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Physical separation of two different forms of human TFIIIB active in the transcription of the U6 or the VAI gene *in vitro*

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Human transcription factor hTFIIIB is necessary to initiate transcription correctly from all RNA polymerase III (pol III) genes which are governed by structurally different promoters, and it is unclear whether hTFIIIB complexes, required for intragenic or 5′-located pol III promoters, are composed of unique or different components. We show here that two different forms of hTFIIIB can be separated physically by ion exchange chromatography. hTFIIIB-α shows strong preference for transcription of the U6 over the VAI gene and does not contain TATA binding protein (TBP). After SDS–PAGE and renaturation of proteins, the transcriptional activity of hTFIIIB-α can be reconstituted by fractions corresponding to a mean M_r of 25, 60 and 90 kDa. Upon gradient centrifugation or gel filtration, the activity of hTFIIIB-α is associated with an M_r of 60 ± 10 kDa, indicating that the components of the complex tend to dissociate. In contrast, hTFIIIB-β is predominantly active on intragenic pol III promoters. It reveals an M_r of 300 ± 30 kDa upon gel filtration and, besides TBP, it contains several associated factors (TAFs). Two of these proteins reveal an M_r of 60 kDa and 90 kDa, and it is conceivable that they are related to polypeptides of similar mass functionally identified in hTFIIIB-α. These proteins are probably required for the recruitment of pol III to the initiation site at 5′-located and intragenic promoters.

Keywords: hTFIIIB-α and -β/*in vitro* transcription/RNA polymerase III/U6 snRNA/VAI RNA

Introduction

RNA polymerase III (pol III) transcribes a diverse family of genes which, in vertebrates, are governed by structurally entirely different promoters. The classical pol III genes, comprising an intragenic promoter, can be subdivided into two types. The 5S rRNA gene has a split promoter of the AC type (type 1) and is recognized by the transcription factors TFIIIA and TFIIIC. The genes of the AB type (type 2; VAI RNA, tRNA, Alu elements) are recognized by TFIIIC2 which, together with TFIIIC1 (Yoshinaga *et al.*, 1987), is sufficient to incorporate TFIIIB. Transcription from these promoters by polymerase III is stimulated by TFIIA (Meissner *et al.*, 1993). The third type of pol III promoters is characterized by regulatory DNA elements which are located entirely in the 5′-flanking region (type 3; U6 snRNA, 7SK RNA) and comprise a TATA, a proximal and a distal sequence element (reviewed by Geiduschek and Kassavetis, 1992; Hernandez, 1992; Willis, 1993). These promoters interact with the proximal sequence element binding protein complex—designated as PBP (Waldschmidt *et al.*, 1991; Simmen *et al.*, 1992a), PTF (Murphy *et al.*, 1992; Yoon *et al.*, 1995) or SNAPc (Sadowski *et al.*, 1993; Henry *et al.*, 1995) by various groups—and the TATA binding protein (TBP). Binding of the latter is facilitated by TFIIA (Waldschmidt and Seifart, 1992). As a result of these processes, TFIIIB can be incorporated into the U6 initiation complex.

Initially, TFIIIB activity was associated with an M_r of 60 kDa in yeast (Klekamp and Weil, 1986) and human cells, according to analysis on glycerol gradients (Waldschmidt *et al.*, 1988). After it had been discovered that TBP is involved in the expression of U6 RNA genes by pol III (Lobo *et al.*, 1991; Margottin *et al.*, 1991; Simmen *et al.*, 1991; Waldschmidt *et al.*, 1991), it was found that it is also indispensable for the transcription from the classical type 1 and 2 pol III promoters (White *et al.*, 1992) as well as the expression of pol I genes (Comai *et al.*, 1992; Cormack and Struhl, 1992; Schultz *et al.*, 1992). In vertebrate cells, TFIIIB is considered currently to represent a multiprotein complex between TBP and several associated factors (TAFs), although the number and nature of these components have been the subject of discussion (Lobo *et al.*, 1992; Simmen *et al.*, 1992b; Taggart *et al.*, 1992; White and Jackson, 1992; Chiang *et al.*, 1993). Polypeptides of 150, 82 and 54 kDa (Lobo *et al.*, 1992) or 190, 96, 87 and 60 kDa (Chiang *et al.*, 1993) as well as 172 kDa and a component described as TAF L of unknown molecular mass (Taggart *et al.*, 1992) have been described to be related to hTFIIIB activity. For higher eukaryotes, none of these components has hitherto been cloned. The characterization of TFIIIB activity is most advanced in yeast cells, in which three proteins are involved (Huet and Sentenac, 1992; Kassavetis *et al.*, 1992). Besides recombinant TBP and recombinant BRF1 [Colbert and Hahn, 1992; also termed PCF4 (López-De-León *et al.*, 1992) or TDS4 (Buratowski and Zhou, 1992)], a purified or renatured B″ component of 90 kDa is required and sufficient to fully reconstitute TFIIIB activity. Remarkably, the same components are required for tRNA as well as U6 promoters (Huet *et al.*, 1994; Joazeiro *et al.*, 1994), and this observation in particular underlines the difference between yeast and higher eukaryotic systems, in which TFIIIB seems to be more complex.

Previous reconstitution studies (Waldschmidt *et al.*, 1991) demonstrated that a purified fraction of hTFIIIB, although capable of reconstituting tRNA transcription, was unable to support U6 expression. More importantly, studies with anti-TBP antibodies showed that while immunodepletion of a nuclear extract eliminated both VAI

and U6 transcription, only the latter could be restored by the addition of recombinant TBP (Lobo *et al.*, 1992; Chiang *et al.*, 1993), implying that different TBP–TAF complexes were utilized in the transcription of pol III genes controlled by internal or external promoters. These different complexes have hitherto not been separated physically. No data are available concerning the composition of the hTFIIIB complex required for the expression of those pol III genes governed by external promoters, although it has been speculated that a fraction eluted from Mono Q with 0.48 M KCl could represent a form of hTFIIIB, sufficient for the transcription of those genes (Sadowski *et al.*, 1993).

Besides hTFIIIB, the fraction eluting from phosphocellulose between 0.1 and 0.35 M KCl also contains a complex between TBP and two associated factors of 170 and 80 kDa, which was designated as B-TFIID. Like hTFIIIB, this complex eluted with an M_r of ~300 kDa upon gel filtration and was thought to be involved in the basal transcription from pol II promoters (Timmers and Sharp, 1991; Timmers *et al.*, 1992). Although it was believed that hTFIIIB was inseparable from TBP and was likely to be closely related to B-TFIID (Simmen *et al.*, 1992b), procedures have been developed allowing separation of hTFIIIB and B-TFIID (White and Jackson, 1992; Meyers and Sharp, 1993) and the exact relationship between these two TBP-containing complexes remains to be clarified (reviewed by Rigby, 1993).

As a first step towards identifying components of hTFIIIB involved in the expression of pol III genes governed by internal or external promoter elements and towards cloning the corresponding DNA sequence, we report here the chromatographic separation of two different forms of hTFIIIB and describe some of their physical properties. One of these complexes (hTFIIIB-β) contains TBP and associated proteins and displays activity predominantly in transcription from the VAI promoter. The other (hTFIIIB-α) is devoid of TBP and is involved in the expression of the U6 gene. We present data from renaturation studies showing that hTFIIIB-α activity is composed of three protein fractions with M_rs of ~25, 60 and 90 kDa.

