Location of Subunit−Subunit Contact Sites on RNA Polymerase II Subunit 3 from the Fission Yeast *Schizosaccharomyces pombe*†

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ABSTRACT: RNA polymerase II from the fission yeast *Schizosaccharomyces pombe* consists of 10 putative subunits. Subunit 3 (Rpb3) is a homologue of prokaryotic α subunit, which plays a key role in the assembly of core enzyme subunits. Previously we indicated that Rpb3 also plays an essential role in subunit assembly because it interacts with at least four subunits, two large subunits (Rpb1 and Rpb2) and two medium-sized subunits (Rpb3 and Rpb5) (*1*), and it constitutes a core subassembly consisting of Rpb2, Rpb3, and Rpb11 (*2*). Using a synthetic mixture of equimolar amounts of individual subunits, which were all purified from cDNA-expressed *Escherichia coli*, we found here that Rpb3 also interacts with Rpb11, another α homologue. By making a set of Rpb3 deletion derivatives, we carried out mapping of the Rpb5- and Rpb11-contact sites on Rpb3. By far-Western blot and GST pull-down assays, we found that the amino acid sequence between residues 105−263 of Rpb3 is involved in binding Rpb5, and the sequence between residues 105−297 is required for binding Rpb11. Although the Rpb5- and Rpb11-contact sites on Rpb3 overlap each other, both subunits are able to associate with Rpb3 simultaneously. The binding of Rpb5 stabilizes the Rpb3−Rpb11 heterodimer.

Eukaryotes contain three forms of RNA polymerase that differ from each other in both structure and function. RNA polymerases I, II, and III are responsible for the synthesis of rRNAs, mRNAs, and small rRNAs and tRNAs, respectively (reviewed in refs *3*−*6*). All three RNA polymerases are multi-subunit enzymes, each consisting of more than 10 different polypeptides, of which some are shared between two or three forms of the RNA polymerase. At present, however, the functional role of each putative subunit remains largely unknown. For instance, the minimum and essential subunits required for the catalytic function of RNA synthesis have not yet been determined.

The RNA polymerase II from the budding yeast *Saccharomyces cerevisiae* is composed of 12 putative subunits (*4*, *6*), whereas the purified RNA polymerase II from the fission yeast *S. pombe* contains 10 polypeptides, lacking components corresponding to subunits 4 and 9 of *S. cerevisiae* (*2*, *7*). The genes coding for all 12 putative subunits of the *S. cerevisiae* RNA polymerase II have been cloned and sequenced (*6*). On the other hand, we and another group have cloned and sequenced the genes and cDNAs encoding all 10 putative subunits of *S. pombe* RNA polymerase II (*1*, *8*−*14*) [the 10 polypeptides associated with the purified *S. pombe* RNA polymerase II are hereafter designated as "subunits"].

The core enzyme of prokaryotic RNA polymerases is composed of three subunits, α, *β* and *β*′, and is assembled sequentially under the order: $2\alpha \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (premature core) → *E* (active core) (reviewed in ref *15*). The α subunit plays a key role in this assembly pathway, by providing the contact surface for both *β*′ and *β* subunits. Among the RNA polymerase II subunits, both subunits 3 (Rpb3) and 11 (Rpb11) contain sequences similar to parts of the prokaryotic α subunits (*10*) and are considered to form the core assembly of RNA polymerase II. Studies with *S. cerevisiae* RNA polymerase II have suggested that subunit 3 (RPB3) forms a homodimer and interacts with subunits 1 (RPB1) and 2 (RPB2), eukaryotic homologues of prokaryotic *β*′ and *β* subunits, respectively, altogether constituting the core complex (*16*), yet some other studies suggested that Rpb3 forms a heterodimer with Rpb11 (*17*, *18*). By stepwise dissociation of the *S. pombe* RNA polymerase, we succeeded in isolating of a core subassembly, which is composed of subunits 2, 3, and 11 (*2*). One short-cut approach to elucidate the assembly mechanism of RNA polymerase II might be to identify the subunit−subunit contact network involving Rpb3. For this purpose, we analyzed in this study the interaction of Rpb3 with other RNA polymerase II subunits by far-Western blot analysis and glutathione *S*-transferase (GST[1]) pull-down assay, and furthermore carried out the mapping of subunit−subunit contact sites on Rpb3.

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[1] Abbreviations: C, carboxyl-terminus; GST, glutathione *S*-transferase; H, His tag; K, protein kinase tag; PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A; PMSF, phenylmethylsulfonylfluoride.

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EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids of RNA Polymerase II Subunits or Subunit Fragments. To make an *E. coli* expression plasmid for full-length Rpb3 fused to a peptide containing protein kinase A (PKA) recognition site and a hexa-histidine (His) tag, pETRB3 (*1*) was treated with *Nde*I and *Xho*I and the resulting *rpb3* fragment was isolated and inserted into pET21b-CK (Ishiguro, A. *et al.*, unpublished experiments) between *Nde*I and *Sal*I sites. The resulting plasmid pETRB3CKH encodes a Rpb3-CKH fusion protein (C, K, and H represent C-terminus, PKA site, and His-tag, respectively) with the sequence, complete Rpb3-DLD(linker)-KLRRASVLE(PKA site)-H_6. To construct *E. coli* expression plasmids for truncated Rpb3 segments fused to PKA recognition site sequence and His tag, truncated *rpb3* sequences were PCR-amplified using pairs of primers, each containing the corresponding boundary sequence of the truncated *rpb3* genes fused with either *Nde*I (5′-primer) or *Sma*I (3′-primer) restriction enzyme sequence. PCR products were first cloned into T-vector (Promega). *Nde*I-*Xho*I fragments were isolated from the resulting plasmids and then inserted into pET21b-CK between the *Nde*I and *Sal*I sites. The resulting plasmids encode Rpb3 fragments with the sequences of truncated Rpb3-LD(linker)-KLRRASVLE(PKA site)-H_6.

