Cell, Vol. 68, 965–976, March 6, 1992, Copyright © 1992 by Cell Press

The TATA-Binding Protein and Associated Factors Are Integral Components of the RNA Polymerase I Transcription Factor, SL1

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Summary

We have previously shown that the TATA-binding protein (TBP) and multiple TBP-associated factors (TAFs) are required for regulated transcriptional initiation by RNA polymerase II. Here we report the biochemical properties of the RNA polymerase I promoter selectivity factor, SL1, and its relationship to TBP. Column chromatography and glycerol gradient sedimentation indicate that a subpopulation of TBP copurifies with SL1 activity. Antibodies directed against TBP efficiently deplete SL1 transcriptional activity, which can be restored with the SL1 fraction but not purified TBP. Thus, TBP is necessary but not sufficient to complement SL1 activity. Analysis of purified SL1 reveals a complex containing TBP and three distinct TAFs. Purified TAFs reconstituted with recombinant TBP complement SL1 activity, and this demonstrates that TBP plus novel associated factors are integral components of SL1. These findings suggest that TBP may be a universal transcription factor and that the TBP-TAF arrangement provides a unifying mechanism for promoter recognition in animal cells.

Introduction

The study of transcriptional initiation has unraveled elegant but complex sets of biochemical interactions between sequence-specific DNA-binding proteins, promoter/enhancer elements, and the basal transcriptional apparatus (Mitchell and Tjian, 1989). However, the molecular interactions that take place between the DNA binding factors and components of the basal apparatus that includes RNA polymerase and a variety of accessory transcription factors have remained elusive. Indeed, our understanding of transcriptional initiation events is incomplete largely because the mechanism by which basal factors communicate with the site-specific regulatory proteins has been difficult to decipher. The major obstacle to a detailed analysis of transcriptional activation mechanisms has been the lack of in vitro transcription reactions reconstituted with well defined and purified components. Even transcription of ribosomal RNA by RNA polymerase I (pol I), which is thought to involve a relatively small number of regulatory factors, has proved to be intransigent (Bell et al., 1988; Learned et al., 1985). Similarly, the essential transcription factors directing RNA pol III transcription remain to be fully characterized (Geiduschek and Tocchini-Valentini, 1988). Recently, progress has been made in isolating and characterizing some of the basal transcription factors responsible for RNA pol II initiation. However, a fully reconstituted reaction with purified components has, as yet, not been achieved, primarily because the initiation complex requires at least 6 separate factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH), each of which may contain multiple subunits (Buratowski et al., 1989; Reinberg and Roeder, 1987a, 1987b; for review, see Sawadogo and Sentenac, 1990). Thus, in all three RNA polymerase systems, at least one transcription factor remains shrouded in mystery.

Transcription by RNA pol I offers some unique advantages in studying the mechanism of promoter recognition and activation (Reeder, 1990; Sollner-Webb and Mougey, 1991). In particular, there is but one type of promoter in each species and at least two transcription factors, promotor selectivity factor (SL1) and upstream binding factor (UBF), that are necessary to direct accurate and promoter-specific transcription of rRNA genes in animal cells (Bell et al., 1988; Learned et al., 1985, 1986). The human UBF has been purified to homogeneity and found to be a 94/97 kd polypeptide that recognizes and binds specifically to the GC-rich upstream control element and the core region of the human rRNA promoter (Bell et al., 1988). The recent molecular cloning of cDNAs encoding UBF identified multiple functional domains including the new high mobility group box DNA binding motif (Jantzen et al., 1990). UBF is the only RNA pol I transcription factor that is necessary for initial promoter binding, and the specificity of this DNA recognition factor is highly conserved across diverse species from man to Xenopus (Bell et al., 1989; Pikaard et al., 1989). Cross-species mixing experiments reveal that both UBF and RNA pol I are interchangeable between closely related species (Bell et al., 1990). The second essential factor necessary for accurate RNA pol I transcription, SL1, does not bind specifically to the human promoter by itself (Learned et al., 1985). However, when both UBF and SL1 are present, a strong cooperative DNA-binding complex with an extended DNA binding region is formed at the human rRNA promoter that is critical for transcriptional initiation (Bell et al., 1990; Learned et al., 1986). It has been demonstrated that transcription of ribosomal RNA by RNA pol I is species-specific (Grummt et al., 1982) and that SL1 is the species-specific factor that directs transcription only of the cognate template (Bell et al., 1990, 1989; Learned et al., 1985). Thus, the key to RNA pol I transcriptional specificity lies with the properties of SL1 and its interaction with UBF and the template DNA. The SL1–UBF complex is reminiscent of the situation that is thought to occur between site-specific upstream enhancer factors and potential interactions with components of the basal RNA pol II transcriptional machinery (Reinberg and Roeder, 1987b). Is there, perhaps, a common mechanistic link between the RNA pol I initiation factor SL1 and components of the initiation complex utilized by RNA pol II?

It has long been known that the essential RNA pol II transcription factor, TFIID, isolated from animal cells, con-

tains an activity (TATA-binding protein [TBP]) that is responsible for recognizing and binding to the TATA box element, a cis-control sequence typically found at position −25 to −30 in most but not all protein coding gene promoters (Horikoshi et al., 1988a, 1988b; Sawadogo and Roeder, 1985; Stringer et al., 1990; for review, see Greenblatt, 1991). Recent biochemical characterization of the partially purified TFIID fraction, which is competent to direct both basal and activated transcription, revealed that TBP actually represents one subunit of a tightly associated, multisubunit complex that includes a variety of TBP-associated factors (TAFs) (Dynlacht et al., 1991; Pugh and Tjian, 1991; Tanese et al., 1991). Most importantly, separation of TAFs from TBP and subsequent reconstituted transcription reactions reveal that some of the TAFs contain coactivator function and are thus able to restore promoter-specific activation directed by a variety of enhancer-binding factors. The finding that TBP is also required for RNA pol III transcriptional initiation of the U6 gene, which contains an upstream AT-rich element, provided the first clue that TBP may be involved in transcription by RNA polymerases other than RNA pol II (Lobo et al., 1991; Margottin et al, 1991; Simmen et al., 1991). These results substantially altered our concepts concerning the role of the TBP and also prompted us to reevaluate the subunit composition and function of other transcription factors involved in initiation. We were particularly intrigued by the hypothesis that TBP associated with different sets of TAFs could direct transcription by different types of templates. If, indeed, TBP and unique subsets of TAFs could function as multisubunit promoter recognition factors, then each type of complex could be responsible for nucleating the initiation reaction on different classes of templates.

Although TBP can participate in the formation of initiation complexes by RNA pol II and some RNA pol III templates, it seemed a remote possibility that TBP would also play a role in the transcription of ribosomal RNA by RNA pol I. The promoter regions of the ribosomal RNA genes are generally GC-rich and do not contain a consensus TATA box element. Moreover, the classical TFIID fraction, isolated from phosphocellulose columns, that contains potent basal factor activity for RNA pol II transcription, displays no detectable activity for transcription of ribosomal promoters by RNA pol I (L. C. and N. T., unpublished data). However, the notion that TBP may also be involved in RNA pol I transcription becomes more appealing when one considers the recent finding that bona fide TATA-less templates also require TBP and TAFs to form productive RNA pol II initiation complexes (Pugh and Tjian, 1990, 1991). Additionally, our characterization of RNA pol I transcription factors had revealed that SL1, which is essential for ribosomal RNA transcription, shares a number of biochemical properties reminiscent of the RNA pol II TFIID complex. For example, although SL1 is not a site-specific DNA-binding protein, it is, nevertheless, responsible for promoter specificity by interacting with UBF and species-specific DNA elements of the promoter (Bell et al., 1990; Learned et al., 1986). This situation is analogous to the requirement for SP1 and the TFIID complex by TATA-less templates (Pugh and Tjian, 1991). Moreover, like the TFIID complex that contains TBP and TAFs, SL1 behaves as a macromolecular complex with a native molecular mass in excess of 200 kd in glycerol gradient sedimentation experiments. In light of this, it seemed reasonable to test the hypothesis that perhaps SL1 consists of a multisubunit complex, and that one of its subunits may be TBP.

