An immunoaffinity purified *Schizosaccharomyces pombe* TBP-containing complex directs correct initiation of the *S.pombe* rRNA gene promoter

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ABSTRACT

The multi-protein complex SL1, containing TBP, which is essential for RNA polymerase I catalyzed transcription, has been analyzed in fission yeast. It was immunopurified based on association of component subunits with epitope-tagged TBP. To enable this analysis, a strain of *Schizosaccharomyces pombe* was created where the only functional TBP coding sequences were those of FLAG–TBP. RNA polymerase I transcription components were fractionated from this strain and the TBP-associated polypeptides were subsequently immunopurified together with the epitope-tagged TBP. An assessment of the activity of this candidate SL1 complex was undertaken cross-species. This fission yeast TBP-containing complex displays two activities in redirecting transcriptional initiation of an *S.pombe* rDNA gene promoter cross-species in *Saccharomyces cerevisiae* transcription reactions: it both blocks an incorrect transcriptional start site at +7 and directs initiation at the correct site for *S.pombe* rRNA synthesis. This complex is essential for accurate initiation of the *S.pombe rRNA* gene: *rRNA* synthesis is reconstituted when this *S.pombe* TBP-containing complex is combined with a *S.pombe* fraction immunodepleted of TBP.

INTRODUCTION

Activation of transcription of eukaryotic rRNA genes entails recognition of the promoter by an essential initiation factor, termed SL1 (1,2) or, alternatively, TIF-IB (3–5), factor D (6,7) or Rib1 (8). This factor is critical in directing association of the catalytic enzyme, RNA polymerase I, with an SL1–rDNA gene promoter complex for initiation of the pre−37−45S rRNA (9,10). In addition, SL1 confers species specificity to transcriptional initiation of eukaryotic rRNA genes, as is evident when even closely related species do not have the capability of directing correct transcriptional initiation of the other species’ rRNA genes (reviewed in 7). The subunit structure of the SL1/TIF-IB factor has been determined in human and mouse and consists of TBP and three TAF I s: TAF I 110 (human), TAF I 95 (mouse) and TAF I 63 and TAF I 48 (human and mouse; 1,11). However, it is not known whether an SL1 complex consisting of TBP and associated subunits is universal in eukaryotes and, if so, whether the subunit composition and mechanism of interaction with species-specific rDNA promoters varies (see for example 12). The first report of a multi-subunit complex required for rRNA synthesis in the yeast *S.cerevisiae* indicated that TBP was not a stably associated subunit (13).

TBP plays a central role in transcription catalyzed by all three nuclear RNA polymerases (14–16), yet forms specific multi-subunit complexes that differ for each of the three polymerases (reviewed in 16). The polymerase II complex, TFII D, bears seven or more TBP-associated factors (TAF I s; 17–19), while the polymerase III complex, TFIIIB, bears two (20–23). While the mouse and human SL1/TIF-IB factors each contain TBP and three TAF I s, evidence suggests that the *Acanthamoeba* polymerase I essential initiation factor, TIF-IB, consists of TBP and four associated polypeptides (13). The candidates for SL1 subunits in bakers yeast, including Rrn6p, Rrn7p and a 66 kDa polypeptide, co-purified in a complex which did not contain TBP (13), although TBP was shown to fractionate with the initiation factor in early stages of purification (24). However, recent analyses revealed that TBP did associate with this polymerase I ‘core factor’ complex and that Rnl1p was the 66 kDa polypeptide (25,26).

An exploration of the composition and activity of the essential initiation factor for rRNA synthesis was undertaken in fission yeast. To this end, the *S.pombe* *tbp* + gene (27,28) was disrupted and a strain of *S.pombe* created whose sole functional TBP was an epitope-tagged version. A complex was immunopurified that displayed SL1-like activity: it directed correct initiation of the *S.pombe* rRNA minigene cross-species in *S.cerevisiae* and repressed incorrect initiation. Reconstitution of *S.pombe* rRNA synthesis using homologous *S.pombe* factors was dependent on this complex.

