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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 Polymerases II and III and Interact with the TATA-Binding Protein

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The proximal sequence element (PSE)-binding transcription factor (PTF) specifically recognizes the PSEs of both RNA polymerase II- and RNA polymerase III-transcribed small nuclear RNA (snRNA) genes. We previously have shown that PTF purified from human HeLa cells is a multisubunit complex of four polypeptides designated PTFα, -β, -γ, and -δ. We now report the isolation and expression of cDNAs encoding PTFγ and PTFδ, as well as functional studies with cognate antibodies that recognize the native PTF complex in HeLa extracts. Immunoprecipitation studies confirm that the four PTF subunits originally found to copurify during conventional chromatography indeed form a tightly associated complex; they further show that the PTF so defined, including the γ and δ subunits specifically, is essential for transcription of both class II and class III snRNA genes. Immunoprecipitation assays also show a weak substoichiometric association of the TATA-binding protein (TBP) with PTF, consistent with the previous report of a PTF-related complex (SNAPc) containing substoichiometric levels of TBP and a component (SNAPc43) identical in sequence to the PTFγ reported here. Glutathione *S*-transferase pulldown assays further indicate relatively strong direct interactions of both recombinant PTFγ and PTFδ with TBP, consistent either with the natural association of TBP with PTF in a semistable TBP–TBP-associated factor complex or with possible functional interactions between PSE-bound PTF and TATA-bound TBP during promoter activation. In addition, we show that in extracts depleted of TBP and TBP-associated factors, transcription from the U1 promoter is restored by recombinant TBP but not by TFIID or TFIIIB, indicating that transcription of class II snRNA genes requires a TBP complex different from the one used for mRNA-encoding genes.

In the nuclei of eukaryotic cells, DNA-dependent RNA polymerases I, II, and III (pol I, pol II, and pol III) direct transcription of different classes (I, II, and III) of genes with distinct promoter structures. Each RNA polymerase recognizes its target promoters via protein-protein interactions with a largely distinct set of basal transcription factors bound to core promoter elements. However, just as several factors binding to upstream regulatory elements have been implicated in transcription from more than one class of genes, so it also has been shown that a key component of the basal transcription factors is required for transcription by all three RNA polymerases (reviewed in references 11 and 16). Thus, the TATA-binding protein (TBP) participates in transcription by all three RNA polymerases by tightly associating with distinct groups of TBP-associated factors (TAFs) to form SL1, TFIID, and TFIIIB (6, 7, 10, 19, 20, 28, 37, 40, 44, 48), which are critical for transcription by pol I, pol II, and pol III, respectively.

Mammalian small nuclear RNA (snRNA) genes contain similar promoter structures, but some (class II) are transcribed by pol II whereas others (class III) are transcribed by pol III (reviewed in references 8, 15, 24, 31, and 34). This finding poses the interesting question as to how specific RNA polymerases are selected for transcription of the different classes of snRNA genes. Both classes of snRNA genes contain a distal sequence element (DSE) and a proximal sequence element (PSE) located near positions −220 and −60, respectively, relative to the start site. The DSE functions as an enhancer and is required for maximal transcription, whereas the PSE is essential for transcription and in class II snRNA genes localizes the transcription start site. The DSE and PSE are functionally interchangeable between the two classes of snRNA genes (26, 30, 33), indicating that the PSE per se is not responsible for polymerase selection. The major difference between promoters of the two classes is the presence of a TATA box at −25 only in class III snRNA genes. Interestingly, while the TATA box (e.g., adenovirus major late promoter) or the PSE (e.g., U1) can separately support pol II transcription, the combination of both elements determines pol III specificity in class III snRNA genes (e.g., U6) (25, 26, 30).

PSE-binding activities have been independently identified and characterized by several laboratories (22, 32, 36, 41). We earlier showed that the PSE is specifically recognized by the PSE-binding transcription factor (PTF) and that binding of PTF to the PSE is increased by Oct-1 bound to an adjacent octamer site (32). More recently we purified PTF to near homogeneity from HeLa cell extracts and showed that it activates transcription from both classes of snRNA genes in a PSE-dependent manner (46). Biochemical analyses demonstrated that human PTF is a stable complex of four polypeptides with apparent molecular masses of 180, 55, 45, and 44 kDa, which are called PTFα, -β, -γ, and -δ, respectively. In addition, UV cross-linking experiments indicated that PTFα is the major DNA-binding subunit of the complex. In addition to PTF, PSE-binding activities called PSE-binding protein (PBP) and snRNA-activating protein complex (SNAPc) have been

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implicated in transcription of class II and class III snRNA genes (4, 36, 41). Interestingly, SNAPc has been reported to be a TBP-TAF complex distinct from SL1, TFIID, and TFIIIB (36). However, our own studies have implicated a 90-kDa subunit (TFIIIB90) of human TFIIIB in transcription of snRNA genes by pol III, although it appears to function in a complex different from the TBP-containing TFIIIB complex involved in transcription of 5S, VAI, and tRNA genes by pol III (42). Hence, the exact involvement of TBP and associated or interacting factors in snRNA transcription remains an important question.

PTF is likely to play a key role in snRNA gene transcription by interacting with transcription factors bound to snRNA enhancers and by communicating directly with both class II and class III general transcription factors. To further study the mechanism of snRNA gene transcription, we have undertaken molecular cloning of cDNAs encoding PTF subunits. Here we report the cloning and characterization of PTFγ and PTFδ cDNAs.

MATERIALS AND METHODS

HeLa cells and extracts. HeLa cells were maintained in Joklik minimal essential medium containing 5% bovine calf serum. Nuclear extracts and cytosolic S100 extracts were prepared as described previously (9). Extracts for U1 and U2 transcription were prepared with a modification described by Bernues et al. (4).

Plasmids. O^+P^+ and O^+P^- 7SK maxigene templates have been described previously (32). The templates pU1*G^-, pU2*G^-, and pU2*$ABCG^-$ (a generous gift from N. Hernandez) have been described by Sadowski et al. (36).

Cloning of cDNAs encoding PTF subunits. PTF was purified as described previously (46). Purified PTF was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on an 8% polyacrylamide gel, and resolved polypeptides were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore). Polypeptide bands corresponding to PTF subunits were excised and digested with endoproteinase Lys-C. After separation of derived peptides by high-pressure liquid chromatography (HPLC), amino acids sequences for several peptides from each PTF subunit were determined at the Rockefeller University protein sequencing facility. Degenerate PCR primers were then designed on the basis of peptide sequences presented in Fig. 1. Nested PCR was performed to clone PTFγ and PTFδ cDNAs directly from a human Namalwa cDNA library, which was used as the template. The outer primers for PTFγ were TTYCARCARGAYYTIGTIAAYG and TTCATRTTYTGRTAR TCRTGIAC. The first-round PCR product was then used as the template for the second-round PCR, for which the inner primers were CARCAYTTYGAYGCI GCITAYA and TCRTGIACRTTIARCATYTCYTC. For PTFδ, the outer primers were CAYCCIGGIGGNCTNCARGG and ATYTCIGGIGCNGGRTCY TC, and the inner primers were GARGCICARCCICCNGCNCC and GGRTC YTCYTGICCICCNGG. PCR products were subsequently used to screen human Namalwa cDNA libraries (1). Final positive phages were excised in vivo and transferred into pBluescript SK (Stratagene) as instructed by the manufacturer and identified by double-stranded DNA sequencing with Sequenase (U.S. Biochemical). The nucleotide sequence of the clones was determined on both strands by synthesizing internal sequencing primers.