An *E. coli* expression plasmid, pGEXRB3, for GST-fused Rpb3 was constructed by Miyao *et al*. (*1*). To construct *E. coli* expression plasmids for the GST-Rpb3 fragment fusions, the truncated *rpb3* sequences were PCR-amplified using pairs of primers, each being linked with either *Nde*I site sequence (5′-primer) or a sequence including opal codon and *Sma*I site (3′-primer). PCR products were first cloned into T-vector (Promega). The *Nde*I-*Sma*I fragment was isolated from each recombinant T-vector plasmid, blunt-ended, and then inserted into pGEX-2TK (Pharmacia) at the *Sma*I site.

Construction of expression plasmids for other His-tagged subunits, Rpb5, Rpb6, Rpb7, Rpb8, Rpb10, Rpb11, and Rpb12, with or without a PKA site sequence is described elsewhere (Ishiguro, A. *et al.*, unpublished experiments).

Purification of Recombinant RNA Polymerase II Proteins. For production of *S. pombe* RNA polymerase II proteins, *E. coli* strain BL21(λDE3) and DH5 were transformed with pET21 and pGEX series expression plasmids, respectively. For expression of the proteins, the transformed cells were grown at 37 °C in LB medium containing ampicillin at 100 μg/mL. When the culture reached 40−60 Klett units, the expression was induced by adding isopropyl-β−D-thiogalactopyranoside (IPTG) to 1 mM. After incubation for 3 h, cells were harvested and stored at −80 °C until use.

Purification of the His-tagged proteins containing a PKA site was performed as follows: Frozen cells were suspended in a lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl and 1 mM EDTA) containing 1 mM phenylmethylsulfonylfluoride (PMSF). After addition of lysozyme (final, 0.3 mg/mL), the cell suspension was incubated on ice for 20 min. After addition of sodium deoxycholate (final, 0.1%), the cell suspension was incubated on ice for additional 20 min and then sonicated with a Heat-Systems sonicator. Inclusion bodies were recovered by centrifugation at 30 000 $\times$ *g* for 20 min at 4 °C, washed with a lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA, and solubilized in TG buffer (50 mM Tris-HCl [pH 8.0] and 5% glycerol) containing 10 mM β-mercaptoethanol (ME) and 6 M urea, and then centrifuged at 80 000 $\times$ *g* for 60 min at 4 °C. Solubilized proteins were applied onto a Ni^{2+}-NTA agarose (QIAGEN) column and, after washing with TG−ME−urea buffer containing 100 mM NaCl, eluted in a stepwise manner by TG−ME−urea buffer containing increasing concentrations of imidazole. The pooled His-tagged protein fraction was dialyzed successively against TGED buffer (50 mM Tris-HCl [pH 8.0], 5% glycerol, 0.1 mM EDTA, and 0.1 mM DTT) containing decreasing concentrations of urea from 5.0, 4.0, 2.5, 1.0, and 0 M, and finally against TGED buffer containing 50% glycerol for storage at −30 °C until use.

For purification of GST-fusion proteins, frozen cells were suspended in phosphate-buffered saline (PBS) containing 1 mM PMSF and sonicated. After addition of Triton X-100 to make 1%, the cell lysate was centrifuged at 30 000 $\times$ *g* for 20 min at 4 °C, and the supernatant was passed through a glutathione-Sepharose 4B column (Pharmacia). The column was washed with 100 mM Tris-HCl buffer [pH 8.0] containing 200 mM NaCl, 10% glycerol, and 0.5% NP-40, and then proteins were eluted with the same buffer containing 100 mM glutathione. Fractions containing GST-fusion proteins were pooled, dialyzed against PBS, and centrifuged at 80 000 $\times$ *g* for 1 h at 4 °C. The proteins were dialyzed against PBS containing 50% glycerol and stored at −30 °C until use. Concentrations of the purified subunit proteins were determined by the Bio-Rad protein assay kit.

Far-Western Blot Analysis. A recombinant subunit mixture was prepared by mixing equimolar amounts of purified Rpb3-CH, Rpb5-CH, Rpb6-CH, Rpb7-CKH, Rpb8-CKH, Rpb10-CKH, Rpb11-CH, and Rpb12-CKH. For far-Western blot analysis, this recombinant subunit mixture was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto nitrocellulose membranes. The membranes were incubated in HGN 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) at 4 °C for 4 h, and then treated with HGN buffer containing 5% skim milk (DIFCO) at 4 °C for 4 h. In far-Western blot experiments with the GST-Rpb5 probe, the membranes were blocked with 75 μg/mL of purified GST. After washing with HGN buffer, the membranes were incubated with 1×10^5 cpm/mL (specific activity, $(1-2) \times 10^6$ cpm/μg) of the probe proteins in HGN buffer containing 150 mM KCl. The membranes were washed with HGN buffer containing 150 mM KCl and then exposed to X-ray film or imaging plate.

^{32}P-Labeled probe proteins were prepared as follows: Proteins carrying the phosphorylation site were labeled by protein kinase A as described previously (*1*) and passed through NAP-5 column (Pharmacia) to remove free radioactive ATP.

