Reconstitution of Yeast RNA Polymerase I Transcription in Vitro from Purified Components

TATA-BINDING PROTEIN IS NOT REQUIRED FOR BASAL TRANSCRIPTION*

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Five purified protein components, RNA polymerase I, Rrn3p, core factor, TBP (TATA-binding protein), and upstream activation factor, are sufficient for high level transcription in vitro from the Saccharomyces cerevisiae rDNA promoter. Rrn3p and pol I form a complex in solution that is active in specific initiation. Three protein components, pol I, Rrn3p, and core factor, and promoter sequence to −38, suffice for basal transcription. Unlike pol II and pol III, yeast pol I basal transcription does not require TBP. Instead, TBP, upstream activation factor, and the upstream element of the promoter together stimulate pol I basal transcription to a fully activated level. The role of TBP in pol I transcription is fundamentally different from its role in pol II or pol III transcription.

Of the three nuclear RNA polymerases, it is RNA polymerase I (pol I)1 that synthesizes large rRNAs. In Saccharomyces cerevisiae, a precursor 35 S rRNA is transcribed and then processed into the mature 18 S, 5.8 S, and 25 S rRNAs found in ribosomes. These rRNAs are encoded by 100–200 direct rDNA repeats on chromosome XII. Each spacer region between the pol I-driven 35 S transcription units contains a gene encoding the remaining rRNA, 5 S rRNA, transcribed by pol III.

The only essential function of pol I in yeast is synthesis of the 35 S rRNA transcript, since the lethal phenotype of a deletion in the second largest subunit of pol I can be rescued by synthesis of the 35 S rRNA transcript by pol II from a GAL promoter placed correctly upstream of the 35 S transcription unit on a high copy plasmid (1). This provided a screen for mutants dependent on pol II-driven synthesis of rRNA from the GAL promoter (2). Such rrr mutants were expected to be defective in pol I activity in vivo, and confirming this, mutations in genes encoding subunits of pol I (those not shared with either pol II or pol III) were isolated (2). Other mutations that also caused defects in rRNA synthesis (as assessed by pulse labeling in vivo) eventually proved to lie in genes encoding (subunits of) pol I transcription factors.

An important advance in the study of yeast pol I was the development of an in vitro transcription system using a crude extract (3–5) and, later, fractionated extracts (6–8). Extracts from rrr mutant strains were not active, and their activity could be restored by addition of fractions from a wild-type extract. This was used as an assay for the purification of pol I transcription factors (6). The availability of cloned RRN genes, which could be tagged with the hemagglutinin antigen (HA) or hexahistidine, greatly facilitated purification. In this way, the multi-subunit factors, core factor (CF), and upstream activation factor (UAF), and the single subunit factor Rrn3p were identified and shown to be necessary for activity in the crude in vitro system as well as in vivo (6, 9–12).

Like higher eukaryotes, the yeast pol I promoter is composed of a core element that is essential for transcription, located roughly between +5 and −40 relative to the start site of transcription, and an upstream element from roughly −60 to −155 which is necessary for high level transcription (13–15). In yeast, the upstream element is required for formation of a stable pre-initiation complex (9). UAF initiates stable complex formation and appears to bind the upstream element tightly, because once bound, it is not readily transferred to a competing template (9). It seems likely that the core histones H3 and H4 present in UAF are important for the stability of UAF binding to the upstream element (16). Also, TATA-binding protein (TBP), which is required for pol I transcription as for pol II and pol III (17–19), was found to interact with UAF in vivo (20) and to be necessary for incorporation of CF into a stable pre-initiation complex (21). Here we report reconstitution of yeast pol I transcription in vitro from highly purified components and, hence, definition of which components are necessary and sufficient for basal transcription and for high level activated transcription. Specifically, we demonstrate rigorously that although TBP is required for high level activated transcription, it is not required for basal transcription.

