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The activity binding to the termination region of several pol III genes represents a separate entity and is distinct from a novel component enhancing U6 snRNA transcription

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ABSTRACT

Human $TFIIIC_1$, a basal transcription factor essentially required for expression of all pol III genes, exerts its function without primarily binding to DNA. We report here the purification of a termination site binding activity (TBA) which was initially described to be contained in fractions designated as $TFIIIC_0$. TBA specifically and strongly binds to the termination region of pol III genes with internal promoters and can be completely separated from $TFIIIC_1$ and a $TFIIIC_1$ related activity ($TFIIIC_{1\text{-like}}$), proving that DNA-binding of TBA is independent of these latter activites. Although TBA is not essentially required for, it strongly stimulates pol III transcription from intragenic promoters. This stimulation strictly depends on the presence of $TFIIIC_1$ and is not observed in conjunction with $TFIIIC_{1\text{-like}}$. We further present the identification of a novel activity, TFIIIU, which is also contained in crude fractions of $TFIIIC_0$. TFIIIU can be separated from TBA by further purification and is essentially involved in transcription of the mammalian U6 gene. TFIIIU cannot be substituted for by any of the established U6 transcription factors and thus represents a novel U6 transcription factor.

INTRODUCTION

Genes transcribed by RNA polymerase III (pol III) can be classified by the promoter structures which control their expression (1). While type 1 genes (5S rRNA) contain internally located A- and C-boxes as well as an intermediate element, type 2 genes (e.g. tRNA and VAI) are controlled by A- and B-boxes within the gene. Expression of type 2 pol III genes requires transcription factors TFIIIC and TFIIIB, whereas synthesis of 5S rRNA additionally requires the primarily DNA-binding protein TFIIIA. Type 3 pol III promoters in higher eukaryotic cells (e.g. U6, 7SK) are governed exclusively by promoter sequences located upstream of the initiation site which include a TATA-box, as well as a proximal sequence element (PSE) and a distal sequence element (DSE). Transcription of these latter genes minimally requires the TATA-binding protein TBP, the PSE-binding protein PBP (2) also designated as PTF (3–5) or SNAPc (6–8), TFIIIB and pol III.

Accurate initiation of transcription of pol III genes is best understood in the yeast system and in the case of the tRNA genes this process depends on the sequence specific interaction of TFIIIC with the B-box promoter element and the subsequent incorporation of the initiation factor TFIIIB and the polymerase itself by protein–protein interactions (1,9–11).

In mammalian cells the situation is more complex. First, it has been shown that human transcription factor IIIB can be separated into functionally different forms TFIIIB-α and TFIIIB-β. The latter is a TBP/TAF complex required for expression of genes with intragenic promoters. In contrast, TFIIIB-α is devoid of TBP and is responsible for the *in vitro* transcription of U6 snRNA (12). Second, $TFIIIC_2$ primarily associates with the B-box but this by itself does not suffice to support efficient transcription in the presence of TFIIIB and the polymerase, implying that additional factors, which participate in transcription complex formation, are required (13,14). The mechanisms of complex formation are hitherto poorly understood in higher eukaryotes and until recently only one of these auxilliary components has been described. This activity was purified from crude fractions of TFIIIC (PC 0.6) and was hence designated as $TFIIIC_1$ (13,15). $TFIIIC_1$ alone does not bind to DNA, but extended the DNAse I footprint over the A-box when added to $TFIIIC_2$ primarily bound to the B-box (13). In contrast to $TFIIIC_2$, which is only required for transcription of type 1 and 2 pol III genes (13,16–18), $TFIIIC_1$ is essentially required for transcription of all pol III genes (3,19). $TFIIIC_1$ positively influences the DNA-binding activity of all three pol III assembly factors: $TFIIIC_2$, TFIIIA and the PSE-binding protein PBP (18,19).

Although $TFIIIC_1$ was consistently reported to be required for transcription, its binding properties to intragenic pol III promoter genes were discussed controversely. $TFIIIC_1$, described by Wang and Roeder (18), was reported to show a strong interaction with the termination site of type 2 genes. In contrast, no termination site binding was found for this factor from the laboratory of Berk (13) and from our laboratory (19). Moreover, we have shown

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recently that $TFIIIC_1$ is chromatographically and functionally distinct from an activity which binds specifically and strongly to the termination region of pol III genes with internal promoters. This DNA-binding activity was initially described to be contained in fractions designated as $TFIIIC_0$ and this fraction could also partially substitute for $TFIIIC_1$ activity (19). Until now, however, it was unclear whether the termination site binding activity (TBA) and the $TFIIIC_1$ related activity ($TFIIIC_{1\text{-like}}$) of these $TFIIIC_0$ fractions were mediated by the same or different components. We show here that TBA can be separated from $TFIIIC_1$ and $TFIIIC_{1\text{-like}}$, demonstrating that the specific DNA-binding of TBA is independent of $TFIIIC_1$ or related activities.

