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The Association of TIF-IA and Polymerase I Mediates Promoter Recruitment and Regulation of Ribosomal RNA Transcription in *Acanthamoeba castellanii*

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Large amounts of energy are expended for the construction of the ribosome during both transcription and processing, so it is of utmost importance for the cell to efficiently regulate ribosome production. Understanding how this regulation occurs will provide important insights into cellular growth control and into the coordination of gene expression mediated by all three transcription systems. Ribosomal RNA (rRNA) transcription rates closely parallel the need for protein synthesis; as a cell approaches stationary phase or encounters conditions that negatively affect either growth rate or protein synthesis, rRNA transcription is decreased. In eukaryotes, the interaction of RNA polymerase I (pol I) with the essential transcription initiation factor IA (TIF-IA) has been implicated in this downregulation of transcription. In agreement with the first observation that rRNA transcription is regulated by altering recruitment of pol I to the promoter in *Acanthamoeba castellanii*, we show here that pol I and an 80-kDa homologue of TIF-IA are found tightly associated in pol I fractions competent for specific transcription. Disruption of the pol I–TIF-IA complex is mediated by a specific dephosphorylation of either pol I or TIF-IA. Phosphatase treatment of TIF-IA-containing *A. castellanii* pol I fractions results in a downregulation of both transcriptional activity and promoter binding, reminiscent of the inactive pol I fractions purified from encysted cells. The fraction of pol I competent for promoter recruitment is enriched in TIF-IA relative to that not bound by immobilized promoter DNA. This downregulation coincides with an altered electrophoretic mobility of TIF-IA, suggesting at least it is phosphorylated.

Key words: Growth control; Polymerase I; Rrn3; rRNA; Transcription; Regulation

RIBOSOMAL RNA (rRNA) transcription can account for up to 60% of all RNA being transcribed in an actively dividing cell. Because of the large amount of energy this transcription requires, it is of utmost importance for the cell to efficiently regulate rRNA transcription. Additionally, recent studies have shown a direct link between the transition from normal to neoplastic growth; indeed, it is not clear whether uncontrolled rRNA transcription might lead to abnormally high cellular growth and division. Thus, uncovering the mechanism(s) that regulate this transcription may lead to an understanding of cellular growth control in general and to possible avenues for attacking uncontrolled cell cycling.

A particular RNA polymerase I (pol I) transcription factor found to be tightly linked to growth rate has been shown in many organisms to be essential for pol I transcription. This factor, transcription initiation factor IA (TIF-IA), was first characterized in the mouse system as being the growth-regulated factor,

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and was later identified in a *Saccharomyces cerevisiae* genetic screen for essential pol I transcriptional components (10,31,37). Termed Rrn3p in yeast (but herein called scTIF-IA), this factor associates with pol I specifically, in the presence or absence of DNA, and is not believed to bind to DNA (23,31,37). ScTIF-IA is required to be associated with pol I for specific transcriptional initiation to occur at the rRNA promoter (23,24,27,37). Less that 2% of the pol I found in a yeast cell is present in this initiation-competent pol I–TIF-IA complex, with the rest of the pol I functioning in elongation or possibly reinitiation (23). When extracts are prepared from cells under unfavorable conditions, such as yeast cells in stationary phase, the protein levels of both pol I and TIF-IA remain constant even though the levels of specific transcription are dramatically decreased (11,23,38). These results suggests that as a cell undergoes the transformation from actively dividing to stationary phase, there must be a modification in either pol I, TIF-IA, or both that disrupts the formation of the pol I–TIF-IA complex.

The association of TIF-IA with pol I that is required for specific transcription initiation is mediated by the direct binding of TIF-IA to a specific pol I subunit, the A43 subunit of pol I in yeast (27). In *Schizosaccharomyces pombe*, the overexpression of the A43 homologue, RPA21, has also been shown to suppress a mutant phenotype of spTIF-IA (19). TIF-IA also makes contact with promoter-associated factors of the pol I machinery. TIF-IA interacts directly with TBP associated factors (TAFs) of the fundamental transcription factor. In yeast, a physical interaction was observed between scTIF-IA and the Rrn6p subunit of core factor (27). An additional interaction has been found in yeast between scTIF-IA and Rrn7p based on two-hybrid and GST pull-down analysis (C. Radebaugh et al., unpublished). Similarly, in mammals, TIF-IA has been shown to interact with the TAFs of SL1/TIF-IB homologous to core factor's Rrn6p and Rrn7p subunits (24,38).

Given that TIF-IA is absolutely required for specific transcription and has been found to associate with both the fundamental transcription factor and pol I, a role for TIF-IA in the recruitment of pol I to the promoter was hypothesized. Reports from the human pol I transcription system provide support for this hypothesis. Affinity-purified antibodies made to peptides of hsTIF-IA are able to block the recruitment of pol I to an rRNA promoter complexed with SL1 (24). Additionally, the same antibodies inhibit specific transcription from the SL1-bound promoter complexes. Together, this indicates a requirement for TIF-IA in recruitment. However, in the yeast system the situation is not so clear. Chromatin immunoprecipitation analysis indicates that pol I recruitment is scTIF-IA dependent (12). In contrast, binding experiments to promoter DNA immobilized on beads have shown that although necessary for transcription initiation, scTIF-IA is not needed for recruiting pol I to the promoter. Bulk pol I can be recruited to a magnetic bead-bound promoter without the presence of scTIF-IA. However, scTIF-IA must be associated with pol I prior to binding for the subsequent complex to be initiation competent (2). These results are similar to the murine system where preinitiation complexes containing pol I are capable of being formed in the absence of TIF-IA, with the factor being required at a subsequent step before transcriptional initiation (30,31). In the latter species, unlike yeast, TIF-IA can be added after pol I recruitment.

The exact requirements for pol I–TIF-IA association are also not clear. The subunits of pol I have been shown to possess multiple phosphorylation sites (7,9). In yeast, the phosphorylation state of the A43 subunit relative to the A190 subunit is significantly altered between free pol I and pol I–TIF-IA complex (14). The A43/A190 phosphorylation ratio is much higher in the pol I–TIF-IA complex than in free pol I. Furthermore, phosphatase-treated yeast pol I does not associate with scTIF-IA. These authors suggest that specific phosphorylations, such as that of A43, are required for the association of pol I with scTIF-IA. Interestingly, this work in yeast also demonstrated that scTIF-IA is capable of forming an initiation competent complex with pol I, independent of its specific phosphorylation state, including bacterially expressed (unphosphorylated) scTIF-IA. In fact, pol I incubated with bacterially expressed scTIF-IA was found to be as transcriptionally efficient as purified pol I–scTIF-IA complex (14).