GST Pull-Down Assay. Mixtures of equimolar amounts of one of the GST-fused truncated Rpb3 proteins and either Rpb5 or Rpb11 were incubated for 60 min on ice and, after addition of glutathione-Sepharose 4B (Pharmacia) equilibrated with 0.1% bovine serum albumin (BSA) in PBS, incubated for additional 60 min on ice. Protein-bound beads were washed with 0.5% NP-40 in PBS and then incubated for 3 min at 95 °C in SDS−PAGE sample buffer. Proteins were separated by SDS−PAGE and directly electroblotted onto poly(vinylidene difluoride) membranes (FluoroTrans

WPVDF Membrane, PULL). The membranes were soaked with 5% skim milk in PBS for 1 h at room temperature, treated with antibodies against Rpb5 or Rpb11, washed with 0.5% Tween 20 in PBS, and incubated with goat anti-rabbit immunoglobulin G antibody conjugated with hydroxyperoxidase (Cappel). The blots were developed with the enhanced chemiluminescence reagent system (ECL, Amersham).

RESULTS

Identification of Rpb3-Contact Subunits: Far-Western Blot Analysis of a Mixture of Isolated Subunits. Previously we indicated direct interactions of Rpb3 with Rpb1, Rpb2, Rpb3, and Rpb5 based on far-Western blot analyses of a purified preparation of *S. pombe* RNA polymerase II using ^{32}P-labeled Rpb3 probe (*1*). However, the identification of interaction between Rpb3 and small-sized subunits was difficult because: (i) purified RNA polymerase II preparations contain a number of degradation products, mostly derived from two large subunits, Rpb1 and Rpb2; (ii) the stoichiometry among 10 putative subunits was not constant; (iii) the purified RNA polymerase contain various levels of accessory proteins and impurities; and (iv) small-sized subunits could not be well-separated on SDS−PAGE. To overcome the ambiguities that arise from the use of purified RNA polymerase II, we constructed a synthetic mixture of equimolar amounts of eight small subunits, 3, 5, 6, 7, 8, 10, 11, and 12, which were all expressed in *E. coli* and purified to near homogeneity [two large subunits, Rpb1 and Rpb2, could not be expressed in intact forms]. For quick purification, radiolabeling, and better separation on SDS−PAGE, all these subunit polypeptides were prepared as fusion proteins with a short peptide tag, either CH (His-tag added at C-terminus) or CKH (kinase-tag for phosphorylation by protein kinase A plus His-tag both added at C-terminus).

This equimolar mixture of small-sized subunits was subjected to far-Western blot analysis using ^{32}P-labeled Rpb3 as a probe. The stained gel, shown in Figure 1, indicates that the staining intensity with Commassie brilliant blue (CBB) is different between subunits. Moreover, Rpb7 could not be detected by CBB staining and thus the migration position of Rpb7 was determined by staining with a silver stain kit (data not shown). The separation of some intact small-sized subunits was difficult on SDS−PAGE (*1, 7*), but better separation of all subunit polypeptides could be achieved by adding the different tags. For instance, intact Rpb8 and Rpb11 migrate to the same position on SDS−PAGE (*1,* 7), but these two subunits could be separated by adding the KH-tag to Rpb8 and the H-tag to Rpb11. Stained but unidentified bands above and below Rpb5 were attributed to impurities included in the Rpb5 preparation used. Far-Western blot clearly showed that Rpb3 interacts with three subunits, Rpb3, Rpb5, and Rpb11, but not with Rpb8. The interaction of Rpb3 with Rpb5, Rpb8, and Rpb11 was confirmed by far-Western blot analysis using Rpb5, Rpb8, and Rpb11 probes (see Figure 4; Ishiguro, A. *et al.*, unpublished experiments). Taken together with our previous observations (*1*) we concluded that Rpb3 interacts with two large subunits (Rpb1 and Rpb2), two medium-sized subunits (Rpb3 and Rpb5), and one small subunit (Rpb11).

Identification of Rpb5- and Rpb11-Contact Sites on Rpb3: Far-Western Blot Analysis using Rpb5 and Rpb11 Probes. To map the subunit−subunit contact sites on Rpb3 (the intact molecule, 297 amino acid residues), we carried out far-Western blot analysis for a set of truncated Rpb3. Truncation was designed based on the location of four conserved regions among subunit 3 from various eukaryotes (Figure 2). Domains A and D are conserved among α subunit homologues from all organisms including both prokaryotes and eukaryotes, whereas domains B and C exist only in eukaryotes (*10*). In this series of experiments, we prepared truncated Rpb3 segments as fusion proteins with both K-tag for labeling with ^{32}P and H-tag for quick purification by Ni^{2+}-affinity chromatography. All the Rpb3 fragments thus prepared were found to be pure, each giving a single-stained band, except Rpb3(73−297)-CKH which gave three bands (Figure 3A). From the N-terminal amino acid sequence analysis, these three bands were found to be

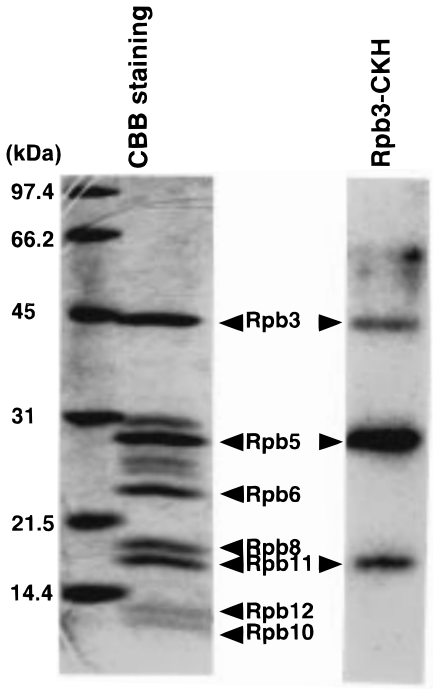

FIGURE 1: Identification of the Rpb3-binding subunits of RNA polymerase II by far-Western blot analysis. A mixture of equimolar amounts of purified recombinant subunits was fractionated by SDS−15% PAGE. One gel was stained with CBB [left] whereas proteins on the other gel were electrophoretically transferred onto a nitrocellulose membrane that was then subjected to far-Western blot analysis using ^{32}P-labeled Rpb3-CKH as a probe [right]. Rpb7 was negatively stained with CBB.