$TFIIIC_1$ functions as a basal pol III factor and thus is also essentially required for transcription of the 5′ regulated 7SK RNA and U6 snRNA genes (3,19). However, Yoon *et al*. (3) reported that $TFIIIC_1$ could only complement a heat-treated extract from human cells lacking TFIIIC activity but not a reconstituted system (consisting of PTF, Oct-1, TBP and TFIIIB) for 7SK transcription. From these findings the authors implied that additional factors, contained in the heat-treated extract, might be necessary. Using an *in vitro* transcription system consisting of PBP, TBP, TFIIIB-α, RNA pol III (2,12) and $TFIIIC_1$ we also find that the mouse U6 gene is poorly transcribed under such conditions. Employing this reconstituted system we identified a novel activity, TFIIIU, which is essentially required for transcription of the mammalian U6 snRNA gene. Although TFIIIU co-elutes with the termination site binding activity TBA upon MQ chromatography, we show in this report that these activities can be separated by further purification and that TFIIIU has no effect on VAI transcription.

MATERIALS AND METHODS

Plasmids

The plasmids $pUmU6_{0.34}$, pUVAI, $pUht_i^{met}$ and pUh5S were as previously described (2,20,21) and contained single copies of the genes coding for mouse U6 snRNA, VAI RNA, human methionine initiator tRNA or synthetic human 5S rRNA.

Preparation of nuclear extract and purification of transcription factors

Buffer A contained 20 mM HEPES, pH 7.9, 10% (v/v) glycerol, 3 mM DTT, 0.2 mM PMSF. Buffer B contained 20 mM Tris–HCl, pH 7.9, 10% (v/v) glycerol, 5 mM $MgCl_2$, 3 mM DTT, 0.2 mM PMSF.

Nuclear extracts from human embryonic kidney (HEK) or HeLa cells were prepared from several batches of 35 l suspension cultures with an index of 7.4×10^5 cells/ml as described previously (22). The nuclear extract was dialyzed against buffer A including 100 mM KCl and was subsequently chromatographed through phosphocellulose (PC) as described previously (23).

The PCC fraction (600 mM KCl) was used to purify human $TFIIIC_0$, $TFIIIC_1$, $TFIIIC_2$ and PBP as described before (19) with the following modifications.

PBP. The PCC fraction was dialyzed against buffer B containing 60 mM KCl, loaded onto a Mono Q column ('MQ', Pharmacia) at 10 mg protein/ml bed volume. After washing the column with the same buffer, bound proteins were eluted with a linear gradient from 60 to 450 mM KCl. Fractions eluting at 300–330 mM KCl contained PBP (0.2 mg protein/ml).

$hTFIIIC_1$. Fractions eluting from 250 to 290 mM KCl in buffer B from Mono Q ($MQ_{0.27}$; 19) were diluted to 200 mM KCl in buffer B and applied to an EMD SO_3^- Fractogel column ('ESF'; Merck, Darmstadt) at 5 mg protein/ml bed volume. After washing the column with 200 mM KCl in buffer B, bound proteins were eluted with a linear gradient from 200 to 600 mM KCl and a 1 M KCl step. $hTFIIIC_1$ activity eluted with 450–500 mM KCl ($ESF_{0.47}$; 0.2 mg protein/ml).

$hTFIIIC_0$. Fractions eluting from 150 to 190 mM KCl in buffer B from the MQ gradient ($MQ_{0.17}$; 19) were applied to an EMD SO_3^- Fractogel column (Merck) at 5 mg protein/ml bed volume. After washing the column with 200 mM KCl in buffer B, bound proteins were eluted with a linear salt gradient from 200 to 600 mM KCl and a 1 M KCl step. Termination region binding activity (TBA) eluted from 390 to 480 mM KCl (0.12 mg protein/ml), TFIIIU eluted from 365 to 415 mM KCl (0.09 mg protein/ml), $TFIIIC_{1\text{-like}}$ eluted from 500 to 545 mM KCl (0.04 mg protein/ml). Alternatively, MQ $TFIIIC_0$ fractions (150–190 mM KCl) were dialyzed against buffer B with 60 mM KCl, applied to a Resource S column ('RS', Pharmacia), washed with buffer B containing 170 mM KCl and eluted with a linear gradient from 170 to 600 mM KCl. TBA eluted with 220–265 mM KCl (0.05 mg protein/ml), $TFIIIC_{1\text{-like}}$ eluted with 320 mM KCl (0.04 mg protein/ml) from this column. The flowthrough, containing TFIIIU, was directly applied onto an EMD SO_3^- Fractogel column (Merck) and eluted with a linear gradient from 200 to 600 mM KCl in buffer B. Fractions eluting from 345 to 410 mM KCl contained TFIIIU.

