Accurate initiation at RNA polymerase II promoters in extracts from *Saccharomyces cerevisiae*

(*in vitro* transcription/*CYC1* and *PYK1* promoters/"TATA" sequences/ α -amanitin/RNA probes)

NEAL F. LUE AND ROGER D. KORNBERG

Department of Cell Biology, Stanford University School of Medicine, Fairchild Science Building, Stanford, CA 94305

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ABSTRACT A yeast nuclear extract supported transcription from the *CYC1* and *PYK1* promoters. Transcription was initiated *in vitro* at or near sites used *in vivo*. Deletion of "TATA" sequences abolished the reaction. α -Amanitin (10 μ g/ml) and chloride (100 mM) were highly inhibitory.

Studies of gene expression in yeast have been hampered by the lack of an *in vitro* transcription system for RNA polymerase II (EC 2.7.7.6). By contrast, extracts from a variety of higher cells will support transcription from RNA polymerase II promoters, allowing the protein factors and mechanisms involved to be investigated (1–4). The transcription reactions catalyzed by these extracts are characterized by initiation at the same sites *in vitro* as those used *in vivo*, by chain elongation for hundreds to thousands of residues, and by inhibition by α -amanitin at concentrations that inhibit purified RNA polymerase II but not RNA polymerase I or III. We report here on a transcription reaction, obtained with an extract from *Saccharomyces cerevisiae*, that exhibits the same characteristics.

Deletion analyses have revealed a similar structure of RNA polymerase II promoters in yeast and higher organisms. Elements of yeast promoters include the following: sequences located hundreds to thousands of base pairs (bp) upstream, which exert positive or negative regulatory effects—for example, upstream activating sequences (UASs) (5); "TATA" sequences, typically 50–100 bp upstream of the transcription start site, which are often essential for transcription (6, 7); and sequences around the start sites, which influence the precise location and frequency of initiation (7, 8). The transcription reaction described here is dependent on TATA sequences but not on regulatory sequences, in the single case so far examined. It should be possible, in future studies, to supplement the reaction with UAS-binding proteins and other components to reveal regulatory effects and reconstitute other features of the yeast transcription process.

MATERIALS AND METHODS

Yeast Strains and Plasmids. *S. cerevisiae* strains BJ926 and YM701 were provided by Elizabeth Jones and Mark Johnston (Washington University, St. Louis), respectively. The pCT family of plasmids (Fig. 1) contained a polylinker upstream of the *S. cerevisiae* *CYC1* promoter [nucleotides –248 to +5 in the numbering scheme of McNeil and Smith (7), lacking the *CYC1* UAS] fused to *lacZ* protein-coding sequences. These plasmids carried, in addition, *URA3* and *SUP11* markers, and *ARS1* and *CEN4* maintenance sequences. In the parental plasmid, pCT136 (a gift of Chris Traver, Stanford University), an oligonucleotide that binds GAL4 protein (9) was inserted between the *Bam*HI and *Eco*RI sites of the poly-

linker, conferring galactose-dependent expression of β -galactosidase *in vivo*. Deletion of the *GAL4* binding site, giving pCTA, reduced the level of expression by two orders of magnitude (A. Buchman, personal communication). In pCTpyk the *GAL4*-binding site was replaced by a 31-bp oligonucleotide containing a sequence derived from the region upstream of the *PYK1* gene [nucleotides –658 to –635 according to Burke *et al.* (10)]. Strains transformed with pCTpyk expressed 2- to 3-fold more β -galactosidase than those harboring pCT136 and grown in galactose (A. Buchman, personal communication). Deletion of a *Xho* I–*Sph* I fragment of the *CYC1* promoter (nucleotides –248 to –139) in pCTpyk gave pCTdp. Further deletion of 27 ± 3 bp of the *CYC1* promoter (nucleotides –138 to –112) with BAL-31 nuclease, removing the major TATA sequence of the promoter, gave pCTbal. Removal of the entire *CYC1* promoter from pCTpyk as an *Eco*RI–*Bam*HI fragment and insertion of an *Eco*RI–*Xba* I fragment containing the *PYK1* promoter (nucleotides –477 to +4) gave pCTPL. Fragments of pCTpyk, extending from *Bgl* I, *Pvu* I, and *Pvu* II sites in the *lacZ* region (11) to *Eco*RI, *Hind*III, and *Eco*RI in the polylinker, were inserted in pSP64, pSP65, and pSP64 (12), to give pSPCTB, pSPCTV, and pSPCTP, respectively. A fragment of pCTPL, extending from the *Bgl* I site in the *lacZ* region to the *Eco*RI site in the polylinker, was inserted in pSP64 to give pSPPL. A plasmid template for synthesis of a readthrough transcript of the *CYC1* promoter was constructed by inserting a fragment of pCTpyk, extending from the *Eco*RI site in the polylinker to the *Cla* I site in the *lacZ* region, into pSP65.

