Preparation and characterization of yeast nuclear extracts for efficient RNA polymerase B (II)-dependent transcription *in vitro*

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ABSTRACT

We present a reproducible method for the preparation of nuclear extracts from the yeast *Saccharomyces cerevisiae* that support efficient RNA polymerase B (II)-dependent transcription. Extracts from both a crude nuclear fraction and Percoll-purified nuclei are highly active for site-specific initiation and transcription of a G-free cassette under the Adenovirus major late promoter. At optimal extract concentrations transcription is at least 5 times more efficient with the yeast extracts than with HeLa whole cell extracts. We show that the transcriptional activity is sensitive to α-amanitin and to depletion of factor(s) recognizing the TATA-box of the promoter. The *in vitro* reaction showed maximal activity after 45 min, was very sensitive to Cl^-, but was not affected by high concentrations of potassium. We find that the efficiency of *in vitro* transcription in nuclear extracts is reproducibly high when spheroplasting is performed with a partially purified β 1,3-glucanase (lyticase). Therefore a simplified method to isolate the lyticase from the supernatant of *Oerskovia xanthineolytica* is also presented.

INTRODUCTION

The regulated expression of genes is an essential feature of all living organisms. In eukaryotes, numerous studies have addressed the mechanisms and factors involved in the regulation of transcription (for recent reviews see 11, 16, 17, 22). An important breakthrough in the understanding of these basic mechanisms was the development of *in vitro* transcription systems from a variety of different organisms: *Drosophila* (14), *Neurospora* (15), chick oviduct (19), silkworm (20), plants (7) and cultured human cell lines (12, 13). Agreement between the initiation sites found *in vivo* and *in vitro* (reviewed in 23, 24) underscores the utility of such *in vitro* systems for the study of interactions between promoter elements and protein factors or RNA polymerase B (II). Such systems also allow the study of tissue-specific gene expression (21).

For many years, studies of transcription in yeast have been limited by the lack of an *in vitro* transcription system. Only recently were extracts developed for *Saccharomyces cerevisiae* that specifically transcribe a given template *in vitro* (2). An important contribution to these studies was the use of a guanine-free cassette, first introduced for the HeLa system (3, 8), which allows a high signal-to-noise ratio, and eliminates non-specific transcripts. In the yeast system, variability and low efficiency of RNA polymerase B (II)-dependent transcription has been observed in many nuclear extracts, perhaps due to the more arduous task of isolating clean, intact yeast nuclei. For this reason we present in this publication new advances in the preparation of a yeast transcription system, based largely on the efficient spheroplast formation of *Saccharomyces cerevisiae* cells by lyticase.

The lyticase activity contains a β 1,3-glucanase and an alkaline protease, purified from the culture medium of *Oerskovia xanthineolytica* (27, 28). The glucanase activity attacks β 1,3-glucan in a random endolytic fashion releasing oligosaccharides, whereas the alkaline protease degrades the protein portion of the cell wall mannoproteins (6). The isolation method, modified from that of Scott and Schekman (6), yields a high lytic specific activity, superior to commercial preparations, especially with regard to nuclease and protease contaminations. Spheroplasts formed under these conditions can be lysed with no loss in the integrity of nuclear structure. We show that nuclear extracts prepared with this method efficiently transcribe a G-free cassette *in vitro* in a site- and promoter-specific manner, dependent on a TATA-binding factor and RNA polymerase B.

MATERIALS AND METHODS

Strains, plasmids and media

The *Saccharomyces cerevisiae* strain C13-ABYS86 (*pra1-1, prb 1-1, prc1-1, cps1-3, ura3*Δ, leu2-3, Δ2-112, his) lacks the

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major vacuolar proteinases and was a kind gift of D. Wolf. Strain 62-5c (MATa, *leu2, his3, lys2, pep4-3*) was kindly provided by H. Riezman. The *pcr*1 and *pep4* phenotypes were regularly tested by a plate-stain assay for carboxypeptidase Y activity (25). The medium used for yeast growth (YPAD) was prepared as described by Sherman *et al.* (5).

The β 1,3-glucanase activity (lyticase) was purified from the culture medium of *Oerskovia xanthineolytica*. The *O. xanthineolytica* strain (obtained from R. Schekman) was grown in M 63 minimal medium (Sistrom's medium) (6, 9) containing 4 g/l glucan, and 1 mg/l each biotin and thiamine. Precultures were grown in the same medium containing 0.4% glucose instead of glucan.

The plasmids $pML(C_2AT)_{19}$ and $p(C_2AT)_{19}$ were obtained from M. Sawadogo, and the plasmids pAlbumin and pT-kinogen were gifts of F. Sierra and R. Walter. These contain 676 bp of the promoter of the mouse albumin gene and nearly 1 kb of the promoter of the rat T-kinogen gene, upstream of G-less cassettes. Plasmid DNA preparation was done by standard methods (4).