Results

Separation of two different hTFIIIB activities

hTFIIIB was purified from cytoplasmic extracts of HeLa cells (S100) by phosphocellulose chromatography (Segall *et al.*, 1980). The fraction which eluted from this column with 0.35 M KCl (PCB) was fractionated further on an EMD–DEAE–Fractogel (EDF) column by elution with a linear gradient from 60 to 500 mM KCl. Individual fractions were assayed subsequently for their hTFIIIB content by testing their ability to reconstitute transcription of either the mouse U6 or VAI gene *in vitro*. It was found that chromatography under these conditions physically separates hTFIIIB activity into two fractions. Those hTFIIIB fractions, which complemented a minimal U6 transcription system consisting of PBP, rhTBP, TFIIA and pol III (Waldschmidt *et al.* 1991), eluted with 200 mM KCl from the EDF column (Figure 1A, lanes 8–9). In contrast, hTFIIIB activity, capable of reconstituting transcription of the VAI gene in the presence of TFIIIC

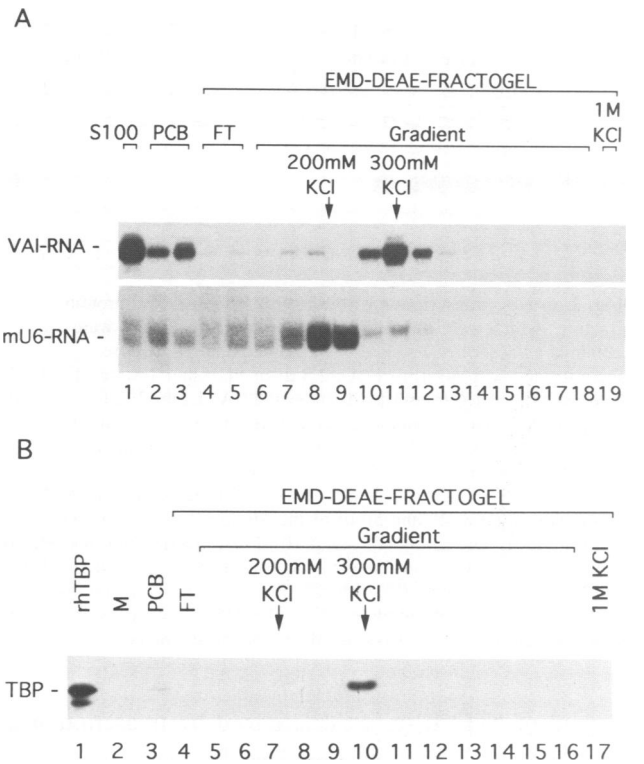

Fig. 1. (**A**) Comparative transcription of the mouse U6 ($pUmU6_{0.34}$) and the VAI (pUVAI) gene. *In vitro* transcription was performed separately for the two genes, as described in Materials and methods. The positions of U6 and VAI transcripts are indicated. 4 μg of $pUmU6_{0.34}$ or 1 μg of pUVAI were incubated with the indicated protein fractions. Lane 1: 5 μl (VAI) or 20 μl of S100 (U6); lanes 2 and 3: 5 and 10 μl (VAI) or 25 and 50 μl (U6) of PCB; lanes 4 and 5: 5 and 10 μl (VAI) or 25 and 50 μl (U6) of EDF flowthrough; lanes 6–18: 10 μl (VAI) or 50 μl (U6) of fractions obtained by elution of the EDF column with a linear gradient from 60 to 500 mM KCl; lane 19: 15 μl of TBP-depleted PCC and 2.5 μl of PCA (VAI) or 40 μl of PCC and 2.5 μl of PCA and 100 ng of rhTBP (U6). The same amounts of protein fraction assayed in lane 19 alone were used to reconstitute the TFIIIB activity in lanes 2–18. (**B**) Western blot analysis of the protein fractions from (A) showing different hTFIIIB activity in the transcription of the mU6 and VAI gene respectively. Proteins were separated over a 12.5% polyacrylamide–SDS gel and transferred to nitrocellulose as described in Materials and methods. The position of TBP is indicated. Lane 1: 100 ng of rhTBP (PC 0.4); lane 2: 1 μg of individual marker proteins; lane 3: 20 μg of PCB; lane 4: 20 μg of EDF flowthrough; lanes 5–17: 50 μg of individual fractions which were obtained by elution of the EDF column with a linear gradient from 60 to 500 mM KCl. The immunoblot was performed using a 1:2000 dilution of mAb-TBP. The antigen–antibody complexes were visualized as described in Materials and methods.

and pol III, eluted with 300 mM KCl (Figure 1A, lanes 10–12), and this latter activity is clearly separated from that supporting U6 transcription. These activities were denoted as hTFIIIB-α and hTFIIIB-β. The activity eluting with 200 mM KCl (hTFIIIB-α), although yielding clear signals for U6 transcription, is only barely detectable when transcribing the VAI gene and, conversely, hTFIIIB-β eluting with 300 mM KCl has an at least 20-fold reduced ability to support transcription of U6 compared with that of the VAI gene. Additional evidence in support of at least two different forms of hTFIIIB stems from fractionated precipitation of PCB fractions with ammonium sulfate. At a saturation of 30%, hTFIIIB-β is quantitatively precipitated (Figure 6A), whilst the same fractions are virtually

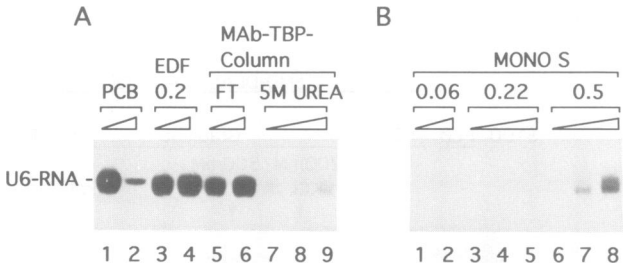

Fig. 2. *In vitro* transcription of the mouse U6 gene. The protein fractions which were assayed for their hTFIIIB-α content were obtained as described in Materials and methods. They were reconstituted with 40 µl of PCC, 2.5 µl of PCA and 100 ng of rhTBP. The position of U6 transcripts is indicated. (**A**) Lanes 1 and 2: 25 and 50 µl of PCB; lanes 3 and 4: 25 and 50 µl of a fraction eluting with 200 mM KCl from the EDF gradient; lanes 5 and 6: 25 and 50 µl flowthrough of the mAb-TBP column (MTA); lanes 7–9: 12.5, 25 and 50 µl of the fraction that was eluted with 5 M urea from the MTA. (**B**) Lanes 1 and 2: 25 and 50 µl of the Mono S flowthrough with 60 mM NaCl; lanes 3–5: 2.5, 5 and 10 µl of a fraction that was eluted with 220 mM NaCl from the Mono S; lanes 6–8: 2.5, 5 and 10 µl of the Mono S eluted with 0.5 M NaCl. The transcription reactions, performed on 4 µg of template DNA ($pUmU6_{0.34}$), were incubated and processed further as described in Materials and methods.

devoid of hTFIIIB-α, as determined by transcription of the U6 gene *in vitro* (data not shown).

hTFIIIB-α contains no detectable TBP

The fractions obtained by elution from an EDF column using a linear salt gradient (Figure 1A) were probed by Western blot analysis with a monoclonal antibody against hTBP (mAb-TBP) and these studies revealed that the peak of TBP content perfectly co-chromatographs with hTFIIIB-β activity (Figure 1B, lane 10) but that fractions with hTFIIIB-α activity did not contain detectable quantities of TBP (Figure 1B, lane 7). To exclude trace amounts of TBP in the latter fractions, they were loaded onto a mAb-TBP column and were step eluted with 60 mM KCl and 5 M urea. hTFIIIB-α was not retained by the antibody column since the flowthrough contained all the activity (Figure 2A, lanes 5 and 6) and none was detectable in the eluate (Figure 2A, lanes 7–9). In contrast, analysis of hTFIIIB-β-containing fractions from the mAb-TBP column in the transcription of the VAI gene, showed no activity in the flowthrough, with the bulk being eluted by 5 M urea from the mAb-TBP column (Figure 7D). These data indicate that hTFIIIB-α and hTFIIIB-β are distinct complexes which differ in their degree of association with TBP.