Here, we report our findings on the purification and characterization of the RNA pol I promoter selectivity factor SL1. In addition, we have explored the potential relationship between SL1, TBP, and TAFs. First, we have tested for the presence of TBP during purification of SL1 by conventional chromatography. Next, we have used specific anti-TBP antibodies to immunodeplete SL1 activity and inhibit RNA pol I transcription. Most importantly, we have purified TAFs from active SL1 fractions following immunoprecipitation of the TBP-containing complex and reconstituted transcription of rRNA in vitro with recombinant TBP, UBF, and purified RNA pol I. Finally, we have carried out reconstituted transcription reactions with mouse and human extracts to determine the species-specific properties of TAFs and TBP. Our findings suggest an interesting relationship between SL1 and a novel TBP–TAFs complex that functions as an RNA pol I coactivator. Thus, the TBP–TAFs arrangement may serve as a universal transcription factor complex involved in initiation of all three classes of RNA polymerase.

Results

The Species-Specific Transcription Factor SL1 Copurifies with a Subpopulation of TBP

Recent studies have indicated that TBP is an important component of the RNA pol II transcription machinery, even for templates that lack a TATA box (Pugh and Tjian, 1991). In addition, transcription of the U6 gene by RNA pol III (Lobo et al., 1991; Margottin et al., 1991; Simmen et al., 1991) also appears to require TBP. Because of these findings, we were interested in determining whether TBP might play a role in RNA pol I transcription. As a preliminary analysis, we determined whether TBP cofractionates with any of the essential components of the RNA pol I transcriptional apparatus. Conventional chromatography of HeLa nuclear extracts over a heparin–agarose column eluted with a linear salt gradient separates the components of the RNA pol I transcription system into three distinct activities: RNA pol I elutes at low salt (0.27 M KCl); the UBF elutes at intermediate salt (0.4 M KCl); and the species-specific SL1, is retained until 0.55 M KCl (Figure 1). Individual fractions from the column were assayed for SL1 activity by using an in vitro transcription reaction containing partially purified RNA pol I and DNA affinity-purified UBF. Aliquots of the same fractions were trichloroacetic acid–precipitated and loaded onto an SDS–polyacrylamide gel, and the presence of the TBP determined by Western blot analysis. Interestingly, the peak of SL1 activity from the heparin column coelutes with a peak of TBP (Figure 2A). This result provides the first clue that TBP may be part of the SL1 fraction, and may therefore be involved in RNA pol I transcription. We find that this peak of TBP represents only

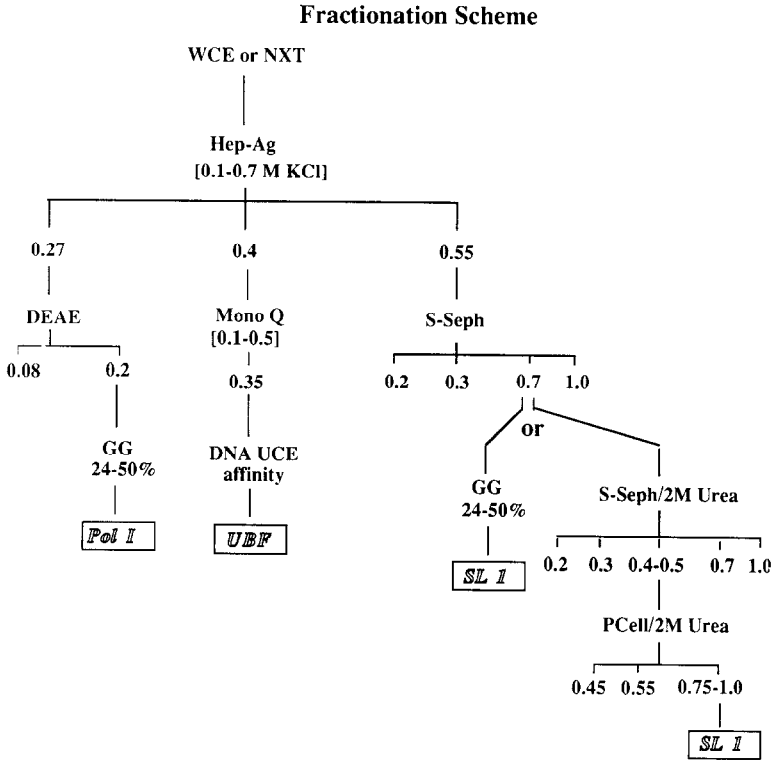

Figure 1. Fractionation Scheme for the RNA Pol I Transcription Factors

Factors used in in vitro transcription assays are enclosed in boxes. Column fractions were tested for SL1 activity as described in Experimental Procedures. For the purification of SL1, two alternate fractionation schemes were used after the S-Sepharose column. Both HeLa whole-cell extract (WCE) and nuclear extract (NXT) were used as the starting material in this work. Salt concentrations indicated within brackets represent a linear gradient of KCl. GG, glycerol gradient sedimentation.

5%–10% of the cellular TBP since most of the protein elutes from the heparin–agarose column between 0.25 and 0.40 M KCl (data not shown).

To examine further a possible relationship between SL1 and TBP, the fractions containing SL1 activity from the heparin column were pooled, concentrated, and subjected to sedimentation in a 24%–50% gradient of glycerol. Various fractions from the gradient were assayed for SL1 activity and TBP protein. Once again, the peak of SL1 activity coincided with the peak of TBP protein (Figure 2B). Both SL1 activity and TBP sediment as a relatively large macromolecular complex with an approximate molecular mass of 230 kd. Because TBP is a 38 kd monomer (Peterson et al., 1990), we infer that it must be associated with one or more additional proteins in the SL1 complex. To determine whether the SL1 complex can be dissociated, we used mild denaturing conditions in the following fractionation steps.

The SL1 fractions from the heparin–agarose column were first concentrated by S-Sepharose chromatography and then subjected to two additional purification steps (see Figure 1). First, SL1 was rechromatographed on S-Sepharose in the presence of 2 M urea and eluted with 0.4–0.5 M KCl. Next, the urea-eluted SL1 fractions were pooled, dialyzed, and subjected to phosphocellulose chromatography, also in the presence of 2 M urea. In both of these sequential purification steps, the SL1 activity consistently copurified with TBP (Figures 3A and 3B). The presence of two distinct SL1/TBP peaks after fractionation over phosphocellulose column may be because of partial disruption of the protein complexes or the fact that the complex elutes at a salt concentration close to 0.75 M KCl. These different purification steps, taken together, indicate that at least a subpopulation of the TBP persistently copurifies with SL1 activity even in the presence of urea and suggest that TBP activity may actually be a component of SL1. However, additional evidence is required to establish that TBP is, in fact, involved in transcription of rRNA by RNA pol I.

Antibodies against TBP Inhibit RNA Pol I Transcriptional Activity

To determine whether TBP plays a role in rRNA transcription, we next tested the ability of the antibodies raised against human TBP to inhibit transcription of rRNA by RNA pol I. These affinity-purified polyclonal antibodies have previously been demonstrated to be specific for TBP, both by Western blot analysis and immunoprecipitation experiments (Pugh and Tjian, 1991; Tanese et al., 1991). Using these highly specific anti-TBP antibodies, a HeLa nuclear extract active for rRNA transcription was depleted of TBP (>70%) by immunoprecipitation. In vitro transcription in the presence of high α-amanitin concentrations revealed that removal of the TBP by immunodepletion resulted in a significant inhibition of transcription by RNA pol I (Figure 4A, lanes 3 and 4). Most importantly, rRNA transcription was completely restored if partially purified SL1 was added back to the TBP-depleted reactions (lanes 5 and 6). As a control, nuclear extracts subjected to immunodepletion using various antibodies directed against unrelated antigens were also assayed for rRNA transcription and found to be unaffected (data not shown). In addition, supple-