Glucan preparation

Insoluble glucan was used as the sole source of carbon to induce production of lyticase by *O. xanthineolytica*. Glucan was prepared from 1 kg yeast as follows: commercially available baker's yeast was suspended in water and centrifuged for 10 min at 5000×g. The cell pellet was suspended to 800 ml 6% NaOH and shaken for 60 min at 75°C. Cold water was then added to a final volume of 2.4 l and the suspension was centrifuged at 12 200×g for 20 min. The supernatant was discarded and the pellet was suspended in 800 ml 3% NaOH and incubated for 60 min at 75°C with agitation. The solution was again diluted, the pellet was recovered by centrifugation as above, suspended once more in 800 ml 3% NaOH, and incubated at 75°C for 60 min. The suspension was then adjusted to pH 7 with HCl, diluted to 2.4 l with water and centrifuged at 12 200×g for 15 min. The glucan pellet was then washed twice with absolute ethanol and once with ether. The pellet was dried completely and ground to a fine powder with mortar and pestle. The yield was 50 to 60 g of glucan per kg of yeast.

Lyticase preparation

The lyticase preparation described by Scott and Schekman (1980) was modified in the following manner. A 15 l culture of *O. xanthineolytica* was grown in a fermentor at 30°C as described above. The activity of the lyticase in the culture supernatant was assayed at intervals using the lytic assay of Scott and Schekman (6). The substrate for this assay was the *S. cerevisiae* strain 62-5c grown to an absorbance of 1 at 600 nm.

The *Oerskovia* culture was harvested when the activity had reached a plateau, after approximately 24 h. The culture medium was separated from the cells by ultrafiltration using a Millipore PDGF filter and the medium was chilled and kept at 4°C for all subsequent steps. It was concentrated 60 fold by ultrafiltration through a 10 kDa cut-off membrane and the retentate was dialysed overnight against 10 mM sodium succinate pH 5.0, 0.002% NaN_3. The dialysate was then loaded onto a CM-52 cellulose column (50 cm in length, 3 cm diameter) equilibrated with 10 mM sodium succinate pH 5.0, 0.002% NaN_3, and the column was washed extensively with the same buffer. Lyticase activity was eluted with the same buffer containing 0.25 M NaCl. The active fractions were pooled and concentrated by dialysis against solid polyethylene glycol 6000 until the volume was reduced to 40–50 ml. The preparation yields about $3-5\times10^6$ units and was kept at 4°C up to one year in the presence of NaN_3. The final enzyme preparation had a lyticase activity of more than 100 000 U/ml. One unit of lyticase is the amount of enzyme necessary to induce a decrease of 10% in the absorbance at 600 nm of a yeast cell suspension (at A600 = 1) in 30 min at 30°C (6).

Isolation of yeast nuclei

Nuclei were isolated according to Amati and Gasser (1988) with modifications mainly in the spheroplast formation step. These modifications are crucial for the reproducible production of nuclear extracts used for *in vitro* transcription. Cells were grown at 30°C to a density of $2-3\times10^7$ cells/ml. They were harvested by centrifugation at 1800×g for 5 min at room temperature, washed once in distilled water and resuspended at 10 ml per g wet cells in 0.1 M K-EDTA, pH 8.0, 10 mM DTT. After incubation for 10 min at 30°C with gentle agitation, the cells were recovered by centrifugation and then thoroughly resuspended in YPD containing 1 M sorbitol at 10 ml per g wet cells. Spheroplasting was initiated by adding 2000 U/ml lyticase (prepared in our laboratory, see above) and 10 μg/ml Zymolyase 100T (Kirin Breweries). Spheroplast formation was followed in the light microscope and was normally completed after 30 min incubation at 30°C with gentle agitation.

Spheroplasts were collected by centrifugation at 1000×g for 10 min at room temperature. They were resuspended at 10 ml/g wet cells in YPD containing 1 M sorbitol. The cells were allowed to recover for 30 min at 30°C with gentle agitation. PMSF was then added to 0.5 mM and spheroplasts were recovered by centrifugation at 1000×g for 10 min at room temperature. After washing gently once in 10 ml/g of YPD with 1 M sorbitol, cells were homogenized on ice by twenty to thirty strokes in a tight Dounce homogenizer in 18% Ficoll, 5 mM Tris-HCl pH 7.4, 20 mM KCl, 2 mM EDTA-KOH, pH 7.4, 0.125 mM spermidine, 0.05 mM spermine, 300 μg/ml benzamidine (Boehringer), 1 μg/ml pepstatin, 2 μg/ml antipain (or Trasylol, Bayer), 0.5 mM PMSF, 1% thiodiglycol (Pierce). Lysis was checked in a light microscope.

A crude nuclear fraction was recovered by differential centrifugation. The homogenate was centrifuged at 5000×g for 12 min at 4°C. The supernatant was recovered and centrifuged once more under the same conditions. These pellets are discarded and the crude nuclear pellet was formed by centrifugation of the supernatant at 24 000×g for 15 min at 4°C. An estimate of the yield of nuclei can be made by reading the absorbance at 260 nm of the nuclear suspension diluted 100-fold in 1% SDS. From 600 to 900 A_{260} units are obtained from 20 g wet cells. Where indicated the nuclei were further purified over a 33% Percoll gradient as described by Amati and Gasser (1988), from which 300 to 500 A_{260} units of nuclei are obtained. While this preparation has been successfully used for a wide variety of haploid strains, centrifugation speeds must be lowered for diploid cells.