In contrast, the current model for mammalian pol I transcription indicates TIF-IA must be phosphorylated in order to bind to the A43 subunit of pol I. TIF-IA produced in Sf9 cells, and therefore believed to be correctly phosphorylated, was shown to physically interact with A43, whereas bacterially expressed TIF-IA could not (11). Interestingly, both TIF-IA preparations were able to bind to the TAFI68 subunit of SL1, indicating a specific phosphorylation event is not necessary for interaction with the fundamental transcription factor. Additionally, phosphatase treatment of TIF-IA inhibited both transcriptional activity and the ability to associate with A43. Cycloheximide treatment of murine cells also inhibited the phosphorylation of TIF-IA, and is associated with the loss of pol I–TIF-IA interaction and transcription (11). It has been shown that two specific serine residues of mam-

malian TIF-IA (S633 and S649) are required to be phosphorylated for transcriptional activity (39). These residues are phosphorylated by ERK and RSK kinases, providing an activation of transcription upon growth factor stimulation. Substitutions of these serines with aspartic acid residues resulted in expression of a TIF-IA with the ability, when added exogenously, to rescue extracts from density-arrested cells, whereas substitution with alanines did not lead to transcriptional rescue. These results are consistent with a requirement for a net negative charge, provided by an amino acid or a phosphate group, at these positions for both transcriptional activity and pol I binding. Unfortunately, the conflicting evidence from the different organisms makes it unclear which phosphorylation target is primary for the formation of the functional pol I–TIF-IA complex. It seems that in lower eukaryotes, such as *S. cerevisiae*, a specific phosphorylation of a pol I subunit (A43) is required for the pol I–TIF-IA complex to form, whereas in mammals specific phosphorylation of TIF-IA itself is required.

Pol I in *A. castellanii* has been purified to near homogeneity and consists of 12 subunits (18). It was first shown in *A. castellanii* that pol I is the component necessary for rRNA transcription that is regulated upon the switch from exponential growth to encystment (5,26). Pol I has been isolated from both encysted and trophozoite cells, and it was found that cyst pol I is deficient for specific transcription and promoter binding (5,26). However, it is shown here that the "pol I" activity our laboratory has reported as being the regulated component likely refers to a pol I–TIF-IA complex. Until the present work, no TIF-IA homologue had been identified from *A. castellanii*.

We report here the characterization of an activity similar to mammalian TIF-IA in the *A. castellanii* system. Bacterially expressed yeast TIF-IA has been shown to stimulate specific transcription in an in vitro reconstituted *A. castellanii* transcription assay. We also have identified an approximately 80-kDa protein associated with transcription competent pol I fractions via cross-reactivity with an anti-TIF-IA polyclonal antibody made to the yeast factor (anti-scTIF-IA). We have found alkaline phosphatase-treated pol I fractions possess similar biochemical properties as those purified from encysted cells: reduced specific transcription activity and an inability to bind promoters. Dephosphorylation by alkaline phosphatase treatment of the pol I–TIF-IA complex was shown to cause a shift in electrophoretic mobility of TIF-IA, suggesting it is phosphorylated when associated with pol I. Furthermore, we report evidence that the *A. castellanii* homologue of TIF-IA is required for pol I recruitment to the rRNA promoter.

MATERIALS AND METHODS

Purification of S. cerevisiae *Proteins*

Pol I, CF, and Rrn3p were purified as previously described (21), with the following exceptions. Polymerase I was purified according to protocol using a protease-deficient cell line, BJ926, with the exception of a 1 ml heparin-Sepharose CL-6B column (Amersham Pharmacia Biotech) that was substituted for the 1 ml HiTrap column. CF was purified according to protocol with the following exceptions. First, the supernatant from 155 g of lysed cells was added to 45 ml of His-Bind resin (Novagen), incubated for 2 h at 4°C, and then poured into a column containing an additional 5 ml of resin. Second, peak-containing fractions from the His-Bind column were loaded onto a 4-ml heparin-Sepharose CL-6B column, then washed and eluted as per protocol. Third, the anti-HA monoclonal antibody affinity column was eliminated. Following the heparin-Sepharose column, peak-containing fractions were pooled and diluted to 240 mM KCl in gradient buffer and then loaded directly onto a 2-ml Q-Sepharose fast-flow column (Amersham Pharmacia Biotech). CF was eluted with a gradient from 240 to 600 mM KCl in gradient buffer, with peak fractions eluting at approximately 340 mM KCl. Recombinant Rrn3p was purified according to protocol.

Purification of A. castellanii *Proteins*

Purification of TIF-IB. TIF-IB was purified from crude nuclear extract as described previously (1,28), and was either from the first or second round of promoter affinity chromatography (1× or 2× DNA affinity TIF-IB).

Purification of Polymerase I. Trophozoite RNA pol I was purified from whole cell extract as previously described (32), with the following exceptions. All buffers used during purification contained 1 mM benzamidine. Cells pelleted in the continuous flow rotor were washed in 2.5 volumes of extraction buffer, instead of 0.15 M KCl. The lysed cell suspension was diluted in buffer as per protocol, with the addition of 12.5% glycerol. The polyethyleneimine (PEI) wash and elution buffer, buffer A and buffer B, contained 12.5% glycerol. The PEI supernatant was loaded onto a 400-ml cake of DEAE-cellulose (DE52) based on the starting cell mass, not based on specific protein concentration of PEI supernatant. The pol I

fractions utilized in these experiments were purified through the heparin-Sepharose chromatography step.

RNA pol I was purified from encysted *A. castellanii* cells as described previously (26), following the methods above with the following modification. After the initial disruption of the cell pellet using the Super Dispax Tissumizer, the unlysed cyst cells were broken open using a Yammato tissue homogenizer (10 rounds). This resulted in 68% lysis of encysted cells.

Purification of TIF-IE. TIF-IE was purified as a side fraction of pol I by rate zonal sedimentation in a glycerol gradient as described previously (1,28).

Plasmids and Templates

For specific transcription assays, pEBH10 (3) was linearized with *Nhe*I to produce a 420 nucleotide transcript. For promoter bead binding experiments the plasmid pJG310 was constructed to introduce a *Nhe*I site upstream of the rRNA promoter. pEBH10 was cleaved immediately upstream of the promoter region with *Eco*RI and *Bam*HI and the 377-bp *Eco*RI–*Bam*HI fragment from pBR322 was cloned into it. This creates a *Nhe*I site 238 bp upstream of the transcription initiation site in pJG310.

