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Cloning and Characterization of the β Subunit of Human Proximal Sequence Element-Binding Transcription Factor and Its Involvement in Transcription of Small Nuclear RNA Genes by RNA Polymerases II and III

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The proximal sequence element (PSE)-binding transcription factor (PTF), which binds the PSE of both RNA polymerase II- and RNA polymerase III-transcribed mammalian small nuclear RNA (snRNA) genes, is essential for their transcription. We previously reported the purification of human PTF, a complex of four subunits, and the molecular cloning and characterization of PTF γ and δ subunits. Here we describe the isolation and expression of a cDNA encoding PTFβ, as well as functional studies using anti-PTFβ antibodies. Native PTFβ, in either protein fractions or a PTF–Oct-1–DNA complex, can be recognized by polyclonal antibodies raised against recombinant PTFβ. Immunodepletion studies show that PTFβ is required for transcription of both classes of snRNA genes in vitro. In addition, immunoprecipitation analyses demonstrate that substantial and similar molar amounts of TATA-binding protein (TBP) and TFIIIB90 can weakly associate with PTF at low salt conditions, but this association is dramatically reduced at high salt concentrations. Along with our previous demonstration of both physical interactions between PTFγ/PTFδ and TBP and the involvement of TFIIIB90 in the transcription of class III snRNA genes, these results are consistent with the notion that a TBP-containing complex related to TFIIIB is required for the transcription of class III snRNA genes, and acts through weak interaction with the four-subunit PTF.

Mammalian small nuclear RNA (snRNA) genes are transcribed either by RNA polymerase II (pol II) (class II, such as U1 to U5 genes) or by RNA pol III (class III, such as U6 and 7SK genes) (for reviews, see references 6, 7, 11, 14, and 18). Both classes of snRNA genes contain a distal sequence element (DSE) around position −220, which bears at least an octamer motif and functions as an enhancer, as well as an essential proximal sequence element (PSE) around position −60. The PSE may also function in transcription start site selection and/or transcript 3′-end formation (8, 16, 17). Interestingly, in addition to the PSE and the DSE, class III genes contain a TATA box around position −25, which functions as the major determinant of RNA pol III specificity (12, 13). The PSEs of the two classes of genes are functionally interchangeable (12, 13, 17). This finding indicates that the PSE per se is not the determinant of RNA polymerase specificity, although the transcription factor(s) bound to the PSE may differentially interact with both pol II and pol III transcription factors to assist in RNA polymerase selection.

We previously showed that the human PSE-binding transcription factor (PTF) binds specifically to the PSE of both classes of snRNA genes and that this binding is greatly stimulated by Oct-1 binding to the adjacent octamer site (15). Human PTF was purified to near homogeneity and shown to be a stable complex containing 180-, 55-, 45-, and 43-kDa subunits, designated PTFα, -β, -γ, and -δ, respectively (26). UV cross-linking experiments indicated that PTFα is the DNA-binding subunit (26). We have also reported the cloning and characterization of PTFγ and PTFδ and the use of corresponding antibodies to verify that PTF is required for transcription of both classes of snRNA genes in a PSE-dependent manner (27). PTFγ and PTFδ were also found to interact physically with TATA-binding protein (TBP) in vitro (27).

Several laboratories have independently reported other PSE-binding activities, including PSE-binding protein 1 (3, 10), PSE-binding protein (23, 25), and snRNA-activating protein complex (SNAPc) (5, 20). Because of similar DNA-binding activities, transcriptional activities, and apparent subunit compositions for PTF and SNAPc, including identities of PTFγ and PTFδ with SNAP43 and SNAP45, respectively (5, 19, 27), it is clear that PTF and SNAPc are closely related. The major difference is that SNAPc was described as a TBP-containing complex analogous to TFIIIB, TFIID, and SL1 (5, 20), whereas TBP was not detected either in highly purified PTF or in complete PTF complexes immunoprecipitated by anti-PTFδ antibodies at 400 mM KCl (27). The results are consistent with the ability of either free TBP or SNAPc, but not PTF, to restore U1 transcription to TBP-depleted extracts (20, 27). However, since TBP was evidently substoichiometric in the SNAPc preparations (5), as well as in low-salt anti-PTFδ immunoprecipitates from nuclear extracts (27), the description of SNAPc as an analog of other stable TBP–TBP-associated factor (TAF) complexes may be inappropriate. Moreover, other studies have also implicated the 90-kDa subunit of TFIIIB, which interacts strongly with TBP, in transcription of class III snRNA genes, most likely in a complex distinct from the conventional TFIIIB complex (22, 24). Hence, the nature of interactions between PTF, TBP, and TFIIIB90, mediated in part by known TBP interactions both with TFIIIB90 and with PTFγ/SNAP43 and PTFδ/SNAP45, and the relationship of these interactions to SNAPc remain important questions. Here

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we report the molecular cloning and characterization of PTFβ and further document the composition of native PTF and the nature of interacting proteins.

MATERIALS AND METHODS

HeLa cell extracts and factors. HeLa cells were maintained in Joklik minimal essential medium with 5% bovine calf serum. Nuclear extracts were prepared as described previously (2). The partially purified PTF used in transcription, electrophoretic mobility shift assay (EMSA), and immunoprecipitation analyses was the Q-Sepharose fraction described previously (27).

Plasmids. O^+P^+ and O^+P^- 7SK maxigene templates were described previously (15). The pU1*G^- and pU2*G^- plasmids (generously provided by N. Hernandez) were described by Sadowski et al. (20). The 5GpMLG$^-$ plasmid (generously provided by D. K. Lee) contains five Gal4 binding sites and the adenovirus major late promoter fused to a G-less cassette.

Cloning of cDNA encoding PTFβ. Human PTF was purified as described previously (26) and separated on a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel. Resolved polypeptides were transferred to a polyvinylidene difluoride membrane (Millipore). The band corresponding to PTFβ was excised and digested with the endoproteinase Lys-C. After separation of derived peptides by high-performance liquid chromatography, amino acid sequences for three peptides for PTFβ subunit were determined at the Rockefeller University sequencing facility. On the basis of peptide sequences shown in Fig. 1, two degenerate oligonucleotide probes were designed for initial screening of a human B-cell cDNA library (1). The probes used were GARGATGGIGARGATCCIGARG TIATICCIGAIAACAC and CTIGGIGARTTYCTIGCITACCCITATGTIGA YCCIGGIACITTCAAC. DNA fragments corresponding to the 5′ regions of the cDNA inserts of the positive clones were generated by PCR. The DNA fragments were then used as probes for subsequent screening of human B-cell and HeLa cDNA libraries. Final positive clones were excised in vivo in the presence of helper phages and transferred into pBluescript SK (Stratagene) or pEXlox (Novagen) vectors as instructed by manufacturers. The nucleotide sequences of the clones were determined by double-stranded DNA sequencing using Sequenase 2.0 (U.S. Biochemical).

