© 1997 Oxford University Press
Nucleic Acids Research, 1997, Vol. 25, No. 8 **1633–1640**

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

Ling Chen[+], Ailan Guo and Louise Pape*

Department of Chemistry, New York University, New York, NY 10003, USA

Received October 11, 1996; Revised and Accepted February 10, 1997

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 TBP-associated factors (TAF_Is): TAF_I110 (human), TAF_I95 (mouse) and TAF_I63 and TAF_I48 (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, TFIID, bears seven or more TBP-associated factors (TAF_{II}s; 17–19), while the polymerase III complex, TFIIIB, bears two (20–23). While the mouse and human SL1/TF-IB factors each contain TBP and three TAF_Is, 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 Rrn11p 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

*To whom correspondence should be addressed. Tel: +1 212 998 8444; Fax: +1 212 260 7905; Email: papel01@mcrcr.med.nyu.edu

[+]Present address: Department of Microbiology, Ohio State University, Columbus, OH 43210, USA

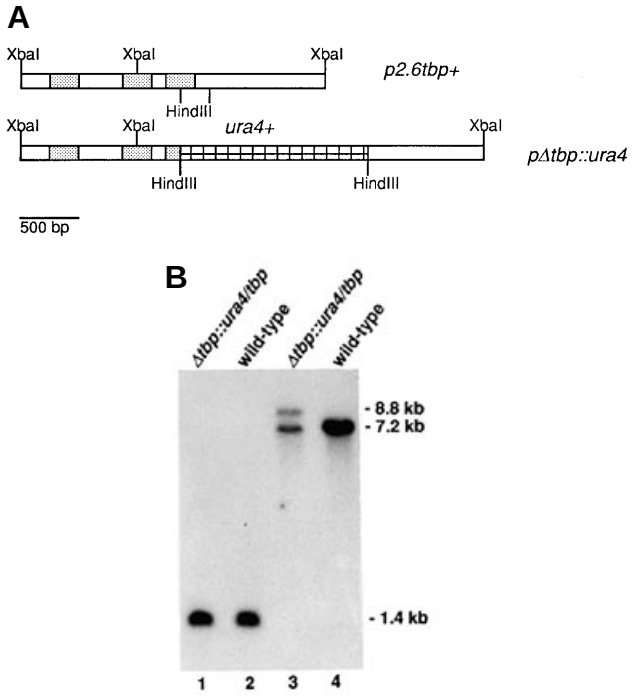

Figure 1. (**A**) Construction of a disrupted *S.pombe tbp*$^+$ gene. The p2.6*tbp*$^+$ plasmid DNA contains an ~2.6 kb partial *Xba*I fragment inserted into pBS SK$^+$ (see positions 12 357–14 991 of *S.pombe* genomic sequence, accession no. Z66525). The three exons of *tbp*$^+$ are shown as stipled boxes. To create the disrupted copy of *tbp*$^+$ coding sequences, a partial *Hin*dIII digest of p2.6*tbp*$^+$ 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 *Hin*dIII fragment bearing *ura4*$^+$ was inserted into this *Hin*dIII site, creating pΔ*tbp*::*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 SP826 (lanes 2 and 4) and from diploid Ura$^+$ transformants (a representative is shown in lanes 1 and 3) and digested with *Eco*RI and *Hin*dIII (lane 1 and 2) or *Eco*RI (lanes 3 and 4). The 7.2 kb *Eco*RI fragment bears the wild-type allele of *tbp*$^+$ and the 8.8 kb *Eco*RI fragment harbors the disrupted *tbp* allele (Δ*tbp*::*ura4*). The Southern blot was hybridized with an ~1 kb *Xba*I probe derived from the 5′ two thirds of the *tbp*$^+$ genomic DNA (see A).

Hoffmann; 27) was partially digested with *Xba*I and a 2.6 kb fragment was gel isolated and subcloned into the pBS SK$^+$ *Xba*I site (corresponding to positions 12 376–14 991; DDBJ/EMBL/GenBank accession no. Z66525). Following partial digestion of the resultant plasmid, p2.6*tbp*$^+$, with *Hin*dIII, a 5.4 kb fragment was gel isolated which lacked 162 bp between the two *Hin*dIII sites of *tbp*$^+$ (see Fig. 1A). This was ligated to a 1.8 kb *Hin*dIII fragment containing the wild-type *S.pombe ura4*$^+$ coding sequences, which was isolated from pREP2 (29,30). The resultant plasmid, pΔ*tbp*::*ura4*, bearing the disrupted *tbp* gene, was linearized with *Xba*I and the insert was transformed into *S.pombe* as described below.