Nuclear Extract. BJ926 was grown in YPD medium (1% yeast extract/2% Bactopeptone/2% glucose) at 30°C to an OD₆₀₀ value of 5–10. Cells were harvested by centrifugation at 5000 $\times$ g for 10 min and converted to spheroplasts by a procedure due to M. J. Fedor (personal communication). The cells (30 g) were suspended in 200 ml of 50 mM Tris-HCl, pH 7.5/30 mM dithiothreitol, shaken slowly at 30°C for 15 min, centrifuged as before, resuspended in 30 ml of YPD medium containing 1 M sorbitol, and digested with 50 mg of Zymolyase 100T (Miles Laboratory), until the OD₆₀₀ in 1% NaDodSO₄ was less than 5% of the starting value. Digestion was stopped by the addition of 300 ml of ice-cold YPD medium containing 1 M sorbitol. The spheroplasts were recovered by centrifugation at 3000 $\times$ g for 5 min, washed with 300 ml of 1 M sorbitol, and lysed in 200 ml of 18% (wt/vol) Ficoll (Pharmacia)/10 mM Tris-HCl, pH 7.5/20 mM KCl/5 mM MgCl₂/3 mM dithiothreitol/1 mM EDTA/0.5 mM spermidine/0.15 mM spermine/1 mM phenylmethylsulfonyl fluoride/2 μ M pepstatin A/0.6 μ M leupeptin with a motor-driven Teflon/glass homogenizer. Unlysed spheroplasts and cell debris were removed by four or five centrifugations at 3000 $\times$ g for 5 min until a uniform white supernatant was obtained. Nuclei were recovered by centrifugation at 25,000

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Abbreviations: UAS, upstream activating sequence; GRFI, general regulatory factor I; nt, nucleotides.

FIG. 1. Templates and RNA probes. The pCT family of plasmids, whose construction is described in *Materials and Methods*, all carried a UAS (filled bar) from the region upstream of the *S. cerevisiae* *PYK1* gene inserted between the *Bam*HI and *Eco*RI sites of a polylinker containing the following restriction sites (clockwise): *Hind*III, *Sph* I, *Pst* I, *Sal* I, *Bam*HI, *Eco*RI, *Sac* I, *Kpn* I, *Sma* I, and *Xho* I. Members of the pCT family differed only in the yeast promoter region (densely stippled bar) to the right of the UAS in the diagram. The promoter and adjacent *lacI-lacZ* region (open bar), enlarged at the top of the diagram, contained various lengths of *CYC1* (pCTbal, pCTdp, pCTpyk) and *PYK1* (pCTPL) promoters. TATA sequences are indicated by filled bars within the promoter regions, and transcription start sites of the *CYC1* and *PYK1* genes *in vivo* are indicated by rightward-pointing arrows. RNA probes complementary to the expected transcripts, synthesized from the pSP family of plasmids, are indicated by leftward-pointing arrows. Restriction sites are abbreviated as follows: B, *Bgl* I; E, *Eco*RI; P, *Pvu* II; S, *Sph* I; V, *Pvu* I; X, *Xho* I.

× *g* for 30 min and suspended in 15 ml of 100 mM Tris acetate, pH 7.9/50 mM potassium acetate/10 mM MgSO_4 /20% (vol/vol) glycerol/3 mM dithiothreitol/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/2 μM pepstatin A/0.6 μM leupeptin. Ammonium sulfate (4 M) was added slowly to give a final concentration of 0.9 M, and the suspension was kept at 4°C for 30 min and centrifuged in a Beckman SW60 rotor at 40,000 rpm for 1 hr at 0°C. The supernatant was adjusted to 75% of saturation with ammonium sulfate by the addition of solid (0.35 g/ml) and then centrifuged in an SW60 rotor at 35,000 rpm for 30 min at 0°C. The pellet was suspended at a concentration of approximately 20 mg of protein per ml in 20 mM Hepes, pH 7.6/10 mM MgSO_4 /10 mM EGTA/20% glycerol/5 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/2 μM pepstatin A/0.6 μM leupeptin and dialyzed against the same buffer until the conductivity was less than 20 mS/cm. The extract was stored in liquid nitrogen and remained active in transcription assays for more than a month.

