Accurate Initiation by RNA Polymerase II in a Whole Cell Extract from Saccharomyces cerevisiae*

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We have developed a simple procedure for isolating a transcriptional extract from whole yeast cells which obviates the requirement for nuclear isolation. Detection of accurate mRNA initiation by RNA polymerase II in the extract requires the use of a sensitive assay, recently described by Kornberg and co-workers (Lue, N. F., Flanagan, P. M., Sugimoto, K., and Kornberg, R. D. (1989) Science 246, 601–604) that involves activation by a GAL4-VP16 fusion protein and a template lacking guanosine residues in the coding strand. The extract is prepared from fresh or frozen yeast cells by disruption with glass beads and fractionation of proteins by ammonium sulfate precipitation. The α-amaminin-sensitive transcripts synthesized in the assay were identical to those produced in a parallel assay using a yeast nuclear extract. The activity of the whole cell extract is lower per mg of protein than a nuclear extract but proportional to the volume of the nucleus relative to the whole cell. The optimal ranges for several reaction components including template, monovalent cations, and nucleotide substrate concentration were determined. Under optimal conditions the whole cell extract produced a maximum of approximately 1 × 10³ transcripts/template molecule in 30 min.

Although it has been clear for many years that the structure of RNA polymerase II has been conserved throughout the eukaryotic kingdom (1), more recent work has demonstrated the conservation of other mRNA transcription factors. Several gene-specific transcriptional activators function both in yeast and animal cells: the yeast GAL4 protein (2, 3); mouse c-fos (4); avian v-jun (5); and rat glucocorticoid receptor (6). General transcription factors from yeast can substitute for HeLa cell factors TFIIA (7) and TFIID (8, 9) in in vitro transcription reactions. This complementation has been used to advantage to circumvent the difficulty of purifying the HeLa cell factors (10, 11). The yeast TFIID equivalent has been highly purified (12, 13), and molecular genetic methods have been applied to clone the yeast gene and map it to a locus known to affect transcription (14–16).

Differences between transcription in yeast and in multicellular eukaryotes, such as in the spacing between the “TATA box” and the transcription initiation site (17–19), exist as well. Elucidation of these similarities and differences by comparative biochemistry of the mammalian and yeast transcription machineries is now possible due to the development of a yeast nuclear extract capable of accurate transcription (20). The levels of transcription in the extract were originally quite low (20), but Kornberg, Ptashne, and their co-workers (21) recently increased the activity of the in vitro reactions by using a GAL4-VP16 fusion protein as a strong activator of a template containing a GALuAs element. This innovation increased the amount of RNA made nearly 100-fold to approximately 10⁻⁵ transcripts/template molecule (21). In addition, the assay procedure has been simplified by the creation of a template containing the GALuAs upstream of the G-minus cassette originally described by Roeder and co-workers (22, 23).

The remaining difficulty in undertaking a biochemical analysis of yeast transcription lay in the nuclear isolation procedure, which required large scale spheroplasting using lytic enzymes and density gradient fractionation of nuclei. Beside being time consuming, spheroplasting introduces proteases to the extract, and the procedure is strain- and growth stage-dependent. In addition, the nuclear fractionation is difficult to carry out on the large scale required for protein purification. We report that prior isolation of nuclei is not necessary to obtain an extract capable of accurate in vitro transcription of mRNA-encoding templates.

MATERIALS AND METHODS

Strains and Plasmids—Whole cell extracts were prepared from two yeast strains: BF338-90 (MATa, ura3, his3, leu2, WH1, can1, G418⁵, obtained from B. Futter, Cold Spring Harbor Laboratory) and BJ2168 (MATa, ura3-52, trpl, leu2, gal2, pep3-5, prb1-1122, prc1-407), obtained through K. Yamamoto, University of California, San Francisco). A nuclear extract was prepared from YJJ199 (MATa, ade1, trpl-289, (leu2), gal4::LEU2, (ura3-52), his3::URA3, gal2g(7,10,11)108) by the procedure of Lue and Kornberg (20). Plasmid pJL2 (21) and Escherichia coli strain XA-90 (Δlac-pro) XIII, ara, nala, argE, thi, rifR/F’ lacZY, proAB) were obtained from J. Leatherwood and M. Ptashne (Harvard University), and the template plasmid pGAL4G-+, with a UASGAL-driven CYC1 promoter fused to “a G-minus cassette” (22, 23), was obtained from D. Chasman and R. Kornberg (Stanford University). Template plasmid was isolated from E. coli NM522 (24) by alkaline lysis and purified through two ethidium bromide cesium chloride equilibrium gradients (25).