Replacement of the chromosomal *S.pombe tbp*$^+$ gene with a disrupted version via homologous recombination

A 3.3 kb *Xba*I fragment containing Δ*tbp*::*ura4* sequences was gel isolated from pΔ*tbp*::*ura4* (see Fig. 1A) and 1 µg was used to transform *S.pombe* SP826 (*h*$^+$/*h*$^+$ *leu l-32//leu1-32 ura-4-D18/ura4-18 ade6-216/ade6-210*; kindly sent by Dr Dave Frendewey). Ura$^+$ transformants were selected (30) and Southern analysis was performed to assess whether a chromosomal allele of *tbp*$^+$ was disrupted in the Ura$^+$ transformants. Genomic DNA was extracted from 10 ml culture using the glass beads method as described (31). One fifth of the extracted nucleic acids from individual *S.pombe* Ura$^+$ transformants was subjected to Southern analysis (31). Following fractionation on a 1% agarose–1× TBE gel and transfer to nitrocellulose, the DNA was cross-linked to the membrane using a GN Gene linker (BioRad) and hybridized in 50% formamide, 6× SSC, 0.1% SDS, 5× Denhardt's reagent, 50 mM Na_3PO_4, pH 6.5, 50 µg/ml single-stranded calf thymus DNA and ~10^6 c.p.m. genomic *tbp*$^+$ probe at 42°C overnight. As seen in Figure 1B, an ~7.2 kb *Eco*RI fragment contains the TBP coding sequences in the parental diploid strain (lane 4), while one-step gene disruption of this diploid results in production of an ~8.8 kb *Eco*RI fragment containing *tbp* sequences (lane 3), due to the *ura4*$^+$ coding sequences present in the disrupted copy, in addition to the 7.2 kb *Eco*RI fragment. Digestion of the genomic DNAs with *Eco*RI and *Hin*dIII liberates the same sized genomic fragment from both the parental and the diploid strains carrying the disrupted *tbp* allele, ~1.4 kb in size, as expected (Fig. 1B, lanes 1 and 2).

Radioactive labeling of the probe by random priming

Aliquots of 100 ng 1 kb *Xba*I fragment containing the 5′ two-thirds of the *S.pombe tbp*$^+$ gene (see Fig. 1A) were labeled by the random priming method in the presence of dA,G and TTPs and 40 µCi [α^{32}P]dCTP (NEN; >3000 Ci/mmol) (31). Random hexamers were obtained from Pharmacia.

Construction of an *S.pombe/Escherichia coli* shuttle vector bearing *S.pombe* FLAG–TBP

The *S.pombe tbp*$^+$ cDNA sequences were amplified utilizing a primer designed to insert coding sequences for the eight amino acid FLAG™ epitope tag (Kodak/IBI) following the initiating methionine at the N-terminus of *tbp*$^+$. This position was shown to be neutral for insertion of an epitope tag in the human TBP coding sequences (33). To this end, the *S.pombe* TBP coding sequences [from the TFIID cDNA clone (27), kindly sent by Drs Alexander Hoffman and M.Horikoshi] were amplified using as forward primer 5′-GCCATATGGATTACAAAGACGATGACGACAAG-GATTTCGCTTTACC, encoding MDYKDDDDKDFAL, and a vector-specific reverse primer. The FLAG™ tag consists of the eight amino acids DYKDDDDK (Kodak/IBI). The PCR products were treated with T4 DNA polymerase to convert the ends to blunt ends and ligated to *Sal*I linkers. Following digestion with *Sal*I and preparative isolation of the fragment, it was subsequently ligated into the *Sal*I site of pBluescript SK$^+$. Due to frequent deletions of the TBP coding sequence, this procedure and screening of *E.coli* Amp^r transformants had to be repeated multiple times until a correct, full-length clone was isolated, pFLAG-*S.p.*TBP. The FLAG–TBP insert was released by digestion with *Nde*I and *Bam*HI and ligated into the *Nde*I and *Bam*HI sites of the *S.pombe*/*E.coli* shuttle vector pRep1 (29; kindly sent by Dr Kinsey Maundrell), creating pRep1/FLAG-*S.p.*TBP.