Estimation of the molecular mass of hTFIIIB-α

In order to determine the M_r of hTFIIIB-α, the corresponding fractions were purified further and concentrated simultaneously on a Mono S column from which the bulk of the activity was step-eluted with 0.5 M NaCl (Figure 2B, lanes 6–8). This fraction was loaded onto glycerol gradients and, as shown in Figure 3A, hTFIIIB-α sediments as a single peak with an apparent M_r of 60 ± 10 kDa, although an appreciable loss of hTFIIIB-α activity is observed. To analyse whether this decrease in activity is due to an additional splitting of hTFIIIB-α upon glycerol gradient centrifugation, the entire gradient was re-assayed in a U6 transcription system containing PBP, rhTBP,

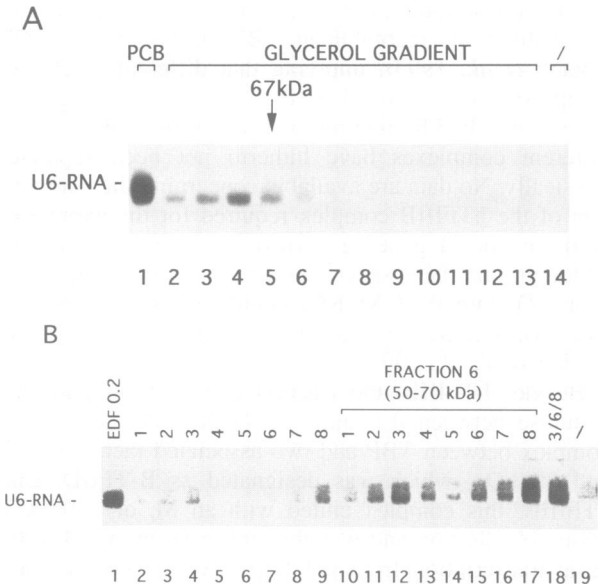

Fig. 3. (**A**) Glycerol gradient centrifugation of hTFIIIB-α. Lane 1: 25 µl of PCB; lanes 2–13: 50 µl of fractions 1–12 obtained by glycerol density gradient centrifugation; lane 14: 40 µl of PCC, 2.5 µl of PCA and 100 ng of rhTBP, which were also used to reconstitute the hTFIIIB-α activity in lanes 1–13. Glycerol density gradient centrifugation, as well as incubation and processing of transcription reactions, were conducted as described in Materials and methods. The position of U6 transcripts is indicated. (**B**) Renaturation of components of hTFIIIB-α obtained after SDS–PAGE. Proteins corresponding to a particular range of molecular mass were excised, eluted and renatured as described in Materials and methods. These proteins were assayed for their ability to reconstitute a minimal U6 transcription system consisting of 40 µl of PCC, 2.5 µl of PCA and 100 ng of rhTBP (lanes 1–19). Addition of components was as follows: lane 19: none; lane 1: 15 µl of the fraction eluted from the EDF column with 0.2 M KCl; lanes 2–9: 15 µl each of renatured eluates 1–8 (Materials and methods); lanes 10–17: the minimal transcription system (see above) was supplemented with 15 µl of eluate 6 containing renatured proteins with an M_r from 50 to 70 kDa. To these samples, 15 µl each of renatured eluates 1–8 were added as indicated; lane 18 contained the minimal system supplemented with 15 µl each of renatured eluates 3 (~25 kDa), 6 (~60 kDa) and 8 (~90 kDa).

TFIIA, pol III and a small amount of the 60 kDa hTFIIIB-α activity (Figure 3A, lane 4; fraction 3) obtained after glycerol gradient centrifugation. Under these conditions, transcription could be stimulated significantly by protein fractions sedimenting at 90 ± 10 kDa (data not shown). To substantiate these findings, the hTFIIIB-α fraction from the EDF gradient was separated by SDS–12.5% PAGE. Regions from the gel corresponding to a particular range of molecular mass were excised and proteins were subsequently eluted, precipitated and renatured by dialysis (Sachs and Kornberg, 1990). The renatured proteins corresponding to mean M_rs of ~90 kDa (80–100 kDa; Figure 3B, lane 9) and ~25 kDa (20–30 kDa; Figure 3B, lane 4) enhanced the activity of a minimal U6 transcription system. Addition of proteins with a mean M_r of 60 kDa (50–70 kDa) led to a concentration-dependent appearance of multiple transcription signals which were difficult to assess quantitatively. However, if renatured proteins with a mean M_r of 25 kDa (lane 12) or 90 kDa (lane 17) were assayed in the background of a minimal system supplemented with the renatured 60 kDa components (lane 7), the enhancement of U6 transcription was very clear. Addition of all three renatured proteins (lane 18) yielded

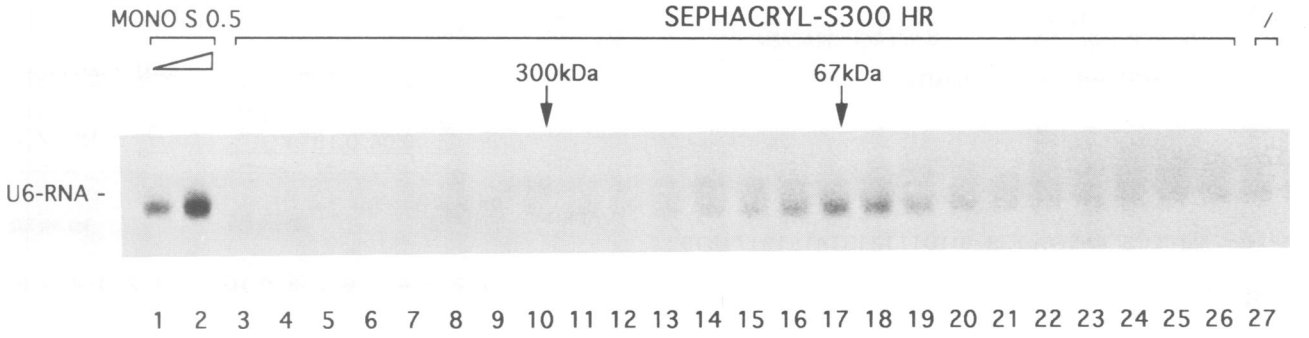

Fig. 4. Estimation of the molecular mass of hTFIIIB-α by Sephacryl-S300 HR gel filtration. The *in vitro* transcriptions of 4 μg of plasmid DNA ($pUmU6_{0.34}$) were reconstituted with 40 μl of PCC, 2.5 μl of PCA and 100 ng of rhTBP (lanes 1–27). Fifty μl of every fraction from the S300 molecular sieve were assayed for hTFIIIB-α activity (lanes 3–26). Lane 27 had no additions and 5 and 10 μl of the Mono S 0.5 load served as control (lanes 1 and 2). The mode of incubation and further processing, as well as the fractionation of proteins used in the transcription, are described in Materials and methods. The position of U6 transcripts is indicated.

a degree of transcription comparable with that achieved with the entire EDF 0.2 fraction (lane 1). These results indicate that hTFIIIB-α comprises more than one component and they show that proteins with M_rs of ~25, 60 and 90 kDa are contained in this complex.

