Cloning, soluble expression, and purification of the RNA polymerase II subunit RPB5 from *Saccharomyces cerevisiae*

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We report the molecular cloning, expression, and single-step homogeneous purification of RNA polymerase II subunit RPB5 from *Saccharomyces cerevisiae*. RPB5 is a 210 amino acid nuclear protein that functions as the fifth largest subunit of polymerase II and plays a central role in transcription. The gene that codes for RPB5 was generated by amplification by polymerase chain reaction. It was then inserted in the expression vector pET28a(+) under the transcriptional control of the bacteriophage T7 promoter and lac operator. BL21(DE3) *Escherichia coli* strain transformed with the rpb5 expression vector pET28a(+) accumulates large amounts of a soluble protein of about 30 kDa (25 kDa plus 5 kDa double His6-Tag at N and C-terminal). The protein was purified to homogeneity using immobilized metal affinity chromatography. RPB5 recombinant protein was further confirmed by immunoblotting with anti-His antibody.

In this study, the expression and purification procedures have provided a simple and efficient method to obtain pure RPB5 in large quantities. This will provide an opportunity to study the role of *S. cerevisiae* RPB5 in gene expression and transcription regulation. Furthermore, it can provide additional knowledge of the interaction partners of RPB5 during various steps of transcription and gene expression.

Introduction

In *Saccharomyces cerevisiae*, the RNA polymerase II (RNAP II) core enzyme is composed of 12 subunits called RNA Polymerase II subunits (RPBs) RPB1–RPB12. RPB1, RPB2, RPB3, RPB4, RPB7, RPB9, and RPB11 encode subunits unique to RNAP II, while RPB5, RPB6, RPB8, RPB10, and RPB12 subunits are the shared components of RNAP I, II, and III.¹ The latter 5 small subunits are required for yeast cell viability and play a central role in RNA transcription as they are present in all the 3 eukaryotic RNAPs.² These essential subunits are assembled along with a unique set of additional subunits to form either of the 3 classes of RNA polymerases I, II, or III.³ Studying the role of these subunits and their specific contribution to regulated gene expression can reveal many puzzles in biological context.

Progress has been made to identify and characterize various transcription factors. In contrast, there is less knowledge related to RPBs.³ RPB5 of *S. cerevisiae* has a molecular weight of approximately 25 kDa and consists of 2 domains, an eukaryote-specific N-terminal domain (16 kDa) and a smaller C-terminal domain (9 kDa).⁴ RPB5 is known to be present as 2 copies in RNAP II, and has been shown to homodimerize under *in vitro* conditions.⁵ RPB5 has been reported to interact with several general transcription factors or specific gene regulators.⁶ Various studies on the human RNAP have suggested that RPB5 binds specific partners of RNAP II. Some previously reported specific partners are TFIIF and Taf15 (TAFII68).⁷ Few studies also showed that transcriptional transcription factor X encoded by the human hepatitis virus may specifically interact with RPB5 to stimulate transcription in virally infected cells.⁸⁹ However, interactions
with these specific partners do not account for the fact that RPB5 is shared by all 3 yeast RNAPs.2,10 Earlier studies also proved the close relation of human RPB5 to the archaeal subunit H.11 and to viral RPB5-like subunits.12 These studies on human RPB5 promote calls for general and specific role of *S. cerevisiae* RPB5 in gene expression.

We have chosen *Escherichia coli* for producing large quantities of recombinant RPB5 protein because of its ability to grow rapidly and at high density on inexpensive substrates, combined with its well-characterized genetics.13-15 The expression of this gene was increased using 3% ethanol (v/v).16 Purified RPB5 can uncover concrete knowledge of the structure, interaction partners, and function of RPB5 in the mechanisms of gene expression operating at the molecular level.