Transcription *in Vitro*. Reaction mixtures (50 μl) contained 10 mM Hepes at pH 7.6, 5 mM MgSO_4 , 5 mM magnesium acetate, 70 mM potassium acetate, 5 mM EGTA, 2.5 mM dithiothreitol, 4 mM phosphoenolpyruvate, 10% glycerol, 0.4 mM each of the four nucleoside triphosphates, 10 mg of protein per ml, and template DNAs as indicated. Reactions

were allowed to proceed at 25°C for 40 min and were stopped by the addition of 200 μl of 0.1 M sodium acetate/10 mM EDTA. The mixtures were extracted four times with equal volumes of phenol/chloroform (1:1, vol/vol), and nucleic acids were precipitated from the aqueous phase by the addition of ammonium acetate (to 2.5 M) and ethanol (2.5 vol). The nucleic acids were dissolved in 60 μl of 50 mM Tris·HCl, pH 7.5/10 mM MgCl_2 /1 mM EDTA, treated with 2 units of DNase I (RQ1 DNase; Promega Biotec, Madison, WI) at 37°C for 20 min, and extracted with an equal volume of phenol/chloroform.

Transcription *in Vivo*. YM701 was transformed with pCTdp, pCTbal, or pCTPL (13) and grown in SD medium (0.6% yeast nitrogen base/2% glucose) to an OD_{600} of 1 and total nucleic acids were isolated according to Elder *et al.* (14). The nucleic acids were processed as described for products of transcription *in vitro*, and 2–5 μg of the resulting RNA was used for RNA probe analysis.

RNA Probes. The pSPCT family of plasmids and pSPPL were linearized by digestion with *Eco*RI (pSPCTP, pSPCTB, and pSPPL) or *Hind*III (pSPCTV) and transcribed with SP6 RNA polymerase (New England Biolabs) in the presence of 12 μM [α - ^{32}P]GTP (410 Ci/mmol, Amersham; 1 Ci = 37 GBq) as described (12). Transcription products and probes (100,000 cpm) were combined, precipitated with ethanol, hybridized,

digested with RNases A and T1 and with proteinase K, and analyzed by gel electrophoresis as described (12).

RESULTS

Extract, Templates, and Probes. Our approach in developing an RNA polymerase II transcription system from yeast was to prepare nuclear extracts, use a promoter driven by a powerful UAS, and detect specific transcripts with highly radioactive RNA probes. In view of the small size of yeast nuclei (far smaller in relation to the cell volume than the nuclei of most higher cells), it seemed that nuclear extracts would be considerably enriched in transcription factors over whole cell extracts. This would be true unless the factors leaked out and were lost during isolation of the nuclei. Pilot experiments with general regulatory factor I (GRFI) (15) showed how such loss could be prevented. GRFI binds specifically to a DNA sequence that occurs upstream of many yeast genes and that functions as a UAS (ref. 15; A. Buchman, personal communication). The level of GRFI in a cell extract is readily determined in nitrocellulose filter-binding assays with 32 P-labeled UAS DNA. Upon lysis of yeast spheroplasts in physiologic salt solutions and centrifugation to pellet nuclei, GRFI is found entirely in the supernatant (no more GRFI is released by extraction with 0.3 M ammonium sulfate). By contrast, when lysis is performed in the presence of a polymer, such as 4% polyvinyl alcohol or 18% Ficoll [commonly used in the preparation of yeast nuclei (16, 17)], GRFI is retained in the pellet. Upon resuspending the nuclei in the absence of polymer and centrifuging again, all of the GRFI is released into the supernatant. Apparently, a polymer causes retention of GRFI in nuclei in a reversible manner, possibly through a macromolecular "crowding" or exclusion effect (18).

Nuclear extracts were prepared from *S. cerevisiae* strain BJ926, which carries mutations reducing the levels of nucleases and protease activities. Cells were converted to spheroplasts in growth medium, allowing fermentation to continue up to the time of lysis. Nuclei were washed in the presence of 18% Ficoll and extracted with ammonium sulfate by a modification of a procedure described for a transcription system from *Neurospora crassa* (19).