Preparation of transcription extract

The transcription extract was prepared essentially according to Lue and Kornberg (2). All following procedures were performed on ice. Crude nuclear pellets or the Percoll-purified nuclei derived from about 20 g of cells, were suspended in 100 mM Tris-acetate pH 7.9, 50 mM potassium acetate, 10 mM $MgSO_4$, 20% glycerol, 3 mM DTT, 2 mM EDTA-KOH pH 7.9, 0.5 mM PMSF, 300 μg/ml benzamidine, 1 μg/ml pepstatin, 2 μg/ml antipain, 0.3 μg/ml leupeptin, and 2 μg/ml chymostatin to 0.6

ml/g wet cells. 4 M $(NH_4)_2SO_4$ was then slowly added to give a final concentration of 0.9 M, the suspension was slowly stirred for 30 min and centrifuged at 215 000×g for 30 min. The supernatant was adjusted to 75% of saturation by adding solid $(NH_4)_2SO_4$ (0.35 g/ml) and neutralized by adding 10 µl 1 M KOH/g $(NH_4)_2SO_4$. The suspension was slowly agitated for 30 min and centrifuged at 165 000×g for 30 min. The pellet was solubilized at a concentration of 10–20 mg protein per ml in 20 mM HEPES-NaOH pH 7.6, 10 mM $MgSO_4$, 10 mM EGTA, 20% glycerol, 5 mM DTT, 0.5 mM PMSF and dialysed against the same buffer. The extract was frozen in liquid N_2 and stored at −80°C. In some cases chymostatin and leupeptin were omitted with no obvious loss in transcription activity.

In vitro transcription

Transcription reactions were carried out using the G-less cassette plasmids (3) in the presence of the chain terminator 3′-O-methyl GTP and RNase T1 in order to avoid read-through from promoters upstream to the Adenovirus major late promoter and to eliminate non-specific transcription products. The standard reaction mixture contained in a final volume of 25 µl buffer E (20 mM HEPES-NaOH pH 7.6, 160 mM KOAc, 12 mM PEP, 1 mM DTT, 0.1 mM K-EDTA, 10% glycerol, 0.25 mg/ml BSA), 5% PEG 6000, $pML(C_2AT)_{19}$ or other plasmid as indicated (0.75 µg), protease inhibitor mix (0.3 mg/ml benzamidine, 1 µg/ml pepstatin A, 0.3 µg/ml leupeptin, 2 µg/ml chymostatin, 0.5 mM PMSF, 2 µg/ml antipain), pyruvate kinase (0.5 µg), 3′-O-methyl-GTP (100 µM), RNasin (0.8 U), RNase T1 (1 U), nucleotide mix (500 µM ATP, 500 µM CTP, 25 µM UTP, 1.25 µCi {α-^{32}P} UTP (400 Ci/mmol, Amersham), 60 µg protein of the crude nuclear extract, and was completed to the final volume with DEPC-treated water. The transcription reaction was stopped after 45 min incubation at 22°C by addition of 300 µl (250 mM NaCl, 1% SDS, 20 mM Tris/HCl pH 7.5, 5 mM EDTA). To this solution, 40 µg Proteinase K was added and incubated for 30 min at 37°C. The RNA was then phenol and chloroform extracted and precipitated with ethanol. After centrifugation, the resulting pellet was washed once with 500 µl 70% ethanol, dried, and resuspended in 90% deionized formamide, heat-denatured, and loaded on a 5% sequencing gel. HeLa whole cell S-100 extracts prepared according to Moncollin *et al.* (31) were kindly provided by V. Moncollin.

Western blots

SDS gels were run according to standard procedures (see 1). The anti-ABF1 antibody was a kind gift of Dr. Peter Rhode (Pasadena), while the other rabbit immune sera were raised in our own laboratories.

RESULTS

Yeast nuclear isolation

A key feature of the extract preparation described here is the integrity of the isolated yeast nuclei. This is in part due to the use of purified lyticase (see Materials and Methods), rather than the more commonly used Zymolyase for spheroplast formation. High levels of nuclease and protease activities in commercially available Zymolyase appear to restrict the efficiency of the transcription extracts. Nuclei isolated as described here have been used successfully for structural studies and have a high level of run-on transcription activity in intact nuclei (data not shown). Consistently, we see a strong enrichment for RNA polymerase B (II) in the nuclear pellet (see lane CN, Figure 1A). In this gel the largest subunit of RNA polymerase B runs at 220 kDa, while the smaller bands consist of breakdown products and other subunits of the holoenzyme (Figure 1A). After extraction of either the crude nuclear pellet or Percoll-purified nuclei with ammonium sulfate, RNA polymerase B is quantitatively recovered in the extracts (compare CNEx with P, PNEx with P′, Figure 1A).

We have also assayed the efficiency of recovery of two abundant nuclear transcription factors, RAP-1 (also called TUF and GRF-1) and ABF-1 (also called GF-1, BAF-1 and SBF-B). These are recovered efficiently in both the crude and the Percoll-purified nuclear fractions (Figure 1B, C, lanes 2–5). Quantitation of the Western blots estimates the copy number of both RAP-1 and ABF-1 at roughly 10^4 molecules/nucleus. The specific enrichment of these proteins in the Percoll-purified nuclei parallels the enrichment for topoisomerase II (Figure 1D), and is an indication that nuclei stay intact throughout the preparation with little loss of abundant nuclear factors. For both ABF-1 and RAP-1 at least 90% of the protein in the nuclear fraction is recovered in the transcription extracts, while for Topoisomerase II, less than 20% is extracted by this procedure (lanes 5–8, Figure 1D).

