Molecular Assembly of Fission Yeast RNA Polymerase II and Mutation Analysis of Rpb6

Akira ISHIGURO

School of Life Science, Graduate University for Advanced Studies, and Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka, Japan

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Abstract

The subunit-subunit contact network within the *Schizosaccharomyces pombe* RNA polymerase II was analyzed using various methods. Far-Western blot analysis of pairs of over-expressed and isolated subunits using six small-sized subunits, Rpb6, Rpb7, Rpb8, Rpb10, Rpb11 and Rpb12, as probes indicated the subunit-subunit interaction for a total of 18 (or 19) combinations. Taken together with our previous analyses, the eight small-sized subunits, Rpb3, Rpb5, Rpb6, Rpb8, Rpb10, Rpb11 and Rpb12, were identified to bind one or both of two large subunits, Rpb1 and Rpb2. In addition, the bimolecular interaction was observed for the combination of Rpb3-Rpb11. The subunit-subunit contact within the assembled RNA polymerase was then analyzed by protein-protein cross-linking using five molecular species of the bifunctional cross-linker with different linker length and specificity. Cross-linking was observed for a total of 19 combinations, including five combinations between small subunits, Rpb3-Rpb10, Rpb3-Rpb11, Rpb5-Rpb6, Rpb6-Rpb7 and Rpb6-Rpb8. The results altogether indicate that two large subunits Rpb1 and Rpb2 provide the platform for assembly of small subunits and besides small subunits interact each other for limited combinations. Direct contact of the two large subunits, Rpb1 and Rpb2, was also demonstrated by cross-linking.

Since Rpb6 was found to make multiple contacts with not only the two large subunits but also three small subunits Rpb5, Rpb7 and Rpb8, genetic analysis was carried out for the common subunit Rpb6 shared by RNA polymerases I, II and III. Deletion and truncation anayses of the *rpb6* gene indicated that Rpb6 consisting of 142 amino acid residues is an essential protein for cell viability, but the essential region is located in the carboxy (C)-terminal proximal half between residues 61-139. After random mutagenesis, a total of 14 temperature-sensitive (Ts) mutants were isolated, each carrying a single (or double in three cases and triple in one case) mutation. Four mutants each carrying a amino acid change in

the essential region were sensitive against 6-azauracil (6AU) that inhibits transcription elongation by depleting the intracellular pool of GTP and UTP. Both 6AU sensitivity and Ts phenotypes of these *rpb6* mutants were suppressed by over-expression of TFIIS, a transcription elongation factor. In agreement with the genetic studies, the direct interaction between TFIIS and RNA polymerase II was observed *in vitro* by pull-down assay using a fusion form of TFIIS with glutathione S-tansferase (GST), and the mutant RNA polymerases containing the mutant Rpb6 subunits showed reduced affinity for TFIIS. Taken together we propose that Rpb6 plays a central role in protein-protein assembly with both the RNA polymerase II subunits and the transcriptiion elongation factor TFIIS.

Introduction

Studies of eukaryotic transcription have been focussed on cis-acting DNA signals affecting transcription regulation, and more recently, on general and specific protien factors affecting transcription (Roeder, 1998; Struhl *et al.*, 1998). In contrast, our knowledge of the structure and function of RNA polymerases is limitted. For instance, the minimal and essential subunits have been determined for none of three forms of the eukaryotic RNA polymerase. In order to get a breakthrough in the RNA polymerase study, I have carried out biochemical and genetical studies of the RNA polymerase II from the fission yeast *Schizosaccharomyces pombe*, in particular focussing on the subunit-subunit interactions within the RNA polymerase II. One reason I chose this research subject was that a lot of knowledge of the subunit assembly of *Escherichia coli* RNA polymerase and experimental systems to solve the problems associated with protein-protein interactions have been accumulated in this laboratory.

The RNA polymerase II of *S. pombe* consists of twelve subunits (Sakurai *et al.*, 1999), corresponding to RPB1 to RPB12 of the *Saccharomyces cerevisiae* RNA polymerase II (Thuriaux and Sentenac, 1992; Woychik and Young, 1994). The genes and cDNA coding for all these twelve subunits have been isolated and sequenced in this laboratory (Azuma *et al.* 1991; Azuma *et al.* 1993; Kawagishi *et al.* 1993; Sakurai & Ishihama 1997; Sakurai *et al.* 1998; Sakurai *et al.* 1999) (Table 1). At present, however, the function of each of these putative subunits remains mostly unsolved. Two large subunits, Rpb1 and Rpb2, are the homologues of the β' and β subunits of bacterial RNA polymerase, while the two small subunits, Rpb3 and Rpb11, carry limited sequene homology with the N(amino)-terminal proximal assembly domain of bacterial α subunit. These four subunits, Rpb1, Rpb2, Rpb3 and Rpb11, altogether are considered to form the enyme core which corresponds to the bacterial core enzyme with the subunit structure $\alpha_a\beta\beta'$ (Kimura *et al.*, 1997; Sakurai *et al.*, 1999). In the case of RNA polymerase formation in *Escherichia coli*, the subunit asssembly proceeds sequentially under the order: $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (core enzyme) $\rightarrow \alpha_2\beta\beta'\sigma$ (holoenzyme)

(Ishihama, 1981). The assembly core of *S. pombe* was identified to be Rpb2-Rpb3-Rpb11 ternary complex, which corresponds to the $\alpha_2\beta$ complex (Kimura *et al.*, 1997). Little is known, however, about the functions of other eight subunits, among which five, Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12, are assembled, as the common subunits, into all three forms of the eukaryotic RNA polymerase (Thuriaux and Sentenac, 1992; Woychik and Young, 1994; Ishihama *et al.*, 1997).

In order to reveal the subunit-subunit contact network in this complex enzyme, our labolatory began the analysis of molecular interactions between subunits using various techniques such as far-Western blotting (Miyao *et al.*, 1996; Yasui *et al.*, 1998), pull-down assay with tagged subunits (Miyao *et al.*, 1996; Yasui *et al.*,1998), and yeast two-hybrid screening (Miyao *et al.*, 1998). The results of analysis for Rpb3 and Rpb5 altogether suggested that: (i) these two subunits contact with both of the two large subunits, Rpb1 and Rpb2; (ii) the two subunits interact each other; and (iii) Rpb5 interacts with Rpb8 and/or Rpb11. In parallel, attempts have been made for step-wise dissociation of the RNA polymerase II with protein denaturants and the initial results indicated that three subunits, Rpb2, Rpb3 and Rpb11, form a core subassembly corresponding to the prokaryotic $\alpha_2\beta$ complex (Kimura *et al.*, 1997).

In this study, I extended the far-Western blot analysis to reveal the bimolecular interactions for all other small-sized subunits, Rpb6, Rpb7, Rpb8, Rpb10, Rpb11 and Rpb12. Since far-Western blotting sometimes fails to detect specific protein-protein interactions due to protein denaturation or detects non-specific protein-protein binding, we also performed the chemical cross-linking of RNA polymerase II with five different cross-linkers, dimethylsuberimidate (DMS), 2-iminothiolane hydrochloride (ILT), *N,N'*-o-phenylene dimaleimide (PDM), dimethyl 3,3'-dithio-bis propionimidate (DTBP) and diepoxybutane (DEB). Taken the results altogether we proposed a model of the subunit topology within the *S. pombe* RNA polymerase II.

The results of far-Western and chemical cross-linking suggested that the small subunits bind the two large subunits, Rpb1 and/or Rpb2, but direct interaction between small subunits was indicated for a small number of combinations. In particular, Rpb6

was indicated to contact with not only two large subunits, Rpb1 and Rpb2, but also three small subunits, Rpb5, Rpb7 and Rpb8. The *S. cerevisiae RPB6* is an essential gene for cell growth, and the RPB6 sequence is highly conserved among eukaryotic RNA polymerases (Woychik *et al.*, 1990; McKune and Woychik, 1994; Sakurai and Ishihama, 1997). The Rpb6 homologues also exist in archael (Langer *et al.*, 1995) and viral RNA polymerases (Lu *et al.*, 1993). These observations altogether suggest that the Rpb6 plays an essential function(s) in the assembly and/or functions of RNA polymerases I, II and III.

A mutant RNA polymerase I prepared from a *Saccharomyces cerevisiae* mutant lacking the A14 subunit (identical with RPB6) is virtually inactive in RNA synthesis *in vitro*, but regains the activity by the addition of RPB6 (Lanzendorfer *et al.*, 1997), supporting the notion that the A14 (or RPB6) subunit is esssential for the catalytic function of RNA polymerases. In agreement with the biochemical evidence of direct Rpb6-Rpb1 interaction within the *S. pombe* RNA polymerase II (Ishiguro *et al.*, 1998), a genetic analysis suggests that an RPB6 mutation of *S. cerevisiae* can suppress a temperature-sensitive mutation of RPB1 (Archambault *et al.*, 1990). Nourini *et al.* (1996) showed that Rpo26 (identical with RPB6; and the *S. pombe* Rpb6 homologue) of *S. cerevisiae* is essential for the assembly of both RNA polymerases I and II.

To get further insight into the structure-function relationship of *S. pombe* Rpb6, I examined the minimum essential segment of Rpb6 by making a set of N(amino)- and C(carboxy)-terminal deletion mutants. I also isolated a number of temperature-sensitive (Ts) *S. pombe* mutants, each carrying a single mutation in the *rpb6* gene, after PCR mutagenesis, and replaced the chromosomal *rpb6* gene by the mutagenized *rpb6* genes. Results indicate that the C-terminal proximal half of Rpb6 is essential for cell viability, but the mutations conferring Ts phenotype clusterred along the entire sequence of Rpb6, presumably reflecting the involvement of Rpb6 in contact with multiple subunits. Some of the *rpb6* mutations in the essential region were found to be suppressed by over-expression of TFIIS, a transcription elongation factor, suggesting the direct protein-

protein contact between Rpb6 and TFIIS. Some biochemical studies support the notion that one of the targets of TFIIS function is the Rpb6 subunit.

Materials and Methods

S. pombe strains and media

S. pombe strains used were: JY741 h^- *ura4*-D18 *leu1 ade6*-M216, and JY746 h^+ *ura4*-D18 *leu1 ade6*-M210. The diploid strain used for disruption of the *rpb6* gene was made by mating these two strains. Cells were grown in medium MM (Alfa *et al.*, 1993) , SD [6.7% yeast nitrogen base (DIFCO) and 2% glucose], YPD [1% yeast extract (Wako), 2% Bacto tryptone (DIFCO Laboratories) and 2% glucose] or YE [1% yeast extract and 3% Glucose].

Purification of RNA polymerase II

RNA polymerase was purified from 200 g of *S. pombe* 972h^- cells grown in YE medium. Cell disruption, polymin P precipitation, elution from polymin P precipitates and ammonium sulfate precipitation were carried out according to Azuma *et al.* (1993). The ammonium sulfate precipitates were dissolved in TGED buffer (50 mM Tris-HCl, pH 7.8 at 4 ^{o}C, 25% glycerol, 0.1 mM EDTA and 1 mM DTT), dialyzed against TGED buffer containing 50 mM ammonium sulfate and loaded onto a DEAE-TOYOPEARL (TOSOH) column (bed volume, 100 ml). After washing the column with the same buffer, proteins were eluted with 800 ml of 50 to 610 mM linear gradient of ammonium sulfate in TGED buffer. RNA polymerase II fraction eluted at 200 mM ammonium sulfate was dialyzed against TGED buffer containing 50 mM ammonium sulfate and loaded onto a Q-Sepharose FF (Pharmacia) column (bed volume, 6 ml). The column was washed with the same buffer and then proteins were eluted with 120 ml linear gradient pf 50 mM to 1 M ammonium sulfate in TGED buffer. RNA polymerase II fractions recovered at about 250 mM ammonium sulfate were dialyzed against TGED buffer containing 50 mM ammonium sulfate, and applied onto a HiTrap Heparin (Pharmacia) column (bed volume, 1 ml) and proteins were eluted with a linear gradient of 50 mM to 1 M ammonium sulfate in TGED buffer. RNA polymerase II was eluted at 340 mM ammonium sulfate. Chromatography on DEAE-TOYOPEARL and Q-

Sepharose FF columns was carried out using Hiroad system (Amersham Pharmacia) while HiTrap Heparin column chromatography was using an HPLC system (TOSOH). The activity of RNA polymerase II was measured using the assay system of non-specific RNA synthesis on denatured DNA templates (Azuma *et al.* 1993). The purified RNA polymerase II was stored at -80 ^{o}C.

For purification of His_8 tagged RNA polymerase II of wild-type 6NH and mutant *S. pombe* strains, carrying the His-tagged wild-type and mutant *rpb6* genes, respectivery,see below, were grown in YE medium supplemented with 75 ml/liter of adenine, uracil and leucine. Cells (20 gm) were disrupted in an extration buffer (50 mM Tris-HCl, pH 7.6 at 4 ^{o}C, 0.5 M NaCl, 1 mM PMSF, and 10% glycerol) with a bead beater. After centrifugation at 15,000 rpm for 39 min, the supernatant was loaded onto Ni2+-NTA agarose column (0.5 ml bed volume). After washing with the extraction buffer containing 0.5% Nonidet P-40, proteins were eluted with the extraction buffer containing 200 mM imidazole. The eluted proteins were dialyzed against buffer A (50 mM Tris-HCl, pH 7.8. 1 mM DTT, 0.1 mM EDTA and 20% glycerol), and loaded on to a DEAE-Sephadex A25 column (1 ml bed volume). Proteins were eluted with 7.5 ml of a linear gradient of ammonium sulfate from 50 to 500 mM. The RNA polymerase II was eluted at about 250 mM ammonium sulfate.

Expression and purification of subunit proteins

To make the expression plasmids of subunits for far-Western blot analysis, full-length cDNAs for all the subunits except Rpb1 and Rpb2 were inserted into pET21b (Novagen) between *Nde*I and *Sal*I sites (for preparation of subunits without PK tag) or pET21bPKC between *Nde*I and *Xho*I sites (for preparation of subunits with PK tag). The pET21bPKC vector contains between *Hin*dIII and *Xho*I sites the sequence 5'AGCTTCGCCGCGCCAGCGTTC3' which encodes the target site sequence (ArgArgAlaSerVal peptide) for heart muscle protein kinase (PK). All the subunit proteins were expressed in *E. coli* BL21(pLysS). Rpb6CH and Rpb6KCH were recovered in the soluble fractions, while Rpb7KCH, Rpb8KCH, Rpb10KCH,

Rpb11CH, Rpb11KCH and Rpb12KCH were recovered in inclusion bodies. These insoluble proteins were solubilized in a buffer (50 mM Tris-HCl, pH 7.8 at 4 ^{O}C, 10 mM 2-mercaptoethanol, 5% glycerol, 0.1 M NaCl and 0.5% NP-40) containing 6 M urea or 6 M guanidium hydrochloride. Rpb7KCH and Rpb8KCH were, however, recovered in the soluble fractions by coexpression of thioredoxin or GroE (Yasukawa *et al.*, 1995). All the recombinant proteins were purified by chromatography on Ni^{2+}-NTA agarose columns and after dialysis against storage buffer (100 mM Tris HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 0.1 mM DTT and 50 % glycerol), stored at -30 ^{O}C.