Antigen and antibody preparation. cDNAs encoding PTFβ were subcloned into pRSET (Invitrogen) vectors to produce His-tagged recombinant PTFβ. The recombinant partial and full-length PTFβs contain, respectively, 32 and 35 additional N-terminal amino acids that are encoded by the vectors. The constructs were introduced into *Escherichia coli* BL21(DE3)pLysS (21). After isopropyl-β-D-thiogalactopyranoside (IPTG) induction, recombinant proteins were purified by nitrilotriacetic acid-resin (Qiagen) affinity chromatography under denaturing conditions and subsequently by electroelution following SDS-polyacrylamide gel electrophoresis (PAGE).

To prepare antiserum, New Zealand White rabbits were injected intradermally with 500 μg of antigen emulsified with an equal volume of Freund's complete adjuvant. Subsequently the rabbits were boosted subcutaneously every 4 weeks with 250 μg of antigen emulsified with an equal volume of Freund's incomplete adjuvant. Blood was collected from dorsal ear veins 10 days after each boost.

The antigens were cross-linked to cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. They were then used to purify corresponding antisera as described previously (4).

In vitro translation. In vitro transcription and translation of PTFβ cDNA clones were performed with TNT lysate (Promega) according to the manufacturer's instructions. To increase the translational efficiency, the second nucleotide upstream of the first ATG (position 10) in the PTFβ cDNA clone was changed from A to C.

EMSA. The O^+P^+ and O^+P^- probes for EMSA were prepared by end labeling the *Hin*dIII restriction fragments of O^+P^+ and O^+P^- plasmids with the Klenow fragment of *E. coli* DNA pol I and [α-^{32}P]dATP. Each 20-μl reaction mixture contained 20 fmol of probe, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 70 to 80 mM KCl, 4% Ficoll 400, 1 mM spermidine, 2 μg of poly(dI-dC) · poly(dI-dC), 1 mM dithiothreitol (DTT), 0.03% Nonidet P-40 (NP-40), and 100 μg of bovine serum albumin (BSA) per ml. The binding reaction was initiated by the addition of protein, and the mixture was incubated for 20 min at 30°C. One microliter of antiserum or purified antibody was then added to the reaction mixture, which was further incubated for 10 min. The reaction mixture was loaded directly onto a 4% (37.5:1, acrylamide/bisacrylamide) nondenaturing polyacrylamide gel with 0.5× TBE (0.045 M Tris-borate, 0.001 M EDTA [pH 8.0]) containing 0.03% NP-40 and run at 150 V for 2 h at room temperature.

Immunoblot analysis. Unless stated otherwise, immunoblot analyses with various antibodies were performed with Amersham's enhanced chemiluminescence system according to the manufacturer's instructions.

In the experiments shown in Fig. 7, immunoblot analysis was performed with the Vistra chemifluorescence system from Amersham and Molecular Dynamics according to the manufacturers' instructions. After incubation with the substrate, the membranes were scanned with the Storm Image system from Molecular Dynamics to quantitate the chemifluorescent signal. Anti-PTFβ and anti-PTFδ antibodies, raised against His-tagged proteins, were first incubated with nitrocellulose coupled with an unrelated recombinant His-tagged protein (generously provided by Y. Tao) to eliminate anti-His tag antibodies. The anti-TBP antibodies were purified with nitrocellulose coupled with glutathione *S*-transferase–TBP (generously provided by Y. Tao).

Immunodepletion of HeLa nuclear extracts with anti-PTFβ antibodies. A 300-μl aliquot of protein A-Sepharose beads (Pharmacia) was incubated with 300 μg of antigen-purified antibodies against partial-length PTFβ antibodies for 2 h at 4°C with constant mixing. After washes with buffer BC (20 mM Tris-HCl [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT) containing 0.03% NP-40 and 100 mM KCl (BC100) and then BC400, the beads were incubated with 1 ml of BC400 containing 0.25 mg of BSA per ml at 4°C for 3 h. The beads were then washed with BC400 and incubated for 9 h at 4°C with 300 μl of HeLa nuclear extract adjusted to 400 mM KCl–5 mM DTT. After centrifugation, the supernatant was incubated for 2 h at 4°C with 150 μl of protein A-Sepharose beads, which had been preincubated with BC400 with 0.25 mg of BSA per ml, to remove any residual immunoglobulins. The supernatant was dialyzed against BC100, and aliquots were frozen.

In vitro transcription. Transcription reactions for adenovirus VA1 and human 7SK genes were performed in a 25-μl reaction mixture with 2.5 μl of HeLa nuclear extract and with 25 ng of VA1 and 0.9 μg of 7SK supercoiled DNA templates. The reaction mixture also contained 10 mM HEPES-KOH (pH 7.9), 5 mM $MgCl_2$, 5 mM creatine phosphate, 70 mM KCl, 1 mM DTT, 500 μM each ATP, CTP, and UTP, 25 μM GTP, 2.5 μCi of [α-^{32}P]GTP (3,000 Ci/mmol), and 2 μg of α-amanitin per ml. After a 1.5-h incubation at 30°C, 125 μl of stop mix containing 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1.1 M ammonium acetate, and 100 μg of yeast tRNA per ml was added. The mixture was extracted with phenol-chloroform and precipitated with ethanol. The precipitates were resuspended in 90% formamide–10 mM EDTA–0.1% bromophenol blue–0.1% xylene cyanol and analyzed on an 8% polyacrylamide denaturing gel.

Transcription reaction mixtures for pU1*G^-, pU2*G^-, and 5GpMLG$^-$ templates (25 μl) contained 9 μl of HeLa nuclear extract, 50 ng of 5GpMLG$^-$, and 1.5 μg of either pU1*G^- or pU2*G^- supercoiled DNA template. The reaction mixtures also contained 10 mM HEPES-KOH (pH 7.9), 2% polyethylene glycol 8000, 3.5 mM $MgCl_2$, 70 mM KCl, 5 mM DTT, 1.2 mM 3′-*O*-methyl-GTP, 1 mM spermidine, 3 U of RNase T_1 (Calbiochem), 20 U of RNasin (Promega), 0.4 mM ATP, 0.4 mM CTP, and 0.26 μM [α-^{32}P]UTP (20 μCi). After 2 h of incubation at 30°C, the mixture was extracted and analyzed on a 5% denaturing polyacrylamide gel as described above.