MATERIALS AND METHODS

Purification of Proteins—RNA polymerase I was purified from NOY203 grown in YEPD medium (3) to A_{600} 1.2. Cells were washed once in breakage buffer (200 mM Tris-Cl, pH 7.8, 10 mM MgCl₂, 20% glycerol, 1 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) and frozen. 80–100 g of paste in breakage buffer was disrupted in a French pressure cell (20,000 pounds/square inch) and centrifuged 100,000 × g for 1 h. The supernatant (360 ml) was added to 160 g of phosphocellulose (Whatman P11) equilibrated in gradient buffer (20 mM Tris-Cl, pH 7.8, 20% glycerol, 0.1 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) with 120 mM (NH₄)₂SO₄, added, stirred 15 min, diluted to bring the (NH₄)₂SO₄ concentration to 120 mM, and stirred 30 min. The mixture was filtered, washed with 4 liters of gradient buffer plus 120 mM (NH₄)₂SO₄, and poured into a column containing an additional 40 g of phosphocellulose. A 1.2-liter gradient from 120 to 360 mM (NH₄)₂SO₄ in gradient buffer was run at 0.25 ml/min, and pol I was eluted at 200 mM (NH₄)₂SO₄. Pooled peak fractions from two preparations (about 275 ml) were added to 3.5 ml of Q-Sepharose (Fast Flow, Amersham Pharmacia Biotech), diluted slowly to 90 mM (NH₄)₂SO₄, stirred 30 min, filtered, and poured into a column containing an additional 2 ml of Q-Sepharose. After washing in gradient buffer plus 90 mM (NH₄)₂SO₄,
a 70-ml gradient from 90 to 300 mm (NH₄)₂SO₄ was run at 0.5 ml/min; pol I eluted at 175 mm (NH₄)₂SO₄. Peak pol I fractions were diluted by addition of one-half volume of gradient buffer without salt and applied to heparin-Sepharose (1-ml HiTrap cartridge, Amersham Pharmacia Biotech). After washing with gradient buffer plus 100 mM KCl, it was eluted with 150 mM Tris-Cl, 500 mM KCl, 10 mM MgCl₂, 0.2 mg/ml acetylated BSA, and 0.05% Tween 20, mixed for 1 h, and centrifuged at 500 g. The peak fraction was diluted into 4 ml of 50 mM Tris-Cl, pH 7.6, 20% glycerol, 100 mM KCl, 250 mM imidazole, 0.2 mM PMSF, and 0.1% Tween 20, disrupted in a French pressure cell (8,000 pounds/square inch), and centrifuged at 30,000 g.

NiSO₄; the mixture was centrifuged at 500 g. Sepharose (Amersham Pharmacia Biotech) that had been charged with addition to the triple HA1 tag at the N terminus of GAL7 sequence SSHHHHHHSS just before the stop codon of a His-Rrn3p eluted at 400 mM KCl. The above two preparations were combined and applied to heparin-Sepharose (5-ml HiTrap cartridge, Amersham Pharmacia Biotech), washed in gradient buffer plus 200 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, and 0.05% Tween 20, then stepwise eluted in the same buffer but with 550 mM KCl. CF-containing fractions were mixed for 1 h with 4 ml of Sepharose 6B cross-linked with 40 mg of 12CA5 monoclonal antibody (purified as described in Ref. 16). The mixture was poured into a column and washed with gradient buffer plus 550 mM KCl, 0.5 mM DTT, 0.05% Tween 20, then in the same buffer but with 250 mM KCl. Before elution, a Q Sepharose column (1-ml HiTrap cartridge, Amersham Pharmacia Biotech) was attached to the outlet of the anti-HA1 column. Peptide elution buffer (gradient buffer plus 240 mM KCl, 0.5 mM DTT, 0.05% Tween 20 and 4 mg/ml HA1 peptide (sequence YPYDVPDYA, from Quality Control Biochemicals, Hopkinton, MA)) was recirculated for 1.5 h at 1 ml/min. The anti-HA column was washed in gradient buffer plus 240 mM KCl, 0.5 mM DTT, and 0.05% Tween 20 (a salt concentration chosen to prevent binding of the peptide to the column) and eluted with a KCl gradient from 240 to 600 mM in the same buffer at 0.25 ml/min. CF eluted, in the only peak detected by A₂₈₀ at 350 mM KCl.

To purify a potentially activated form of pol I, 66 µg of pol I and 19 µg of Rrn3p were mixed in 1.1-ml dilution buffer and incubated 18 h at room temperature. This represents a molar ratio of pol I to Rrn3p of 0.4 (see Ref. 16). 1 ml of the mixture was applied to a 1-ml heparin-Sepharose column (HiTrap, Amersham Pharmacia Biotech), which was eluted with a gradient from 190 to 640 mM KCl in 4.5 ml at 0.2 ml/min, and 200 µl-fractions were collected. The single peak detected by A₂₈₀ contained pol I as expected. As a control, the chromatography was repeated exactly, except that the mixture of pol I and Rrn3p was applied immediately to the heparin column. The transcription reactions employed the minimal (~35) rDNA promoter template and were as described below with the following exceptions: the reaction volume was 40 µl; the CF used had been purified by anti-HA chromatography and heparin chromatography and was free of other pol I factors but was not as pure as the preparation shown in Fig. 1; µl of pol I (peak fraction from heparin, either that shown in lane 3 or lane 4 of Fig. 2A) was added to each reaction as indicated, estimated to be about 20 ng per reaction; when added, Rrn3p was 17 ng per reaction, and precinuculation of pol I and Rrn3p prior to transcription (where indicated) was 2 h at room temperature.