MATERIALS AND METHODS

Disruption of the *S.pombe* *tbp* + coding sequences

A clone containing the genomic *tbp* + gene (with an ~10 kb genomic insert in pDB248; kindly provided by Dr Alexander

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isolated from pREP2 (29,30). The resultant plasmid, p2.6 kb partial digestion of the genomic sequence, accession no. Z66525). The three exons of tbp\(^+\) are shown as stippled boxes. To create the disrupted copy of tbp\(^+\) coding sequences, a partial HindIII digest of p2.6 kb released a 162 bp fragment coding for the essential C-terminal 14 amino acids of tbp\(^+\) and a 3′-untranslated region. A 1.8 kb HindIII fragment bearing ura4\(^+\) was inserted into this HindIII site, creating p\(\text{Δ}tbp::\text{ura4}\). A diagram of the disrupted tbp\(^+\) gene is shown below, with the 1.8 kb ura4\(^+\) coding sequences crosshatched. (B) Southern analysis of the S.pombe strain bearing a disrupted tbp allele. Genomic DNA was isolated from the parental diploid strain S.pombe SP826 (lanes 2 and 4) and from diploid Ura\(^+\) transformants (a representative is shown in lanes 1 and 3) and digested with EcoRI and HindIII (lane 1 and 2) or EcoRI (lanes 3 and 4). The 7.2 kb EcoRI fragment bears the wild-type allele of tbp and the 8.8 kb EcoRI fragment harbors the disrupted tbp allele (\(\text{Δ}tbp::\text{ura4}\)). The Southern blot was hybridized with an ~1 kb XbaI probe derived from the 5′ two thirds of the tbp\(^+\) genomic DNA (see A).

Hoffmann; 27) was partially digested with XbaI and a 2.6 kb fragment was gel isolated and subcloned into the pBS SK\(^+\) vector-specific reverse primer. The PCR products were treated with T4 DNA polymerase to convert the ends to blunt ends and ligated to SaII linkers. Following digestion with SaII and preparative isolation of the fragment, it was subsequently ligated into the SaII site of pBluescript SK\(^+\). Due to frequent deletions of the TBP coding sequence, this procedure and screening of E.coli Amp\(^+\) transformants had to be repeated multiple times until a correct, full-length clone was isolated, pFLAG-S1p.TBP. The FLAG–TBP insert was released by digestion with NdeI and BamHI and ligated into the NdeI and BamHI sites of the S.pombe/E.coli shuttle vector pRep1 (29; kindly sent by Dr Kinsey Maundrell), creating pRep1/FLAG-S1p.TBP.

Introduction of a tagged version of tpb\(^+\) cDNA into the \(\text{Δ}tbp::\text{ura4}\) diploid strain of S.pombe

Plasmid pRep1/FLAG-S1p.TBP was introduced into a diploid S.pombe strain bearing a disrupted tbp\(^+\) allele (\(\text{Δ}tbp::\text{ura4}\)).
and Leu\(^+\) transformants were selected. Following sporulation and selective killing of non-sporulating diploid cells, Leu\(^\ast\), Ura\(^\ast\), Ade\(^-\) haploids were isolated. This procedure (suggested by Dr Henry Levin, NIH) involved treating ~1 ml diploid cells (~10\(^7\) cells/ml) overnight with 20 \(\mu\)l 1:10-fold dilution of glusulase; removing the glusulase and incubating the cells for 30 min in 30% ethanol before plating onto solid medium lacking leucine and uracil. Southern analysis revealed that these haploids contained the disrupted \(\Delta\text{tp}:\Delta\text{ura4}\) chromosomal allele and the extrachromosomal FLAG–TBP coding sequences (data not shown). The resultant strain is \(\text{SpATBP} (\Delta\text{leu} 1-32 \text{ura}-4-D18 \text{ade}6 \Delta\text{tp}:\Delta\text{ura4} \text{pRep1/FLAG-5p.TBP}\). The extrachromosomal plasmid \(\text{pRep1/FLAG-S.p.TBP}\) did not segregate during growth in rich medium, as expected, since it carried the only viable TBP coding sequences.