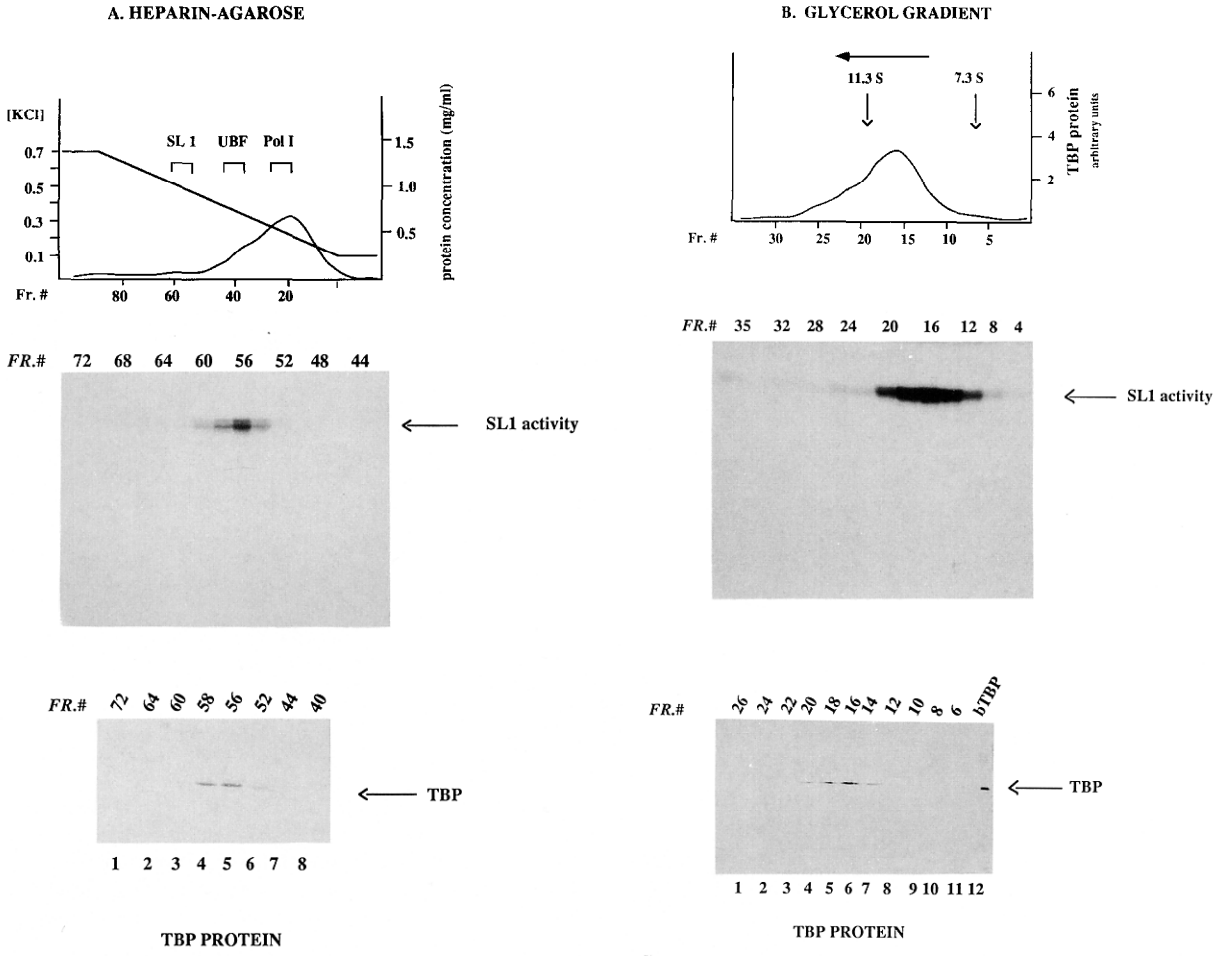

Figure 2. SL1 Activity and a Subpopulation of TBP Protein Cofractionate during Purification on a Heparin–Agarose Column and Glycerol Gradient

(A) (Top) Total protein and salt elution profile of the heparin–agarose column used to purify pol I, UBF, and SL1. (Middle) Individual fractions (from 5 to 10 ng) eluted between 0.35 and 0.7 M KCl were assayed for SL1 activity in an in vitro reconstituted transcription assay, as described in Experimental Procedures, and the products were detected by S1 analysis (indicated by an arrow). (Bottom) Western blot of the indicated column fractions (~2.5 μg of protein per lane) incubated with anti-TBP antibodies.

(B) (Top) Glycerol gradient sedimentation analysis. The graph shows the sedimentation profile of TBP. Approximate amounts of TBP present in each fraction were estimated from a Western blot. Horizontal arrow indicates the direction of sedimentation. The position of two molecular mass standards (catalase, 232 kd; aldolase, 158 kd) were determined from a parallel glycerol gradient, and their sedimentation coefficients are indicated. (Middle and Bottom) The fractions collected from the gradient (60 μl) were tested for SL1 activity and TBP protein as in (A). Four microliters of each fraction was used in transcription assays (Middle), and 50 μl was loaded in each lane of the Western blot. Eight nanograms of the recombinant TBP was loaded in lane 12 (Bottom).

menting the depleted extract with RNA pol I and UBF in the absence of SL1 did not restore transcription (data not shown). These results indicate that removal of TBP and possibly TBP-associated factors in the SL1 preparations indeed affect RNA pol I transcription and that SL1 can functionally substitute for the immunodepleted TBP.

To extend this finding, we also carried out transcription of rRNA by using nuclear extracts that had been heat-treated for 15 min at 47°C, a procedure previously reported to inactivate cellular TBP activity (Nakajima et al., 1988). The heat-treated nuclear extract, like the immunodepleted preparations, was dramatically reduced in its ability to transcribe rRNA (Figure 4A, lanes 7 and 8). As before, supplementing the heat-treated transcription reaction with preparations of SL1 completely restored activated transcription of rRNA by RNA pol I (lanes 9 and 10). Interestingly, the addition of purified recombinant TBP did not restore SL1 activity (lanes 11 and 12). Thus, although TBP appears to be an important component of the RNA pol I transcription machinery, it is not sufficient to complement SL1 activity. Apparently other heat-labile components are necessary to reconstitute the activity provided by the SL1 fraction.

To study in more detail the role of TBP in rRNA transcription, we immunoprecipitated TBP from the phosphocellulose-purified SL1 preparation. After immunodepletion of TBP from these SL1 fractions, the supernatant was tested for its ability to direct rRNA transcription in the presence of purified RNA pol I and UBF. Treatment of the partially purified SL1 fraction with anti-TBP antibodies se-

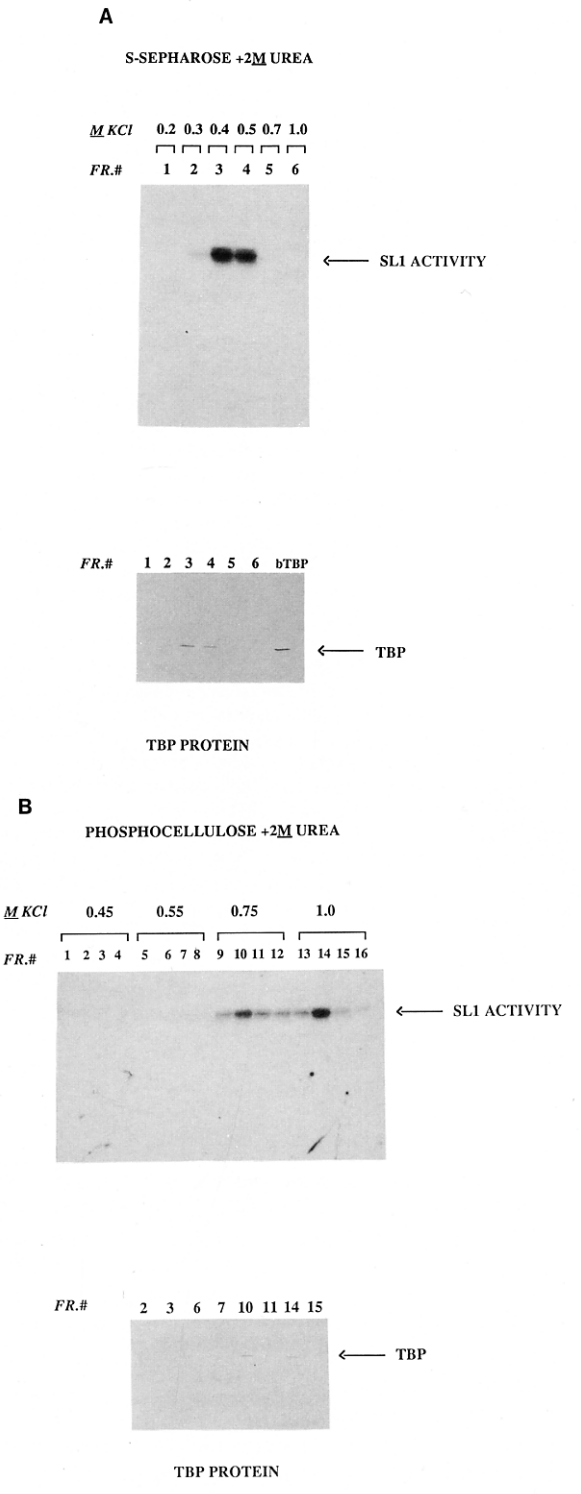

Figure 3. Cofractionation of SL1 Activity and TBP Protein under Denaturing Conditions