In addition to these data, the size of the hTFIIIB-α activity, contained in fraction Mono S 0.5, was estimated by gel filtration on a Sephacryl-S300 HR molecular sieve. The results show that the activity of hTFIIIB-α elutes from this column in a region corresponding to an M_r of 60 ± 10 kDa (Figure 4). As was observed for glycerol gradients, the 60 kDa fraction likewise had lost more activity after gel filtration than could simply be explained by dilution of these protein fractions, and this gives support to the idea that dissociable components are separated from each other.

Further characterization of hTFIIIB-α

Protein fractions enriched in hTFIIIB-α obtained from gel filtration (Figure 4) were subsequently chromatographed on Cibacron Blue–agarose. hTFIIIB-α binds to this column, from which the bulk of activity can be eluted at 60 mM NaCl (Figure 5, lanes 3 and 4), while a lesser amount is collected in the 2 M NaCl/5 M urea step (lanes 5–7). When loading the Cibacron Blue 0.6 fraction onto a Ni-NTA column, most of its hTFIIIB-α activity is retained and can be eluted with 200 mM imidazole (Figure 5, lanes 9–11), a behaviour that had been observed previously during the purification of yeast TFIIIB (Huet *et al.*, 1994) and that possibly reflects a similarity in some components of these factors from yeast and mammalian cells. The protein fraction eluting with 200 mM imidazole from the Ni-NTA–agarose column was analysed for its peptide composition by SDS–PAGE (Figure 9, lane 1) and for its content of TBP by Western blotting. As observed before (Figure 1B), it was found that none of these hTFIIIB-α active fractions contained detectable quantities of TBP (data not shown). Although highly purified by conventional techniques involving seven different steps, silver staining of hTFIIIB-α revealed that this fraction still contains multiple polypeptides (Figure 9, lane 1) and, although renaturation studies delineated three ranges of molecular mass to be functionally important, it is presently impossible to associate individual polypeptides with the activity of hTFIIIB-α.

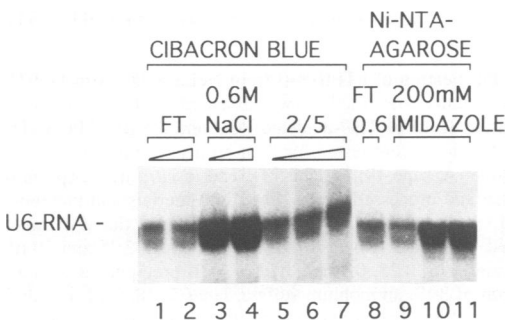

Fig. 5. Characterization of hTFIIIB-α by Cibacron Blue–agarose and Ni-NTA–agarose chromatography. The proteins were purified from HeLa S100 extracts as described in Materials and methods. The hTFIIIB-α activity of the individual fractions indicated was tested for its ability to reconstitute 40 μl of PCC, 2.5 μl of PCA and 100 ng of rhTBP in a U6 *in vitro* transcription assay (lanes 1–11). Incubation and processing of the samples is described in Materials and methods. The position of the U6 transcripts is indicated. Lanes 1 and 2: 25 and 50 μl of the Cibacron Blue–agarose flowthrough; lanes 3 and 4: 25 and 50 μl of the fraction eluting with 600 mM NaCl from Cibacron Blue–agarose; lanes 5–7: 12.5, 25 and 50 μl of the fraction that eluted with 2 M NaCl and 5 M urea from Cibacron Blue–agarose; lane 8: 50 μl Ni-NTA–agarose flowthrough; lanes 9–11: 12.5, 25 and 50 μl of the fraction eluting with 200 mM imidazole from Ni-NTA–agarose.

Properties of hTFIIIB-β

As was shown in Figure 1A, hTFIIIB-β, obtained by chromatography on EDF columns, supports transcription of the VAI gene *in vitro* when reconstituted with TFIIIC and pol III. Separation of hTFIIIB-β and hTFIIIB-α can also be achieved by several alternative methods, and the results support the conclusions obtained by EDF separation.

It was found that precipitation of phosphocellulose fraction B with 30% ammonium sulfate retained all the hTFIIIB-β activity in the precipitate (Figure 6A, lanes 4–6) but contained virtually no hTFIIIB-α activity, assessed by transcription of the U6 gene (not shown). Glycerol gradient centrifugation of the precipitated hTFIIIB-β activity (Figure 6A, lanes 7–18) reproducibly revealed a slightly faster sedimentation of hTFIIIB-β (Figure 3A), corresponding to that of the bovine serum albumin (BSA) standard. This region from the gradient containing hTFIIIB-β activity was pooled and subjected to gel filtration on a Sephacryl-S300 HR column from which it was

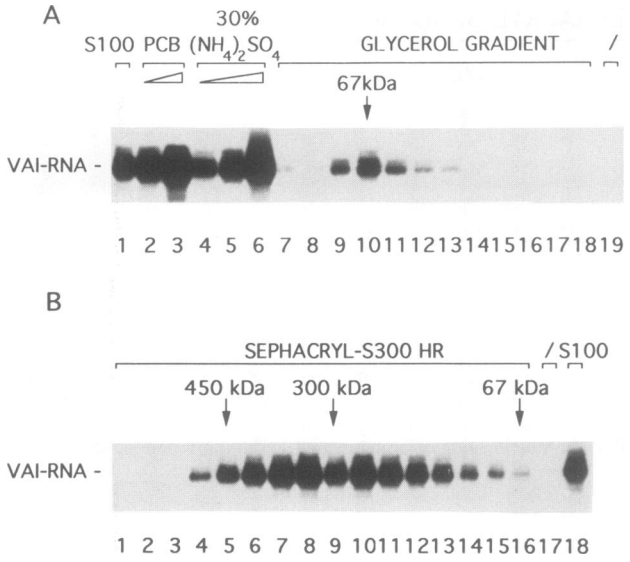

Fig. 6. Purification of hTFIIIB-β from HeLa S100 extracts. hTFIIIB-β activity of individual fractions was assessed by their ability to reconstitute 15 µl of TBP-depleted PCC and 2.5 µl of PCA. These protein fractions were tested for their ability to transcribe the VAI gene alone (A: lane 19; B: lane 17). The *in vitro* transcription was incubated and processed as described in Materials and methods using 1 µg of template DNA (pUVAI). The position of the VAI transcript is indicated. (**A**) Lane 1: 4 µl of S100; lanes 2 and 3: 5 and 10 µl of PCB; lanes 4–6: 0.25, 0.5 and 1 µl of PCB precipitated with a saturation of 30% ammonium sulfate; lanes 7–18: 3 µl of individual fractions that had been obtained by glycerol density gradient centrifugation as described in Materials and methods. (**B**) Lanes 1–16: 25 µl of fractions obtained by Sephacryl-S300 HR gel filtration as described in Materials and methods; lane 18: 4 µl of S100. The relative position of marker proteins is appropriately indicated.

