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Use of RNA Yeast Polymerase II Mutants in Studying Transcription Elongation

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Studies of the elongation phase of RNA polymerase II transcription have benefited greatly from complementary genetic and biochemical studies in the budding yeast *Saccharomyces cerevisiae*. The tools of molecular biology have enabled the generation of mutant yeast strains and have enabled analysis of the biological consequences of the mutations on cell growth, viability, and transcript elongation. Preparative amounts of genetically altered RNA polymerase II have been purified and assayed for biochemical function using conventional chromatography or affinity separation.^{1,2} The roster of the components of cellular machinery that participate in elongation has been expanded through the study of genetic interactions using a drug-sensitive phenotype in concert with demonstrated elongation defects in RNA polymerase II. As a result, proteins involved in DNA repair and chromatin structure (as well as others) have been linked to elongation.^{3,4,5} The mechanistic basis of defects in mutant RNA polymerases can be appreciated in the context of a recently developed, atomic-level structural model of the RNA polymerase II elongation complex.⁶

History and Rationale

One of the earliest-identified and best-studied transcription elongation factors for RNA polymerase II is SII (also known as TFIIS).⁷ Mutation of *DST1* (also known as *PPR2*), the non-essential gene encoding SII in yeast, leads to reduced growth rates in the presence of 6-azauracil (6AU).^{8,9} 6-Azauracil is an inhibitor of IMP dehydrogenase (IMPDH), the rate-limiting enzyme in *de novo* GTP synthesis. Treatment of cells with 6AU results in depletion of intracellular nucleotide pools.⁹ This cardinal finding has led to the use of 6AU-sensitivity as a phenotypic marker for a compromised elongation machinery in yeast. It has been suggested that depressed intra-cellular nucleotide pools stress the elongation machinery by reducing the levels of these RNA substrates below RNA polymerase's K_m .¹⁰ When the concentration of nucleotides available to RNA polymerases is restricted *in vitro*, elongation rates are correspondingly reduced. RNA polymerase molecules that elongate slowly are prone to becoming arrested, a state reversed by SII.^{7,11,12} Mutation and/or deletion of a number of RNA polymerase II subunit genes has also been shown to confer 6AU-sensitivity.^{10,13,14,15} Following purification, biochemical defects were observed in the elongation behavior of mutant yeast RNA polymerase II.^{13,15,16} Hence, it is attractive to think that due to its role as an IMPDH inhibitor, 6AU imposes on the cell an increased requirement for optimal elongation factor function.¹⁰ Presumably, this requirement cannot be met by cells with mutated elongation factors or RNA polymerase II, which results in slowed growth.

Mycophenolic acid (MPA) has also become useful to assay for mutations in the elongation machinery. This drug is a well-characterized and specific inhibitor of IMPDH. There are

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CTP, GTP, and UTP (lane 4) and incubated at 22°C for 5 min before RNA was isolated from each for gel electrophoresis. The 15% (w/v) polyacrylamide/50% urea (w/v) gel shown was dried and exposed to XOMAT (Dupont) film. For reference purposes, lane 1 shows the starting 10-base oligoribonucleotide labeled with γ -³²P-ATP and polynucleotide kinase.

detailed pharmacological studies in human cells and an atomic-level structural model of MPA in a complex with mammalian IMPDH.¹⁷ Yeast strains lacking a functional SII protein are MPA sensitive, as are elongation-defective RNA polymerase II mutants.^{9,18}

Thus, starting with mutations in genes encoding RNA polymerase II subunits, it is possible to assay for: (1) growth rate changes in the presence of drugs that inhibit nucleotide synthesis and (2) intrinsic or elongation factor-related defects in elongation by mutant RNA polymerase II enzymes *in vitro*. Conversely, a collection of mutagenized yeast can be screened for drug-sensitive growth and candidates can be tested for a role in transcription elongation. This approach has been used to successfully screen for 6AU and MPA sensitive mutants in a collection of haploid yeast deleted for nonessential genes.^{19,20}

This chapter addresses the application of 6AU- and MPA-sensitivity as a genetic assay and will provide procedures for the purification of RNA polymerase II for use in *in vitro* transcription assays.

Drug-Sensitive Growth Assays

6-Azauracil

One of the limitations of the 6AU-sensitivity assay is that cells must be tested on uracil-free media since uracil competes with and neutralizes the drug's effect. Therefore, the strain must be URA⁺. Synthetic complete medium lacking uracil (SC-ura) is favored over rich medium such as YPD. Standard recipes for these media have been described.²¹

1. Media should be prepared, autoclaved, and allowed to cool to the touch.
2. Prepare a 5 mg/ml stock of 6-AU (Sigma, Catalog #A-1757) in water. The drug will take a number of hours to go into solution with constant stirring at 30° C. Sterilize by passing the fully dissolved solution through a 0.2 μ m filter (Gelman, Product #4192).
3. Add the drug to media while stirring. A series of liquid cultures or plates can be made with varying amounts of the drug to assess sensitivity; effective concentrations range from 50–100 μ g/ml (w/v). The drug is stable for days or weeks in media.