Immunoprecipitation from HeLa nuclear extracts with anti-PTFβ antibodies. One milligram of antigen-purified anti-PTFβ antibodies was first bound to 0.5 ml of protein A-Sepharose beads (Pharmacia) and then covalently cross-linked to the beads with dimethyl pimelimidate as described previously (4). As a control, preimmune immunoglobulin G was purified from preimmune serum with protein A-Sepharose beads as described previously (4). In the experiments shown in Fig. 6, 50 μl of antibody-coupled beads was incubated for 2 h at 4°C with 1 ml of HeLa nuclear extract in buffer BC (20 mM Tris-HCl [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT) containing 100 mM KCl–0.05% NP-40, 200 mM KCl–0.05% NP-40, 300 mM KCl–0.05% NP-40, or 300 mM KCl–0.1% NP-40. After the beads were washed extensively with the same buffer, the bound proteins were eluted twice with 50 μl of 0.1 M glycine (pH 2.5). The eluted proteins were pooled and neutralized with 10 μl of 1.5 M Tris-HCl (pH 8.8), and aliquots were analyzed by immunoblotting. For the experiments shown in Fig. 7, the immunoprecipitation reaction was scaled up to use 10 ml of HeLa nuclear extract. The eluate was precipitated with 10% trichloroacetic acid and resuspended in 250 μl of 1× SDS loading buffer.

RESULTS

Cloning of cDNA encoding PTFβ. PTF was purified from HeLa S100 extracts (5 liters, 100 g of protein) as described previously (26), and individual polypeptides were transferred to a membrane after separation by SDS-PAGE. The polypeptide corresponding to PTFβ was subjected to peptide microsequencing. Three peptide sequences were obtained, and two of them were used to design degenerate oligonucleotide probes to screen a human cDNA library (Fig. 1A). The initial positive cDNA clones were used as PCR templates to prepare DNA probes for further library screening. The longest cDNA clone obtained had an insert of 2.5 kb with an open reading frame of 411 amino acids and a calculated molecular mass of 46.75 kDa (Fig. 1A). The predicted amino acid sequence contains all three PTFβ peptide sequences obtained via microsequencing.

The deduced open reading frame of PTFβ is generally acidic, with an isoelectric point of 4.95. There are potential leucine zipper and/or zinc finger motifs in the carboxyl-terminal region. Database searches revealed that PTFβ is a novel protein. It has a weak sequence relationship with a deduced

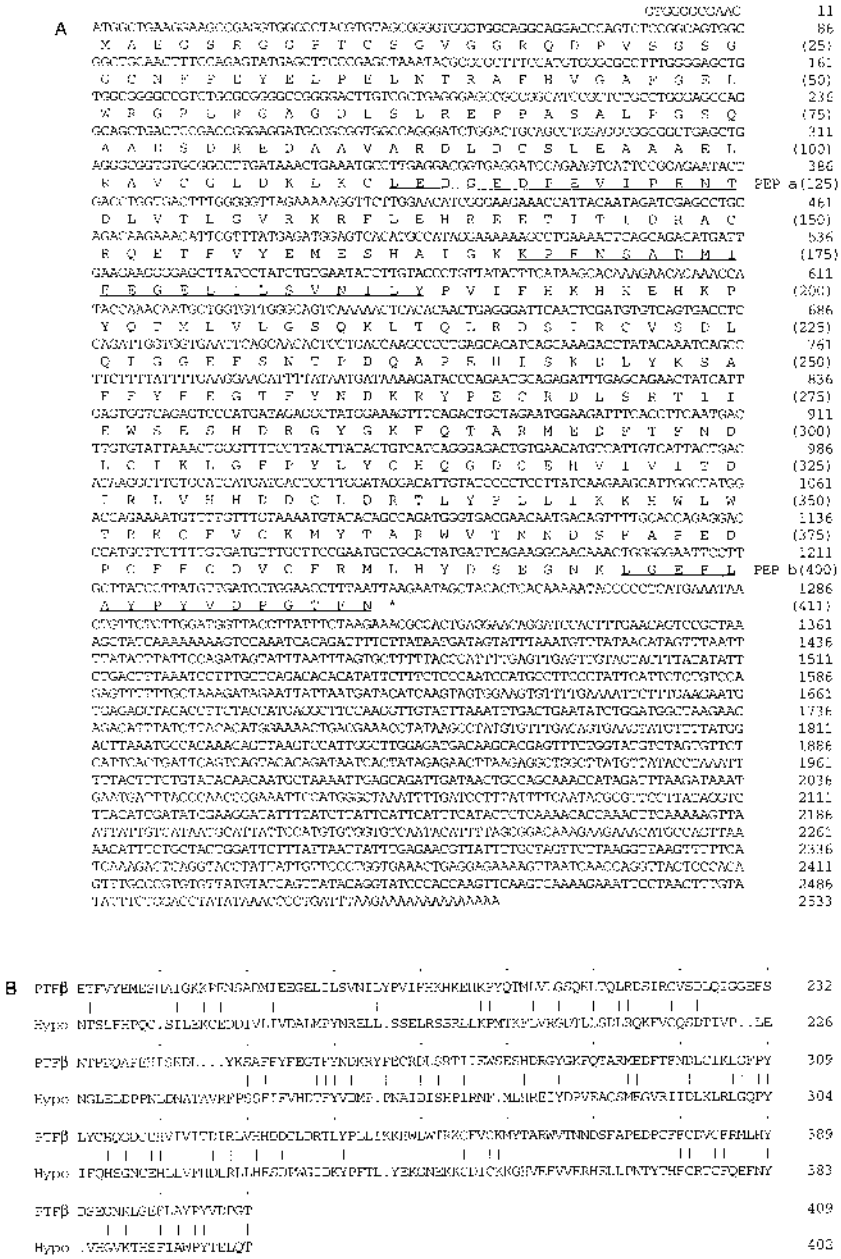

FIG. 1. Sequence of PTFβ. (A) cDNA and deduced amino acid sequences of PTFβ. Peptide sequences obtained from microsequencing of the purified protein are underlined. Peptides a and b are the peptide sequences used to design probes for screening a human cDNA library to obtain the initial partial clone. The regions between amino acids 312 and 334 and between amino acids 354 and 383 represent potential zinc finger motifs, while the region between amino acids 321 and 356 represents a potential leucine zipper motif. (B) Sequence comparison between human PTFβ and a *C. elegans* hypothetical protein (GenBank accession number Z35603). Amino acids 153 to 409 of PTFβ are aligned with amino acids 151 to 402 of the *C. elegans* hypothetical protein (Hypo). Lines represent sequence identity.