In vitro transcription assay

Extracts were prepared both from the crude nuclear pellet and from nuclei purified over a Percoll gradient. Transcripts generated during incubation with plasmid $pML(C_2AT)_{19}$ were analyzed by polyacrylamide gel electrophoresis. Consistently two major transcripts between 350 and 370 nucleotides long were seen (Figure 2, lanes 1–8). Although some yeast strains yield extracts more active than others, in general the yeast nuclear extracts prepared as described here transcribed the template nearly ten times more efficiently than HeLa whole cell extracts (compare Figure 2 lanes 1–4 and 9, 10). Efficiency was calculated by the total radioactivity incorporated into transcripts using a fixed concentration of template and optimal extract concentrations. Background was determined by the incorporation using a control template in the presence of α-amanitin. The calculated efficiency of the yeast extract is 3×10^{-3} transcripts per template. This compares favorably with the maximal transcription levels (10^{-4} to 10^{-3} transcripts per template) reported previously for yeast extracts (33).

Extracts prepared from Percoll gradient nuclei were also more efficient than the HeLa extracts tested, yet showed a weaker signal per µg protein than the crude yeast nuclear extracts (Figure 2, lanes 1–4 compared with 5–8). This may reflect the fact that the nuclear protein was generally more dilute during the extraction procedure (roughly four-fold), possibly leading to disruption of important complexes. Despite the efficient recovery of RAP-1, ABF-1 and RNA polymerase B in the Percoll-purified nuclei, we can not rule out loss of certain other soluble components that augment transcription efficiency, during the Percoll purification. Proteins such as RAP-1, which are highly sensitive to proteases, are not significantly more degraded after Percoll purification (Figure 1B), thus degradation does not seem to be the cause of its lower efficiency. Titrations of different extracts show that a protein concentration between 60 to 80 µg per assay is optimal for maximal transcription efficiency.

Specificity of the *in vitro* transcription

The *in vitro* reaction showed a high sensitivity to α-amanitin at 10 µg/ml, a concentration that inhibits RNA polymerase B (Figure 3). This strongly suggests that the observed signals derive from

