.*, 1996). On the other hand, upon brief treatment of the cell extracts at high temperatures or by transcription assay at high temperatures, the activity of mutant cell extracts decreased much faster than the wild-type cell extract (Fig. 16). The rate of heat inactivation seems to be faster for the mutants, Ts3-53, Ts3-176 and Ts3-231, which carry mutations in either region A or D. Taken together we propose that the regions A and D play more critical roles in the subunit assembly.

(i) Conclusion

For identification of the *in vivo* roles of Rpb3 in the assembly and function of RNA polymerase II, in this study, we isolated, 9 temperature-sensitive (Ts^-) or 3 cold-sensitive (Cs^-) *S. pombe* mutants with mutations throughout the *rpb3* gene by PCR mutagenesis. After sequence analysis each mutant was found to carry a single (or double in a few cases) amino acid substitution. Most of the mutations were located within the four conserved regions A to D. A total of 4 Ts^- and 3 Cs^- mutations were located in the region A,

indicating the importance of region A for the function of Rpb3, in agreement with the most important role of the corresponding region in the assembly of prokaryotic RNA polymerase. In fact, one of the mutation in region A was found to reduce *in vitro* stability of the core assembly consisting Rpb3, Rpb11 and Rpb2. Measurement of the metabolic stability for the mutant Rpb3 proteins indicated that the amounts of all the mutant Rpb3 protein are severely reduced at non-permissive temperature. Growth characteristics of each mutant correlates with the metabolic in stability of RNA polymerase II. From these results we concluded that the region A is involved in the assembly of RNA polymerase II core structure as in the case of prokaryotic RNA polymerase. Supporting this conclusion, the Ts^- phenotype of all the mutants were suppressed by overexpression of Rpb11, the pairing subunit in the initial stage of RNA polymerase II assembly.

In addition to the role of Rpb3 in the assembly of RNA polymerase II, we analyzed the possible role of Rpb3 in transcription activation function. For the assessment of transcription activation by the mutant RNA polymerases, we established an activator-dependent *in vitro* transcription system for *S. pombe*. The results of transcription assays using the mutant cell extaracts indicated that the decrease in activation transcription level was more remarkable for the *rpb3* mutants with mutations in the regions B and C. This is in contrast with the findings that the *rpb3* mutants with mutations in the regions A and D are more thermolabile. Taken together we propose that the regions A and D play more critical roles in the subunit assembly and the region B and C may be involved in transcription activation.

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Strain	Mutation[a]	Nucleotide substitution	Amino acid substitution	Region	Growth rate[b] (doubling/hr/30°C)
Wild-type	*rpb3*$^+$	-	-	-	0.315
Ts3-24	*rpb3-24*	A70C	T24P	A	nd[c]
Ts3-30	*rpb3-30*	A131T	N30Y	A	0.313
Ts3-31	*rpb3-31*	C94T, C135T, C138T	S31L	A	nd
Cs3-39(Ts)	*rpb3-39*	G158A	E39K	A	nd
Cs3-41(Ts)	*rpb3-41*	C165T	P41L	A	nd
Ts3-53	*rpb3-53*	A246G	N53D	A	0.303
Cs3-59(Ts)	*rpb3-38/3-59*	C156T, A265T	A38V, D59V	A	nd
Ts3-84	*rpb3-84*	T340C	L84S	B	0.240
Ts3-154	*rpb3-154*	T550C	I154T	C	0.333
Ts3-176	*rpb3-176*	G615C	A176P	C	0.263
Ts3-229	*rpb3-229*	T775C	F229S	D	0.238
Ts3-231	*rpb3-231*	T781C	M231T	D	0.294

Table 1 Sites of the *rpb3* mutations and growth rates of the *rpb3* mutants

[a] DNA sequence of the *rpb3* genes from the mutants was determined after PCR amplification.

[b] The growth rate was determined by measuring the turbidity at 595 nm of cells grown in YE(+Ade +Ura) medium at 30°C.

[c] nd, not determined.

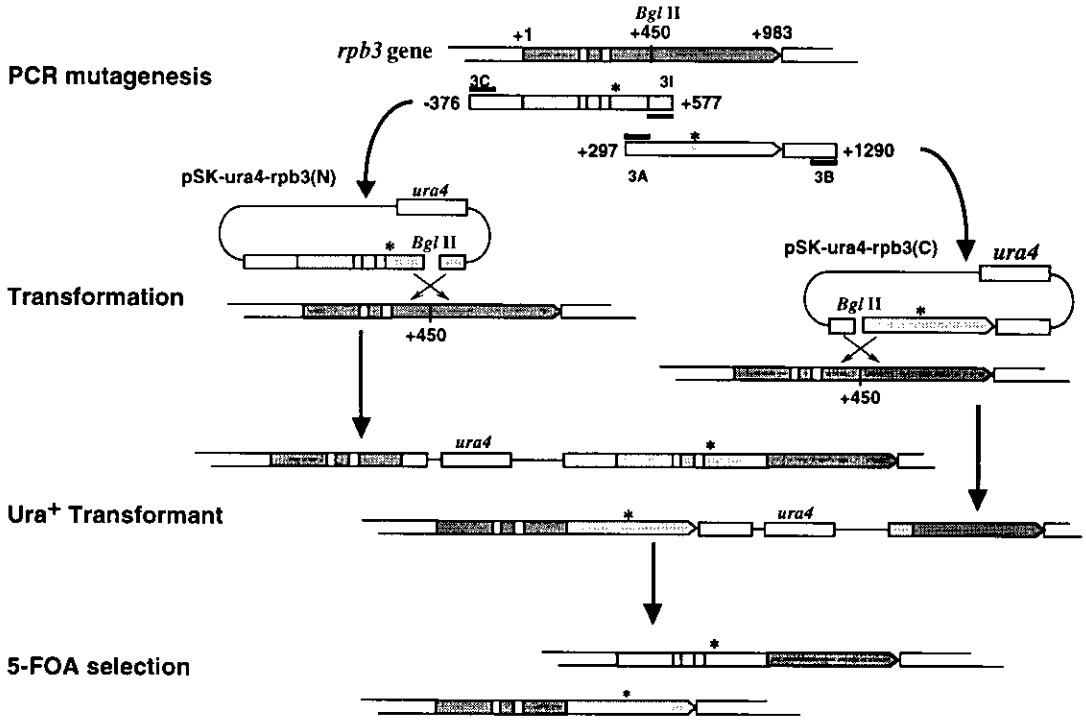
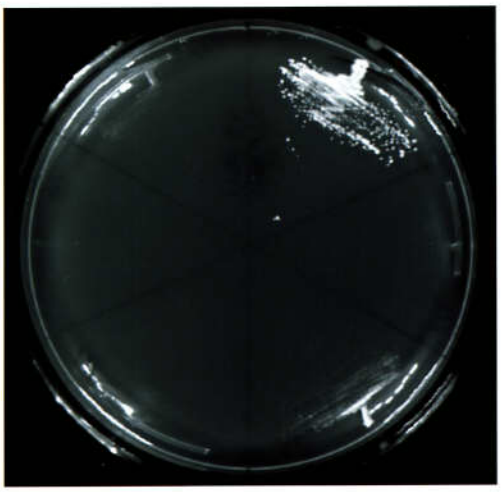
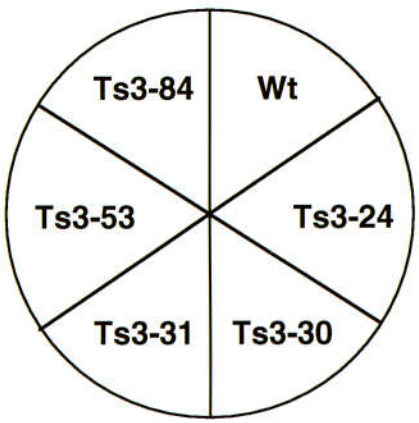