Introduction of a tagged version of *tbp*$^+$ cDNA into the Δ*tbp*::*ura4*/*tbp*$^+$ diploid strain of *S.pombe*

Plasmid pRep1/FLAG-*S.p.*TBP was introduced into a diploid *S.pombe* strain bearing a disrupted *tbp*$^+$ allele (*tbp*/Δ*tbp*::*ura4*)

and Leu$^+$ transformants were selected. Following sporulation and selective killing of non-sporulating diploid cells, Leu$^+$, Ura$^+$, 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 µl 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 Δ*tbp*::*ura4* chromosomal allele and the extrachromosomal FLAG–TBP coding sequences (data not shown). The resultant strain is SpΔTBP (*h*$^+$ *leu l-32 ura-4-D18 ade6*$^-$Δ*tbp*::*ura4* pRep1/FLAG-*S.p.*TBP). The extrachromosomal plasmid 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

SpΔTBP was grown in thiamine-deficient EMM medium, to ensure maximal expression of FLAG–TBP (30), with constant vigorous shaking at 30°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°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°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 Pharmacia 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™ 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™ M2 monoclonal antibody; Kodak/IBI) was added at 1:300 dilution, following standard protocols (31,33). Detection utilized enhanced chemiluminiscent reagents from Amersham 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:XH (Fig. 3) has been described previously (32). p5′Δ-243/3′Δ+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′Δ-243/3′Δ+89 (32) was used in the transcription assays shown in Figure 7 (at a final concentration of 0.5 µg/ml). *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 be 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′-^{32}P-labeled *Hpa*II fragments derived from pBR322. The 5′-end-labeled probe used to detect transcription supported by template p-243:XH was prepared by labeling at the unique *Xba*I 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′Δ-243/3′Δ+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′-243/3′Δ+89 probe was labeled at a unique *Xho*I site at +135 on the template strand. The initiation site for *in vitro S.pombe* rRNA synthesis was shown to be the same *in vitro* as *in vivo* (32; see also Fig. 6C).

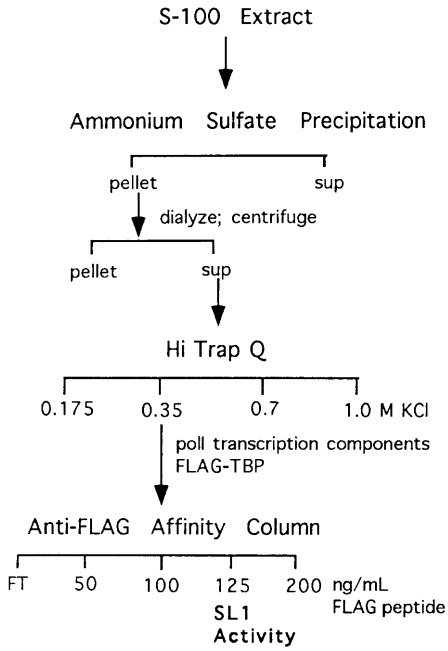

Figure 2. Fractionation scheme for purification of *S.pombe* TBP-containing essential initiation factor for RNA polymerase I transcription.

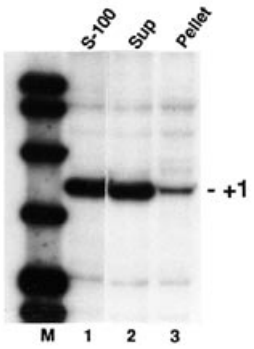

Figure 3. Transcriptional analysis of fractionated *S.pombe* RNA polymerase I components. Following ammonium sulfate precipitation of *S.pombe* S-100, the pellet was extensively dialyzed and subjected to centrifugation (24) and the resultant supernatant and pellet assessed for RNA polymerase I transcription components. Correctly initiated transcription supported by the template p-243:XH, bearing a full rDNA promoter and rDNA termination sites, was assessed using control S-100 extract (lane 1), supernatant (lane 2) and pellet (lane 3) (~25 µg each). An S1-protected fragment of 340 nt represents correctly initiated RNA (32) and is marked +1. Markers (M) are ^{32}P-labeled pBR/*Hpa*II fragments.

S1-protected fragments representing rRNAs initiated *in vitro* in *S.cerevisiae* or *S.pombe* S-100 extracts were electrophoresed on sequencing gels next to Maxam–Gilbert sequencing reactions for mapping the start site (see Fig. 6C; 31).

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 l-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* (r_{k-}, m_{k+}), *supE44*, *thi-1*, λ$^-$, *recA1*, *gyrA96*, *relA1*, *lac*, (F′, *proAB*, *lacI*^{q}*ZΔM15*, Tn*10*, (tet^r)] and SURE™ [*mcrA*, Δ(*mcrBC-hsdRMS-mrr)171*, *endA1*, *supE44*, *thi-a*, λ$^-$, *gyrA96*, *relA1*, *lac*, *recB*, *recJ*, *sbcC*, *umuC*::Tn*5*, (kan^r), *uvrC*, (F′, *proAB*, *lacI*^{q}*ZΔM15*, Tn*10*, (tet^r)] (Stratagene). 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 pΔ*tbp*::*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, pRep1/FLAG-*S.p.*TBP, was constructed and introduced into this Δ*tbp*::*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 dialyzed, 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.