(A) The pooled SL1 fraction from the S-Sepharose column (400 μl) was diluted in TM buffer to 0.1 M KCl and loaded onto a second S-Sepharose column (see Figure 1). Column fractions (500 μl) were collected from salt steps (0.2, 0.3, 0.4, 0.5, 0.7, and 1.0 M KCl) in the presence of 2 M urea, dialyzed, and assayed for SL1 activity (1 μl per lane [Top]) and tested for TBP on a Western blot (8 μl per lane [Bottom]). Twenty nanograms of the bacterial recombinant TBP were loaded in the last lane of the Western blot.

(B) Fractions 3 and 4 from the S-Sepharose column were pooled and applied to a phosphocellulose column. Elution was carried out with salt steps (0.45, 0.55, 0.75, and 1.0 M KCl) in the presence of 2 M urea and after dialysis each fraction (4 fractions per salt step, 250 μl each) was tested for SL1 activity (1 μl per lane [Top]) and TBP protein (5 μl per lane [Bottom]). The presence of two distinct SL1 activity–TBP protein peaks may reflect either the heterogeneity of the SL1 complex when fractionated under denaturing conditions or the complex eluting at a salt concentration close to 0.75 M KCl (see Results).

verely inhibited transcription (Figure 4B, lanes 3 and 4). By contrast, the supernatant from a mock-depleted SL1 fraction retained full transcriptional activity (lanes 1 and 2). As expected, the addition of purified TBP was not sufficient to restore transcriptional activation (lanes 5 and 6). Consistent with these results, TBP also cannot substitute for SL1 in an in vitro reconstituted transcription reaction containing purified UBF and RNA pol I (lane 7). As an additional test of antibody specificity, we determined that treatment of the SL1 fraction with antibodies directed against another RNA pol II basal factor, TFIIB, failed to inhibit SL1 activity (lanes 8 and 9). These results strongly implicate TBP as an important component of the species-specific RNA pol I transcription factor, SL1. Significantly, other factors coprecipitating with TBP from the SL1 fraction appear to be necessary in order to reconstitute SL1 activity.

SL1 Is a Protein Complex Composed of TBP and Three Novel TBP-Associated Polypeptides

Our results thus far suggest that TBP in the SL1 fraction is likely to be associated with other essential factors. This hypothesis is consistent with the glycerol-gradient sedimentation data that revealed that TBP and SL1 actually sediment as a high molecular weight complex (see Figure 2B). To identify the subunit components of this putative SL1 complex, TBP was immunoprecipitated from the SL1 fraction and the products analyzed by SDS–polyacrylamide gel electrophoresis. Silver staining of the immunoprecipitated proteins reveals the presence of three novel polypeptides, of approximate molecular mass 110 kd, 63 kd, and 48 kd, that coprecipitated with TBP and therefore appear to be tightly associated with TBP (Figure 5). These three TBP-associated factors, or TAFs, are analogous to those factors found in the TFIID complex for RNA pol II transcription (Pugh and Tjian, 1991; Tanese et al., 1991). Preparation of purified TAFs largely depleted of TBP were generated by eluting the immunocomplex with either 1 M guanidine–HCl or 2.5 M urea. We estimate that TBP and the SL1–TAFs have been purified approximately 40,000-fold (Table 1) from the whole-cell extract. The deduced molecular mass of the complex (roughly 250 kd) correlates well with the observed molecular mass of SL1 as determined by glycerol-gradient sedimentation. Monoclonal antibodies directed against the C-terminal portion of Drosophila TBP crossreact with the human TBP on a Western blot and immunoprecipitate recombinant human TBP (N. T., unpublished data). They do not, however, immunoprecipitate TBP from the SL1 fraction (Figure 5, lanes 3 and 4). This finding suggests that the epitopes recognized by these two monoclonal antibodies are most

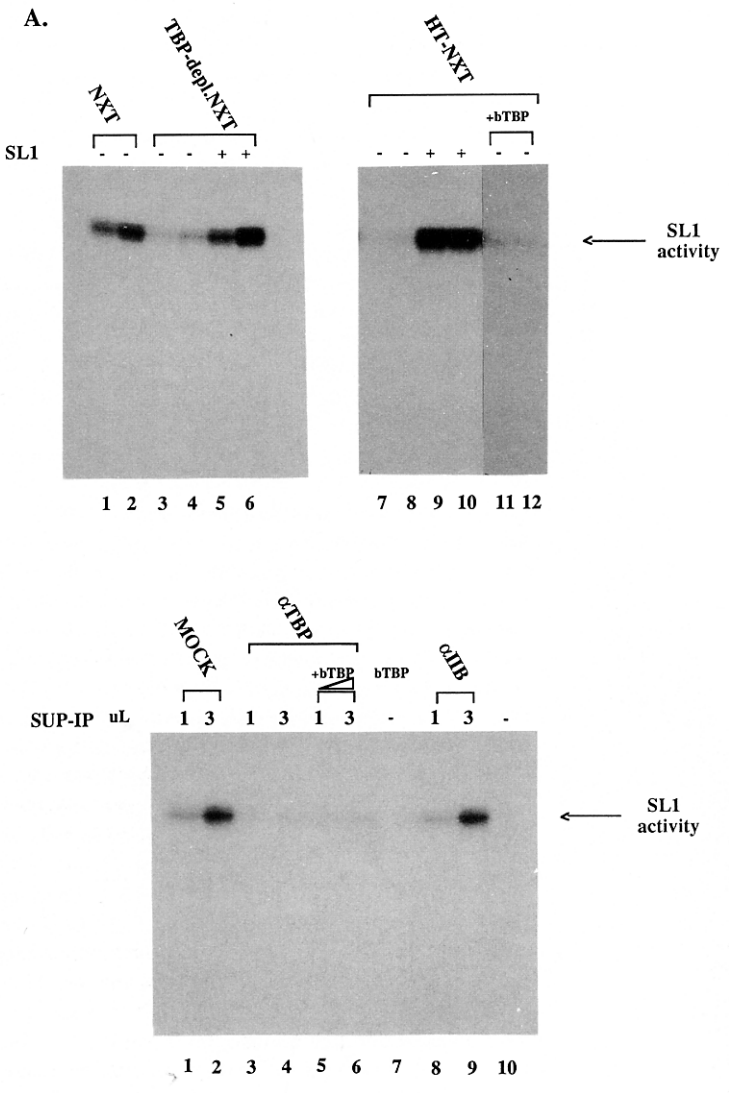

Figure 4. Immunodepletion of TBP from HeLa Nuclear Extract or a Partially Purified SL1 Fraction Results in Inhibition of Pol I Transcription

(A) Immunodepletion or heat inactivation of TBP in a nuclear extract inhibits pol I transcription that can be restored with the addition of an SL1 fraction. Mock-depleted (lanes 1 and 2), TBP-depleted (lanes 3–6), or heat inactivated (47°C, 15 min; lanes 7–12) nuclear extracts were assayed for pol I transcription. Odd numbered lanes contain 40 μg of extract; even numbered lanes contain 80 μg. In lanes 5 and 6 and 9 and 10, approximately 15 ng of a partially purified SL1 fraction (from the glycerol gradient) was added to the reaction mixture. In lanes 11 and 12, bacterial recombinant TBP was added (10 and 20 ng, respectively). The heat-treated nuclear extract was supplemented with a partially purified pol I–UBF fraction (8 μg per lane) (see Experimental Procedures).