Antigen and antiserum preparation. To produce recombinant proteins used as antigens, cDNAs encoding PTFγ and PTFδ were subcloned into pRSET (Invitrogen). The resulting plasmids were introduced into *Escherichia coli* BL21 (DE3) containing pLysS (39). After induction with isopropyl-β-D-thiogalactopyranoside (IPTG), proteins were purified by nitrilotriacetic acid-resin affinity chromatography (17) and subsequently by electroelution following SDS-PAGE.

New Zealand White rabbits (3 kg) initially were injected intradermally with 300 μg of antigen emulsified with an equal volume of complete Freund's adjuvant. Rabbits were boosted subcutaneously every 4 weeks with 100 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.

Preparation of antisera against PTFα and PTFβ will be published elsewhere (2, 45).

EMSA. The probe for electrophoretic mobility shift analysis (EMSA) was prepared by end labeling the *Hin*dIII restriction fragment of O^+P^+ with the Klenow enzyme and [α-^{32}P]dATP. Double-stranded oligonucleotide competitors had the following sequences: wild-type mouse U6 PSE, ACAAAAGGAAACT CACCCTAACTGTAAAGTAATTGTGTGTT; and mutant mouse U6 PSE, AC AAAAGGAAACTAAGATCTGCTGTAAAGTAATTGTGTGTT. Twenty femtomoles of probe was used in a 20-μl reaction mixture containing 4% Ficoll type 400, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) KOH (pH 7.9), 70 to 80 mM KCl, 1 mM spermidine, 100 μg of bovine serum albumin per ml, 0.03% Nonidet P-40, 1 mM dithiothreitol, and 2 μg of poly(dI-dC). Reactions were initiated by the addition of protein, and mixtures were incubated for 20 min at 30°C. Antiserum (1 μl) was then added to the reaction mixture, which was further incubated for 10 min. Samples were loaded directly onto a 4% (37.5:1, acrylamide/bisacrylamide) nondenaturing polyacrylamide gel with 0.5× Tris-borate-EDTA and 0.03% Nonidet P-40 and electrophoresed at 150 V for 2 h at room temperature.

Immunoblot analysis. Immunoblotting with different antibodies was performed by using the Amersham enhanced chemiluminescence system according to the manufacturer's instructions.

In vitro transcription. Transcription reactions for adenovirus VAI and human 7SK genes were performed for 1 h at 30°C in a 25-μl reaction mixture with 8 μl of HeLa cell nuclear extract (8 mg/ml), 400 ng of 7SK, and 4 ng of VAI supercoiled DNA templates. The standard reaction mixtures contained 10 mM HEPES-KOH (pH 7.9), 70 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 500 μM each ATP, CTP, and UTP, 25 μM GTP, 2.5 μCi of [α-^{32}P]GTP (3,000 Ci/mmol), 5 mM creatine phosphate, and 2 μg of α-amanitin per ml. Following incubation, 200 μl of stop mix (10 mM Tris-HCl [pH 7.8], 1 mM EDTA, 1.1 M ammonium acetate, 100 μg of yeast tRNA per ml) was added, and the mixture was extracted with phenol-chloroform and ethanol precipitated. The precipitates were resuspended in 90% formamide–10 mM EDTA–0.1% bromophenol blue–0.1% xylene cyanol, heat denatured, and analyzed on 6% polyacrylamide gels with 8 M urea.

Transcription reactions from the pU1*G^-, pU2*G^-, and pU2*$ABCG^-$ templates were performed in a final volume of 25 μl containing 11 μl of HeLa nuclear extract, 20 mM HEPES-KOH (pH 7.9), 5.0 mM dithiothreitol, 1.0 mM 3′-*O*-methyl GTP, 500 μM ATP, 500 μM CTP, 0.26 μM [α-^{32}P]UTP (20 μCi), 2.0 U of RNase T_1, and 1.0 μg of a supercoiled template. Transcription proceeded for 1 h at 30°C, and RNA products were extracted and analyzed as described above.

Immunoprecipitation of PTF and TBP. Anti-PTFδ antibodies were purified from rabbit sera by using protein A-Sepharose (Pharmacia) as described previously (13). Antibodies (2 mg of protein per ml of resin) were cross-linked to protein A-Sepharose by incubation with dimethyl pimelimidate (20 mM in 0.2 M sodium borate [pH 9.0]) for 30 min. After the residual active groups were blocked by 0.2 M ethanolamine, the non-cross-linked immunoglobulins were removed by washing the resin in 0.2 M glycine (pH 2.5). In the experiments shown in Fig. 5, 30 μl of antibody-coupled resin was incubated for 2 h at 4°C with 400 μl of nuclear extract in buffer BC (20 mM Tris-HCl [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.03% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) containing either 100 (BC100) or 400 (BC400) mM KCl. After the resin was washed five times with the same buffer, the bound proteins were eluted with 60 μl of 0.2 M glycine (pH 2.5).

For immunoprecipitation of TBP, 400 μl of nuclear extract prepared from HeLa cells constitutively expressing the FLAG-tagged TBP (6) was incubated for 6 h at 4°C with 20 μl of the anti-FLAG M2–agarose (IBI/Kodak). After the beads were washed five times in BC100, the bound proteins were eluted twice by incubation at 4°C for 20 min each time with 30 μl of BC100 containing 0.2 mg of the FLAG peptide per ml. Eluted proteins were pooled, and aliquots were analyzed by immunoblotting.

Protein-protein interaction assays. Glutathione *S*-transferase (GST), GST-TBP, GST-N terminus of TBP (residues 1 to 163; GST-TBP_N), and GST-TBP core domain (residues 154 to 335; GST-TBP_C) fusion proteins were expressed in *E. coli* (18) and purified with glutathione-Sepharose. Labeled FLAG-tagged PTFγ and PTFδ were produced by coupled in vitro transcription-translation in the presence of [^{35}S]methionine as described by the manufacturer (Promega). GST and GST fusion proteins (1 μg bound to 5 μl of glutathione-Sepharose resin) were incubated with 5 μl of translated FLAG-PTFγ and FLAG-PTFδ in 100 μl of BC100 or BC400 for 1 h at room temperature. After extensive washing with the same buffer, bound proteins were resolved by SDS-PAGE and analyzed by fluorography.