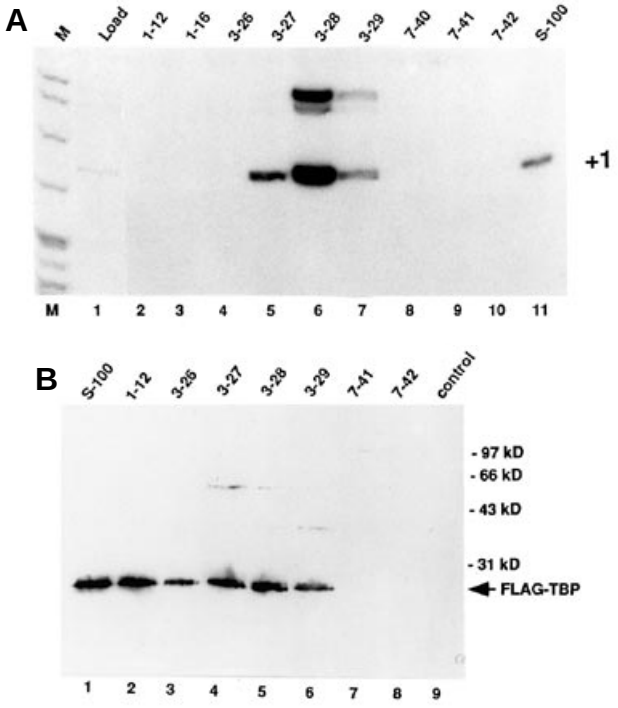

Figure 4. (**A**) Transcriptional analysis of RNA polymerase I components following chromatographic separation on a HiTrap-Q column. Following HiTrap-Q chromatographic separation, fractions eluted at 0.175, 0.35 and 0.7 M KCl were assessed for transcriptional capacity. Five microliters of each fraction 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.

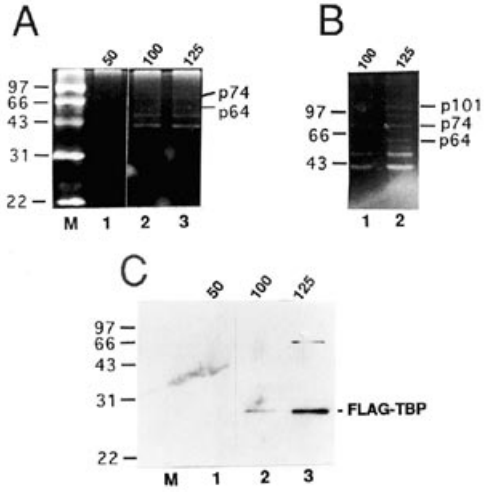

Figure 5. Immunoaffinity purification of *S.pombe* TBP-associated polypeptides from RNA polymerase I transcription components. The peak fractions containing RNA polymerase I transcription components were pooled and subjected to immunoaffinity purification via an anti-FLAG M2 affinity gel. (**A**) Resolution 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, 25 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.

Fractions eluted between 0.175 and 0.35 M KCl contained all components required for correct initiation (see Fig. 4A, lanes 5–7). In addition, FLAG–TBP is detected in these peak fractions, as expected (Fig. 4B, lanes 4–6). Although fraction 3-26 (lane 3) contains FLAG–TBP, it does not support initiation; this fraction may lack a required component, such as activated polymerase, αTIF-IC activity (37–40; Guo,A. and Pape,L., unpublished data) or another factor. FLAG–TBP eluted at lower KCl concentration is presumably in the TBP-containing initiation complex for RNA polymerase II or III catalyzed transcription, TFIID (17–19) or TFIIIB (20–23), or is free TBP (Fig. 4B, lane 2).

The fractions containing the peak rDNA transcriptional activity were combined for further resolution. Immunoaffinity purification of the FLAG–TBP-associated factors present in the fractions active for RNA polymerase I catalyzed initiation was performed using an anti-FLAG antibody-conjugated matrix and the composition and activity of TBP and its associated factors assessed. Polypeptides eluted from the immunoaffinity column with increasing concentrations of FLAG peptide were subjected to fractionation by SDS–PAGE. Western analysis of TBP and TBP-associated polypeptides is shown in Figure 5C (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.