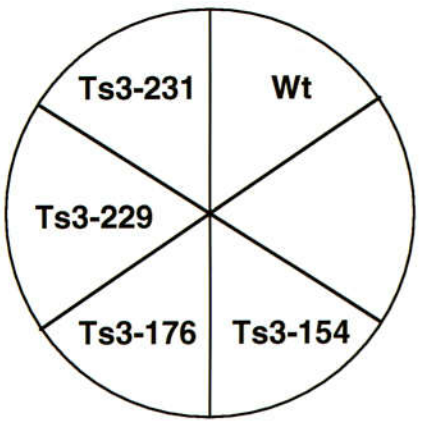
Fig. 1 Construction of *S. pombe rpb3* mutants. The N-terminal (-376 to +577) and C-terminal (+297 to +1290) segments of the *S. pombe rpb3* gene were PCR-amplified separately, and cloned into pSKura4 to construct two independent libraries, pSK-ura4-rpb3(N) and pSK-ura4-rpb3(C). After treatment with *Bgl*II, the linearlized plasmids were transformed into strain JY741 for screening Ura^+ transformants. Starting from the Ura^+ transformants, Ts^- and Cs^- mutants were isolated. After confirmation of the *rpb3* mutations by sequencing of PCR-amplified DNA, Ura^- Ts^- strains were isolated after screening on 5-FOA plate.

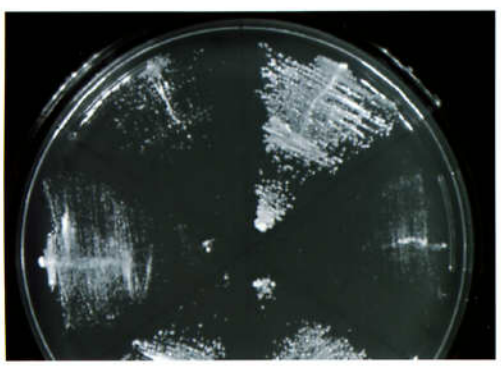
YE(+Ade +Ura) 37°C 5days



YE(+Ade +Ura) 37°C 3days







YE(+Ade +Ura) 18°C 7days

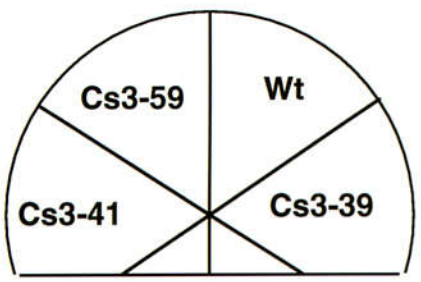


Fig. 2 Effect of temperature on growth of 9 Ts^- and 3 Cs^- strains. Isolated Ts^-Ura^+ strains were pre-incubated at 30 °C on EMM(+Ade, -Ura) for 2 days, and then cells were streaked ontoYE(+ Ade, +Ura) plates. Incubation time and temperature are indicated on each panel.

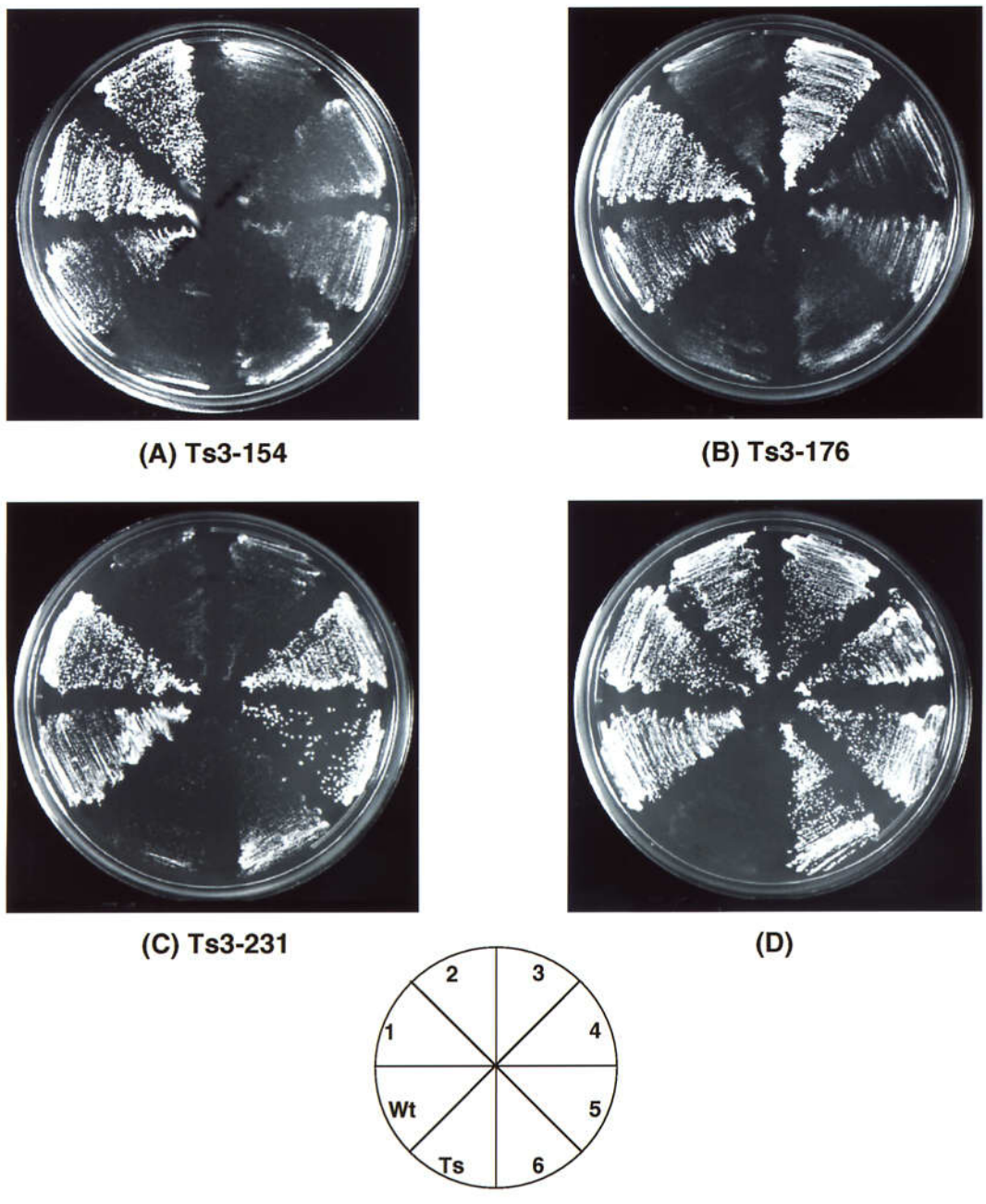


Fig. 3 Re-transformation of the *rpb3* genes recovered from Rpb3 mutants. Plasmids were recoverd from Ts3-154 (A), Ts3-176 (B), Ts3-231 (C) and then re-transformed into JY741. Ura^+ transformants were checked for growth at non-permissive temperature. The plasmid of panel D was recovered from Ts^- candidate which did not have any mutation in the *rpb3* gene. All transformants in this experiment showed Ts^+ phenotype.