Immunodepletion of PTF and TBP from HeLa nuclear extracts. To deplete PTF, 300 μl of anti-PTFδ antiserum was incubated with 100 μl of protein A-Sepharose (Oncogene Science) for 2 h at 4°C with constant mixing. After three washes with BC100 and three washes with BC400, the resin was incubated for 2 h with 400 μl of HeLa nuclear extract adjusted to 400 mM KCl. After centrifugation, the supernatant was incubated for 1 h with 50 μl of protein A-Sepharose beads to remove residual immunoglobulins. The supernatant was dialyzed against BC100, and aliquots were quickly frozen in liquid nitrogen.

The procedure for immunodepletion of TBP was essentially the same except that nuclear extract was incubated twice with anti-TBP antibody-coated protein A-Sepharose resin.

RESULTS

Cloning of cDNAs encoding PTF subunits. PTF was purified from HeLa S100 extracts (5 liters, 100 g of protein) as described previously (46), and individual polypeptides were transferred to a membrane after separation by SDS-PAGE. Major polypeptides corresponding to the four PTF subunits were subjected to peptide microsequencing analysis. Peptide sequences derived from PTFγ and PTFδ were subsequently

used to design degenerate primers for PCR amplification, and derived PCR products were used as probes to screen human cDNA libraries for corresponding cDNAs. Nucleotide sequences of the longest cDNA clones for PTFγ (λ12-1) and PTFδ (λ23-2) indicated that PTFγ and PTFδ cDNAs encoded open reading frames of 368 and 334 amino acids with calculated molecular masses of 43 and 36 kDa, respectively (Fig. 1). The putative translation initiation codons were assigned to the first ATGs of each clone, which closely match the Kozak consensus sequence (23). The deduced protein sequences contain all PTFγ and PTFδ peptide sequences that were obtained by microsequencing.

The predicted amino acid sequence of PTFγ indicates that it is a highly charged (36% of total amino acids) and overall basic protein with an isoelectric point of 9.5. The hydropathy profile of PTFγ shows a high degree of hydrophilicity in the carboxy-terminal half of the protein, reflecting the more frequent occurrence of charged amino acids (data not shown). Secondary structure plots predict that PTFγ has a predominantly α-helical structure. Interestingly, the amino-terminal region of PTFγ has a substantial aromatic amino acid content, with 20 phenylalanines and tyrosines in the first 150 amino acids. Its significance is not known.

The deduced amino acid sequence of PTFδ shows that it is both acidic (pI of 5.7) and rich in proline (13.8% of all residues). The region between residues 141 and 196 is especially proline rich (26.6%). Plots predicting protein secondary structure show potential turns in the proline-rich region (data not shown). Protein database searches scored sequence similarities of this region to other proline-rich proteins, including the carboxyl-terminal domain of RNA pol II.

Antibodies raised against recombinant PTF subunits recognize native PTF. To facilitate the analysis of various functions of PTF in transcription, rabbit antisera were prepared against portions of PTFγ and PTFδ expressed in bacteria. Both anti-PTFγ and anti-PTFδ antibodies reacted strongly with protein bands of 45 and 44 kDa, respectively, in purified PTF (data not shown) and in HeLa nuclear extracts (see Fig. 6B) via immunoblotting assays. Since the calculated molecular mass of PTFδ (36 kDa) is substantially smaller than its apparent size (43 to 44 kDa) in the purified PTF, the cDNA encoding PTFδ was transcribed and translated in rabbit reticulocyte lysates, and the mobility of the translated protein in an SDS-polyacrylamide gel was compared with that of the native protein in the HeLa nuclear extract by immunoblot analysis. Recombinant PTFδ as well as recombinant PTFγ had the same mobilities as their natural counterparts (data not shown), indicating that the cDNA clones encode full-length PTFγ and PTFδ subunits.

To examine whether natural equivalents of the cloned proteins were present in the specific PTF-PSE complex, the effect of the anti-recombinant PTF subunit antibodies on the protein-DNA complex was monitored by an Oct-1–PTF supershift assay. As shown in Fig. 2, incubation of PTF and Oct-1 with a probe containing both the 7SK PSE and an adjacent octamer element resulted in an Oct-1–PTF–DNA supershift complex in addition to an Oct-1–DNA complex (lane 4). The supershift complex, but not the Oct-1–DNA complex, was greatly reduced by competition with an excess of an oligonucleotide containing the wild-type mouse U6 PSE but not with one containing the mutant site (lanes 5 and 6), indicating that the formation of the supershift complex is dependent on the presence of the PSE. When immune serum against Oct-1 was added to the reaction, both Oct-1–DNA and Oct-1–PTF–DNA complexes were abolished and an antibody-dependent supershift complex specific to the immune serum (lanes 7 and 8) appeared on top of the gel, confirming that both complexes contained Oct-1. Addition of anti-PTFγ (lane 10) or anti-PTFδ (lane 12) antiserum to the reaction supershifted the Oct-1–PTF–DNA complex but did not affect the Oct-1–DNA complex. In addition to the supershifted complex (which was more readily apparent in a longer autoradiographic exposure; data not shown), anti-PTFδ antibodies generated a band with a mobility slightly greater than that of the Oct-1–PTF–DNA complex (lane 12). It is possible that this represents a complex lacking the PTFδ subunit as a result of its removal by the antibodies. Addition of preimmune sera did not affect the protein-DNA complexes (lanes 9 and 11). These results demonstrate that the cloned proteins represent subunits of the native PTF and that the anti-PTF antibodies can recognize PTF in solution and are suitable for functional studies.

PTF is required for transcription from both class II and class III snRNA genes. To investigate the requirement of PTF for transcription from both pol II- and pol III-transcribed snRNA genes, HeLa nuclear extracts were incubated with anti-PTFδ preimmune or immune serum to remove endogenous PTF. The immunoblotting analysis in Fig. 3A shows that PTFδ was efficiently depleted by the immune serum but not by the preimmune serum. Other PTF subunits were also efficiently and specifically removed by anti-PTFδ antibodies, suggesting that PTF subunits exist as a stable complex in solution (data not shown; see Fig. 6B). Immunodepletion of PTFδ greatly reduced the level of transcription from the 7SK gene but did not affect the transcription from the adenovirus VAI gene used as a control pol III promoter (lanes 1 and 3). Addition of increasing amounts of highly purified PTF activated transcription from the 7SK gene in a dose-dependent manner without affecting VAI gene transcription (lanes 5 to 7). Transcriptional activation by PTF was dependent on the intact PSE site, since a template with a PSE mutation that eliminates PTF binding (32) was inactive both in the mock-depleted extract (lane 2) and in the exogenous PTF-dependent assay (lane 8).

We next examined the effect of PTF depletion on the transcription of pol II-dependent snRNA genes in nuclear extracts. As shown in Fig. 4, removal of PTFδ inhibited transcription from both U1 and U2 snRNA promoters (lanes 2 and 8). U1 and U2 transcription was restored in a dose-dependent manner by addition of purified PTF to depleted extracts (lanes 3 to 5 and 10) and was dependent on an intact PSE (lanes 10 and 11). Transcription from the adenovirus major late promoter control was not affected by the depletion or readdition of PTF (data not shown). These results indicate that PTF is required for the transcription of both pol II- and pol III-dependent snRNA genes.