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

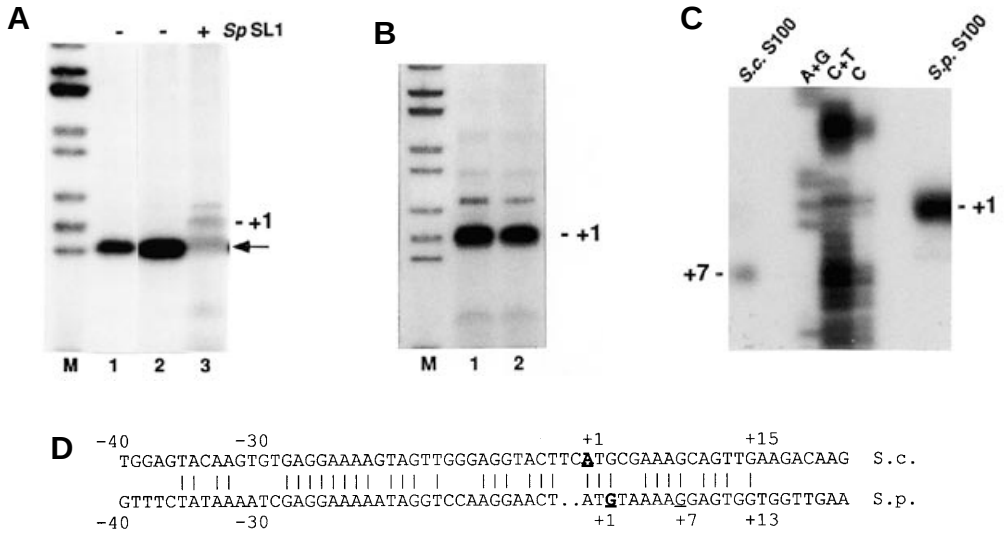

Figure 6. (**A**) Cross-species transcriptional analysis of the RNA polymerase I transcription complex of FLAG–TBP and associated polypeptides. To assess for *S.pombe* SL1 activity, the components that co-eluted with TBP were added to *S.cerevisiae* RNA polymerase I *in vitro* transcription reactions and RNA polymerase I catalyzed transcriptional initiation supported by the *S.pombe* rDNA template p5′Δ-243/3′Δ+31 was assessed (lane 3). The control lanes (1 and 2) show S1 analysis of transcription reactions conducted in the absence of added *S.pombe* SL1, with respectively 10 µl (~50 µg) and 20 µl (~100 µg) *S.cerevisiae* S-100 extract. Lane 3 shows S1 analysis of a reaction conducted with 20 µl *S.cerevisiae* S-100 extract plus ~1 ng *S.pombe* TBP-containing polymerase I complex (+ *Sp*SL1). The arrow points to the alternate transcription start (at +7) on the *S.pombe* rRNA gene promoter and +1 marks the S1 resistant fragment representing correctly initiated *S.pombe* rRNAs. The radioactive probe is a single-stranded DNA molecule derived from p5′Δ-243/3′Δ+31 5′-end-labeled with ^{32}P at +77 on the template strand. (**B**) Transcription of the same *S.pombe* rDNA template, p5′Δ-243/3′Δ+31, in an *S.pombe in vitro* transcription reaction is shown, with the S1 resistant fragment representing correctly initiated rRNAs marked with +1. The minor S1-protected fragment of ~92 nt, representing initiation at ~–15, is also seen for *in vivo* synthesized rRNAs; other minor S1-protected fragments may represent non-specifically initiated rRNAs. (**C**) S1-protected-fragments representing the alternative polymerase I start on the *S.pombe* rDNA promoter using *S.cerevisiae* transcription components (left lane) and the wild-type start using the homologous components (right lane) flank lanes containing Maxam–Gilbert sequencing reactions. The sequencing ladders are A+G, C+T and C reactions (31). (**D**) Initiation region of the rRNA genes of *S.cerevisiae* and *S.pombe*. The top line shows the sequence of the *S.cerevisiae* rRNA gene promoter, with the initiation site shown in bold and underlined (+1). The bottom sequence is that of the *S.pombe* rRNA gene. The initiation site is underlined and in bold and the alternate transcription start at +7 utilized cross-species is underlined.

of the alternate start and in direction of initiation at the correct site (marked +1; see Fig. 6A, lane 3). Thus, addition of the *S.pombe* TBP complex revealed two inherent activities: repression of the incorrect start and direction of a correct start on the *S.pombe* rRNA gene promoter (Fig. 6A, lane 3).