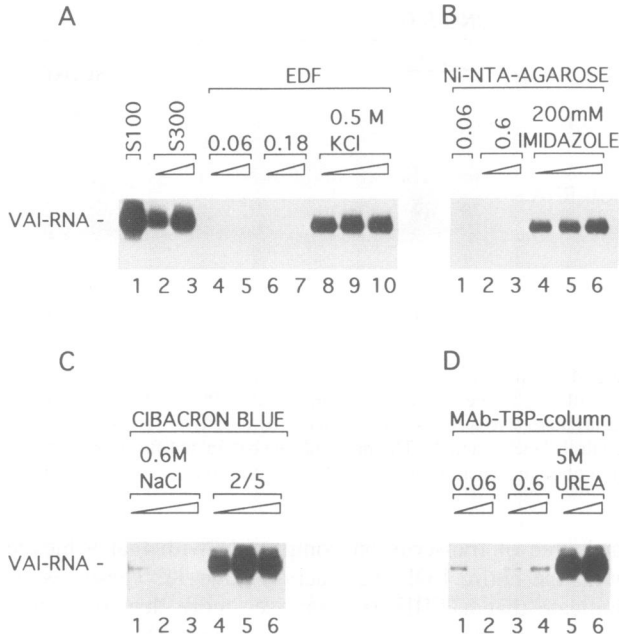

Fig. 7. Characterization of hTFIIIB-β activity on EDF, Ni-NTA–agarose, Cibacron Blue–agarose and affinity chromatography on a monoclonal anti-TBP column (MTA). The position of VAI transcripts is indicated. The proteins were purified as described in Materials and methods. *In vitro* transcription reactions were reconstituted with 15 µl of TBP-depleted PCC and 2.5 µl of PCA. They were incubated and processed as described in Materials and methods. (**A**) Chromatography of hTFIIIB-β on an EDF column. Lane 1: 4 µl of S100; lanes 2 and 3: 25 and 50 µl of fractions that eluted with 300 kDa from the S300 gel filtration; lanes 4 and 5: 25 and 50 µl of the EDF flowthrough with 60 mM KCl; lanes 6 and 7: 10 and 20 µl of EDF 180 mM KCl; lanes 8–10: 5, 10 and 20 µl of EDF 500 mM KCl. (**B**) Chromatography on Ni-NTA–agarose. Lane 1: 10 µl of Ni-NTA–agarose flowthrough with 60 mM KCl; lanes 2 and 3: 5 and 10 µl of Ni-NTA–agarose eluate with 600 mM KCl; lanes 4–6: 5, 10 and 20 µl of Ni-NTA–agarose eluate with 200 mM imidazole. (**C**) Chromatography on Cibacron Blue–agarose. Lanes 1–3: 5, 10 and 20 µl of the fractions eluted with 600 mM NaCl from Cibacron Blue–agarose; lanes 5–7: 5, 10 and 20 µl of the fractions eluted with 2 M NaCl and 5 M urea from Cibacron Blue–agarose, which were renatured by dialysis (Waldschmidt *et al.*, 1988) against a 1000-fold excess of buffer 2 containing 60 mM KCl. (**D**) Immuno-affinity chromatography. Lanes 1 and 2: 10 and 20 µl of the 60 mM KCl flowthrough of the mAb-TBP column (MTA); lanes 3 and 4: 10 and 20 µl of the MTA eluate with 600 mM KCl; lanes 5 and 6: 10 and 20 µl of the MTA eluate with 5 M urea, which were renatured by dialysis against a 1000-fold excess of buffer 2 containing 60 mM KCl.

recovered in a fairly broad peak corresponding to an M_r of 300 ± 30 kDa. Importantly, almost no transcription was observed by fractions eluting at 67 kDa (Figure 6B). Subsequently, these hTFIIIB-β-containing fractions from the S300 column were loaded onto an EDF column from which the activity was step-eluted with 500 mM KCl (Figure 7A; lanes 8–10). As observed before (Figure 1A), the fraction eluting with 180 mM KCl contained no hTFIIIB-β activity (lanes 6 and 7). The hTFIIIB-β activity, eluted in the 0.5 M KCl fraction, was next chromatographed in parallel either on Cibacron Blue–agarose or on Ni-NTA–agarose. As was already observed for hTFIIIB-α (Figure 5), hTFIIIB-β likewise binds to Ni-NTA–agarose from which it can be step-eluted with 200 mM imidazole (Figure 7B, lanes 4–6).

The chromatographic behaviour of hTFIIIB-β on Cibacron Blue–agarose differs from that of hTFIIIB-α on the same exchanger and is another parameter by which these two activities can be differentiated. hTFIIIB-β elutes almost entirely with 2 M NaCl/5 M urea with barely no activity in the 0.6 M NaCl step (Figure 7C), whereas previous experiments (Figure 5) had shown that the bulk of hTFIIIB-α activity elutes from this column at 0.6 M NaCl and a smaller portion at 2 M NaCl/5 M urea. It is conceivable that hTFIIIB-α is a partial complex of hTFIIIB-β and that both complexes share mutual peptides. It was of interest, therefore, to investigate whether hTFIIIB-α activity could be generated from pre-purified hTFIIIB-β fractions, and the differential behaviour of these complexes on Cibacron Blue–agarose was exploited to this end. The fractions described in Figure 7C were thus re-assayed for their hTFIIIB-α activity. The hTFIIIB fraction chromatographed on Cibacron Blue–agarose in Figure 7C was selectively enriched in hTFIIIB-β activity by prior precipitation with ammonium sulfate, size selection on Sephacryl-S300 HR and selective elution from EDF at 0.5 M KCl and it was consequently devoid of hTFIIIB-α activity. However, if the fractions eluted from Cibacron Blue–agarose were re-assayed for their ability to support U6 transcription, it was evident that a clearly detectable quantity of hTFIIIB-α was now eluted at 0.6 M from Cibacron Blue–agarose (Figure 8, lanes 1–4) with a lesser activity in the fraction eluted with 2 M NaCl/5 M urea (Figure 8, lanes 5–8), which is in accordance with the elution properties of hTFIIIB-α (see Figure 5). This result lends strong support to the assumption that the

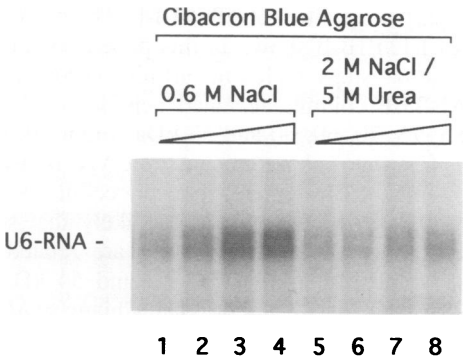

Fig. 8. hTFIIIB-α can be generated partially from a pre-purified fraction of hTFIIIB-β and it can be recovered from a Cibacron Blue–agarose column. The fractions obtained by Cibacron Blue–agarose chromatography in the course of hTFIIIB-β purification and renatured as described in Figure 7C were re-assayed for their ability to support transcription of the mouse U6 gene. Lanes 1–4: 5, 10, 20 and 50 µl of the Cibacron Blue–agarose fraction, eluted with 600 mM NaCl; lanes 5–8: 5, 10, 20 and 50 µl of the Cibacron Blue–agarose fraction, eluted with 2 M NaCl and 5 M urea. Fractions were reconstituted with 40 µl of PCC, 2.5 µl of PCA and 100 ng of rhTBP. The position of U6 transcripts is indicated.

hTFIIIB-α activity found here (Figure 8, lanes 1–4) was generated by partial dissociation from hTFIIIB-β. This is probably not a very efficient process, since the amount of hTFIIIB-α generated was low, as evidenced by the fact that the gel shown in Figure 8, lanes 1–8 had to be exposed for 96 h, as opposed to the standard exposure time of ~12 h, which is clearly reflected in a higher background of this particular gel.

Association of hTFIIIB-β with TBP

Protein fractions with hTFIIIB-β activity, purified as described here, were analysed by immunoblot for their TBP content and, as was reported earlier (Lobo *et al.*, 1992; Simmen *et al.*, 1992b; Taggart *et al.*, 1992; White and Jackson, 1992b; Chiang *et al.*, 1993), hTFIIIB-β activity co-fractionated strictly with TBP (data not shown). As an additional purification step, fractions from the EDF column were applied subsequently to a column to which mAb-TBP had been coupled. hTFIIIB-β activity could be eluted from this column with 5 M urea (Figure 7D, lanes 5 and 6) and its peptide composition was analysed by SDS–PAGE (Figure 9, lane 5). Besides TBP, 10 additional peptides are detectable, which could comprise hTFIIIB-β activity, but their functional significance obviously remains to be established. To evaluate this last hTFIIIB-β purification step, a control chromatography of the identical protein fraction was conducted with a monoclonal antibody directed against an irrelevant protein (ribosomal protein S3). The results show (Figure 9, lanes 3 and 4) that the polypeptide patterns, eluted with 5 M urea from the TBP and the non-specific Mab column, are clearly different. Although the latter column also seems to retain polypeptides weakly in a mass region of 50–68 kDa, these signals, which are equally present in the wash step of the column (lane 4), could also be due to an artefact of the silver staining procedure (Ochs, 1983).