Far-Western analysis

For preparation of far-Western probes, the purified recombinant subunit proteins were phosphorylated using [γ-^{32}P]ATP and heart muscle protein kinase (Sigma) according to Kaelin *et al.* (1992). Labeled probes were purified by gel filtration using NAP^{TM} 5 columns (Amersham Pharmacia). The conditions for far-Western analysis were as described in Miyao *et al.* (1996). In brief, an equal amount (10 pmol) of each purified subunit was blotted on to nitrocellulose membrane (Protein nitrocelluose; Schleicher & Schuell) using slot-blotter (Schleicher and Schuell). The membrane was washed with 200 µl of slot blotting buffer (10 mM CAPS-Noah, pH 11, and 10% Methanol), blocked with GED buffer (50 mM HEPES-NaOH, pH 7.5, 50 mM KCl, 7.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 % NP-40, 0.1 mM DTT and 10 % glycerol) containing 5 % skim milk at room temperature for 2 hr, and then incubated with the radio-labeled Rpb6, Rpb7, Rpb8, Rpb10, Rpb11, Rpb12 or GST at room temperature for 2 hr. After washing, the membranes were exposed to imaging plate (Fuji) for 4 hr, and the plates were analyzed with BAS 2000 image analyzer (Fuji).

Protein-protein cross-linking

Dimethylsuberimidate (DMS), 2-iminothiolane hydrochloride (ITL), N,N'-*o*-phenylene-dimaleimide (PDM) and dimethyl 3,3'-dithio-bis propionimidate (DTMP) were products of Sigma while diepoxybutane (DEB) was obtained from Aldrich Chem.

For each cross-linking experiment, RNA polymerase II was dialyzed against 50 mM HEPES (pH 8.0), 300 mM KCl, 0.1 mM EDTA and 5% glycerol. The protein concentration used for cross-linking experiments was 0.2 mg/ml. The conditions for cross-linking with DMS was as described in Walleczek *et al.* (1989) and Redl *et al.* (1989). A stock DMS solution (30 mg/ml dimethylfulfoxide) was added to the RNA polymerase II to a final concentration of 3 mg/ml, and the mixture was incubated for 10 min at 25 ^{o}C. The reaction was terminated by adding 0.2 M ammonium chloride. Cross-linking with PDM was carried out as described in Chang & Flaks (1972). PDM (75 mM dissolved in dimethylformamide) was added to the RNA polymerase II to a final concentration of 7.5 mM, and after incubation for 1 min at 37 ^{o}C, the reaction was terminated by adding 2-mercaptoethanol at a final concentration of 0.1 M. Cross-linking with ITL was performed as described in Walleczek *et al.* (1989) and Kenny *et al.* (1979). 2-Iminothiolane was added to the RNA polymerase II to a final concentration of 12 mM. After incubation for 60 min at 0 ^{o}C, 40 mM H_2O_2 was added to the solution and incubation was continued for additional 30 min at 0 ^{o}C. Cross-linking of RNA polymerase II with DTBP was carried out as described in Cornell *et al.* (1989). DTBP was added to the RNA polymerase II to a final concentration of 0.8 mg/ml. After incubation for 1 min at 37 ^{o}C, the reaction was terminated by adding 100 mM ammonium acetate. Cross-linking of the RNA polymerase II with DEB was performed according to Brockmoller & Kamp (1986). Solid DEB was added to the RNA polymerase II to make a final concentration of 1% (v/v). After incubation for 20 min at 37 ^{o}C, glycinamide hydrocloride was added at the final concentration of 50 mM and incubation was continued for additional 40 min at 37 ^{o}C.

Cross-linked proteins were identified by Western blotting. After transfer of the proteins to PVDF membranes (Fluorotrans; PALL) by electroblotting (Trans blot cell; Bio Rad), the membranes were incubated with blocking buffer (10 mM Tris-HCl, pH 7.8 at 4 ^{o}C, 137 mM NaCl and 5% skim milk) and then with antibodies against RNA polymerase II subunits as indicated in each experiment. After washing with 20 mM Tris-HCl, pH 7.8 at 4 ^{o}C, 137 mM NaCl and 0.1% Tween 20, the membranes were

incubated with anti-rabbit (or mouse in the case of anti-CTD) IgG antibody conjugated with horse raddish peroxidase. After repeated washing, the membranes were treated with ECL Western blotting detection reagents (Amersham) and exposed to X-ray films (Fuji RX-U).

The anti-CTD antibody used was mouse 8WG16 monoclonal antibody (Thompson *et al.*, 1989). Antibodies against other subunits were raised in rabbits against the respective His_6-tagged full-length subunits expressed in *E. coli* (Ishiguro *et al.*, 1998)

Disruption of the *rpb6* gene

Plasmid pRpb6::ura4 used for construction of *S. pombe rpb6* disruptants was prepared as follows: The *ura4* coding sequence was PCR-amplified and inserted into pBluescript II SK(+) at *Bam*HI site; about 1 kpb DNA fragment including the *rpb6* 5'-flanking sequence between -1032 and -12 was inserted betweem *Eco*RI and *Pst*I; and about 1 kbp fragment including the *rpb6* 3'-flanking sequence between +648 to +1637 was inserted between *Not*I and *Sac*I sites (see Fig. 7). The *Eco*RI-*Sac*I smaller fragment including the *rpb6* 5'-flanking sequence, *ura4* coding sequence and *rpb6* 3'-flanking sequence was used for transformation. Ura^+ transformants were selected, and the integration of *ura4* at the *rpb6* locus of the *S. pombe* chromosome was confirmed by PCR. One of the transformants was used as the *rpb6* disruptant *S. pombe rpb6::ura4*.

Complementation assay of the *rpb6* disruptants

For construction of the Rpb6 expression plasmids, the entire or partial sequences of *rpb6* coding sequence were amplified by PCR using Pfu DNA polymerase, template pRpb6::$Rpb6NH_8$ and various sets of primers. The resulting DNA segments were inserted into pREP81 vector between *Nde*I and *Bam*HI. pREP81-rpb6 contains a full-length *rpb6* cDNA, while pREP-rpb6(g) contains the *rpb6* genomic DNA in the same

pREP81 vector between NdeI and *Bam*HI. The expression plasmids for Rpb6 deletion mutants are summarized in Table 2.

Construction of temperature-sensitive *rpb6* mutants

Plasmid pRpb6::Rpb6NH$_8$ used as the PCR template for generation of the recombinant gene coding for Rpb6 fused to His$_8$ at N terminus was prepared as follows: a PCR amplified DNA segment containing the entire coding sequence of *rpb6* (except for the initiation codon) and the 3'-flanking sequence down to +1164 was inserted into pBluescript II KS(+) between *Bam*HI and *Sac*I; an *rpb6* 5'-flanking sequence between -302 and -12 was inserted at the 5' terminus of *rpb6* coding sequence between *Eco*R1 and *Bam*H1; and then a sequence coding for octahistidine (His$_8$) including the initiation codon ATG was inserted at *Bam*H1 site. PCR amplification was carried out using the resulting plasmid pRpb6::Rpb6NH as template and a set of primers (5' primer, 5'-AAGAATTCAAAGTAATAGTAACAAATAGAC-3'; 3' primer, 5'-AAGAGCTCATTATACCTTGTAAATTTCGC-3'). PCR products were transformed into *S. pombe rpb6*::*ura4* to yield *S. pombe* 6NH strain carrying the recombinant *rpb6* gene for production of wild-type Rpb6 with H$_8$ tag at N terminus.

For generation of *S. pombe* strains carrying *rpb6* mutants, mutagenesis of *rpb6* was performed by PCR in the presence of 20 mM $MnCl_2$ using pRpb6::pRpb6NH as template and a set of primers (5' primer sequence 5'-AAGAATTCAAAGTAATAGTAACAAATAGAC-3', and 3' primer sequence 5'-AAGAGCTCATTATACCTTGTAAATTTCGC-3'). The PCR-amplified DNA sequence including the coding sequence for His-tagged Rpb6 were transformed into *S. pombe rpb6*::*ura4* using the electroporation method. The tranformed cells were screened for viable colonies on SD plate lacking leucine but containing 5-FOA, 20 mM thiamine and 0.2 mg/ml phloxin B. After incubation at 30 ^{o}C for 4 days, the temperature was raised to 36 ^{o}C, and temperature-senstive (Ts) colonies were selected by the phloxin B color selection (see Fig. 7).

Expression and purification of TFIIS

The *E. coli* expression plasmid pGEX2T-SpIIS for GST-TFIIS fusion was constructed by inserting the TFIIS-coding sequence into pGEX2T at the *Bam*H1site. *E. coli* DH5 containing the expression plasmid for GST-TFIIS or GST was growin in LB medium. Expression of the recombinant proteins was induced by adding (IPTG). Cells were disrupted in a lysis buffer (50 mM Tris-HCl pH 7.8, 5% glycerol 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 0.1% NP-40 and 0.3 mg/ml lysozyme). Crude extract was mixed with glutathione-Sepharose 6B beads (Phamacia) and the bead-bound proteins were eluted with an elution buffer (50 mM Tris-HCl pH 7.8, 5% glycerol 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 0.1% NP-40 and 5 mM glutathione). For use in transcription assaay, the GST-TFIIS fusion protein was cleaved by thrombin.

GST pull-down assay of TFIIS complexes

Affinity beads were prepared by mixing purified GST or GST-TFIIS proteins at a protein concentration of 2 mg/ml with glutatione-Sepharose 4B beads (Amersham Phalmacia). Clude extracts of witd-type and mutant *S. pombe* were preparated essentially as described by Azuma *et al.* (1993) with a slight modification : the ammonium sulfate precipitates were dialized against a pull-down buffer (50 mM Tris-HCl, pH 7.8, 10% grycerol, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 0.1% TritonX-100). The samples (approximately 50 μg proteins/100 μl) were mixed with 10 μl of the affinity beads. After incubation at 4 ^{O}C for 60 min, the beads were harvested by centrifugation, washed 3 times with 0.5 ml each of the pull-down buffer or the buffer containing 150 mM NaCl.

Results

I. Analysis of the subunit-subunit contact network within RNA polymerase II

1-1. Detection of subunit-subunit interactions by far-Western blotting

Previously, we carried out far-Western blot analysis of *S. pombe* RNA polymerase II, separated into individual subunits by SDS-PAGE, with radio-labeled Rpb3 and Rpb5 probes (Miyao *et al.*, 1997; Yasui *et al.*,1998). For identification of the subunit-subunit interaction involving other small-sized subunits, we carried out far-Western blotting using radio-labeled small subunits, Rpb6, Rpb7, Rpb8, Rpb10, Rpb11, and Rpb12, as probes. For this purpose, we constructed a set of expression plasmids for all these small-sized subunits fused to a phosphorylation site sequence, ArgArgAlaSerVal, by heart muscle protein kinase (PK), and expressed the fusion proteins in *Escherichia coli*. The purified recombinant proteins were phosphorylated *in vitro* with [γ-^{32}P]ATP and heart muscle protein kinase.

The purified RNA polymerase II contains various levels of degradation products arisen from the two large subunits, Rpb1 and Rpb2, and some contaminating proteins albeit at low levels (Sakurai *et al.*, 1996; Sakurai & Ishihama, 1997). Moreover, the separation of small-sized subunits was difficult on SDS-PAGE. To overcome these problems, we expressed recombinant subunits, Rpb3CH, Rpb5CH, Rpb6CH, Rpb7KCH, Rpb8KCH, Rpb10KCH, Rpb11CH and Rpb12KCH in *E. coli* [C, H and K represent carboxyl(C)-terminus, His-tag and PK tag, respectively], and purified them to apparent homogeneity. Since the expression of intact Rpb1 and Rpb2 in *E. coli* was difficult, we expressed and isolated three fragments of Rpb1 [Rpb1N (residues 1-289), Rpb1M (290-1003) and Rpb1C (1004-1752)] and two fragments of Rpb2 [Rpb2N (residues 1-712) and Rpb2C (597-1210)]. The purified subunits or subunit fragments were directly slot-blotted onto membranes and subjected to far-Western blot analysis

using radio-labeled Rpb6KCH, Rpb7KCH, Rpb8KCH, Rpb10KCH, Rpb11KCH and Rpb12KCH as probes.

The far-Western slot-blot patterns, shown in Fig. 1, indicate that: (i) all the small-sized subunits bound to Rpb1M, Rpb1C and Rpb2C, and in addition, (ii) the interaction took place between the radio-labeled Rpb11KCH and unlabeled Rpb3. Since the Rpb3-Rpb11 interaction was also detected using the radio-labeled Rpb3 probe (Yasui *et al.*, 1998) and since the ^{32}P-labeled GST probe exhibited no signal against all the subunits analyzed, the observed far-Western signals were considered to include specific interactions. Among 80 combinations of bimolecular interaction analyzed, including the combinations analyzed with Rpb3 and Rpb5 probes (Miyao *et al.*, 1996; Yasui *et al.*, 1998), the subunit-subunit contact was observed for a total of 18 (or 19) combinations, *i.e.*, interaction of all eight small-sized subunits with both of the two large subunits, Rpb1 and Rpb2, and the interaction between small-sized subunits for three combinations of Rpb3-Rpb5, Rpb5-Rpb8 or Rpb11 (Miyao *et al.*, 1996; Yasui *et al.*, 1998) and Rpb3-Rpb11 (Ishiguro *et al.*, 1998). Using the slot-blot system employed, however, we failed to detect the interaction of Rpb5 with both Rpb8 and Rpb11 probes.

1-2. Detection of subunit-subunit interactions by chemical cross-linking

In order to confirm the results obtained by far-Western blotting and moreover to get insight into the subunit arrangement within RNA polymerase II, we carried out inter-subunit cross-linking experiments using various chemical cross-linkers listed in Table 3. Preliminary analysis of cross-linked RNA polymerase II with 2-iminothiolane hydrochloride (ITL; the maxim lengh of cross-linking, 14.5Å) by two-dimensional electrophoresis followed by far-Western blotting using mono-specific antibodies against each subunit indicated that most of the small subunits could be cross-linked to one or both of the two large subunits (data not shown).