The PCB fraction (350 mM KCl) was used to purify human TFIIIB-α, TFIIIB-β and RNA polymerase III (pol III) as described before (12,19). Recombinant human TBP (rhTBP) was expressed in *Escherichia coli* and purified as described (24). Recombinant human TFIIIA (rhTFIIIA) was expressed in *E.coli* and purified as described (19,25).

In vitro transcription

The assays were as described previously (19,23). For type 1 and 2 pol III genes, 0.2 or 1 µg of plasmid DNA was used in 50 µl reactions. For U6 transcription, 0.5 or 2 µg of plasmid DNA was used in 70 µl reactions. All reactions were conducted in the presence of 10 U Ribonuclease Inhibitor (Stratagene, Heidelberg). *In vitro* synthesized RNA products were electrophoretically separated on 6% (VAI RNA) or 7.5% denaturing urea sequencing gels and autoradiographed for at least 1 day at –80°C with intensifying screens.

DNAse I footprint analysis

Fragments were generated by PCR amplification of the VAI gene from pUVAI with 5′ end-labelled 17mer sequencing primer, unlabelled 17mer reverse sequencing primer (M13/pUC; Boehringer, Mannheim) and Goldstar DNA Polymerase (Eurogentec). Individual protein fractions were pre-incubated with 2 µg poly (dIdC) and 0.5 µg pUC 9 DNA as non-specific competitor in a total volume of 50 µl for 15 min at 30°C. Subsequently 15 000 c.p.m. of the labelled fragment were added and incubation was prolonged for 90 min at 30°C. Samples were cleaved with 40 ng of DNAse I for 1 min at room temperature, reactions were stopped with 50 µl of stop solution and further processed as described before (19,21). An end-labelled *Msp*I

digest of pBR322 was used as a size marker to assign the protected regions.

RESULTS

Purification of the termination site binding activity TBA from $TFIIIC_0$ fractions

Starting from phosphocellulose fraction C (PCC), derived from nuclear extracts of human cells, we could recently enrich an activity by MQ FPLC chromatography which showed a strong interaction with the termination region of type 1 and 2 pol III genes (19). This activity eluted at 170 mM KCl from the MQ column and such MQ fractions were denoted as $TFIIIC_0$ (Materials and Methods) to distinguish them from $TFIIIC_1$ and $TFIIIC_2$. $TFIIIC_1$ activity eluting at 270 mM KCl from MQ columns did not bind to DNA and never showed DNase I protection on pol III genes with internal promoters (19).

Since the MQ $TFIIIC_0$ fraction is comparatively crude, it was further purified in this report by FPLC chromatography on ESF columns. Bound proteins were eluted with a linear gradient from 200 to 600 mM KCl, and the DNA-binding activity of individual fractions was analyzed by footprint experiments. As shown in Figure 1A the activity which displayed strong DNase I protection over the termination region of the VAI gene from +154 to +182 was eluted from this column by 390–480 mM KCl (lanes 6–10) and it was hence denoted as termination site binding activity (TBA). The boundaries of the footprint are very distinct and no protection of the A- and B-boxes is observed.

The $TFIIIC_0$ fraction additionally contains a $TFIIIC_1$ related activity ($TFIIIC_{1\text{-like}}$)