(B) Depletion of TBP from a purified SL1 fraction inhibits pol I transcription. The SL1 fraction (0.75 M KCl) from the phosphocellulose–2M urea column was incubated with either TM buffer (lanes 1 and 2), anti-TBP antibodies (lanes 3–6), or anti-TFIIB antibodies (gift of E. Maldonado and D. Reinberg) (lanes 8 and 9). Protein A–Sepharose was then added, and the immune complex was pelleted by centrifugation. The supernatant (volume in μl indicated in the figure) from each immunoprecipitation reaction was then assayed for SL1 activity as described in the Experimental Procedures. Recombinant TBP was added to the reactions shown in lanes 5 and 6 (4 ng and 12 ng, respectively). In lane 7, 12 ng of the recombinant TBP was present in the reaction with no supernatant from the immunoprecipitation of the SL1 fraction. In lane 10, no SL1 or TBP was added to the transcription reaction. Identical results were obtained using the 1.0 M KCl SL1 fraction from the phosphocellulose–2 M urea column or SL1 purified on a glycerol gradient.

likely masked by the presence of the TAFs. A comparison of the TAFs found in the SL1 fraction with TAFs in the TFIID complex reveals no common polypeptides shared between these two distinct complexes, with the exception of the TBP (lanes 6 and 7). Apparently, the TAFs of the SL1 complex represent novel regulatory factors specific for RNA pol I transcription. These results together with our other findings suggest that TBP is an integral subunit of both TFIID and SL1 complexes. We therefore infer that promoter and RNA polymerase specificity is most likely dictated by the presence of these novel TAFs.

SL1 Activity Can Be Reconstituted with Recombinant TBP and Purified TAFs

The presence of three novel TAFs coimmunoprecipitated with TBP from the SL1 fraction raises the exciting possibility that these TAFs can mediate promoter-specific transcription in an in vitro reconstituted system. To test this hypothesis, we have purified the three TAFs by eluting the immunocomplex attached to Sepharose A resin under denaturing conditions in a manner similar to the one that had previously been successful for purifying RNA pol II TAFs. Both 2.5 M urea and 1 M guanidine–HCl selectively dissociate the TAFs from the protein A–immunoglobulin–TBP complex (Figure 5, lanes 8 and 9). Although purified TAFs are largely devoid of TBP, we often see a small amount of TBP eluting with the TAFs. A second round of immunoprecipitation of the eluted TAFs with anti-TBP antibodies was effective in removing most of the residual TBP (data not shown). The eluted TAFs were renatured by dialysis into transcription buffer, and then tested for their ability to activate RNA pol I transcription in a reconstituted system containing purified RNA pol I, UBF, and recombinant TBP. As shown in Figure 6, the addition of purified SL1–TAFs and TBP to the reaction mixture strongly activates transcription. In the absence of exogenous TBP but in the presence of added TAFs, there is some low level transcription that is most likely caused by trace amounts of endogenous TBP still present in the TAF fraction. By contrast, addition of the recombinant TBP without the

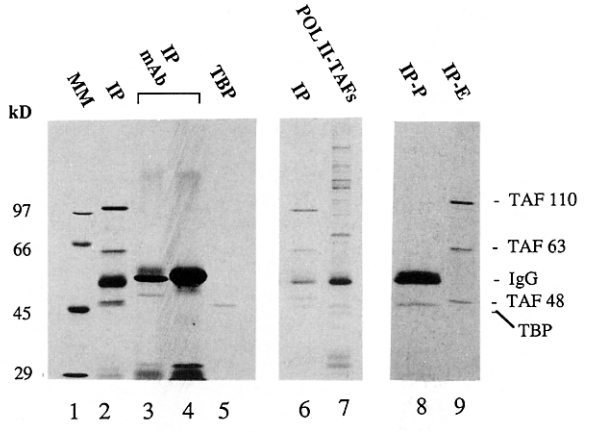

Figure 5. Immunopurification of TBP from the SL1 Fraction Reveals a Protein Complex Consisting of TBP and Three Associated Factors

Immunopurification was carried out as described in Experimental Procedures. Lane 2 shows the products of a typical immunoprecipitation reaction carried out with approximately 70 μg of a partially purified SL1 fraction and polyclonal anti-TBP antibodies. In lanes 3 and 4, two different monoclonal antibodies (gift of R. Weinzierl) that recognize TBP on a Western blot were added to the immunoprecipitation reaction with SL1. Lane 5 contains 40 ng of recombinant TBP. Lanes 6 and 7 compare the profiles of the immunoprecipitation products from the SL1 fraction and the TFIID fraction required for pol II transcription (Tanese et al., 1991). Lanes 8 and 9 show the pellet and the eluted fraction, respectively, after incubation of the immunoprecipitated protein complex with a buffer containing 1 M guanidine–HCl (see Experimental Procedures). The TAF polypeptides have been designated according to their estimated molecular weights. All samples were electrophoresed on 8% SDS–polyacrylamide gels and visualized by silver staining. Lane 1 shows the molecular weight standards. No additional polypeptides smaller than 29 kd were observed when the immunoprecipitated proteins were analyzed on higher percentage SDS–polyacrylamide gel.

TAFs results in no detectable transcription. As expected, the level of transcriptional activation is proportional to the amounts of TAFs and TBP added to the reconstituted reactions (Figure 6). These results establish, first of all, that TBP is indeed playing an important role in RNA pol I transcription. Moreover, our findings demonstrate that the SL1 TAFs behave like coactivators and are essential to reconstitute accurate RNA pol I transcription.

TBP and TAFs Can Reprogram Transcription in a Species-Specific Manner

An intrinsic property of the promoter-selective transcription factor, SL1, is its species specificity in directing RNA pol I transcription. Thus, transcription of human rRNA requires human SL1, while transcription of the mouse rRNA template requires mouse SL1. By contrast, human and mouse UBF are interchangeable, as is RNA pol I, between these two species. In addition, human SL1 can reprogram the mouse transcription system to direct synthesis of the human rRNA (Figure 7, lane 1) (Bell et al., 1990). To determine the species specificity of the TBP–TAF complex, purified human TBP and TAFs were tested for their ability to activate transcription from the human ribosomal promoter in a mouse nuclear extract. As expected, addition of purified recombinant human TBP to a transcriptionally active mouse nuclear extract failed to activate transcription of the human gene (Figure 7, lane 3). Similarly, adding only the purified human TAFs to the mouse extract failed to direct transcription of the human template (lane 4). By contrast, addition of both human TBP and human TAFs to the mouse system strongly activated transcription of the human template (lane 5). The activity of this reconstituted reaction is comparable to that observed by adding bona fide human SL1 fractions to the mouse extract (Figure 7, lane 1). The ability of the human TAFs plus human TBP to reprogram mouse nuclear extracts provides additional evidence that the TBP–TAF complex, indeed, constitutes the species-specific RNA pol I transcription factor, SL1.

Table 1. Summary of the Purification of SL1 Complex from HeLa Whole-Cell Extract (WCE)

	Amount Protein[a] (μg)	Activity	Fold Purification
WCE	300,000	100%	
Hep-Ag	70	50%	2,000
Immunopurification	0.2	6%	40,000

[a] Protein concentration was determined by Bradford assays, except for the immunopurified proteins. Concentration of TBP–TAFs after immunopurification was estimated by silver staining of SDS–polyacrylamide gels.
Activity was estimated from in vitro transcription assays.

Discussion

Recent molecular cloning and biochemical characterization of the basal transcription factors as well as RNA polymerase subunits have begun to identify interesting structural and functional relationships. For example, although RNA pol I, II, and III systems have traditionally represented different classes of enzymes with unique properties and divisions of labor in transcribing different sets of genes in the cell, their subunit composition suggests a certain underlying conservation of structure as well as function (Murphy et al., 1989; Woychik et al., 1990). Another particularly striking example is the finding that an unusual RNA pol III transcription unit that contains an upstream AT-rich element, actually utilizes the RNA pol II TBP for its initiation (Lobo et al., 1991; Margottin et al., 1991; Simmen et al., 1991). This intriguing finding opened the possibility that the TBP may be a more universal transcription factor than had previously been recognized.