In order to identify the subunits which were cross-linked to each of the two large subunits, the cross-linked RNA polymerase by ITL was directly applied onto SDS-10% slab-gel with a 10 cm-width well and after electrophoresis, the proteins on the slab gel were directly transferred onto PVDF membrane. For separate immunostaining of the single and same membrane with different antibodies, we used a Miniblotter (Immunetics) which divides the membrane into a number of vertical slots. Each slot was treated with one of the anti-subunit antibodies. As shown in Fig. 2, some small-sized subunits migrated slower than the uncross-linked Rpb2 or even Rpb1, suggesting that these subunits were cross-linked to Rpb2 and/or Rpb1. Six small subunits, Rpb5, Rpb6, Rpb7, Rpb10, Rpb11 and Rpb12, formed bands, which migrated between Rpb1 and Rpb2 on SDS-PAGE (Fig. 2, lanes 5, 6, 7, 10, 11 and 12). Since the efficiency of cross-linking under the conditions employed was at most 10%, it is unlikely that these slowly migrating bands represent cross-linked complexes of several small subunits. Since some of Rpb2 migrated slower than the uncross-linked Rpb2 between Rpb1 and Rpb2 (Fig. 2, lane 2). Thus, we concluded that at least these six subunits were cross-linked to Rpb2 (Table 4).

Five small subunits, Rpb5, Rpb6, Rpb8, Rpb11 and Rpb12, formed bands which migrated slower than Rpb1 and most of these small subunits except Rpb8 formed two bands, one migrating between Rpb1 and Rpb2 (cross-linking to Rpb2) and the other above Rpb1 (Fig. 2, lanes 5, 6, 8, 11 and 12). Therefore we concluded that these five small subunits were cross-linked to Rpb1. In agreement with this prediction, some of Rpb1 migrated slower than uncross-linked Rpb1 (Fig. 2, lane 1).

In addition, Rpb3 also formed three or four slowly migrating Rpb3 bands, one major band migrating close to Rpb1 and the others above Rpb1 (Fig. 2, lane 3). Since Rpb3 is known to form stable complexes with Rpb2 (Kimura *et al.*, 1997), we interpreted this observation to suggest that the major band migrating close to Rpb1 represents a cross-linked Rpb2-Rpb3 complex. Taken together with the results of far-Western analysis and cross-linking using other bifunctional cross-linkers, we also

interpreted that one of the super-shifted bands migrating slower than Rpb1 represent cross-linked Rpb1-Rpb3 complexes.

The efficiency of cross-linking depends on various factors including the linker length of bifunctional cross-linking agents and the target amino acid residues for conjugation. I then tried the cross-linking using other four agents (Table 3), dimethylsuberimidate (DMS; the maximum length of cross-linking, 11Å), N,N'-*o*-phenylene-dimaleimide (PDM, 5.2Å), dimethyl 3,3'-dithio-bis propionimidate (DTBP, 11Å) and diepoxybutane (DEB, 4Å). The results of cross-linking are summarized in Fig. 3. In these experiments, a freshly prepared RNA polymerase II was used, which contained a high level of Rpb1a (Rpb1a represents subunit 1 with carboxy-terminal domain (CTD) whereas Rpb1b represents the same subunit without CTD). Cross-linking to Rpb1a and Rpb1b was observed for Rpb3 (DMS and ITL), Rpb5 (ITL), Rpb6 (DMS, ITL, DTBP and DEB), Rpb8 (DMS, ITL and DTBP), Rpb11 (ITL), Rpb12 (ITL), while cross-linking to Rpb2 was observed for Rpb3 (ITL), Rpb5 (DMS and ITL), Rpb6 (DMS, ITL, DTBP and DEB), Rpb7 (DMS, ITL, PDM and DTBP), Rpb10 (DMS, ITL and DTBP), Rpb11 (ITL) and Rpb12 (DMS and ITL). Although separation of the large subunits cross-linked with small subunits from the respective uncross-linked subunits was not clear in some cases, we propose that in all these cases, the small subunits are cross-linked to the large subunits because the formation of big aggregates between small subunits is unlikely based on the low efficiencies of intermolecular cross-linking.

For some small-sized subunits, the cross-linking to the two large subunits was detected using only when specific cross-linking reagents were used. For instance, the cross-linking of Rpb3 to Rpb1 was observed using DMS and ITL, and the cross-linking of Rpb3 to Rpb2 was detected only with ITL. Likewise, the Rpb12-Rpb1 and Rpb12-Rpb2 cross-linking was observed with ITL and DMS/ITL, respectively. The failure of cross-linking with certain cross-linkers is often observed because of the lack of unique amino acid residues for cross-linking within the appropriate distance.

1-3. Cross-links between two large subunits Rpb1 and Rpb2

In the case of *E. coli* RNA polymerase, two large core subunits, β and β' do not form stable complexes under isolated state, but are located in close vicinity after assembly into the core enzyme (reviewed in Ishihama, 1981). In contrast, no clear biochemical evidence has been reported for direct contact between the two large subunits within the eukaryotic RNA polymerase II. For detection of cross-linking between the two large subunits, RNA polymerase II was treated with DMS and separated by SDS-7.5% PAGE. The gel was treated for Western blotting using monospecific antibodies against Rpb1 and Rpb2. As shown in Fig. 4, two clear bands were identified, which migrated slower than uncross-linked Rpb1 proteins and reacted with both anti-Rpb1 and anti-Rpb2 antibodies (Fig. 4B and 4C). When the membrane was treated with an antibody against the Rpb1 CTD, only the upper band was stained (Fig. 4A). Thus, we concluded that the two large subunits were cross-linked each other with DMS, forming Rpb1a-Rpb2 and Rpb1b-Rpb2 complexes.

1-4. Identification of small subunits cross-linked to Rpb3

Rpb3 and Rpb11 share similar sequence with prokaryotic α subunits (Azuma *et al.*, 1993; Sakurai and Ishihama, 1997). Previously we demonstrated the interaction between Rpb3 and Rpb11 by far-Western analysis and GST-Rpb3 fusion protein pull-down assay (Yasui *et al.*, 1998). Formation of heterodimers has been demonstrated between the two α-like subunits from *Arabidopsis thaliana* (Ulmasov *et al.*, 1996) and from *S. cerevisiae* (Larkin and Guilfoyle, 1997). To confirm the results by a chemical cross-linking method and to identify Rpb3-contact subunits other than Rpb11, RNA polymerase II was treated with DEB which is the crosslinker with the shortest chain length (4Å) among those used in this study. Subunits were separated by SDS-10% PAGE and transferred onto PVDF membrane. After far-Western blotting, a band was detected, which was stained with both anti-Rpb3 and anti-Rpb11 antibodies (Fig. 5A). The apparent molecular mass estimated from the migration distance on SDS-PAGE was close to the theoretical value calculated for a complex consisting of one molecular each

of Rpb3 and Rpb11. The result clearly showed that Rpb3 and Rpb11 indeed make direct contact each other.

Rpb10 is the smallest among the *S. pombe* RNA polymerase II subunits (Sakurai and Ishihama, 1997), and associates with the Rpb2-Rpb3-Rpb11 core subassembly (Kimura *et al.*, 1997). In good agreement with this finding, Rpb10 was cross-linked to Rpb2 with various cross-linkers (Fig. 2 and 3). For detection of possible interaction of Rpb10 with Rpb3, RNA polymerase II was treated with PDM, separated by SDS-10% PAGE and transferred onto PVDF membrane. After Western blotting, a band was detected, which cross-reacted against both anti-Rpb3 and anti-Rpb10 antibodies (Fig. 5B). The mobility of this complex is close to that estimated from the molecular mass of Rpb3-Rpb10 binary complex. Essentially the same result was obtained using other cross-linkers DTBP and ITL (data not shown).

1-5. Cross-link between small subunits

Rpb5, Rpb6 and Rpb8 are the common subunits shared among all three nuclear RNA polymerases, while Rpb7 is an RNA polymerase II-specific subunit (Sentenac *et al.*, 1992). To detect cross-linking between small subunits, RNA polymerase II was treated with ITL and cross-linked products were analyzed as above using four kinds of antibodies against Rpb5, Rpb6, Rpb7 and Rpb8 (Fig. 5C). Cross-linking was detected for the combinations of Rpb5-Rpb6, Rpb6-Rpb7 and Rpb6-Rpb8, which all formed super-shifted bands. Bimolecular interaction for these three combinations was, however, not detected by far-Western analysis (Miyao *et al.*, 1996; also see above).

II. Mutation analysis of the roles of Rpb6 in protein-protein contacts

2-1. Genetic analysis of *rpb6* mutants

2-1-1. Deletion mapping of Rpb6

In order to define the functional map of Rpb6, we constructed a mutant *S. pombe* *rpb6*::*ura4* by replacement of the *rpb6* gene on the chromosome by the *ura4* coding sequence. This *rpb6* disruptant can survive only under the supply of Rpb6 by an expression plasmid (see Fig.7 for the construction procedure). Since the *rpb6* gene on the plasmid is under the control of *nmt1* promoter, Rpb6 is synthesized only in the absence of thiamine addition (Maundrell, 1990). In the presene of thiamine, the synthesis of Rpb6 is repressed, and as a result, *S. pombe* is unable to grow, indicating that *rpb6* is an essential gene in *S. pombe*.

Into this *rpb6* disruptant, we introduced a set of compatible plasmids expressing various degrees of both N- and C-terminal deletion mutant Rpb6 (see Table 2), and tested the functions of truncated Rpb6 proteins in the presene of thiamine (or in the absence of intact Rpb6). As summarized in Fig. 8, the N-terminal deletion down to residue 61 did not affect the Rpb6 function as measured by the cell viability, while the C-terminal deletion of 6 amino acid residues made Rpb6 inactive. The results indicate that the region essential for Rpb6 function is located at the C-terminal proximal half of Rpb6 molecule between residues 61-139. The sequence of this region is highly conserved among Rpb6 homologues from seven organisms so far determined (see Fig. 9). The dispensable nature of the N-terminal proximal region down to residue 43 has been observed for the *S. cerevisiae* RPB6 subunit consisting of 155 amino acid residues (Nouraini *et al.*, 1996). Based on the deletion mapping of *S. pombe* Rpb6, we also constructed a minimum fragment between 61-139 residues, lacking both N- and C-terminal dispensable regions. This minimum fragment was, however, unable to support the cell growth.

2-1-2. Isolation of Rpb6 mutants

In order to isolate *S. pombe* mutants carrying a mutation in the *rpb6* gene, the *ura4* gene was replaced by PCR-mutagenized *rpb6* by homologous recombination, and recombinants were isolated on 5-fluoroorotic acid (5-FOA)-containing plate. For quick isolation of mutant RNA polymerases, an octahistidine (His_8) tag sequene was added at the N-terminus of *rpb6*. Starting from 10,000 Ura$^+$ colonies, we have so far isolated fourteen indepeendent temperature-sensitive (Ts) mutants at 36 ^{O}C, each carrying a single (or multiple in a few cases) mutation in the *rpb6* gene (see Fig. 7).

The entire Rpb6-coding region was PCR-amplified from all fourteen Ts mutants, and sequenced. As shown in Fig. 9 and Table 4, eight mutants carried a single (or triple for one mutant) mutation in the N-terminal proximal region between residues 5-23. In particular, mutations were clustered in a narrow region from residues 10 to 14. This is unexpected because the N-terminal proximal region is dispansable for cell growth (see above), and because none of the *S. cerevisiae RPB6* Ts mutants carried mutations in the N-terminal dispensable region (Nouraini *et al.*, 1996).

Six mutants carried a single (or double for one case) mutation in the C-terminal proximal essential region downstream from residue 61 (Table 5). Mutations in the most conserved region of Rpb6 critical for functions must have rendered *S. pombe* lethal as in the case of *S. cerevisiae* (Nouraini *et al.*, 1996).

2-1-3. Growth charateristics of the Rpb6 mutants

Growth was monitored for all the Ts mutants on a rich medium plate after up-shift from permissive (30 ^{O}C) to non-permissive (36 ^{O}C) temperature. Five mutants stopped cell growth after 5 days, while others continued to grow albeit at reduced rates (Table 5). Detailed analysis was then carried out for seven mutants, Ts1 (Ala63Thr), Ts89 (Asp135Asn and Glu139Ala), Ts113 (Ala81Thr), Ts118 (Tyr78Asn), Ts158 (Val99Ala) and Ts159 (Met112Thr), which carried a single mutation (or doulbe in the case of Ts89) in the essential region, and one mutant, Ts127, carrying a Thr45Ile mutation in the non-essential region.

Growth was monitored for these seven mutants, together with the parental strain 6NH, in a liquid minimal medium containing adenine, leucine and uracil after temperature up-shift from 30 to 36 ^{O}C. As shown in Fig. 10, four mutants, Ts-1, Ts-127, Ts-158 and Ts-159, stopped cell growth after the temperature up-shift, but the other three leaky mutants continued to grow at reduced rates. The growth characteristics were essentially the same between agar plate and liquild media (data not shown).

Growth of the Rpb6 mutants was also examined on a plate containing 6 azauracil (6AU), which inhibits IMP dehydrogenase and leads to limitations of GTP and UTP pools (Exinger & Lacroute, 1992; Lanzendorfer *et al.*, 1997). After five days at the permissive temperature (30 ^{O}C), the growth of three mutants, Ts-1, Ts-158 and Ts-159, was significantly reduced (Table 5), suggesting that Rpb6 plays a role in the catalytic activity of RNA synthesis. However, other four mutants grew as fast as the wild-type parent even in the presence of 6AU.

2-2. Functional interaction of Rpb6 with TFIIS

2-2-1. Suppression of rpb6 mutations by TFIIS

The *S. cerevisiae* mutants lacking the *PPR2* gene encoding the elongation factor TFIIS (or SII) are sensitive to 6AU (Nakanishi *et al.*, 1995), because of the elongation arrest of RNA chains due to limitation of nucleotide pools (Extinger and Lacroute, 1992). Likewise, some RPB1 and RPB2 mutants of *S. cerevisiae* are sensitive to 6AU (Archambault *et al.*, 1992; Lennon *et al.*, 1998), suggesting that these RNA polymerase II mutants are defective at the step of RNA chain elongation. The 6AU sensitivity of some *rpb6* mutants suggests the involvement of Rpb6 in the transcription elongation step. We then tried to suppress the 6AU-sensitive phenotype of *S. pombe rpb6* mutants by introducing multicopies of the *S. pombe TFIIS* gene (Williams & Kane, 1992). As shown in Fig. 11, the 6AU-sensitive charateristics of three mutants, Ts-1, Ts-158 and Ts-159, was suppressed in the presence of multicopy plasmid p41-SII encoding TFIIS.

The growth of 6AU-insensitive mutant Ts-127 was, however, not affected by overexpression of TFIIS. The Ts phenotype of the same set of three *rpb6* mutants was also suppressed by introducing multiple copies of the TFIIS expression plasmid. These obsevations altogether indicate the functional interaction between Rpb6 and TFIIS.