MQ $TFIIIC_0$ fractions containing the DNA-binding activity TBA were initially described to be able to partially substitute for $TFIIIC_1$ activity in transcription reactions (19). Therefore, individual fractions of the ESF gradient (Fig. 1A) were assayed for their ability to reconstitute transcription of the VAI, human methionine initiator transfer RNA and human 5S rRNA genes *in vitro* in a system deficient in $TFIIIC_1$. As demonstrated in Figure 1B, fractions containing the termination site binding activity TBA failed to reconstitute transcription of any of these genes in the absence of $TFIIIC_1$ (compare Fig. 1A and B, lanes 6–10). However, transcription could be restored in all cases by fractions 11–13 of the same gradient eluting from 500 to 545 mM KCl (Fig. 1B, lanes 11–13). Conversely, the latter fractions failed to bind the termination region of the VAI gene (Fig. 1A, lanes 11–13). Because the transcriptional activity, stemming from a $TFIIIC_0$ fraction, can complement a $TFIIIC_1$ deficient system, but is separated from $TFIIIC_1$ by MQ chromatography, we have denoted this activity as $TFIIIC_1$ like activity ($TFIIIC_{1\text{-like}}$). It should be pointed out that the maximal level of transcription mediated by $TFIIIC_{1\text{-like}}$ fractions corresponds to that observed for the $TFIIIC_0$ input-fraction, but is much less than found for $TFIIIC_1$ (see also Fig. 2).

TBA stimulates VAI transcription only in a system containing $TFIIIC_1$

Since TBA which binds with high affinity to the termination region of the VAI gene fails to restore transcription of this gene in the absence of $TFIIIC_1$ (Fig. 1B, lanes 6–10), we tested individual fractions of the ESF gradient in a transcription system which contained $TFIIIC_1$ transcriptional activity (Fig. 2A, lane 2). Under such conditions, the highest level of VAI RNA synthesis was observed upon addition of fractions 7–10 (Fig. 2A, compare lanes 8–11 with lane 2) which corresponded to the termination region binding activity TBA (Fig. 1A, lanes 6–10).

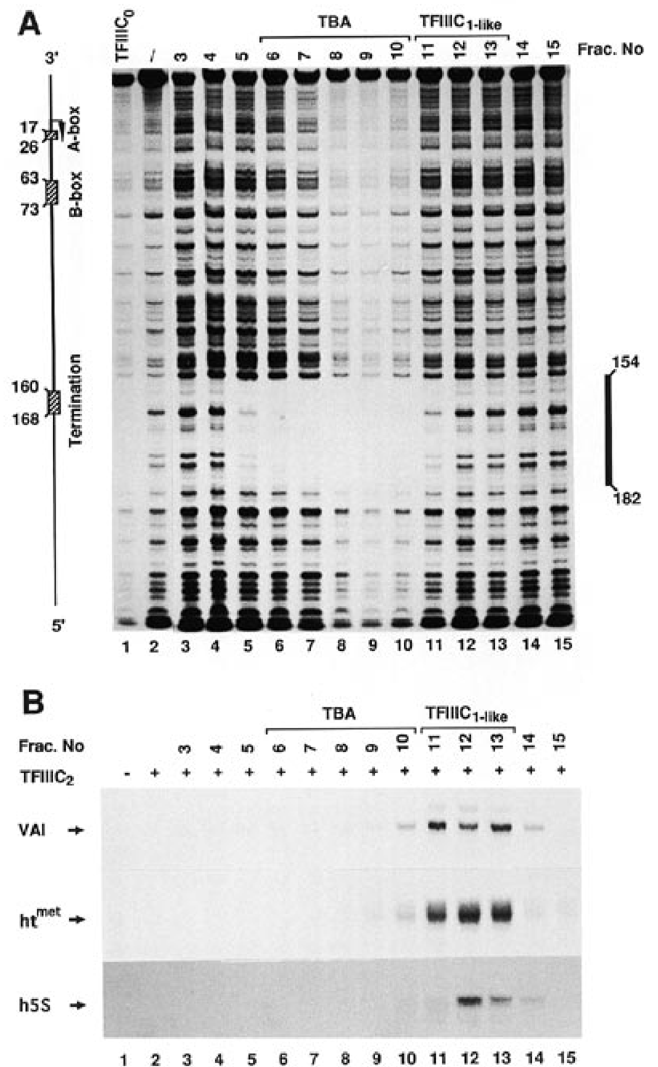

Figure 1. Purification of the MQ $TFIIIC_0$ fraction by ESF chromatography. (**A**) DNAse I protection of the VAI termination region by TBA. A 321 bp fragment of the VAI gene, labelled at the 5′ end of the coding strand, was incubated with 2 µg of $TFIIIC_0$ ($MQ_{0.17}$) fraction (lane 1) or 40 µl of the individual fractions of the gradient (lanes 3–15) for footprint analysis as described in Materials and Methods. Lane 2 served as a control reaction without protein. The protected region of the VAI gene, spanning sequences from +154 to +182, is appropriately indicated. (**B**) TBA does not support transcription in a $TFIIIC_1$ deficient system. Fractions of the ESF-gradient were assayed for their ability to complement VAI, ht_i^{met} or h5S rRNA transcription. *In vitro* transcription was performed separately for the three genes as described in Materials and Methods, and the positions of the individual transcripts are indicated. One microgram of each template was incubated with 50 ng of pol III ($ESF_{0.55}$) and 4 µg of TFIIIB-β ($ESF_{0.31}$, lanes 1–15). For lanes 2–15, 0.3 µg of $TFIIIC_2$ ($MQ_{0.5}$) and for lanes 3–15, 20 µl of the individual fractions of the ESF gradient were added as indicated. For *in vitro* transcription of the h5S gene all samples were supplemented with 1 µl rhTFIIIA.