Preparation of the GAL4-VP16 Fusion Protein—GAL4-VP16 fusion protein was isolated from strain XA-90 (pJL2) essentially as described by Chasman et al. (21) through the 35% (NH₄)₂SO₄ precip. This material was resuspended in a small volume of 20 mM HEPES, pH 7.6, 20 mM 2-mercaptoethanol, 10 μM zinc acetate, 1 mM PMSF, 2 mM benzamidine hydrochloride, 0.5 μg/ml leupeptin, 0.4 μg/ml pepstatin, 2 μg/ml chymostatin and was dialyzed against the same buffer containing 20% glycerol for 1 h at 4 °C. This material was 30–50% pure as judged by Coomassie Blue staining of a sodium dodecyl sulfate-polyacrylamide gel and was stored in aliquots in liquid nitrogen. It could be thawed and frozen repeatedly.

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The abbreviations used are: TF, transcription factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; EDTA, [ethylenebis(oxyethylenenitrito)]tetracetic acid.
Preparation of the Whole Cell Extract—Yeast were grown in 1% yeast extract, 2% peptone (Difco), 2% glucose (red filter) of 100–300 ml, harvested, and washed once in cold sterile distilled water. All further steps were carried out at 0–4 °C. The cell pellet was resuspended in 1 ml/g of 100 mM Tris acetate, pH 7.9, 50 mM potassium acetate, 10 mM MgSO_4, 20% (v/v) glycerol, 3 mM DTT, 2 mM EDTA, and the following protease inhibitors: 1 mM PMSF, 2 mM benzamidine hydrochloride, 0.5 μg/ml leupeptin, 2.5 μg/ml antipain, 0.35 μg/ml bestatin, and 0.4 μg/ml pepstatin. These cells could be used directly or frozen in liquid nitrogen and stored at -70 °C. The suspension was diluted with an equal volume of the same buffer, and cells were broken with glass beads in a Dyno-Mill (Glen Mills) with a Neslab cooling unit set at -10 °C. By alternating three 1-min bursts of grinding with 1 min of slower mixing, the temperature of the lysate rose to 10 °C. The suspension was diluted with an equal volume of the same buffer, alternating four 30-s bursts of grinding with 1 min of slower mixing; the temperature of the lysate was kept between 1 and 4 °C. One functional unit was collected by centrifugation at 31,000 rpm in an SW 40 rotor for 30 min. The pellets could be frozen in liquid nitrogen and stored at -70 °C. Cells were recovered in a minimal volume in a Beckman SW 40 rotor. After digestion of the top white layer, the supernatant was removed and 0.35 g/ml solid (NH_4)_2SO_4 was added over the course of 1 h. 1 M KOH was also added at a ratio of 10 μl/g (NH_4)_2SO_4. This solution was stirred for 30 min, and the precipitate was collected by centrifugation at 31,000 rpm in an SW 40 rotor for 30 min. The pellets were resuspended in 1 ml/g of 100 mM Tris acetate, pH 7.9, 50 mM potassium acetate, 10 mM MgSO_4, 5 mM DTT and the same protease inhibitors listed above. The extract was then dialyzed extensively against the same buffer lacking all the protease inhibitors except PMSF until the conductance of a 1:200 dilution was below 100 microsiemens/cm. Protein concentrations were initially measured as described by Bradford (27). The dialyzed extract was stored in aliquots in liquid nitrogen and could be thawed and frozen repeatedly.

Transcription Reactions—The transcription reactions were initially based on those reported by Lue et al. (23). The template bears no guanosine in the transcribed portion. Omission of GTP during the reaction and subsequent treatment with RNase T1 greatly reduce the background of nonspecific transcription. 30-μl reactions contained 0.6 μg of template DNA (pGAL4CG-), 0.5 unit of Inhibit-ACE (5'-3'), 0.5 μl of GAL4-VP16 fusion protein, 50 mM HEPES, pH 7.6, 20% (v/v) glycerol, 10 mM EGTA, 10 mM MgSO_4, 5 mM DTT and the same protease inhibitors listed above. The extract was then dialyzed extensively against the same buffer lacking all the protease inhibitors except PMSF until the conductance of a 1:200 dilution was below 100 microsiemens/cm. Protein concentrations were initially measured as described by Bradford (27). The dialyzed extract was stored in aliquots in liquid nitrogen and could be thawed and frozen repeatedly.

Template concentration had only a small effect within the range of 20-50 pg/ml. The level of stimulation held constant over a further 10-fold increase in GAL4-VP16 protein. In addition, each preparation of the GAL4-VP16 fusion protein had to be titrated in the reactions. Optimal amounts of template/reaction and one whole cell extract at 4.7 mg/ml. The level of stimulation held constant over a further 10-fold increase in GAL4-VP16 fusion protein.

We optimized conditions further using saturating amounts of one fusion protein preparation at approximately 80 ng of protein/reaction and one whole cell extract at 4.7 mg/ml. Template concentration had only a small effect within the range of 20-50 μg/ml (data not shown). Transcription was significantly reduced when template was below 20 μg/ml. In Fig. 2 we show the result of varying the magnesium concentration; the optimal range was between 15 and 19 mM magnesium acetate. This range is higher than that reported for the nuclear extract (20) but similar to the values reported for the whole cell extract (21). The determination of the nuclear extract, fractions (1 and 5%, Fig. 1) of the nuclear extract reaction were compared with the entire whole cell extract reaction. From these data, measurements of protein concentrations, and calculations of transcription efficiencies, we estimate that the whole cell extract was 25-40-fold less active than the nuclear extract in terms of the number of transcripts per μg of protein.