2-2-2. Interaction *in vitro* of RNA polymerase II with TFIIS

In order to confirm the genetic interaction between Rpb6 and TFIIS, and to examine the possiblity of direct protein-protein interaction between RNA polymerase II and TFIIS, we expressed a GST (glutathione S-transferase) fusion form of TFIIS in *E. coli* and purified the recombinant TFIIS to apparent homogeneity by glutathione-Sepharose column chromatography. The purified GST-TFIIS was mixed with partially purified RNA polymerase II from the wild-type *S. pombe* and the Rpb6 Ts mutants. Protein complexes containing the GST-TFIIS were then isolated by using glutathione-Sepharose beads. The recovery of RNA polymerase II in the unbound and column-bound fractions was measured by Western blot analysis using anti-Rpb1, Rpb6 and anti-Rpb7 antibodies. As shown in Fig. 12(A), the RNA polymerase II of wild-type 6NH and Ts-127 mutant *S. pombe* was recovered in the GST-TFIIS fraction, while the yield of RNA polymerase II in the complex fraction was signifiantly redued for Ts-1, Ts-158 and Ts-159 [compare lanes I (input) and S (GST-SII complex); lane I corresponds to 10% of the lane S].

If the observed interaction between RNA polymerase II and TFIIS was attributed to the direct contact between Rpb6 and TFIIS, the complex formation must be interfered by the addition of Rpb6 protein. To test this possibility, increasing amounts of the purified recombinant Rpb6 were added to the complex formation assay. As shown in Fig. 12(B), the addition of free Rpb6 interfered with the formation of RNA polymerase II-TFIIS complex, as detected by immunostaining using anti-Rpb1, anti-Rpb6 and anti-Rpb7 antibodies.

2-2-3. Transcription stimulation *in vitro* by TFIIS

TFIIS can stimulatie *in vitro* transcription of RNA polymerase II by direct binding with the enzyme. We purified these mutant polymerases (Fig.13A) and measured the activity in the absence or presence of recombinant TFIIS (Fig. 13B). Non-specific transcription activity was measured for wild-type and four mutant RNA polymerases. TFIIS stimulated the 6NH and Ts127 RNA polymerases activities 1.7 and 1.9 fold, respectively, but the stimulation level of Ts1, Ts158 and Ts159 was only 1.4, 1.1 and 1.2 fold, respectively by addition of TFIIS (0.4μg) (Fig. 13B). The weak stimulation of the rpb6 mutant RNA polymerase suggests the weak affinity between TFIIS and mutant Rpb6.

Discussion

1. Subunit-subunit contact network

Previously we identified the subunits which interact with Rpb3 and Rpb5 by far-Western blotting and GST-pull down assays (Miyao *et al.*, 1996; Yasui *et al.*, 1998). I extended in this study the analysis so as to determine the subunit-subunit contact involving all other small-sized subunits, *i.e.*, Rpb6, Rpb7, Rpb8, Rpb10, Rpb11 and Rpb12. The far-Western analysis provided positive signals for binding of all the small-sized subunits with the two large subunits (Fig. 1). Taken together with the previous observations (Miyao *et al.*, 1996; Yasui *et al.*, 1998), we detected the binding of all eight small-sized subunits, Rpb3 to Rpb12, to both of the two large subunits, Rpb1 and Rpb2. In the previous experiments, we detected the interaction between small subunits for two combinations, *i.e.*, Rbp3-Rpb5 and Rpb5-Rpb8/Rpb11. In addition, we detected here the interaction for another combination between Rpb3 and Rpb11. The interaction between Rpb3 and Rpb5 was observed using both Rpb3 (Yasui *et al.*, 1998) and Rpb5 (Miyao *et al.*, 1996) probes, and confirmed by pull-down experiments. Previously we detected the interaction of Rpb5 with either Rpb8 or Rpb11 using Rpb5 probe (Miyao *et al.*, 1996), but here neither Rpb8 nor Rpb11 probe bound to Rpb5. The discrepancy might be due to the difference in far-Western blotting methods: in the previous experiments, we blotted RNA polymerase subunits separated by SDS-PAGE onto membranes [thus proteins were once denatured with SDS] but in this experiment, purified individual subunit proteins were directly blotted onto membranes. Thus, one possibility is that the signal detected in the previous experiment was due to non-specific interaction between Rpb5 probe with denatured Rpb8 or Rpb11. Otherwise, the binding affinity of Rpb8 or Rpb11 probes to native Rpb5 fixed on the membrane is too weak to be retained. It is also not excluded yet that the signal detected using the Rpb5 probe was due to a contaminated degradation product(s) of either Rpb1 or Rpb2 migrating on SDS-PAGE to the similar positions with Rpb8 and Rpb11.

To confirm the results of far-Western analysis and to get some more information on the spatial arrangement of small subunits, we performed the subunit-subunit cross-linking studies using various cross-linkers. This method has been successfully employed for identification of the spatial arrangement of multi-subunit complexes such as *E. coli* RNA polymerase (Hillel and Wu, 1977; Ishihama *et al.*, 1979), *E. coli* 30S (Chang and Flaks, 1972; Lambert *et al.*, 1983) and 50S ribosomes (Redl *et* al., 1989; Walleczek *et al.*, 1989a; 1989b), *S. cerevisiae* 60S ribosomal subunit (Xiang and Lee, 1989) and rat river phosphocholine cytidyl transferase (Cornell, 1989). Using five species of bifunctional cross-linkers, each differing in the linker length between the two reactive sites and in the target amino acid residues for conjugation, we found the cross-linking of six small subunits Rpb3, Rpb5, Rpb6, Rpb8, Rpb11 and Rpb12 with the largest subunit Rpb1, and of seven small subunits Rpb3, Rpb5, Rpb6, Rpb7, Rpb10, Rpb11 and Rpb12 with the second largest subunit Rpb2 (Fig. 3; and Table 2). The results, summarized in Table 2 and Fig. 6, essentially agreed with those obtained by far-Western blot (Fig. 1; and also Miyao *et al.*, 1996; Yasui *et al.*, 1998) and GST pull-down assay (Miyao *et al.*, 1996; Yasui *et al.*,1998). Thus, we concluded that one or both of the two large subunits provide the contact surface for all small-sized subunits (Rpb3 to Rpb12). For three combinations, Rpb1-Rpb7, Rpb1-Rpb10 and Rpb2-Rpb8 (dotted lines in Fig. 6), the bimolecular interaction was detected only by far-Western blotting but not by cross-linking. For detection of the cross-linking for these pairs, other cross-linking reagents or reaction conditions may be required. Otherwise, the observed far-Western signals for these combinations may represent artifacts arisen from non-specific protein-protein interactions under denatured state.

Recently, Acker *et al.* (1997) reported a systematic study of the bimolecular interactions *in vivo* between the human RNA polymerase II subunits after analysis of binary complex formation between pairs of subunits, one gluthathione S-transferase-tagged and the other untagged subunits, expressed in the same cells. The results indicated that both Rpb3 and Rpb5 constitute the nucleation center for the assembly of other subunits. By cross-linking and far-Western blot analyses, however, we failed to

detect the interaction for the combinations, Rpb3-Rpb7, Rpb3-Rpb12, Rpb5-Rpb7 and Rpb5-Rpb10. One possibility is that the bimolecular interaction for these combinations take place during the intermediate step(s) of subunit assembly, but the two subunits are not closely located in the assembled RNA polymerase II. The distance between two α subunits of *E. coli* RNA polymerase is known to alter during the assembly pathway, being the shortest for the α dimer (reviewed in Ishihama, 1981). Acker *et al.* (1997) were unable to specify the Rpb2-contact subunits because Rpb2 was not expressed without co-expression of Rpb1. Here we succeeded to demonstrate the interaction of Rpb2 with not only small subunits but also the largest subunit Rpb1. We provided the first experimental evidence which suggests that the two large subunits are arranged as to make direct contact. In the case of *E. coli* RNA polymerase, the two large subunits, β and β', do not form stable complexes under isolated states but are in close contact within the core enzyme (reviewed by Ishihama, 1981).

Upon stepwise dissociation of the *S. pombe* RNA polymerase II with increasing concentrations of urea, we identified the core subassembly consisting of Rpb2 (β homologue), Rpb3 (α homologue) and Rpb11 (α homologue) (Kimura *et al.*, 1997), which is analogous to the assembly intermediate $\alpha_2\beta$ complex of *E. coli* RNA polymerase (Ishihama, 1981). In the stepwise dissociation studies, we also identified the association of Rpb8 to Rpb1. In agreement with this finding, the Rpb1-Rpb8 interaction was detected by both far-Western blotting and cross-linking with three different reagents. These observations may suggest that Rpb1 associates with Rpb8 prior to association with the Rpb2-Rpb3-Rpb11 core subassembly (Kimura *et al.*, 1997). On the other hand, Rpb10, one of the five common subunits shared among RNA polymerase I, II and III, was suggested to be loosely associated with the Rpb2-Rpb3-Rpb11 core subassembly (Kimura *et al.*, 1997). The interaction of *S. cerevisiae* RPB10 (ABC10β) with both AC40 (RPB3 homologue in RNA polymerases I and III) and AC19 (RPB11 homologue in RNA polymerases I and III) was also suggested by mutant studies (Lalo *et al.*, 1993). In this study, we found that Rpb10 can be cross-linked with both Rpb2 and Rpb3. This observation is consistent with the hypothesis that Rpb10 is

the subunit which binds to the core subassembly before the association of other small subunits.

Rpb6 is one of the five common subunits among three forms of RNA polymerase and essential for cell viability, suggesting its important role in either RNA polymerase function or assembly (reviewed by Sentenac *et al.*, 1992). In this study, we found that Rpb6 could be cross-linked with not only the two large subunits but also at least three small subunits, *i.e.* two subunits, Rpb5 and Rpb8, shared among RNA polymerases I, II and III, and one RNA polymerase II-specific subunit Rpb7 (see Fig 6). This observation suggests that Rpb6 is located in the central core of assembled RNA polymerase II in close contact with a number of subunits.

2. Roles of Rpb6 in protein-protein interactions

The RNA synthesis *in vitro* by at least the RNA polymerases I and II from *S. cerevisiae* is inhibited by the addition of anti-RPB6 antibodies (Sawadogo *et al.*, 1980; Breant *et al.*, 1983). Likewise the *S. cerevisiae* RNA polymerase I lacking A43, A14 and ABC23 (RPB6) is defective in basal transcription activity *in vitro*. However, the activity of RNA polymerase devoid of RPB6 could be restored by the addition of recombinant ABC23 (RPB6) up to 50 % of the wild type level (Lanzendorfer *et al.*, 1997). Mutant studies herewith described indicate that the Rpb6 is an essential subunit for RNA polymerase functions. In support of the prediction, the Rpb6 homologues exist in wide varieties of RNA polymerases from eukaryotes, archaes and some DNA viruses (Lu *et al.*, 1993; Langer *et al.*, 1995; Shpakovski *et al.*, 1995), and the RPB6 of *S. cerevisiae* is functionally exchangeable with the corresponding subunits from human and fission yeast (Shpakovski, 1994; McKune and Woychhik, 1994; Shpakovski *et al.*, 1995).

Deletion analysis indicated that the essential region for the Rpb6 function is located in the C-terminal half of Rpb6 (see Fig. 8). The dispensable nature of the N-terminal proximal region of down to residue 43 has been observed for the *S. cerevisiae* RPB6 subunit consisting of 155 amino acid residues (Nouraini *et al.*, 1996) However,

Ts mutants isolated in this study was found to carry mutations in not only the C-termial region but also the N-terminal half. In particular, mutations are clustered within a narrow region between residues 10-20 near the N-terminus. Since this region is not present in the Rpb6 homologue of archae and since this region is not highly conserved among eukaryotes (see Fig. 9), this N-terminal protruding tail may not have an essential function, but it may have a unique regulatory function specific for *S. pombe* or a controlling function of the Rpb6 structure. Such a functional map of *S. pombe* Rpb6 is in good agreement with the functional map of *S. cerevisiae* RPB6 (Nouraini *et al.*, 1996). In agreement with these findings, the sequence is more conserved at the C-terminal proximal region (see Fig. 9).

One novel finding in our mutant studies is the functional interaction of Rpb6 with transcription elongation factor TFIIS (or S-II). TFIIS was originally isolated as a stimulation factor of transcription elongation process by the RNA polymerase II (Sekimizu *et al.*, 1979) and is present throughout eukaryotes, archaes (Langer *er al.*, 1995), and a group of DNA viruses (Ahn *et al.*, 1990; Rodriguez *et al.*, 1992; Dixon *et al.*, 1994). During trnascription elongation, TFIIS enforces cleavage of nascent RNA at the pause or arrest sites and there by enhances transcription elongation (Reines *et al.*, 1989; Reines, 1992; Awrey *et al.*, 1997). As in the case of bacterial GreA and GreB proteins, TFIIS binds to the RNA polymerase directly and stimulates its RNA synthesis activity by cutting off nascent RNA chains at 3' ends (Sawadogo *et al.*, 1980; Sopta *et al.*, 1985; Izban & Luse, 1992). The TFIIS of *S. cerevisiae* is composed of three domains, I, II and III, among which the NMR structures have been solved for the C-terminal proximal domains II and III (Olmsted *et al.*, 1998). The domains II and III are known to be essential for interaction with the RNA polymerase II (Agarwal *et al.*, 1991; Shimoaraiso *et al.*, 1997: Awrey *et al.*, 1998).

S. cerevisiae mutants carrying mutations in the *PPR2* gene for TFIIS have been isolated, which showed high level sensitivity to 6AU that inhibits IMP dehydrogenase and ultimately results in limitation in GTP and UTP pools (Exinger and Lacroute, 1992). Pevious genetic studies of *S. cerevisiae* RNA polymerase II indicated functional

interactions of TFIIS with two large subunits, RPB1 (Archambaut *et al.*, 1992) and RPB2 (Powell and Reines, 1996; Lennon *et al.*, 1998). In ageement with this prediction, a mutant RPB1 was isolated, which showed decreased binding affinity to TFIIS (Wu *et al.*, 1996). Since some of the *S. pombe* Rpb6 mutants herein isolated showed high-level sensitivity to 6AU, I predicted the functional interaction between Rpb6 and TFIIS (in addition to its interaction with Rpb1 and Rpb2). In fact, over-expression of *S. pombe* TFIIS supressed the 6AU-sensitive phenotype (see Fig. 11). Four Ts mutants, Ts-1, Ts-155, Ts-158 and Ts-159, were sensitive to 6AU, but Ts-127 was not. The mutations of the 6AU-sensitive mutants are located in the C-terminal essential region of Rpb6, but the mutation in Ts-127 is located in its N-terminal-proximal region. Thus, the contact site of TFIIS might be located in the C-terminal essential region of Rpb6. To confirm the genetic interaction between Rpb6 and TFIIS, I carried out the GST pull-down assay using the GST-TFIIS recombinant fusion protein. The wild-type intact Rpb6 formed complexes with the GST-TFIIS, but the affinity for TFIIS was signignificantly reduced for the Ts-mutant Rpb6 proteins (see Fig. 12A). Moreover, I detected evident competition between Pol II and recombinant Rpb6CH proten by the GST pull-down assay of TFIIS binding (see Fig. 12B).