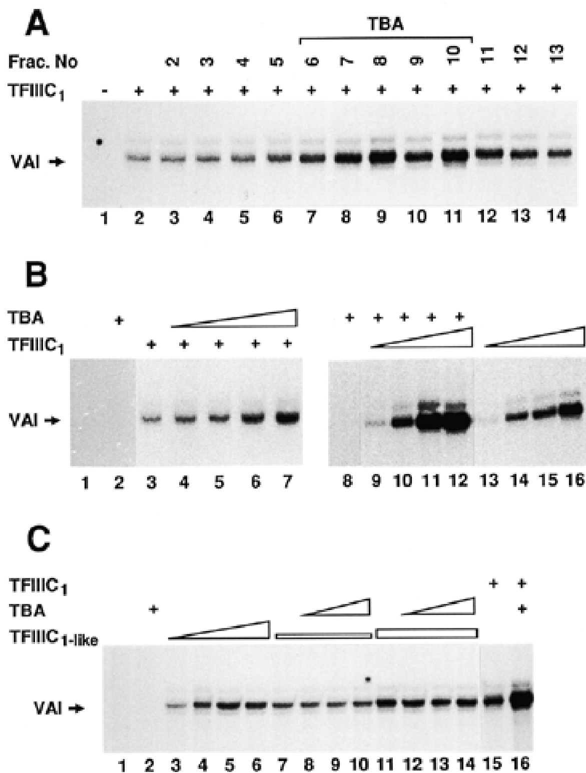

Figure 2. (**A**) TBA stimulates VAI transcription in a system containing $TFIIIC_1$. ESF-gradient fractions were analyzed in a $TFIIIC_1$ containing reconstitution system. The *in vitro* transcription of 0.2 µg of pUVAI was conducted in the presence of 10 ng of pol III ($ESF_{0.55}$), 0.8 µg of TFIIIB-β ($ESF_{0.31}$), 60 ng of $TFIIIC_2$ ($MQ_{0.5}$) and 200 ng of $TFIIIC_1$ ($ESF_{0.47}$). For lane 1, $TFIIIC_1$ was omitted. Five microlitres of individual ESF gradient fractions 2–13 were added as indicated (lanes 3–14) and *in vitro* transcription was performed as described in Materials and Methods. The position of the VAI transcript is indicated. (**B**) Transcriptional activity of TBA depends on the presence of $TFIIIC_1$. *In vitro* transcription of 0.2 µg of pUVAI was performed in the presence of highly purified pol III, TFIIIB-β and $TFIIIC_2$ (lanes 1–16) as described for (A). For lanes 3–7, 0.2 µg of $TFIIIC_1$ ($ESF_{0.47}$) were analyzed in the presence of increasing amounts of TBA (0.13, 0.25, 0.5 and 1 µg of fraction ESF-Grad. 430 mM KCl, lanes 4–7). For lanes 2 and 8, 1 µg of TBA (ESF-Grad. 430 mM KCl) were added alone. For lanes 9–16, increasing amounts of $TFIIIC_1$ (0.1, 0.25, 0.5 and 1 µg of fraction $ESF_{0.47}$) were analyzed in the presence of 1 µg of TBA (ESF-Grad. 430 mM KCl, lanes 9–12) or were added alone to the basal transcription system (lanes 13–16). (**C**) $TFIIIC_{1\text{-like}}$ cannot fully substitute for $TFIIIC_1$ activity. *In vitro* transcription of 0.2 µg of pUVAI was performed in the presence of highly purified pol III, TFIIIB-β and $TFIIIC_2$ (lanes 1–16) as described for (A). Increasing amounts of TBA [0.25 µg (lanes 8 and 12), 0.5 µg (lanes 9 and 13) and 1 µg (lanes 2, 10 and 14) of fraction ESF-Grad. 430 mM KCl] were analyzed in the presence of 0.24 µg (lanes 7–10) or 0.48 µg (lanes 11–14) of $TFIIIC_{1\text{-like}}$ (ESF-Grad. 520 mM KCl) or 0.5 µg of $TFIIIC_1$ ($ESF_{0.47}$, lane 16). For lanes 3–6, 0.06, 0.12, 0.24 and 0.48 µg of $TFIIIC_{1\text{-like}}$ (ESF-Grad. 520 mM KCl), for lane 15, 0.5 µg of $TFIIIC_1$ ($ESF_{0.47}$) were added alone to the basal transcription system.