Caenorhabditis elegans protein (Fig. 1B). The hypothetical *C. elegans* protein contains 418 amino acids, with a calculated molecular mass of 48.9 kDa and an isoelectric point of 4.99. Approximately 250 amino acids at the carboxyl-terminal regions of the two sequences have a similarity of 53.0% and an identity of 28.1%. The significance of this relationship is not known.

Antibodies raised against recombinant PTFβ recognize native PTFβ. In immunoblot assays, affinity-purified antibodies raised against recombinant PTFβ expressed in bacteria reacted strongly with a 55-kDa peptide in both a HeLa nuclear extract (Fig. 2, lane 3) and a partially purified PTF fraction (see Fig. 6A, lane 1, and Fig. 7A, lane 1). Since the apparent size of PTFβ in purified native PTF (55 kDa) is larger than the calculated molecular mass of the cloned PTFβ (46.75 kDa), the PTFβ cDNA was transcribed and translated in rabbit reticulocyte lysates. In an immunoblot analysis (Fig. 2), recombinant in vitro-translated PTFβ showed the same mobility in SDS-PAGE as native PTFβ, indicating that the cDNA clone encodes the full-length PTFβ.

To confirm the presence of the natural counterpart to the cloned PTFβ within the specific PTF-PSE complex, the effect

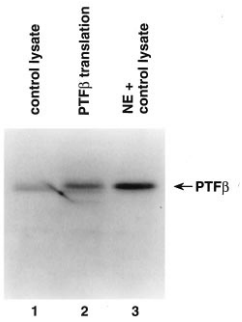

FIG. 2. Recombinant PTFβ and the native PTFβ have the same mobilities in SDS-PAGE. In vitro translation of the PTFβ clone was performed as described in Materials and Methods. One microliter of control in vitro translation reaction mixture (lane 1), 1 μl of in vitro translation reaction mixture for the PTFβ cDNA clone (lane 2), and 1 μl of HeLa nuclear extract (NE) mixed with 1 μl control in vitro translation reaction mixture (lane 3) were subjected to SDS-PAGE on a 10% polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane, and immunoblotting was performed with antigen-purified anti-PTFβ antibodies.

of anti-PTFβ antibodies on an Oct-1–PTF–DNA complex was tested by EMSA. As shown in Fig. 3, incubation of PTF and Oct-1 with a DNA probe containing both the 7SK PSE and an adjacent octamer element resulted in an Oct-1–PTF–DNA supershift complex in addition to the Oct-1–DNA complex (lane 8). Mutation of the PSE element in the probe eliminated the supershift complex but not the Oct-1–DNA complex (lane 4), indicating that the formation of the supershift complex is

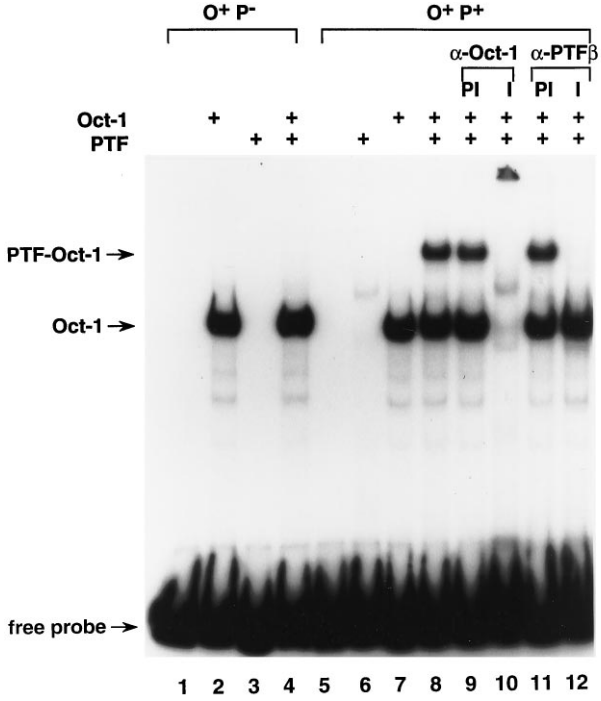

FIG. 3. PTFβ is present in the Oct-1–PTF–DNA complex. EMSA was performed with partially purified PTF (1 μl) and partially purified Oct-1 (1 μl), using end-labeled *Hin*dIII restriction fragments of O^+P^+ or O^+P^- as the probe. Preimmune (PI) and immune (I) sera (1 μl) against Oct-1 (lanes 9 and 10) and purified preimmune (PI) and immune (I) antibodies (1 μl) against PTFβ (lanes 11 and 12) were incubated with the reaction mixture after sufficient time for complex formation. The arrows indicate the positions of Oct-1–DNA and Oct-1–PTF–DNA complexes.

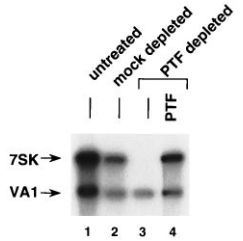

FIG. 4. PTFβ is required for activation of the human 7SK gene. In vitro transcription of adenovirus VA1 and human 7SK genes was performed with untreated HeLa nuclear extract (lane 1), mock-depleted HeLa nuclear extract (lane 2), PTFβ-depleted HeLa nuclear extract (lane 3), or PTFβ-depleted HeLa nuclear extract supplemented with 1 μl of partially purified PTF (lane 4). Each reaction mixture contained 0.9 μg of 7SK and 25 ng of VA1 supercoiled DNA templates. The positions of 7SK and VA1 transcripts are indicated.

PSE dependent. Immune serum against Oct-1 (lane 10), but not preimmune serum (lane 9), eliminated both Oct-1–DNA and Oct-1–PTF–DNA complexes and resulted, instead, in the formation of an antibody-dependent supershift complex at the top of the gel, confirming that both complexes contained Oct-1. Anti-PTFβ antibodies (lane 12), but not preimmune antibodies (lane 11), abolished preformed Oct-1–PTF–DNA complexes but did not affect preformed Oct-1–DNA complexes. These results indicate that the cloned cDNA encodes a polypeptide which corresponds to the native β subunit of PTF.

PTFβ is essential for transcription of both class II and class III snRNA genes. Immunodepletion assays were used to test the requirement of PTFβ for transcription of the two classes of snRNA genes. HeLa nuclear extracts were incubated with protein A-Sepharose beads coated with purified anti-PTFβ antibodies in order to deplete endogenous PTFβ. As shown in Fig. 4, immunodepletion of PTFβ specifically diminished transcription of the 7SK gene but did not affect transcription of the VA1 gene, which is also transcribed by pol III (lanes 2 and 3). The addition of partially purified PTF to the PTF-depleted extract restored transcription of the 7SK gene but had no obvious effect on transcription of the VA1 gene (lane 4).

