

# Subunits of the *Schizosaccharomyces pombe* RNA polymerase II: Enzyme purification and structure of the subunit 3 gene

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## ABSTRACT

To improve our understanding of the structure and function of eukaryotic RNA polymerase II, we purified the enzyme from the fission yeast *Schizosaccharomyces pombe*. The highly purified RNA polymerase II contained more than eleven polypeptides. The sizes of the largest, the second-, and the third-largest polypeptides as measured by SDS-polyacrylamide gel electrophoresis were about 210, 150, and 40 kilodaltons (kDa), respectively, and are similar to those of RPB1, 2, and 3 subunits of *Saccharomyces cerevisiae* RNA polymerase II. Using the degenerated primers designed after amino acid micro-sequencing of the 40 kDa third-largest polypeptide (subunit 3), we cloned the subunit 3 gene (*rpb3*) and determined its DNA sequence. Taken together with the sequence of parts of PCR-amplified cDNA, the predicted coding sequence of *rpb3*, interrupted by two introns, was found to encode a polypeptide of 297 amino acid residues in length with a molecular weight of 34 kDa. The *S.pombe* subunit 3 contains four structural domains conserved for the  $\alpha$ -subunit family of RNA polymerase from both eukaryotes and prokaryotes. A putative leucine zipper motif was found to exist in the C-terminal proximal conserved region (domain D). Possible functions of the conserved domains are discussed.

## INTRODUCTION

Knowledge of the structure and function of RNA polymerase is essential to understand the molecular mechanisms of transcriptional regulation. In eukaryotes, three classes of nuclear RNA polymerases, I(A), II(B) and III(C), exist, each responsible for transcription of large rRNA, mRNA, and small rRNA plus tRNA, respectively (for reviews see refs. 1,2). Each class of purified RNA polymerase is composed of two large and about 10 small polypeptides (1,2).

The genes coding for the largest (subunit 1) and the second-largest (subunit 2) subunits of the three classes of RNA

polymerase have been cloned from a number of organisms (3,4; and other work cited there). Sequence analyses indicate that these subunits share notable homologies with the  $\beta'$  and  $\beta$  subunits, respectively, of prokaryotic RNA polymerases. The gene encoding the third-largest subunit (henceforth designated as subunit 3) has so far been cloned from only four organisms (5–8), but it appears that the subunit 3 has partial similarity in primary structure with the  $\alpha$  subunit of prokaryotic RNA polymerases (8). The roles of these three major subunits of eukaryotic RNA polymerases can therefore be predicted from the knowledge of the functions of *E.coli* RNA polymerase subunits: the catalytic site is located on  $\beta$ , while  $\beta'$  has non-specific binding activity to DNA; subunit  $\alpha$  links these two large subunits into core enzyme structure (reviewed in refs. 9,10) and in addition, carries the contact site with various transcription factors (reviewed in refs. 11,12). In addition to these three core enzyme subunits, prokaryotic RNA polymerase holoenzyme contains one of the multiple species of s subunit, which plays a major role in promoter recognition (13,14). In eukaryotes, low levels of homology to the s subunit were found in general transcription factor, TFIID, and RNA polymerase II subunit RPB4 (15,16). Determination of the role(s) for each subunit of eukaryotic RNA polymerases was hitherto difficult due to the complexity in structure and to the low yield of purified enzymes, but recent progress in cloning of the genes encoding RNA polymerase subunits has provided an experimental basis for detailed characterization of each subunit polypeptide.

For the molecular anatomy of eukaryotic RNA polymerase II, we have been concerned with cloning of the genes for the putative subunits of RNA polymerase II from the fission yeast *Schizosaccharomyces pombe*. We chose *S.pombe* because genetic analysis is as easy as *S.cerevisiae*, nevertheless the strategies for gene expression such as transcription initiation mechanism (17) and splicing pattern (18,19) are similar to those in higher eukaryotes. Up to the present, we have isolated the genes, *rpb1* and *rpb2*, encoding the subunit 1 and subunit 2, respectively (3,4). In this report, we describe the protein composition of purified *S.pombe* RNA polymerase II and cloning of the gene, *rpb3*, for subunit 3. By sequence analyses of both the genomic clones and

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parts of PCR-amplified cDNA, we determined the gene organization and the protein structure of the *S.pombe* RNA polymerase II subunit 3 and compared the structure with those of the corresponding subunits from other organisms.