The *S.pombe* TBP fraction does not direct initiation on its own (Fig. 7B, lane 1), but reprograms initiation of the *S.pombe* rRNA gene promoter in conjunction with other required transcription factors supplied in the *S.cerevisiae* extract. Thus, the complex of TBP and associated factors purified from active *S.pombe* RNA polymerase I transcription components represents SL1 activity: the *S.pombe* TBP and TBP-associated factors were able to direct correct initiation of their own species' rRNA 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 *S.pombe* SL1 complex and an *S.pombe* upstream rDNA promoter binding complex.

Further evidence that the *S.pombe* TBP complex contains SL1 activity comes from reconstitution analysis. Immunodepletion of the *S.pombe* polymerase I 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 *in vitro* transcription of the *S.pombe* rRNA gene promoter requires both this immunodepleted fraction and the immunopurified *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, Rib1 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 *Acanthamoeba* (44) and apparently by an upstream activating factor, UAF (45), in *S.cerevisiae*. An rDNA transcriptional stimulatory activity of *S.pombe* forms a stable complex with the rDNA promoter and may also promote association of SL1 (Chen,L., Zhao,A., Liu,Z., Boukghalter,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 *S.cerevisiae* (13) than was the case for mammalian SL1 complexes (1; TIF-IB; 4). The TFIID initiation factor for RNA polymerase II catalyzed transcription was initially isolated as the TBP monomer from yeast (46), but both TFIIIB and TFIID were later shown to consist of multiple subunits (47–49), akin to the analogous complexes in higher eukaryotes (17).

In *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

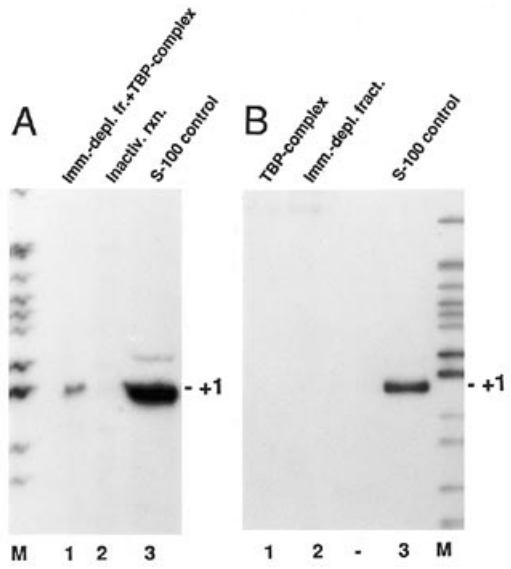

Figure 7. The *S.pombe* TBP complex is essential for reconstitution of rDNA transcriptional activity. (**A**) Transcription reactions utilizing an *S.pombe* fraction immunodepleted of TBP (Imm.-depl.fr.) together with the fraction containing *S.pombe* TBP and TBP-associated factors (TBP-complex) reconstituted the activity required for directing correct initiation of the *S.pombe* rDNA template 5′Δ-243/3′Δ+89 (lane 1). This activity was inhibited by competition with 2.5 μg/ml rDNA template (lane 2, inactiv. rxn.). Lane 3 is a control lane showing the S1-protected fragment representing correctly initiated rRNAs synthesized in a reaction containing unfractionated S-100 extract (S-100 control). The S1-protected fragment of 135 nt (noted with +1) represents correctly initiated rRNAs. (**B**) The ability of the TBP complex alone (lane 1, TBP-complex) or the immunodepleted fraction (Imm.-depl.fract., lane 2) to direct correct initiation was assessed. Lane 3 is a control lane showing the S1-protected fragment representing correctly initiated rRNAs (S-100 control). Size markers are end-labeled pBR322/*Hpa*II fragments. The radioactive probe is a single-stranded DNA molecule derived from p5′Δ-243/3′Δ+89 5′-end-labeled with ^{32}P at +135 on the template strand.

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 subunits of the essential initiation factor for rRNA synthesis show a similar polypeptide profile. Comparison of the profile of polypeptides co-eluting with TBP and with the profile of polypeptides in the human and mouse SL1 complex (1,4,11), as well as in the multi-subunit initiation complex for *S.cerevisiae* (13,25,26), suggests that the three TBP-associated polypeptides that may be the *S.pombe* SL1 TAF_Is are the ~64, 74 and ~101 kDa polypeptides, however, assignment awaits sequence determination of these polypeptides. Polypeptides present that are not bona fide TAF_Is could be contaminating polypeptides that are detected upon affinity purification of TBP-associated factors (48) and 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 are and whether they share homology with human TAF_I110, TAF_I63 and TAF_I48 (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* (51,52) 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 TAF_I-encoding 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 TAF_Is or simply that the genomic region encoding the TAF_Is has not been sequenced. While the *S.cerevisiae* TAF_{II}s 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 TAF_I110 and TAF_I48 or TAF_I63 polypeptides (1,55). This lends further evidence to differences in factors and mechanisms involved in species-specific rDNA 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.