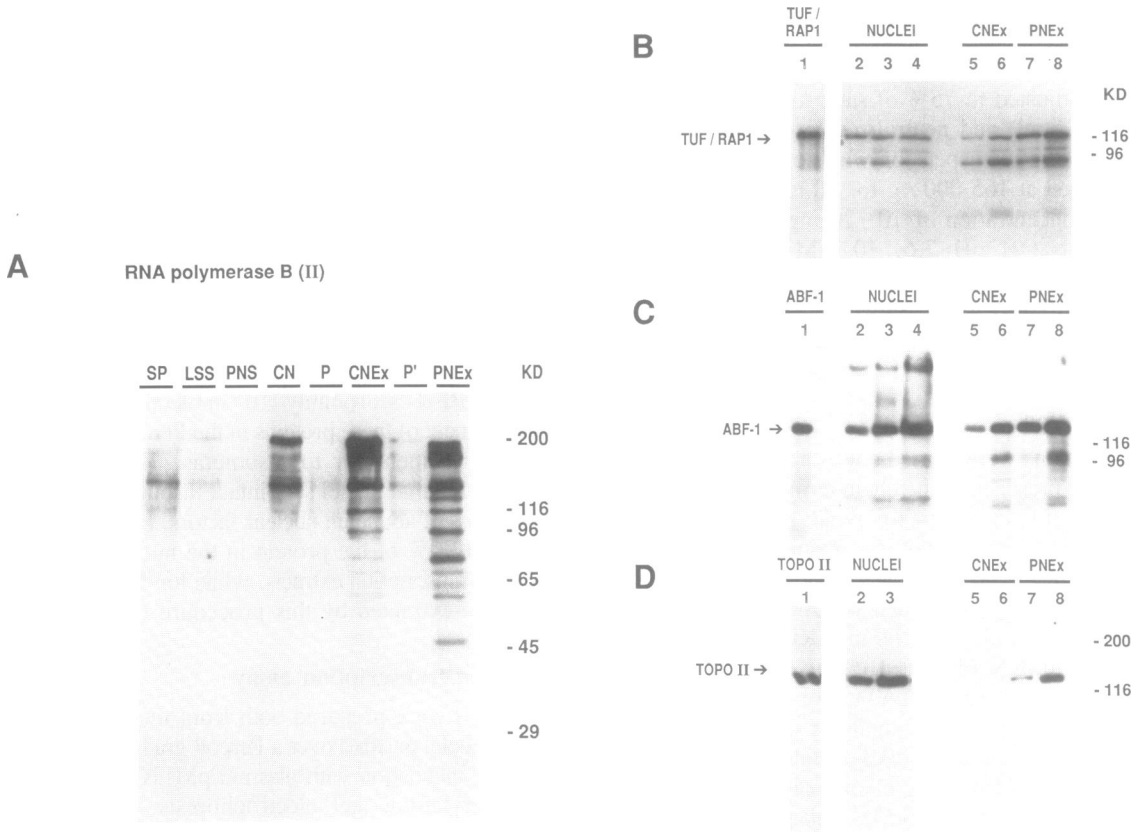

Figure 1. A. RNA polymerase B is enriched in the nuclear fractions and is quantitatively recovered in extracts. Yeast cells were subfractionated as described and fractions were denatured in SDS for gel electrophoresis. 100 μg of total spheroplast protein (SP), the low-speed supernatant after lysis (LSS), post-nuclear supernatant (PNS), crude nuclear pellet (CN), the ammonium sulfate extract from crude nuclei (CNEx), that from Percoll-purified nuclei (PNEx) and equal proportions of the remaining pellet of crude nuclei (P) and Percoll-purified nuclei (P') were run on a 7.5–15% polyacrylamide gradient gel. This was transferred to nitrocellulose and reacted with anti-RNA polymerase B antiserum and iodinated secondary antibody. The large subunit of RNA polymerase B is the slowest migrating band at 220 kDa, while the others are most probably breakdown products and other subunits of the holoenzyme. A 1 M urea wash was used on the blot to remove non-specific interactions of the antibody. **B.–D.** RAP-1 (TUF) and ABF-1 are recovered in nuclei and in nuclear extracts. In each panel Percoll-purified nuclei, crude nuclear extracts and Percoll nuclear extracts prepared as described in Materials and Methods, were run on 9% polyacrylamide gels, transferred to nitrocellulose and reacted with affinity purified antibodies (panel B, anti-RAP1; panel C, anti-ABF1; panel D, anti-Topoisomerase II). Autoradiographs are shown. Quantitation of the blots was done by directly counting the ^{125}I emission from the secondary antibody in cut nitrocellulose bands. In the case of RAP-1 the major breakdown products at 105 kDa and 92 kDa were included in the quantitation. Lane 1 of panel B contains 140 ng of purified RAP-1, lanes 2, 3, and 4 contain 0.2, 0.6 and 2 A_{260} units of purified nuclei, respectively; lanes 5 and 6 contain 11 and 55 μg protein of crude nuclear extract, derived from 0.24 and 1.2 A_{260} units of crude nuclei. Lanes 7 and 8 contain 21 and 62 μg protein of Percoll nuclear extract, respectively, derived from 1 and 3 A_{260} units of Percoll-purified nuclei. In panel C, lane 1 contains 100 ng of purified ABF-1, lanes 2, 3, and 4 contain 0.5, 1 and 1.5 A_{260} units of purified nuclei, and lanes 5 through 8 are exactly equivalent to those in panel B. In panel D, lane 1 contains 160 ng of purified yeast topoisomerase II, lanes 2 and 3 contain 1 and 2 A_{260} units of purified nuclei, respectively. Lanes 5 and 6 contain 73 and 240 μg protein of crude nuclear extract, respectively, derived from 1.6 and 5 A_{260} units of crude nuclei. Lanes 7 and 8 contain 8 and 72 μg protein of Percoll nuclear extract, derived from 0.8 and 3 A_{260} units of starting Percoll-purified nuclei. Molecular weight markers are indicated on the right.

transcription by RNA polymerase B. With yeast nuclear extracts, the initiation site of transcription was found to be 30 to 40 nucleotides downstream from the initiation site used in the HeLa whole cell extracts (Figures 2 and 3). This observation agrees with previous results obtained *in vivo* and *in vitro* (8, 18).

The initiation of transcription was totally dependent on the presence of the Adenovirus major late promoter. Plasmids lacking these sequences [$p(C_2AT)_{19}$] or containing other mammalian promoters that are tissue-specific (pAlbumin and pT-kinogen), did not allow efficient site-specific initiation of transcription (Figure 3). Weak transcription signals were detectable for these latter promoters in the yeast extract (lanes 7 and 9, Figure 3), yet the fact that the transcript observed with the pAlbumin template has the same size as the HeLa control (Figure 3, lane 6) suggests read-through transcription rather than a specific initiation. In the case of pT-kinogen, the presence of bands with the size expected for the specific transcripts (Figure 3, lane 9) suggests weak binding of yeast trans-acting factors on this rat promoter.

The Adenovirus major late promoter contains binding sites for the yeast factor UEFy (CP-1 or CBF-1), which is an abundant nuclear factor thought to activate genes involved in methionine metabolism. CP-1 is present in the nuclear extracts and its depletion abolishes transcription for the Adenovirus major late promoter (32). The absence of transcription from the other promoters is probably due to the fact that no yeast factors have high-affinity binding sites in these promoter sequences. Similarly, the tissue-specific promoters were not transcribed to a significant level in HeLa cell extracts (lanes 8 and 10, Figure 3).