In the case of pol II transcription, depletion of PTFβ from nuclear extracts specifically diminished transcription of both the U1 (Fig. 5A, lanes 2 and 3) and U2 (Fig. 5B, lanes 2 and 3) genes but did not affect transcription of the adenovirus major late promoter. Addition of partially purified PTF to the PTF-depleted extracts restored transcription of the U1 (Fig. 5A, lane 4) and U2 (Fig. 5B, lane 4) genes, again with no obvious effect on transcription of the major late promoter. These results indicate that PTFβ is specifically required for transcription of both classes of snRNA genes.

Equimolar amounts of TBP and TFIIIB90 can loosely associate with PTF. As discussed earlier, studies of the interactions and relationships between PTF, TBP, and TFIIIB90 are critical both for understanding the function of these components in U6/7SK transcription and for resolving the reported differences between PTF and SNAPc. Toward this objective, we immunoprecipitated nuclear extracts with antigen-purified anti-PTFβ antibodies under four conditions. The bound proteins were eluted and analyzed by immunoblot assays after SDS-PAGE. Specificity was evident from coimmunoprecipitation by immune but not preimmune antibodies. The amounts of immunoprecipitated PTFβ and PTFδ were essentially unchanged at all conditions (Fig. 6A), confirming previous indications (26, 27) that PTF is a highly stable multisubunit complex. In the cases of TFIIIB90 and TBP (Fig. 6B), high levels of each were immunoprecipitated at 100 mM KCl–0.05% NP-40 (lanes 2

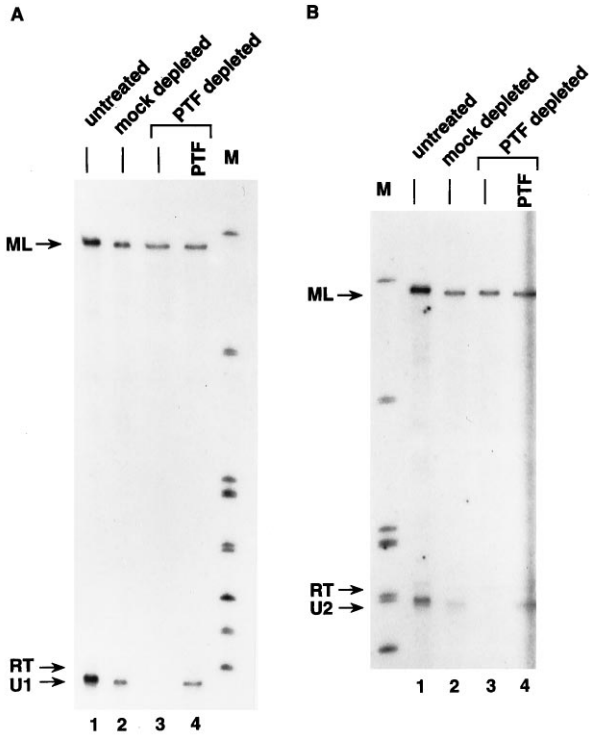

FIG. 5. PTFβ is required for activation of human U1 and U2 genes. (A) PTFβ is required for activation of a human U1 gene. In vitro transcription reactions were performed with untreated HeLa nuclear extract (lane 1), mock-depleted nuclear extract (lane 2), PTFβ-depleted HeLa nuclear extract (lane 3), or PTFβ-depleted HeLa nuclear extract supplemented with 1 μl of partially purified PTF (lane 4). Each reaction mixture contained 1.5 μg of pU1*G$^-$ and 50 ng of 5GpMLG$^-$ supercoiled DNA templates. (B) PTFβ is required for activation of a human U2 gene. In vitro transcription reactions were performed with untreated HeLa nuclear extract (lane 1), mock-depleted nuclear extract (lane 2), PTFβ-depleted HeLa nuclear extract (lane 3), or PTFβ-depleted HeLa nuclear extract supplemented with 1 μl of partially purified PTF (lane 4). Each reaction mixture contained 1.5 μg of pU2*G$^-$ and 50 ng of 5GpMLG$^-$ supercoiled DNA templates. The positions of readthrough (RT) and correctly initiated U1 and U2 promoter transcripts and the major later (ML) promoter transcripts are indicated. Lanes M, size markers.

and 3). These levels were greatly reduced, by similar proportions, at 200 mM KCl–0.05% NP-40 (lanes 4 and 5). When the KCl concentration was increased to 300 mM, almost no TBP or TFIIIB90 could be detected in the immunoprecipitates (lanes 6 to 9). These results indicate that TFIIIB90 and TBP associate with PTF only through weak interactions that are easily disrupted at higher salt concentrations.

We next quantitated the levels of coimmunoprecipitated PTFβ, PTFδ, TBP, and TFIIIB90 at 100 mM KCl–0.05% NP-40 by immunoblot analysis. Figure 7A shows an immunoblot analysis for quantitation of PTFβ and PTFδ. Three different amounts of immunoprecipitated sample were analyzed (lanes 2 to 4), along with variable amounts of a mixture containing equimolar concentrations of His-tagged PTFβ and His-tagged PTFδ as standards (lanes 5 to 13). As a control, partially purified native PTF was also included (lane 1). Similarly, Fig. 7C shows an immunoblot analysis for quantitation of TBP and TFIIIB90. The immunoprecipitated sample (lane 2) was analyzed along with variable amounts of a mixture containing equimolar concentrations of His-tagged TBP (9) and Flag-tagged TFIIIB90 (24) (lanes 3 to 11). As a control, a highly purified glycerol gradient fraction of TFIIIB (24) was included in lane 1. Results from the recombinant PTFβ-PTFδ (Fig. 7B) and recombinant TBP-TFIIIB90 (Fig. 7D) protein analyses were used to plot standard curves of the amounts of the respective proteins versus the intensities of the Western blot (immunoblot) signals; the latter were quantitated by the Storm Image system and are plotted as relative fluorescent units (rfu). On the basis of these standard curves, the amounts of corresponding native proteins in the samples were determined.

The standard PTF fraction (Fig. 7A, lane 1) contained 5.4 nM PTFβ and 5.7 nM PTFδ, while the standard TFIIIB fraction (Fig. 7B, lane 1) contained 2.8 nM TFIIIB90 and 2.7 nM TBP. For each complex, the calculated molar ratio of the two components analyzed was roughly 1:1, as expected, indicating the validity of the approach. The anti-PTFβ antibody-immunoprecipitated sample, analyzed in Fig. 7, contained 10 nM PTFβ, 5.0 nM PTFδ (Fig. 7A, lanes 3 and 4; lane 2 was not considered because its PTFδ signal fell outside the range of the standard curve), 3.9 nM TBP, and 3.4 nM TFIIIB90 (Fig. 7C, lane 2). Thus, the PTFβ/PTFδ/TBP/TFIIIB90 molar ratio in the immunoprecipitated sample was roughly 1:0.5:0.39:0.34. The higher molar amount of PTFβ than of other polypeptides most likely reflects dissociation of some of the PTF complexes by the polyclonal anti-PTFβ antibodies used in the immunoprecipitation. This idea is consistent with the EMSA data in Fig. 3 demonstrating dissociation of the PTF–Oct-1–DNA complex by anti-PTFβ antibodies. These data indicate that substantial and similar molar amounts of TBP and TFIIIB90 can associate with PTF under low-salt conditions.