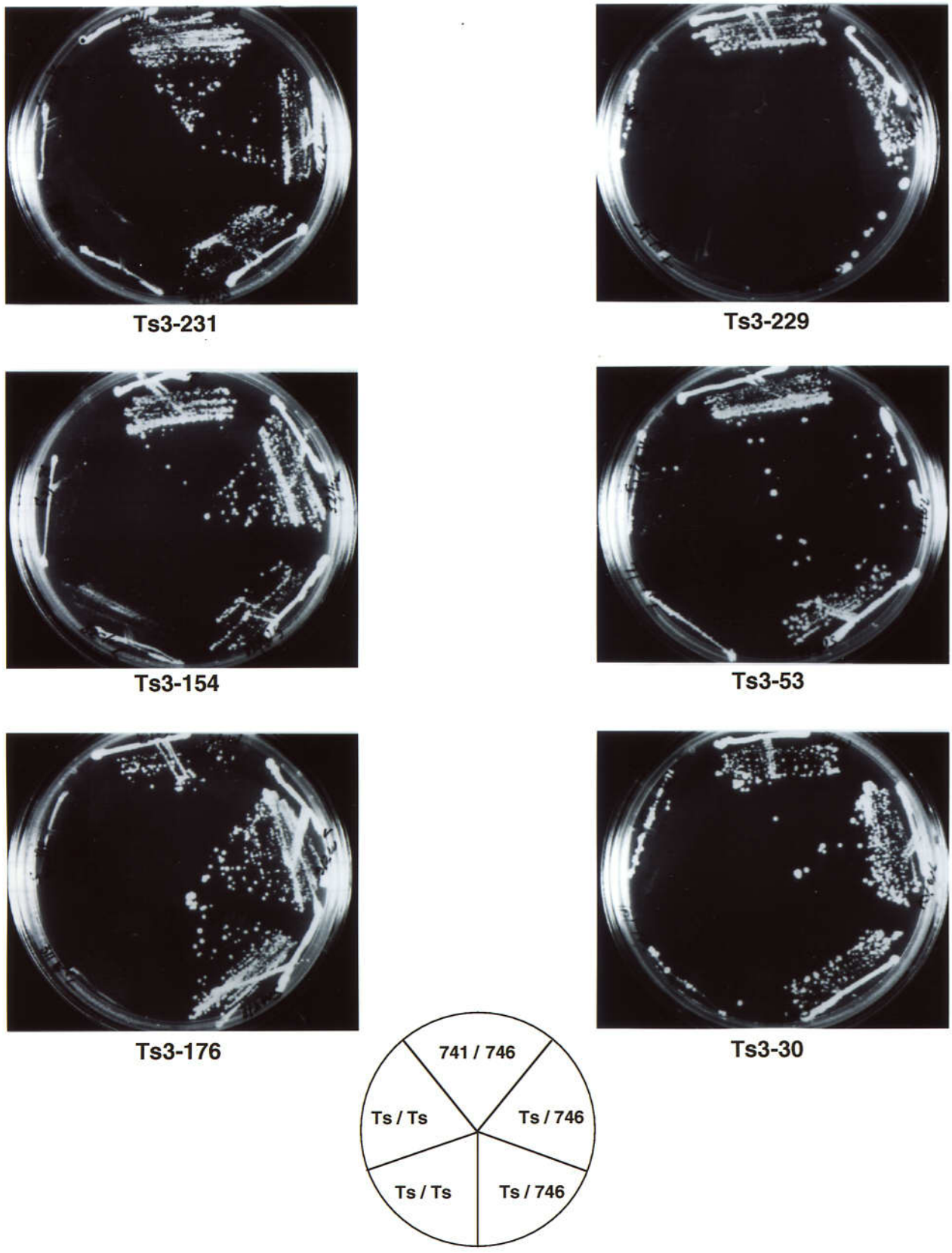


Fig. 4 Test of temperature sensitivity for *rpb3* heterozygote diploids. 6 *rpb3* heterozygote diploids were produced by mating with h^+ version of each Ts^- strains with wild-type JY741 [$Ts^-(h^+)$ / JY741], and homozygote diploids of each *rpb3* mutaion [$Ts^-(h^+)$ / $Ts^-(h^-)$], wild-type *rpb3* [JY746 / JY741] were also produced and checked for growth at the non permissive temperature.