## MATERIALS AND METHODS

### *S.pombe* strain and medium

A wild-type strain 972h<sup>-</sup> of *S.pombe* provided by M. Yamamoto (Univ. Tokyo) was used throughout this study, and was grown in YPD medium [2% yeast extract, 1% Bacto-peptone (Difco Laboratories), 2% glucose].

### Buffers and reagents

TGED buffer contained 50 mM Tris-HCl (pH 7.8 at 4°C), 25% glycerol, 0.1 mM EDTA, and 1 mM DTT. Buffer A contained 75 mM Tris-HCl (pH 7.8 at 4°C), 6% glycerol, 0.15 mM EDTA, 1.5 mM DTT, and 150 mM ammonium sulfate, while buffer B consists of 50 mM Tris-HCl (pH 7.8 at 4°C), 0.1 mM EDTA, 1 mM DTT and 200 mM ammonium sulfate. Protease inhibitor mixture contained (per liter): 100 mg benzamidine-HCl (Wako Chemicals), 10 mg chimostatin (Sigma), 10 mg aprotinin (Funakoshi), 10 mg pepstatin A (Funakoshi), 10 mg leupeptin (Funakoshi), 10 mg antipain (Sigma), 50 mg N- $\alpha$ -p-tosyl-L-lysine chloromethylketone (Sigma), 10 mg N-tosyl-L-phenylalanine chloromethylketone (Sigma), and 174 mg phenylmethanesulfonyl fluoride (PMSF) (CLABIOCHEM).  $\alpha$ -amanitin (CALBIOCHEM) was added to a non-specific transcription assay at 50  $\mu$ g/ml.

The medium used for reverse phase HPLC column chromatography was 0.1% trifluoroacetic acid (Wako Chemicals) containing either 10 or 60% acetonitrile. Electro-elution buffer of proteins from gels contained 0.125 M Tris-HCl (pH 6.8), 0.1% SDS and 5% glycerol. V8-digestion buffer contained 50 mM Tris-HCl (pH 7.8) and 0.1% SDS.

### Purification of RNA polymerase II

*S.pombe* RNA polymerase II was purified by the modified method of RNA polymerase purification from wheat germ (20). In brief, *S.pombe* was grown at 30°C with aeration to  $5 \times 10^7$  cells per ml. About 250 g of the cells was harvested by centrifugation (8,000 $\times$ g for 10 min at 4°C) from 25 l of the culture, suspended in two volumes (about 500 ml) of buffer A containing protease inhibitors, and disrupted repeatedly with Mini Lab (RANNIE) under 90 MPa pressure for each 4 min. Crude extract was obtained after removing particulate materials by centrifugation at 28,000 $\times$ g for 30 min at 4°C. One percent volume of 10% Polymin P solution was added to the crude extract and after stirring for 1 hour at 4°C, the precipitates were recovered by centrifugation at 15,000 $\times$ g for 20 min, and then extracted with 1 l of buffer B containing protease inhibitors. After centrifugation at 15,000 $\times$ g for 20 min, 36.1 g of solid ammonium sulfate per 100 ml was added to the supernatant. After stirring for 20 min at 4°C, the precipitates were collected by centrifugation (15,000 $\times$ g for 20 min) and resuspended in 200 ml of TGED containing protease inhibitors. After the ammonium sulfate concentration was reduced to 100 mM by adding TGED, the sample was centrifuged at 28,000 $\times$ g for 20 min and loaded onto a DEAE-Sephadex A-25 column (bed volume, 300 ml) equilibrated with TGED containing 50 mM ammonium sulfate and 1 mM PMSF. After washing the column with six times

volume of TGED containing 50 mM ammonium sulfate and 1 mM PMSF, proteins were eluted with a 1.8 l linear gradient of ammonium sulfate from 50 to 400 mM in TGED buffer. Each fraction was assayed for non-specific transcription activity. Pooled RNA polymerase II fractions eluted at about 150 mM ammonium sulfate were applied onto a Q Sepharose FF column (20 ml; Pharmacia), and eluted with a 100 ml linear gradient of 150–450 mM ammonium sulfate in TGED. RNA polymerase II was eluted at about 250 mM ammonium sulfate. In some experiments, RNA polymerase II was further purified by Superose 6 gel filtration column chromatography in TGED containing 100 mM ammonium sulfate.