In conclusion, several lines of the *in vivo* and *in vitro* evidence indicated the direct protein-protein contact between Rpb6 and TFIIS. Since Rpb6 of *S. pombe* interacts with both Rpb1 and Rpb2 (Ishiguro *et al.*, 1998), one possibility is that TFIIS interacts with the RNA polymease II at the boundary surface formed among Rpb1, Rpb2 and Rpb6.

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Table 1. Subunit composition of the *Schizosaccharomyces pombe* RNA polymerase II

Subunit	Number of amino acids	Protein mass (kDa)	Number of amino acids (sequence identity, %) *S. cerevisiae*	*H. sapiens*	*A. thaliana*
Rpb1	1,752	194	1,733 (63)	1,970 (53)	1,841 (56)
Rpb2	1,210	138	1,224 (68)	1,174 (60)	1,188 (62)
Rpb3	297	34	318 (48)	275 (36)	319 (30)
Rpb4	135	15	221 (26)	142 (36)	138 (31)
Rpb5	210	24	215 (57)	210 (44)	205 (42)
Rpb6	142	16	155 (56)	127 (49)	144 (44)
Rpb7	172	19	171 (54)	172 (48)	176 (46)
Rpb8	125	14	146 (39)	150 (36)	146 (32)
Rpb9	113	13	122 (47)	125 (44)	134 (41)
Rpb10	71	8.3	70 (72)	67 (68)	71 (70)
Rpb11	123	14	120 (44)	117 (43)	116 (39)
Rpb12	63	7.2	70 (39)	58 (46)	51 (44)

The subunit compositions of RNA polymerase II are as described in the followings:
S. cerevisiae (Thuriaux & Sentenac, 1992; Woychik & Young, 1994); *H. sapiens* (Khazak *et al.*, 1998); and *A. thaliana* (Larkin *et al.*, 1999).

Table 2. Plasmids used in this study

Plasmid	Construction
pRpb6::ura4	pBluescript containing *rpb6* 5'- flanking seqeunce, *ura4*-coding sequence, and *rpb6* 3'-flanking sequence
pRpb6::Rpb6NH$_8$	pBluescript containing *rpb6* 5'flanking sequence, His$_8$-*rpb6* coding sequence, and *rpb6* 3'-flanking sequence
pREP81-Rpb6	pREP81 containing intact *S. pombe rpb6*-coding sequence
pREP41-SpIIS	pREP41 containing *S. pombe* TFIIS-coding sequence
pAI-ARS	pBluescript containg *ura4* at *Hin*dIII site, *ARS1* at *Eco*R1 site, and *rpb6* 5'- and 3'-flanking sequences between *Eco*R1 and *Sac*I sites
pRpb6WT	pAI-ARS containing intact *rpb6*-coding sequence
pRpb6NTM (-40)	pAI-ARS containing N-terminal 40 residue deletion *rpb6*
pRpb6NTM (-50)	pAI-ARS containing N-terminal 50 residue deletion *rpb6*
pRpb6NTM (-60)	pAI-ARS containing N-terminal 60 residue deletion *rpb6*
pRpb6NTM (-70)	pAI-ARS containing N-terminal 70 residue deletion *rpb6*
pRpb6CTM (-3)	pAI-ARS containing C-terminal 3 residue deletion *rpb6*
pRpb6CTM (-6)	pAI-ARS containing C-terminal 6 residue deletion *rpb6*
pRpb6CTM (-60/-3)	pAI-ARS containing N-terminal 60 and C-terminal 3 residue deletion *rpb6*
pGEX2T-SpIIS	pGEX2T containing the *S. pombe* TFIIS gene
pET21b-Rpb6CH	pET21b containing *S. pombe rpb6* cDNA

Table 3. Bifunctional chemical cross-linkers used in this study

Cross-linker	Abbreviation	Linker length (A)	Targer amino acid
Dimethulsuberimidate	DMS	11.0	Lys
2-Iminothiolane hydrochloride	ITL	14.5	Lys
N,N'-*o*-Phenylene-dimaleimide	PDM	5.2	Cys
Dimethyl 3,3'-dithio-bis propionimidate	DTBP	11.0	Lys
Diepoxybutane	DEB	4.0	Lys/Cys

Table 4. Subunit-subunit cross-linking

Cross-linked subunit pair		Cross-linking reagent
Rpb1	Rpb2	DMS, ITL, PDM, DTBP, DEB
Rpb1	Rpb3	DMS, ITL
Rpb1	Rpb5	ITL
Rpb1	Rpb6	DMS, ITL, DTBP, DEB
Rpb1	Rpb8	DMS, ITL, DTBP
Rpb1	Rpb11	ITL
Rpb1	Rpb12	ITL
Rpb2	Rpb3	ITL
Rpb2	Rpb5	DMS, ITL, PDM
Rpb2	Rpb6	DMS, ITL, DTBP, DEB
Rpb2	Rpb7	DMS, ITL, PDM, DTBP
Rpb2	Rpb10	DMS, ITL, PDM, DTBP
Rpb2	Rpb11	ITL
Rpb2	Rpb12	DMS, ITL
Rpb3	Rpb10	PDM, DTBP, ITL
Rpb3	Rpb11	DEB, ITL
Rpb5	Rpb6	ITL
Rpb6	Rpb7	DMS, ITL
Rpb6	Rpb8	ITL

Table 5. Fission yeast *rpb6* mutants

Mutant sensitivity	*rpb6* mutation	Rpb6 change	Growth (36 C)	6AU
6NH	wild-type	wild-type	Normal	Resistant
Ts1	G198A, T288A	A63T,	Ts	Sensitive
Ts89	G403A, A419C	D135N, E140A	Ts (leaky)	Resistant
Ts91	G40T, G132A, A348G	G14S	Ts (leaky)	Resistant
Ts113	G241A	A81T	Ts (leaky)	Resistant
Ts115	G37T	D13N	Ts (leaky)	Resistant
Ts118	T232A	Y78N	Ts (leaky)	Resistant
Ts124	A14G, G43A, A68T	E5G, A15T, E23V	Ts (leaky)	Resistant
Ts127	C134T	Y45I	Ts	Resistant
Ts128	T30A	F10L	Ts (leaky)	Resistant
Ts155	T335C, A402G	M112T	Ts	Sensitive
Ts158	T296C	V99A	Ts	Sensitive
Ts159	T335C	M112T	Ts	Sensitive
Ts161	A34G,G41A, T120C	M12V, G14D	Ts (leaky)	Resistant
Ts233	A20G	D7G	Ts (leaky)	Resistant

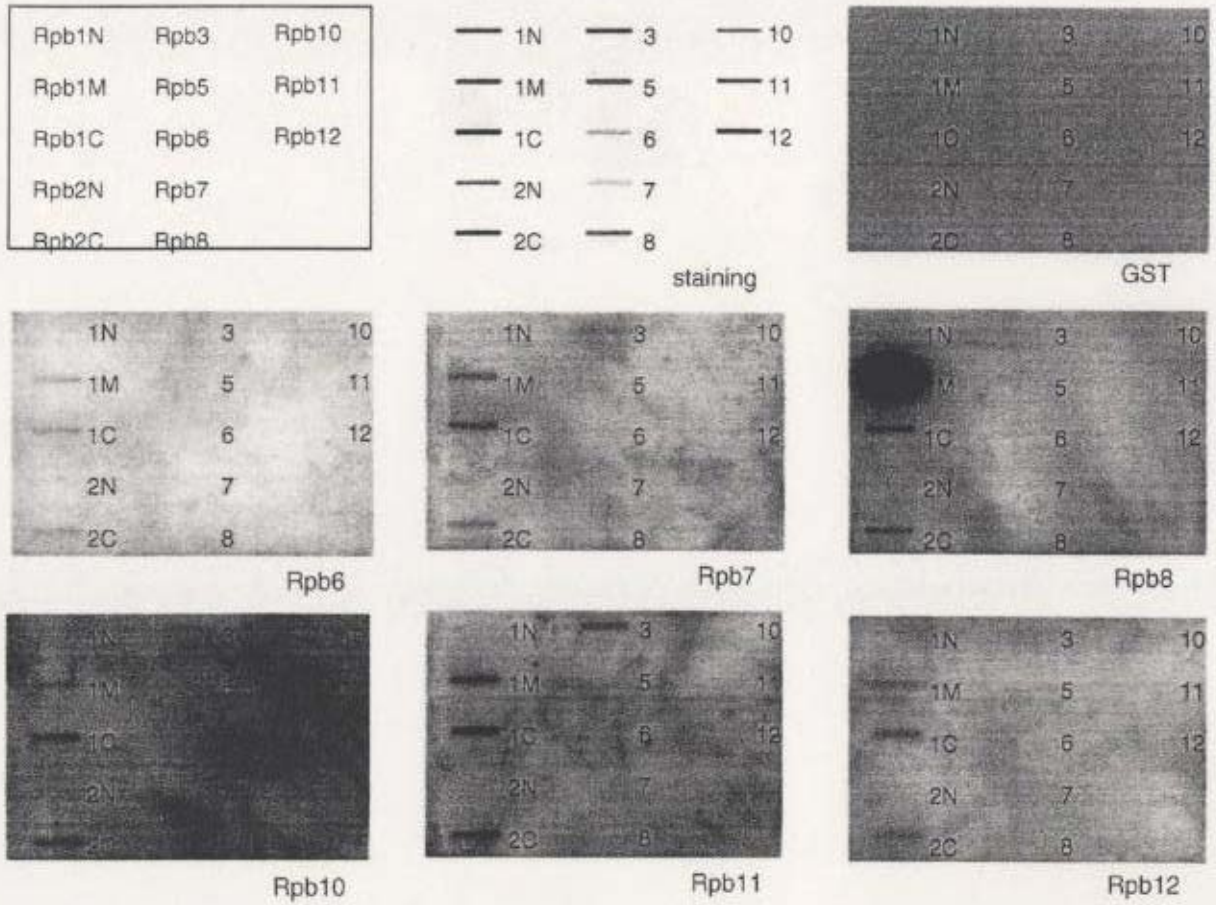

Figure 1. Far-Western analysis of bimolecular interaction between two isolated RNA polymerase II subunits. Isolated subunits (Rpb3 to Rpb12) or subunit fragments (Rpb1N, Rpb1M, Rpb1C, Rpb2N and Rpb2C) (10 pmol aech) were blotted onto nitrocellulose membranes at the positions as indicated on the left panel of the top lane. The middle panel of the top lane shows the stained membrane with colloidal gold (Protogold; British Bio Cell International), while other membranes were treated for far-Western blotting using one of 32P-labeled subunit probes as indicated at the bottom of each panal.

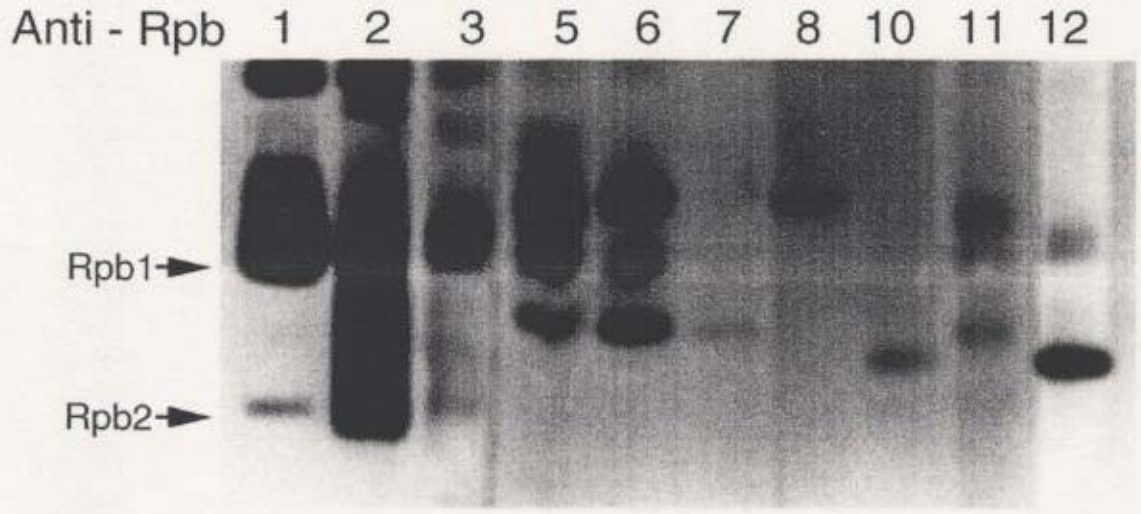

Figure 2. Identification of subunit-subunit cross-links within ITL-treated RNA polymerase II. Purified RNA polymerase II (10 μg) was treated with ITL, applied into a 10 cm-width well on a 7.5% polyacrylamide slab gel. After SDS-gel electrophoresis, proteins were transferred onto a nitrocellulose membrane and the membrane was probed with 10 different subunit-specific antibodies. Antibodies used are indicated on the top of each lane.