In this report, we have purified and characterized the human RNA pol I transcription factor, SL1, and found that this promoter-selective factor consists of a multi-subunit complex containing the 38 kd TBP tightly associated with three distinct polypeptides that are essential to reconstitute transcription in vitro. This finding simultaneously defines the biochemical nature as well as subunit composition of SL1 and provides strong evidence to support the coactivator model for mediating transcriptional specificity of enhancer and promoter binding factors. In particular, these results with SL1 validate the notion that different

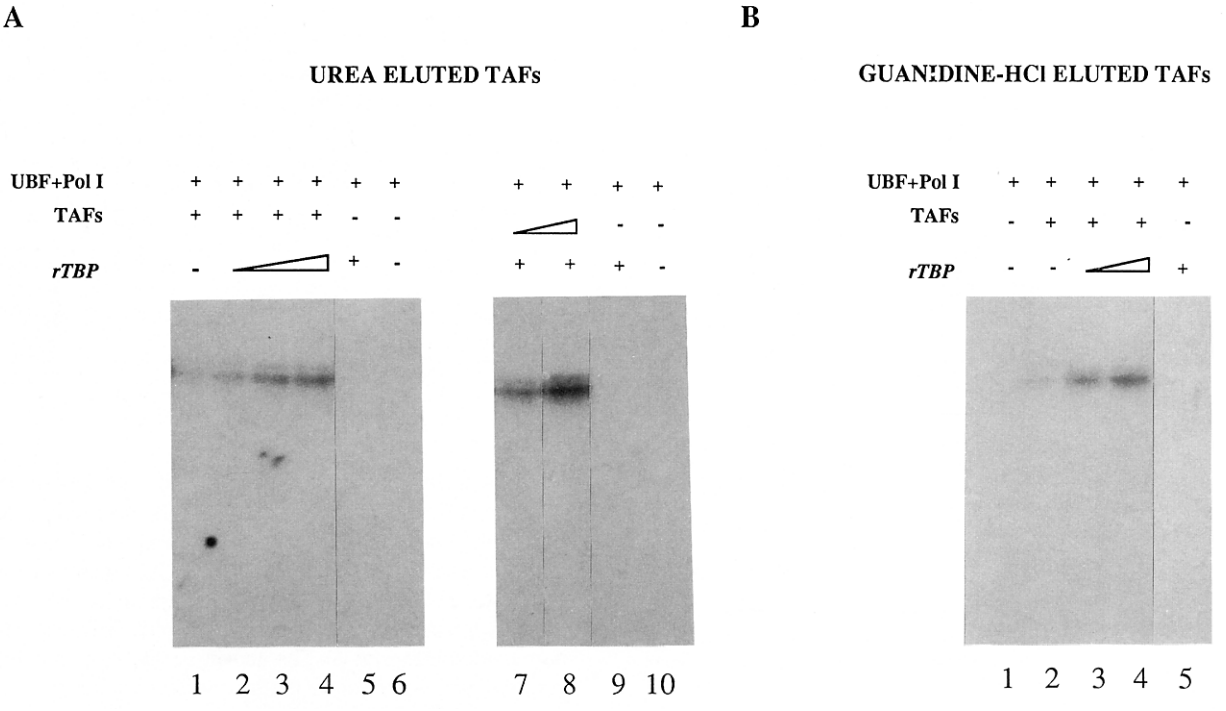

Figure 6. TBP and Associated Factors Reconstitute Pol I Transcription

In vitro transcription reactions were reconstituted with pol I and UBF as described in Experimental Procedures.

(A) Reactions shown in lanes 1–4, 7, and 8 contain TAFs eluted with 2.5 M urea (see Results and Experimental Procedures). Lanes 1–4 contain approximately 10 ng of eluted TAFs. Lanes 2, 3, and 4 contain 4, 8, and 12 ng of recombinant TBP, respectively. Increasing the amount of TBP results in stimulation of transcription. Lanes 7 and 8 contain approximately 10 and 25 ng of eluted TAFs, respectively. The addition of increasing amounts of TAFs to these reactions increases pol I transcriptional activity. Lanes 5, 7, 8, and 9 contain 12 ng of recombinant TBP.

(B) Reactions shown in lanes 2–4 contain approximately 30 ng of TAFs eluted with 1 M guanidine–HCl. Lanes 3, 4, and 5 contain 4 ng, 12 ng, and 12 ng of the recombinant TBP, respectively.

TBP–TAF complexes are responsible for mediating activation by different upstream site-specific DNA-binding proteins, perhaps serving as adaptor molecules or intermediaries.

In the case of the UBF–SL1 complex, the interaction between these two components has been well characterized. First, it has been documented that at least in human cells, UBF is the primary component responsible for recognizing and binding specific ribosomal promoter sequences (Bell et al., 1988). The SL1 complex, by contrast, has no demonstrable sequence-specific DNA binding capabilities to the ribosomal RNA promoter on its own. However, in the presence of UBF, SL1, which we now know consists of TBP and three TAFs, can extend the DNAase protection region by forming a stable complex with UBF and DNA (Bell et al., 1988). Analysis of various clustered point mutations in the human ribosomal promoter indicates that the extended sequences contacted by the UBF–SL1 complex are important for promoter function. Since SL1 contains TBP, it is perhaps not surprising that some component of SL1 should contact DNA. However, our experiments indicate that adding the purified recombinant TBP to the UBF–DNA complex does not complement SL1 transcription activity, and it fails to generate the extended footprint pattern (H.-M. Jantzen, unpublished data). Thus, it seems likely that one or more of the TAFs must either alter the DNA binding properties of TBP to interact with the ribosomal DNA sequences, or alternatively TBP does not contact the DNA at all under these circumstances. Instead, the SL1-specific contacts are likely to be mediated by the TAFs directly. Thus, it seems plausible that TBP functions predominantly as a core subunit decorated with different TAFs that dictate the promoter specificity of the complex. A similar situation is thought to occur with transcription of TATA-less templates by the TFIID complex, SP1 and RNA pol II (Pugh and Tjian, 1990). Indeed, it appears that TBP can act by two distinct modes: either as a site-specific DNA-binding protein or as a scaffold protein decorated with TAFs.

We speculate that TBP and TAFs essentially serve a promoter recognition function that is at least formally analogous to that of prokaryotic σ factors, which are responsible for programming RNA polymerase to transcribe different templates (Helmann and Chamberlin, 1988; Losick and Pero, 1976). Like σ, the TBP–TAFs complex does not bind DNA when tested in isolation but may do so in a complex with other components of the transcriptional apparatus. For example, σ and core RNA polymerase can form a stable complex with DNA while σ alone cannot. In animal cells, interaction of the TBP–TAF complex with the promoter can be stabilized by interaction with site-specific DNA-binding proteins (i.e., UBF, Sp1) as well as with RNA

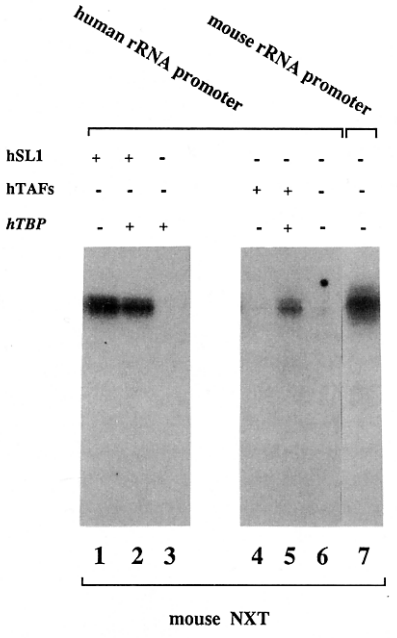

Figure 7. Human TBP and TAFs Reprogram Pol I Transcription from a Human rRNA Promoter in a Mouse Nuclear Extract

Mouse nuclear extract was used in the presence of the indicated human factors to transcribe either the human (lanes 1–6) or the mouse (lane 7) rRNA gene template. Twelve nanograms of recombinant human TBP was added to the reactions 2, 3, and 5. TAFs eluted in 1 M guanidine–HCl (30 ng) were used in the reactions shown in lanes 4 and 5.