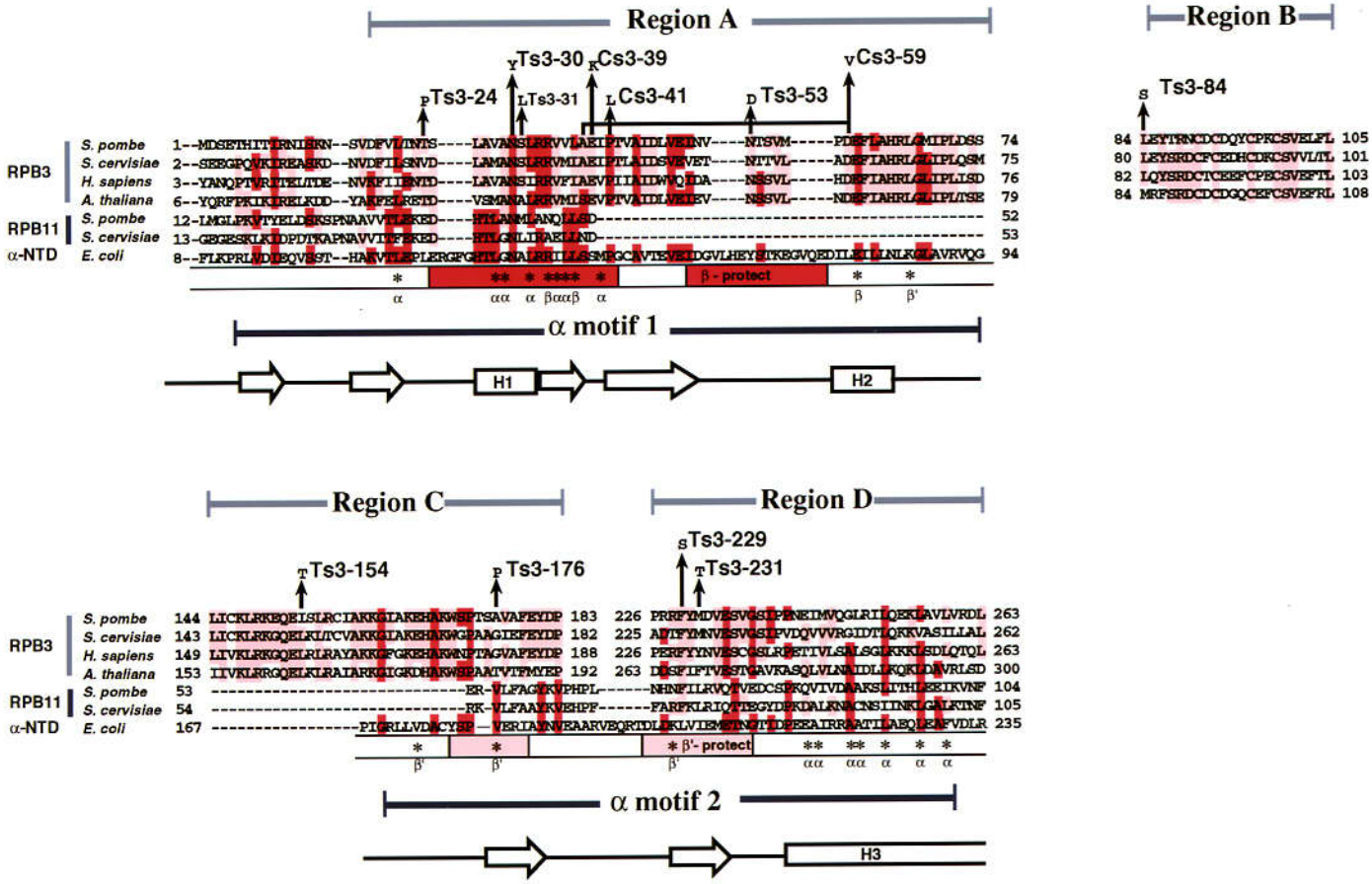


Fig. 5 Locations of the *rpb3* mutations in the *S. pombe* Rpb3 Ts⁻ and Cs⁻ mutants. The amino acid sequences predicted from the nucleotide sequences are aligned as to match the published alignment system (Zhang and Darst, 1998) using program Gene Works (Inteli Genetics). Amino acids that are identical to that of *E. coli* α subunit are shaded with red, while those that are eukaryotic RNA polymerase subunit 3 are shaded with pink. Dashed lines represent gaps that are inserted to make the maximum identity. The *rpb3* mutations identified in this study are shown on the top of sequence alignment. The *E. coli* α subunit sequences that are protected by the β and β' subunits from hydroxyl-radical cleavage are indicated by the red and pink bars, respectively, shown below the sequence alignment, and the *E. coli rpoA* mutations affecting the association with α (dimerization), and β or β' subunits are indicated by asterisks. The predicted secondary structure of *S. cerevisiae* RPB3 subunit is shown on the bottom line (the putative α helices H1, H2 and H3 and the β sheets) (Zhang and Darst, 1998). The amino acid substitution mutations in the *RPB3-1* allele of *S. cerevisiae* are located at residues corresponding to the *S. pombe* residues 29 and 176 (Kolodziej and Young, 1989).

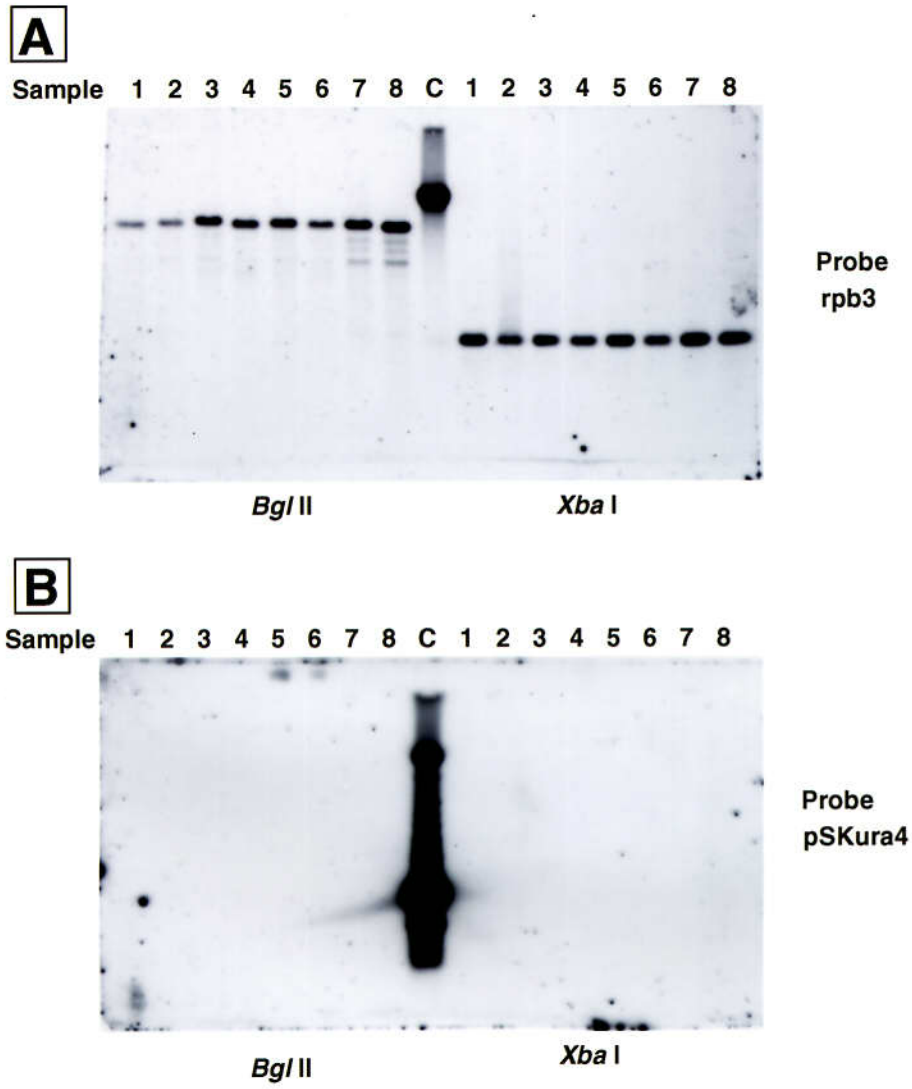


Fig. 6 Southern blot of the *rpb3* gene. Genomic DNA from 5-FOA resistant colonies were digested with *Bgl*II and *Xba*I and subject to Southern brot hybridzation analysis. Probes used were : PCR amplified *rpb3* coding sequence [**A**] or integration vector pSKura4 without insertion [**B**]. Samples analyzed were 1, Ts3-30; 2 , Ts3-53; 3, Ts3-84; 4, Ts3-154; 5, Ts3-176; 6, Ts3-229; 7, Ts3-231; 8, JY741; C, genom DNA of Ura^+ strain (non-digested) + 1 kb-plus^{TM} size marker.

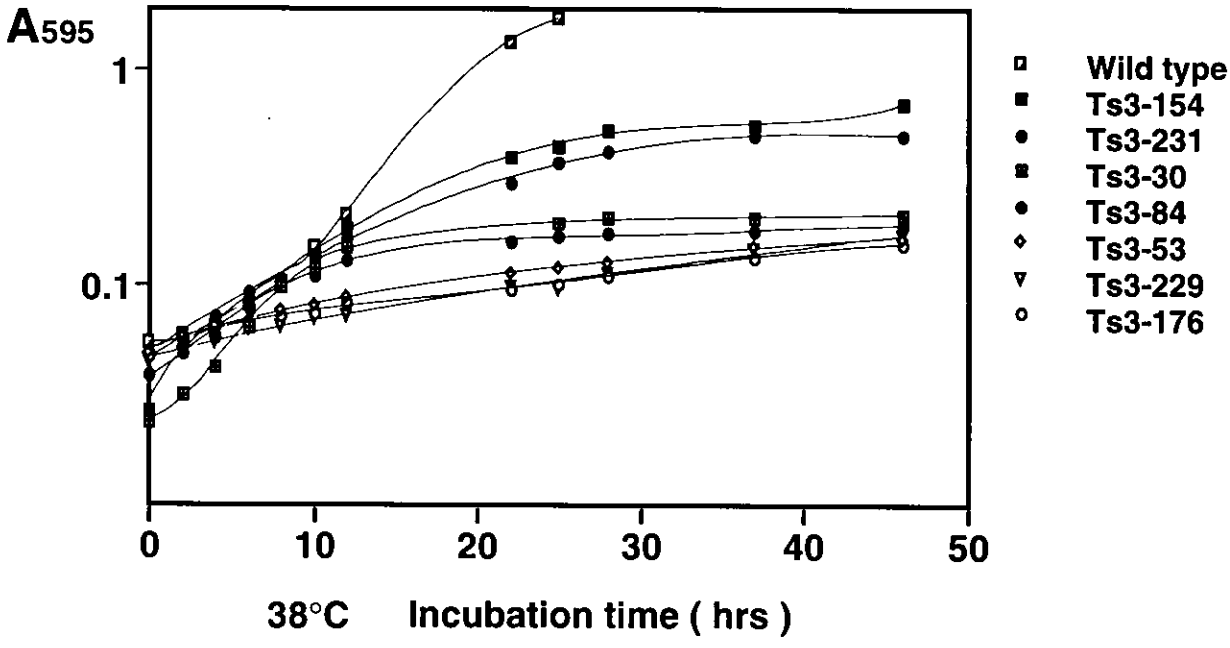


Fig. 7 Growth curve of the Rpb3 Ts$^-$ mutants. 7 Ts$^-$ mutants of *S. pombe* carrying amino-acid substitution mutations grown in YE (+Ade, +Ura) medium at 30 °C were transferred to a fresh YE(+Ade, +Ura) medium at time 0, and the incubation was continued at 38 °C. The growth was monitored by measuring the turbidity at 595 nm.