**Results and Discussion**

**PCR amplification and cloning of full-length rpb5 gene**

*S. cerevisiae* rpb5 gene was generated by PCR amplification using gene-specific primers. A single PCR product of the expected size (648 bp) was obtained (Fig. 1, lane 2) and cloned to pSK+ vector. In order to verify the identity of the isolated fragment, the construct was digested with BamHI and HindIII. Two bands of expected sizes 3 kb and 648 bp were obtained (Fig. 1, lane 3) confirming the identity of the rpb5 gene. Positive clones were sequenced and compared with the rpb5 sequences from *Saccharomyces* genome database. The rpb5 gene was subcloned into pET28a(+) expression vector (Fig. 1, lane 4). The final construct was named as pET28a(+)-rpb5.

**RPB5 protein expression and solubility optimization**

In the present study, both IPTG concentrations from low (0.5 mM) to high (1 mM) have shown almost same level of RPB5 protein expression (data not shown). Whereas, in the presence of 3% alcohol, increased fold of overexpression of RPB5 was found. In this study, we demonstrate the influence of temperature on RPB5 protein expression and solubility at 25°C and 37°C. We found that soluble expression at 25°C was higher than 37°C (data not shown). SDS-PAGE results for overexpressed RPB5 protein is shown in Figure 2, lane 3. The overexpressed protein was not detected in the un-induced control sample (Fig. 2, lane 2).

Recombinant bacterial cells were collected by centrifugation and lysed by sonication. The supernatant and the pellet were collected and subjected to 12% SDS-PAGE analysis. The results show that the molecular weight of the recombinant product is 30 kDa, which corresponds to the predicted size of pET28a(+)–rpb5. RPB5 protein was predominantly present in soluble fraction (Fig. 2, lane 5) in comparison with the pellet fraction (Fig. 2, lane 4).

**Purification of RPB5 protein by affinity chromatography**

Purification of recombinant *S. cerevisiae* RPB5 protein was accomplished by immobilized metal affinity chromatography (IMAC) on a Ni–NTA resin column. The SDS-PAGE analysis (Fig. 2, lane 5) showed a single protein band with the expected molecular mass of the recombinant protein (30 kDa), based on the molecular mass of the *S. cerevisiae* RPB5 (25 kDa plus 5 kDa double His6-Tag at N and C-terminal).17 Following IMAC, recombinant RPB5 protein was purified to homogeneity. The yield of purified recombinant RPB5 per liter of culture was approximately 45 mg/L. Table 1 shows the amount of cells and purification fold after each step. The presence of recombinant protein was confirmed with immunoblotting using anti-His antibody (Fig. 3, lane 2).

For structural, functional, and biochemical studies pure recombinant protein in active form is always a prerequisite.

![Figure 1](https://www.tandfonline.com/Bioengineered)
RPB5 is a 210 amino acid nuclear protein that functions as the fifth largest subunit of polymerase II and plays a central role in transcription. RPB5 is well conserved among all the 3 RNA polymerases. In RPB5 protein expression experiment, we noticed an increased expression rate under ethanol treatment.

Genomic DNA isolation from S. cerevisiae
Genomic DNA of S. cerevisiae was manually isolated by method mentioned by Hoffman, et al.

Amplification of rpb5 gene
Gene-specific primers, 5'-CGGGATCCATGGGACCAAG-3' (forward) and 5'-CCAGCTTTCTACATAAGATT-3' (reverse), were designed for PCR amplification of the S. cerevisiae rpb5 gene. Primers introduced BamHI and HindIII restriction sites on 5' and 3' ends, respectively. Synthetic oligonucleotides were purchased from Xcleris lab (Gujarat, India). The PCR reaction was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, USA) with the following cycle parameters. Initial denaturation temperature of 98°C for 120 s, 30 cycles of 98°C for 40 s, 52°C for 15 s, and 72°C for 15 s, followed by a final extension of 72°C for 5 min. PCR products were resolved using 1.0% agarose gel electrophoresis, and amplion of the expected size (648 bp) was purified with the QIAquick Gel Extraction Kit (Qiagen, USA).