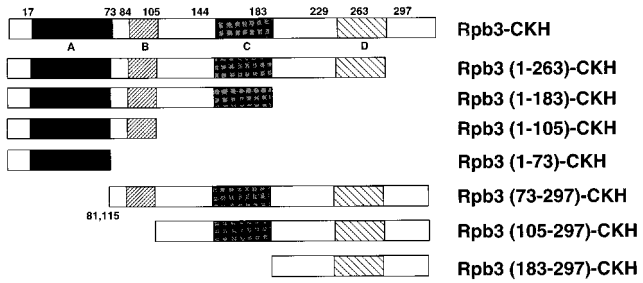

FIGURE 2: Preparation of truncated Rpb3 fragments. A set of expression plasmids was constructed, each carrying the coding sequence for Rpb3 segment fused at its C-terminus to the phsophorylation site by PKA, designated K, and the His-tag sequence, designated H. Truncation of Rpb3 was designed as to include one or more of the four conserved regions among eukryotic RNA polymerases.

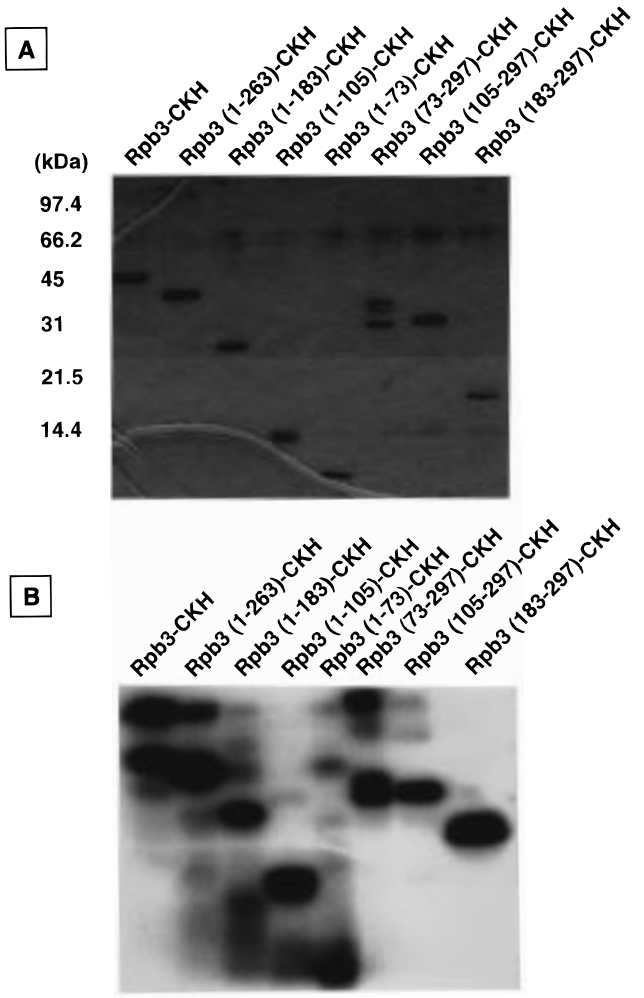

FIGURE 3: Purification of Rpb3 fragments. (A) Rpb3 fragments shown in Figure 2 were expressed in *E. coli*, purified by Ni^{2+}-agarose affinity column chromatography, and separated by SDS−15% PAGE. The gel was stained with CBB. (B) Purified Rpb3 proteins were labeled with ^{32}P. Aliquots with ∼20 000 cpm radioactivity were separated by SDS−15% PAGE, and the gel was exposed to an X-ray film.

Rpb3(73−297)-CKH and two degradation fragments Rpb3-(81−297)-CKH and Rpb3(115−297)-CKH (data not shown).

To determine the regions on Rpb3 responsible for interaction with Rpb5 and Rpb11, we carried out far-Western blot analysis for each truncated Rpb3 fragment thus prepared. ^{32}P-Labeled GST-Rpb5 bound to the full-length Rpb3-CKH and at least four truncated fragments, Rpb3(1−183)-CKH, Rpb3(1−263)-CKH, Rpb3(115−297)-CKH [the fastest migrating band included in the Rpb3(73−297)-CKH preparation], and Rpb3(105−297)-CKH (Figures 4A and B; indicated by arrows) [however, Rpb3(1−183) probe did not bind to Rpb5 (see Figure 5) and GST-fused Rpb3(1−183) did not form a binary complex with Rpb5 (see Figure 6)]. The sequence between residues 115 and 183 are included in all these Rpb3 fragments, suggesting that the Rpb5 contact site is located in this region that includes the conserved region C and the spacer between B and C regions. Failure to detect the interaction for two fragments, Rpb3(73−297) and Rpb3-(80−297), both including Rpb3(115−297) sequence, might be due to low concentrations of these fragments (see Figure 3A). Otherwise, a sequence between 73 and 114 interferes with Rpb5 binding. ^{32}P-Labeled GST without Rpb5 used as a control probe gave no binding signal (data not shown). On the other hand, ^{32}P-labeled Rpb11 probe bound to Rpb3-CKH, Rpb3(73−297)-CKH [and its cleaved fragments Rpb3-(80−297)-CKH and Rpb3(115−297)-CKH], and Rpb3(105−297)-CKH (Figures 4C and D), suggesting that Rpb11 binding requires an entire region of Rpb3 downstream from residue 115, which is wider than that required for Rpb5 binding.