### Non-specific transcription assay

Reaction mixture contained in 40  $\mu$ l: 50 mM Tris-HCl (pH 7.8 at 37°C), 2 mM MnCl<sub>2</sub>, 0.5 mM DTT, 50 mM ammonium sulfate, 0.5 mM each of ATP, GTP, CTP, 7  $\mu$ M UTP, 0.2  $\mu$ Ci [<sup>3</sup>H]UTP (Amersham), 3  $\mu$ l of the enzyme in TGED, and 2  $\mu$ g of heat-denatured calf thymus DNA.  $\alpha$ -amanitin was added at 50  $\mu$ g/ml. Non-specific transcription assays were carried out for 20 min at 30°C and terminated by adding 0.9 ml stop solution containing 5% trichloroacetic acid and 10 mM pyrophosphate. TCA precipitates were collected on a GF/C glass-filter, and washed three times with the stop solution and then with 100% ethanol. The filters were counted for radioactivity with a liquid scintillation spectrometer. One unit of the enzyme activity represents the incorporation of 1 nmole labeled substrate into TCA precipitates under the standard assay conditions. Protein concentration was determined according to Lowry *et al.* (21).

### Isolation of RNA polymerase II subunits

About 30  $\mu$ g of the RNA polymerase II subunit 3 was separated from other polypeptides by SDS-gel electrophoresis according to the method of Laemmli (22) using a discontinuous separation gel consisting of 15 (bottom) and 7.5% (top) polyacrylamide. Electro-elution of proteins from the gel was performed by the method of Toda and Ohashi (23). In brief, about 0.2 ml suspension of homogenized pieces of the subunit 3 gel band was incubated in 1 ml of the electro-elution sample buffer for 30 min at room temperature and then boiled for 5 min at 70°C. The gel suspension was poured into a disposable column (7 $\times$ 60 mm) sealed with dialysis tube at the bottom; after electrophoresis for 3 hours at 2 mA using the Laemmli's electrode reservoir solution (22), the subunit 3 eluted from the gel pieces was recovered in 50  $\mu$ l sample buffer in the dialysis tube. The recovery of subunit 3 was more than 70%.

### Amino acid sequencing

Proteins dialyzed against V8-digestion buffer for 2 hours at 4°C were treated with 1 mg of V8 protease per 10 mg sample protein for 12 hours at 37°C. Digested polypeptide fragments were separated by reverse phase chromatography on RPC18 column (Pharmacia). Amino acid sequences of the purified fragments were determined using an Applied Biosystem model 977 system attached on-line with HPLC model 120 protein sequencer.

### Gene cloning and sequencing

To isolate the *S.pombe* *rpb3* gene encoding the RNA polymerase II subunit 3, PCR amplification of parts of cDNA was carried out using the degenerated primers designed from the amino acid sequences. Amplified cDNA fragments of *rpb3* were cloned into

M13mp18 phage vector and sequenced. Using one of the cDNA fragments as a probe, Southern hybridization was performed, and one hybridized DNA fragment containing the entire coding region of *rpb3* was cloned into M13mp18.

### RNA analysis

Poly(A)<sup>+</sup> RNA was prepared from *S.pombe* by the standard RNA extraction procedure (24). Northern hybridization analysis was performed after denaturation of poly(A)<sup>+</sup> RNA with glyoxal and dimethyl sulfoxide (24). For primer extension analysis, primers were designed based on the sequence of genomic DNA, end-labeled at 5' termini with [ $\gamma$ -<sup>32</sup>P]ATP, and elongated using reverse transcriptase (25). To determine splice junctions and poly(A) sites, the test regions of cDNA were amplified by PCR and the PCR products were cloned into M13mp18 for sequencing.

