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ISOLATION AND PURIFICATION OF DNA-DEPENDENT RNA-POLYMERASE II FROM *Saccharomyces cerevisiae*

RAMESH CHANDRA*

Department of Chemistry, University of Delhi, Delhi-110007, India

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An improved and rapid procedure for the isolation and purification of RNA polymerase II from *Saccharomyces cerevisiae* is described. The procedure may be completed in less than 48hrs and resultant enzyme is judged to be more than 85 per cent pure. Total RNA polymerase activity was solubilized from sixty gram yeast cells by sonication in extraction buffer and RNA polymerase II was purified by phosphocellulose slurry treatment, polyamin P fractionation, ammonium sulphate precipitation and chromatography on DEAE cellulose (DE-52) and phosphocellulose (p-11).

Key Words : RNA Polymerase II; *Saccharomyces cerevisiae*; Ion Exchange Resins; DEAE-Cellulose; Polyamin-P; Phosphocellulose

INTRODUCTION

THE DNA-dependent RNA polymerase is the key enzyme responsible for the read out of all the genetic informations stored in a cell. Under the direction of DNA template, it catalyzes the sequential assembly of four nucleoside triphosphates into RNA. In addition to the requirement of extrinsic metal ion, RNA polymerase II also possesses intrinsic metal ions. In 1971, Acrutton, Wu and Goldthwait[1] first discovered that RNA polymerase from *E. coli* is a metalloenzyme containing g-atoms of tightly bound zinc^{2+} ions per mole of enzyme. In fact information is the process by which naked DNA is introduced into cell, resulting in a heritable change. An understanding of the molecular mechanism which regulate the transcription in eukaryotic cells will require the duplication of specific transcription events in soluble *in vitro* system utilizing purified components. Weil *et al.*[2] described the first *in vitro* system capable of supporting specific transcription initiation on eukaryotic class II genes. Since this system is dependent on the addition of purified RNA polymerase II. Matsui *et al.*[3] were subsequently able to demonstrate that in addition to RNA polymerase II, four chromatographically separable soluble factors are required for specific transcription initiation *in vitro*. These soluble proteins appear to be general factors that are necessary and sufficient for specific transcription initiation but that do not affect *in vitro* the regulatory events observed *in vivo*.[4]

The genetic complexity of yeast *Saccharomyces cerevisiae* is 100-fold lower than higher eukaryotes; therefore, it is an attractive model system for the study of

**Address for communication*:
Dr Ramesh Chandra, UGC Research Scientist (Reader), Department of Chemistry, University of Delhi, Delhi-110007

eukaryotic transcriptional regulation. A biochemical analysis of transcriptional regulatory proteins would be greatly facilitated, if they were first characterized genetically. *S. cerevisiae* is highly amenable to genetic analysis and the regulation of expression of several genes has already been extensively studied genetically.[5,6] This development of yeast transformation systems[7-9] makes genetic analysis of yeast even more tractable. Additionally, these information systems allow the reintroduction of modified genes into the living cell in order to assess the *in vivo* facets of the *in vitro* mutagenesis. The characterization of transcriptional regulatory proteins will require complementation assays for specific transcription. Mammalian RNA polymerase II does not support specific initiation of transcription. However, if the purified enzyme is supplemented with soluble proteins (which do not contain detectable RNA polymerase II) from hypotonically lysed human KB cells, selective and accurate initiation of transcription at the adenovirus 2 major late promoter is promoted by RNA polymerase II; such a system allows the use of factors from different cell types (e. g., genetically different strains) in complementation reactions with purified RNA polymerase II. To develop a similar system from yeast, a new and rapid procedure for purification of *S. cerevisiae* DNA-dependent RNA polymerase II has been developed. This is a very rapid method than those previously published with a high yield of homogeneous RNA polymerase II.

Materials and Methods

Cells

S. cerevisiae, 20B-12 (PEP4-3) from Yeast Genetic Stock Center, Berkley, California (U.S.A) was grown with aeration in commercial fermenter at 37 °C in YEPD† media. Cells were harvested on ice from the commercial fermenter during the last growth cycle. After one wash at 4 °C, pellets were suspended in 2 volumes of buffer A. Aliquots were frozen in a dry ice/ethanol bath and stored at −70 °C.

Buffers

A 20mM Tris-HCl; *p*H 7.9, 10mM β-Mercaptoethanol, 0.5mM EDTA†, 10 per cent (v/v) Glycerol.