DISCUSSION

The PSE is an essential element for transcription of mammalian snRNA genes. PTF specifically recognizes the PSE, interacts with upstream activators (such as Oct-1) to activate basal transcription, and assists in type-specific RNA polymerase selection by interacting with both pol II and pol III transcription factors. To study PTF function and mechanisms of snRNA gene transcription, we have undertaken the molecular cloning and characterization of PTF subunits. The cloning and characterization of the δ and γ subunits of PTF (27), equivalent to SNAP45 and SNAP43, respectively (5, 19), were reported previously. In this report, we present related studies of

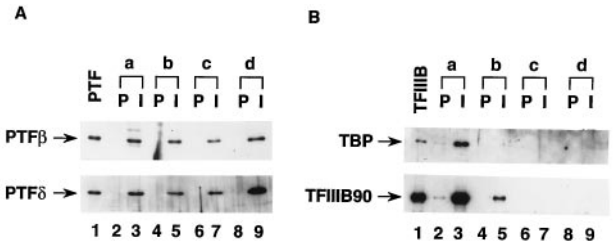

FIG. 6. Association of TBP and TFIIIB90 with PTF. Immunoprecipitations from HeLa nuclear extract by both preimmune (PI) and immune (I) anti-PTFβ antibodies were performed as described in Materials and Methods under four conditions: 100 mM KCl–0.05% NP-40 (a), 200 mM KCl–0.05% NP-40 (b), 300 mM KCl–0.05% NP-40 (c), and 300 mM KCl–0.1% NP-40 (d). Five-microliter aliquots of the 0.1 M glycine eluate from each immunoprecipitate (A, lanes 2 to 9; B, lanes 2 to 9) were analyzed by electrophoresis on an SDS–8% polyacrylamide gel, transferred to nitrocellulose membranes, and subjected to immunoblot analysis. (A) Immunoprecipitation of PTFβ and PTFδ by anti-PTFβ antibodies. Five microliters of partially purified PTF was loaded in lane 1. (B) Immunoprecipitation of TBP and TFIIIB90 by anti-PTFβ antibodies. Five microliters of purified TFIIIB was loaded in lane 1. The arrows indicate the positions of TBP and TFIIIB90. In the upper and lower portions of both panels, aliquots of the same immunoprecipitate were subjected to electrophoresis in separate gels and blotted with the indicated antibodies.