Dependence on TATA-box factor

To show the dependence of the transcription on a TATA-box factor, we performed competition experiments using

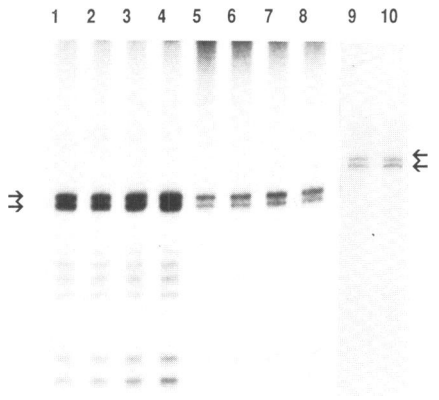

Figure 2. Transcription efficiency of yeast nuclear extracts. Crude nuclear extracts from 62-5c (lanes 1–4) and extracts from Percoll gradient purified nuclei (lanes 5–8), and HeLa whole cell extracts (lanes 9–10) were tested for transcription of pML$(C_2AT)_{19}$. The transcription reactions shown in lanes 1–10 were done in duplicate. *In vitro* transcription used standard conditions as described in Materials and Methods and contains 60 μg protein from the yeast extracts. Lanes 1, 2, 5 and 6 contained 0.31 μg template instead of the standard 0.75 μg. The two arrows left of lane 1 indicate the position of the two major transcription products obtained with the yeast extracts, and the two arrows to the right identify the two major transcripts in the HeLa extract. The gel was exposed to autoradiography for one hour at −70°C with an intensifying screen.

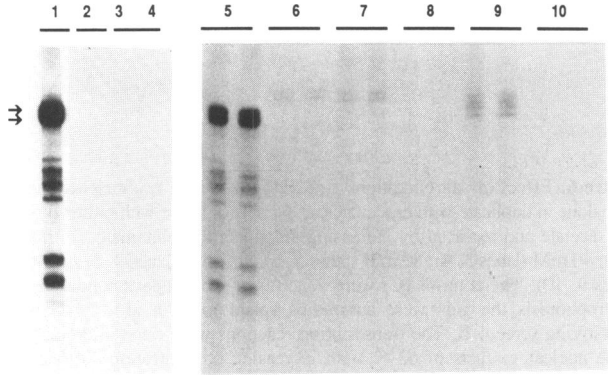

Figure 3. Transcription is dependent on the Adenovirus major late promoter and is α-amanitin-sensitive. Crude nuclear extracts from 62-5c were tested for *in vitro* transcription using plasmid pML$(C_2AT)_{19}$ (lanes 1–2) or p$(C_2AT)_{19}$ (lanes 3–4) as a template, in the presence (lanes 2 and 4) or in the absence (lanes 1 and 3) of α-amanitin (10 μg/ml). In addition plasmids containing tissue-specific promoters for mouse albumin (pAlbumin, lanes 7 and 8) and rat T-kinogen gene (pT-kinogen, lanes 9 and 10) were used in the standard transcription reaction. The reaction using pML$(C_2AT)_{19}$ as a template is shown for both yeast (lanes 5) and HeLa extracts (lanes 6). The mouse and rat promoters gave negative results both in yeast extracts (lanes 7, 9), and in HeLa extracts (lanes 8, 10). The latter reactions were done in duplicate, resulting in two samples for each condition described. Gels were exposed to autoradiography for one hour at −70°C with an intensifying screen.

oligonucleotides containing the wild-type TATA-box from the U6 promoter region. When added to the nuclear extract, the wild-type TATA-box oligonucleotide inhibits transcription (lanes 2–6, Figure 4), while addition of an unrelated oligonucleotide had no inhibitory effect (lanes 7–11, Figure 4). As an additional control we have used an oligonucleotide from positions −53 to +7 of the Adenovirus promoter in which the TATA-box is replaced by a stretch of T-residues. In this case again no inhibition of

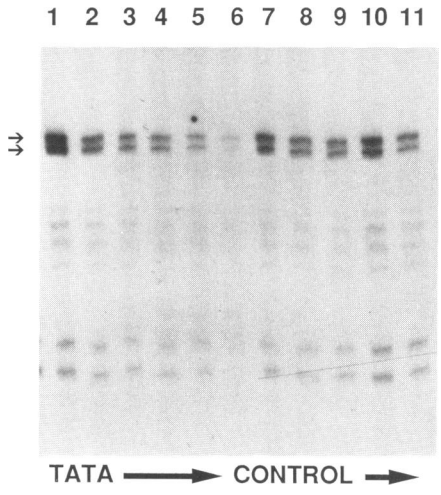

Figure 4. Transcription is inhibited by an excess of oligonucleotide containing a TATA-box. Transcription with the 62-5c crude nuclear extract was performed in the absence of added oligonucleotide (lane 1); in the presence of increasing amounts of TATA-box oligonucleotide (lanes 2–6), or unrelated nucleotide (lanes 7–11). Lanes 2–6 contain the oligonucleotide AAAAAACATTTATTTATA-CTAGCCGAAAA at 0.18 μg, 0.45 μg, 0.90 μg, 2.25 μg, and 4.50 μg respectively. Lanes 7–11 contain the oligonucleotide GAACAGTGGTAAAA-GTATTTCGT at 0.14 μg, 0.34 μg, 0.68 μg, 1.7 μg, and 3.4 μg, respectively. The gel was exposed to autoradiography for 1.5 hours at −70°C with an intensifying screen.

specific transcription was observed, while the nonmutated oligonucleotide blocked transcription (data not shown).

Effects of time and temperature and salt concentration

The transcription reaction was usually done at 22°C. Higher temperatures, such as 30°C or 37°C, resulted in partial or complete loss of transcription. While transcript amounts increased during 45 minutes of incubation at 22°C, Figure 5 shows that increasing the incubation time to 120 min did not substantially increase the amount of specific transcription products.