Immunoblotting

The presence of specific pol I subunits or TIF-IA was detected by immunoblotting. Protein samples were resolved by electrophoresis on either 10% or 11% SDS polyacrylamide gels and then transferred to PVDF membrane (Pall Life Science) using a Genie electrophoretic blotter as specified by the manufacturer (Idea Scientific). The membranes were blocked in TBS with 0.05% Tween 20 and 5% milk (TBST-milk) for 3 h at room temperature then incubated overnight at 4°C with either anti-scTIF-IA polyclonal, anti-ABC22.5 polyclonal, or anti-AC39 monoclonal antibodies (see below) at a dilution of 1:1000 in TBST-milk. The membranes were then washed twice briefly, once for 20 min and twice for 5 min with TBST. The membranes were incubated with either anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase (Sigma) at a dilution of 1:30,000 in TBST-milk for 5 h at room temperature. The membranes were washed as described above and antibody cross-reactivity was detected using ECF reagent (Amersham) on a STORM860 scanner (Molecular Dynamics). The anti-scTIF-IA polyclonal antibody was made to full-length recombinant scTIF-IA. The anit-ABC22.5 polyclonal antibody and the anti-ABC39 monoclonal antibody were made using purified *A. castellanii* ABC22.5 and AC39 subunits, respectively.

Specific Transcription Runoff Assay

The standard specific transcription assays for the *A. castellanii* system were carried out as previously described (28), with the following exceptions. Typically 1 μl of 2× DNA affinity or 2 μl of 1× DNA affinity TIF-IB were used in the transcription reactions with 30 mU of pol I. Nucleic acids were precipitated by addition of 75 μl of 4.68 M ammonium acetate, 0.33 mg/ml linear polyacrylamide, and 450 μl of 95% ethanol. Nucleic acids were then pelleted for 30 min at 14,000 rpm in an Eppendorf centrifuge. Pellets were washed with 500 μl of 70% ethanol, dried under vacuum, resuspended in 5 μl formamide dye, and electrophoresed as previously described (28). Specific transcriptions assays for the *S. cerevisiae* system were conducted as previously described (21).

Nonspecific Transcription Assays

The nonspecific transcriptional activity of pol I was measured by a RNA polymerase assay as previously described (32).

Factor Binding to Promoter-Bound SA-PMPs

The pJG310 plasmid was cleaved with *Nhe*I and *Pvu*II, and the 461-bp fragment containing the promoter element was purified as follows. The DNA cleavage reactions were electrophoresed through a 1.5% agarose gel, and the 461-bp fragment was excised and purified with a QIAEX II Gel Extraction Kit (Qiagen). The *Nhe*I cleavage left a 5′ overhang that was labeled with biotin-dCTP in a fill-in reaction. A typical 50-μl fill-in reaction contained 0.1 mM dNTPs (A, T, and G), 5 units of Klenow fragment exo- (Fermentas), 0.5 mM biotin-dCTP, and 580 ng of the 461 bp fragment in a 1× concentration of Klenow buffer (Fermentas). The reactions were incubated at 37°C for 30 min, followed by an incubation at 70°C for 10 min to heat kill the enzyme. Free NTPs and enzyme were removed by size exclusion chromatography in a Chroma Spin+TE-100 column (BD Biosciences) at $700 \times g$ for 5 min. Biotin-labeled promoter fragments were bound to a 40-μl suspension of Streptavadin MagneSphere® Paramagnetic Particles, SA-PMPs, (Promega) according to manufacturer's protocol. The bead–DNA mixture was then equilibrated with protein binding buffer, 30 mM HEPES, pH 7.9, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40, 0.5 mg/ml insulin, 10% glycerol, 100 mM KCl, and either 2.5 ng/μl linearized (*Nru*I) or 3 ng/

μl pBR322 nonspecific competitor DNA. Typical binding reactions contained the equilibrated beads, 2–10 μl of 1× DNA affinity-purified TIF-IB, and 100–800 mU heparin-Sepharose-purified pol I in binding buffer in a 50–100-μl reaction volume. The binding reactions were incubated for 30 min at 25°C. The protein-bound beads were then collected using a magnetic separator. Nonspecifically bound or trapped proteins were removed by washing the beads one or two times with 50–100 μl of binding buffer. Bound proteins were then eluted with 50–100 μl binding buffer containing 1 M KCl or by suspension in 1× SDS-PAGE sample buffer. If necessary, proteins were chloroform/methanol precipitated as previously described (36). Precipitated proteins were suspended in 1× SDS-PAGE sample buffer. The protein samples were then analyzed by immunoblotting.

To assay for the presence of factors bound to the promoter beads following transcription, TIF-IB and pol I were bound to promoter beads as above. Following the 30-min incubation, the protein-bound beads were collected as above and washed once. To PIC-bound beads, 50–100 μl of binding buffer containing 0.5 mM NTPs was added and transcription was allowed to proceed for 30 min at 25°C. Released proteins were extracted using a magnetic separator; the beads were washed once more and proteins remaining bound were eluted as above.

Alkaline Phosphatase Treatment of A. castellanii *pol I Fractions*

Calf intestinal alkaline phosphatase covalently attached to agarose beads was purchased from Sigma. The alkaline phosphatase beads were washed twice with two volumes distilled H_2O and then twice with pol I dialysis buffer: 50 mM HEPES, pH 7.8, 10% glycerol, 0.1 mM EDTA, 0.2% NP-40, 100 mM KCl, and 0.2 mg/ml BSA. Pol I (300–800 mU) was incubated with 5 U alkaline phosphatase at 30–37°C for the times indicated. The beads were collected by centrifugation at $14{,}000 \times g$ for 2 min and the supernatants containing alkaline phosphatase-treated pol I were removed. The fractions were then used in transcription, promoter bead binding, and immunoblotting assays.

RESULTS

Yeast TIF-IA Stimulates Transcription in an In Vitro Reconstituted Transcription Assay Containing Purified A. castellanii *Factors*

TIF-IA is required for specific rDNA transcription from several species. Additionally, this factor possesses a high level of conservation, whereby the human protein is able to function in a yeast strain containing a lethal deletion of scTIF-IA, and TIF-IA purified from mouse and human cells is able to complement transcriptionally deficient extracts from the reciprocal species (25,31). Based on these results, we asked whether the yeast transcription factor scTIF-IA would have an effect on the *A. castellanii* transcription system. We incubated pol I fractions with either purified bacterially expressed scTIF-IA or a control protein, BSA, for 2 h at 4°C and 30°C, before using this pol I in a reconstituted in vitro transcription reaction. At each preincubation temperature, transcription from pol I that had been preincubated with the yeast transcription factor was greater than with the pol I that had been preincubated with the control protein (Fig. 1A, compare lanes 1 with 2 and 3 with 4). Additionally, in both *A. castellanii* and *S. cerevisiae* reconstituted transcription systems, incubating pol I with increasing amounts of scTIF-IA resulted in a linear increase in the amount of specific transcript produced (Fig. 1B). The fact that yeast TIF-IA is able to stimulate transcription in a reconstituted *A. castellanii* system indicates that not only may there be a homologue to scTIF-IA in *A. castellanii*, but also that the conservation between the two species must be fairly high. It is also consistent with the lack of need for phosphorylation of the yeast factor for function (14,27).