Similar immunodepletion studies with anti-PTFγ antibodies generated results which paralleled those observed with anti-PTFδ antibodies. The antibodies coprecipitated other PTF subunits and specifically impaired transcription from both the 7SK and U1 genes (data not shown). In an effort to assign gene-specific functions to each PTF subunit, purified antibodies against PTFγ and PTFδ were directly added to transcription reactions for both pol II- and pol III-dependent snRNA genes. However, addition of antibodies failed to reveal any differential roles for the various subunits in transcription, since anti-PTFγ antibodies specifically impaired transcription of both class II and class III snRNA genes whereas anti-PTFδ antibodies had no effect on transcription of either class (data not shown). The latter result does not contradict the results presented above, since the immunoreactive PTF complexes were not removed from the reaction.

Interaction of PTF with TBP. Our studies involving purification of PTF have indicated that PTF is composed of four polypeptides which are present in a roughly equimolar ratio

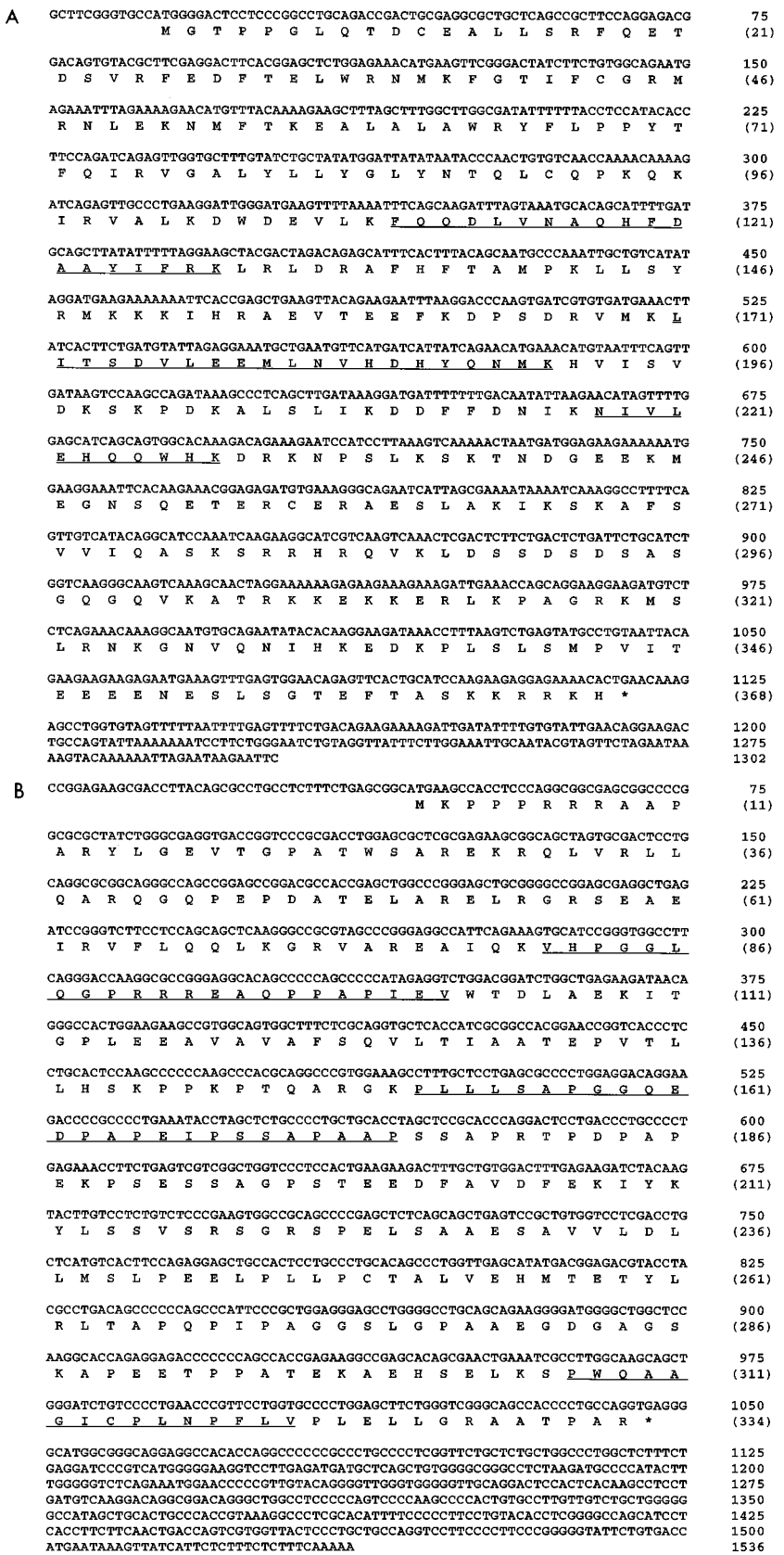

FIG. 1. Nucleotide and deduced amino acid sequences of PTFγ (A) and PTFδ (B). The numbers at the right indicate the nucleotide and amino acid positions. The peptide sequences obtained from microsequencing analysis of purified PTF are underlined.

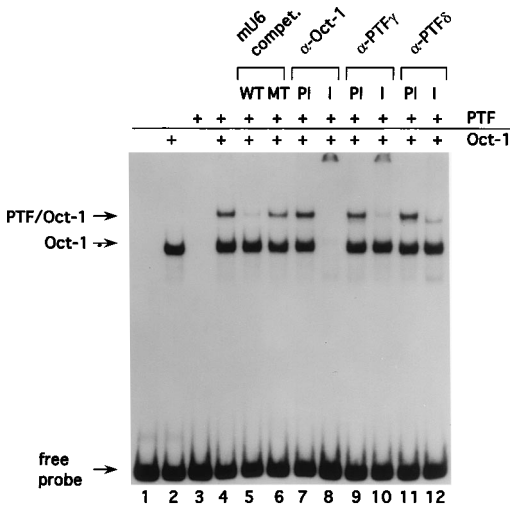

FIG. 2. PTFγ and PTFδ are present in the Oct-1 · PTF–DNA complexes. EMSAs were performed with partially purified PTF (0.25 μl of the Q-Sepharose fraction) and recombinant Oct-1 (0.5 ng), using the end-labeled *Hin*dIII restriction fragment of O^+P^+ as a probe. Unlabeled competitors (compet.; 50-fold molar excess over probe) containing a wild-type (WT) or mutant (MT) PSE sequence of mouse U6 were added to the reactions in lanes 5 and 6. Preimmune (PI) and immune (I) sera (1 μl) against Oct-1 (lanes 7 and 8), PTFγ (lanes 9 and 10), or PTFδ (lanes 11 and 12) were incubated with the reaction mixture after the addition of DNA probe. The positions of free probe and of Oct-1–DNA and Oct-1–PTF–DNA complexes are indicated at the left.