The yeast *in vitro* transcription was found to require potassium, and to be very sensitive to Cl^- and ammonium sulfate. This latter observation confirms results obtained earlier by Lue and Kornberg (2). Specific transcription was reduced drastically in the presence of 50 mM of KCl, NaCl or ammonium sulfate and was completely abolished with 100 mM of these different salts (not shown). In contrast, when Cl^- was replaced by other anions, such as acetate (not shown) or glutamate (lanes 3–10, Figure 6A), no inhibition of the transcription reaction was detected up to 160 mM K-acetate or 400 mM K-glutamate. The nature of the sensitivity to Cl^- or ammonium sulfate remains unclear. *In vitro* transcription with HeLa extracts is notably less sensitive to KCl (3).

Omission of potassium from the standard transcription buffer reduced transcription efficiency by at least 100 fold (Figure 6A, lanes 3–4). The optimal concentration for potassium was found to be 160 mM, but higher amounts did not affect the transcription. Transcription was reduced by 2 mM EDTA and completely inhibited by 4 mM (lanes 6–10, Figure 6B), indicating requirement for divalent cations. However, supplementing the transcription mixture with Mg^{2+} ions in the forms of Mg-acetate over the concentration already contributed by the nuclear extract (2 mM) did not increase the transcriptional efficiency (lanes 1–5, Figure 6B).

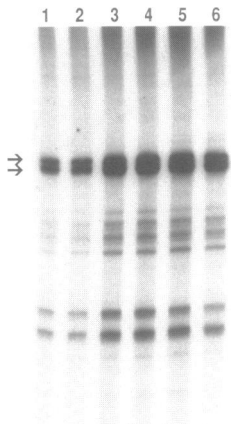

Figure 5. Transcription is maximal at 45 minutes. Transcription reactions were done in duplicate with crude nuclear extracts of strain 62-5c under standard conditions with variation in the incubation time. Samples were incubated at 22°C for 15 minutes (lanes 1, 2), 45 minutes (lanes 3, 4) and 120 minutes (lanes 5, 6).

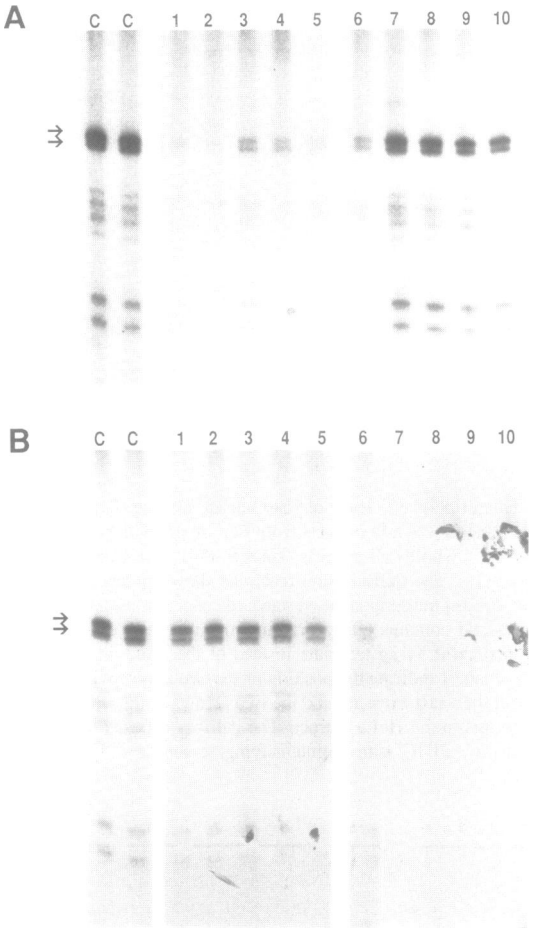

Figure 6. Effect of salt conditions and EDTA. **A.** The transcription reactions were done in duplicate with crude nuclear extracts of 62-5c with buffer B depleted of K-acetate and replaced by increasing amounts of K-glutamate: 0 mM (lanes 1, 2), 40 mM (lanes 3, 4), 80 mM (lanes 5, 6), 200 mM (lanes 7, 8) and 400 mM (lanes 9, 10). The controls (C) were done under the standard conditions. After electrophoresis the gels were autoradiographed for 1 h at −70°C with an intensifying screen. **B.** The transcription reactions were done in duplicate with crude nuclear extracts of 62-5c with increasing concentrations of Mg-acetate (lanes 1−5) or EDTA/K (lanes 6−10). Control mixtures contained 2 mM $MgSO_4$ contributed by the nuclear extracts. The Mg-acetate concentrations were 4 mM (lane 1), 6 mM (lanes 2, 3) or 12 mM (lanes 4, 5). EDTA concentrations were 2 mM (lane 6), 4 mM (lanes 7, 8) and 10 mM (lanes 9, 10).