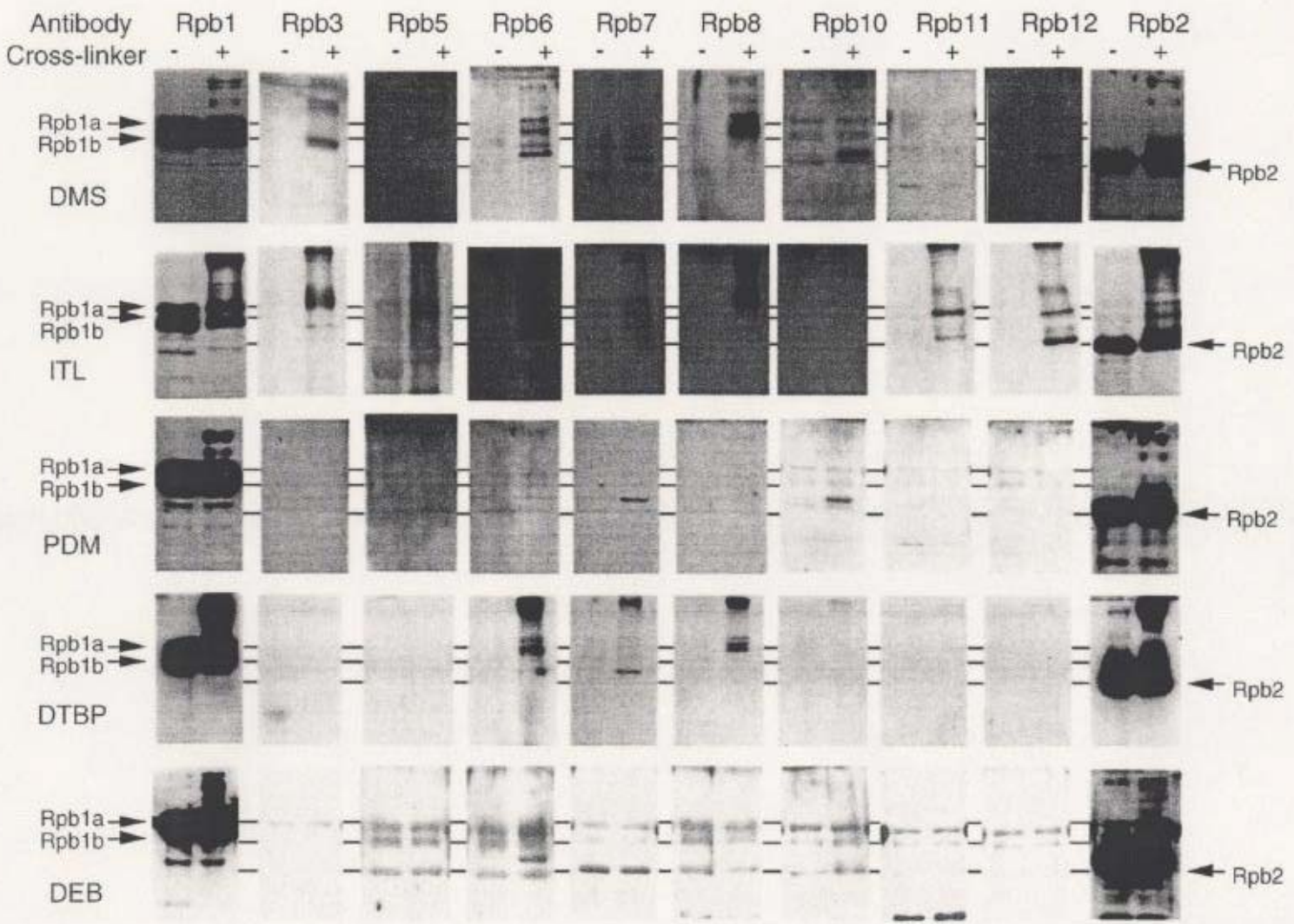

Figure 3. Identification of small-sized subunits cross-linked to two large subunits. Purified RNA polymerase II (1 μg) was incubated in the absence (cross-linker, -) or presence (cross-linker, +) of five species of cross-linkers as indicated at the left side, and the cross-linked products were separated from uncross-linked subunits by SDS-7.5% PAGE. Locations of individual subunits were detected by Western blotting using 10 different subunit-specific antibodies as indicated on the top.

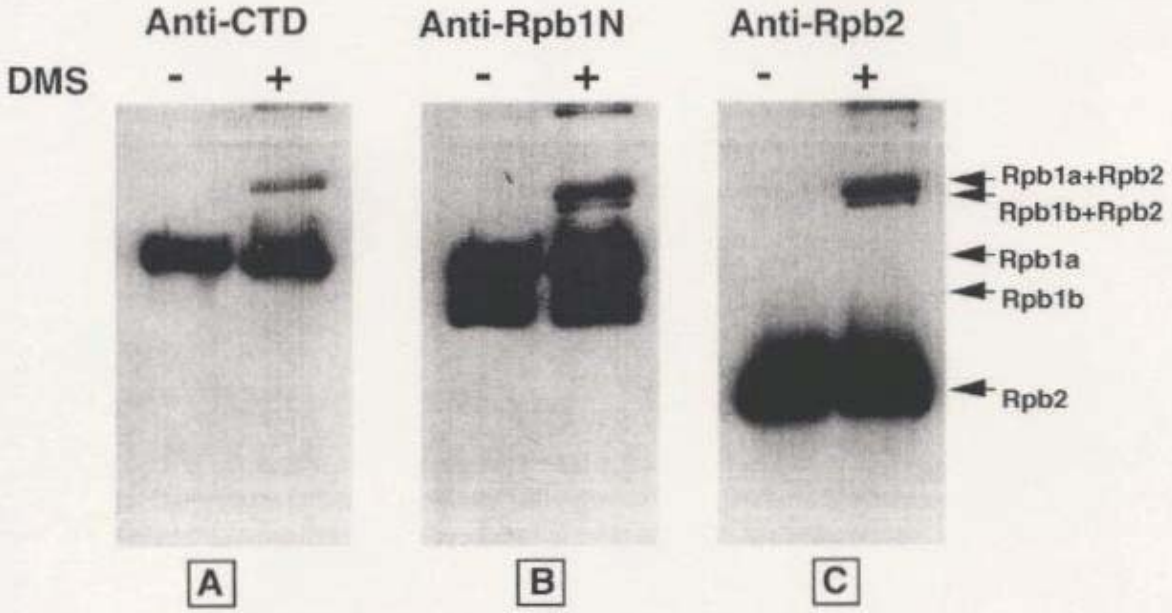

Figure 4. Cross-linking betweeen two large subunits. RNA polymerase (1 μg) was incubated in the absence (-) or presence (+) of DMS and then subjected to SDS-7.5% PAGE. The gel was treated for Western blotting using three different antibodies as indicated on each panel. Parts of the gels are shown. The migration positions of uncross-linked and cross-linked Rpb1 and Rpb2 are indicated at the right.

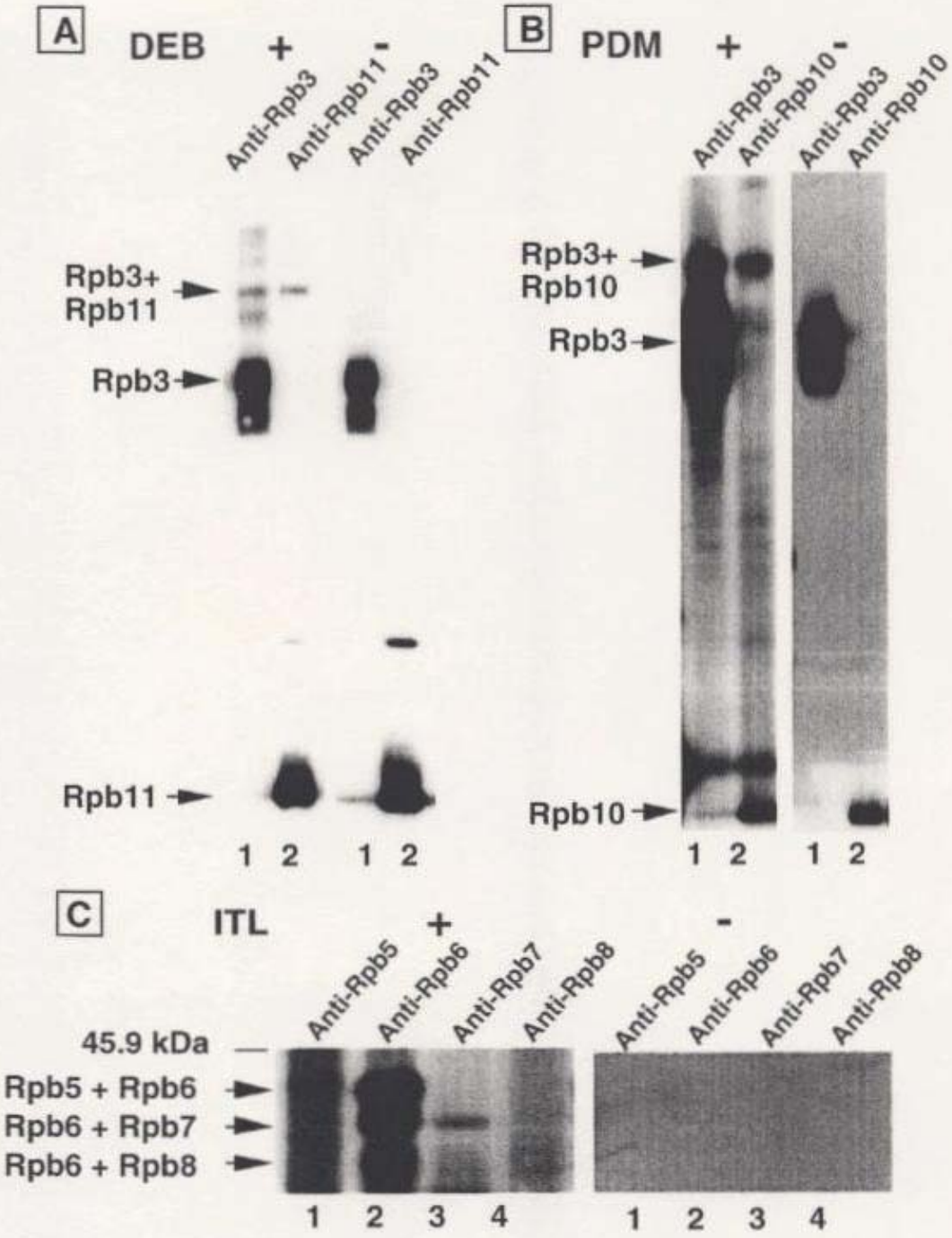

Figure 5. Cross-linking between small-sized subunits. [A] RNA polymerase II (10 μg) was incubated in the presence (+) or absence (-) of DEB, and subjected to SDS-10% PAGE. The gel was analyzed by Western blotting using anti-Rpb3 and anti-Rpb11 antibodies. [B] RNA polymerase II (10 μg) was incubated in the presence (+) or absence (-) of PDM, and subjected to SDS-10% PAGE. The gel was analyzed by Western blotting using anti-Rpb3 and anti-Rpb10 antibodies. [C] RNA polymerase II (10 μg) was incubated in the presence (+) or absence (-) of ITL, and subjected to SDS-10% PAGE. The gel was analyzed by Western blotting using antibodies againt Rpb5, Rpb6, Rpb7 and Rpb8

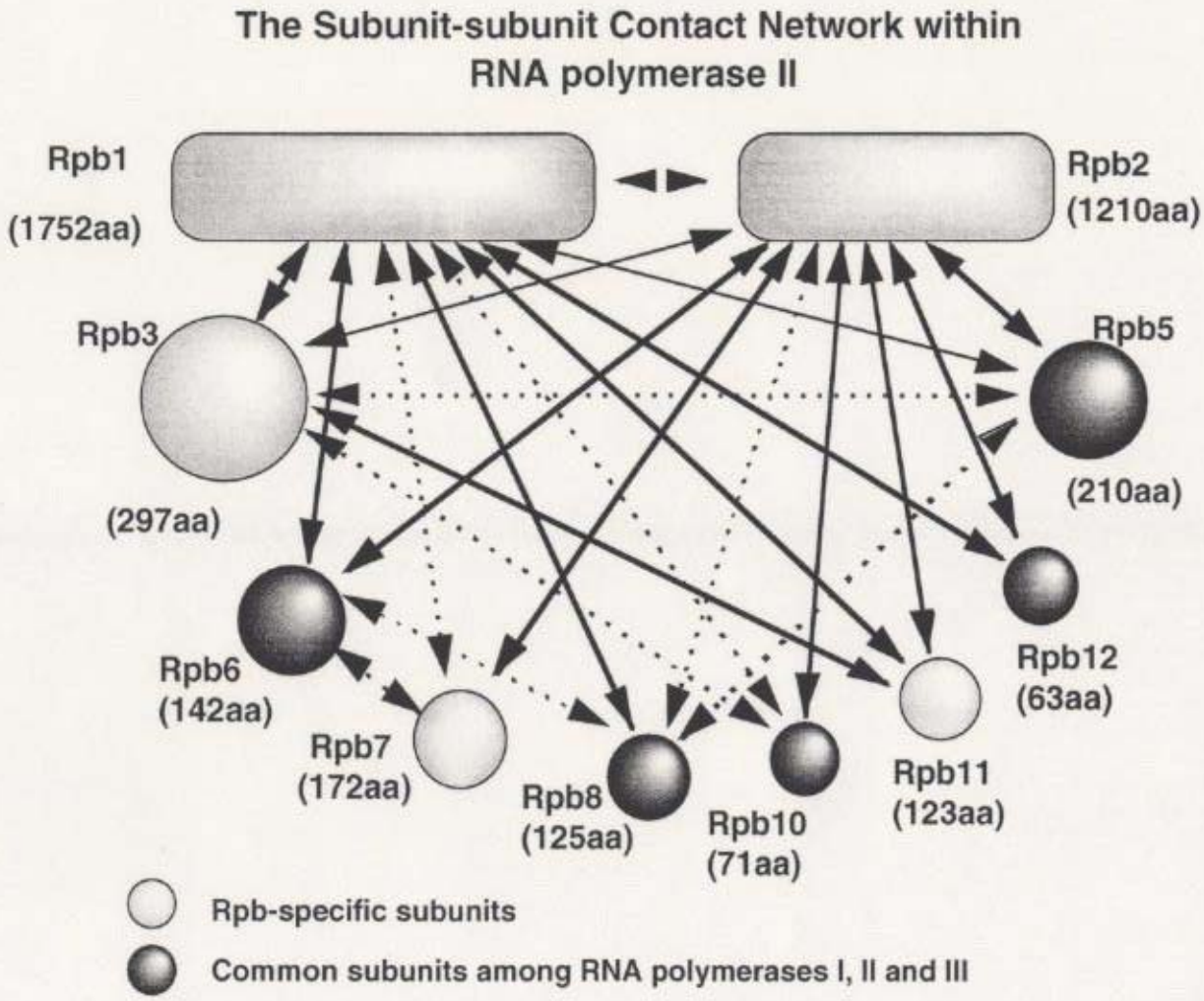

Figure 6. The subunit-subunit contact network within RNA polymerase II. Solid lines represent subunit-subunit contacts detected by both far-Western blotting and cross-linking while dotted lines represent subunit-subunit contacts detected by only one of the two methods. Subunits 4 and 9 are not included in the *S. pombe* RNA polymerase. RNA polymerases I, II and III share five common subunits marked by star. The numbers in parentheses represent amino acid residues for each subunit.