## RESULTS AND DISCUSSION

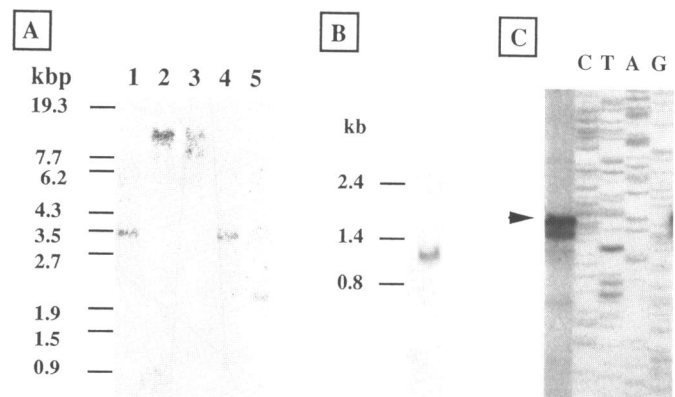
### Purification of RNA polymerase II

Cell lysates were prepared from wild-type *S.pombe* by disruption with a high pressure French press (Mini Lab). Purification of the RNA polymerase II was achieved in four steps: precipitation with Polymin P and elution from the Polymin P precipitates with ammonium sulfate solution; separation of the RNA polymerase II from other class RNA polymerases by DEAE-Sephadex column chromatography (Fig. 1A); fractionation by Q Sepharose FF column chromatography; and final-step purification by Superose 6 gel filtration. Starting from 25 l of cell culture, about 1.1 mg of RNA polymerase II was obtained at the step of Q-Sepharose FF chromatography (Table 1). Inhibition of 50% activity of the purified RNA polymerase II was achieved at 2.5  $\mu$ g/ml  $\alpha$ -amanitin. The  $\alpha$ -amanitin sensitivity was as high as that of *S.cerevisiae* RNA polymerase II (26), but was significantly lower than that of RNA polymerase II from higher eukaryotes. Substitutions of one amino acid (aa) residue in the subunit 1 of RNA polymerase II render the enzyme resistant to  $\alpha$ -amanitin (27,28). This essential aa residue affecting  $\alpha$ -amanitin sensitivity of both *S.pombe* and *S.cerevisiae* RNA polymerase II is different from that of other eukaryotic RNA polymerases (for details see ref. 3).

The highly purified *S.pombe* RNA polymerase II contained more than eleven polypeptides as analyzed by SDS-polyacrylamide gel electrophoresis. The molecular weights of these components were estimated to be 210 (subunit 1), 150 (subunit 2), 40 (subunit 3), 34, 33, 25, 20, 16, 15, 13.5 and 13 kDa (Fig. 1B), although the staining intensity of some components is different between two dyes. This observation agreed with the proposal that the *S.cerevisiae* RNA polymerase II is composed of more than 11 subunits (29). The molar stoichiometry of the three large subunits was 1:1:2 for subunit 1:2:3, as measured with a laser densitometer ULTROSAN XL (LKB) and for both silver- and Coomassie brilliant blue-stained SDS-polyacrylamide gels. After prolonged storage of the purified RNA polymerase II, the subunit 1 was partially converted to a degradation product of 190 kDa devoid of the C-terminal repeated domain (CTD).

### Sequencing of the subunit 3 and cloning of the *rpb3* gene

The *S.cerevisiae* RPB3 gene encoding the RNA polymerase subunit 3 did not cross-hybridize with the *S.pombe* gene (data not shown) as analyzed by Southern hybridization under the same



**Figure 1.** Purification of *S.pombe* RNA polymerase II. [A] Separation of three forms of RNA polymerase. Ammonium sulfate-step fraction was fractionated on a DEAE-Sephadex column (2.6 $\times$ 57 cm) at a flow rate of 200 ml/hour. Non-specific transcription assay was performed for 3  $\mu$ l aliquots under the standard reaction conditions. RNA polymerase activity, ●—●; ammonium sulfate concentration, —. Flow-through fractions contained the RNA polymerase I activity which was resistant to 50  $\mu$ g/ml  $\alpha$ -amanitin, while the RNA polymerase II activity was sensitive at 2.5  $\mu$ g/ml. [B] Polypeptide composition of the purified RNA polymerase II. The Superose 6 fraction was electrophoresed on a 15% polyacrylamide gel under denatured conditions (22) and stained with either a silver staining kit (a) or Coomassie brilliant blue (b).

