RNA Polymerase II Subunits 2, 3, and 11 Form a Core Subassembly with DNA Binding Activity*

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RNA polymerase II purified from the fission yeast Schizosaccharomyces pombe consists of 10 species of subunit polypeptide. We introduced a histidine cluster tag sequence into the chromosomal rpb1 and rpb3 genes, which encode subunit 1 (Rpb1) and subunit 3 (Rpb3), respectively, and purified the RNA polymerase by Ni²⁺ affinity chromatography. After stepwise dissociation of the Rpb1- and Rpb3-tagged RNA polymerases fixed on Ni²⁺-resin by increasing concentrations of urea or guanidium hydrochloride, Rpb2-Rpb3-Rpb11 or Rpb2-Rpb3-Rpb11-Rpb10 complexes were obtained. Since the complex consisting of Rpb2, Rpb3, and Rpb11 cannot be dissociated even after treatment with 6 M urea buffer, we propose that this complex represents a core subassembly of the RNA polymerase II, analogous to the αβ complex in the assembly of Escherichia coli RNA polymerase. Both the Rpb2-Rpb3-Rpb11 complex and the free Rpb1 protein showed DNA binding activity, although the affinity was weaker compared with the intact RNA polymerase.

Eukaryotic nuclear RNA polymerases are all multisubunit enzymes consisting of some 10 species of polypeptide. Among three types of nuclear RNA polymerase, RNA polymerase II (pol II) is involved in the synthesis of mRNA and thus plays a central role in gene transcription. The genes coding for all the putative subunits have been cloned and sequenced for three organisms, human, the budding yeast Saccharomyces cerevisiae, and the fission yeast Schizosaccharomyces pombe; but for all these enzymes, little is known about the function(s) of each polypeptide.

DNA blotting, ultraviolet cross-linking, and antibody inhibition studies indicated the involvement of subunit 1, the homologue of the E. coli RNA polymerase β′ subunit, in DNA binding (1–5), whereas subunit 2, the homologue of the E. coli β subunit, has binding sites for nucleotide substrates (6–9) and DNA (10, 11). S. cerevisiae pol II subunit 3 (RPB3) and the corresponding subunits in other organisms have a limited sequence similarity to the corresponding subunits from other organisms have a limited sequence similarity to the corresponding subunits from other organisms (12, 13). The α-like sequence also exists in S. cerevisiae RPB11 and the corresponding subunits from other eukaryotes (14–16). The RPB3 and RPB11 homologues of Arabidopsis thaliana pol II associate in vitro with each other (15). Molecular interaction between S. cerevisiae AC40 and AC19, which are RPB3 and RPB11 homologues present in both RNA polymerases I and III, was also suggested by genetic analysis (17).

pol II purified from S. pombe contains 10 species of subunit (18), named Rpb1 to Rpb12 after the S. cerevisiae subunit nomenclature (19), lacking two subunits corresponding to RPB4 and RPB9 of S. cerevisiae pol II. The S. pombe genes coding for all 10 subunits have been cloned and sequenced (16, 20–25). In the case of S. cerevisiae pol II, RPB4 and RPB7 are readily dissociated from the enzyme and form a heterodimer with transcription initiation stimulatory activity (12, 26), whereas purified S. pombe pol II contains only Rpb7 and lacks Rpb4 (16, 18). Using expressed and purified subunit proteins, we have identified the subunit-subunit contact network involving Rpb1, Rpb2, Rpb3, and Rpb5 (23). Analysis of the subunit-subunit contact network has been extended to include other subunits. In parallel, we initiated analysis of the subunit assembly mechanism. Here, we show the biochemical isolation of two forms of core subassemblies, one containing Rpb2, Rpb3, and Rpb11 and the other containing Rpb10 in addition to these three core subunits. We also show DNA binding activity for free Rpb1 and the Rpb2-Rpb3-Rpb11 complex in aqueous solution.

EXPERIMENTAL PROCEDURES

Plasmids Used for Gene Replacement—Plasmid pBS-rpb1H-ura4 (see Fig. 1A) was used to construct a His-tagged rpb1 strain of S. pombe. This plasmid was constructed using the previously reported rpb1 gene clone (20) as follows. Oligonucleotide primers for polymerase chain reaction were synthesized to generate a NheI site and an ApaI site downstream of the termination codon. By ligating three polymerase chain reaction-amplified fragments, each corresponding to the KpnI-ApaI, ApaI-HindIII, or HindIII-PstI region, a 1.5-kb KpnI-PstI fragment covering the rpb1 C-terminal portion and 5′-noncoding region was generated and introduced into pBluescript KS+, A 1.7-kb HindIII-HindIII fragment containing the rpb1 gene was then inserted into the HindIII site. Finally, a synthetic oligonucleotide duplex with the sequence corresponding to the 10-histidine codon sequence was inserted between the NheI and ApaI sites.