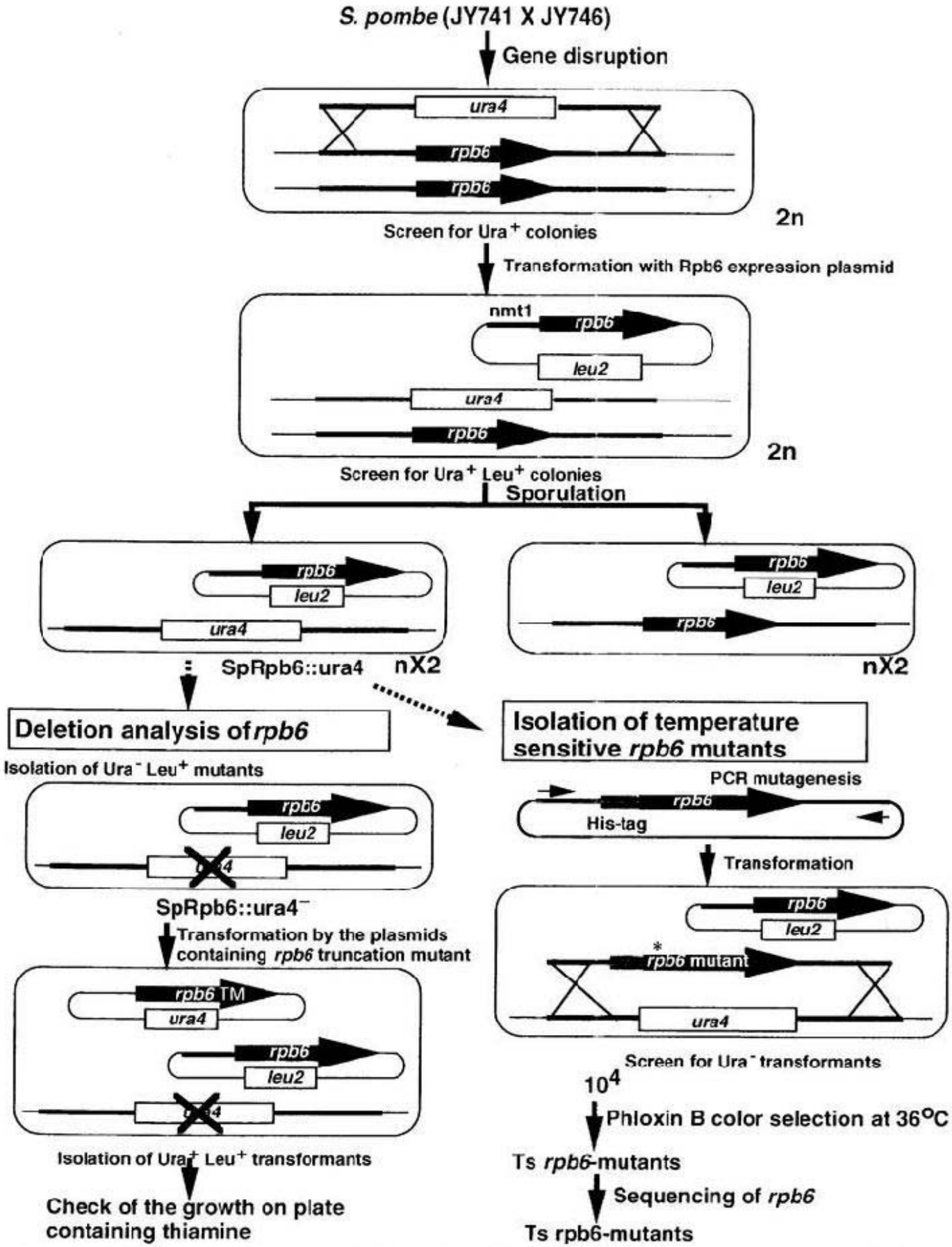

Figure 7. Genetic manupulations of the *S. pombe rpb6* gene. The *rpb6* disruptant was constucted by homologous recombination after transformation of plasmid pRpb6::ura4 into diploid *S. pombe* (JY741 X JY746) *ura4- leu1-*, and screening for Ura+ transformants. The haploid containing the *ura4+* allele was used for the functional analysis of various extents of the *rpb6* deletion and the generation of temperature-sensitive *rpb6* mutants. Details are described in Materials and Methods.

Mutant	Size	Viability	
		Thiamine(-)	Thiamine(+)
WT	1 – 142	+	+
NTM-40	41 – 142	+	+
NTM-50	51 – 142	+	+
NTM-60	61 – 142	+	+
NTM-70	71 – 142	+	-
CTM-3	1 – 139	+	+
CTM-6	1 – 136	+	-
TM-61-139	61 – 139	+	-

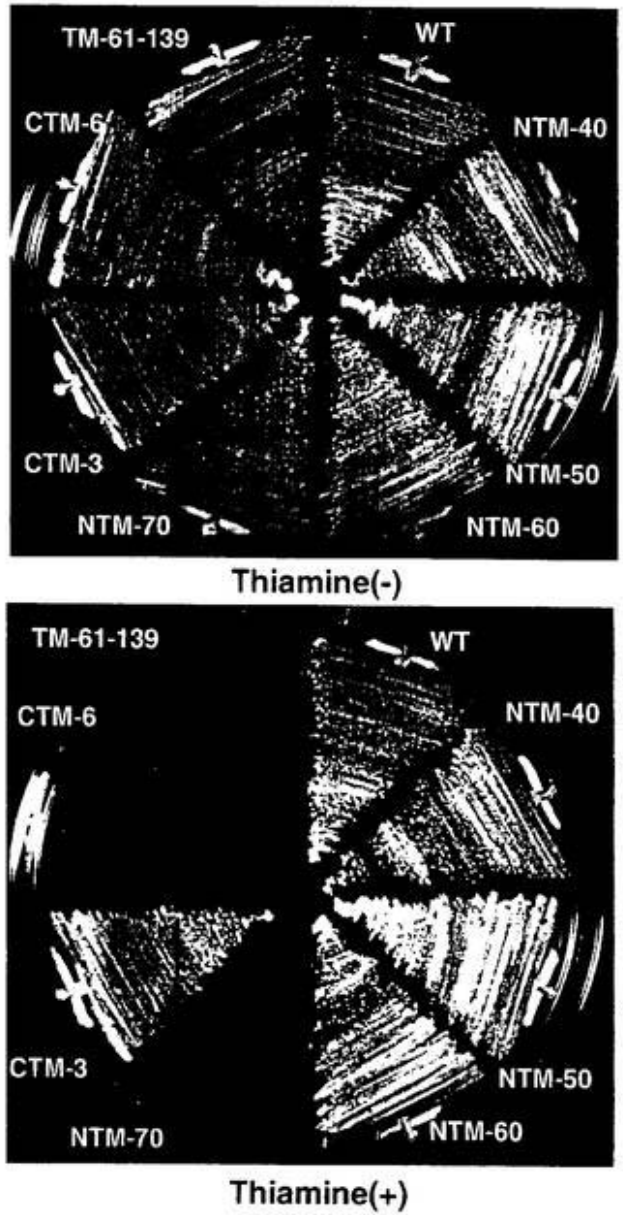

Figure 8. Functional analysis of the truncated mutants of Rpb6. A set of the expresion plasmids for N- and C-terminal deletion mutants of *rpb6* was constructed using vector pAI-ARS and transformed into *S. pombe* SpRpb6::ura4- containing pREP81-Rpb6 (see Table 1 for plasmids). In the absence of thiamine, the intact Rpb6 is expressed from pREP81-Rpb6 (agar plate, upper panel) while its expression is turned off by the addition of thiamine (agar plate, lower panel). The functional integrity of truncated Rpb6 mutants could be tested in the absence of intact Rpb6.

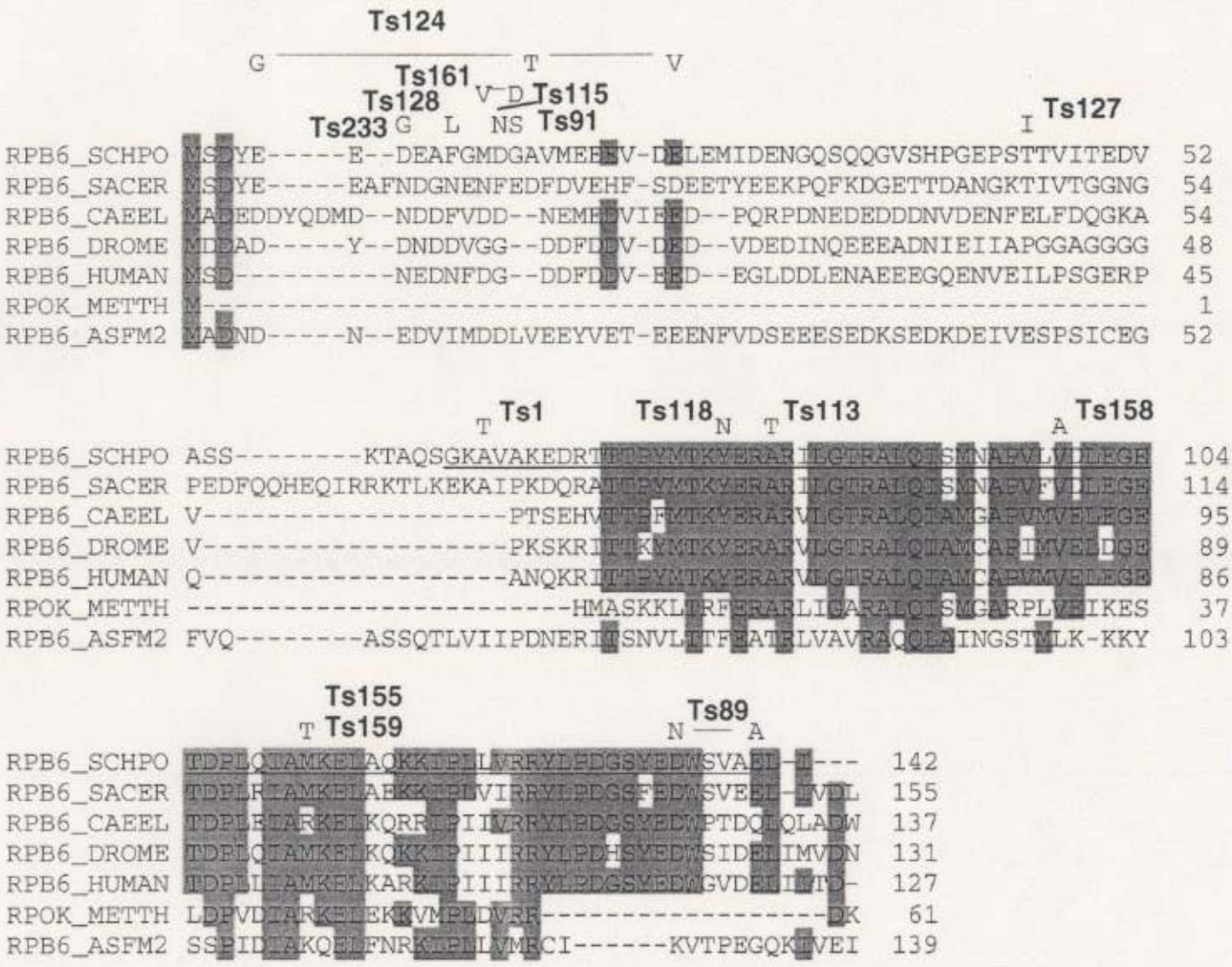

Figure9. Sequence of the *rpb6* gene from the temperature-sensitive *rpb6* mutants. The *rpb6* gene was PCR-amplified from total DNA of all 14 temperature-sensitive *rpb6* mutants isolated in this study, and sequenced. The positions of mutations are shown along the *S. pombe rpb6* gene together with those of all the known *rpb6* homologues (SACER = *Saccharomyces cerevisiae*; CAEEL, ; DROME = *Drosophilla* melanogaster; HUMAN = *Homo* sapiens; METTH = *Methanococcus* ; ASFM2 = African swine fever virus gene 2). The conserved sequences among 4 species of the *rpb6* homologues are shaded.

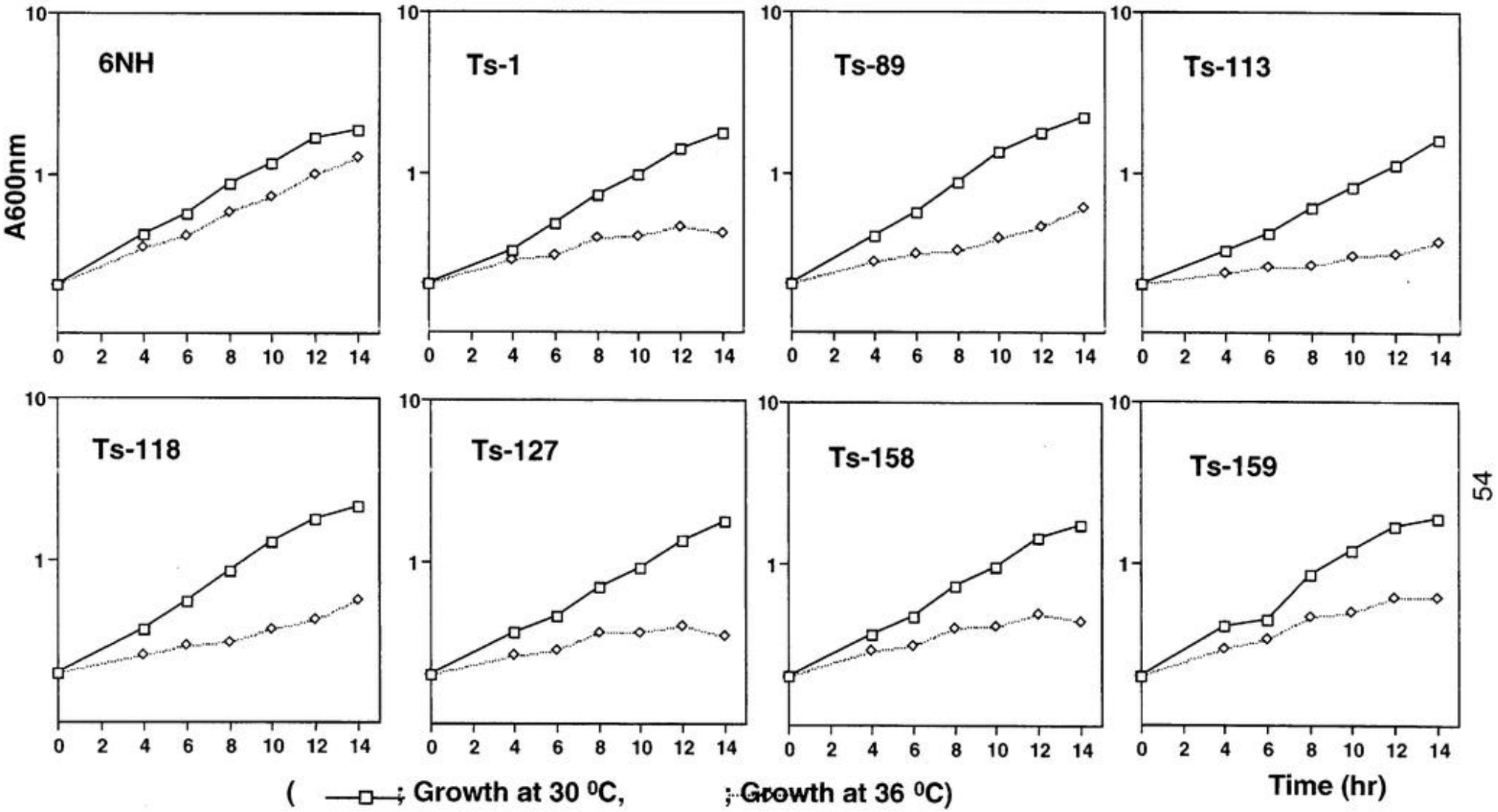

Figure 10. Growth curve of the temperature-sensitive *rpb6* mutants. Seven species of the representative Ts *rpb6* mutants were, together with the wild-type parental strain 6NH, were grown in a liquid medium at 30 °C. After dilution in to a fresh medium, the growth was continued at both 30 and 36 °C. Growth was monitored by measuring the cell turbidity at 600 nm.