**Preparation of S-100 extract and ammonium sulfate precipitation**

\(\text{SpATBP}\) was grown in thiamine-deficient EMM medium, to ensure maximal expression of FLAG–TBP (30), with constant vigorous shaking at 30\(^\circ\)C and cells were collected while in mid logarithmic growth phase. S-100 was prepared from 40 l cells as described (32). Aliquots of 195 mg S-100 (total protein concentration; S-100 was made from 50 g pelleted and frozen cells) were adjusted to ammonium sulfate 60% saturation, centrifuged (15 000 \(g\), 4\(^\circ\)C, 15 min) and suspended in TA buffer (20 mM Tris-acetate, pH 7.5) as described (25). Following 4 h dialysis, the solution was diluted to 20–30 mg/ml and centrifuged at 10 000 \(g\) (25). The pellet was suspended in 0.2 ml buffer [20 mM HEPES–KOH, pH 7.9, 50 mM KCl, 5 mM EGTA, 0.05 mM EDTA, 2.5 mM dithiothreitol (DTT), 20% glycerol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. The suspended pellet (35 mg/ml) and the supernatant (1.5 ml with a protein concentration of 59 mg/ml) were stored at −75\(^\circ\)C.

**HiTrap-Q column elution of low salt supernatant of ammonium sulfate fractionation**

A sample of 31 mg protein (the supernatant following the second centrifugation, above) was adjusted to 1× loading buffer [25 mM HEPES, pH 7.9, 0.2 mM EDTA, 5 mM MgCl\(_2\), 20% glycerol (v/v), 1 mM PMSF, 1 mM DTT], 0.1 M KCl and loaded onto a 5 ml Phacma HiTrap-Q column pre-equilibrated with 0.1 M KCl, 1× loading buffer. This was followed by step elution of fractions at 1× loading buffer with 0.175, 0.35, 0.7 and 1.0 M KCl, at a flow rate of 1 ml/min (controlled by a Pharmacia Gradifrac System). All steps were repeated with a second batch of ~30 mg protein.

**Immunopurification of polymerase I–TBP complex using an anti-FLAG M2 affinity column**

The peak fractions for RNA polymerase I transcription components were pooled from two trials (~10 mg total protein). The transcriptional activity was assessed as described below (see also Fig. 4A). The KCl concentration was adjusted to 0.15 M and the samples were loaded onto a 1 ml anti-FLAG\(^\text{TM}\) M2 affinity gel (Kodak/IBI; binding capacity 25 nmol FLAG protein/ml gel), as recommended. The flow-through was collected and re-loaded; the column was washed three times with phosphate-buffered saline, pH 7.4. Elution of FLAG–TBP and associated polypeptides was conducted using 1 ml elution buffer with increasing concentrations of FLAG peptide (50, 100, 125 and 200 ng/ml), followed by 1 ml 0.1 M glycine–HCl, pH 3.0. The fraction containing unbound polypeptides was demonstrated to lack TBP by Western analysis (data not shown) and served as the fraction immunodepleted of TBP (see Fig. 7A and B).

**Western blot analysis of TBP complexes using anti-FLAG antibody**

An aliquot of 20 µg protein from S-100 extract, or fractions as indicated, was fractionated by 15% SDS–PAGE and transferred to nitrocellulose using a BioRad SD Semi-Dry Transfer Cell. The anti-FLAG antibody (anti-FLAG\(^\text{TM}\) M2 monoclonal antibody; Kodak/IBI) was added at 1:300 dilution, following standard protocols (31,33). Detection utilized enhanced chemiluminescent reagents from Amershams and included sheep horseradish peroxidase-linked anti-mouse Ig whole antibody as the secondary antibody.

**SYPRO orange protein stain**

The SDS–PAGE minigel containing fractionated TBP and associated polypeptides (Fig. 5A and B) was immersed in 50 ml 1:5000 dilution of SYPRO orange protein stain (BioRad) in 7.5% (v/v) acetic acid and stained and photographed under UV light (34).