Identification of Rpb5- and Rpb11-Contact Sites on Rpb3: Far-Western Blot Analysis using Rpb3 Probes. To confirm the results, we carried out far-Western blot analysis in opposite combinations; that is, blotting of Rpb5 and Rpb11 using ^{32}P-labeled Rpb3 fragments as probes. For this purpose, Rpb3 fragments were isolated as fusions with both H and K tags, and the purified Rpb3 fragments were labeled with ^{32}P. As shown in Figure 3B, labeling of these truncated Rpb3 proteins with protein kinase A gave mostly single radiolabeled bands, but in the case of full-sized Rpb3 and two truncated forms, Rpb3(1−263) and Rpb3(73−297), portions of the phosphorylated forms formed aggregates (Figure 3B). Figure 5 shows the far-Western blot analysis of increasing amounts of Rpb5 or Rpb11. ^{32}P-Labeled Rpb3-(1−263)-CKH lacking C-terminal 34 amino acid residues bound to Rpb5 but not Rpb11. Further truncation of its C-terminal region rendered Rpb3 inactive in binding with both Rpb5 and Rpb11. On the other hand, Rpb3(73−297)-CKH lacking N-terminal 72 amino acid residues and Rpb3-(105−297)-CKH lacking N-terminal 104 amino acid residues bound to both Rpb5 and Rpb11. Further deletion, however, made Rpb3 inactive in binding both Rpb5 and Rpb11. The results of Rpb3-Rpb5 and Rpb3-Rpb11 interactions using Rpb3 probes essentially agreed with those obtained using Rpb5 and Rpb11 probes (see Figure 4). Thus, we concluded that: (i) an internal fragment of Rpb3 between amino acid residue 115 and 263 is enough for binding of Rpb5; but (ii) a long C-terminal fragment from amino acid residue 115 to the C-terminus (residue 297) is needed for binding of Rpb11.

Identification of Rpb5- and Rpb11-Binding Sites on Rpb3: GST Pull-Down Assay. To further confirm the results of far-Western blot analyses, we carried out a GST pull-down assay. The GST-fused Rpb3 fragments were overexpressed in *E. coli* and purified by glutathione-Sepharose (Figure 6A; indicated by dots). In addition to the GST-fused Rpb3 fragments, a number of protein bands including GST were detected for each sample, mainly because the overexpressed and metabolically unstable Rpb3 proteins were rapidly degraded. Each of the GST-Rpb3 proteins was mixed with the purified Rpb5 or Rpb11 and after incubation, glutathione-Sepharose beads were added to the mixture. Proteins tightly bound to the beads were fractionated by SDS−PAGE and analyzed by Western blotting with antibodies against Rpb5 or Rpb11. As shown in Figure 6B, the full-sized GST-Rpb3 formed complexes with both Rpb5 and Rpb11, supporting the conclusion that Rpb3 interacts with both Rpb5 and Rpb11. Among the C-terminal truncated Rpb3 fragments, GST-Rpb3(1−263) formed complexes with Rpb5 but not with Rpb11. Further truncation of the C-terminal region made Rpb3 inactive in binding both Rpb5 and Rpb11. On the other hand, two N-terminal truncated Rpb3 fragments, GST-Rpb3(73−297) and GST-Rpb3(105−297), bound both Rpb5 and Rpb11. However, neither Rpb5