To follow the time course of pol I-Rrn3p complex formation, 128 ng of His-tagged, HA1-tagged pol I was combined with 15 ng of Rrn3p in 175 µl (final volume) dilution buffer plus 100 mM KAc, giving a molar ratio of pol I to Rrn3p of 0.4. After the indicated times at 30 °C, 40-µl samples were added to 40 µl of a 50% slurry of heparin-Sepharose (gradient buffer plus 100 mM KCl). After 15 min mixing and three 200-µl washes in the same buffer, proteins were step-eluted in 40 µl of gradient buffer plus 1 mM KCl and then trichloroacetic acid-precipitated and electrophoresed. In Vitro Transcription—The templates for all in vitro transcription experiments reported here (except in Fig. 4A) were pNOY378, referred to as the rRNA1 promoter, or pNOY310, referred to as the minimal (~35) promoter. The latter was constructed by L. Vui. The wild-type (−210) template, pNOY378, carried the 1.15-kilobase pair NcoI-HindIII fragment from pNIRT, a pSIRT derivative in which a linker was inserted in the rRNA1 promoter. The three templates included a StuI site upstream of the rDNA promoter (see Ref. 13), between the Smal and HindIII sites of pbS II K5 (Strawberry), and pNOY378 was identical except the rDNA promoter sequence extended only to ~38. The templates were linearized with StuI (giving a transcript length of 533 nucleotides) and then separated by agarose gel electrophoresis followed by purification using glass powder (26) and quantitation by A₂₈₀.

The templates used in Fig. 4A included Ec/I36I-linearized pNOY3237 (wild type, −210), in which the Smal·XbaI fragment of rDNA is cloned into pbS KS +, and pNOY32240 (−122), a Smal·XbaI deletion of pNOY3237. Templates ~119 to ~38 were PvuII·StuI fragments of the corresponding pSIRT plasmids (13). In vitro transcription was at room temperature (usually about 22 °C) in 20 µl. The reactions contained 25 mM Tris acetate, pH 7.9, 4% glycerol, 100 mM K⁺ glutamate, 8 mM magnesium acetate, 2 mM DTT, 0.025 units/µl RNasin (Promega), 0.25 mg/ml acetylated BSA, and 10 mM Tris chloride. The reactions were started at 30 °C. The dilution buffer used to adjust concentrations of protein components (20 mM Tris acetate, pH 7.9, 20% glycerol, 0.2 mg/ml acetylated BSA, and 1 mM DTT). The reactions also contained about 14 mM KCl from the protein preparations. To a 2.5-fold concentrated mixture of buffers, salts, and RNasin was added DNA template, then protein components, except for pol I and Rrn3p, pol I and Rrn3p were mixed and allowed to

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preincubate at 30 °C for at least 2 h, and then 9 volumes of dilution buffer were added, and 2 μl of the mixture was added to the other components just prior to initiation of transcription by addition of NTPs. The standard “complete” reaction contained 130 ng (0.2 μl of pol I, 13 ng (0.04 μl) of Rrn3p, 10 ng (0.01 μl) of TBP, 0.1 μl of UAF, 0.5 μl of CF, and nucleotides as follows: 200 μM each ATP, CTP, GTP, and TTP, 15 μM GTP, and 2–10 μCi [32P]GTP per 20-μl reaction. The 5′-labeling experiment shown in Fig. 5B used 10 μM ATP, 200 μM each GTP, UTP, and CTP, and where present, 50 μCi of [γ-32P]ATP or 20 μCi [32P]GTP per 20-μl reaction. Transcription was for 20–30 min and then 1 μl of 1 mg/ml heparin was added; after another 5 min transcription was stopped by addition of 40 μl of phenol/CHCl₃ and 60 μl of 50 mM EDTA, pH 7.5. 1.6 mg/ml glycerol. 75 μl of the aqueous phase was recovered and added to 300 μl of 1x ammonium acetate in 95% ethanol and kept in dry ice for 10 min. After a 10-min centrifugation, the supernatant was withdrawn with a fine-tipped pipette. To the visible pellet, 8 μl of loading dye (90% formamide, 1x TBE, 0.05% each xylene cyanol, and bromphenol blue) was added, placed at 80 °C for 10 min before application to a 5% TBE urea gel, and electrophoresed at 650 V until bromphenol blue dye ran off the bottom. Transcripts were visualized by PhosphorImager (Molecular Dynamics).

Reverse Transcription of In Vivo and In Vitro Generated RNA—The in vivo “wild-type” RNA that was reverse-transcribed in Fig. 5 was prepared from a derivative of the yeast strain ts2 (18) carrying a reporter plasmid, pNOY386 or pNOY387, constructed by L. Vu. pNOY386 has the NcoI (−210) to HindIII wild-type rDNA promoter fragment from pNIRT (which includes a non-yeast sequence used as a reporter tag; see Ref. 13) cloned between the SalI and NheI sites of yeast24 (using the SalI and XbaI sites in the pBSII KS+ polynucleotide). Upstream of this fragment in pNOY386 lies an identical rDNA promoter fragment except that it has promoter sequence only to −38, and its unique non-yeast sequence tag is different from that of the wild-type rDNA promoter fragment. This upstream rDNA promoter plays no significant role in the experiment reported in Fig. 5.) The RNA used for the lane in Fig. 5 marked “Δ” was from the same host strain carrying a control plasmid, pNOY387, identical to pNOY386 but with promoter DNA from −210 to +8 deleted.