**DNA templates and in vitro transcription reactions**

p-243:3XH (Fig. 3) has been described previously (32). p5\(^\Delta\text{243}/3\Delta\text{+31}\) is a similar template containing promoter sequences from −243 to +31, but in the cloning vector pBS (32); it was used in the transcriptional assays shown in Figure 6A–C. Template p5\(^\Delta\text{243}/3\Delta\text{+89}\) (32) was used in the transcription assays shown in Figure 7 (at a final concentration of 0.5 µg/µl). In vitro transcription reactions were 40 µl and contained 10 µl extract (~50 µg protein) or indicated amounts of fractionated RNA polymerase I transcription components, template DNA (0.025–1.25 µg/ml, as indicated), 20 mM HEPES–KOH, pH 7.9, 70–90 mM KCl, 10 mM MgCl\(_2\), 5 mM EGTA, pH 7.9, 0.05 mM EDTA, pH 7.9, 10% glycerol, 1.3 mM DTT, 100–500 µM each of the four ribonucleoside triphosphates (Pharmacia) and 10 µg/ml α-amanitin (Sigma) and were incubated for 45 min at 26°C (32). Transcription in an \(S.cerevisiae\) S-100 extract (32,35) was performed as above, except that ~1 ng FLAG–TBP complex was added, with the final concentration of transcription buffer adjusted to the same as above. The reconstitution assays (shown in Fig. 7) utilized 5 µl \(S.pombe\) fraction depleted of TBP and/or 1.0 µl (~1 ng) FLAG–TBP complex. The RNA was isolated and S1 analysis conducted as described (32). S1-protected fragments were resolved by electrophoresis on 4% acrylamide–9 M urea gels; the size markers were 5–32P-labeled \(Hpa\)II fragments derived from pBR322. The 5′-end-labeled probe used to detect transcription supported by template p-243:3XH was prepared by labeling at the unique \(Xba\) site (+340) on the template strand and converting the DNA to single-stranded as described (32). For preparation of the probe used to detect transcription supported by p5\(^\Delta\text{243}/3\Delta\text{+31}\), 5′-end-labeling was at position +77 on the template strand (at a unique \(Xho\)I site). The probe used to detect transcription supported by p5\(^\Delta\text{243}/3\Delta\text{+89}\) probe was labeled at a unique \(Xho\)I site at +135 on the template strand. The initiation site for \(\text{in vitro}\) \(S.pombe\) RNA synthesis was shown to be the same \(\text{in vitro}\) as \(\text{in vivo}\) (32; see also Fig. 6C).
Strains, media and transformation

Schizosaccharomyces pombe S-100 extract made from wild-type strain 972 (h−, kindly sent by Dr H.Levin) was used in control transcription reactions (32). The diploid strain used for disruption of *tbp* was *S.pombe* SP826 h*+/h*+ leu 1-32 *ura*-4-D18/ura4-18 ade6-216/ade6-210 (kindly sent by Dr Dave Frendewey, NYU Medical Center). The bacterial strains used included: XL1-Blue [endA1, hsdR17 (rK−, mK+)], supE44, thi-1, lacI−, recA1, gyrA96, relA1, lac (F′, proAB, lac-proAB, lacF218proAB, lacZAM15, Tn10, (tetR)) and SURE™ [mcrA, S/mcrBC-hsdRMS-mrr]171, endA1, supE44, thi-a, lac−, gyrA96, relA1, lac, recB, recJ, sbcC, umuC, Tn5, (kan′), uvrC, (F′, proAB, lac-proAB, lacZAM15, Tn10, (tetR)] (Strategene). The *S.cerevisiae* S-100 extract was made from *S.cerevisiae* W303 [MATα, ade2-1, his3-11,15, leu-23,112, trp1-1, ura3-1, can1-100 (R.Rothstein)]. Media used included EMM (31) and SC medium (32). Bacterial cells were transformed by electroporation using a BioRad Gene Pulser (32). The lithium acetate transformation method was used for introduction of plasmid DNAs into yeast (30,31).

RESULTS

Our strategy to determine whether the essential initiation factor for rRNA synthesis in fission yeast was stably associated with TBP was to introduce a tagged copy of TBP into *S.pombe*. This would facilitate detection and localization of TBP during fractionation of RNA polymerase I transcription components and enable immunoaffinity purification of associated factors. To ensure that all TBP coding sequences were epitope tagged, the chromosomal copy of the *tbp*+ gene was inactivated in a diploid strain using one-step gene disruption (36). Such a strain would also facilitate analysis of interactions of the essential initiation factor for RNA polymerase I, SL1, with other RNA polymerase I transcription factors and with the regulatory regions of the *S.pombe* rRNA gene.

A plasmid was constructed that contained a disrupted copy of the *S.pombe* TBP coding sequences, named pΔtbp::ura4 (see Fig. 1A and Materials and Methods for details). One-step gene disruption of the chromosomal *tbp*+ allele was conducted in diploid strain SP826 of *S.pombe*, since TBP is an essential gene. Southern analysis confirmed that gene replacement was successful and that a diploid strain was constructed containing one wild-type and one disrupted allele of TBP (see Fig. 1B). A plasmid bearing an epitope-tagged version of *tbp*+ cDNA, pRepl1/FLAG-S.p.TBP, was constructed and introduced into this Δtbp::ura4/tbp+ diploid strain of *S.pombe* (see Materials and Methods for details on construction of the FLAG™ epitope-tagged TBP). To ensure high levels of expression, the TBP coding sequences were placed under the control of the nmt promoter (29,30).