ACKNOWLEDGEMENTS

This work was supported by NSF grant MCB-9219220 to L.K.P. and in part by NYU Research Challenge Fund grants. NSF is also thanked for its support of the computing resources through grant BIR-9318128. We thank Drs Alexander Hoffman, Masami Horikoshi and Robert Roeder for *S.pombe* TBP genomic and cDNA clones and suggestions and we thank Drs Henry Levin and Dave Frendewey for useful suggestions and discussions.

REFERENCES

1 Comai,L., Tanese,N. and Tjian,R. (1992) *Cell*, **78**, 965–976.
2 Smith,S.D., Oriahi,E., Lowe,D., Yang-Yen,H.-F., O'Mahony,D., Rose,K., Chen,K. and Rothblum,L.I. (1990) *Mol. Cell. Biol.*, **10**, 3105–3116.
3 Eberhard,D., Tora,L., Egly,J.-M. and Grummt,I. (1993) *Nucleic Acids Res.*, **21**, 4180–4186.
4 Rudloff,U., Eberhard,D., Tora,L., Stunnenberg,H. and Grummt,I. (1994) *EMBO J.*, **13**, 2611–2616.
5 Radebaugh,C.A., Matthews,J.L., Geiss,G.K., Liu,F., Wong,J.M., Bateman,E., Camier,S., Sentenac,A. and Paule,M.R. (1994) *Mol. Cell. Biol.*, **14**, 597–605.