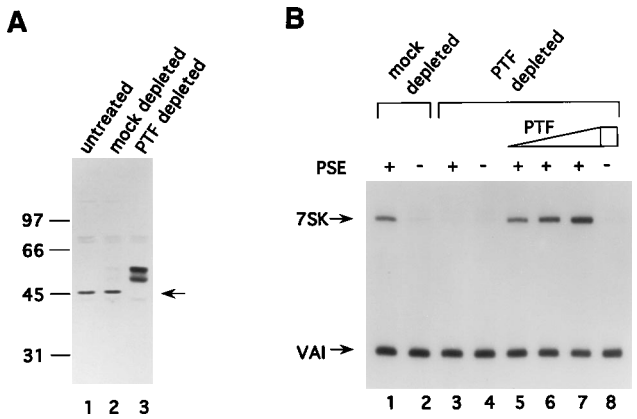

FIG. 3. PTFδ is required for activation of the human 7SK gene. (A) Immunodepletion of PTFδ from HeLa nuclear extracts. HeLa nuclear extracts (3 μl of each, 24 μg of protein) treated as indicated above the lanes were separated by electrophoresis on an SDS–10% polyacrylamide gel and transferred to nitrocellulose. The blot was incubated with a 1:5,000 dilution of a rabbit antiserum raised against the recombinant PTFδ. Antigen-antibody complexes were visualized as described in Materials and Methods. The positions of protein molecular weight markers are indicated at the left in kilodaltons. The arrow indicates the position of PTFδ. Residual immunoglobulin G is visible in lanes 2 and 3. (B) In vitro transcription of VAI and 7SK genes in a PTFδ-depleted HeLa nuclear extract. In vitro transcription reactions were performed with human 7SK (400 ng) and adenovirus VAI (4 ng) templates in either mock-depleted (lanes 1 and 2) or PTFδ-depleted (lanes 3 to 8) HeLa nuclear extracts. Reactions in lanes 5 to 8 were supplemented with highly purified PTF (0.25 μl in lane 5; 0.5 μl in lane 6; 1 μl in lanes 7 and 8). The positions of 7SK and VAI transcripts are indicated at the left. The 7SK templates used are indicated above the lanes. PSE + and PSE − represent O^+P^+ and O^+P^-, respectively.

(46). Given that the PSE is the only common essential proximal promoter element of snRNA genes and that TBP is involved in transcription by all three polymerases, PTF may interact with TBP. In fact, Sadowski et al. (36) recently reported that SNAPc, a PSE-binding activity closely related to PTF, is a unique TBP-TAF complex different from SL1, TFIID, or TFIIIB, although TBP was found in markedly substoichiometric amounts (14). To investigate a possible interaction or association between PTF and TBP, we immunopurified PTF from HeLa cell nuclear extracts by using preimmune or immune anti-PTFδ antibodies cross-linked to protein A-agarose beads. The bound proteins were eluted and subjected to SDS-PAGE, and the presence of TBP was analyzed by immunoblotting. When immunoprecipitation was performed in buffer containing 0.1 M KCl (Fig. 5A), PTF was efficiently and specifically purified from nuclear extracts with the immune antibodies (lanes 2 to 5), and a significant amount of TBP was coprecipitated with PTF (lane 5). However, when immunoprecipitation was carried out in buffer containing 0.4 M KCl (Fig. 5B), TBP was not detectable in the pellet fraction even though PTF was still efficiently precipitated by the immune antibody-immobilized beads (lanes 4 and 5). The interaction between PTF and TBP was also examined in a reciprocal experiment in which the epitope (FLAG)-tagged TBP and its associated proteins were immunopurified with the anti-FLAG antibody-conjugated agarose from nuclear extracts prepared from HeLa cells constitutively expressing the tagged TBP. Similar methods have been previously used to purify TFIID and TFIIIB (6). The Western blotting (immunoblotting) with anti-TBP antibodies showed that most tagged TBP was precipitated with the antibody-coated resin (Fig. 5C, lane 6). Although some PTF was specifically coimmunoprecipitated with tagged TBP (lane 6), overall recovery (less than 1%) was too little relative to that for TBP (near 100% of tagged TBP, which consists of about 50% of the total TBP) to account for an equimolar association of PTF subunits with TBP. These results indicate that the interaction between TBP and PTF in solution is sensitive to high salt, and possibly to the TBP-antibody interaction, and thus that TBP is not tightly associated with PTF.

Since the interaction between TBP and PTF was detected by immunoprecipitation, GST pulldown assays were performed to examine potential direct interactions between TBP and individual PTF subunits. As shown in Fig. 5D, both PTFγ and

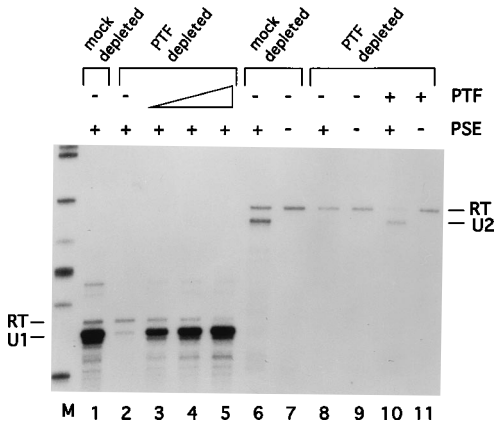

FIG. 4. PTFδ is required for the activation of human U1 and U2 genes. In vitro transcription reactions were performed in either mock-depleted (lanes 1, 6, and 7) or PTFδ-depleted (lanes 2 to 5 and 8 to 11) nuclear extract as described in Materials and Methods. The templates used are the wild-type U1 plasmid (pU1*G^-; lanes 1 to 5), the wild-type U2 plasmid (pU2*G^-; lanes 6, 8, and 10), or the mutant U2 plasmid (pU2*$ABCG^-$; lanes 7, 9, and 11). Reactions in lanes 3, 4, 5, 10, and 11 were supplemented with highly purified PTF (0.25 μl in lane 3; 0.5 μl in lane 4; 1 μl in lanes 5, 10, and 11). Marker lane M is derived from ^{32}P end labeling of *Msp*I-digested pBR322. The positions of correctly initiated U1 and U2 transcripts (U1 and U2) and readthrough transcripts (RT) are indicated.