pUC-rpb3H-ura4 (see Fig. 1B) was the plasmid used to make a His-tagged rpb3 strain. The plasmid was constructed using the previously reported rpb3 gene clone (22) as follows. Oligonucleotide primers were synthesized to insert a NheI site and an ApaI site tandemly between codons 1 and 2. Two polymerase chain reaction-amplified fragments, each corresponding to the KpnI-ApaI or ApaI-EcoRI region, were ligated, and the resultant 1.3-kb KpnI-EcoRI fragment covering the 5′-noncoding region and the N-terminal portion of rpb3 was introduced into pUC18. Three fragments, a 0.5-kb PstI-KpnI fragment covering the rpb3 5′-noncoding region, a 0.5-kb EcoRI-EcoRV fragment covering the rpb3 C-terminal portion and 3′-noncoding region with a

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‡ The abbreviations used are: pol II, polymerase II; kb, kilobase; PAGE, polyacrylamide gel electrophoresis.

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The resulting ura4 (see Fig. 1) strain was constructed by a two-step method. The plasmid pUC-rpb3H-ura4 at 4 °C, proteins in the supernatant were precipitated by 60%
20% glycerol, 10 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride) and loaded onto a Ni 2
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The precipitate was dissolved in TGM buffer (50 mM Tris-HCl (pH 8.0),
washed with a 20
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agarose column equilibrated with the same buffer. The column was
covered the region from codon 1462 to the

The His-tagged pol II—The His-tagged rpb1 strain was constructed by one-step gene replacement. The plasmid pBS-rpb1H-ura4 (see Fig. 1A) was digested with Kpn1 and Pst1 and then introduced into strain JY741 (h ade6-M216 ura4-D18 leu1). ura4-

The resultant ura4-

Construction of S. pombe Carrying His-tagged Pol II—The His-tagged rpb1 strain was constructed by one-step gene replacement. The plasmid pBS-rpb1H-ura4 was digested with Kpn1 and Pst1 and then introduced into strain JY741 (h ade6-M216 ura4-D18 leu1). ura4-

Transformation of His-tagged RNA Polymerases—The strains constructed as described above were grown at 30 °C in YE (plus Ade and

Fig. 1. Plasmids used for construction of His-tagged pol II strains. A, pBS-rpb1H-ura4. A Kpn1-Pst1 fragment of rpb1 covering the region from codon 1462 to the Pst1 site in its 3'-noncoding region and containing a 10-histidine codon sequence between the Nhel and Apal sites generated at the N-terminal end of the carboxyl-terminal domain (CTD) and an ura4 gene within its 3'-noncoding region at a HindIII site was inserted into pBluescript KS' to generate pBS-rpb1H-ura4. The thick black arrow indicates the C-terminal portion of the rpb1 gene. The black line indicates the 3'-noncoding region. The white arrow indicates the ura4 gene. The plasmid was digested with Kpn1 and Pst1 and used for one-step gene replacement. B, pUC-rpb3H-ura4. A fragment of the ura4 gene from the Pst1 site in the 3'-noncoding region at the Nhel site and Apal sites generated at the N-terminal end and a fragment carrying the ura4 gene were inserted into pUC18 at the indicated positions, leading to the generation of pUC-rpb3-ura4. The thick black arrow indicates the rpb3 gene, and the black line indicates its 5'- and 3'-noncoding regions. The white arrow indicates the ura4 gene. The plasmid was digested with Kpn1 and used for two-step gene replacement. aa, amino acid; UTR, untranslated region.

linker sequence at the EcoRV terminus, and a HindIII-HindIII ura4 fragment, were inserted stepwise into the resultant plasmid. Finally, a synthetic oligonucleotide duplex with the 10-histidine codon sequence was inserted between the Nhel and Apai sites.

the same buffer containing the same concentration of denaturant resins was applied onto a Superose 12 PC 3.2/30 column at a flow rate of 30 ml/min in buffer containing 0.3 M NaCl, 0.5% Nonidet P-40, and 50 mM Tris-HCl (pH 8.0), 10% glycerol, and 0.2 ml EDTA at 4 °C using Smart System (Pharmacia Biotech Inc.). Fractions of 50 μl were collected and analyzed by SDS-PAGE.