Preparation of RNA from yeast cells (500 ml grown at 30 °C to A₆₀₀ 0.8 to 0.9) was by a published protocol (27), except that cycloheximide was not used, and the extraction buffer was 0.2% Tris-Cl, pH 7.5, 0.5% LiCl, 10 mM EDTA, and 1% SDS. RNA concentration was determined by A₆₀₀, and relative RNA concentrations were confirmed by visual inspection of ethidium bromide-stained formaldehyde agarose gels. 20 μl of RNA prepared from yeast cells was used per primer extension reaction.

In vitro transcribed RNA (which also includes the non-yeast reporter sequence) was from standard transcription reactions as described above, with the components indicated in the legend to Fig. 5, and were processed through the extraction and precipitation steps but lacked labeled nucleotide and were scaled up 2-fold. 0.2 pmol of end-labeled primer (sequence 5′GACATTCTCGAGACGGTGTG; hybridizes to the non-yeast sequence tag) was allowed to hybridize to each RNA sample for 1.5 h at 35 °C in 15 mM containing 40 μM Tris·Cl, pH 7.5, 20 mM MgCl₂, 50 mM NaCl, 10 mM DTT, and 20 units of RNasin. After cooling slowly to room temperature, 35 μl containing 30 mM Tris·Cl, pH 8.3, 4 mM MgCl₂, 15 mM DTT, 200 units of Moloney murine leukemia virus reverse transcriptase (Promega), and the four dNTPs at 0.28 mM each, was added to each sample. Following extension for 60 min at 42 °C, 100 μl of 10 mM Tris·Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 100 μg/ml herring sperm DNA, and 20 μg/ml DNase-free RNase A was added and incubated 15 min at 37 °C. After extraction with phenol/CHCl₃/isooamyl alcohol and precipitation with 0.1 M NaCl, 70% ethanol (final concentration), the samples were resuspended in formamide loading buffer, heated to 70 °C 3 min, and electrophoresed in a 9% sequencing gel, and reverse transcription products were detected by PhosphorImager.

RESULTS

Components Necessary for Reconstitution of rDNA Transcription in Vitro—In our first attempts to reconstitute yeast pol I transcription, we fractionated a crude extract and then assayed combinations of the fractions together with purified pol I for activity. The fractions necessary for transcription were purified further and eventually were substituted with highly purified factors. In this manner, the following five components were found to be sufficient for high level transcription in vitro (see below): pol I, Rrn3p, CF, TBP, and UAF. Some of our previous preparations of pol I purified by a classical protocol (28) had apparently been contaminated with small amounts of CF or UAF, so pol I purification was made more stringent. pol I was purified from yeast crude extracts by gradient elution from each of four columns, phosphocellulose, Q Sepharose, heparin-Sepharose, and MonoQ, yielding a preparation that was essentially pure as judged by visual inspection of silver-stained gels (Fig. 1), and free of any other pol I factors (by transcriptional assay, see below). pol I that had been His-tagged on the second largest subunit (A135) was purified by nickel affinity chromatography followed by gradient elution from heparin and MonoQ resins. The His-tagged preparation was of similar purity to the first and behaved identically in reconstitution experiments (data not shown). Rrn3p and TBP were both His-tagged, expressed in E. coli, and then purified (Fig. 1), ensuring that they are not contaminated by yeast proteins and have not been post-translationally modified by any yeast regulatory systems. The purification of the UAF preparation used for these experiments (Fig. 1) was described previously (16). CF was His-tagged and HA1-tagged on the Rrn7p subunit and was purified from yeast by nickel affinity, heparin-Sepharose, anti-HA1 monoclonal antibody affinity, and anion exchange chromatography steps (Fig. 1). Although only the peak fractions used for reconstitution experiments are shown in Fig. 1; fractions flanking the peak from the final column in the purification of each of the components were also assayed for transcriptional activity. In all cases, transcriptional activity correlated with the relative amount of the component visible in each column fraction on a gel, indicating that the activity of each component was due to a single species (data not shown).

When the five purified components were mixed with a linearized wild-type rDNA template and a mixture of nucleoside triphosphates (NTPs), relatively strong transcription resulted (Fig. 2, complete reaction). If pol I or CF was omitted, no transcript was detectable. Omission of Rrn3p often resulted in no detectable transcript as well, but in some experiments (as in Fig. 2) a very weak transcript could be seen, about 0.3% that seen for the complete reaction. Omission of TBP, UAF, or both TBP and UAF decreased transcription, but still a significant level remained, usually 2–10% of the complete reaction (Fig. 2). Thus, pol I, CF, and Rrn3p are sufficient for a basal level of transcription; addition of UAF and TBP stimulates transcription about 10–50-fold.