Transcription was carried out initially with templates comprising a GRFI-binding sequence from the region upstream of the *S. cerevisiae* pyruvate kinase gene, the TATA sequences and RNA start sites of the *S. cerevisiae* *CYC1* gene, and the *Escherichia coli* *lacZ* protein-coding sequence (Fig. 1). Such a template, incorporated in the centromeric plasmid pCTpyk, supported a high level of expression of β -galactosidase *in vivo*. Deletion of 106 bp between the GRFI-binding and TATA sequences, giving plasmid pCTdp, resulted in even higher expression (about 5-fold more *CYC1-lacZ* RNA and β -galactosidase than with pCTpyk) (A. Buchman and N.F.L., unpublished). The strength of the GRFI-binding sequence as a UAS *in vivo* and the abundance of GRFI in extracts motivated the choice of these plasmids as templates for transcription *in vitro*. It later emerged that the use of the *CYC1* promoter was the chief feature of the templates responsible for the success of the experiments.

Transcripts were detected by annealing with 32 P-labeled RNA probes, followed by RNase digestion and analysis of protected fragments in polyacrylamide gels. The probes were synthesized by SP6 RNA polymerase and were extended from *Bgl* I, *Pvu* I, and *Pvu* II sites in the *lacZ* sequence (Fig. 1, probes pSPCTB, pSPCTV, and pSPCTP, respectively) through the *CYC1* promoter to sites immediately upstream (pSPCTV) or downstream (pSPCTB and pSPCTP) of the UAS. The regions of the probes homologous to the expected transcripts were derived largely from *lacZ* sequences, dimin-

ishing the possibility of interference from transcripts of cellular genes present in the extracts.

Transcription Reaction. The products of transcription reactions with pCTdp as template protected fragments of probe pSPCTP about 150 residues in length, corresponding well with the distance of 152 bp from the major *CYC1* start site *in vivo* (7) to the *Pvu* II site in the *lacZ* sequence (Fig. 2). The protected fragments were clearly attributable to transcription *in vitro* by RNA polymerase II, since they were not found when template was omitted from the reaction or when α -amanitin was added at a concentration (10 μ g/ml) that selectively inhibits polymerase II. [Incorporation of ribonucleotides into trichloroacetic acid-insoluble form by purified yeast RNA polymerase II is 95% inhibited by α -amanitin at 10 μ g/ml, whereas RNA polymerase I activity is only 50% inhibited at 300 μ g/ml and RNA polymerase III activity is essentially unaffected (20).] Further evidence for accurate initiation at the major *CYC1* start site came from mapping transcription products with other probes and from comparison with *CYC1* RNA synthesized *in vivo* (Fig. 3). Protected fragments of approximately 275 and 245 residues were expected in the case of probes pSPCTB and pSPCTV, respectively, and fragments of these lengths were found for products of transcription both *in vitro* and *in vivo*. There were multiple protected fragments, indicative of multiple start sites, with some differences in location and relative amount between the transcripts made *in vitro* and *in vivo*.

A control experiment was performed to test for the artefactual appearance of fragments of the expected size, due to RNase digestion of hybrids of probes and readthrough transcripts at an internal location near the expected initiation site. RNA corresponding to the hypothetical readthrough transcript was synthesized with SP6 RNA polymerase, mixed

FIG. 2. Transcription from the *CYC1* promoter *in vitro*. Templates (numbers indicated times 0.25 μ g) were pCTdp (lanes 1–6, 9) and pCT Δ (pCTpyk from which the UAS was deleted by cleavage; lane 8). Some reaction mixtures contained α -amanitin (10 μ g/ml, lanes 4–6) or 150 ng of the GRFI-binding oligonucleotide also present as a UAS in the templates (lane 9). RNA probe was from pSPCTP. nt, Nucleotides.

FIG. 3. Transcription of the *CYC1* promoter *in vitro* and *in vivo*. Reactions *in vitro* were performed with 1 μ g of pCTdp in the presence (lanes 1, 2) or absence (lanes 3, 4) of α -amanitin at 10 μ g/ml. RNA made *in vivo* (lanes 5, 6) was from YM701 transformed with pCTdp. RNA probes were from pSPCTB (lanes 1, 3, 5) or pSPCTV (lanes 2, 4, 6).

with extract, deproteinized, annealed with probe pSPCTV, and digested and analyzed as described above. No fragments approximately 245 residues in length were observed (data not shown), ruling out the possibility of an RNase-sensitive region near the transcription start site.

Maximal transcription of the *CYC1* promoter was obtained with the most concentrated extracts, corresponding to about 10 mg/ml of protein in the reaction mixture. The optimal level of plasmid DNA was 20–40 μ g/ml; lower levels gave little of the desired transcript (Fig. 2), whereas higher levels gave excessive nonspecific transcription. Similar results were obtained with linear and circular plasmid templates. There were well-defined optima of pH (7.6), potassium acetate concentration (100 mM), and magnesium concentration (12 mM). Substitution of chloride for acetate inhibited transcription by at least 90%.