The essential initiation factor for polymerase I catalyzed transcription was fractionated from the resultant haploid strain of *S.pombe*, based on its presence in transcriptionally active fractions and on affinity purification via the epitope-tagged TBP. The fractionation scheme for purification of the essential initiation factor for rRNA synthesis is outlined in Figure 2. Polypeptides that precipitated at 60% ammonium sulfate were collected and diazylated, as described in Riggs et al. (24). However, the *S.pombe* RNA polymerase I transcription components behaved differently from those of *S.cerevisiae*, where required RNA polymerase I transcription components formed a sedimentable complex following dialysis of the suspended ammonium sulfate precipitated polypeptides (24). In the case of the *S.pombe* RNA polymerase I transcription factors, they were largely present in the ‘low salt supernatant’ (Fig. 3, lane 2), although a fraction did form a sedimentable complex (Fig. 3, lane 3).

The RNA polymerase I components required for initiation were further fractionated on a Pharmacia HiTrap-Q anion exchanger.
TIF-IC activity (37–40; Guo, A. and Pape, L., unpublished data)

α may lack a required component, such as activated polymerase, contains FLAG–TBP, it does not support initiation; this fraction as expected (Fig. 4 B, lanes 4–6). Although fraction 3-26 (lane 3) contains all polymerase II or III catalyzed transcription, TFIID (17–19) or TFIIIB (20–23), or is free TBP (Fig. 5 A, lane 2).

Fractions eluted between 0.175 and 0.35 M KCl were assessed for transcriptional capacity. Five microliters of each fraction were tested for ability to support accurate initiation, using the p-243:XH template (0.1 µg/ml, 32). The first number of the name of the fractions, 1-, 3- or 7-, refers to the KCl concentration of the step cut (0.175, 0.35 or 0.7) and the second number is the fraction number. The S1-resistant fragment representing correctly initiated rRNA is 340 nt in length and is marked (+1). (B) Western analysis of TBP-containing fractions. Approximately 20 µg (unless stated otherwise) of each fraction assessed in (A) were separated on a 15% SDS–PAGE gel, transferred to nitrocellulose membrane and challenged with anti-FLAG monoclonal antibody M2 (Kodak/IBI). The amounts in lanes that differ from the standard –20 µg include: for fraction 12, eluted at 0.1 M KCl (1-12; lane 2), 10 µg; 7-41, 10 µg (lane 7); 7-42 (lane 8), 6.5 µg. Lane 9 contains 20 µg control S-100 prepared from wild-type S.pombe cells.

To test whether the TBP and its associated polypeptides (seen in Fig. 5A, lane 3, and B, lane 2) harbor activity for directing correct initiation of rRNA genes, a cross-species assay was performed. Transcriptional initiation of eukaryotic rRNA genes is species specific, with the critical species-specific factor being SL1 (reviewed in 7). Thus, correct transcriptional initiation of an S.pombe rRNA gene promoter in a S.cerevisiae RNA polymerase I transcription extract is not apparent (Fig. 6A). However, an alternate rRNA transcript starting at +7 is produced (see Fig. 6A, lanes 1 and 2, and C, left lane), while the S.pombe extract directs initiation at the wild-type start site (see Fig. 6B, lanes 1 and 2, and C, right lane). Addition of the S.pombe FLAG–TBP complex to the heterologous transcription components resulted in repression

Figure 4. (A) Transcriptional analysis of RNA polymerase I components following chromatographic separation on a HiTrap-Q column. Following HiTrap-Q chromatographic separation, fractions eluted at 0.175, 0.35 and 0.7 M KCl were assessed for transcriptional capacity. Five microliters of each fraction were tested for ability to support accurate initiation, using the p-243:XH template (0.1 µg/ml, 32). The first number of the name of the fractions, 1-, 3- or 7-, refers to the KCl concentration of the step cut (0.175, 0.35 or 0.7) and the second number is the fraction number. The S1-resistant fragment representing correctly initiated rRNA is 340 nt in length and is marked (+1). (B) Western analysis of TBP-containing fractions. Approximately 20 µg (unless stated otherwise) of each fraction assessed in (A) were separated on a 15% SDS–PAGE gel, transferred to nitrocellulose membrane and challenged with anti-FLAG monoclonal antibody M2 (Kodak/IBI). The amounts in lanes that differ from the standard –20 µg include: for fraction 12, eluted at 0.1 M KCl (1-12; lane 2), 10 µg; 7-41, 10 µg (lane 7); 7-42 (lane 8), 6.5 µg. Lane 9 contains 20 µg control S-100 prepared from wild-type S.pombe cells.