The Active Form of pol I Is Complexed with Rrn3p—Maximal activity in these reconstituted transcription reactions depended on preincubation of Rrn3p with pol I, and the preincubation...
The complete reaction included 130 ng of pol I, 13 ng of Rrn3p, 10 ng of components using a wild-type (Fig. 3, panel A, lane 2) did not show any further increase of transcriptional activity in the presence of another preincubation period prior to transcription. However, the majority of the Rrn3p was found in the fractions that passed through the column, as expected. This pol I peak fraction containing copurified Rrn3p was active in reconstituted transcription without added Rrn3p (Fig. 3B, lane 1). Further addition of Rrn3p either in the absence or in the presence of another preincubation period prior to transcription did not show any further increase of transcriptional activity (Fig. 3B, lanes 2 and 3, respectively). As a control, the same chromatography procedure was performed on a mixture of pol I and Rrn3p that was not preincubated but was applied to the heparin-Sepharose column immediately after mixing. In this case, Rrn3p was not detected in the pol I peak fraction (Fig. 3A, lane 4), and very little transcriptional activity was observed for this fraction without further addition of Rrn3p (Fig. 3B, lane 4). However, this fraction showed weak activity after addition of Rrn3p (Fig. 3B, lane 5), and preincubation of the additional Rrn3p with the pol I fraction stimulated transcription further (Fig. 3B, lane 6), showing that the pol I recovered from the control column was still intact and activatable. The simplest interpretation of these experiments is that when incubated together, pol I and Rrn3p form a complex that is stable enough to survive column chromatography, and the complex is the active form of the polymerase in specific transcription.

The time course of pol I-Rrn3p complex formation was assessed by small scale batch purification of samples taken at various times from a mixture of pol I and Rrn3p. Immediately after mixing, Rrn3p that copurified with pol I was nearly undetectable by silver staining (Fig. 3C, lane 3), similar to a sample of pol I without Rrn3p (Fig. 3C, lane 2). After 1 h of incubation together, a significant amount of Rrn3p was complexed with pol I (Fig. 3C, lane 4). Additional incubation for up to 4 h did not apparently increase complex formation (Fig. 3C, lanes 5 and 6). The Rrn3p recovered after chromatography was estimated to be 30–50% of the pol I on a molar basis, by comparison to standard amounts of the two components (data not shown). As expected, Rrn3p in the absence of pol I was not detectable by silver staining in the heparin eluate (Fig. 3C, lane 1), nor by more sensitive immunoblot (data not shown). Thus, under the conditions of this experiment, pol I-Rrn3p complex formation is maximized after 1 h incubation.

The Minimal rDNA Promoter and Components Necessary for Basal Transcription—The wild-type template used in these experiments includes rDNA promoter sequences extending to -210 relative to the start site of transcription. A template carrying the entire intergenic (non-transcribed) region of the rDNA repeat, including the enhancer/terminator element, gave the same level of transcription as a -210-truncated template in a crude in vitro system (3, 15) and in reconstituted transcription using purified components (data not shown).

To determine the minimum sequence necessary for reconstituted pol I transcription, equal amounts of several truncated promoter templates were tested. In the presence of all five required protein components, promoter templates truncated from upstream to -119, -101, -91, or -76 supported a level of transcription similar to that of the wild-type (-210) template.
whereas truncation to -60 or -38 decreased transcription substantially (Fig. 4A). Truncation to -26 abolished transcription, and when UAF and TBP were omitted from the transcription reactions, all the templates were transcribed equally (data not shown).

Since the -38 promoter was the shortest of those tested that still showed activity, it was chosen for an omission experiment to determine which protein components were required for its transcription. When all five protein components were present in the same amounts as in transcription reactions, all the templates were transcribed equally (data not shown).

The Start Site of rDNA Transcription Is the Same in Vitro and in Vivo—One test of the faithfulness of the in vitro system is to examine whether basal and activated transcription in vitro initiates the same position as in vivo. This was assessed by reverse transcription of RNA synthesized in vitro and of RNA isolated from yeast cells. The templates for in vitro transcription contained a unique 22-base pair rDNA sequence tag downstream of the start site. The wild-type (-210) template containing the tag was cloned into a high copy yeast plasmid and transformed into S. cerevisiae. RNA prepared from this strain, or transcribed in vitro using either the wild-type (-210) or the minimal (-38) promoter template, was reverse transcribed with a labeled primer complementary to the sequence tag. The start site for the in vivo RNA sample corresponded to the previously published in vivo start site (29, 30), the “A” of the sequence ATGC (Fig. 5A). The start sites from the wild-type or minimal promoters transcribed by the reconstituted pol I in vitro system both mapped to the same position as in vivo (Fig. 5A). To confirm the identity of the 5’ nucleotide as A, in vitro transcription was performed with [γ-32P]ATP as the labeled nucleotide instead of the usual [α-32P]GTP. Transcripts from both the minimal and wild-type promoters could be labeled, indicating that A was indeed the initiating nucleotide (Fig. 5B).