A Putative acTIF-IA Is Associated With A. castellanii *pol I*

Pol I fractions were analyzed in a Western blot assay for the presence of TIF-IA by cross-reactivity to a polyclonal antibody produced against cloned scTIF-IA expressed in *Escherichia coli*. A cross-reacting polypeptide paralleled transcription activity in the pol I fractions, with an apparent molecular weight of approximately 80,000, similar to the size reported for TIF-IA homologues in other species (6,25,31,37). Pol I fractions were analyzed for both specific and nonspecific transcription activity, as well as for antibody cross-reactivity. Figure 2 shows the peak of TIF-IA protein corresponds with the peaks of both specific and nonspecific transcription activities. These results are similar to those in yeast, where the presence of scTIF-IA corresponded to specific transcription. However, in yeast, the peaks of specific and nonspecific transcription activity are not aligned; scTIF-IA cross-reactivity parallels specific but not nonspecific transcription, presumably revealing the pol I–TIF-IA complex (23). Given that nonspecific and specific transcriptional activities are coincidental during the purification of pol I from *A. castellanii*, it has proven impossible to separate the pol I–TIF-IA complex from free pol I in the *A. castellanii* system.

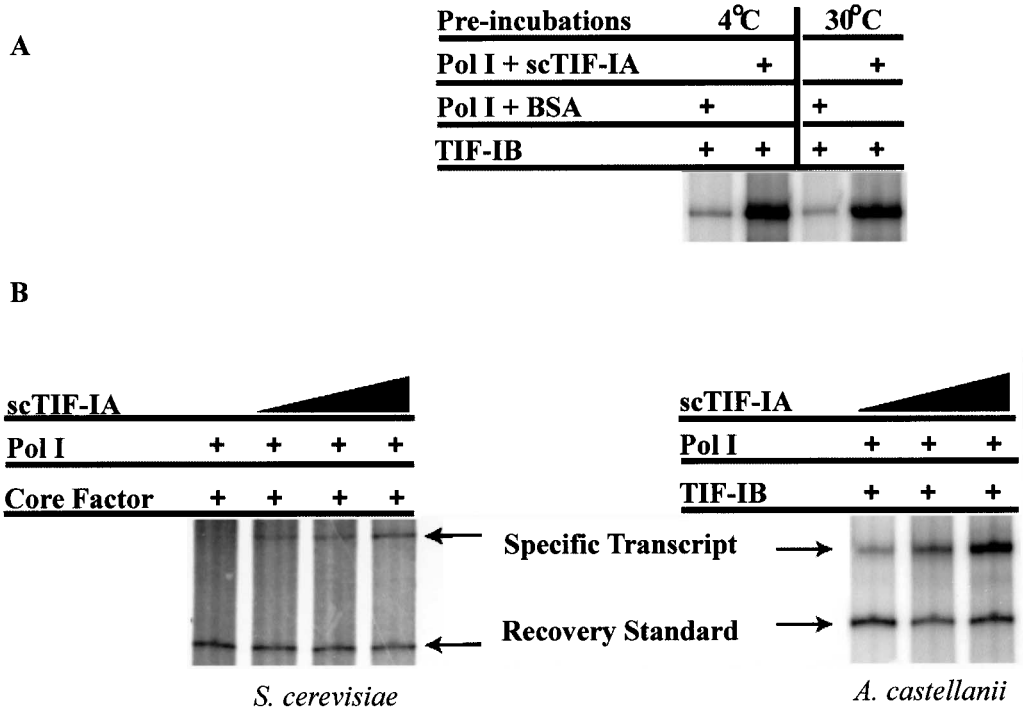

Figure 1. Yeast TIF-IA stimulates transcription in a reconstituted *A. castellanii* transcription system. (A) Pol I heparin-Sepharose fractions (80 mU) were preincubated with 0.175 μg of either MonoQ-purified recombinanat scTIF-IA or BSA for 2 h at 4°C or 30°C. Pol I (30 mU) was then added to a transcription assay containing 1 μl of 2× DNA affinity TIF-IB. (B) Yeast MonoQ-purified scTIF-IA (15.6, 46.7, and 140 ng) was preincubated with 8 μl *S. cerevisiae* heparin-Sepharose pool pol I and 80 mU *A. castellanii* heparin-Sepharose pol I for 2 h at 30°C. The pol I–TIF-IA complexes were then used in their respective in vitro transcription assays containing 0.75 μl Q-Sepharose core factor or 1 μl of 2× DNA affinity TIF-IB.

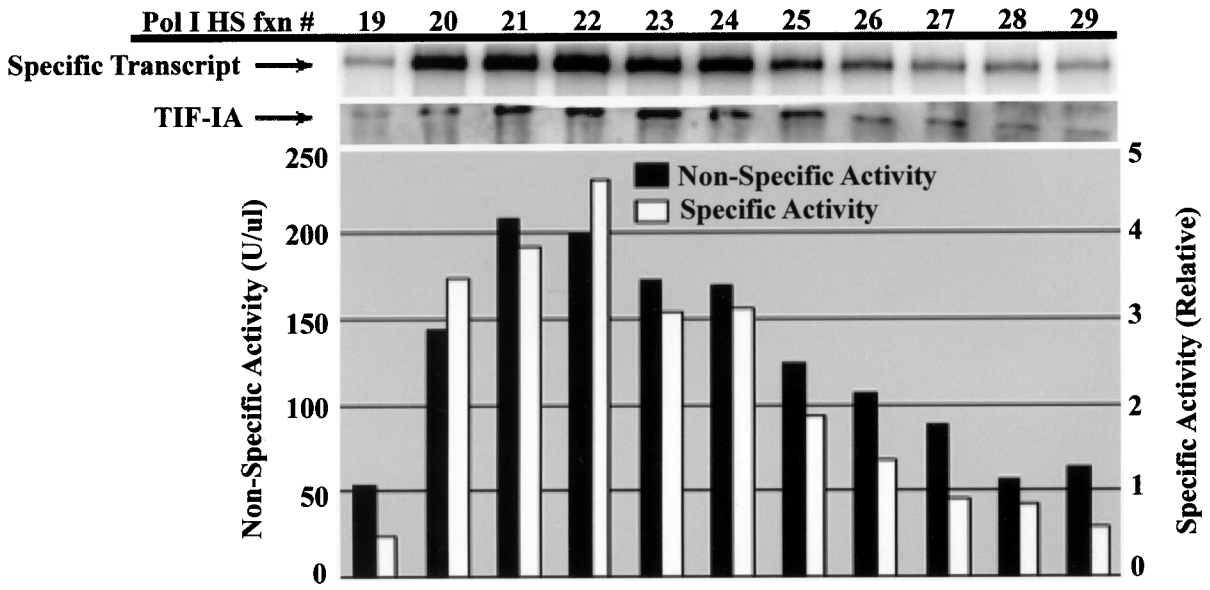

Figure 2. TIF-IA corresponds to the peaks of specific and nonspecific transcriptional activities. Heparin-Sepharose pol I fractions were assayed for specific (0.15 μl) and nonspecific (1.5 μl) transcription activity as well as cross-reactivity with the anit-scTIF-IA pAb in an immunoblot (3 μl).