The effect on transcription mediated by TBA strictly depends on and is related to the quantity of $TFIIIC_1$ present. Results from titration experiments depicted in Figure 2B show that TBA had no effect in the absence of $TFIIIC_1$ (lanes 2 and 8), but increasing amounts of TBA in the presence of $TFIIIC_1$ clearly led to a potentiation of transcription (compare lanes 4–7 with lane 3). To analyze more rigorously the interdependence of TBA and $TFIIIC_1$, the inverse experiment was conducted in lanes 8–16 in which the amount of $TFIIIC_1$ was titrated in the presence of the highest concentration of TBA tested in lane 7. The results imply that a particular relation of the two components is required for their optimal interaction (compare lanes 9–12 with lanes 13–16). Nevertheless, efficient VAI RNA synthesis could be observed with high amounts of $TFIIIC_1$ in the absence of TBA (lanes 13–16), suggesting that transcription can be augmented by, but does not depend on, TBA. Identical results were found for 5S rRNA transcription (data not shown).

As demonstrated in lanes 3–6 of Figure 2C, transcription of the VAI gene in the presence of TFIIIB-β (12), pol III and $TFIIIC_2$ could be partially complemented by $TFIIIC_{1\text{-like}}$, although the extent of RNA synthesis was much lower than observed for reactions which contained $TFIIIC_1$ instead of $TFIIIC_{1\text{-like}}$ (compare with lanes 13–16 of Fig. 2B). Importantly, addition of TBA containing fractions strongly stimulated VAI RNA synthesis only in the presence of $TFIIIC_1$ (compare lane 16 with lane 15) but not in conjunction with $TFIIIC_{1\text{-like}}$ (compare lanes 8–10 with lane 7 and lanes 12–14 with lane 11). These results suggest that $TFIIIC_{1\text{-like}}$ represents an incomplete form or a subcomplex of $TFIIIC_1$ which cannot fully substitute for $TFIIIC_1$ activity.

Identification of an activity necessary for U6 snRNA transcription

$TFIIIC_1$ is essentially required for transcription of all pol III genes including the U6 gene. However, expression of the mouse U6 snRNA gene cannot be fully restored by $TFIIIC_1$ in the presence of PBP, pol III, TFIIIB-α and rTBP as will subsequently be dealt with in more detail (Fig. 3B and C).

We found previously that transcription of the mU6 snRNA gene was more efficiently reconstituted by the MQ $TFIIIC_0$ fraction than by the MQ $TFIIIC_1$ fraction (19). In order to check whether the U6 gene requires an additional activity besides $TFIIIC_1$, which could possibly be contained in the $TFIIIC_0$ fraction of the MQ gradient, individual fractions of the ESF gradient were analyzed with respect to their ability to reconstitute transcription of the mU6 gene *in vitro* in the presence of $TFIIIC_1$, PBP, pol III, TFIIIB-α and rTBP. Under such conditions U6 snRNA synthesis was strongly stimulated by an activity, eluting from 365 to 415 mM KCl (fractions 5–7) from this gradient (Fig. 3A, compare lanes 5–7 with lane 1). This activity is distinct from TBA (as will be discussed later) and is subsequently referred to as TFIIIU, because it stimulates transcription of the U6 snRNA gene containing an upstream promoter. It has previously been described (12,26–28) that *in vitro* transcription of the U6 gene yields a characteristic double-band which results from two specific U6 transcripts although the mechanism by which these transcripts are generated is presently unclear. Figure 3 also shows that their relationship to each other is not influenced by TFIIIU.