The relative efficiency of labeling of transcripts from the two promoters was similar whether [γ-32P]ATP or [α-32P]GTP was supplied as the labeled nucleotide (Fig. 5B), showing that the low concentration of ATP necessary for 5’ labeling with [γ-32P]ATP did not preferentially favor initiation at an alternative site. A previous determination of the start site in a crude yeast pol I in vitro system showed most 5’ ends at +5 relative to the start site, in addition to a small fraction ending at the start site, perhaps because of degradation of transcripts in the crude extract (3).

TBP Is Not Required for Basal Transcription—Because CF was required for basal transcription, and because a previous report concluded that TBP was a subunit of CF (11), it was important to determine rigorously whether TBP was required for basal transcription. First, the sensitivity of reconstituted transcription to added TBP was assayed (Fig. 6A). All the reactions contained pol I, Rrn3p, and CF and the wild-type promoter template. In the absence of UAF, even large amounts of TBP (25 or 100 ng) failed to stimulate transcription, whereas in the presence of added UAF, these amounts stimulated pol I transcription 30–50-fold (Fig. 6A). This reinforces the conclusion that UAF is required together with TBP for an activated level of transcription.

Next, the physical presence of TBP in the purified pol I or CF preparations used for reconstituted transcription was assayed by immunoblot. 0.04 ng of the His-tagged TBP could be de-
pol I lane

Figure 6. TBP does not stimulate basal transcription. A, reconstituted in vitro transcription from the wild-type (−210) rDNA promoter in the absence or presence of UAF with the indicated amounts of His-tagged TBP. All reactions included pol I, Rrn3p, and CF. B, immunoblot of purified pol I, CF, and varying amounts of His-tagged TBP probed with antibody against TBP. The weak band near 30 kDa in the pol I lane is the ABC27 subunit of pol I and was visible by Coomassie staining of the blot.

Discussion

Five purified components, pol I, Rrn3p, CF, TBP, and UAF, are sufficient for high level transcription from the yeast rDNA promoter in vitro. Several lines of evidence indicate that the in vitro system faithfully replicates the in vivo mechanism. Omission of each component individually showed that each is necessary for high level activity in this purified transcription system, as shown previously for crude systems (5, 6, 9, 12). This single-component omission experiment has essentially been replicated in vivo by using yeast strains with null mutations in genes encoding (subunits of) four of these components: pol I, Rrn3p, CF, and UAF. Such mutations have been detected in yeast crude extracts and in more purified pol I preparations (8).

The minimal requirements for correctly initiated (basal) transcription in vitro are as follows: promoter DNA extending to −38 upstream of the start site and the protein components pol I, Rrn3p, and CF. These components transcribe the −38 and wild-type (−210) promoters with equal efficiency. The pol I-Rrn3p complex, like pol I alone, is capable only of nonspecific transcription (12); thus it seems likely that the requirement for CF is to direct the pol I-Rrn3p complex to the correct start site.

The basal level of transcription observed in this minimal pol I in vitro system does not require TBP and is not stimulated by addition of TBP, even in large quantities. Rather, TBP and UAF together activate basal transcription 10–50-fold. This activated level of transcription cannot be achieved from the −38 truncated promoter; it requires sequences upstream of −60. However, a promoter truncated to −76 is sufficient for activation.

Our data show that the mechanism of action for TBP at the pol I promoter is fundamentally different from that at pol II or pol III promoters. In pol II transcription, TBP is required for basal transcription whether or not the TATA element is present, and when it is present, TATA is usually located around positions −25 to −35 (33). (There is an exception known where TBP is dispensable and transcription depends on the YY1 protein binding the initiator sequence near the start site (34).) Recently, another report of pol II transcription without TBP has appeared (35). In pol III transcription, TBP contained in the factor TFIIIB interacts around position −35 (36). Once bound to its site, TFIIIB is sufficient to direct initiation by pol III (37). The role of TBP in yeast pol I transcription, to participate in activation with UAF, requires an upstream element that is distinct from the “core” region of the promoter required for basal transcription and is different from the role of TBP in pol II and pol III transcription.

A previous report defined CF as including TBP, based on physical association of TBP with the known subunits of CF, Rrn6p, Rrn7p, and Rrn11p (11). However, that report could not be directly compared to our data because it did not use a purified complex of pol I and Rrn3p as a template and other proteins (12). This preincubation step became unnecessary (and did not stimulate transcription) if a purified complex of pol I and Rrn3p was substituted for the template and other proteins (12). The recombinant His-tagged Rrn3p purified from E. coli did not contain detectable TBP, as expected (not shown). From the immunoblot, we calculate that the amount of TBP that could have been added to the transcription reactions as a contaminant of the CF or CF preparations would have been less than 0.002 ng. This is 50 times less than the amount of His-TBP (0.1 ng; Fig. 6A) that barely stimulated transcription when added in the presence of UAF. In the absence of UAF, even large amounts of added TBP did not stimulate transcription. This reinforces the conclusion that pol I, Rrn3p, and CF are sufficient for basal transcription, and that TBP is not required.