DNA Binding Assay—HocIII-digested salmon testis DNA was dephosphorylated with alkaline phosphatase, end-labeled with T4 polynucleotide kinase in the presence of less than stoichiometric amounts of [γ-32P]ATP, and purified on a Sephadex G-25 spin column. One μg of the labeled DNA and appropriate amounts of pol II or the subassembly were mixed in the assay buffer for RNA polymerase activity and incubated at 30 °C for 10 min. In the competition assay, 5 μg of unlabeled DNA was included in addition to the labeled DNA. The mixture was filtrated through a nitrocellulose filter (Millipore HAWP00924) pre-
soaked in 20 mM Tris-HCl (pH 7.5), 0.1 mM KCl, and 0.2 mM EDTA using a vacuum apparatus and washed with the same buffer. Radioactivity on the filter was measured with a liquid scintillation counter.

RESULTS

Partial Dissociation of Pol II—To isolate not only fully assembled pol II, but also its subassemblies from S. pombe, we introduced a histidine cluster tag sequence into the chromosomal rpbl or rpb3 gene. In the case of rpbl, the tag was inserted immediately downstream of the glyceraldehyde-3-phosphate dehydrogenase (Gapl) gene, because a proteinase cleavage sequence can be inserted in this position of S. cerevisiae RPB1 (27). On the other hand, in the case of rpb3, the tag was inserted to fuse the His tag at the N terminus of Rpb3 (Fig. 1B), the site where the hemagglutinin tag could be inserted in S. cerevisiae RPB3 (12). From both of the S. pombe variants carrying the modified rpbl or rpb3 gene, pol II was effectively purified by a combination of Ni
t-nt-nitrilotriacetic acid-agarose beads, and the complexes were treated with increasing concentrations of urea or guanidium hydrochloride. After washing with the same dissociation buffer followed by soaking with the buffer without denaturants, the proteins retained on the resin were eluted with imidazole and subjected to SDS-PAGE (Fig. 2A). The nature of subunits in the eluted fractions was identified by Western blotting using specific antibodies against each subunit (Fig. 2B). Upon treatment with 2 M urea buffer, both pol II preparations remained intact without release of any subunit, and the enzymes eluted from the resin were as active as the control enzyme similarly treated with urea-free buffer. After treatment of Rpb1-tagged pol II with 4 M urea buffer, virtually all the subunits, except Rpb1 and a small amount of Rpb8, were dissociated (Fig. 2B). On the other hand, when pol II was fixed through His-tagged Rpb3, three core subunits (Rpb2, Rpb3, and Rpb11) and a reduced level of Rpb10 remained bound to the Ni
t-resin even after treatment with 4 M urea. Rpb10 was clearly detected when the gel was stained with silver (Fig. 2A, lower right panel), although it was hardly detected by Western blotting owing to the low reactivity of the anti-Rpb10 antiserum used (Fig. 2B). Since Rpb1, Rpb5, Rpb6, Rpb7, Rpb8 and Rpb12, were partially retained on the pol II complex after treatment with 3 M urea buffer (data not shown), 3 M is the critical concentration of urea for the dissociation of these subunits from the core assemblies. After treatment with 6 M urea, Rpb10 was completely dissociated, but both Rpb2 and Rpb11 still remained bound to Rpb3. Dissociation of pol II was also examined using other protein denaturants. The same core subassembly forms were obtained by treatment with 1 M guanidium hydrochloride, 2 M KSCN, or high pH (12.5) (data not shown), indicating that the core subunit complex is resistant to a variety of denaturants. However, Rpb2 and Rpb11 were dissociated from His-tagged Rpb3 by treatment with 4 M guanidium hydrochloride buffer.