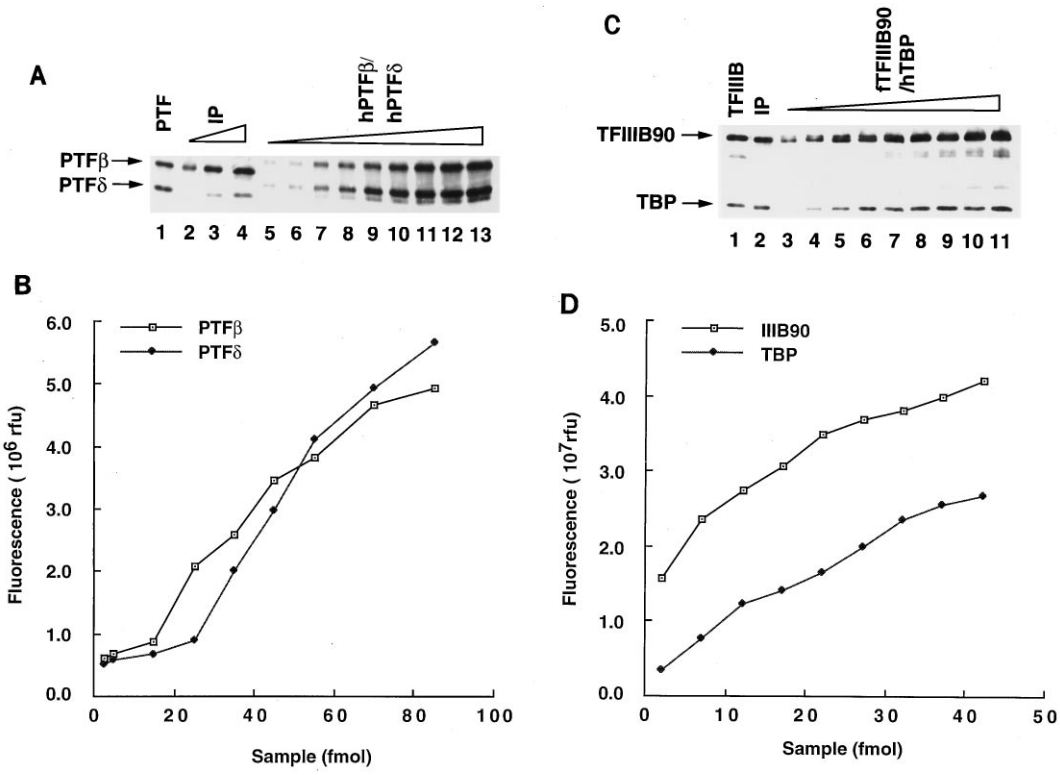

FIG. 7. Quantitation of PTFβ, PTFδ, TBP, and TFIIIB90 coimmunoprecipitated by anti-PTFβ antibodies at 100 mM KCl–0.05% NP-40. Immunoprecipitation from HeLa nuclear extract by anti-PTFβ antibodies was performed as described in Materials and Methods. Immunoprecipitated samples, native protein fractions, and recombinant protein standards were subjected to immunoblot analysis using the Vistra chemifluorescence system and the Storm Imaging system. (A) Immunoblot analysis to quantitate PTFβ and PTFδ. The samples analyzed were 7 μl of partially purified PTF (lane 1); 1, 3, and 6 μl (lanes 2 to 4) of immunoprecipitation sample (IP); and 0.5, 1, 3, 5, 7, 9, 11, 14, and 17 μl (lane 5 to 13) of a mixture containing 5 nM recombinant full-length His-tagged PTFβ (hPTFβ) and 5 nM His-tagged PTFδ (hPTFδ). Immunoblotting was performed with a mixture of anti-PTFβ antibodies and anti-PTFδ antibodies. (B) Standard curves for the amounts of recombinant PTFβ and PTFδ versus Western blot signal intensities quantitated by the Storm Imaging system. The 7-μl PTF sample and the 1-, 3-, and 6-μl immunoprecipitation samples from panel A gave PTFβ and PTFδ signals, respectively, of 2.7 and 2.4; 0.97 and 0.35; 2.4 and 0.65; and 4.5 and 1.6 (10^6 rfu). (C) Immunoblot analysis to quantitate TBP and TFIIIB90. The samples analyzed were 8 μl of highly purified native TFIIIB fraction (lane 1); 5 μl of immunoprecipitation sample (lane 2); and 1, 3, 5, 7, 9, 11, 13, 15, and 17 μl (lanes 3 to 11) of a mixture containing 2.5 nM His-tagged TBP (hTBP) and 2.5 nM Flag-tagged TFIIIB90 (fTFIIIB90). Immunoblotting was performed with a mixture of anti-TBP antibodies and anti-TFIIIB90 antibodies. (D) Standard curves for the amounts of recombinant TBP and TFIIIB90 versus Western blot signal intensities quantitated by the Storm Imaging system. The 8-μl TFIIIB sample from panel C gave signals of 3.5 and 1.6 (10^7 rfu) for TFIIIB90 and TBP, respectively, while the 5-μl immunoprecipitation sample gave corresponding signals of 2.9 and 1.5 (10^7 rfu), respectively. The concentrations of stock solutions of recombinant PTFβ, PTFδ, TBP, and TFIIIB90 were measured by Coomassie blue staining using BSA as standard following SDS-PAGE.

PTFβ and further document interactions of functionally relevant general factors with PTF.

Cloning of cDNA encoding PTFβ. As described previously (26), human PTF was purified to near homogeneity from HeLa cell extracts. Peptide sequences were obtained and used to clone a cDNA corresponding to the 55-kDa subunit of PTF. A number of independent cDNA clones were isolated for PTFβ, and all were derived from a single mRNA species. All three peptide sequences obtained from the 55-kDa protein band were found within the predicted amino acid sequence based on PTFβ cDNA, indicating that the 55-kDa band in purified PTF represents only a single polypeptide.

Database searches revealed that PTFβ is a novel polypeptide with a weak sequence relationship with a hypothetical *C. elegans* protein of similar peptide length, molecular mass, and isoelectric point. It is possible that the hypothetical *C. elegans* protein represents the *C. elegans* counterpart of human PTFβ. No other significant sequence relationship was found with other known peptide sequences. Because there are many patches of hydrophobic amino acids in PTFβ, and because antibodies against PTFβ can disrupt the Oct-1–PTF–DNA complex, it may be speculated that PTFβ occupies a central position within the PTF complex. There are potential zinc finger and leucine zipper motifs in the carboxyl-terminal region, indicating that PTFβ may interact with DNA, although our previous UV cross-linking experiments (26) suggested that PTFα is the primary PSE-binding subunit within the PTF complex.

Antibodies raised against bacterially expressed recombinant PTFβ recognize native PTFβ in HeLa nuclear extracts, in purified PTF fractions, and in the preformed Oct-1–PTF–DNA complex. Depletion of PTFβ from HeLa nuclear extracts diminished both pol II and pol III transcription of snRNA genes, and the addition of partially purified PTF restored transcription. These results confirm that PTFβ is required for transcription of both class II and class III snRNA genes and that cloned PTFβ corresponds to a component of the native PTF as previously defined.

Composition of PTF and its association with TBP and TFIIIB90. Our past and present studies indicate that the α, β, γ, and δ subunits of PTF copurify through various chromatographic steps, are present in the most purified fraction in similar molar amounts, remain together in an Oct-1–PTF–DNA complex, and are coimmunoprecipitated by either anti-PTFβ or anti-PTFδ antibodies at high-salt conditions. Altogether, these studies demonstrate that these four polypeptides are

tightly associated, genuine subunits of PTF. Consistent with the observations that recombinant TBP can interact physically with recombinant PTFγ (SNAP43) and PTFδ (SNAP45) in vitro (5, 19, 27) and that SNAPc preparations contain substoichiometric amounts of TBP (5), we show here that very substantial (and potentially stoichiometric) amounts of TBP and TFIIIB90 may associate with PTF under low-salt (100 mM KCl) conditions in nuclear extracts. However, these associations are weak and dramatically reduced at higher salt concentrations. Furthermore, TBP and TFIIIB90 are coimmunoprecipitated in equimolar amounts under the low-salt condition, and both are proportionally dissociated from PTF at the higher salt concentrations. Given the previous demonstration of a tight association (resistant to at least 1 M KCl) between TFIIIB90 and TBP in natural or core TFIIIB complexes (24), these results suggest that most of the TBP associated with PTF is not in a free form but rather is in a TFIIIB90-containing complex. Therefore, free TBP is apparently not a genuine subunit of PTF or the related SNAPc. It also seems inappropriate to consider either SNAPc or the low-salt-isolated PTF complex as conventional TBP-TAF complexes because they do not show both the stoichiometric and the highly stable subunit interactions characteristic of well-established TBP-TAF complexes such as SL1, TFIID, and TFIIIB. It is more likely that the previously described SNAPc preparations represent mixtures of PTF and substoichiometric amounts of an interacting TFIIIB-like TBP-TAF complex containing TBP, TFIIIB90, and perhaps other components. The discrepancy between the highly purified PTF and SNAPc preparations may be attributed to the different procedures for preparation of cell extracts and fractionation of protein samples.

Since PTF is involved in transcription not only of U6 and 7SK genes by RNA pol III but also of U1 to U5 genes by RNA pol II, our finding of anti-PTFβ antibody-precipitated complexes with near stoichiometric amounts of PTF subunits and TFIIIB-related components is somewhat surprising. However, for the following reasons, the present data are not incompatible with dual PTF functions and the existence of analogous PTF-accessory factor complexes specific for the U1 to U5 genes. First, the nuclear extracts from which the PTF-TBP-TFIIIB90 complexes were immunoprecipitated are far more active for U6/7SK transcription than for U1/U2 transcription. This finding suggests that PTF-interacting factors involved in pol II transcription may not be efficiently retained in or extracted from isolated nuclei or that they may not be stable during extract preparation (thus allowing preferential interactions of TFIIIB-related components with PTF). Second, while the data presented are reproducible and representative, the quantitative measurements may not be sufficiently accurate (because of possible systematic errors) to exclude a subpopulation of complexes containing PTF and factors specific for U1 to U5 genes. More definitive studies on this point await the identification of such factors and a more quantitative analysis of PTF-containing complexes.