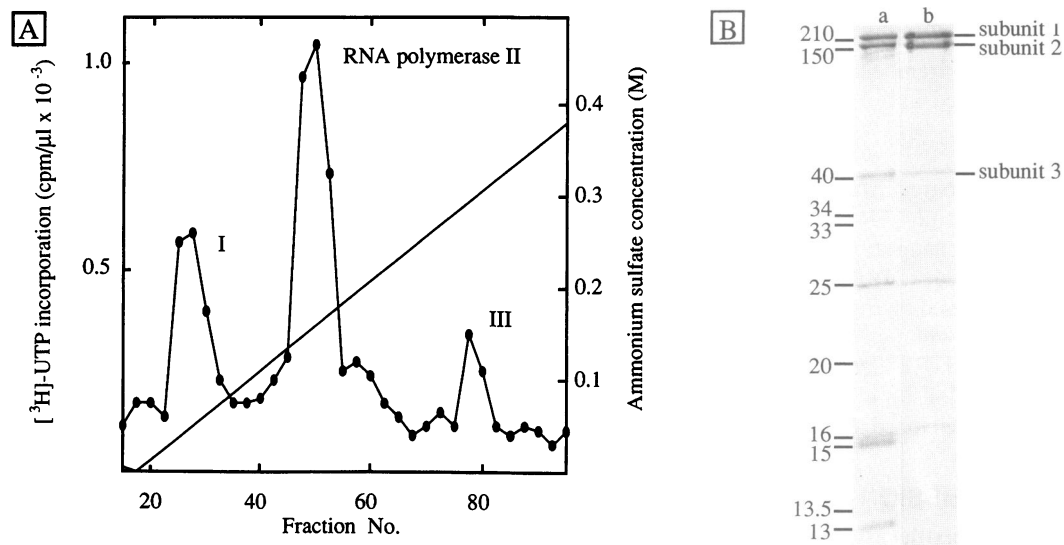
**Table 1.** Purification of RNA polymerase II from *S.pombe*

Purification step	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units/mg)	Purification (fold)
Crude extract	10600	18000	100	0.17	1.0
Polymin P eluate	2200	13000	73	0.59	3.7
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> eluate	1500	12000	68	0.80	4.9
DEAE-Sephadex	19	4900	27	25.8	154
Q-Sepharose FF	1.1	4100	23	373	2200
Superose 6	0.5	3300	18	660	3900

RNA polymerase II was purified from 250 g (wet weight) of *S.pombe*.

conditions used for cloning of the *rpb1* and *rpb2* genes (3,4). For cloning the *S.pombe* gene for the subunit 3, we then carried out aa sequence analysis of polypeptide fragments generated by limited digestion of the isolated subunit 3 with *Staphyrococcus aureus* V8 protease (the sequences determined are indicated in Fig. 2B). Two species of 20-mer oligodeoxynucleotides with the sequences of (5')ATICCIACIGTIGCIATIGA(3') and (5')(A/G)TCIA(A/G)IGGIATCATICCI(3') were designed from the aa sequences, IPTVAID and LGMIPLD. Using these oligonucleotides as primers, we carried out RT(reverse transcription)-PCR and obtained a single major product of amplified cDNA. After DNA sequence analysis, this fragment of 97 bp (F1 in Fig. 2A) was found to have one open reading frame, which is homologous in aa sequence to a part of *S.cerevisiae* RNA polymerase II RPB3 (60% identity from aa 41 to 73).

For isolation of a complete genomic clone carrying the entire subunit 3-coding sequence, total *S.pombe* DNA was digested with various restriction enzymes and analyzed by Southern hybridization using the PCR-amplified cDNA fragment F1 as a probe and under high stringency conditions (for details see ref.



**Figure 2.** Structure of the *rpb3* gene. [A] Filled and open boxes represent exons and non-coding sequences including two introns, respectively. F1 shows the 97 bp cDNA fragment amplified using the primers designed from the amino acid sequences of V8 fragments of the subunit 3. F2 was cloned using F1 as a probe, while F2 was used as a probe for cloning F3. [B] Parts of the complete nucleotide sequence of *PstI-EcoRV* F3 fragment are shown. The coding sequence of the RNA polymerase II subunit 3 starts at nt 988 and ends at nt 1966. This sequence is interrupted near the N-terminal proximal region by two introns which are shown by small letters (nt 1068–1110 and 1161–1206). The consensus sequences for the exon-intron boundaries are boxed. Both the 5' (nt 792) and 3' (nt 2043 and 2044) ends of the *rpb3* transcript are marked by shadowed boxes, and the putative poly(A) signal is shown by a double-underline. The aa sequences experimentally determined are shown by shadow boxes on the aa sequence. The primers designed from these aa sequences are indicated by waved lines. The primers # 1 (nt 877–894) and # 2 (nt 1833–1852) used in the transcript analysis are underlined. The complete nt sequence has been deposited in DNA database (D15070).