Pol I Fractions Purified From Encysted A. castellanii *Cells Exhibits Reduced Promoter Binding and Specific Transcription Activities*

Pol I was purified from both trophozoite and cyst *A. castellanii* cells, and their activities were measured in a promoter bead binding assay. When *A. castellanii* cells are starved for nutrients in an encystment medium, there is a cessation of cellular growth and division (33) that coincides with a downregulation of rRNA transcription (5). Pol I purified from encysted cells possesses the ability to transcribe DNA nonspecifically in a manner equivalent to that of trophozoite pol I; however, cyst pol I is downregulated in both specific transcription and promoter binding as assayed by DNase I footprinting (5). We asked whether cyst pol I exhibited a reduction in binding to bead-bound promoter DNA under conditions similar to those used for promoter binding in a footprinting assay. When equal amounts, based on their nonspecific transcription activity, of heparin-Sepharose pol I purified from cysts or trophozoites were assayed in a promoter bead binding assay, cyst pol I exhibited a reduced ability to bind to the promoter (Fig. 3; 80% of trophozoite binds; 48% of cyst binds), consistent with its reduced in vitro and in vivo transcription activity. In the presence of the large amount of competitor DNA present in the assay, pol I was shown to not bind nonspecifically to the beads containing no DNA nor to promoter DNA beads in the absence of TIF-IB (data not shown). Thus, the binding being measured is a reflection of the ability of the pol I–TIF-IA complex to be recruited specifically to the promoter by bound factors.

Alkaline Phosphatase Treatment Reduces the Specific Transcription and Promoter Binding Activities of pol I

Specific phosphorylations are reported to be necessary for the formation of the specific transcription-competent pol I–TIF-IA complex in yeast and mammals (11,14,22,39). We analyzed how phosphatase treatment of *A. castellanii* pol I affected the ability of the enzyme, or more specifically the pol I–TIF-IA complex, to participate in specific transcription and binding to TIF-IB-bound rRNA promoter. First, pol I fractions were incubated with increasing amounts of agarose bead-bound calf intestinal alkaline phosphatase. As a fixed amount of pol I was incubated with increasing amounts of alkaline phosphatase, the level of specific transcription decreased in a dose-dependent fashion (Fig 4, white bars). When the same pol I fractions were assayed for their ability to transcribe DNA nonspecifically from calf thymus DNA, polymerase activity was not affected as severely (Fig. 4, black bars). Although there was a slight decrease initially in nonspecific activity, increased units of alkaline phosphatase did not cause additional reduction in activity as with specific transcription. Additionally, the amount of pol I recovered from the phosphatase beads was measured in a Western blot by cross-reactivity with a monoclonal antibody specific to the AC39 pol I subunit. It is clear from the Western blot that the reduction in nonspecific activity was due to a slight loss of total pol I protein, while the loss in specific activity was significantly greater, demonstrating a targeted effect on promoter-dependent transcription (Fig. 4).

The effect of phosphatase treatment of pol I on its ability to bind to the promoter was evaluated by promoter bead binding assay. Pol I was incubated at 30°C with or without alkaline phosphatase for the times shown, equalized for nonspecific transcription activity, and added to the binding reaction. Incubation of pol I with alkaline phosphatase inhibited the ability of pol I to bind to the TIF-IB-bound promoter beads in a time-dependent manner (Fig. 5). To show that TIF-IB binding was not affected by the ability of pol I to bind the promoter, fractions from the bead binding reaction were immunoblotted and probed for

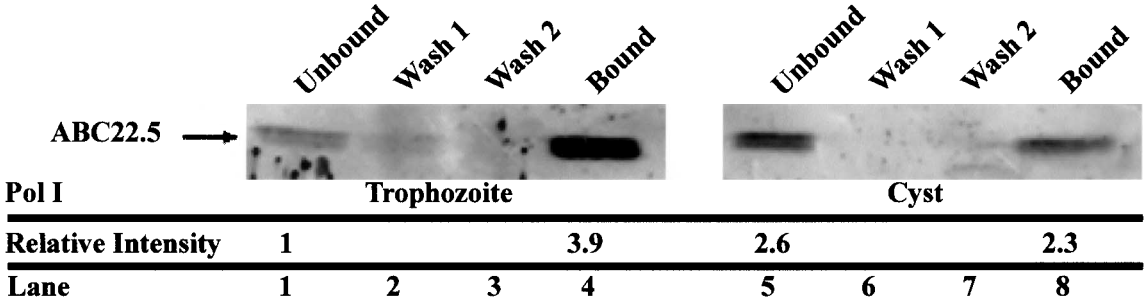

Figure 3. Cyst pol I is deficient for promoter binding and acTIF-IA association. Equal amounts of heparin-Sepharose trophozoite and cyst pol I (150 mU) were added with 6 μl 1× DNA affinity TIF-IB to bead binding reactions. Samples were subjected to immunoblotting and the presence of pol I was detected by cross-reactivity to the anti-ABC22.5 pAb.