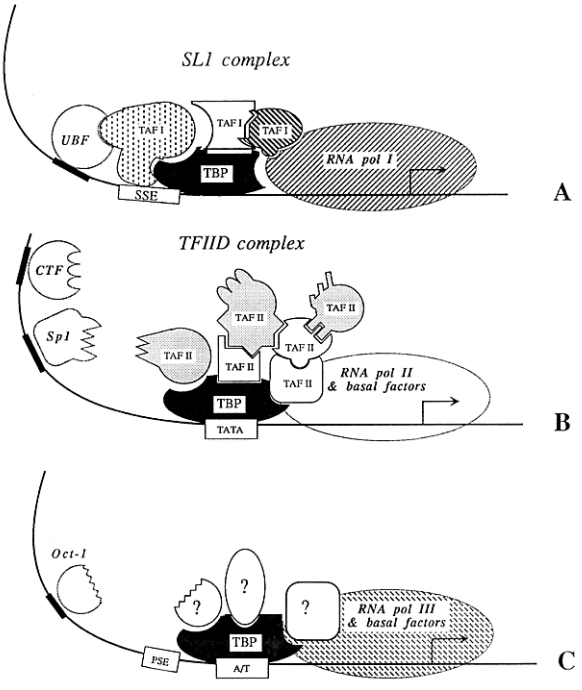

Figure 8. Schematic Model for the Involvement of TBP–TAFs Complex in Pol I, II, and III Transcription

We propose that TBP serves as a "core" transcription factor that participates in the initiation of transcription by all three classes of polymerases and that TBP–TAFs complex specific for each class is responsible for promoter recognition.

(A) For RNA pol I, formation of a preinitiation complex requires, in addition to pol I, transcription factor UBF bound specifically to the ribosomal promoter sequences in the template DNA and SL1, which interacts strongly with UBF. For simplicity, only the proximal promoter region is shown. Our studies indicate that SL1 is a multisubunit complex consisting of TBP and three distinct TAFs. Since SL1 confers species specificity on pol I transcription, one or more of its components is thought to interact with the species-specific element in the promoter.

(B) Transcriptional initiation by RNA pol II requires at least six separate basal factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH). TFIID has been shown to be a large multisubunit complex, composed of TBP, that binds to the TATA box element and at least six tightly associated factors (TAFs). Some or all of the TAFs function as coactivators that mediate transcriptional activation by the DNA-bound upstream regulators such as Sp1 and CTF. Other activators may work through direct contacts with TBP or TFIIB (Greenblatt, 1991).

(C) The initiation complex assembled on an RNA pol III promoter (such as U6) appears to consist of RNA pol III, basal transcription factors, and the octamer motif-binding protein Oct-1 bound to the upstream sequences in the template. Recent studies have demonstrated a requirement for TBP in transcribing the U6 gene. Thus, it is possible that a unique TBP–TAFs complex interacting with the essential elements of the promoter (AT-rich sequences) is an integral component of the pol III initiation complex. However, at present, there is no evidence that TBP involved in pol III transcription is associated with TAFs. Although we depict various specific contacts between TBP, TAFs, and template DNA in the model, these are merely hypothetical as the specific protein–protein and protein–DNA interactions have not yet been determined.

polymerase (Figure 8). In both situations, the primary function of σ factors and TBP–TAF complexes is to direct template recognition and promoter specificity of the RNA polymerases. In prokaryotes, most σ factors consist of a single subunit that forms a tight complex with RNA polymerase and may mediate regulation by upstream binding factors. In eukaryotes, the greater demand for promoter diversity requires a multi-subunit "σ factor" consisting of TBP plus TAFs, which also serves to mediate regulation by upstream site-specific factors. The interaction of TBP and TAFs with subunits of RNA polymerase remains to be studied.

Apparently, promoters characteristic of the three general classes of genes transcribed by pol I, II, and III have evolved to retain the use of the core subunit TBP, but each type of transcription machinery has recruited class-specific TAFs to direct and mediate transcription in a promoter-selective manner. Indeed, phosphocellulose chromatography of HeLa nuclear extracts revealed the presence of TBP in the 0.3 M, 0.5 M, and 0.7 M KCl eluates (F. Pugh, unpublished data; Timmers and Sharp, 1991). The TFIID activity was found within the 0.5–0.7 M KCl fraction and represented approximately 25% of the TBP in the nucleus. By contrast, the TBP associated with SL1 was predominantly eluted at even higher salt fractions and represented approximately 5%–10% of the cellular TBP. Thus, TBP appears to be present in different pools, presumably as a result of its association with different sets of TAFs, and each type of complex directs the transcription of a different class of promoter. The existence of different TBP pools had previously gone undiscovered because the transcription properties of these different TBP complexes

were essentially promoter selective. Consequently, SL1 fractions containing TBP were largely inactive for RNA pol II transcription and the TFIID fractions were inactive for RNA pol I transcription. The ability to track TBP by antibodies, however, revealed the more extensive distribution of this "core" transcription factor and its potential involvement in the initiation by all three classes of RNA polymerase.

A schematic diagram depicting the potential participation of different TBP–TAF complexes in transcriptional initiation by RNA pol I, II, and III is shown in Figure 8. Our results indicate that for RNA pol I there is only one DNA binding factor, UBF, that interacts specifically with the ribosomal RNA promoter. SL1 consists of TBP plus three distinct TAFs, one or more of which function as a coactivator essential for reconstituting transcription (Figure 8A). In the presence of UBF, the SL1 complex binds template and extends the DNAase footprint to include the species-specific element (SSE) (Bell et al., 1990, 1988). Interestingly, the three TAFs associated with SL1 appear to be distinct from the major TAFs found in the TFIID complex used by RNA pol II. Thus, it seems evident that the presence of different sets of TAFs may be the mechanism by which distinct initiation complexes can be assembled along with TBP as a common core component. We envision that the TAFs most likely act as adaptor molecules that interact with the upstream activator (UBF) and the basal transcription machinery. However, unlike enhancers for RNA pol II, which can function from long distances in either orientation, the position of the UBF binding site relative to species-specific element and the initiation site are spatially restricted (Haltiner et al., 1986). Thus, UBF bound to the ribosomal RNA promoter is likely to be in a tight fit with SL1. It is possible that one or more of the TAFs contacts the species-specific element of the ribosomal RNA promoter. In addition, we envision that one of the TAFs will contact UBF and another will interface with RNA pol I. We have also depicted TBP making some contact with RNA polymerases. This untested idea is prompted by the observation that several of the "small" RNA polymerase subunits are common to all three enzymes (Woychik et al., 1990), and thus TBP would be a logical target for interaction with these conserved domains. Direct evidence for the activation mechanism of the TBP–TAF complex and the specific protein–protein interactions will require additional biochemical experiments.

Our previous work with RNA pol II transcription and the characterization of the TFIID complex revealed a more elaborate assembly of TAFs and TBP than was observed for SL1. In both human and Drosophila cells, TBP appears to be tightly associated with at least 6 TAFs that are present in approximately 1:1 stoichiometry with TBP (Figure 8B). In addition, the immunoprecipitation of the TFIID complex from human cells reveals other TAFs that are present in substoichiometric amounts (Pugh and Tjian, 1991; Tanese et al., 1991). Thus, it is possible that even within the RNA pol II class, there are multiple distinct TBP complexes that are responsible for mediating transcription by different types of activators. This more elaborate arrangement involving multiple TBP–TAF complexes might be expected, since RNA pol II is responsible for directing the transcription of 50–200,000 genes by interacting with perhaps a few thousand site-specific transcription factors that function in a temporally regulated and spatially restricted manner. It will be of great interest, therefore, to determine whether some TAFs exhibit cell-type specificity and/or developmentally regulated patterns of expression.

Although there has, thus far, been no direct evidence for TAFs involved in RNA pol III transcription, it seems likely that a similar arrangement could also be utilized, since it is clear that transcription, at least of the U6 gene by RNA pol III, requires TBP (Figure 8C). Recently, the involvement of TBP was extended to include classic RNA pol III transcription units such as tRNA and 5S RNA promoters, which do not contain TATA boxes (White et al., 1992). Most notably, these studies indirectly implicate the requirement for additional components, possibly TAFs/coactivators, that are required to reconstitute transcription in vitro. Further characterization of TFIIIB and TFIIIC and perhaps immunoprecipitation of TBP complexes from fractions competent for RNA pol III transcription may reveal the presence of TAFs.