6 Mishima,Y., Financsek,I., Kominami,R. and Muramatsu,M. (1982) *Nucleic Acids Res.*, **10**, 6659–6669.
7 Sollner-Webb,B. and Tower,J. (1986) *Annu. Rev. Biochem.*, **55**, 801–830.
8 McStay,B., Hu,C.H., Pikaard,C.S. and Reeder,R.H. (1991) *EMBO J.*, **10**, 2297–2303.
9 Kownin,P., Bateman,E. and Paule,M.R. (1987) *Cell*, **50**, 693–699.
10 Tower,J. and Sollner-Webb,B. (1987) *Cell*, **50**, 873–883.
11 Zomerdijk,J.C., Beckmann,H., Comai,L. and Tjian,R. (1994) *Science*, **266**, 2015–2018.
12 Gong,X., Radebaugh,C.A., Geiss,G.K., Simon,M.N. and Paule,M.R. (1995) *Mol. Cell. Biol.*, **15**, 4956–4963.
13 Keys,D.A., Vu,L., Steffan,J.S., Dodd,J.A., Yamamoto,R.T., Nogi,Y. and Nomura,M. (1994) *Genes Dev.*, **8,** 2349–2362.
14 Schultz,M.C., Reeder,R.H. and Hahn,S. (1992) *Cell*, **69**, 697–702.
15 Cormack,B.P. and Struhl,K. (1992) *Cell*, **69**, 685–696.
16 Hernandez,N. (1993) *Genes Dev.*, **7**, 1291–1308.
17 Tanese,N. and Tjian,R. (1993) *Cold Spring Harbor Symp. Quant. Biol.*, **58**, 179–185.
18 Meyers,R.E. and Sharp,P.A. (1993) *Mol. Cell. Biol.*, **13**, 7953–7960.
19 Zhou,Q., Boyer,T.G. and Berk,A.J. (1993) *Genes Dev.*, **7**, 180–187.
20 Buratowski,S. and Zhou,H. (1992) *Cell*, **71**, 221–230.
21 Kassavetis,G.A., Nguyen,S.T., Kobayashi,R., Kumar,A., Geiduschek,E.P. and Pisano,M. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 9786–9760.
22 Lopez-de-Leon,A., Librizzi,M., Puglia,K. and Willis,I.M. (1992) *Cell*, **71**, 211–220.
23 McBryant,S.J., Meier,E., Leresche,A., Sharp,S.J., Wolf,V.J. and Gottesfeld, J.M. (1996) *Mol. Cell. Biol.*, **167**, 4639–4647.
24 RiggsD.L., Peterson,C.L., Wickham,J.Q., Miller,L.M., Clarke,E.M., Crowell,J.A. and Sergere,J.C. (1995) *J. Biol. Chem.*, **270**, 6205–6210.
25 Lalo,D., Steffan,J.S., Dodd,J.A. and Nomura,M. (1996) *J. Biol. Chem.*, **271**, 21062–21067.
26 Lin,C.W., Moorefield,B., Payne,J., Aprikian,P., Mitomo,K. and Reeder,R.H. (1996) *Mol. Cell. Biol.*, **16**, 6436–6443.
27 Hoffmann,A., Horikoshi,M., Wang,C.K., Schroeder,S., Weil,P.A. and Roeder,R.G. (1990) *Genes Dev.*, **4**, 1141–1148.
28 Fikes,J.D., Becker,D.M., Winston,G. and Guarente,L. (1990) *Nature*, **346**, 291–294.
29 Maundrell,K. (1993) *Gene*, **123**, 127–130.
30 Moreno,S., Klar,A. and Nurse,P. (1991) *Methods Enzymol.*, **194**, 795–823.
31 Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1994) *Current Protocols in Molecular Biology.* John Wiley & Sons, New York, NY.
32 Liu,Z., Chen,L., Zhao,A. and Pape,L. (1997) *Nucleic Acids Res.*, **25**, 659–667.
33 Zhou,Q., Lieberman,P.M., Boyer,T.G. and Berk,A.J. (1992) *Genes Dev.*, **6**, 1964–1976.
34 Schagger,H. and von Jagon,F. (1987) *Anal. Biochem.*, **166**, 367.
35 Schultz,M.C., Choe,S.Y. and Reeder,R.H. (1990) *Proc. Natl. Acad. Sci. USA*, **88**, 1004–1008.
36 Rothstein,R.J. (1983) *Methods Enzymol.*, **101**, 202–210.
37 Schnapp,G., Schnapp,A., Rosenbauer,H. and Grummt,I. (1994) *EMBO J.*, **13**, 4028–4035.
38 Yamamoto,R.T., Nogi,Y., Dodd,J.A. and Nomura,M. (1996) *EMBO J.*, **15**, 3964–3973.
39 Bateman,E. and Paule,M.R. (1986) *Cell*, **47**, 445–450.
40 Brun,R.P., Ryan,K. and Sollner-Webb,B. (1994) *Mol. Cell. Biol.*, **14**, 5010–5021.
41 Jantzen,H.M., Chow,A.M., King,D.S. and Tjian,R. (1992) *Genes Dev.*, **6**, 1950–1963.
42 Hempel,W.M., Cavanaugh,A.H., Hannan,R.D., Taylor,L. and Rothblum,L.I. (1996) *Mol. Cell. Biol.*, **16**, 557–563.
43 Schnapp,A. and Grummt,I. (1991) *J. Biol. Chem.*, **266**, 24588–24595.
44 Yang,Q., Radebaugh,C.A., Kubaska,W., Geiss,G.K. and Paule,M.R. (1995) *Nucleic Acids Res.*, **23**, 4345–4352.
45 Keys,D.A., Lee,B.-S., Dodd,J.A., Nguyeu,T.T., Vu,L., Fantino,E., Burson,L.M., Nogi,Y. and Nomura,M. (1996) *Genes Dev.*, **10**, 887–903.
46 Hahn,S., Buratowski,S., Sharp,P.A. and Guarente,L. (1989) *Cell*, **58**, 1173–1181.
47 Poon,D. and Weil,P.A. (1993) *J. Biol. Chem.*, **268**, 15325–15328.
48 Kwon,H. and Green,M.R. (1994) *J. Biol. Chem.*, **269**, 30140–30146.
49 Poon,D., Bai,Y., Campbell,A.M., Bjorklund,S., Kim,Y.J., Zhou,S., Kornberg,R.D. and Weil,P.A. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 8224–8228.
50 Steffan,J.S., Keys,D.A., Dodd,J.A. and Nomura,M. (1996) *Genes Dev.*, **10**, 2551–2563.
51 Kulkens,T., Riggs,D.L., Heck,J.D., Planta,R.J. and Nomura,M. (1991) *Nucleic Acids Res.*, **19**, 5363–5370.
52 Choe,S.Y., Schultz,M.C. and Reeder,R.H. (1992) *Nucleic Acids Res.*, **20**, 279–285.
53 Ruppert,S., Wang,E.H. and Tjian,R. (1993) *Nature*, **362**, 175–179.
54 Pape,L.K., Windle,J.J. and Sollner-Webb,B. (1990) *Genes Dev.*, **4**, 52–62.
55 Comai,L., Zomerdikj,J.C.l, Beckmann,H., Zhou,S., Admon,A. and Tjian,R. (1994) *Science*, **266**, 1966–1972.