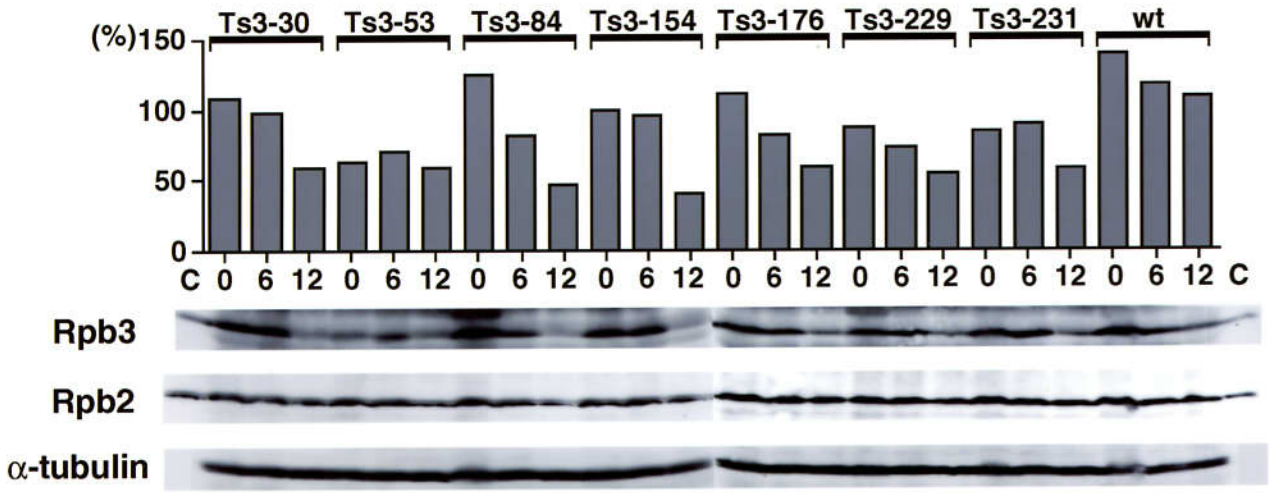


Fig. 8 The intracellular concentrations of Rpb3 proteins in the *S. pombe* Ts$^-$ mutants carrying the mutations in the *rpb3* gene. The *S. pombe* Rpb3 Ts$^-$ mutants were cultured as described in Fig. 7, and at the time of 0, 6 and 12 h after the temperature up-shift, the amounts of Rpb3 proteins were measured for the whole cell extracts using the quantitative Western blot analysis. As internal controls, the level of Rpb2 and α-tubulin was measured for the same extracts. The intensity of Rpb3 band was measured with a densitometer, and corrected for the density of α-tubulin. Lane C represents the purified RNA polymerase II from *S. pombe*. The measurement was repeated at least three times to determine the level of Rpb3 proteins.

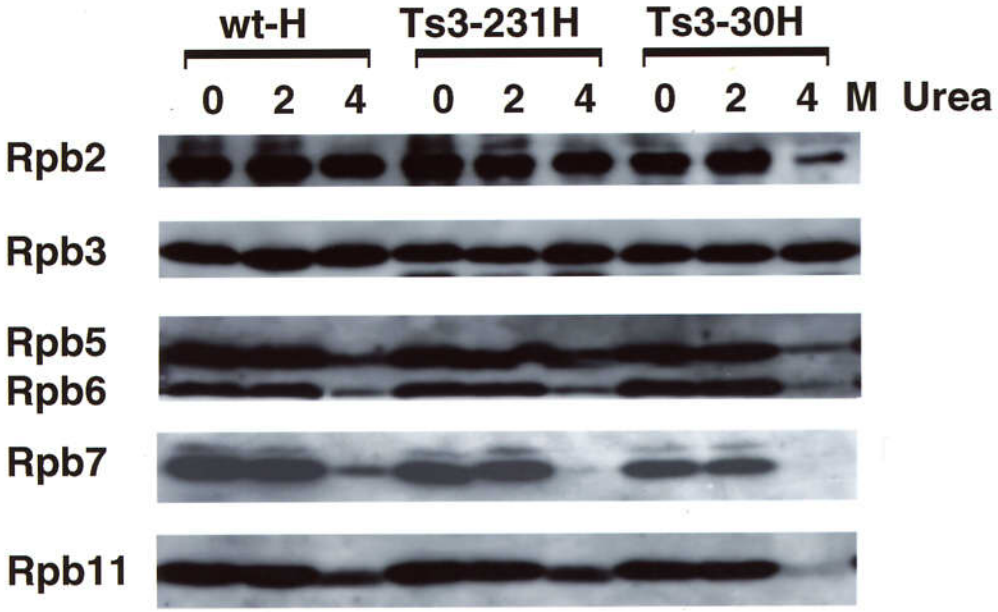


Fig. 9 Characterization of the RNA polymerase II purified from two Rpb3 Ts$^-$ mutants. RNA polymerase II containing His$_6$-tagged Rpb3 was isolated by Ni^{2+}-NTA affinity purification method from the wild-type *S. pombe* producing His$_6$-tagged Rpb3 (Kimura *et al.*, 1997) and two Ts$^-$ mutants, Ts3-30H and Ts3-231H. The purified RNA polymerase was bound to Ni $^{2+}$-NTA resin and treated at 4 °C with the binding buffer with or without 2 or 4 M urea. The proteins remaining bound with the Ni $^{2+}$-NTA resin were analyzed by SDS-PAGE.