Discussion

Initiation from all known pol III promoters requires the participation of TFIIIB. The promoter elements of these

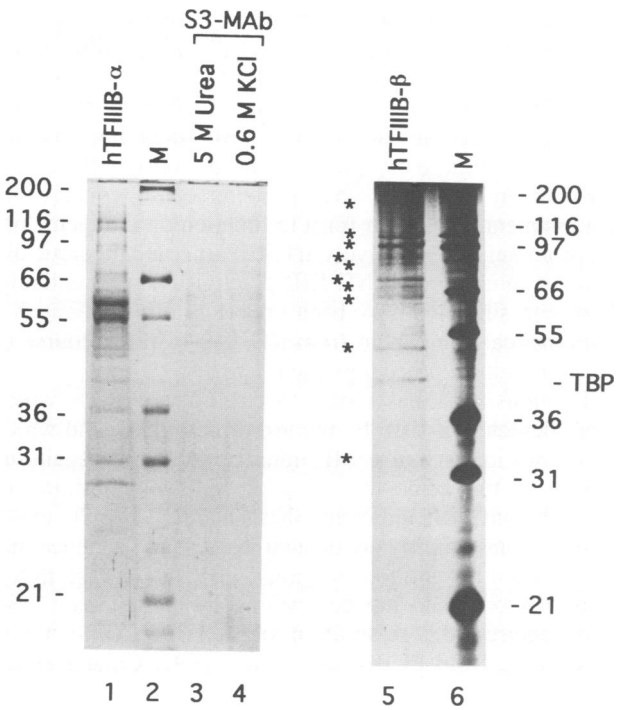

Fig. 9. SDS–PAGE analysis of hTFIIIB-α and hTFIIIB-β. Protein fractions showing hTFIIIB-α and hTFIIIB-β activity were separated, purified and silver stained as described in Materials and methods. Lane 1: the fraction eluting with 200 mM imidazole from the Ni-NTA–agarose showing hTFIIIB-α activity (4 µg protein); lanes 2 and 6: the migration of 0.5 and 1 µg of marker proteins is appropriately indicated. The hTFIIIB-β fraction, obtained after elution by 0.5 M KCl from the EDF column, was applied to a column containing mAb-TBP and subsequently eluted with 5 M urea. Lane 5 contained 100 µl (1 µg protein) of this eluate, and individual peptides are indicated by an asterisk. Alternatively, the same EDF 0.5 M KCl fraction was applied to a control column to which an irrelevant monoclonal antibody (directed against ribosomal protein S3) had been coupled (Harlow and Lane, 1988). This latter column was washed with 0.6 M KCl (lane 4) and likewise eluted with 5 M urea (lane 3). 100 µl of both eluates were applied to the SDS gel.

genes differ in their architecture and they are recognized by distinctly different DNA binding proteins. For human cells, it has not yet been clarified whether hTFIIIB activity is exhibited by the same or by unique protein complexes during the formation of pre-initiation complexes on 5′-regulated or intragenic pol III promoters. Here we show biochemical evidence that distinct forms of hTFIIIB exist, which are selectively active in the transcription of the U6 or the VAI gene.

hTFIIIB-α is separable from TBP

Chromatography of a phosphocellulose fraction B (PCB) over an EDF anion exchange column and elution with an appropriate gradient reveals two forms of hTFIIIB. One of these elutes from the column with 200 mM KCl and is devoid of TBP (hTFIIIB-α). The other hTFIIIB activity appears with 300 mM KCl (hTFIIIB-β) and strictly co-elutes with TBP over the next five columns. hTFIIIB-α functions predominantly as an initiation factor in the context of the 5′-located pol III promoters, exemplified in this paper by transcription of the mouse U6 gene. It displays a very low activity on the intragenic pol III promoters assayed by transcription of the VAI gene. This

could be due to the possibility that hTFIIIB-α is a partial activity of hTFIIIB-β, lacking TBP and possibly other factors. However, when testing hTFIIIB-α in the transcription of the VAI gene, its low activity cannot be enhanced by adding recombinant human TBP (data not shown), showing that these complexes differ not only in their TBP content. In addition, other proteins could conceivably complement hTFIIIB-α for it to function on intragenic pol III promoters. Alternatively, hTFIIIB-α could theoretically contain an inhibitor of hTFIIIB-β activity. This is unlikely, however, since we have been unable to dissociate such a hypothetical component from hTFIIIB-α in the course of the purification procedure used here.

Previous work has shown that cell-free extracts which were depleted of TBP by the use of antibodies and which were thus inactive in pol III transcription could regain the ability to transcribe the U6 gene by the re-addition of recombinant TBP, indicating that a hTFIIIB activity exists which is not tightly associated with, and is hence not precipitated by, antibodies against TBP. In contrast, tRNA transcription could not be restored by recombinant TBP and required the re-addition of a TBP–TAF complex originating from PCB (Lobo *et al.*, 1992; Chiang *et al.*, 1993).

Several research groups have fractionated PCB and have characterized protein fractions which were devoid of TBP but which were required to restore VAI transcription in conjunction with other protein fractions. Taggart *et al.* (1992) described a protein (TAF L) of unknown molecular mass only loosely associated with—and hence dissociable from—TBP. Controversial results have been presented in the literature concerning the separation of components of hTFIIIB by chromatography on Mono Q into a TBP-containing and a TBP-free fraction which were only jointly able to complement TFIIIC and pol III in the transcription from class 1 and 2 pol III promoters. Interestingly, these two TBP-free activities were described as eluting either with 0.48 M (Lobo *et al.*, 1992) or 0.21 M KCl (Chiang *et al.*, 1993) from the same exchange material, possibly reflecting the segregation of different proteins from a complex or the use of different reconstitution systems, but the exact interrelationship of these two fractions is presently unclear. On the basis of indirect evidence, it was assumed previously that the 0.48 M Mono Q fraction discussed above could represent the component of hTFIIIB required for U6 transcription (Sadowski *et al.*, 1993; Hernandez, 1993), and it is thus possible that this component bears a relationship to hTFIIIB-α described here, but definitive data to prove the identity of these components have not yet been obtained.

hTFIIIB-β activity co-elutes with TBP

hTFIIIB activity, assayed in the transcription from intragenic promoters, is tightly associated with TBP (Figures 1B and 7D). As discussed above, it has nevertheless been described that chromatography of a PCB over Mono Q (Lobo *et al.*, 1992; Chiang *et al.*, 1993) or depletion with antibodies raised against TBP (Taggart *et al.*, 1992) could separate the TBP-free fractions mentioned above from TBP–TAF complexes. The latter components alone were unable to transcribe the tRNA or VAI genes and additionally required the fraction which was devoid of TBP. For that reason, the TAFs described in these studies can only represent partial activities of hTFIIIB-β. The most purified fraction of hTFIIIB-β shown in this paper, obtained after six purification steps including affinity chromatography on a mAb-TBP column, revealed peptides of 190, 110, 97, 90, 85, 75, 70, 60, 50 and 33 kDa (Figure 9, lane 5). Since this fraction is able to reconstitute VAI transcription efficiently by a TBP-depleted phosphocellulose fraction C (Figure 7D; lanes 5 and 6), it is likely that some of these peptides (190, 97, 90, 60, 50) are related to the previously published TAFs (150, 82 and 54 kDa, Lobo *et al.*, 1992; 190, 96, 87 and 60 kDa, Chiang *et al.*, 1993; 172 kDa, Taggart *et al.*, 1992). The other proteins which have not been described until now (110, 85, 75, 70 and 33 kDa) could be contained in the TBP-free hTFIIIB preparations used in the other studies and which were required to reconstitute VAI transcription.