In order to further analyze whether TFIIIU is essentially required for transcription of the mU6 snRNA gene, appropriate controls were conducted in Figure 3B. The maximal level of U6 snRNA synthesis could be observed upon addition of TFIIIU in the presence of highly purified pol III, TFIIIB-α (12), PBP (2), rTBP (24) and $TFIIIC_1$ (lanes 6–8). In contrast, TFIIIU had no effect in a $TFIIIC_1$ deficient reconstitution system (lane 9). Moreover, even high amounts of $TFIIIC_1$ in the absence of TFIIIU only slightly restored U6 transcription, indicating that $TFIIIC_1$ is required but not sufficient for efficient U6 synthesis

Figure 3. A novel component, TFIIIU, is involved in *in vitro* transcription of the mU6 snRNA gene. (**A**) Influence of ESF gradient fractions on reconstituted U6 transcription *in vitro*. Ten microlitres of the individual ESF-gradient fractions 2–15 (lanes 2–15) were analyzed for their ability to reconstitute U6 transcription in the presence of 15 ng of pol III ($ESF_{0.55}$), 2 µg of TFIIIB-α ($ESF_{0.5}$), 0.5 µg of PBP ($MQ_{0.32}$), 0.12 µg of $TFIIIC_1$ ($ESF_{0.47}$) and 20 ng of rhTBP (lanes 1–15). *In vitro* transcription was carried out as described in Materials and Methods using 0.5 µg $pUmU6_{0.34}$ as template DNA. The position of the U6 transcript is indicated. (**B**) TFIIIU strongly enhances U6 transcription. *In vitro* transcription of 0.5 µg of $pUmU6_{0.34}$ was performed in a basal system containing highly purified pol III, TFIIIB-α, PBP and rhTBP (lanes 2–13) as described for (A). For lane 1, PBP was omitted. For reconstitution of U6 transcription, TFIIIU activity (0.9 µg of ESF-Grad. 370 mM KCl, lane 9) was analyzed in the presence of increasing amounts of $TFIIIC_1$ (0.2, 0.4 and 0.8 µg of fraction $ESF_{0.47}$, lanes 6–8) or of $TFIIIC_{1\text{-like}}$ (0.06, 0.12 and 0.24 µg of fraction ESF-Grad. 520 mM KCl, lanes 10–12). For lanes 3–5, 0.2, 0.4 and 0.8 µg of $TFIIIC_1$ ($ESF_{0.47}$), for lane 13, 0.24 µg of $TFIIIC_{1\text{-like}}$ (ESF-Grad. 520 mM KCl) were added alone to the basal reconstitution system. (**C**) TFIIIU is required for efficient U6 transcription. To verify whether TFIIIU is distinct from the hitherto established and required human U6 transcription factors, each component of a fully reconstituted U6 transcription system was individually omitted as indicated above each lane. The reactions were incubated and processed as described in Materials and Methods using 2 µg of $pUmU6_{0.34}$, 60 ng of pol III ($ESF_{0.55}$, lanes 1–4 and 6–10); 8 µg of TFIIIB-α ($ESF_{0.5}$, lanes 1–5 and 7–10); 2 µg of PBP ($MQ_{0.32}$; lanes 2–6, 8 and 10); 0.4 µg of $TFIIIC_1$ ($ESF_{0.47}$, lanes 1–6 and 9); 0.9 µg (lane 3) and 1.8 µg (lanes 4–7 and 9–10) of TFIIIU (ESF-Grad. 370 mM KCl). All reactions were supplemented with 80 ng of rhTBP.

(lanes 3–5) in this reconstitution system. U6 snRNA synthesis could be reconstituted, albeit to a much lower extent, by $TFIIIC_{1\text{-like}}$ in the presence of TFIIIU (compare lanes 10–12 with lanes 6–8), supporting the observation that $TFIIIC_1$ and $TFIIIC_{1\text{-like}}$ are functionally related but not identical. $TFIIIC_{1\text{-like}}$ had no effect in a TFIIIU deficient reconstitution system (lane 13).

Additional controls were conducted in Figure 3C. Confirming the data derived from Figure 3B, a dramatic stimulation of U6 transcription was mediated by high amounts of TFIIIU in a $TFIIIC_1$ containing transcription system (compare lanes 3 and 4 with lane 2). This reconstitution system also depends on each of the known U6 transcription factors, PBP (lanes 1, 7 and 9), TFIIIB-α (lane 6), $TFIIIC_1$ (lanes 7, 8 and 10) and pol III (lane 5), because no U6 synthesis was observed when one of these factors was omitted. Since U6 transcription also depends on exogenously added TBP (6,12,29,30) we tested the TBP content of the TFIIIU fraction by western blot but found no detectable TBP in this fraction (data not shown). This finding indicates that TFIIIU can functionally not be substituted for by any of these activities. Thus, our data functionally characterize TFIIIU as a novel factor which is required for transcription of the mU6 gene *in vitro* in a reconstitution system containing purified pol III, TFIIIB-α, PBP, $TFIIIC_1$ and rTBP.