Mycophenolic acid

MPA is effective in rich medium or synthetic dropout medium. However, it is somewhat unstable, so it is recommended that plates be poured as close to use as possible and that stock solutions be made fresh prior to addition to liquid media.

1. Medium should be prepared, autoclaved, and allowed to cool to the touch.
2. Prepare a 15 mg/ml stock of MPA (Sigma, Catalog #M-5255) in dimethylsulfoxide and sterilize through 0.2 μ m filters (Gelman, Product # 4192).
3. Add the drug to media with stirring. A series of liquid cultures or plates can be made with varying drug concentrations. Effective concentrations range from 5–30 μ g/ml (w/v).

Cell Growth

Growth inhibition is a relative measure. At a high enough concentration, even wildtype cells show reduced growth rates for a drug such as MPA. Therefore, it is important to have a quantitative measure of this parameter. Strains to be compared should be obtained from a culture in the logarithmic stage of growth. Growth on solid medium can be gauged by

diluting cells to an optical density (600 nm) of 0.001 ($\approx 5 \times 10^7$ cells/ml/OD). Ten μ l of 4 to 10-fold serial dilutions from this stock are spotted onto agar plates for incubation at 30° C. For liquid cultures, the cell density is monitored and the time it takes for the number of cells in a liquid culture to double is calculated (slope of the logarithmic portion of the growth curve divided by log[2]). A typical strain with a disruption of the *DST1* gene shows a 1.4 to 1.9-fold increase in doubling time in SC-ura with 75 μ g/ml 6AU.¹⁸ Using doubling time as a gauge, synthetic effects of combining mutations can be readily observed.¹⁸ It should be noted that the relative drug sensitivity of different strains can vary. Drug-sensitive cells should grow well when guanine is also included in the medium, demonstrating the specificity of the phenotype since this base can be converted into GTP by a salvage pathway, bypassing the pathway blocked by 6AU and MPA.

Induction of IMP Dehydrogenase

After exposing wildtype yeast to 6AU or MPA there is a robust transcriptional response of at least one of the four genes encoding IMPDH (*IMD2*; also known as *PUR5*).^{22,23,24} Furthermore, this induction is compromised in a number of transcription elongation mutants.²² Thus, measurements of *IMD2* induction using northern blotting have been an asset in understanding phenotypes of candidate elongation mutants.^{25,26}

RNA Polymerase II Purification and Transcription

Once a candidate RNA polymerase II mutant has been identified by the drug-sensitive phenotype, the enzyme can be readily purified and subjected to *in vitro* transcription assays.

Conventional Chromatography (after Edwards et al., 1990)²

1. Cells are grown at 30° C in 6 L of rich medium (YPD), pelleted, washed with cold water, and resuspended in 0.2 volumes of 0.25 M Tris-HCl, pH 7.9, 5 mM EDTA, 2.5 mM DTT, 10 mM sodium pyrophosphate, 5% (v/v) dimethylsulfoxide, and 50% (v/v) glycerol, and frozen at -80° C.
2. Thawed cells are mixed at 4° C with 1.25 volumes glass beads and disrupted by fifteen 30-s bursts of a bead beater (Biospec Products, Bartlesville, OK) with 90-s of cooling between bursts.
3. The lysate is diluted into 0.6 volumes of buffer A (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.5 mM DTT, 10 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol) and protease inhibitors (per liter: 10 mg of aprotinin, 320 mg of benzamidine, 1 mg of pepstatin, 10 mg of leupeptin, 5 mg L-1-chloro-3-[4-tosylamido]-4 phenyl-2 butanone, 24 mg of L-1-chloro-3-[4-tosylamido]-7 amino-2-heptanone hydrochloride, 174 mg of phenylmethylsulfonyl fluoride). Glass beads and cell debris are removed by centrifugation at $27,500 \times g$ for 40 min at 4° C. The supernatant is filtered through 3MM paper (Whatman, Catalog #3030917).
4. Apply the supernatant to a 40-ml heparin-Sepharose CL-6B column (Pharmacia Biotech Inc.). The column is washed with buffer A containing 0.1 M KCl and eluted with buffer A containing 0.6 M KCl. Peak protein fractions can be identified by spot immunoblotting with monoclonal antibody 8WG16²⁷ directed against the largest subunit of RNA polymerase II. Peak fractions are pooled and precipitated with 50% (w/v) saturated ammonium sulfate at 4° C. This precipitate is collected by centrifugation at $34,000 \times g$ for 30 min at 4° C, dissolved in buffer B (40 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride), and dialyzed versus buffer B containing 0.1 M KCl.