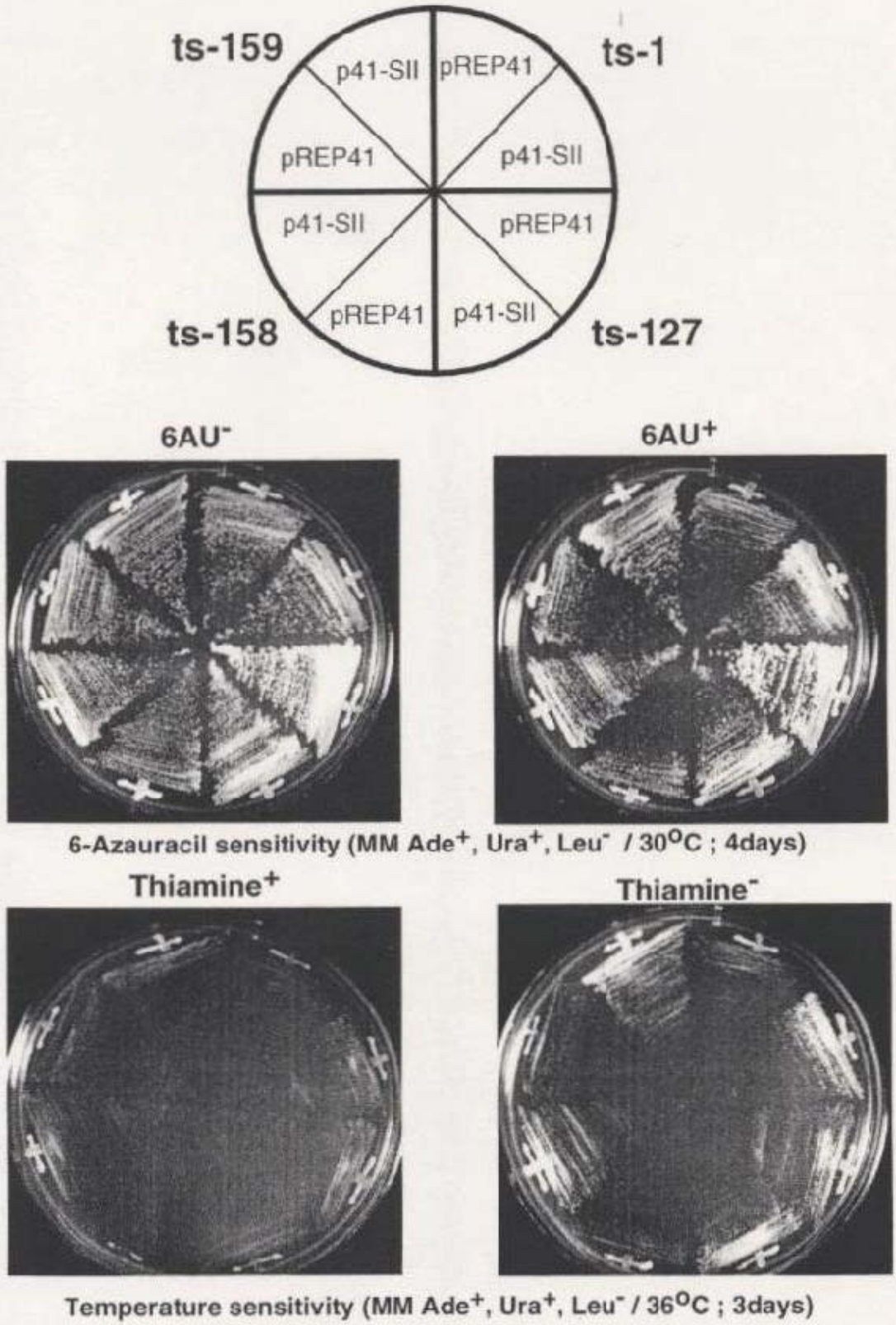

Figure 11. Multi-copy suppression of the sensitivities of *rpb6* mutants to 6-azauracil and high temperature by introduction of a TFIIS expression plasmid. Three *rpb6* mutantsshowing 6AU-sensitive and Ts phenotypes (Ts1, Ts158 and Ts159) and one 6AU-insensitive Ts mutant (Ts1 27) were transformed into *S. pombe* carrying either TFIIS-expression plasmid pREP41-TFIIS or conrol plasmid pREP41. The transformants were growtn on plate with or without 6AU, and at either 30 or 36 °C.

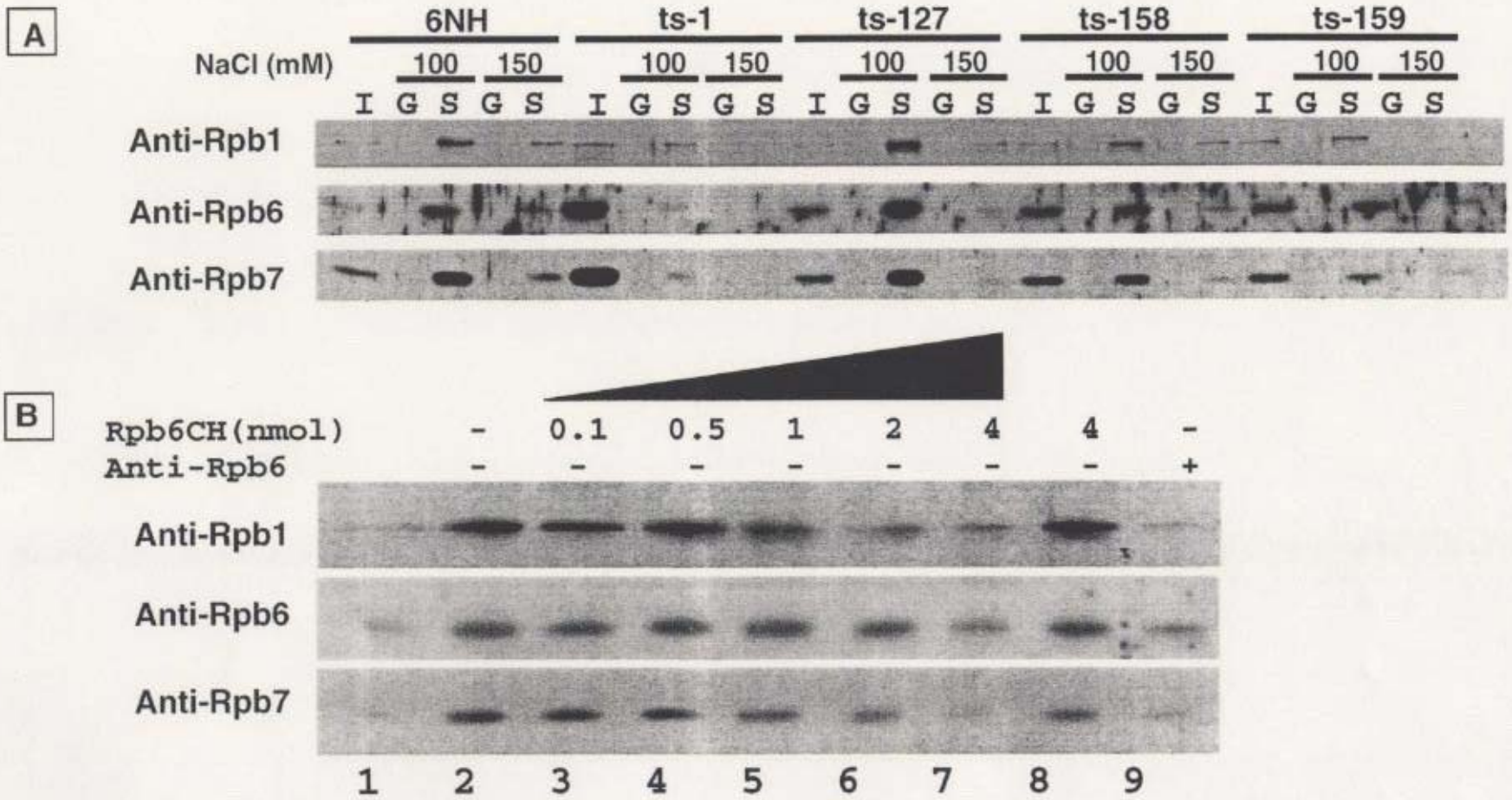

Figure 12. GST pull-down assay of the mutant RNA polymerase II. [A] Crude extracts of four *rpb6 S. pombe* mutants were mixed *in vitro* with the purified GST-TSIIS fusion protein. Complexes formed in the presence of 100 or 150 mM NaCl were isolated by using glutathione-Sepharose beads, and separated by SDS-12.5% PAGE, followed by Western blot analysis using anti-Rpb1, anti-Rpb6 and anti-Rpb7 antibodies. Lanes I, crude extrancts; lanes G, the eluted fractions from GST-conjugated beads; S, the eluted fractions from the GST-TFIIS fusion protein-conjugated beads. [B] Competition assay of TFIIS complex formation. Purified GST-TFIIS was treated for 120 min with increasing amounts of the purified Rpb6CH protein (lanes 3 to 7, 0.1 to 4 nmol of recombinant Rpb6) prior to the addition of crude extracts. The amounts of RNA polymerase II-GST-TFIIS complexes bound to the affinity column were measured by Western blot analysis using anti-Rpb1, anti-Rpb6 and anti-Rpb7 antibodies. Lane 8, 4 μmol of Rpb6 was added after 60 min incubation with Rpb6CH. Lane 9, the cell extract was treated for 30 min with anti-Rpb6 antibody prior to the competition assay. The bound proteins were separated by 12.5% PAGE. After blotting to nitrocellurose memblane, Western blotting was carried out using polycronal anti bodies, anti-Rpb1, anti-Rpb6 and anti-Rpb7, and detected by ECL Western blotting detection reagents (Amersham Pharmacia) as described Ishiguro *et al.* (1998).

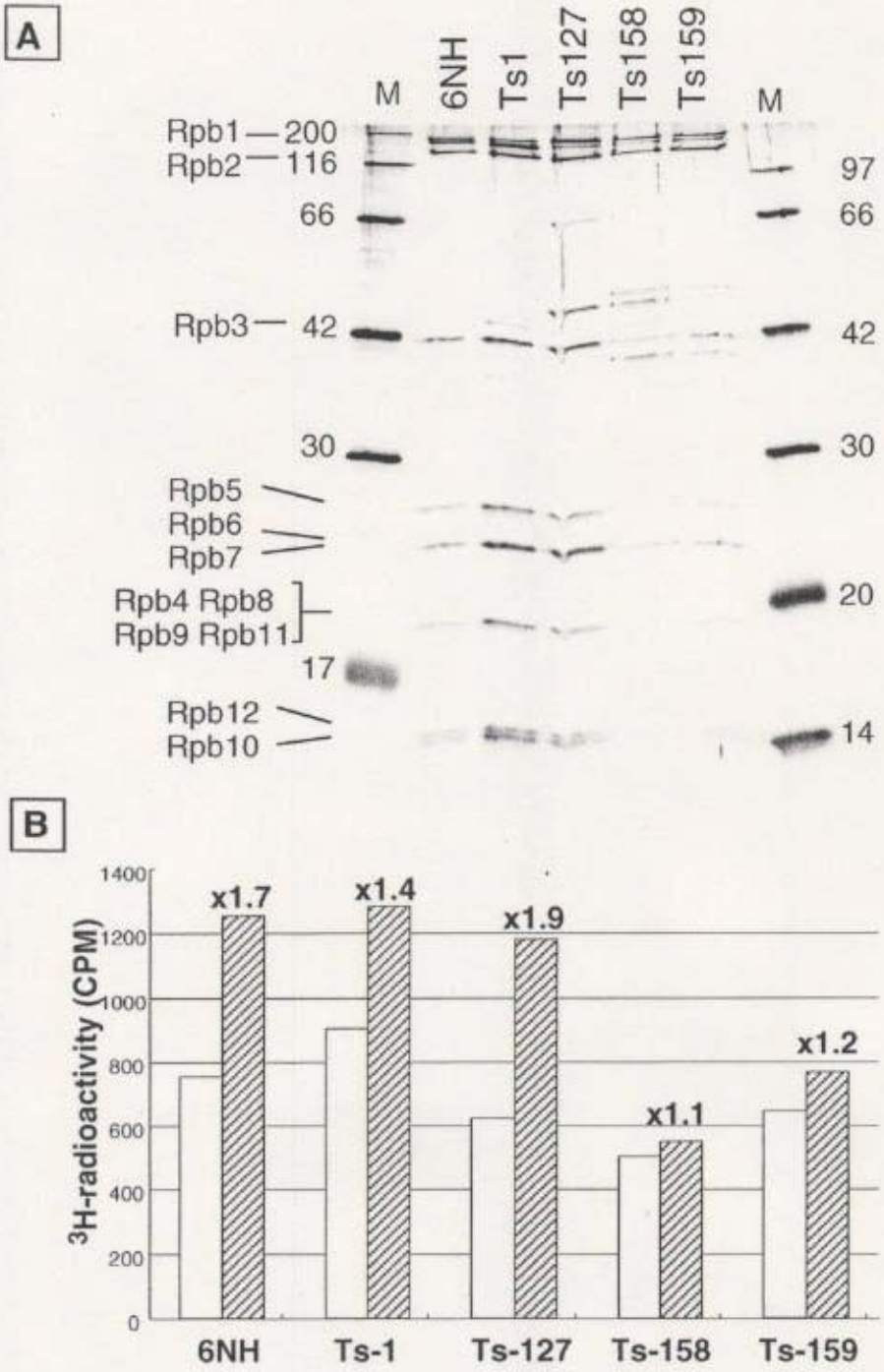

Figure 13. Transcription stimulation by TFIIS of wild-type and mutant RNA polymerase II. Non-specific RNA synthesis was carried out using the partially purified RNA polymerase II from wild-type (6NH) and four species of Ts *rpb6* mutants (Ts-1, Ts-127, Ts-158, Ts-159). The reaction mixtures and the reaction conditions were as described in Materials and Methods.