hTFIIIB-α and hTFIIIB-β differ in size

Estimation of the molecular mass of hTFIIIB-β by different techniques yielded an interesting result: separation on a glycerol density gradient showed the peak of hTFIIIB-β activity in fractions which co-sedimented with BSA (67 kDa). If these fractions were loaded onto a Sephacryl-300 HR molecular sieve, the maximum activity appeared with an apparent M_r of 300 ± 30 kDa. Although we cannot offer a convincing explanation, it confirms our original observation (Waldschmidt *et al.*, 1988) that hTFIIIB activity sedimented with an M_r of ~67 kDa on glycerol gradients. Possibly the interactions of the proteins in the hTFIIIB-β complex are destabilized by increasing concentrations of glycerol and the centrifugal force, which leads to the simulation of a smaller molecular mass. After centrifugation, the components can re-assemble to form the complete hTFIIIB-β complex which then elutes from gel filtration according to its real mass of ~300 kDa, as has been observed repeatedly by other authors (Simmen *et al.*, 1992b). Alternatively, glycerol gradients may be entirely unsuitable for analysing the native molecular mass of large, unstable protein complexes, since it has also been reported that the multisubunit factor g from yeast cells exerts a lower mass upon glycerol density gradient centrifugation than that estimated by gel filtration (Henry *et al.*, 1992). Whatever the true explanation is for this startling phenomenon, the sizing of hTFIIIB-β in two entirely different windows of apparent molecular mass by different techniques provides a discriminating purification procedure. hTFIIIB-α, in contrast, shows the same behaviour on glycerol density gradient centrifugation and Sephacryl-S300 HR gel filtration. Its peak of activity always elutes with an M_r of 60 ± 10 kDa, which is slightly lower than that of hTFIIIB-β upon glycerol density gradient centrifugation. This may also indicate that components of hTFIIIB-α are partially separated on the glycerol density gradient and the maxima of transcriptional activity reflect the overlap of individual components. Renaturation of hTFIIIB-α activity after SDS–12.5% PAGE strengthens this assumption, since proteins of 25, 60 and 90 kDa were described which clearly had an effect on U6 transcription. Since these components could dissociate easily in the absence of TBP, the estimation of the native mass of 60 kDa could be interpreted as an overlap of the three individual maxima. In addition, such an explanation could serve as a model

to understand the loss of hTFIIIB-α activity upon glycerol gradient centrifugation.

Other properties of hTFIIIB-α and hTFIIIB-β

hTFIIIB-α and hTFIIIB-β differ in size and TBP content and, accordingly, in differential ability to bind to an affinity column containing monoclonal antibodies against TBP. In addition, they show different affinities to Cibacron Blue–agarose. Whilst the bulk of hTFIIIB-α can be eluted from this column with 600 mM NaCl, the major portion of hTFIIIB-β can only be recovered from this exchange material using 2 M NaCl/5 M urea. Purification of yeast TFIIIB by Klekamp and Weil (1986) showed TFIIIB activity only in the 2 M NaCl/5 M urea step, whereas another published purification scheme used a buffer with 50% glycerol, resulting in the elution of yeast TFIIIB activity with 700 mM NaCl from Cibacron Blue–agarose (Kassavetis *et al.*, 1989). Extended studies from the latter group suggested that the same components of yeast TFIIIB are necessary and sufficient for the transcription of tRNA and U6 genes. Nevertheless, it remains possible that ryTBP, ryBRF1 and renaturated B″ as TFIIIB components are sufficient but not optimal for the transcription of a yeast tRNA gene, and only the addition of other proteins, which could be contained in a TBP–TAF complex (Poon and Weil 1993), restores full transcriptional activity to this promoter. Alternatively, the observation that the same components are required for expression of the yeast U6 and tRNA genes (Huet *et al.*, 1994; Joazeiro *et al.*, 1994) may reflect the similarity in the architecture of the yeast tRNA and U6 promoters, possibly classifying the latter yeast gene as a classical pol III gene.

It is possible that hTFIIIB-α and hTFIIIB-β activities are partially attributable to similar or identical proteins. They both bind to Ni-NTA–agarose and are eluted by 200 mM imidazole, as has been observed already for yeast TFIIIB. This chromatographic behaviour could be due to proteins which are shared between hTFIIIB-α and hTFIIIB-β. This observation could also suggest a relationship between these proteins and one of the components of the yeast factor. Moreover, some hTFIIIB-α activity is recovered in the 600 mM NaCl wash step of the 6th column (Cibacron Blue–agarose) in the course of hTFIIIB-β purification, indicating that hTFIIIB-α can be split off from hTFIIIB-β (Figure 8, lanes 1–8). The exact composition of hTFIIIB-α remains to be determined, but it minimally contains proteins of 25 ± 5, 60 ± 10 and 90 ± 10 kDa.

Based on the data presented in this paper, we suggest that hTFIIIB-α contains all the polypeptides necessary to function as a proper initiation factor for pol III. In the case of the intragenic pol III promoters, these proteins must be supported by several other factors associated with TBP to contact TFIIIC which is a prerequisite to obtain a high level of specific initiation. These TAFs could in turn sterically hinder the incorporation of hTFIIIB-β into the U6 pre-initiation complex, thereby limiting transcription of the gene. By definition, TFIIIB functions as an initiation factor proper of RNA polymerase III (Kassavetis *et al.*, 1990). This ability must thus be exerted by components common to hTFIIIB-α and hTFIIIB-β, which is in agreement with our observation that hTFIIIB-α can be generated from hTFIIIB-β as discussed above. Renaturation studies have identified proteins of ~60 and 90 kDa to be functionally essential for U6 transcription and, since polypeptides of the same molecular mass are also contained in hTFIIIB-β, it is highly probable that one (or both) of these components is responsible for correct initiation from all pol III promoters. As is the case for TFIIB in the pol II transcription system, these peptides are not necessarily tightly associated with TBP and they cannot thus be considered as pol III TAFs in the strict sense of the word.

Materials and methods

HeLa cells and extracts

Cytoplasmic extracts (S100) from HeLa cells were prepared from several batches of 20 l suspension cultures with an index of 5×10^5 cells/ml as previously described (Waldschmidt *et al.*, 1988). Extracts with a protein concentration of 15 mg/ml were stored at –80°C.

Plasmids

The plasmids pUVAI and $pUmU6_{0.34}$ were as previously described (Schneider *et al.*, 1989; Waldschmidt *et al.*, 1991) and contained a single copy of the genes coding for VAI RNA and mouse U6 snRNA respectively.

Purification of hTFIIIB-α

Buffer 1: 20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 3 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF); buffer 2: 20 mM Tris–HCl, pH 7.9, 10% (v/v) glycerol, 5 mM $MgCl_2$, 3 mM DTT, 0.2 mM PMSF; buffer 3: 50 mM Tris–HCl, 50 mM borate, pH 8.3, 0.01% SDS, 10% methanol.