Figure 5. Immunoaffinity purification of S.pombe TBP-associated polypeptides from RNA polymerase I transcription components. The peak fractions containing RNA polymerase I transcription components were pooled and subjected to immunoaffinity purification via an anti-FLAG M2 affinity gel. (A) Resolution of TBP-associated polypeptides eluted from the anti-FLAG affinity matrix at increasing concentrations of FLAG peptide: Lane 1, 25 µl fraction eluted with 50 ng FLAG peptide; lane 2, 235 µl fraction eluted with 100 ng FLAG; lane 3, 235 µl (1 ng/µl) eluted with 125 ng FLAG. The gel was a 15% SDS–PAGE gel (31). Polypeptides were visualized with SYPRO orange (BioRad; 34). The protein standards were from Sigma (M). (B) Aliquots of 25 µl of fractions eluted with the indicated amounts of FLAG peptide (as in A) were resolved by 12% SDS–PAGE and visualized as in (A). (C) Western analysis of the same fractions as in (A). Lane 1, fraction eluted with 50 ng FLAG peptide; lane 2, 100 ng; lane 3, 125 ng. The blot was challenged with anti-FLAG M2 monoclonal antibody (Kodak/IBI) and detected as described in Materials and Methods.

TBP-associated polypeptides is shown in Figure 5 C (the composition of the polypeptides fractionated by SDS–PAGE and transferred to membrane is seen in Fig. 5A). Detection of FLAG–TBP was via the anti-FLAG antibody M2 (Kodak/IBI) and the peak TBP-containing fraction eluted with 125 ng FLAG peptide (Fig. 5C, lane 3; fractions eluted at 0.2 mg/ml FLAG peptide or with glycine contained significantly less FLAG–TBP; data not shown). The composition of polypeptides eluting with TBP is seen in Figure 5A, lane 3, and B, lane 2. While multiple polypeptides co-fractionated with TBP, three are marked in Figure 5B (lane 2) as appearing approximately equimolar and as having sizes correlating with the subunit size of TBP-associated factors present in mammalian SL1/S.cerevisiae polymerase I core factor. An additional four to five polypeptides are also present, including prominent polypeptides of ~42 and ~47 kDa.
of the alternate start and in direction of initiation at the correct site (marked +1; see Fig. 6A, lane 3). Thus, addition of the \textit{S. pombe} TBP complex does not direct initiation on its own (Fig. 7B, lane 1), but reprograms initiation of the \textit{S. pombe} RNA gene promoter in conjunction with other required transcription factors supplied in the \textit{S. cerevisiae} extract. Thus, the complex of TBP and associated factors purified from active \textit{S. pombe} RNA polymerase I transcription components represents SL1 activity: the \textit{S. pombe} TBP and TBP-associated factors were able to direct correct initiation of their own species’ rDNA gene promoter in a heterologous RNA polymerase I transcription system. While the efficiency of initiation site utilization appears to be low, this may be due to requirements for interactions between an \textit{S. pombe} SL1 complex and an \textit{S. pombe} upstream rDNA promoter binding complex.

Further evidence that the \textit{S. pombe} TBP complex contains SL1 activity comes from reconstitution analysis. Immunodepletion of the \textit{S. pombe} pol III synthetic machinery of TBP and TBP-associated factors abolishes its ability to direct correct rDNA transcriptional initiation (see Fig. 7B, lane 2). Reconstitution of correct \textit{in vitro} transcription of the \textit{S. pombe} RNA gene promoter requires both this immunodepleted fraction and the immunopurified \textit{S. pombe} TBP complex (see Fig. 7A, lane 1).