FIG. 6. TBP does not stimulate basal transcription. A, reconstituted in vitro transcription from the wild-type (−210) rDNA promoter in the absence or presence of UAF with the indicated amounts of His-tagged TBP. All reactions included pol I, Rrn3p, and CF. B, immunoblot of purified pol I, CF, and varying amounts of His-tagged TBP probed with antibody against TBP. The weak band near 30 kDa in the pol I lane is the ABC27 subunit of pol I and was visible by Coomassie staining of the blot.

Discussion

Five purified components, pol I, Rrn3p, CF, TBP, and UAF, are sufficient for high level transcription from the yeast rDNA promoter in vitro. Several lines of evidence indicate that the in vitro system faithfully replicates the in vivo mechanism. Omission of each component individually showed that each is necessary for high level activity in this purified transcription system, as shown previously for crude systems (5, 6, 9, 12). This single-component omission experiment has essentially been replicated in vivo by using yeast strains with null mutations in genes encoding (subunits of) four of these components: pol I, Rrn3p, CF, and UAF. Such mutations have a lethal or nearly lethal phenotype, but the phenotype can be rescued by transcription of rRNA from a pol II promoter, indicating that the defect is in pol I transcription (1, 2, 6, 9, 10, 12, 31, 32). Thus, pol I, Rrn3p, CF, and UAF are necessary for transcription in vivo as well as in vitro. In addition, some but not all TBP mutants are defective in rRNA synthesis at their non-permissive temperature, indicating that TBP is also necessary for rRNA synthesis both in vivo and in vitro (18, 19). Interestingly, the phenotype of UAF null mutants is slightly leaky, and they exhibit a low level of residual rRNA synthesis from the pol I promoter in vivo (9). Correlating with this, when UAF is omitted from the reconstituted in vitro system, only a basal level of transcription is seen. By contrast, pol I, Rrn3p, and CF null mutants are not leaky (2, 6, 10, and 12); they do not detectably transcribe rRNA from the pol I promoter in vivo, and these components are required for both high level transcription and basal transcription in vitro. Finally, the start site of pol I transcription reconstituted in vitro is identical to the start site in vivo. The correlations between pol I transcription in vivo and that reconstituted from purified components in vitro argue that conclusions drawn from the in vitro purified system may reflect in vivo mechanisms.

Yeast rDNA transcription in vitro is greatly stimulated by incubation of pol I and Rrn3p together before addition to the template and other proteins (12). This preincubation step became unnecessary (and did not stimulate transcription) if a purified complex of pol I and Rrn3p was substituted for the individual components. Formation of the pol I-Rrn3p complex prior to purification by column chromatography depended on preincubation of the individual components together, just as stimulation of transcription using the individual components. We conclude that the active form of pol I for specific transcription is the pol I-Rrn3p complex. Under our conditions, 30–50% of the pol I was комплексed with Rrn3p in 1 h. Since recombinant Rrn3p was used, unmodified Rrn3p is most likely functional in transcription. A complex of pol I and Rrn3p, probably representing only a few percent of the total pol I in the cell, has been detected in yeast crude extracts and in more purified pol I preparations (8).

The minimal requirements for correctly initiated (basal) transcription in vitro are as follows: promoter DNA extending to −38 upstream of the start site and the protein components pol I, Rrn3p, and CF. These components transcribe the −38 and wild-type (−210) promoters with equal efficiency. The pol I-Rrn3p complex, like pol I alone, is capable only of nonspecific transcription (12); thus it seems likely that the requirement for CF is to direct the pol I-Rrn3p complex to the correct start site.

The basal level of transcription observed in this minimal pol I in vitro system does not require TBP and is not stimulated by addition of TBP, even in large quantities. Rather, TBP and UAF together activate basal transcription 10–50-fold. This activated level of transcription cannot be achieved from the −38 truncated promoter; it requires sequences upstream of −60. However, a promoter truncated to −76 is sufficient for activation.

Our data show that the mechanism of action for TBP at the pol I promoter is fundamentally different from that at pol II or pol III promoters. In pol II transcription, TBP is required for basal transcription whether or not the TATA element is present, and when it is present, TATA is usually located around positions −25 to −35 (33). (There is an exception known where TBP is dispensable and transcription depends on the YY1 protein binding the initiator sequence near the start site (34).) Recently, another report of pol II transcription without TBP has appeared (35.) In pol III transcription, TBP contained in the factor TFIIIB interacts around position −35 (36). Once bound to its site, TFIIIB is sufficient to direct initiation by pol III (37). The role of TBP in yeast pol I transcription, to participate in activation with UAF, requires an upstream element that is distinct from the “core” region of the promoter required for basal transcription and is different from the role of TBP in pol II and pol III transcription.