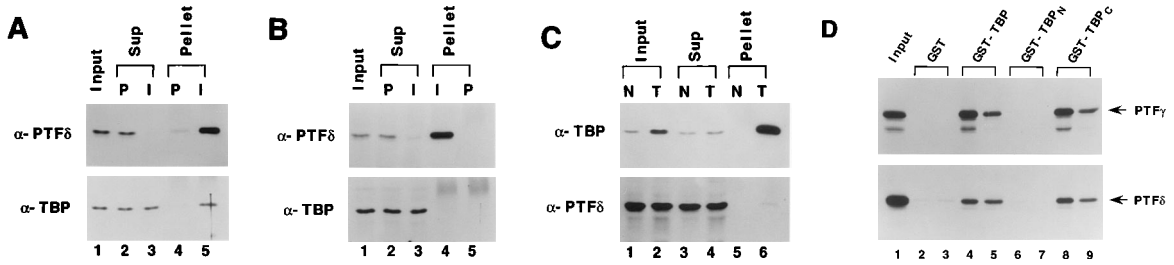

FIG. 5. PTF interacts with TBP. (A) Immunoprecipitation of PTF performed in buffer containing 100 mM KCl. Immunoprecipitation was performed as described in Materials and Methods with preimmune (PI) and immune (I) antibodies against PTFδ. Aliquots of input (4 μl of nuclear extract; lane 1), supernatants (Sup; 4 μl; lanes 2 and 3), and eluates (10 μl; lanes 4 and 5) were analyzed by electrophoresis on an SDS–10% polyacrylamide gel and transferred to nitrocellulose. The blot was first probed with anti-TBP antibodies (lower panel) and then reprobed with anti-PTFδ antibodies (upper panel). (B) Immunoprecipitation of PTF performed in buffer containing 400 mM KCl. (C) Immunoprecipitation of TBP. TBP and its associated proteins were immunoprecipitated from extracts prepared from normal HeLa cells (N) or cells expressing the FLAG-tagged TBP (T) as described in Materials and Methods, using anti-FLAG monoclonal antibodies. Aliquots of input (4 μl of extract; lanes 1 and 2), supernatants (4 μl; lanes 3 and 4), and eluates (10 μl; lanes 5 and 6) were analyzed by immunoblotting following electrophoresis on an SDS–10% polyacrylamide gel. The blot was first probed with anti-PTFδ antibodies (lower panel) and then reprobed with anti-TBP antibodies (upper panel). (D) Independent binding of PTFγ and PTFδ to the core domain of TBP. GST pulldown assays were performed in either 100 mM KCl (lanes 2, 4, 6, and 8) or 400 mM KCl (lanes 3, 5, 7, and 9) as described in Materials and Methods, using the GST fusion protein indicated above each lane. Aliquots of input (2 μl of labeled in vitro-translated FLAG-tagged PTFγ or PTFδ; lane 1) and bound proteins (from 5 μl of input per reaction) were resolved by SDS-PAGE and analyzed by fluorography. Interaction assays were conducted individually with either PTFγ (top panel) or PTFδ (bottom panel).

PTFδ were retained by immobilized GST-TBP (lanes 4 and 5) and by GST-TBP_C (lanes 8 and 9) but not by GST alone (lanes 2 and 3) or by GST-TBP_N (lanes 6 and 7). The interactions were strong enough to survive washing in 400 mM KCl in this assay (lanes 5 and 9). These results show that PTFγ and PTFδ each have the capacity to interact directly with the core domain of TBP, although the interactions with TBP may be stronger (more salt resistant) for the free subunits than for the bound (within PTF) subunits.

PTF is a stable complex of four subunits. We previously defined four polypeptides of 180, 55, 45, and 44 kDa as PTF subunits by virtue of their copurification with PTF activities through multiple chromatographic steps (46). To test whether these polypeptides form a tightly associated complex in solution, a partially purified PTF fraction (0.3M KCl fraction from the Q-Sepharose column in the second chromatographic step) was incubated with anti-PTFδ antibody-conjugated beads. After extensively washing the beads, we analyzed the bound proteins by SDS-PAGE and silver staining. As shown in Fig. 6A (see also the figure legend), the four polypeptides were precipitated specifically by the immune antibodies but not by the preimmune antibodies. From these data, we conclude that the polypeptides form a tightly associated complex, since the complex was resistant to the high salt (0.4 M KCl) and detergent (0.1% Nonidet P-40) conditions used during the immunopurification.

To more clearly identify the polypeptide bands in the immunopurified complex, we performed immunoblotting analysis using antibodies against individual PTF subunits and TBP. As shown in Fig. 6B, antibodies against PTFα, -β, -γ, and -δ reacted efficiently with polypeptides of 180, 55, 45, and 44 kDa, respectively, in the HeLa nuclear extract (lanes 1, 3, 5, and 7). Protein bands of the same molecular sizes were detected in the immunoprecipitate obtained with anti-PTFδ antibodies (lanes 2, 4, 6, and 8), showing that the four polypeptides correspond to the PTF subunits originally determined by conventional purification. However, although TBP was present in the partially purified PTF fraction used for immunopurification (data not shown), it was not detected in the high-salt immunoprecipitate (lanes 9 and 10), even after 10-fold-longer exposure (lanes 11 and 12). This result indicates that PTF subunits do not interact as strongly with TBP as they do with one another.

TBP requirements for pol II-transcribed snRNA genes. In an effort to establish an in vitro transcription system for snRNA genes reconstituted with purified or recombinant components and to thereby dissect the mechanism of differential selection of RNA polymerases in snRNA gene transcription, we determined the TBP requirement for class II snRNA genes. HeLa nuclear extracts were incubated with polyclonal anti-TBP antibodies to deplete TBP. TBP and TBP-containing complexes were then added to the depleted extracts to test for restoration of U1 transcription. As shown in Fig. 7, TBP depletion greatly reduced the transcription from the U1 promoter compared with the mock depletion (lanes 2 and 3), in agreement with previous reports (4, 36). Addition of recombi-

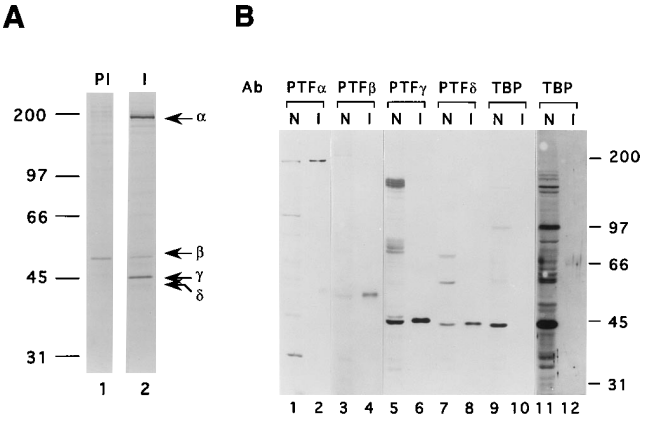

FIG. 6. PTF is composed of four polypeptide subunits. (A) SDS-PAGE analysis of immunoprecipitated protein with anti-PTFδ antibodies. Partially purified PTF (200 μl of the Q-Sepharose fraction) was incubated in BC400–0.1% Nonidet P-40 with protein A-Sepharose beads (20 μl) coated with antibodies purified from preimmune (PI) and immune (I) sera of PTFδ. After the resin was washed extensively, the bound proteins were eluted with 0.2 M glycine (pH 2.5) and analyzed by SDS-PAGE. The polypeptides were visualized by silver staining. The positions of protein size markers are shown on the left in kilodaltons. PTF subunits are indicated on the right. PTFβ was negatively stained with silver and does not show up well in this figure. A band corresponding to PTFδ is only faintly visible in the figure but clearly visible in the original stained gel. (B) Immunoblotting analysis of proteins immunoprecipitated with anti-PTFδ antibodies. HeLa nuclear extract (N) proteins and proteins immunoprecipitated in BC400–0.1% Nonidet P-40 (I) were resolved by SDS-PAGE (10% polyacrylamide gel) and transferred to nitrocellulose. The blots were probed with the antibodies (Ab) indicated above the lanes. Lanes 11 and 12 show a 10-times-longer exposure of lanes 9 and 10. The positions of protein size markers are shown on the right in kilodaltons.