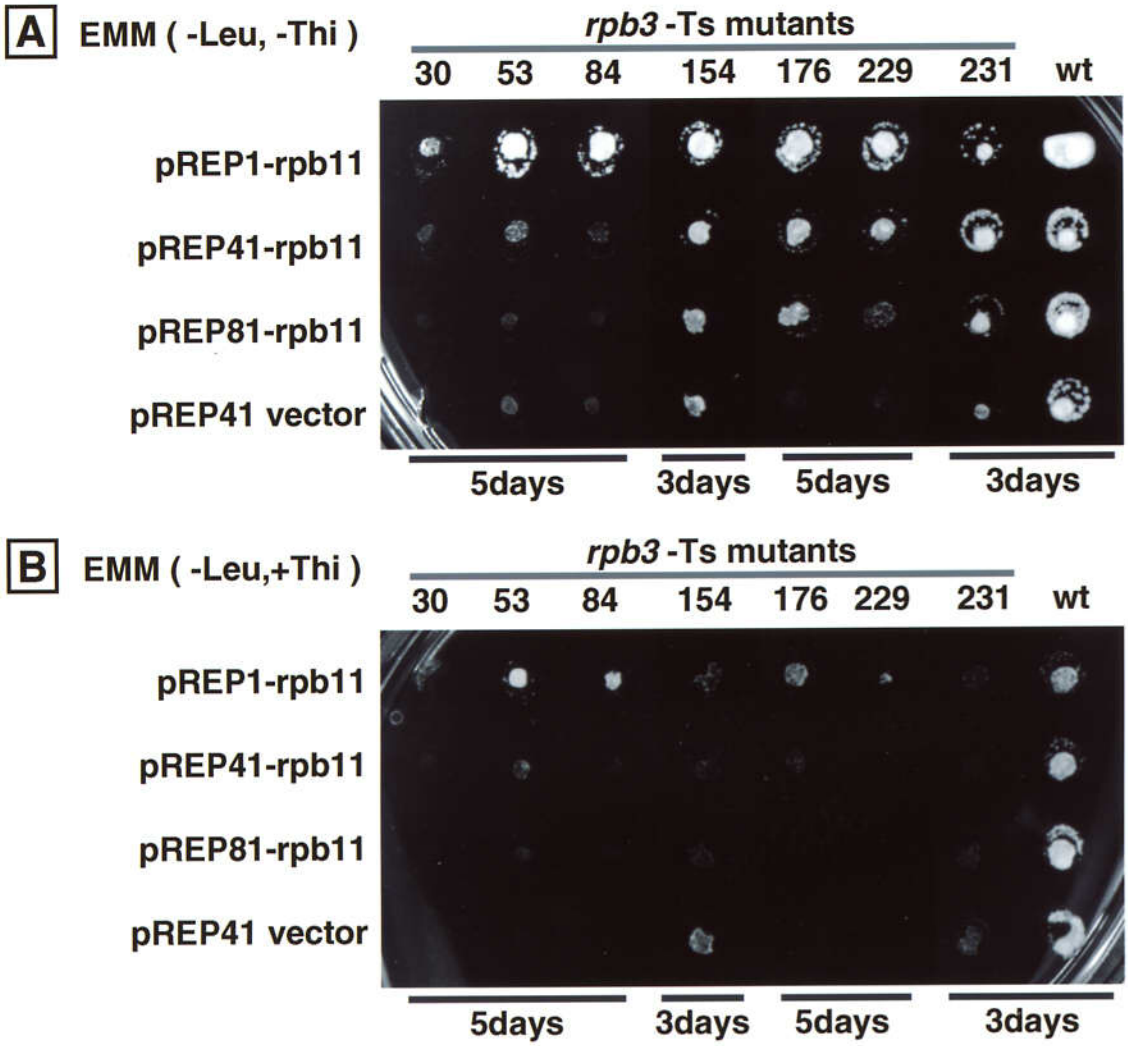


Fig. 10 Suppression of the *rpb3* mutations by overexpression of Rpb11. [**A**] The Rpb3 Ts$^-$ mutants were transformed by each of the Rpb11 expression plasmids, pREP1(rpb11), pREP41(rpb11) or pREP81(rpb11). As a control, the vector pREP41 without the *rpb11* gene was transformed. An aliquot of each transformant culture was spotted on to EMM (+Ade, +Ura, -Leu) plate. The cell growth at the non-permissive temperature of 37 °C under the induced condition (in the absence of thiamine) for Rpb11 expression was monitored after 3 or 5 days depending on the growth rate. The order of the expression level of Rpb11 protein was: pREP1 > pREP41 > pREP81.

[**B**] The same volume of each transformant culture was also spotted on to EMM(+Ade, +Ura, -Leu, +Thi) plate for observation of the cell growth in the absence of Rpb11 expression. The low level of cell growth for pREP1 transformant might be due to leaky expression of the Rpb11 protein.

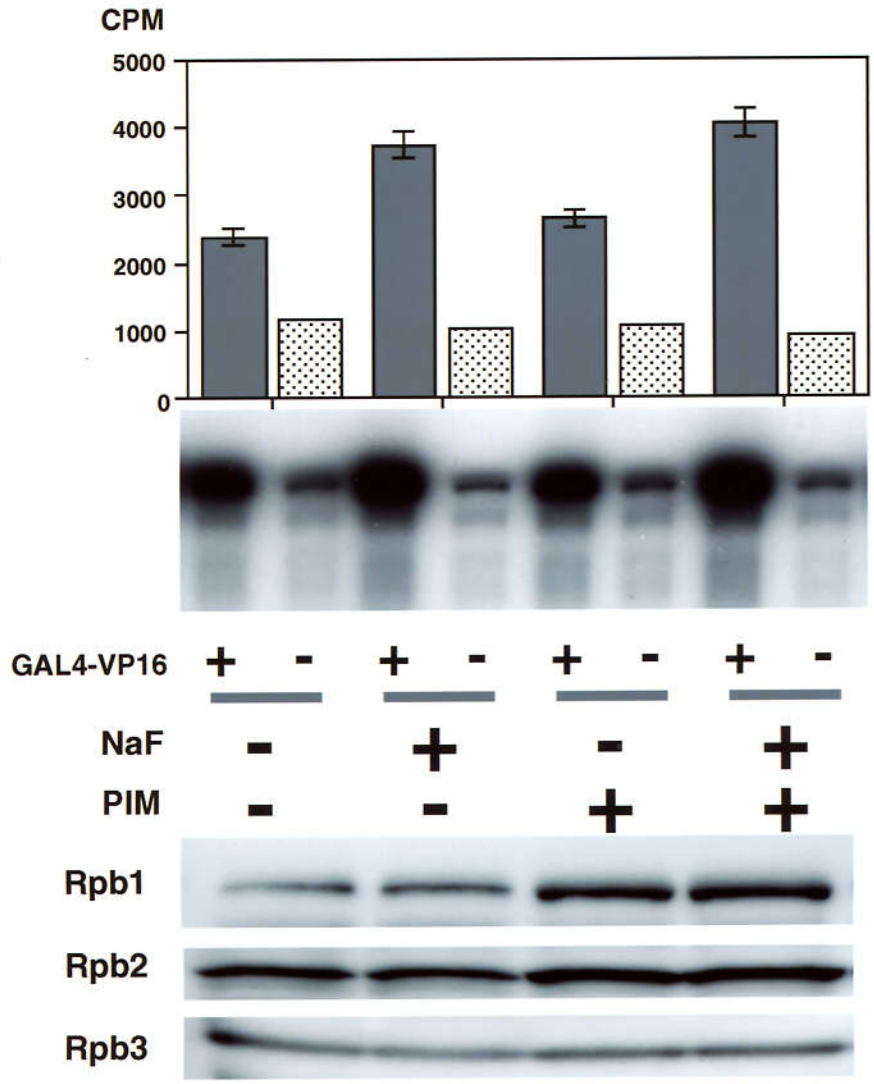


Fig. 11 GAL4-VP16-activated *in vitro* transcription by various 4 preparations of wild type *S. pombe* extracts. The addition of sodium fluoride [NaF] and high concentration of protease inhibitors [PIM] were indicated by + or -. The protein concentrations of 4 transcription extracts used ranged from 20 to 25 mg/ml. The transcription reactions were performed under standard condition (see materials and methods) using 125 µg each of the cell extracts. The radioactivity of each transcription product was measured with a phosphoimager. Dark gray bars indicate GAL4-VP16 dependent transcriptions. Basal transcriptions are indicated by light gray bars. GAL4-VP16 dependent transcription was repeated two times, error bars indicate maximum and minimum values of experiments. 125 µg of same samples were also subjected to 10 % SDS-PAGE and analyzed Western blotting by specific antibody against Rpb1, Rpb2 and Rpb3.