HeLa cell extract (~100 ml S100) was dialysed against 100 volumes of buffer 1 including 100 mM KCl and applied to a phosphocellulose column at 15 mg of protein/ml bed volume. The flowthrough (PCA; 5 mg protein/ml) was collected and the column was washed extensively with the same buffer. It was subsequently eluted with buffer 1 containing 0.35 M KCl (PCB; 3 mg protein/ml), and 0.6 M KCl (PCC; 1.5 mg protein/ml). Fifty ml of PCB were dialysed against 100 volumes of buffer 2 including 60 mM KCl and then applied to a 20 ml EMD-DEAE-Fractogel (EDF) column supplied by Merck, Darmstadt. It was washed with the same buffer, and proteins were eluted with a linear gradient of 60–500 mM KCl. Fractions eluting with 200 mM KCl from several EDF columns (25 ml of fraction EDF 0.2; 1 mg protein/ml) subsequently were subjected to chromatography over protein A–Sepharose to which mAb-TBP had been covalently coupled (Harlow and Lane, 1988). This column was washed with buffer 2 including 200 mM KCl [fraction mAb-TBP column (MTA) flowthrough; 1 mg protein/ml] and bound proteins were eluted with buffer 2 including 5 M urea (MTA 5 M urea; 5 μg protein/ml). Twenty-five ml flowthrough of the MTA column were dialysed against 100 volumes of buffer 1 including 60 mM NaCl and loaded onto a HR 5/5 Mono S column, which was then washed with 10 volumes of the same buffer to yield the Mono S 0.06 fraction (0.2 mg protein/ml). It was subsequently eluted stepwise with 220 mM NaCl (Mono S 0.22; 1 mg protein/ml) and 500 mM NaCl (Mono S 0.5; 2.5 mg protein/ml). Three ml of Mono S 0.5 were dialysed against 100 volumes of buffer 2 including 60 mM KCl and applied to a 150 ml Sephacryl-S300 HR gel filtration column. Thirty individual 4 ml fractions were collected, representing M_rs from 1.5 MDa (void) to 10 kDa. Alternatively, 200 μl of the dialysed Mono S 0.5 were layered on a linear 12.5–30% glycerol gradient. Twelve ml (700 μg of protein) obtained by Sephacryl-S300 HR gel filtration and representing an M_r of 60 ± 10 kDa were pooled and subjected to chromatography over a 1 ml Cibacron Blue–agarose column which was equilibrated with buffer 2 including 60 mM NaCl. The flowthrough of that column, eluted with buffer 2 and 60 mM NaCl, contained 30 μg protein/ml. Bound proteins were eluted by addition of 0.6 M NaCl (CB 0.6; 40 μg protein/ml) or 2 M NaCl and 5 M urea to the equilibration buffer (CB 2/5; 10 μg protein/ml). The fraction CB 0.6 was applied directly to a 1 ml Ni-NTA–agarose column supplied by QIAGEN. The flowthrough was eluted with buffer 2 including 0.6 M NaCl (Ni 0.6; 20 μg protein/ml) and the column was then eluted with buffer 2 including 200 mM imidazole (10 μg protein/ml).

Recombinant human TBP was prepared as described (Waldschmidt *et al.*, 1992) with the following modifications: fraction HS 0.6 was dialysed against 100 volumes of buffer 1 with 100 mM KCl and loaded

onto a 1 ml phosphocellulose column. Bound rhTBP was eluted with buffer 1 containing 400 mM KCl (PC 0.4).

Purification of hTFIIIB-β

PCB fractions (100 ml) were pooled and precipitated at a saturation of 30% (176 g/l) ammonium sulfate. The pellet was resuspended in 3 ml of buffer 2 including 60 mM KCl and dialysed against 100 volumes of the same buffer. Two hundred µl of this fraction [PCB 30% $(NH_4)_2SO_4$; 20 mg protein/ml] were layered onto multiple glycerol density gradients. Three ml (4 mg) of fractions sedimenting with 67 kDa were pooled and subjected to chromatography over a 150 ml Sephacryl-S300 HR gel filtration column. This column was eluted with buffer 2 containing 60 mM KCl, and 30 individual fractions of 4 ml each were collected. Two-hundred ml (5 mg protein) fractions of several preparations which eluted with 300 kDa from the molecular sieve were pooled and applied to a 1 ml EDF column. This column was washed with buffer 2 including 60 mM KCl (EDF 0.06; 15 µg protein/ml) and eluted with 200 mM KCl (EDF 0.2; 300 µg protein/ml) and 500 mM KCl (EDF 0.5; 200 µg protein/ml). Five ml of EDF 0.5 were dialysed against 100 volumes of buffer 2 containing 60 mM KCl and loaded on a 250 µl protein A–Sepharose column to which mAb-TBP had been covalently coupled (MTA column; Harlow and Lane, 1988). The flowthrough was obtained by washing the column with buffer 2 and 60 mM KCl (MTA 0.06; 200 µg protein/ml). Bound proteins were eluted with buffer 2 and 600 mM KCl (MTA 0.6; 3 µg protein/ml) and 5 M urea (MTA 5 M urea; 5 µg protein/ml). Alternatively, dialysed EDF 0.5 fractions were subjected to chromatography over a 1 ml Cibacron Blue–agarose column and step-eluted with 60 mM (150 µg protein/ml) and 600 mM NaCl (35 µg protein/ml). The remainder was then eluted with 2 M NaCl and 5 M urea (10 µg protein/ml). Additionally, an EDF 0.5 fraction was further purified by Ni-NTA–agarose chromatography. This fraction was applied to a 1 ml column in buffer 2 containing 60 mM KCl and step-eluted with 60 mM KCl (140 µg protein/ml), 600 mM KCl (25 µg protein/ml) and with 200 mM imidazole (20 µg protein/ml). In order to obtain a phosphocellulose C (PCC) fraction suitable for the reconstitution of hTFIIIB-β, it was necessary to deplete TBP from this fraction. The TBP-depleted PCC was prepared by chromatography of 10 ml PCC (15 mg protein) over the MTA column. The flowthrough of this column (1.5 mg protein/ml) was 95% depleted of hTBP.

In vitro transcription

The *in vitro* transcription reactions were performed as previously described (Jahn *et al.*, 1987) but with the following modifications: 1 µg of pUVAI template DNA was incubated for 120 min in a final volume of 60 µl. Four µg of $pUmU6_{0.34}$ were incubated for 180 min at 30°C in a final volume of 110 µl. *In vitro* synthesized RNA products were separated electrophoretically on 6% denaturating urea sequencing gels and autoradiographed for at least 12 h at –80°C with an intensifying screen.

Rate zonal centrifugation on glycerol gradients

Analysis by glycerol density gradient centrifugation was performed as described in Waldschmidt *et al.* (1991).

SDS–PAGE

SDS–PAGE was carried out as described by Laemmli (1970). Proteins were visualized by silver stain (Morrissey, 1981).

Western blot analysis

After fractionation on SDS–PAGE, proteins were transferred to nitrocellulose in buffer 3 using the BIO RAD transblot cell. After transfer, the proteins on the filter were stained with Ponceau S and further processed as described in Waldschmidt *et al.* (1990). Detection of antigen–antibody complexes was achieved by employing ECL (Amersham).

Renaturation of proteins after SDS–PAGE

The SDS gels were cut into individual slices of 1 cm width each. From these slices, proteins were eluted and renatured as described (Sachs and Kornberg, 1990). The numerically annotated eluates represent approximate ranges of the following M_rs: eluate 1, <15 kDa; eluate 2, 15–20 kDa; eluate 3, 20–30 kDa; eluate 4, 30–40 kDa; eluate 5, 40–50 kDa; eluate 6, 50–70 kDa; eluate 7, 70–80 kDa; eluate 8, 80–100 kDa

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