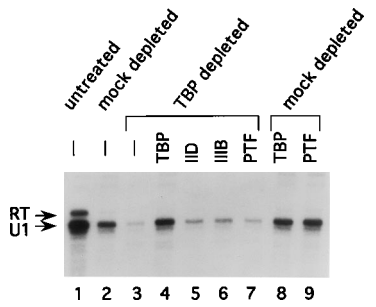

FIG. 7. Distinct TBP complex requirements for U1 transcription. In vitro transcription reactions were performed with a human U1 template (pU1*G$^-$) in untreated (lane 1), mock-depleted (lanes 2, 8, and 9), or TBP-depleted (lanes 3 to 7) HeLa nuclear extracts. Reactions in lanes 4, 5, 6, and 8 were supplemented with recombinant human TBP or TBP-containing complexes (3 ng of TBP) as indicated above the lanes. Reactions in lanes 7 and 9 were supplemented with 1 μl of highly purified PTF. Recombinant human TBP was purified as described previously (17). Purification of epitope-tagged TBP complexes from 0.3 M (TFIIIB; lane 6) and 0.85 M (TFIID; lane 5) KCl P11 fractions was performed as described previously (6). The positions of U1 and readthrough (RT) transcripts are indicated at the left.

nant TBP restored U1 transcription to a level comparable to that in the mock-depleted extract (lane 4). However, TFIID or TFIIIB isolated by affinity purification of epitope-tagged complexes from HeLa cells did not restore U1 transcription (lanes 5 and 6), even though they fully restored transcription of the adenovirus major late and VAI promoters in the same extract (data not shown; see reference 46). Moreover, the observation that TFIID and TFIIIB did not alter transcription in a mock-depleted extract (data not shown) argues against the possibility that they contain a U1-specific inhibitor. Purified PTF, which is free of TBP, although active in transcription assayed in PTF-depleted extracts, did not activate U1 transcription in the TBP-depleted extracts (lane 7). TBP and PTF were not limiting in the mock-depleted extracts, since further additions did not substantially increase transcription (lanes 8 and 9). These results indicate that neither TFIID nor TFIIIB can fulfill the TBP requirement of U1 transcription and thus that the TBP in these complexes is, as previously reported (6, 10, 28, 40, 48), stably bound to the corresponding TAFs. These results parallel those observed for 7SK and U6 RNA transcription in response to TBP, TFIID, and TFIIIB in TBP-depleted extracts (46).

DISCUSSION

The PSE is a key promoter element common to all snRNA-type genes and plays a major role in snRNA gene transcription. The PSE site is recognized by PTF, a factor which can interact with the enhancer-bound transcription factors (e.g., Oct-1) to stimulate the basal transcription, communicate with both class II and class III basal transcription factors, and thereby assist in recruiting an appropriate RNA polymerase to the target promoter. In an effort to study the function of PTF, and to understand the mechanism of snRNA gene transcription in particular, we have cloned and expressed cDNAs encoding two PTF subunits.

Cloning of cDNAs encoding PTFγ and PTFδ. Large-scale purification of human PTF made it possible to obtain enough peptide sequence information to clone cDNAs corresponding to PTF subunits. Ponceau S staining performed following the transfer of purified protein to a membrane revealed four major polypeptides with an approximate 1:1:1:1 stoichiometry, in agreement with the estimation from the peptide profile following HPLC purification and consistent with the idea that PTF contains equimolar amounts of four subunits (α, β, γ, and δ). All peptide sequences obtained from 45- and 44-kDa protein bands were found in the amino acid sequences deduced from PTFγ and PTFδ cDNAs, indicating that each band in purified PTF contains mainly one polypeptide species. For PTFγ and PTFδ, we obtained a group of independent cDNA clones which were apparently derived from a single mRNA species, as judged by partial sequencing.

Protein database searches with the predicted amino acid sequence of PTFδ failed to reveal extensive sequence similarity to other known sequences. However, there is weak similarity between the proline-rich region of PTFδ and the carboxyl-terminal domain of RNA pol II, which is manifested by the conserved spacing of prolines but of unknown significance. Recombinant PTFδ does not show any independent PSE-binding activity (data not shown), consistent with our earlier observation (46) that only the largest subunit of PTF is UV cross-linked to DNA. Together with the fact that the deduced sequence of PTFδ does not contain any known DNA-binding motifs, these data suggest that PTFδ is not involved in DNA binding.

While this report was in preparation, Henry et al. (14) reported purification of SNAPc and cloning of a subunit designated SNAPc43. A sequence comparison revealed that PTFγ is identical to SNAPc43, indicating that PTF/SNAPc is the major PSE-binding activity in the cell. The deduced amino acid sequence of PTFγ does not have significant similarity to other sequences in the available protein databases. Although PTFγ is a highly charged protein which contains patches of basic amino acids that could interact with DNA, in vitro-translated PTFγ does not bind the PSE. Rather, PTFγ may interact with other factors involved in snRNA gene transcription, an idea supported by the observation that transcription from both class II and class III snRNA genes is specifically inhibited by direct addition of purified anti-PTFγ antibodies which presumably interfere with an interaction(s) between PTF and another transcription factor(s) in the reaction (data not shown). Indeed, the GST pulldown assays (Fig. 5D; reference 14) indicate that PTFγ may directly interact with TBP.

In addition to PTF, PBP and SNAPc have been shown to bind the PSE and to activate transcription from both classes of snRNA genes (4, 32, 36, 41, 46). The finding that the sequence of PTFγ is identical to that of SNAPc43 demonstrates that PTF is identical or closely related to SNAPc. Highly purified SNAPc was found to contain mainly 50-, 45-, and 43-kDa polypeptides, with trace amounts of 200- and 54-kDa proteins (14). Although the precise relationship between the two factors remains to be determined, on the basis of the reported composition of PTF and SNAPc, it is likely that the 200-, 54-, and 45-kDa protein bands of SNAPc correspond to PTFα, -β, and -δ subunits, respectively.