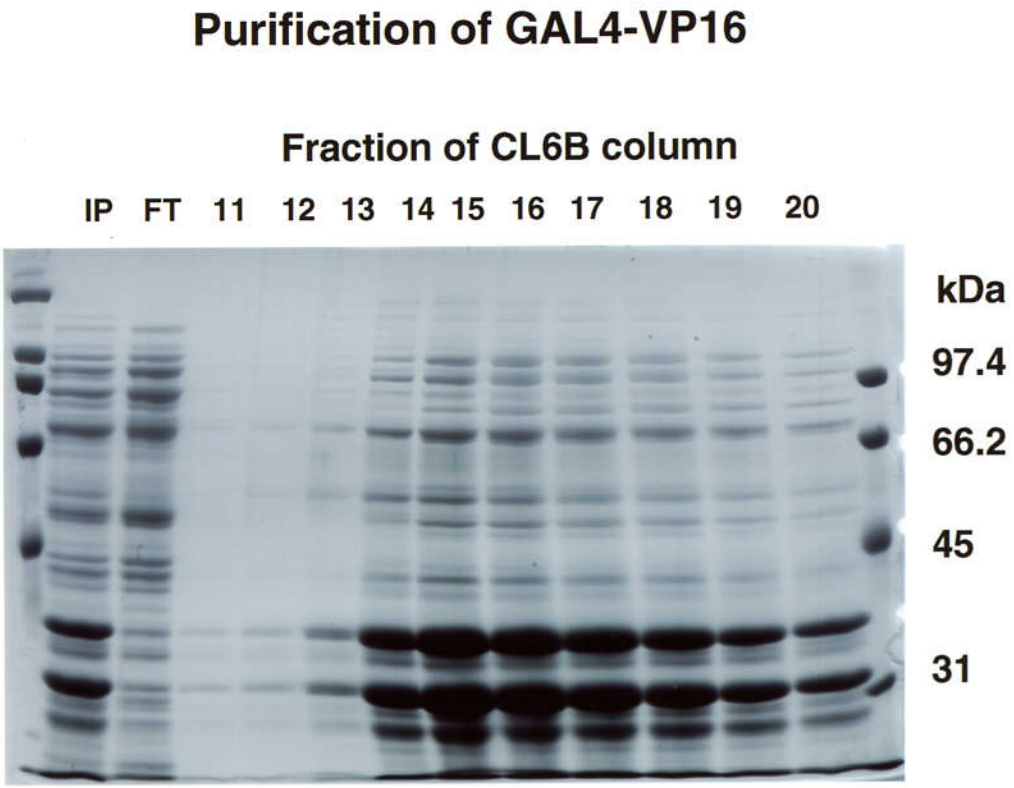


Fig. 12 Purification of GAL4-VP16. GAL4-VP16 was expressed in *E. coli* and purified as described in Materials and Methods. After heparin sepharose column chromatography, the composition of GAL4-VP16 was checked by 10% SDS-PAGE, The gel was stained with CBB. IP, input of heparin sepharose CL-6B column; FT, flow through; Fraction No,15 was used for the *in vitro* transcription system.

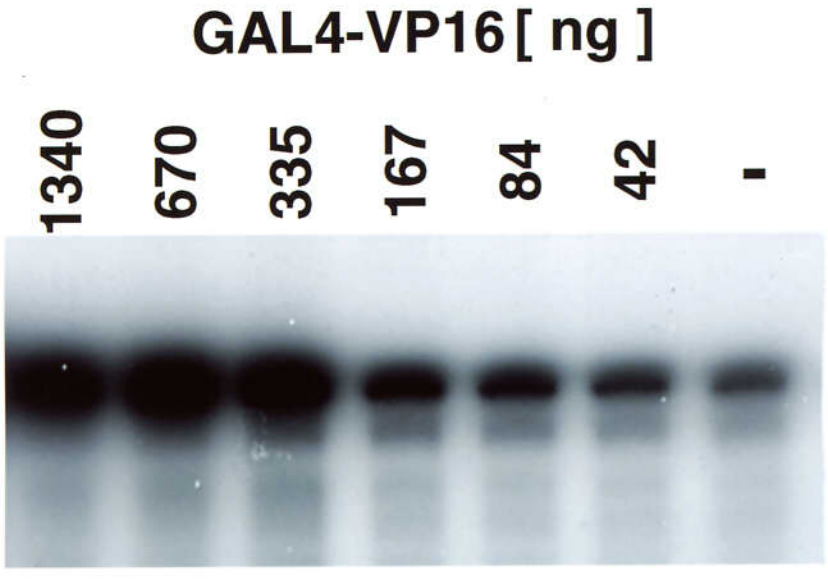


Fig. 13 The effect of GAL4-VP16 concentration on activated transcription. Transcription *in vitro* was carried out in the presence of increasing amount of GAL4-VP16. The amounts of GAL4-VP16 in each transcription reactions [ng] are indicated on the top of the panel.

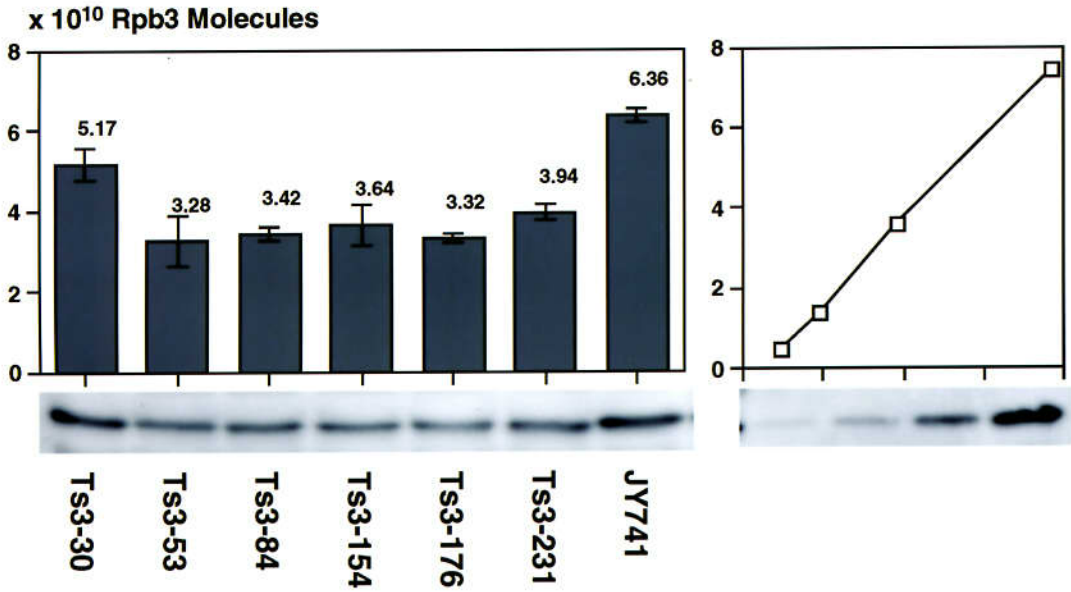


Fig. 14 The number of Rpb3 molecules in the transcription extracts Transcription extracts from *S. pombe* Rpb3 Ts$^-$ mutants were prepared as described in material and methods, the amounts of Rpb3 proteins were measured for 125 μg of transcription extracts using the quantitative Western blot analysis. The intensity of Rpb3 bands were measured with a densitometer, and the number of molecules in the 125 μg transcription extracts were calculated by standard curve with authentic purified *S. pome* RNA polymerase II which also visualized on the same blot.