Optimization of the Reaction Conditions—The original assay conditions were based on those of Lue et al. (23) which were derived from those described by Tyler and Giles (28) for Neurospora whole cell extracts. We have varied several parameters of the assay, the most important being template and extract concentrations, and we have increased the overall activity of the reaction approximately 50-fold relative to the conditions used in Fig. 1. Preliminary tests revealed optimum conditions at 20°C (33) with sizes of 1425, 521, 516, 506, 396, 344, 298, 245, 221, 220, and 219 nucleotides. We optimized conditions further using saturating amounts of one fusion protein preparation at approximately 80 ng of protein/reaction and one whole cell extract at 4.7 mg/ml. Template concentrations had only a small effect within the range of 20-50 μg/ml. The level of stimulation held constant over a further 10-fold increase in GAL4-VP16 fusion protein.
FIG. 2. Magnesium optimum. Incubations were carried out at the indicated concentrations of magnesium acetate. Maximal incorporation of UMP was similar to that seen in Fig. 3B (30-min time point), approximately 10 fmol. Densitometry of autoradiographs (Kodak XRP film with intensifying screens) was used to generate this graph with the highest value set to 100%. Several different exposures were used to ensure linearity of the film's response. The extract contributed 0.6 mM magnesium to each reaction in the form of sulfate.

FIG. 3. Kinetic analysis of the transcription reaction. Panel A, 10-μl aliquots were removed from a larger reaction at the times indicated above each lane and processed as described. This panel shows the time course under conditions of 70 mM potassium acetate, 5 mM magnesium sulfate, and 5 mM magnesium acetate. A duplicate autoradiograph was used as a template to excise the bands from a dried gel. Panel B, radioactivity in gel slices was determined by scintillation counting and the results converted to femtomoles of UMP incorporated per 30-μl reaction. Open circles indicate reaction conditions which include 70 mM potassium acetate, 5 mM magnesium sulfate, and 5 mM magnesium acetate. Closed circles indicate the same reactions performed with 100 mM potassium glutamate and 15 mM magnesium acetate.

FIG. 4. Comparison of extracts from WHI1+ and WHI1-1 strains. 30-μl assays were analyzed as described under "Materials and Methods." The relevant genotype of the strain used to make each extract and the volume of extract used in a reaction are indicated above each lane; each extract was approximately 40 μg/ml protein. The transcripts are indicated by their sizes of 375 and 350 nucleotides. The material indicated by the arrow is larger than 1400 nucleotides and was variably present in reactions incubated with nuclear and whole cell extracts; the labeled material is sensitive to RNase A and resistant to DNase and to 10 μg/ml α-amanitin (see Fig. 1).

At optimal extract, template, and magnesium concentrations, the potassium concentration exhibited a broad optimum. Tests of various counterions indicated that potassium acetate from 50 to 100 mM and potassium glutamate from 70 to 120 mM were virtually indistinguishable. Potassium chloride above 20 mM inhibited the reaction, and ammonium sulfate could not substitute for a potassium salt (data not shown). In all of the reactions shown in this work, the labeled nucleotide (UTP) was at 1 μM. Analysis of early time points by densitometry of autoradiographs has revealed continued increases in transcription in response to increasing UTP concentrations, with 50 μM UTP (the apparent Kₘ for the selective transcription reaction) supporting approximately 30-fold higher rates of synthesis than 1 μM UTP (data not shown).

Time Course of the Transcription Reaction—A comparison of reaction kinetics under the original and optimal mono- and divalent cation concentrations is shown in Fig. 3. Incorporation was linear for 15 min and reached a maximum by 20–40 min. The approximately 50–100 attomol of transcripts produced were largely stable for an additional 50 min. A third transcript of approximately 400 nucleotides was also visible in the later time points of Fig. 3A. This transcript was large enough to have traversed the entire G-minus cassette. Appearance of this transcript was variable but sensitive to 10 μg/ml α-amanitin. Lue et al. (23) report that a transcript of similar size seen in reactions with nuclear extract is suppressed by inclusion of the chain terminator 3'-O-methyl GTP, suggesting readthrough from an upstream initiation site.

Extract Production Is Not Strain-dependent—We originally made a whole cell extract from a WHI1-1 mutant (BF338-9b), reasoning that the reduced cytoplasmic volume of these cells (30) might improve the recovery of transcription factors. We later tested wild-type WHI1+ cells, hoping to produce extracts from currently available mutants without requiring introduction of the WHI1-1 allele. Indeed, extracts made in parallel from the wild type and the mutant were both func-
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