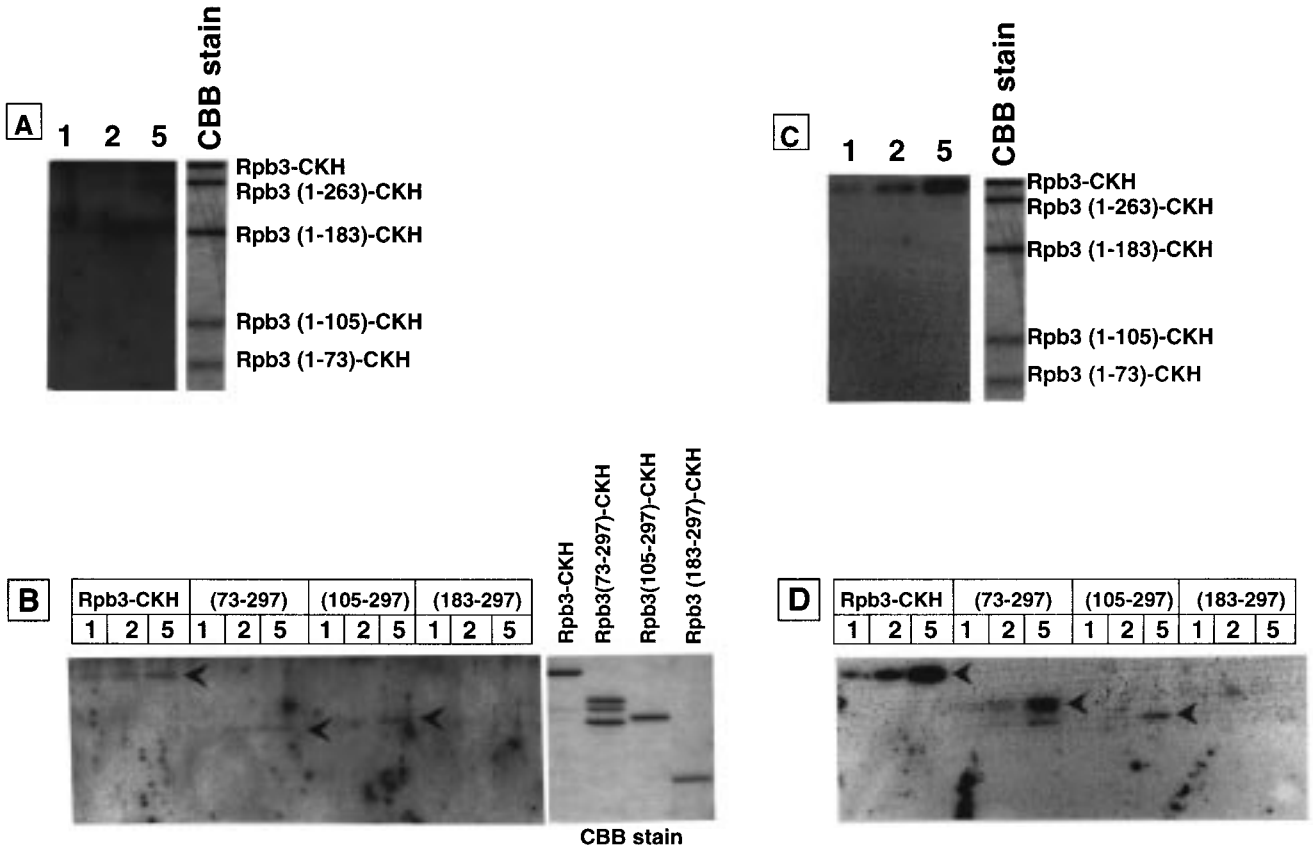

FIGURE 4: Mapping of the Rpb5- and Rpb11-contact regions on Rpb3. Far-Western blot analysis of Rpb3 fragments was carried out using either ^{32}P-labeled Rpb5 (A and B) or Rpb11 (C and D) probe. (A and C) Increasing amounts of the equimolar mixture of N-terminal Rpb3 fragments were fractionated by SDS−15% PAGE (from left to right, 1,0, 2.0 and 5.0 pmol each) and transferred onto nitrocellulose membranes. (B and D) Increasing amounts of C-terminal Rpb3 fragments were fractionated by SDS−12.5% PAGE (from left to right, 1.0, 2.0, and 5.0 pmol) and then transferred to nitrocellulose membranes. Positive bands detected by far-Western blot analysis are indicated by arrows.

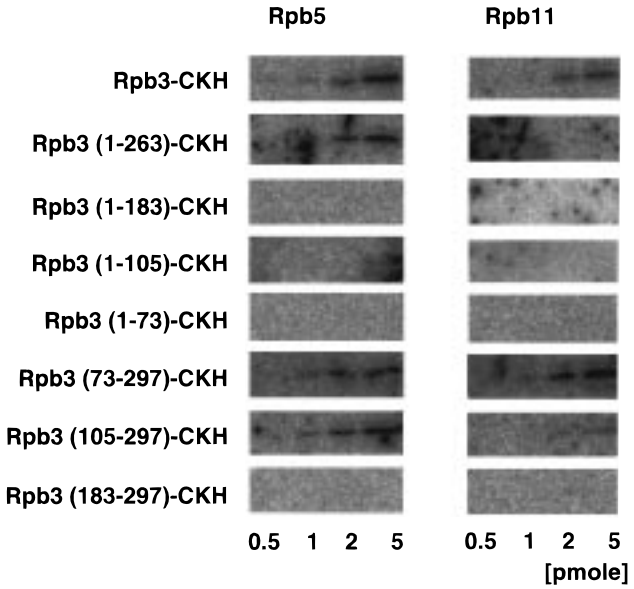

FIGURE 5: Mapping of the Rpb5- and Rpb11-contact regions on Rpb3. Increasing amounts (from left to right, 0.5, 1.0, 2.0, and 5.0 pmol) of Rpb5 (left panel) and Rpb11 (right panel) were blotted onto nitrocellulose filters, and the filters were subjected to far-Western blot analysis using the ^{32}P-labeled Rpb3-CKH probes as indicated on left.

nor Rpb11 could bind GST-Rpb3(183−297). These results of the pull-down assay in solution completely agreed with the subunit contact site map of Rpb3 obtained by far-Western blot analyses.

Stimulatory Effect of Rpb5 on Rpb11 Binding to Rpb3. The experiments just noted clearly indicated that Rpb3 interacts with both Rpb5 and Rpb11. We then examined the formation of ternary complexes by mixing three subunits. For this purpose, two series of experiments were carried out: (i) the increasing amounts of Rpb11 were added to a mixture of equimolar amounts of Rpb3 and Rpb5; and (ii) the increasing amounts of Rpb5 were added to a mixture of equimolar amounts of Rpb3 and Rpb11. In both cases, the third subunit, either Rpb11 in the first series experiments or Rpb5 in the second series experiments, was found to bind to the binary complexes. Because the binding of Rpb5 and Rpb11 to Rpb3 is not competitive with each other, the contact sites on Rpb3 are not completely overlapped between Rpb5 and Rpb11.