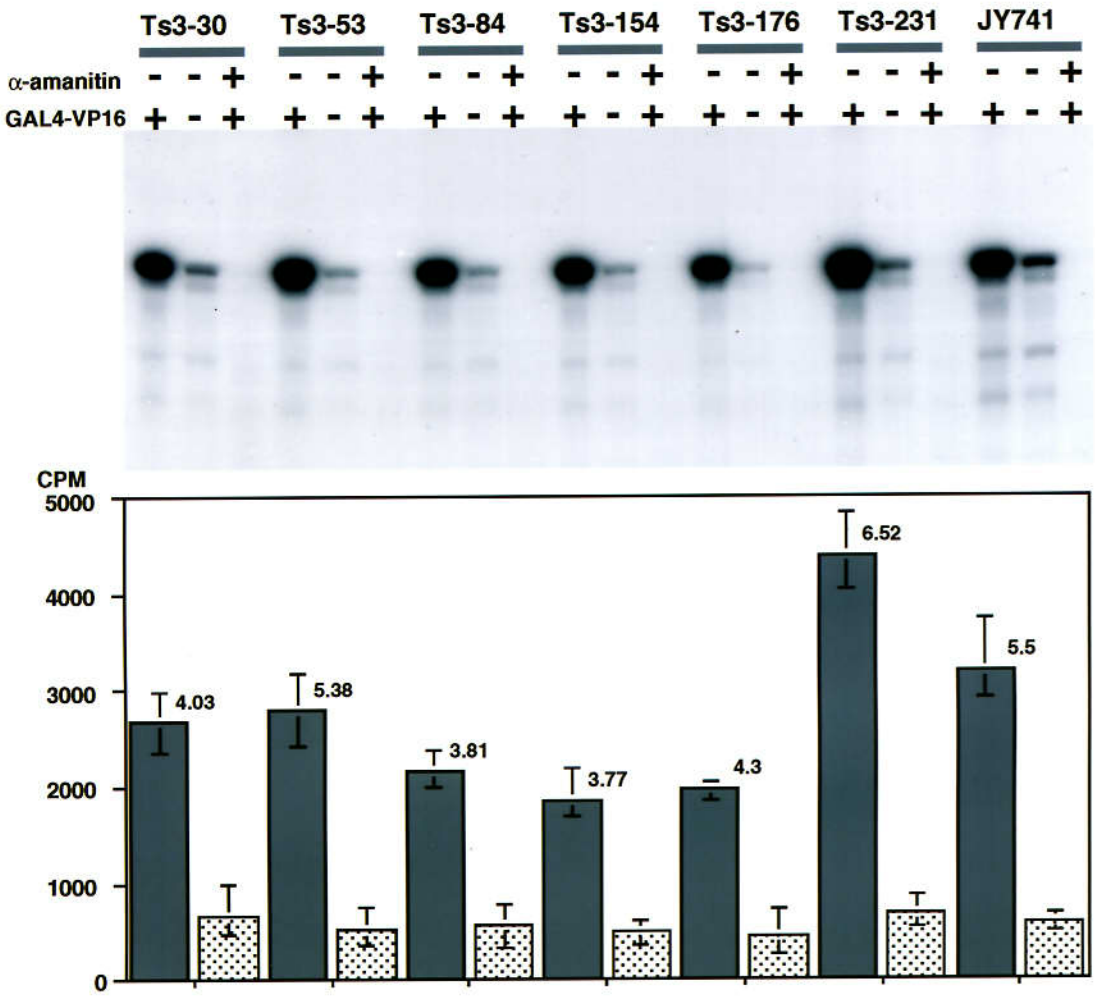


Fig. 15 GAL4-VP16 activated *in vitro* transcription by *S. pombe* mutant transcription extracts. Transcription *in vitro* were carried out in the presence or absence of 335 ng GAL4-VP16 or 1 μg α-amanitin, under the standard reaction conditions as described in materials and methods, using cell extracts from *S. pombe* mutant T3-30, Ts3-53, Ts3-84, Ts3-154, Ts3-176, Ts3-231 and wild type JY741 strains. The radioactivity of each transcription product was measured with a phosphoimager. The bars indicate the average of three independent mesurements while the error bars indicate the range of maximum and minimum values. GAL4-VP16 dependent transcriptions are indicated by dark gray bars. Basal transcriptions are indicated light gray bars. Fold activation values were indicated at the top of dark gray bars.

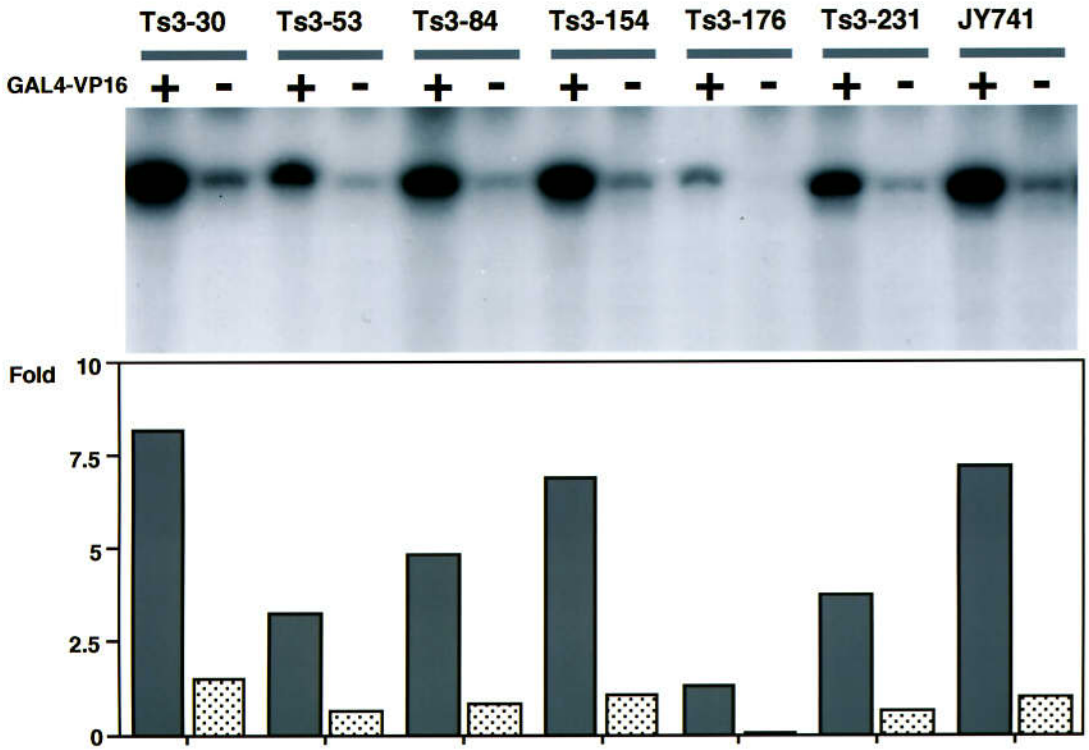


Fig. 16 Effect of heat treatment and reaction at the non-permissive temperature on *in vitro* transcription of *S. pombe* mutant extracts. Extracts of *S. pombe* mutant strains and wild-type JY741 were treated for 1 min at 41 °C, and then subject to GAL4-VP16-activated *in vitro* transcription assay at 37 °C for 30 min. After transcription reaction, the samples were treated by standard condition. The gel was exposed to X-ray film (10 times longer than the other experiments) and the intensity of each band was measured by BAS station (Fuji Film). The relative values against basal transcription of wild type were indicated in the bar graph. GAL4-VP1 6 dependent transcriptions are indicated by dark gray bars. Basal transcriptions are indicated light gray bars.

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