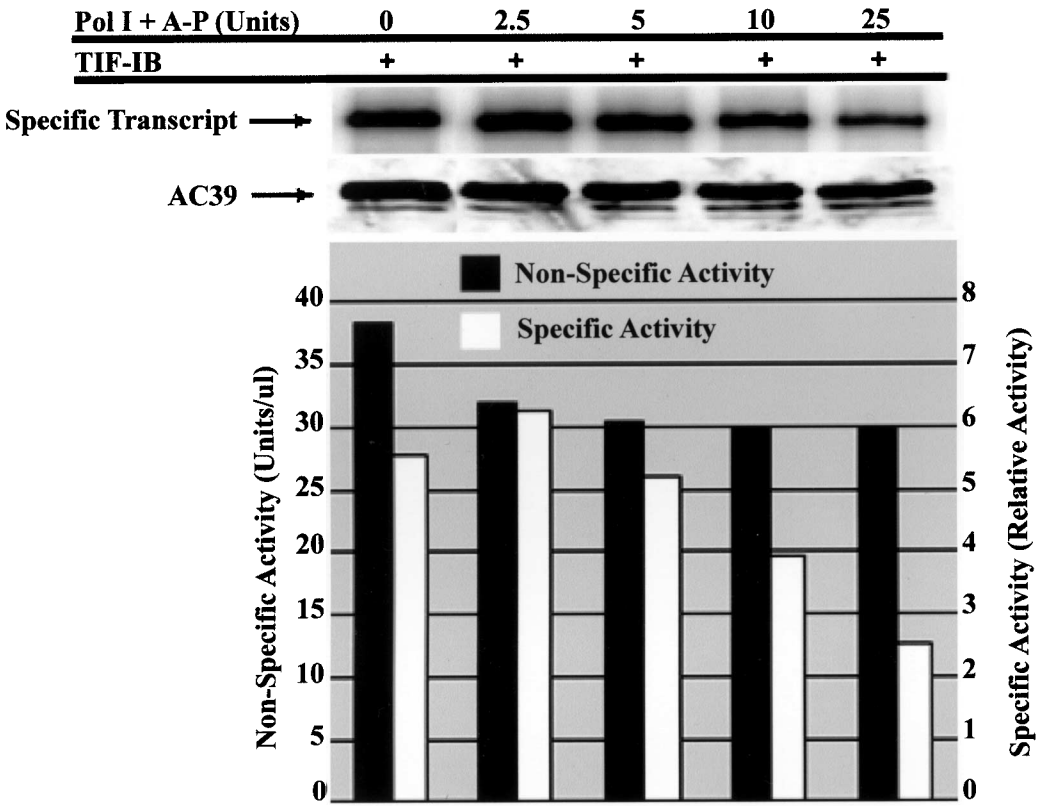

Figure 4. Alkaline phosphatase treatment of pol I causes a decrease in specific transcription. Heparin-Sepharose pol I (1500 mU) was incubated with the indicated amounts of alkaline phosphatase and then used in specific (34 mU), nonspecific (408 mU), and immunoblot (612 mU) assays. Pol I recovery from the agarose beads was measured by cross-reactivity to the anti-AC39 mAb.

a TIF-IB subunit with anti-TBP polyclonal antibodies. As can be seen in Figure 5, the decrease in pol I binding to the beads was not due to a decrease in TIF-IB binding. These results suggest that phosphatase treatment inhibits specific transcription, by possibly initiating a disruption of the pol I–TIF-IA complex. This reduces the ability of pol I to be specifically recruited to the promoter element. These characteristics of the phosphatase-treated enzyme are similar to the pol I isolated from encysted cells and clearly imply a role for phosphorylation/dephosphorylation in the regulation of rRNA transcription in *A. castellanii*.

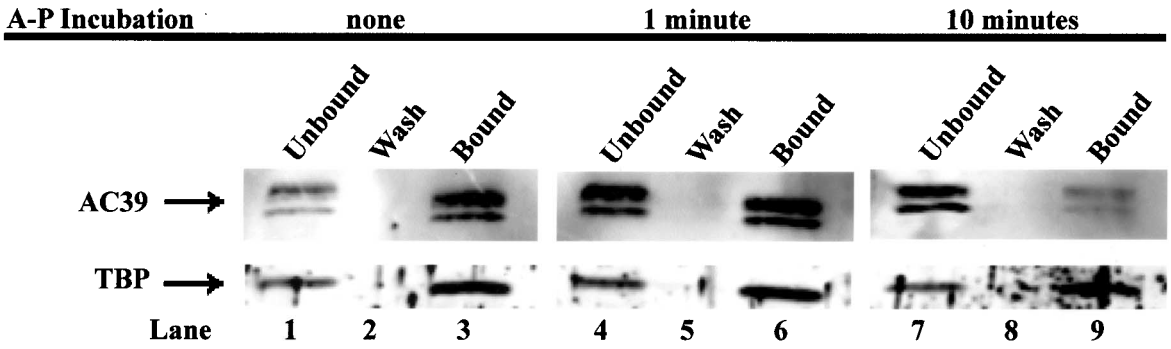

Figure 5. Alkaline Phosphatase inhibits pol I promoter binding. Heparin-Sepharose pol I (610 mU) was incubated without alkaline phosphatase for 10 min, or with 5 U of alkaline phosphatase for 1 and 10 min at 30°C. Pol I fractions (180 original mU) were then added with 12 μl 1× DNA affinity TIF-IB to promoter beads in a bead binding reaction. Samples were subjected to immunoblotting and probed for the presence of pol I (AC39) or TIF-IB (TBP) with anti-AC39 mAb, and anti-TBP pAb, respectively.

Alkaline Phosphatase Treatment of the pol I–TIF-IA Complex Causes an Electrophoretic Mobility Shift of TIF-IA

Mammalian TIF-IA has been shown to possess different electrophoretic mobilities based on its state of phosphorylation (39). To examine the effects of alkaline phosphatase treatment on acTIF-IA, we assayed phosphatase-treated pol I fractions in an immunoblot. Pol I fractions from the heparin-Sepharose column, competent for specific transcription, were incubated with or without alkaline phosphatase for 30 min at 37°C. Upon alkaline phosphatase treatment, the fractions were then subjected to SDS-PAGE and immunoblotting using anti-scTIF-IA polyclonal antibody. Figure 6A shows that when treated with alkaline phosphatase, the electrophoretic mobility of the TIF-IA found in complex with pol I was altered, resulting in a faster migrating band. The alteration in migration due to the dephosphorylation of TIF-IA could be responsible for the loss of specific transcription ability as well as the loss of promoter binding. A pool of free TIF-IA was found in an early fraction during pol I purification. As shown in Figure 6B, this free TIF-IA found in the non-pol I-containing PEI supernatant also migrated faster than the pol I-associated TIF-IA found in the heparin-Sepharose pol I fraction, further indicating a physical difference between the active, pol I-associated TIF-IA and the inactive, free TIF-IA pool. However, a cause and effect relationship based solely on TIF-IA dephosphorylation cannot be proven because the pol I was also treated with phosphatase in this assay.