Extraction Buffer

20mM Tris-HCl *p*H 7.9 buffer, 10mM β-Mercaptoethanol, 0.5mM EDTA†, 10 per cent (v/v) Glycerol, 1mM $MgCl_2$, 1 per cent (v/v) DMSO†, 0.3mM PhSOF (added just before use).

Biochemicals

Unlabelled nucleoside triphosphates were purchased from P. L. Biochemicals. [^{3}H] labelled UTP (40–50ci/m.mol) was from Amersham, Bovine serum albumin and calf thymus DNA were from Sigma chemical company, Zymolyase 5000 was from Kirin Brewery Japan.

Preparation of Ion Exchange Resins

Ion exchange resins, DEAE-Cellulose (whatman DE-52), phosphocellulose (whatman P-11) were prepared as reported by Bitter.[10]

Assay for RNA Polymerase

RNA polymerase II was assayed in 25μl reactions containing 50mM Tris HCl; *p*H 7.9; 2mM $MnCl_2$; 600μM each of GTP†, CTP† and ATP†; 50mM [^{3}H] UTP†; 120μl/ml heat denatured calf thymus DNA and 50mM $(NH_4)_2SO_4$. After incubating at 37 °C for 10 minutes; the entire reaction was stopped by placing the tubes in an ice bucket. After that we added 0.1ml of 0.1M sodium-pyrophosphate, mixed on vertex for *ca.* 10 seconds, then, added 5ml of 5 per cent TCA† solution to precipitate out any possible nucleic acid (RNA) formed. Let it stand for 10 min. Filter each tube mixture on glass fiber filters (GF/C, 24mm diameter); wash the filters five times (5ml wash) with cold 1 per cent TCA†, followed by cold 95 per cent ethanol and ether. Pin filter paper on a styraform covered with aluminium foil. Add two dry filters and put 10μl of standard, [^{3}H] UTP and then count the activity.

One unit is defined as the amount of activity which incorporates one picomole of UMP† into RNA† under the above conditions.

TABLE I

Purification of RNA polymerase II from 60gm Saccharomyces cerevisiae

Fraction	Protein* (mg)	RNA Polymerase II (units)	Sp. Activity (units/μg)	Yield (per cent)
F 1	11,100	3885,000	0.35	100%
F 2	10,360	3833,000	0.37	99%
F 3	5,928	2295,000	6.2	88%
DEAE-Cellulose	68.3	2275,000	42.6	85%
Phosphocellulose	2.0	2275,000	1190.0	42%

*The Protein concentration was determined by Biored assay, using BSA as standard.

Salt Concentration Measurements

The concentration of Ammonium Sulphate enzyme samples and chromatographic fractions were determined: a 5μl sample was mixed with 1ml of water and the conductivity was measured at 23 °C with Radiometer CDM-2 conductivity meter. Salt concentrations were read from standard curves generated with known ammonium sulphate solutions in buffer A.

Polyacrylamide Gel Electrophoresis

The subunit structure of purified Yeast RNA polymerase II was examined in 10 per cent polyacrylamide gels containing SDS† as described by Laemmli (11).

Purification Methods

All the operations were at 4 °C except where indicated. All centrifugations were done in Sorvall GSA rotor. Sixty grams of cells in 120ml extraction buffer, added 5mg/gm of cells Zymolyase 5000 and the suspension incubated at 20 °C for about one hour. Spheroplasting was monitored by diluting an aliquot of the suspension 400-fold in H_2O and measuring the A_{600} (spheroplasting is complete when the A_{600} drops to less than 40 per cent of the starting value), during this extraction *p*H was adjusted to 7.9 by adding 0.3M Ammonium sulphate. The suspension was then chilled at 4 °C and was made 0.3M Ammonium sulphate and then the suspension was sonicated with a heat system Ultrasonic Model W185, at 25 cycles, output of 5V and 50 per cent cycles efficiency for four 30-s bursts with 30-s cooling periods between sonications. The solution was then centrifuged for 30 min at 20,000 rpm. Pellet was discarded and supernatant was diluted to half by addition of same volume of buffer A, and mixed with 25–40 gm of phosphocellulose slurry equilibrated in buffer A. After stirring for 4-6 hrs, it was filtered and to the supernatant, added 5μl of 10 per cent Polyamin-P† per mililiter of solution. After 10 minutes, the solution was centrifuged for 30 min at 10,000 rpm; supernatant was discarded and pellet was resuspended in extraction buffer ($\frac{1}{2}$ of the original) by gentle homogenisation in a Dounce homogenizer with a Type A pestle. After 10 minutes, the suspension was centrifuged at 10,000 rpm for 20min. The supernatant was dialyzed with buffer A + 0.15M Ammonium sulphate for four hours or till the conductivity comes down to equivalent of 0.15M Ammonium sulphate. This protein suspension was loaded on a 60-ml DEAE-Cellulose column.