A previous report defined CF as including TBP, based on physical association of TBP with the known subunits of CF, Rrn6p, Rrn7p, and Rrn11p (11). However, that report could not

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Footnotes:

7 J. Keener, C. A. Josaitis, J. A. Dodd, and M. Nomura, unpublished data.

8 R. Yamamoto and M. Nomura, unpublished data.
resolve whether TBP was required for transcriptional activity of CF, since TBP was also present in the crude extracts used for transcription. In view of the results here showing the activity of CF in basal transcription without TBP, the affinity of TBP for CF subunits (11, 21) should probably be interpreted as an interaction important for activation of basal transcription by TBP plus UAF. Indeed, TBP, as well as UAF, is necessary for incorporation of CF into a stable preinitiation complex (21), and interaction between TBP and UAF is required for rDNA transcription \textit{in vivo} (20). The mechanism for activation of basal transcription by TBP plus UAF may be stabilization of CF binding to the promoter.

Mammalian pol I transcription has been extensively studied \textit{in vitro}. Full activity \textit{in vitro} from both mouse and human rDNA promoters requires pol I, upstream binding factor (UBF), and the TBP-containing transcription initiation factor TIF-IB/SL1 (38). Two additional factors required in the mouse system, TIF-IA and -IC, associate with pol I prior to template binding and perhaps are part of the human pol I preparation (39). Although UBF might be functionally analogous to UAF plus TBP of the yeast pol I system in that it appears to be stimulatory rather than essential (39–41), there are several important differences between mammalian UBF and yeast UAF. First, UBF is a homodimer containing high mobility group domains, whereas yeast UAF is a multi-subunit complex that includes the core histones H3 and H4. There is no amino acid similarity between UBF and any of the five characterized subunits of UAF. Second, UBF does not appear to mediate stimulation by the upstream promoter element but rather stimulates transcription whether the upstream element is present or not (40). Third, UBF is a relatively abundant protein, at least 100 times more abundant than TIF-IB/SL1 (40, 42). By contrast the intracellular amount of UAF is similar to that of CF, a few hundred molecules per cell, an amount roughly comparable to the number of rDNA promoters per cell (unpublished experiments cited in Ref. 9). Thus, UBF may play an architectural role (42, 43) distinct from the function of UAF in yeast. It is possible that an undiscovered mammalian homologue of UAF exists which might mediate the stimulatory activity of the upstream element.

TIF-IB/SL1 in mammalian systems seems analogous to yeast CF in that it is essential for both basal and UBF-stimulated transcription \textit{in vitro}, and sequence extending to −45 upstream of the start site suffices for basal transcription (40). Although there is obvious amino acid sequence similarity between subunits of the human and mouse factors TIF-IB and SL1 (44), no similarity is apparent between these factors and yeast CF. Another incongruity is that TBP is a subunit of TIF-IB/SL1 and appears to be required for transcriptional activity of the complex (45), but as shown here, TBP is not required for the activity of yeast CF in basal transcription. However, the requirement of TBP for TIF-IB/SL1 transcriptional activity was shown using full human rDNA promoter and UBF (45). A formal possibility remains that TBP is not required for a basal level of human rDNA transcription.

The results here confirm the proposed model for yeast pol I transcription (see Ref. 21; see Fig. 7). pol I binds Rrn3p in solution, forming a complex that is active in specific transcription. The pol I-Rrn3p complex, together with CF and promoter DNA extending to −38 suffice for a basal level of transcription. An activated level of transcription requires UAF and TBP, in addition to the components necessary for basal transcription, and requires an upstream element that includes promoter DNA between −60 and −155. Perhaps such a relatively large region is required if the DNA is wrapped in nucleosome-like fashion by UAF as might be suggested by the presence of histones H3 and H4. All of the five protein components required for high level transcription of rDNA \textit{in vitro} have been shown to be necessary for transcription \textit{in vivo} as well. We believe this pol I transcription system reconstituted from purified components, which is faithful to transcription \textit{in vivo}, represents the basic pol I initiation machinery and may be a

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{model.png}
\caption{Model for pol I transcription from the rDNA promoter of yeast. TBP is placed to indicate contacts deduced between TBP and CF and TBP and UAF (11, 21, 20). There are no data to indicate where or if TBP interacts with promoter DNA directly.}
\end{figure}
useful tool to search for regulatory inputs and influences of nucleolar structure on rDNA synthesis.

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Reconstitution of Yeast RNA Polymerase I Transcription in Vitro from Purified Components: TATA-BINDING PROTEIN IS NOT REQUIRED FOR BASAL TRANSCRIPTION

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