5. This protein is applied to a DEAE 5-PW column (TosoHaas; 75 × 7.5 mm, part #07164) using a Pharmacia FPLC system. Bound material is eluted with a 9-ml gradient from 0.1–0.5 M KCl at a flow rate of 0.6 ml/min. Peak fractions are identified by immunoblotting and assayed for nucleotide incorporation using calf thymus or salmon sperm DNA as a template.²⁸ Nucleotide incorporation is typically greater than 90% α -amanitin-sensitive (100 μ g/ml), and the specific activity ranges from 18–42 units/mg protein (1 unit = 1 nmol nucleotide incorporated per minute into an acid insoluble form). This procedure results in a preparation that is >10 to 20% pure using specific activity and silver staining of SDS gels.

Affinity Chromatography

The convenience of affinity purification is preferable to conventional chromatography for analyzing multiple mutants. If native (i.e., untagged) polymerase is required, affinity purification of RNA polymerase II using an immobilized monoclonal antibody (8WG16) against the carboxyl terminal domain of the largest subunit is effective²⁷ and has enabled the purification of milligram amounts of enzyme.^{2,6} One limitation of this approach is the availability of large amounts of the monoclonal antibody.

If modified polymerase is acceptable, a high affinity ligand binding site can be added to a subunit using an appropriate genetically engineered strain. Recombinant plasmid encoding a subunit such as Rpb3p with a peptide extension recognized by a monoclonal antibody (12CA5), can be readily introduced into a yeast strain.¹ Similarly, a fusion of Rpb3p to glutathione-S-transferase can be used to purify the enzyme on glutathione agarose.^{29,30} Alternatively, the tag can be added by introducing a piece of DNA encoding a ligand binding site into the end of a subunit's open reading frame in the chromosome.³¹ Purification of such tandem affinity purification (TAP)-tagged multiprotein complexes is rapid and efficient and facilitates a large-scale analysis of mutant RNA polymerases. The TAP-tag can be engineered such that the tag "polypeptide" can be removed by a specific protease if native protein is desired.

For an analysis of elongation rates there are two useful experimental systems that do not require promoter-sequences, enabling the analysis of purified polymerase in the absence of general initiation factors. The first employs DNA duplexes extended at their 3' ends with deoxycytidylate (also known as, "tailed" templates).^{13,32} Tailed templates can be used to study elongation on specific cloned DNA sequences of choice for template lengths of a few kilobases. The influence of elongation factors upon transcription rates can also be assayed. A detailed discussion of tailed templates can be found in an earlier volume in this series.³³

Elongation complexes can also be assembled directly from two synthetic oligodeoxynucleotides that represent template and non-template strands, a small oligoribonucleotide "primer," and purified yeast RNA polymerase II.³⁴ This can be a very useful transcription system for quickly characterizing elongation by many tagged, affinity-purified mutant yeast RNA polymerases. One limitation of this method is that only relatively small lengths of template can be transcribed unless additional sequences are ligated onto pre-initiated complexes.³⁵ An advantage of the synthetic oligodeoxynucleotide templates over the use of tailed templates is that the nascent RNA is displaced from the template strand and the parameters of DNA rewinding and RNA-DNA melting are comparable to that seen for "normal" elongation complexes.³³ Elongation complexes formed on tailed templates may not displace the RNA chain, resulting in an extended RNA-DNA hybrid.³³ Purified yeast RNA polymerase II prepared using the conventional chromatographic procedure described can be "walked" stepwise down such a synthetic oligodeoxynucleotide template. As an example, wildtype enzyme was used to extend a 10-base

oligoribonucleotide (Fig. 1, lane 1) by a single CMP residue in the presence of α -³²P-CTP (lane 2), by four bases in the presence of CTP and UTP (lane 3), or to completion in the presence of all four nucleoside triphosphates (lane 4). This system enables the study of the microscopic steps involved in nucleotide incorporation. In this example, DNA sequences representing the adenovirus major late promoter have been selected. To study more complex interactions, such as those between RNA polymerase II and the basal machinery, the mutant enzyme can be added to a reconstituted reaction in which RNA polymerase initiates transcription from a standard double-stranded promoter using recombinant general transcription factors.³⁶

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Fig. 1.

Assembly and function of a synthetic elongation complex composed of purified yeast RNA polymerase II, DNA oligonucleotides, and an RNA oligonucleotide. The template strand DNA and 10-base RNA shown were annealed and mixed with 2 μ g of purified yeast RNA polymerase II (DEAE fraction) as described.³⁴ The non-template DNA was annealed to the template strand and the resulting complexes were provided with 10 μ Ci of α -³²P-CTP (400 Ci/mmol) for 5 min at 22° C. An aliquot of RNA was prepared for gel electrophoresis (lane 2). The reaction was split and adjusted to 30 μ M UTP (lane 3) or 30 μ M each of ATP, CTP, GTP, and UTP (lane 4) and incubated at 22° C for 5 min before RNA was isolated from each for gel electrophoresis. The 15% (w/v) polyacrylamide/50% urea (w/v) gel shown was dried and exposed to XOMAT (Dupont) film. For reference purposes, lane 1 shows the starting 10-base oligoribonucleotide labeled with γ -³²P-ATP and polynucleotide kinase.