The formation of the Rpb3−Rpb5 complex was not influenced by the addition of Rpb11 (data not shown), whereas the level of Rpb3-bound Rpb11 significantly increased in the simultaneous presence of Rpb5 in a concentration-dependent manner (Figure 7). Because Rpb3 and Rpb11 bind to Rpb2 and form the core subassembly (*2*), this finding suggests that the Rpb3−Rpb11 interaction in the core subassembly is stabilized by the association of Rpb5.

DISCUSSION

The establishment of an *in vitro* reconstitution system of RNA polymerase II from isolated wild-type and mutant subunits is a short-cut way for identification of the functional

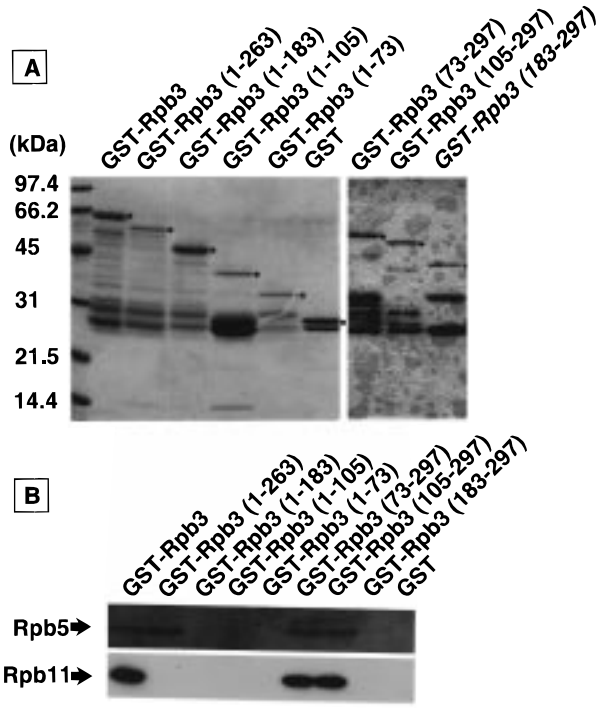

FIGURE 6: Mapping of the Rpb5- and Rpb11-contact regions on Rpb3. The GST pull-down assay was carried out using purified GST-fused Rpb3 fragments. (A) Overexpressed and purified GST-Rpb3 fragment fusion proteins were separated by SDS−12.5% PAGE and stained with CBB. (B) Aliquots of 100 pmol each of the purified GST-Rpb3 proteins were mixed with 100 pmol of either Rpb5 or Rpb11. Glutathione-Sepharose 4B beads were then added, and proteins bound to the beads were analyzed by SDS−12.5% PAGE. Gels were subjected to immunoblot analysis using antibodies against Rpb5 or Rpb11. Immunostained bands are indicated by dots.

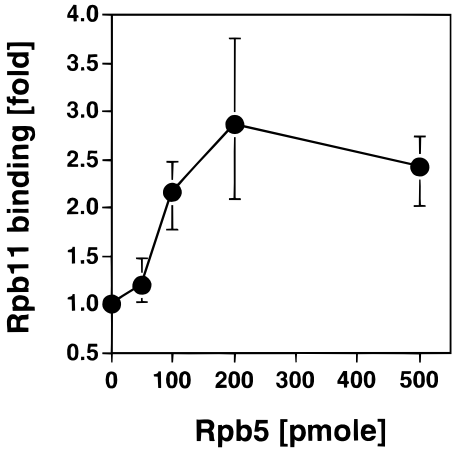

FIGURE 7: Effect of Rpb5 on Rpb3-Rpb11 interaction. Purified GST-Rpb3 proteins (100 pmol each) were mixed with 100 pmol of Rpb11 in the presence of indicated of Rpb5. After 60 min on ice, glutathione-Sepharose 4B was added and proteins bound to the beads were separated by 12.5% SDS−PAGE. Gels were subjected to immunoblot analysis using antibodies against Rpb11. The band intensities were measured with a PDI image analyzer with a white light scanner and are shown as the relative values to that obtained in the absence of Rpb11. The values represent the average of three independent experiments.

role of each putative subunit. Our attempts of reconstitution by mixing all 10 subunits of *S. pombe* RNA polymerase II have so far been unsuccessful. Toward this end we have carried out analyses of the subunit−subunit interaction network within the RNA polymerase II from *S. pombe* using various methods such as far-Western blot analysis (*1*; this paper), pull-down assay of binary subunit complexes (*1*; this paper), subunit−subunit cross-linking with chemical cross-linkers (Ishiguro, A. *et al*., unpublished experiments), stepwise dissociation with protein denaturants (*2*), and yeast two-hybrid assay between subunit pairs (Miyao, T. *et al*., unpublished experiments). In the far-Western blot analysis of a purified RNA polymerase II using ^{32}P-labeled Rpb3, and Rpb5 probes (*1*), we found that: (i) Rpb3 interacts with Rpb1, Rpb2, Rpb3, and Rpb5, and (ii) Rpb5 interacts with Rpb1, Rpb2, Rpb3 and Rpb5. In this paper, we confirmed the results by far-Western blot analysis of a synthetic mixture of equimolar amounts of subunits that are expressed in *E. coli* and purified to apparent homogeneity. With use of this subunit mixture, we could overcome background noises of far-Western blot, which arose from the presence of degradation products of subunits, subunit−associated proteins, and impurities in purified RNA polymerase II preparations. In addition, by adding different tags, we succeeded in separating all the small-sized subunits on SDS−PAGE. As a result, we identified that Rpb3 interacts with Rpb11 in addition to Rpb1, Rpb2, Rpb3, and Rpb5 (Figures 1 and 5).