DEAE-Cellulose Chromatography

The above sample suspension was loaded (90ml/hr) on a 60ml DEAE-Cellulose (Whatman DE-52) column (2.5 × 12.5cm), which had previously equilibrated with buffer A containing 0.15M Ammonium sulphate. After sample application, the column was washed with 200ml of buffer A with 0.3M Ammonium sulphate at a flow rate of 90ml/hr. Fractions of 5ml each were collected and their A_{280} was recorded. Biored assay was done for peak fractions to estimate protein concentration and radioactive assay was done for enzyme activity by filter binding method.

Phosphocellulose Chromatography

The peak fractions of RNA polymerase II activity from DEAE-cellulose chromatography described above, were pooled and dialyzed versus 500 ml of buffer A containing 0.03M Ammonium sulphate for 4–5 hrs. The dialysate was collected and loaded (40 ml/hr.) on to a 10 ml Phosphocellulose (Whatman P-11) column (1.5 × 8cm), which had been previously equilibrated with buffer A containing 0.03M Ammonium sulphate. RNA polymerase II was eluted with 0.14M Ammonium sulphate in buffer A. Fractions of 1.1 ml were collected. The peak fractions containing RNA polymerase II activity were split into several aliquots and stored in storage buffer at —70 °C or in liquid nitrogen.

Results and Discussion

The activity present in Yeast tissue spheroplasts is solubilized and rendered DNA-dependent by sonication at high salt concentrations.[12] The yield of RNA polymerase II per gm of cells is considerably as high as in previously published methods,[13,14] indicating that conversion of cells to spheroplasts do not result in loss of RNA polymerase II activity. Our purification method needs the smallest possible column volumes, which enabled us to keep the enzyme more concentrated, which increases the stability. The polyamin-P has been used to completely precipitate RNA polymerase II activity using its minimum amount. Approximately 50-fold purification was achieved by P. Cell slurry treatment and Polyamin-P fractionation. The binding capacity of Polyamin-P varies from batch to batch.[15]

The eluted proteins were concentrated by Ammonium sulphate precipitation and resuspended in column chromatogarphy buffer. RNA polymerase I and III do not bind to DEAE-cellulose column and are recovered in flow through fractions. The peak fractions were pooled and diluted until conductivity equalled that of 0.15M Ammonium sulphate. After dialysis, the pooled fractions were chromatographed on phospho-cellulose. It has been observed that if RNA polymerase II was applied to Phosphocellulose, which had been equilibrated in buffer, having 0.05M Ammonium sulphate, low recovery of RNA polymerase II resulted. Fractions of Phosphocellulose column were split into several aliquots, quick frozen and stored at −70 °C.

The purity of RNA polymerase II after Phosphocellulose chromatography was determined in SDS†-polyacrylamide gels. The enzyme showed a sub unit structure as reported.[16–18] It has been confirmed that two forms of RNA polymerase II exists in yeast, which differ in molecular weight of large sub unit (220,000 or 180,000), and 220,000 sub units may be especially cleaved to the 180,000 subunit *in vitro* using a Yeast-protease extract. Purification of the RNA polymerase II is possible and it takes less than 48 hours for the whole isolation and purification process, minimum time so far as record.

In this paper, a new and rapid procedure is reported for the purification of Yeast RNA polymerase II, and it takes less than 48hrs., minimum time so far on record and having more than 85 per cent purity.

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†*Abbreviations used*:
SDS, Sodium dedecyl sulphate; TCA, Trichloro acetic acid. TGE, Tris-HCl/Glycerol/EDTA; PhSOF, Phenyl sulphonyl chloride EDTA, Ethylene dichloride tetraacetic acid; YEPD-media, 2 per cent Yeast Extract, 4 per cent Bactophenone, 4 per cent Dextrose; GTP, Guanine triphosphate CTP, Cytidine triphosphate; ATP, Adenosine triphosphate; UTP, Uridine triphosphate; DNA, Deoxyribonucleic acid; UMP, Uridine monophosphate.

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