3). As shown in Fig. 3A, one major band was identified for all the restriction enzymes used. A 2.0 kbp *PstI-EcoRI* fragment (lane 5 in Fig. 3A; indicated as F2 in Fig. 2A) was size-selected by agarose gel electrophoresis, and cloned into M13mp18. The sequence determination revealed the presence of all the aa sequences determined for the subunit 3 fragments within this cloned DNA fragment (see Fig. 2B). A region of high degree of aa sequence homology (56% identity; the conserved domain A in Fig. 4) was found between this major open reading frame and the *S.cerevisiae* RNA polymerase II RPB3 (aa 46 to 248). The result strongly suggested that the cloned DNA fragment contained a part of the RNA polymerase II subunit 3 (*rpb3*) gene of *S.pombe*. In order to clone the complete gene, we next cloned a 2.5 kbp *PstI-EcoRV* genomic DNA fragment (F3 in Fig. 2A) into M13mp18 using the *PstI-EcoRI* fragment as a hybridization probe.

### Structure of the *rpb3* gene

Nucleotide (nt) sequence was determined for the continuous genomic DNA segment of 2,348 bp between *PstI* and *EcoRV* sites. The outline of this sequence is shown in Fig. 2. The coding frame spanning from nt position 988 to 1,966 encodes a polypeptide of 297 amino acids in length with a high degree of aa sequence homology to the *S.cerevisiae* RNA polymerase II subunit 3 (see Fig. 4 for aa sequence comparison). We then propose to designate this gene as *rpb3*, according to the nomenclature for the *S.cerevisiae* RNA polymerase genes proposed by Nonet *et al.* (30). After comparison of the sequences of the cDNA clone F1 and the genomic DNA clones covering the corresponding region, the *rpb3* gene was found to contain at least one intron (from nt 1,161 to 1,206). This finding is in good agreement with the result of direct aa sequence analysis of one V8 fragment covering aa 40–48 (Fig. 2B).

Systematic analysis of the exon-intron organization was then extended to other regions by sequencing cDNA clones, which cover different parts of *rpb3* mRNA, with use of primers designed from the genomic DNA sequence of *rpb3*. Results indicated that the coding frame of *rpb3* was interrupted by two introns (another from nt 1,068 to 1,110). In both 5' and 3' boundaries of these two introns, there are the consensus sequences of the *S.pombe* intron-exon junctions (boxed sequences in Fig. 2B). In sharp contrast to *S.cerevisiae*, the *rpb1*, *rpb2* and *rpb3* genes of *S.pombe* all contain several introns at their N-terminal proximal regions (3,4; this paper).

Detailed Southern analysis of the genomic DNA digested with various restriction enzymes showed that this gene is present as a single copy in the *S.pombe* genome (Fig. 3A).

### Transcription organization

Northern analysis demonstrated that the size of *rpb3* transcript is about 1.2 kb in length (Fig. 3B). The start site of the transcript determined by primer extension analysis using primer # 1 (nt 877–894) was located at nt 792 (Fig. 3C). We concluded that ATG at nt 988 is the start codon because: 1) the predicted start codon is located within the first exon (no intron was found between the transcription start site and this putative start codon) (see Fig. 2B); 2) no other ATG codon exists upstream the putative start codon on the same open reading frame; 3) the N-terminal proximal region of subunit 3 from the initiator Met to the first conserved domain (domain A in Fig. 4) is as large as that of *S.cerevisiae* RNA polymerase II subunit 3, even though homology is not so high in this region between *S.pombe* and *S.cerevisiae*; and 4) the sequence near the putative initiation codon fits well to the Kozak's rule (31) of the consensus sequence for translation initiation.

Finally, we determined the nt sequence near the 3' end of *rpb3*



may be related to function(s) specific for RNA polymerase II. According to the recent studies with the  $\alpha$ -subunit C-terminal deletion mutants of *E. coli* RNA polymerase (36), the N-terminal fragments of  $\alpha$  subunit containing the domain D can still be assembled into enzymatically active pseudo-core enzymes but further deletion from the C-terminus renders mutant  $\alpha$  inactive in the core assembly. These observations suggest that the domain D is required for the formation and/or stability of RNA polymerase. In the domain D sequence of *S. pombe* (and also *H. sapiens*), we discovered a leucine zipper-like motif (aa 249–263). This finding may support a role of the domain D in subunit-subunit contact. The C-terminal region of *E. coli* RNA polymerase  $\alpha$  subunit downstream from aa 235 carries the protein-protein contact site I with the class I activator proteins (37,38). Since the subunit 3 of eukaryotic RNA polymerases lack the corresponding region, the molecular communication between RNA polymerase and transcription factors may be different between prokaryotes and eukaryotes. It is not excluded yet that this leucine zipper-like motif in the domain D of eukaryotic RNA polymerases plays a role in contact with transcription factors.

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