Cloning and subcloning of rpb5 gene
The purified PCR product of rpb5 gene was ligated to pSK+ vector. The ligation product was used to transform in competent E. coli DH5α cells. The plasmid was isolated from the positive clones and sequenced to ensure sequence fidelity. Nucleotide sequencing was carried out by Xcleris lab (Gujarat, India). The plasmid mid was isolated from the positive clones and then spread onto agar plate containing ampicillin (100 μg/mL) to allow selection of colonies that successfully incorporated the plasmids. Plasmid DNA extraction was performed using the QiAprep Midiprep plasmid purification kit (Qiagen, USA).

Materials and Methods
Materials
The molecular biology kits and NTA agarose were purchased from Qiagen, CA, USA. The dNTPs and enzymes were purchased from New England Biolabs, MA, USA. All other reagents and chemicals were purchased either from Sigma-Aldrich Chemical Company, St. Louis, MO, USA, or Sisco Research Laboratories, Mumbai, India and were of the highest purity available. Bacterial culture media was purchased from Himedia Laboratories, Mumbai, India.
Optimization of recombinant RPB5 protein expression

Recombinant cells harboring pET28a (+)-rpb5 plasmid were screened on selective Luria Broth (LB) agar plates supplemented with kanamycin. A positive clone was picked up and grown overnight in 5 mL LB broth containing 50 μg/mL ampicillin at 37°C with shaking (180 rpm). First, optimal expression conditions were standardized using 5 mL cultures. Two isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations (0.5 mM and 1 mM) and 2 different expression temperatures (25°C and 37°C) were tested at 18 h and 4 h incubation time course, respectively. We tested and used 3% ethanol (v/v) to increase the expression fold of the recombinant gene according to Chhetri et.al.16 A 2 mL primary culture was inoculated in 400 mL of sterile LB broth supplemented with 50 μg/mL kanamycin and incubated at 37°C with shaking (180 rpm) until the OD600 reached 0.5. Another 5 mL LB broth culture tube containing above-mentioned antibiotics was inoculated with the primary culture and was kept as un-induced control. After the OD600 reached, culture was induced with 0.5 mM IPTG and incubated for 18 h at 25°C with continuous shaking. After 24 h, cells were harvested by centrifugation at 4°C, and the pellet was resuspended in 20 mL of 50 mM Tris (pH 8.0) and 300 mM NaCl containing protease inhibitor cocktail.

Purification of recombinant RPB5 protein

Homogenized cells were lysed on ice using sonication at 50% amplitude for 30 cycles (30 s pulse on, off). The crude lysate was then clarified by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was filtered through a 0.45 μm pore PVDF membrane before affinity chromatography. Matrix was extensively washed with 10 bed volumes of equilibration buffer (50 mM Tris (pH 8.0), 300 mM NaCl). The supernatant was passed through the Ni–NTA agarose matrix (Qiagen, USA) and was washed with increasing concentration of imidazole. Elution of RPB5 recombinant protein was accomplished with elution buffer (50 mM Tris (pH 8.0), 300 mM NaCl, and 300 mM Imidazole). The purity was analyzed by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) stained with 0.05% Coomassie brilliant blue R-250. The protein was dialyzed overnight against dialysis buffer (20 mM phosphate pH 8.0, 150 mM NaCl) at 4°C. Quantitation of recombinant proteins was carried out using Bradford method using BSA as a standard.

Western blotting

His-tagged recombinant RPB5 purified protein was subjected to SDS-PAGE on 12% polyacrylamide gel and transferred to 0.45 μm polynvinylidene fluoride (PVDF) membrane (Whatman) for 90 minutes at 50 mV. Membrane was next blocked in 5% skimmed milk and incubated with mouse anti-His antibody (1/3000 dilution). After 3 times washing with PBS, pH 7.4, membrane was then incubated with anti-mouse IgG alkaline phosphatase (1:1000). Both primary and secondary antibodies were diluted in PBS containing 3% BSA. Membranes were then 3 times washed with PBS, pH 7.4. Signals were detected with the BCIP/NBT-Blue Liquid Substrate (Sigma-Aldrich Co, St. Louis, USA) and analyzed using gel doc system.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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