Dependence on Promoter Sequences. A number of findings demonstrated a lack of dependence of the transcription reaction on DNA elements upstream of the TATA sequence. First, deletion of the GRFI-binding site from pCTpyk had no effect on transcription (Fig. 2, lane 8). Second, the addition of a 50-fold molar excess of a GRFI-binding oligonucleotide (see *Materials and Methods*) as competitor was also without effect (Fig. 2, lane 9). Finally, the levels of transcription obtained with pCTpyk and the derivative pCTdp, lacking sequences between the GRFI-binding site and TATA sequence, were the same (not shown).

By contrast, transcription was completely dependent on TATA sequences. When the deletion in pCTdp was extended by 27 bp in pCTbal, removing the major TATA sequence of the *CYC1* promoter, transcription was abolished. No initiation at the *CYC1* start sites could be detected either *in vitro* or *in vivo* (Fig. 4).

Not all TATA sequences and start sites would support the transcription reaction. Transcripts were obtained from the *CYC1* promoter, as described, and also from the *PYK1* promoter, but not from the *GALI*, *ADH1*, or *DED1* promoters. A *PYK1* template, pCTPL (Fig. 1), was constructed by substituting a 481-bp fragment, containing the TATA and initiation sequences of the *PYK1* proximal promoter, for the *CYC1* promoter in pCTpyk. An RNA probe, pSPPL, extending from the *Bgl* I site in the *lacZ* sequence to the GRFI-binding site, was used for detection. A protected fragment of the probe about 264 residues in length was expected on the basis of mapping of the 5' ends of transcripts of the natural *PYK1* gene (8). Fragments of this length were obtained from transcription of pCTPL, both *in vitro* and *in vivo* (Fig. 5). The appearance of these fragments was dependent on template and was blocked by α -amanitin at 10 μ g/ml *in vitro*.

DISCUSSION

Our success in developing a yeast RNA polymerase II transcription system may be attributed to the following: the preparation of nuclear extracts, the choice of the *CYC1* promoter, detection by hybridization with uniformly labeled probes, and the use of acetate rather than chloride salts. The inhibitory effect of chloride may be worth investigating in other transcription systems.

FIG. 4. TATA dependence of transcription *in vitro* and *in vivo*. Reactions *in vitro* were performed with 1 μ g of pCTdp (lanes 1, 2) or pCTbal (lanes 3, 4) in the presence (lanes 2, 4) or absence (lanes 1, 3) of α -amanitin at 10 μ g/ml. RNA made *in vivo* was from YM701 transformed with pCTdp (lane 5) or pCTbal (lane 6). RNA probe was from pSPCTV.

FIG. 5. Transcription of the *PYK1* promoter *in vitro* and *in vivo*. Reactions *in vitro* were performed with (lanes 1–4) or without (lane 5) 1 μ g of pCTPL in the presence (lanes 3, 4) or absence (lanes 1, 2, 5) of α -amanitin at 10 μ g/ml. RNA made *in vivo* (lane 6) was from YM701 transformed with pCTPL. RNA probe was from pSPPL.

The correspondence of transcription start sites *in vitro* with those found *in vivo* and the α -amanitin sensitivity of the *in vitro* reaction are the main lines of evidence for the authenticity of the yeast RNA polymerase II transcription system. Even apparent incongruities, such as the slight differences in location and frequency of *CYC1* starts *in vitro* and *in vivo*, and the detection of transcripts from only two of five promoters tested, are in keeping with the behavior of other polymerase II transcription systems (20). For example, *in vitro* transcription of the simian virus 40 early promoter in HeLa cell extracts starts mainly downstream of the replication origin, whereas *in vivo* late in infection upstream start sites are preferentially utilized (21, 22). This difference is probably due to repression by tumor (T) antigen of downstream initiations. Comparable factors and effects will doubtless emerge to explain the vagaries of the yeast transcription system.

The low efficiency of the yeast transcription reaction (10^{-3} to 10^{-4} transcripts per template) may be due to limiting amounts of essential factors, and it could also reflect the weak constitutive activity of the promoters used in the absence of enhancement by a UAS. Some evidence for limiting components in the extract has come from apparent competition between the *CYC1* and *PYK1* promoters in mixing experiments (N.F.L., unpublished data). Nitrocellulose filter-binding assays and electrophoretic mobility shift analyses have revealed proteolysis of GRFI in the extract (A. Buchman, personal communication). It remains to enrich the various components and correct these deficiencies.

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