DISCUSSION

Reliable and efficient *in vitro* transcription systems for RNA polymerase B are essential for the study of the molecular mechanisms of gene regulation. These are yet more powerful when combined with the genetic analysis available in yeast. Despite the obvious advantages of yeast for genetic approaches, cell fractionation and *in vitro* studies have generally lagged behind advances made in other organisms. The lack of an efficient and protease-free lytic enzyme for degradation of the yeast cell wall was an important limiting factor for spheroplasting and subsequent lysis of yeast cells. For these reasons we present in this publication a simplified method for the partial purification of lyticase from *O. xanthineolytica*. This activity is sufficiently depleted of proteases and nucleases that nuclear integrity is maintained during both spheroplasting and nuclear isolation. Transcription factors and RNA polymerase B are highly enriched in the yeast nuclear extracts described here.

We have prepared nuclear extracts from different protease-deficient yeast strains (see Materials and Methods), all of which are highly active for *in vitro* transcription. The variation in efficiency and yield of crude nuclear extracts may reflect the ease with which different yeast strains can be spheroplasted and lysed. Purification of nuclei over Percoll gradients prior to extraction did not result in loss of essential factors involved in transcription, yet this step did not enhance transcriptional efficiency. The major changes we found necessary to introduce for reproducible isolation of active transcription extracts are the methods of spheroplasting and nuclear purification, both described here in detail.

In our extracts an oligonucleotide containing a TATA-box competed with the template, diminishing the rate of transcription, and the reaction was shown to be sensitive to α-amanitin at 10 μg/ml. Together these results provide strong evidence that the observed signals derive from transcription by RNA polymerase B. Comparison with HeLa whole cell extracts shows that the initiation of transcription takes place in a species-specific rather than in a template-specific manner. That is, transcription of the Adenovirus major late promoter in yeast was found to start 30 to 40 nucleotides downstream from the initiation site used in HeLa whole cell extracts. This observation agrees with previous results obtained *in vivo* and *in vitro* (8).

Transcription experiments realized with the plasmid $p(C_2AT)_{19}$ showed that the transcription is dependent on the presence of the Adenovirus major late promoter. In control experiments using purified RNA polymerase B alone or together with TFIID, no signal was observed (data not shown). At the present time it is unclear which factor (s) of the transcription machinery determines the initiation point. It is evident, however, that the template DNA itself does not define the exact site of transcriptional initiation, since the same DNA is transcribed differently in the two systems tested. In addition, TFIID is not the determining factor, since replacing the TFIID of HeLa with the yeast analog resulted in transcription initiation at the HeLa-specific start site (18).

The transcription reaction in the yeast extract is very sensitive to Cl^-, whereas even in the presence of high amounts of potassium (400 mM) no inhibitory effect was observed. The

nature of this very pronounced Cl^- sensitivity remains unclear. *In vitro* transcription systems derived from plants also show a pronounced sensitivity to high amounts of Cl^- (7). In addition, the DNA polymerase III of *E. coli* is inhibited by Cl^-, but can tolerate high concentrations of glutamate (30). Supplementing the transcription with additional Mg^{2+} did not increase transcription, whereas EDTA strongly inhibited it. This shows that the reaction is strongly dependent on divalent cations, which in addition to Mg^{2+} could also be Mn^{2+} or Zn^{2+}, an essential cofactor for the activity of zinc-finger containing transcription factors *in vitro* and *in vivo* (7, 12). *In vitro* experiments using whole cell extracts from yeast showed an optimal Mg^{2+} concentration between 10–20 mM (29).

In our reaction the amount of DNA is not rate limiting, because a 2.5 fold increase of template did not result in an increased transcription efficiency. The reaction was saturated after 45 min of incubation, and breakdown products do not appear to accumulate as a function of time. The smaller transcripts may therefore reflect pause sites rather than degradation of the transcripts. Initiation at sites downstream of the major late promoter is unlikely as small RNA products were not seen with plasmids lacking the Adenovirus major late promoter. In view of this, we think that the amount of transcription product remains constant due to loss of activity of the nuclear extract and/or a limitation in the number of initiation cycles, rather than transcript degradation. Addition of exogenous non-specific DNA did not enhance transcription rates, nor did the addition of template DNA after 15 minutes, even though the template DNA is rapidly relaxed in the crude nuclear extracts.

The observed transcription is dependent on the nuclear factor UEFy (also termed CP-1 or CBF-1) which binds the upstream element in the major late promoter (32) and which can be replaced by the human homolog UEFh (32). This and other experiments (33, 34) show that the *in vitro* transcription system responds to trans-acting factors and underscores the conservation of fundamental aspects of transcription in eukaryotes. A similar observation has been made in an *in vitro* reconstitution experiment of a mammalian transcription system where TFIID was replaced by the yeast equivalent (18), and transcription initiation took place as expected for a typical mammalian promoter (26). The elucidation of the molecular mechanisms underlying transcriptional activation in yeast is now possible by fractionation and detailed analysis of these transcriptionally active nuclear extracts.

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ABBREVIATIONS

DEPC: diethylpyrocarbonate; EDTA: ethylenediaminetetraacetate; G-less: guanine less; kDa: kilodalton; PEG: polyethyleneglycol; PEP: phosphoenolpyruvate; PMSF: phenylmethyl-sulfonylfluoride; SDS: sodium dodecylsulfate; BSA: bovine serum albumin; DTT: dithiothreitol; HEPES: 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; KOAc: potassium acetate.

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