Mechanism of RNA pol III transcription of snRNA genes. As discussed previously (27), an important issue regarding the mechanism of RNA pol III transcription of snRNA genes is whether, as proposed by Sadowski and colleagues (20), the presumed SNAPc complex binds to and functions through the PSE while another molecule of TBP binds to and functions at the TATA box (thereby involving two TBP molecules), or whether, as we have suggested (27), PTF and a distinct TFIIIB-like complex, consisting of TBP, TFIIIB90, and possibly other components (24), have the potential to interact separately with the two promoter elements. Our past and current observations strongly support the later model. The weak associations between PTF and the presumptive TFIIIB-like complex (tentatively designated TFIIIB′), presumably mediated, at least in part, by interactions of TBP with PTFγ and PTFδ (5, 19, 27), could have a synergistic effect on their binding to the PSE and the TATA box and thus facilitate the assembly of the complete preinitiation complex. At the present time, it remains an interesting possibility that a PTF-TFIIIB′ complex exists in vivo in the absence of DNA binding and that such a complex is a natural or obligate precursor for formation of a functional preinitiation complex on class III snRNA promoters. More definitive studies on these questions await the further identification and purification of the complete set of factors involved in snRNA gene transcription.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1993. Current protocols in molecular biology. Greene Publishing Associates and Wiley Interscience, New York.
2. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
3. **Gunderson, S. I., M. W. Knuth, and R. R. Burgess.** 1990. The human U1 snRNA promoter correctly initiates transcription in vitro and is activated by PSE1. Genes Dev. **4:**2048–2060.
4. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
5. **Henry, R. W., C. L. Sadowski, R. Kobayashi, and N. Hernandez.** 1995. A TBP-TAF complex required for transcription of human snRNA genes by RNA polymerase II and III. Nature (London) **374:**653–656.
6. **Hernandez, N.** 1992. Transcription of vertebrate snRNA genes and related genes, p. 281–313. *In* S. McKnight and K. R. Yamamoto (ed.), Transcriptional regulation. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
7. **Hernandez, N.** 1993. TBP, a universal eukaryotic transcription factor? Genes Dev. **7:**1291–1308.
8. **Hernandez, N., and R. Lucito.** 1988. Elements required for transcription initiation of the human U2 snRNA gene coincide with elements required for snRNA 3′ end formation. EMBO J. **7:**3125–3134.
9. **Hoffmann, A., and R. G. Roeder.** 1991. Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. Nucleic Acids Res. **19:**6337–6338.
10. **Knuth, M. W., S. I. Gunderson, N. E. Thompson, L. A. Strasheim, and R. R. Burgess.** 1990. Purification and characterization of proximal sequence element-binding protein 1, a transcription activating protein related to Ku and TREF that binds the proximal sequence element of the human U1 promoter. J. Biol. Chem. **265:**17911–17920.
11. **Kunkel, G. T.** 1991. RNA polymerase III transcription of genes that lack internal control regions. Biochim. Biophys. Acta **1088:**1–9.
12. **Lobo, S. M., M. Tanaka, M. L. Sullivan, and N. Hernandez.** 1992. A TBP complex essential for transcription from TATA-less but not TATA-containing RNA polymerase III promoters is part of the TFIIIB fraction. Cell **71:**1029–1040.
13. **Mattaj, I. W., N. A. Dathan, H. D. Parry, P. Carbon, and A. Krol.** 1988. Changing the RNA polymerase specificity of U snRNA gene promoters. Cell **55:**435–442.
14. **Murphy, S., B. Moorefield, and T. Pieler.** 1989. Common mechanisms of promoter recognition by RNA polymerase II and III. Trends Genet. **5:**122–126.
15. **Murphy, S., J.-B. Yoon, T. Gerster, and R. G. Roeder.** 1992. Oct-1 and Oct-2 potentiate functional interaction of a transcription factor with the proximal sequence element of small nuclear RNA genes. Mol. Cell. Biol. **12:**3247–3261.
16. **Neuman de Vegvar, H. E., and J. E. Dahlberg.** 1989. Initiation and termination of human U1 transcription requires the concerted action of multiple flanking sequence elements. Nucleic Acids Res. **17:**9305–9318.

17. **Parry, H. D., and I. W. Mattaj.** 1990. Positive and negative functional interactions between promoter elements from different classes of RNA polymerase III-transcribed genes. EMBO J. **9:**1097–1104.
18. **Reddy, R., and R. Singh.** 1991. Synthesis of small nuclear RNAs. Prog. Mol. Subcell. Biol. **12:**1–36.
19. **Sadowski, C. L., R. W. Henry, R. Kobayahi, and N. Hernandez.** 1996. The SNAP45 subunit of the small nuclear RNA (snRNA) activating protein complex is required for RNA polymerase II and III snRNA gene transcription and interacts with the TATA box binding protein. Proc. Natl. Acad. Sci. USA **93:**4289–4293.
20. **Sadowski, C. L., R. W. Henry, S. M. Lobo, and N. Hernandez.** 1993. Targeting TBP to a non-TATA box cis-regulatory element; a TBP-containing complex activates transcription through the PSE. Genes Dev. **7:**1535–1548.
21. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
22. **Teichmann, M., and K. H. Seifart.** 1995. Physical separation of two different forms of human TFIIIB active in the transcription of the U6 or the VA1 gene in vitro. EMBO J. **14:**5974–5983.
23. **Waldschmidt, R., I. Wanandi, and K. H. Seifart.** 1991. Identification of transcription factors required for the expression of mammalian U6 genes in vitro. EMBO J. **10:**2595–2603.
24. **Wang, Z., and R. G. Roeder.** 1995. Structure and function of a human TFIIIB subunit that is evolutionarily conserved and contains both TFIIB and HMG2 related domains. Proc. Natl. Acad. Sci. USA **92:**7026–7030.
25. **Wannandi, I., R. Waldschmidt, and K. H. Seifart.** 1993. Mammalian transcription factor PBP. J. Biol. Chem. **268:**6629–6640.
26. **Yoon, J.-B., S. Murphy, L. Bai, Z. Wang, and R. G. Roeder.** 1995. Proximal sequence element-binding transcription factor (PTF) is a multisubunit complex required for transcription of both RNA polymerase II- and RNA polymerase III-dependent small nuclear RNA genes. Mol. Cell. Biol. **15:**2019–2027.
27. **Yoon, J.-B., and R. G. Roeder.** 1996. Cloning of two proximal sequence element-binding transcription factor subunits (γ and δ) that are required for transcription of small nuclear RNA genes by RNA polymerase II and III and interact with the TATA-binding protein. Mol. Cell. Biol. **16:**1–9.