We have made antibodies to cloned PTF subunits for probing PTF function. Anti-PTFγ and PTFδ antibodies recognize PTF in solution, as judged by EMSA (Fig. 2), showing that the cDNA clones encode bona fide PTF subunits. More importantly, transcription from both pol II- and pol III-dependent snRNA genes was inhibited by depletion with both anti-PTFγ and anti-PTFδ antibodies but restored by addition of highly purified PTF (Fig. 3 and 4 and data not shown). These results provide strong evidence that PTFγ and PTFδ are both involved in transcription of class II and class III snRNA genes. Together with the observation that individual PTF subunits (α, β, γ, and δ) are coimmunoprecipitated (Fig. 6A) and apparently derived from single mRNA species, these results also argue for the involvement of a single PTF species in these processes.

Interaction of PTF with TBP. Recent studies from several laboratories have indicated that all three eukaryotic RNA polymerases require TBP for transcription from both TATA-containing and TATA-less promoters. TBP also has been shown to be required for transcription from both pol II-dependent (4, 36) and pol III-dependent (27, 38, 46) snRNA genes. Given that the PSE may be considered as a core promoter element for both classes of snRNA genes and that TBP participates in transcription in association with factors (TAFs) that confer particular functional activities to distinct TBP-TAF complexes such as SL1, TFIID, and TFIIIB (16), the potential interaction or association between PTF and TBP was investigated. Immunoprecipitation data (Fig. 5) indicate that PTF can interact with TBP in solution. However, the interaction between TBP and PTF is relatively weak, showing a marked sensitivity to high salt, and the TBP/PTF ratio is clearly substoichiometric. This finding contrasts significantly with what has been observed for the very stable SL1, TFIID, and TFIIIB TBP-TAF complexes and leaves open the question (see below) as to whether the SNAPc/PTF-TBP complexes should be considered formally equivalent to the more stable TAF-TBP complexes containing stoichiometric levels of TBP.

Purified SNAPc was shown by immunoblot analysis to contain TBP which comigrated with a 45-kDa polypeptide. However, it is likely that TBP was only a minor component of purified SNAPc, since the 45-kDa band yielded only peptide sequences unrelated to TBP (14). Unlike SNAPc, highly purified PTF does not contain TBP that can be detected by immunoblot analysis. Furthermore, immunopurification of PTF (Fig. 6) has demonstrated that the four subunits of PTF are tightly associated with each other and that TBP is apparently absent from the PTF complex. In addition, anti-TBP antibodies did not affect the mobility of the PTF-PSE shift in EMSA, although antibodies against each PTF subunit did supershift the complex (45). Considering the fact that a substoichiometric level of TBP coimmunoprecipitates with PTF in low salt (Fig. 5; reference 14) and that a detectable amount of TBP copurifies with PTFγ/SNAPc43 under certain purification conditions (14), it is possible that PTF can loosely associate with TBP to form SNAPc, which might thus be regarded as a TAF-TBP complex specific for snRNA gene transcription. Relevant to this point, it is noted that free TBP and a pol II-specific TAF complex have been separately isolated in yeast cells (35), whereas TBP of higher eukaryotes is tightly and quantitatively complexed with TAFs in the corresponding TFIID. The integrity of a putative TBP-PTF complex may be affected by the methods used to prepare cell extracts and by the protein fractionation conditions. The more important question is whether, as proposed by Sadowski and colleagues (36), the presumed SNAPc/PTF-TBP complex binds to and functions through the PSE of U6 and 7SK genes while another molecule of TBP binds to and functions at the TATA box, or whether, as we suggest, PTF and TBP (or a distinct TBP-TAF complex; see below) can interact separately with the two promoter elements. In the latter case, subsequent interactions between PTF subunits and TATA-bound TBP could contribute to the overall activation mechanism (including stabilized binding), and the physical interaction data presented here indicate that such interactions may be mediated by both PTFγ (Fig. 5D; reference 14) and PTFδ (Fig. 5D). However, the observed interactions of TBP with PTFγ and PTFδ could also be relevant to the stable binding of a PTF-TBP or SNAPc complex at the PSE (independently of the TATA element). Reconstitution of PTF and SNAPc with recombinant subunits and biochemical dissection of in vitro transcription systems for both classes of snRNA genes should resolve this question.

Factor requirements for human snRNA gene transcription. We previously have shown (46) that the TBP requirement for U6 and 7SK genes cannot be supplied by highly purified TFIIIB or TFIID preparations which are fully functional for transcription of VAI and typical pol II-dependent genes, respectively. Functional TFIIIB containing only TBP and a 90-kDa subunit (TFIIIB90) has been purified recently from HeLa cells, and the cDNA encoding TFIIIB90 has been isolated (42). Interestingly, TFIIIB90 appears to be required for transcription of U6 and 7SK genes as well as the VAI gene, since immunodepletion of TFIIIB90 impaired transcription from both promoters. However, the core TFIIIB (TBP-TFIIIB90 complex) failed to restore U6 and 7SK transcription, although it was sufficient to restore VAI transcription (42). These results suggest that transcription of U6 and 7SK genes may require an additional component(s) associated with TFIIIB90 in a modified TFIIIB complex. This presumed component is not TFIIIC1 (47) or 0.48M-TFIIIB (28), since both are required for transcription of the VA1, U6, and 7SK genes. Relevant to the foregoing discussion, the weak substoichiometric association of such a complex with PTF might account for the presence of trace amounts of TBP in low-salt immunoprecipitates of PTF (Fig. 5) and in SNAPc (14, 36).

As an extension of our previous studies on factor requirements for class III snRNA genes, we have tested the TBP requirement for U1 transcription (Fig. 7). Our finding that neither TFIID nor TFIIIB can fulfill the TBP requirement for U1 transcription is in agreement with reports from other laboratories (4, 36) and parallels the results of similar studies on U6 and 7SK transcription. Unlike SNAPc, which is reported to restore U1 transcription when added to TBP-depleted extracts, PTF fails to restore U1 transcription in such an assay. Instead, TBP alone can fully support U6 and 7SK transcription. However, whether free TBP rather than a TBP-TAF complex does function on the U1 promoter in intact cells is a matter of conjecture. It is possible that TBP is stoichiometrically but weakly associated with specific TAFs in vivo and that it is easily dissociated by anti-TBP antibodies in vitro. Our data are compatible with the idea that either PTF or other (e.g., TFIIIB-interacting) components may function as loosely associated TAFs.

TFIIB and BRF, the yeast homolog of TFIIIB90, have been shown to interact with pol II and III, respectively (3, 5, 12, 21, 29, 43). In light of evidence that TFIIIB90 is involved in transcription of class III snRNA genes (42) and that TFIIB is involved in transcription of class II snRNA genes (4, 45), it is likely that the recruitment of either TFIIIB90 or TFIIB to snRNA promoters is one of the key steps in determining polymerase specificity. The availability of recombinant PTF subunits will allow us to examine the interplay between the key factors and to gain a better understanding of the mechanism involved in polymerase selection in snRNA gene transcription.

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