**Discussion**

Formation of the complex assembly of factors required to direct correct initiation of eukaryotic rRNA genes involves association of the essential initiation factor SL1 (also called TIF-IB, Rb1 and factor D; 1–8) at an early step in this process (7,41). This association is promoted by UBF in vertebrates (8,41–43), by an enhancer binding factor in \textit{Acanthamoeba} (44) and apparently by an upstream activating factor, UAF (45), in \textit{S. cerevisiae}. An rDNA transcriptional stimulatory activity of \textit{S. pombe} forms a stable complex with the rDNA promoter and may also promote association of SL1 (Chen, L., Zhao, A., Liu, Z., Boukhalter, B. and Pape, L., submitted for publication).

While TBP is a component of the essential initiation complex for all three nuclear RNA polymerases in yeast (14,15), its association with the essential initiation factor for rRNA synthesis initially appeared less stable in the yeast \textit{S. cerevisiae} (13) than was the case for mammalian SL1 complexes (1; TIF-IB; 4). The TFIIId initiation factor for RNA polymerase II catalyzed transcription was initially isolated as the TBP monomer from yeast (46), but both TFIIIB and TFIIID were later shown to consist of multiple subunits (47–49), akin to the analogous complexes in higher eukaryotes (17).

In \textit{S. cerevisiae}, three of the subunits of an essential transcription factor for rRNA synthesis are Rrn6p, Rrn7p (13) and Rrn11p (25,26). Very recent results demonstrate that these subunits...
associate with TBP (25,26). In this paper, we have shown that a fission yeast complex can be immunopurified from active RNA polymerase I transcription components consisting of a tagged TBP and TBP-associated polypeptides. Furthermore, this complex is capable of repressing an incorrect transcriptional start site on a S. pombe rDNA promoter and promoting the correct start cross-species. It is of interest that the yeast, human and mouse essential initiation factor for rRNA synthesis in SL1 TAFs are and whether they share homology with yeast TFIID (49) or subunits of other polymerase–TBP complexes or subunits of a polymerase I UAF-like complex (50).

It remains to be determined what the primary sequence of the S. pombe SL1 TAFs is and whether they share homology with human TAF110, TAF63 and TAF48 (11). The subunits of the essential initiation factor for rRNA synthesis in S. cerevisiae, Rrn6p, Rrn7p and Rrn11p (p66) (13,25,26), are unrelated in primary sequence to the mammalian SL1 subunits and efforts to isolate coding sequences for the S. pombe subunits utilizing heterologous mammalian or S. cerevisiae probes have been unsuccessful, suggesting that their primary sequences may also vary significantly from other SL1/core factor subunits.

The association of the essential RNA polymerase I initiation factor with the rDNA core promoter region is critical for rRNA synthesis, but stimulatory factors are required to stabilize this interaction (8,41–45,50). We have found that S. pombe SL1 can form a weak complex with the S. pombe rDNA promoter (data not shown). Figure 6D shows a comparison of the core rDNA promoter sequences of S. pombe (32) with those of S. cerevisiae (31,52) and may explain why a transcription start, albeit aberrant, is seen in cross-species transcription of an S. pombe rDNA promoter with S. cerevisiae polymerase I transcription components (Fig. 6A). Conserved regions extending between −26 and −14 and between −10 and −3 may direct basal level cross-species initiation dependent on this core rDNA promoter, but at an altered initiation site (54). Addition of the putative S. pombe SL1 complex results in correct recognition of and association with its own species promoter to direct initiation at the natural start site.

It has not been possible to identify homologous TAFI-encoded genomic sequences in S. pombe as of yet by searching S. pombe sequence databases. This may be due to sequence heterogeneity for all of the TAFIs or simply that the genomic region encoding the TAFIs has not been sequenced. While the S. cerevisiae TAFIs are highly homologous to their human counterparts (49), none of the subunits of the S. cerevisiae essential RNA polymerase I transcription factor, Rrn6p, Rrn7p or Rrn11p (13,25,26), show any defining homology to the TAFI110 and TAFI48 or TAFI63 polypeptides (1,55). This lends further evidence to differences in factors and mechanisms involved in species-specific rRNA promoter activation. The identity of the interactions directing species-specific RNA polymerase I transcriptional initiation will further our understanding of the evolution of species-specific cis-acting regulatory elements of eukaryotic rRNA genes and, in turn, of the corresponding RNA polymerase I transcriptional machinery that correctly transcribes only its target genes, in both a polymerase class- and a species-specific manner.

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