Composition and Activities of Core Assemblies—Purified pol II from both S. cerevisiae and S. pombe probably contains two molecules of Rpb3 (12, 22), although there is a controversy as to its stoichiometry (28). The stoichiometry of Rpb11 was estimated to be 1 as determined from the band intensity of the stained gels. Assuming the stoichiometry of Rpb3 to be 2 in S. pombe RNA polymerase II, three possibilities exist as to the molecular composition of the minimum core subassembly: (i) a single species of complex consisting of Rpb2, Rpb11, and two Rpb3 molecules; (ii) a mixture of Rpb2-Rpb3 and Rpb3-Rpb11 complexes; and (iii) a ternary complex consisting of one molecule each of the three subunits plus a free Rpb3 molecule. To identify the nature of the core assembly, the Ntt-resin fraction was subjected to gel filtration chromatography (Fig. 3). All three core subunits eluted together in high molecular mass fractions, forming a peak at fractions 10–12 with a mean molecular mass of ~300 kDa as estimated from the elution positions of 440- and 232-kDa markers. The subassembly eluted broadly because it forms various levels of aggregates and/or it interacts weakly with the gel matrix. The apparently broader distribution of the Rpb2 subunit is due to the high staining intensity of this large protein compared with the small subunits. None of the three core subunits eluted, however, in fractions expected for the unassembled free forms. This indi-
When the core subassembly was isolated from the Rpb2-Rpb3-Rpb11 complex and the free Rpb1 or Rpb3 subunits were subjected to a filter binding assay (Fig. 4). Various amounts of tagged pol II, the subassembly, or the free subunits were incubated with radioactive double-stranded DNA fragments, and the DNA-protein complexes formed were recovered by filtration through filters. As shown in Fig. 4A, free Rpb1 and Rpb1-tagged pol II, both prepared as described for the experiment in Fig. 2, exhibited strong DNA binding activity, although the affinity of free Rpb1 was a little lower than that of pol II. The addition of unlabeled DNA to the mixture inhibited the filter binding of the labeled DNA, indicating that the observed binding is specific. As shown in Fig. 4B, the Rpb2-Rpb3-Rpb11 complex exhibited DNA binding activity, even though the activity of the complex was much weaker than that of pol II containing His-tagged Rpb3. In contrast, the free Rpb3 subunit showed virtually no DNA binding. We conclude that both Rpb1 and the Rpb2-Rpb3-Rpb11 subassembly have lower levels of DNA binding activity compared with pol II.

**DISCUSSION**

Previously, we showed subunit-subunit contact between Rpb1 and Rpb3, Rpb1 and Rpb5, Rpb2 and Rpb3, Rpb2 and Rpb5, and Rpb3 and Rpb5 by far Western blot analysis (23). In this work, we proved directly that three subunits (Rpb2, Rpb3, and Rpb11) form a core subassembly that cannot be dissociated even after treatment with high concentrations of urea. The previously identified Rpb1-Rpb3, Rpb1-Rpb5, and Rpb3-Rpb5 interactions seem to be weaker than the interactions among Rpb2, Rpb3, and Rpb11 because Rpb1 and Rpb5 were dissociated with 4 M urea buffer. Likewise, small subunits except Rpb10 and Rpb11 were dissociated even though their interactions with both or either Rpb1 or Rpb2 were detected by far Western blotting or cross-linking.4 This core subassembly with the composition of Rpb2-Rpb3-Rpb11 is reminiscent of the αβ complex of E. coli RNA polymerase, which is an intermediate subassembly in the following pathway: α → α2 → αβ → αββ′ (reviewed in Ref. 29). The first step of the assembly is dimerization of the α subunit, and the dimeric form of the α subunit plays an important role in the subsequent pathway of β and β′ subunit assembly (30–33). This finding is in good agreement with the sequence similarities between the β and Rpb2 subunits and between the α and Rpb3 subunits. Rpb11 also carries a sequence similar to part of the α subunit (14–16). These findings agree with the observation that the RPB2-RPB3 complex is formed in vivo in S. cerevisiae (13). Besides this form of core subassembly, we also isolated a complex consisting of Rpb2, Rpb3, Rpb11, and Rpb10, although the stoichiometry was <1 for Rpb10. The association of Rpb10 with the Rpb2-Rpb3-Rpb11 complex is consistent with the genetic study indicating interaction among the AC40, AC19, and ABC10β subunits in S. cerevisiae (17). The AC40 and AC19 subunits are shared between pol I and pol III and have sequence similarities to Rpb3 and Rpb11, respectively, whereas ABC10β is identical to RPB10.

In this study, we also found that both the free Rpb1 subunit and the Rpb2-Rpb3-Rpb11 complex exhibit DNA binding activity, albeit at low affinity. Again, this agrees with the case of E. coli RNA polymerase. Both the free β′ subunit and the αβ complex exhibit DNA binding activity, even though free α and β subunits are virtually inactive in DNA binding (reviewed in Ref. 29). The DNA-binding component within the core subassembly may well be Rpb2 because DNA could be cross-linked to this subunit within assembled pol II (1, 10). The DNA binding activity of individual subunits has so far been mapped by cross-linking or antibody inhibition using assembled pol II or by Southwestern blotting against subunits separated by PAGE (1–5, 10, 11). Here, we succeeded in observing the direct binding of the isolated Rpb1 protein and of the Rpb2-Rpb3-Rpb11 subassembly to DNA in aqueous solution, and thus, we could compare the DNA binding affinity among free Rpb1, the subassembly, and the pol II enzyme. As a result, it is clear that the affinity for DNA is weaker for both the Rpb1 subunit and the Rpb2-Rpb3-Rpb11 subassembly than for intact pol II, suggesting that both the Rpb1 and Rpb2 subunits in intact pol II participate in the high affinity binding of pol II to DNA.

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