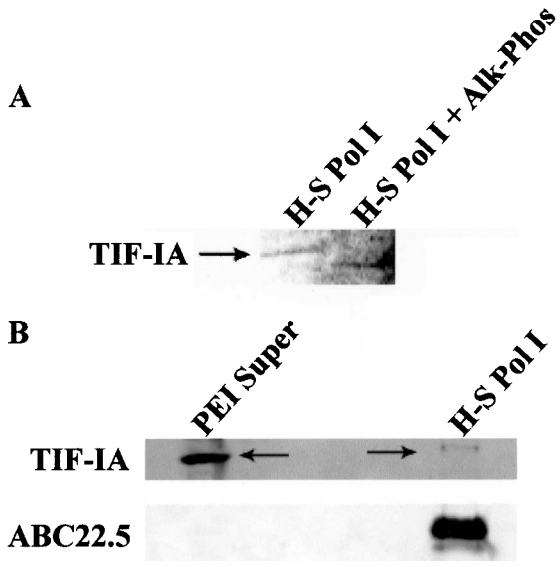

Figure 6. Alkaline phosphatase treatment of the pol I–TIF-IA complex leads to a shift in the molecular weight of TIF-IA. (A) Heparin-Sepharose pol I (858 mU) was incubated with and without alkaline phosphatase at 37°C for 30 min, then 650 mU was subjected to immunoblotting and TIF-IA was detected by cross-reactivity with an anti-scTIF-IA pAb. (B) PEI supernatant (10 μl) and heparin-Sepharose pol I (10 μl, 1246 mU) were subjected to immunoblotting and probed for the presence of pol I (AC39) and TIF-IA with anti-AC39 mAb and anti-scTIF-IA pAb, respectively.

TIF-IA Is Required for pol I Promoter Binding

Whether pol I recruitment to the promoter requires TIF-IA is controversial (2,24,31). In vitro in yeast, both free pol I and the pol I–TIF-IA complex are recruited to the promoter. This results in the formation of preinitiation complexes (PICs) that are transcriptionally inactive or active, respectively (2). Upon the addition of nucleotide triphosphates, PICs containing transcriptionally active pol I are able to initiate transcription, resulting in the release of pol I and scTIF-IA from bead-bound template DNA. Pol I from inactive PICs remains bound. In contrast, past studies from our laboratory have shown that nearly all of the pol I stably bound to the *A. castellnaii* rRNA promoter is competent for promoter clearance and specific transcription. DNase I and MPE footprinting assays have shown that the stable pol I footprint is translocated downstream of the start site upon the addition of nucleotide triphosphates (4,5). Additionally potassium permanganate, MPE.Fe(II), and DEPC footprinting show the presence of a pol I footprint as the pol I is walked down the promoter by the specific addition of required nucleotides (20). This suggests all components required for initiation are associated with pol I. We tested whether this actively recruited *A. castellanii* pol I is associated with acTIF-IA. Pol I was incubated with TIF-IB-bound promoter DNA immobilized on paramagnetic beads, in the presence of excess nonspecific competitor DNA. Any nonspecifically bound protein was further removed by washing the promoter DNA-bound beads with additional nonspecific DNA in binding buffer. Template-bound PICs were eluted with SDS. The samples were then subjected to immunoblotting and probed for the presence of pol I and TIF-IA. TIF-IA was present in the bound sample (Fig. 7A, lane 5). The fraction of pol I found not to be recruited to the promoter consisted of pol I not associated with TIF-IA (Fig. 7A, lane 2). Pol I bound to immobilized DNA was shown to be initiation competent and was released upon addition of nucleotide triphosphates (Fig. 7B). These results show that in contrast to the in vitro yeast promoter bead binding experiments (2), all the pol I bound to the *A. castellanii* promoter is associated with TIF-IA and competent for specific transcription initiation.

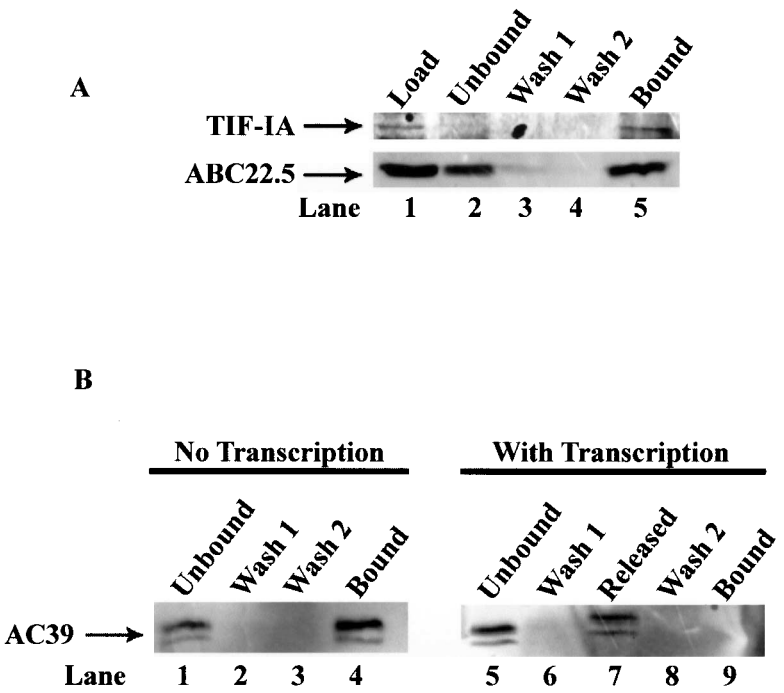

Figure 7. TIF-IA is required for pol I recruitment to the rRNA promoter; this pol I is transcriptionally competent. (A) Heparin-Sepharose pol I (680 mU) and 10 µl 1× DNA affinity TIF-IB were added to promoter-bound beads in a bead binding reaction. Samples were subjected to immunoblotting and probed for the presence of pol I (ABC22.5) and TIF-IA with anti-ABC22.5 and anti-scTIF-IA polyclonal antibodies. (B) Heparin-Sepharose pol I (210 mU) and 6 µl 1× DNA affinity TIF-IB were added to promoter-bound beads in two separate bead binding reactions. After a 30-min incubation the reactions were washed and eluted as per protocol; the second reaction was washed then incubated in binding buffer containing NTPs. Transcription was allowed to occur for 30 min at 25°C. The released proteins were removed and the beads were washed and eluted as per protocol. Samples were subjected to immunoblotting and probed for the presence of pol I (AC39) with an anti-AC39 mAb.

DISCUSSION

The transcription of the ribosomal genes in vitro is species specific (16); however, not all of the pol I transcription factors exhibit this strict species specificity. The fundamental transcription factor, TIF-IB/SL1, has been shown to confer species specificity to the pol I transcriptional apparatus (13,29). TIF-IA, on the other hand, is promiscuous. The human TIF-IA gene can functionally substitute for the yeast gene in vivo (25). Additionally, purified TIF-IA from both the mouse and human systems can function in transcription in the reciprocal species (31). We have taken advantage of the functional conservation of the TIF-IA factor to assay for TIF-IA-related activity in *A. castellanii*. We report here that purified, recombinant yeast TIF-IA is capable of stimulating transcription in a reconstituted in vitro transcription assay from *A. castellanii* (Fig. 1). Furthermore, antibodies to the yeast protein cross-react with an 80-kDa protein found tightly associated with *A. castellanii* pol I active in both specific transcription (Fig. 2) and promoter binding (Fig. 7).