Protein−protein contact signals detected by far-Western blot analysis are sometimes attributed to artifacts from protein denaturation. The results of Rpb3−Rpb5 and Rpb3−Rpb11 contacts obtained by far-Western blot analyses were, however, confirmed by the GST pull-down assay of subunit−subunit binary complex formation in solution. In the pull-down assay, however, we added a GST tag to one of the test subunits. Thus, it is necessary to show that GST-fused subunits remain biologically functional with respect to subunit assembly. In the case of Rpb3, we showed that the wild-type *rpb3* gene on the *S. pombe* chromosome could be replaced by a mutant gene encoding Rpb3 fused to His-tag (*2*).

Rpb3 is suggested to interact with a number of subunits, including Rpb1, Rpb2, Rpb5, Rpb11, and Rpb3 itself. In the case of bacterial RNA polymerases, two molecules of the α subunit exist, each interacting with one of the two large subunits, β and β' (*15*). If this were the case for the RNA polymerase II, each Rpb3 should not necessarily interact with all these subunits together. However, disagreement exists with respect to the copy number of Rpb3 in eukaryotic RNA polymerase II. Kolodziej and Young (*19*) reported that *S. cerevisiae* RPB3 forms a homodimer that might subsequently interact with one molecule each of two large subunits, RPB1 and RPB2, as in the case of prokaryotic RNA polymerase (*15*). In agreement with this finding, it was estimated from the results of SDS−PAGE that *S. cerevisiae* (*16*) and *S. pombe* (*10*) RNA polymerase II contain two copies of subunit 3. Recently, however, the formation in vitro of a heterodimer between Rpb3 and Rpb11 was suggested for RNA polymerase II from *S. cerevisiae*, *Arabidopsis thaliana*, and mouse (*17*, *18*, *20*). The formation in vivo of heterodimers was also supported using a yeast two-hybrid system (*17*, *21*). Here, we found that *S. pombe* Rpb3 interacts with not only Rpb11 but also Rpb3 by far-Western blot analysis even though Rpb3−Rpb3 interaction was weaker than Rpb3−Rpb11 interaction (Figure 1). The signal of homodimer formation in far-Western blot analysis is, however, often weaker than that of heterodimer formation, presumably because blotted proteins should form self-aggregates on membranes. Thus, it still remains unsolved

whether eukaryotic RNA polymerase II also contains two molecules of subunit 3. The possibility that a single molecule of subunit 3 binds both subunits 3 and 11 remains to be examined. It should also be noted that the subunit−subunit contacts observed between two isolated subunits do not necessarily indicate that the two subunits are in close contact even after the final step of assembly, because the subunit arrangement in certain multi-subunit protein complexes changes in the pathway of subunit assembly. For instance, the distance between two α subunits of *E. coli* RNA polymerase changes depending on the pathway of subunit assembly (*15*).

In agreement with the Rpb3−Rpb11 heterodimer formation, Rpb11 also has a limited amino acid sequence homology with the α subunits of prokaryotic RNA polymerases. The short segments of amino acid sequence present in both subunits 3 and 11 have since been referred to as α-motifs (*5*, *21*). In subunit 3, the α-motifs exist in the conserved regions A and D. Mutant studies of *E. coli* α subunit have shown that the regions A and D including the α-motifs play essential roles in the enzyme assembly (*22*−*25*). In the case of *E. coli* α subunits, the four conserved regions A−D are all included in the amino-terminal domain with the activity of core enzyme assembly (*26*). Thus, the carboxy-terminal regulatory domain for interaction with DNA UP elements and class-I transcription factors is not necessary for RNA polymerase assembly (*27*), and is missing in subunit 3 of eukaryotic RNA polymerase II (*10*). The sites essential for β-subunit binding are located on the A region whereas the sites for β'-subunit binding are located on the D region (*22*−*25*). The dimerization of α subunits involves mutltiple sites in the amino-terminal assembly domain as indicated by mutant studies (*22*−*25*) but the major contact sites for homodimer formation are located within the region A as analyzed by contact-dependent protein cleavage with α-conjugated Fe-EDTA (*28*). Here we demonstrated that the entire sequence of *S. pombe* Rpb3 downstream from residue 105 is necessary for Rpb11 binding but the region A [residue 17−73 (*10*)] is not necessary for interaction with both Rpb5 and Rpb11. In the case of *Arabidopsis thaliana* α-like large subunit, however, all four conserved regions including the region A has been proposed to be necessary for binding of α-like small subunit as analyzed by complex formation of *in vitro* co-translated proteins (*17*). This apparent disagreement might be due to the difference in detection method of subunit−subunit interactions. We detected subunit−subunit complex formation by both far-Western blot and GST pull-down assays using high concentrations of subunit proteins. Under those conditions, protein complexes involving weaker interactions could be detected.

The region B of subunit 3 contains a putative metal-binding sequence, $CXCX_3CX_2C$, whereas the region D includes a leucine zipper motif (*10*). The regions B and C exist only in eukaryotic RNA polymerases and thus are considered to be involved in functions specific for eukaryotes (10). The amino acid sequence 105−263 of Rpb3 is required for Rpb5 binding and the sequence 105−297 is needed for Rpb11 binding, both including the regions C and D. Because both Rpb5 and Rpb11 are the eukaryote-specific subunits, one possibility is that the motif C plays a role in contact with Rpb5 and/or Rpb11. Since both Rpb5 and Rpb11 can simultaneously interact with Rpb3, the sequence 264−297 downstream from the region D must be important for binding Rpb11.

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