A hallmark of rRNA transcription by RNA pol I is species specificity. Earlier work established that although transcription was species-specific between mouse and man, both RNA pol I and UBF were functionally interchangeable between species. By contrast, the SL1 fraction was species-specific and would only function in conjunction with the correct template. The studies reported here confirm that the TBP–TAF complex isolated from human cells behaves like human SL1 and thus can reprogram a mouse transcription apparatus (i.e., mouse pol I and UBF) to transcribe the human rRNA in vitro. Because promoter specificity must be at least in part dictated by specific protein–DNA interactions, our results suggest that one or more of the TAFs is likely to be responsible for the reprogramming activity. However, it is also possible that TBP contributes to the species specificity through the less conserved N-terminal sequences of the mouse and human proteins (Tamura et al., 1991). The finding that TAFs play a major role in dictating species specificity of RNA pol I transcription is entirely consistent with the model that TAFs govern promoter selectivity by altering the activity of the TBP core subunit. Why has TBP been preserved as an integral component in all three types of transcription systems in eukaryotes? One attractive hypothesis is that TBP is responsible for coordinating the specificity of the three different transcription systems. It is also appealing to consider TBP as an ancestral σ factor that has evolved to contain multiple TAFs. It will be important to determine the primary function of each TAF in the SL1 complex, and how they communicate with UBF and RNA pol I. These and other fundamental mechanistic questions may now be addressed as we proceed to purify, clone, and characterize the TBP-associated TAFs. Ultimately, it should be possible to reconstitute the activity of these regulatory proteins in a completely defined transcription system.

Experimental Procedures

Protein Purification

RNA pol I, UBF, and SL1 were purified as follows. A HeLa whole-cell extract (or nuclear extract) was applied to a heparin–agarose column

(column volume, 100 ml; column dimensions, 3 × 20 cm) equilibrated in TM buffer (50 mM Tris–HCl [pH 7.9], 12.5 mM $MgCl_2$, 20% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol) containing 0.1 M KCl, washed with TM, 0.2 M KCl and then eluted with a continuous salt gradient (0.2 M–0.7 M KCl in TM) (flow rate, 40 ml/hr; fraction size, 8.2 ml). The fractions containing the RNA pol I activity (approximately 0.27 M KCl) were pooled, dialyzed against TM, 0.05 M KCl buffer, and then applied to a diethylaminoethyl–Sepharose column equilibrated with TM, 0.1 M KCl buffer. The column was washed with TM buffer containing 0.08 M KCl and then step-eluted with TM, 0.2 M KCl buffer. Fractions containing the RNA polymerase activity (0.2 M KCl) were pooled, dialyzed and frozen in 100 μl aliquots at −70°C. RNA pol I used in the reconstitution experiments (shown in Figures 4, 6, and 7) had been further purified by loading the eluate from the diethylaminoethyl column (100 μl aliquot) onto a 2 ml linear glycerol gradient (24%–50%) prepared in TM^+, 0.2 M KCl containing 0.1 mM sodium metabisulfite and 0.1 mM phenylmethylsulfonyl fluoride. Gradients were centrifuged at 50,000 rpm for 7 hr at 4°C in a Beckman TLS55 rotor. A total of 32 fractions containing 60 μl each was collected. Fractions containing pol I activity were divided into aliquots and stored at −70°C. UBF (0.4 M KCl eluate from the heparin–agarose) was purified to homogeneity as previously described (Bell et al., 1988). Fractions from the heparin–agarose containing SL1 activity (0.55 M KCl) were pooled, dialyzed against TM, 0.2 M KCl buffer, and chromatographed over a small S-Sepharose column (300 μl). The column was washed with TM, 0.2 M KCl buffer, and step-eluted (1.2 ml) with TM buffer containing 0.3 M KCl and 0.7 M KCl. The concentrated pool of SL1 (0.7 M KCl step) was diluted with TM buffer to 0.1 M KCl, loaded onto a second S-Sepharose column in TM, 0.1 M KCl, and eluted with salt steps (0.2, 0.3, 0.4, 0.5, 0.7 and 1.0 M KCl) in the presence of 2.0 M urea. SL1 activity was recovered in the 0.4–0.5 M KCl steps and immediately dialyzed against TM buffer containing 0.2 M KCl. The buffer of the pooled SL1 fractions was changed to PC buffer (20 mM HEPES–KOH [pH 7.9], 2 mM $MgCl_2$, 10 μM $ZnSO_4$, 1 mM dithiothreitol, 10% glycerol, 0.3 M KCl), by another round of S-Sepharose chromatography prior to loading onto a phosphocellulose P11 column equilibrated in PC buffer containing 0.3 M KCl. The column was subsequently washed and step eluted with PC buffer containing 0.45 M, 0.55 M, 0.75 M, and 1.0 M KCl in the presence of 2.0 M urea. The SL1 activity eluted at 0.75 and 1.0 M KCl was dialyzed in TM, 0.1 M KCl containing 0.1% NP-40. All column fractions were assayed for SL1 activity using a pol I/UBF fraction purified over heparin–agarose and diethylaminoethyl columns as described in Learned et al. (1985). Alternatively, 100 μl aliquots of SL1 fraction (heparin–agarose- and S-Sepharose-purified) were subjected to glycerol gradient sedimentation using identical conditions as described above for the purification of RNA pol I. Fractions containing SL1 were pooled and stored in aliquots at −70°C. Recombinant TBP was purified as described in Pugh and Tjian, 1991. Proteins were quantitated by Bradford assays using γ-globulin as a standard or estimated from silver-stained SDS–polyacrylamide gels.

In Vitro Transcription

In vitro transcription reactions were carried out as previously described (Bell et al., 1990, 1988; Learned et al., 1986) in the presence of 100 μg/ml α-amanitin. In vitro synthesized RNAs were detected by S1 nuclease analysis using 5′ end–labeled single-stranded DNA oligomers (Bell et al., 1988). Data shown are representatives of the transcription reactions that have been repeated several times.

Antibodies and Immunoprecipitation

Rabbit polyclonal antisera raised against the bacterially produced hTBP (gift of G. Peterson) were affinity-purified according to the published procedures (Dynlacht et al., 1991). Polyclonal antisera against hTFIIB were a generous gift of E. Maldonado and D. Reinberg, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey. Immunoprecipitation reactions were carried out as described (Dynlacht et al., 1991) with the following modifications. All the extracts were in TM, 0.1 M KCl buffer, and the washes were performed in the same buffer supplemented with 0.1% NP-40 (TM^+, 0.1 M KCl). To elute the TAFs from the TBP–antibody complex two different buffers were used. A: 1 M guanidine–HCl, TM^+, 0.1 M KCl, 0.2 mg/ml insulin. B: 2.5 M urea, TM^+, 0.1 M KCl, 0.2 mg/ml insulin. To visualize the proteins in the immune complex, the immunoprecipitated products were solubilized in 0.1% SDS and loaded onto an 8% SDS–polyacrylamide gel. In a typical reaction, 50–100 μg of partially purified SL1 (S-Sepharose fraction) was mixed with 1–1.2 μg of affinity-purified anti-TBP antibodies and incubated on ice for approximately 1 hr. Thirty microliters of a 50% slurry of protein A–Sepharose was then added to the reaction and incubated for another hour at 4°C. To elute the TAFs from the antigen–antibody complex, the complexes were incubated on ice with 50 μl of elution buffer A or B (see above) for approximately 30 min and centrifuged at 3000 rpm. The supernatant containing the TAFs was then dialyzed against TM^+, 0.1 M KCl for 2 hr at 4°C, subjected to a second immunoprecipitation reaction using 200 ng of anti-TBP antibodies and then used directly in transcription assays. Concentration of TAFs in the eluate is estimated at 5–10 ng/μl as judged by silver staining.

Acknowledgments

First, we are indebted to M. Learned and S. Bell for conducting the initial studies with SL1 and to Greg Peterson for suggesting that TBP might be associated with SL1. We thank S. Bell, B. Dynlacht, M. Jantzen, G. Peterson, F. Pugh, and R. Weinzierl for sharing their valuable experimental reagents for this work. We especially thank all the members of the Tjian laboratory for helpful discussions throughout this work and critical reading of the manuscript. We also thank K. Ronan for the assistance in the preparation of the manuscript. This work was supported in part by a National Institutes of Health grant to R. T. L. C. is supported by a Damon Runyon–Walter Winchell Cancer Research Fund Fellowship, DRG-1130, and N. T. is a recipient of an American Cancer Society, California Division fellowship, S-60-90.

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Received December 6, 1991; revised January 8, 1992.

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