Ribosomal RNA transcription is regulated by numerous mechanisms. In the mammalian cell cycle, rRNA synthesis is shut down during mitosis by modification of both the fundamental transcription factor, SL1/TIF-IB, and the activating factor, UBF (15). However, many studies have shown that the activity of the fundamental transcription factor is not the target of modification in growth-related rRNA transcriptional repression (10,23,26). Depending on the particular system, the target for this regulatory mechanism has been shown to be either pol I or TIF-IA (5,11, 22,34,38). As a cell encounters unfavorable growth conditions, either pol I itself or its essential transcription factor, TIF-IA, must be modified. In *A. castellanii*, the switch from exponential growth to encystment correlates with the inactivation of rRNA transcription. Pol I isolated from cyst cells transcribes DNA nonspecifically as efficiently as pol I isolated from trophozoite cells, but exhibits reduced specific transcriptional activity resulting from reduced promoter binding (5,26) (Fig. 3). Additionally, we show here that trophozoite pol I–TIF-IA complex treated with alkaline phosphatase has reduced specific transcription and promoter binding ability (Figs. 4 and 5), similar to cyst pol I. We conclude that as *A. castel-*

lanii cells undergo encystment, dephosphorylation of TIF-IA or pol I is likely responsible for the decrease in transcriptional activity. This loss of activity corresponds to an altered electrophoretic mobility of TIF-IA, indicating that a dephosphorylation of TIF-IA correlates with the disruption of the pol I–TIF-IA complex (Fig. 6A). An alteration of the electrophoretic mobility of TIF-IA has similarly been shown to correspond to a decrease in transcriptional activity in the mouse system (39). Additionally, cross-reactivity with an anti-scTIF-IA polyclonal antibody has been observed in purification fractions not associated with pol I, possibly identifying a large pool of free TIF-IA (Fig. 6B). This free TIF-IA also possesses the faster electrophoretic mobility characteristic of dephosphorylated TIF-IA in the artificially inactivated pol I–TIF-IA complex, suggesting but not proving the modification is necessary for association with pol I.

These results do not rule out the possibility, however, that *A. castellanii* pol I could also be a target of a growth-dependent regulatory modification, as in yeast (14). A central role for TIF-IA phosphorylation in *A. castellanii* is open to some doubt because scTIF-IA expressed in *E. coli* stimulates *A. castellanii* pol I even though the factor is not properly phosphorylated. Reports examining the ability of recombinant TIF-IA to function in pol I subunit binding, pol I recruitment, and/or transcriptional initiation have been contradictory. Cavanaugh et al. (1) reported the loss of interaction between human TIF-IA and mouse A43 when recombinant hsTIF-IA was purified from *E. coli*. However, hsTIF-IA expressed in insect cells does bind to the mouse A43 subunit, suggesting a specific posttranslational modification occurs in eukaryotic cells that facilitates the association between pol I and hsTIF-IA. In contrast, Yuan et al. (38) have shown human TIF-IA expressed in *E. coli* binds to GST-tagged rpa43, even though they later reported specific phosphorylation of S633 and S649 is necessary for transcription (39). Moreover, recombinant yeast TIF-IA expressed in *E. coli* is capable of associating with yeast pol I, via its A43 subunit, to yield a transcriptionally active complex (14,27), similar to the *A. castellanii* results presented here. In yeast, it is the polymerase that must be phosphorylated for association with TIF-IA (14). In fact, dephosphorlyated scTIF-IA is believed to be associated with phosphorylated pol I while the free scTIF-IA pool is the phosphorylated population (14). These apparent differences may have arisen due to contaminating kinases or phosphatases in the polymerase or factor preparations used by the various laboratories, or to differences in conditions used in interaction assays. Alternately, it is possible these differences are real and species related, with two extremes evolving from a primordial more general scheme involving phosphorylation of both the factor and the polymerase. Mammals have retained the need for factor phosphorylation, and perhaps also for pol I phosphorylation, which has not been exhaustively tested in highly purified preparations. Such a dual set of targets might lend a greater diversity of targets for regulatory modifications in these more complex metazoans. Yeast may have evolved such that no specific factor phosphorylation is needed for binding to pol I, but has retained the need for pol I A43 phosphorylation. This model could explain why bacterially expressed yeast TIF-IA functions in the highly purified yeast and *A. castellanii* systems without specific phosphorylation.

There also are differences between laboratories concerning the TIF-IA requirement for pol I recruitment. In mouse, pol I can be recruited to the promoter without TIF-IA in vitro (31). In yeast, in vitro promoter binding experiments suggest that TIF-IA is not required for pol I recruitment, but is required for subsequent initiation steps, whereas in vivo chromatin immunoprecipitation analysis indicates that TIF-IA is required for pol I recruitment (2,12,14). In the human system, pol I does not bind to the PIC without hsTIF-IA (24). *A. castellanii* pol I bound to the promoter is competent for specific transcription and, therefore, in parallel with other species, must be associated with TIF-IA (4,5,20). By direct measurement, we show here that the transcriptionally competent pol I (Fig. 7B) that binds to the rRNA promoter is associated with TIF-IA (Fig. 7A). In this experiment, TIF-IA was depleted from the pol I fraction that did not bind the promoter, showing that only the pol I associated with TIF-IA is capable of binding the promoter (i.e., TIF-IA is required for promoter recruitment in *A. castellanii*).

Regardless of what step in transcriptional initiation TIF-IA is required, a functional pol I–TIF-IA complex is necessary for specific transcription. Numerous studies have shown that the active complex is used stoichiometrically in crude systems (8,17,23), suggesting a modification of one of its components by other components present in the extracts, presumably by a phosphatase. In highly purified systems, in the absence of the putative phosphatase, multiple rounds of transcription are more the norm (31). In vivo, following each round of transcription, TIF-IA and/or pol I needs to be rephosphorylated to allow replenishment of the active complex. In rapidly growing cells, the rate of rRNA transcription is so high that conversion of the pool of active complex into dephosphorylated components can occur extremely rapidly. In growing yeast, for example, complexes are deactivated at a rate of 2000 per minute (35); the entire complement of 50,000 pol I molecules could be inactivated in 25

min, or if the steady state estimate that only 2–10% are in active form (23), only 2.5 min would be needed to shut down rRNA transcription by this mechanism. Clearly, discerning the target(s) and enzymes that mediate these interconversions is important to fully understand the mechanisms responsible for rRNA transcriptional regulation.

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