TFIIIU is distinct from TBA

Although TFIIIU eluted at 365–415 mM KCl from the ESF column (Fig. 3A, lanes 5–7) and the elution profile of this factor hence overlapped with that of the DNA-binding activity TBA (390–480 mM KCl; Fig. 1A, lanes 6–10), both activities are clearly different. Using an alternative ion exchange resin, Resource S (RS), we could completely separate TFIIIU from TBA. While the specific DNA-binding activity TBA bound to the Resource S column and eluted at 220–265 mM KCl (Fig. 4A, lanes 5–7), TFIIIU was contained in the flowthrough of this column (Fig. 4B, compare lanes 3 and 4 with lane 2) which did not show any DNAse I protection of the VAI gene (Fig. 4A, lanes 2 and 3). To verify that the stimulation of U6 snRNA synthesis by the RS flowthrough is in fact mediated by TFIIIU, we re-chromatographed this fraction on an ESF column. It was found that the U6 specific activity displayed the same elution profile (data not shown) as was found when MQ $TFIIIC_0$ fractions were applied directly to the ESF column, confirming that the RS flowthrough in fact contained TFIIIU.

It should be emphasized that TFIIIU had no effect on VAI transcription, neither in a $TFIIIC_1$ deficient (Fig. 4C, lanes 2 and 3 compared with lane 1) nor in a $TFIIIC_1$ containing reconstitution system (compare lanes 6–8 with lane 5 and also compare with lanes 3 and 4 of Fig. 4B). These data not only prove that TFIIIU and TBA are distinct activities but also show that TFIIIU acts on the U6 and not on the VAI gene.

DISCUSSION

In yeast cells the same six polypeptides of the multiprotein complex TFIIIC are sufficient for DNA-binding and transcription (31–35). The mammalian counterpart of this factor, however, appears to be more complex. Human TFIIIC activity can be separated by MQ chromatography into three functionally distinct activities: $TFIIIC_0$, $TFIIIC_1$ and $TFIIIC_2$ (13,18,19). MQ $TFIIIC_0$ fractions show a strong interaction with the termination region of several pol III genes and can also partially substitute for $TFIIIC_1$ activity (19). In the course of further purification studies of such $TFIIIC_0$ fractions presented here, we identified three chromatographically and functionally distinct activities: the termination region binding activity TBA, a transcriptional

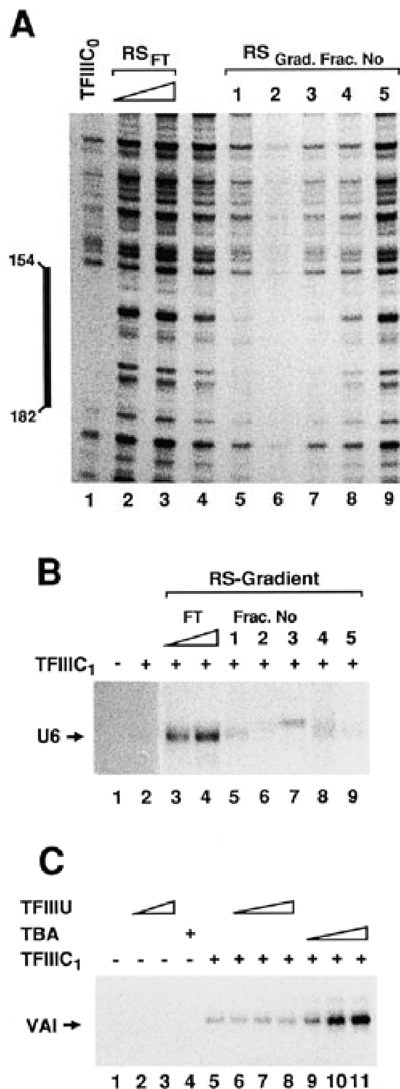

Figure 4. TFIIIU is distinct from TBA. (**A**) Footprint analysis of fractions obtained by RS chromatography. A 321 bp fragment of the VAI gene, labelled at the 5′ end of the coding strand, was incubated with either $TFIIIC_0$ (2 µg of the input $MQ_{0.17}$ fraction, lane 1), 25 or 50 µl of the 170 mM KCl flowthrough of the RS column (lanes 2 and 3) or with 40 µl of the RS eluate fractions 1–5 (lanes 5–9) for footprint analysis as described in Materials and Methods. Lane 4 served as a control reaction without protein. The protected region is appropriately indicated relative to the VAI gene. (**B**) The flowthrough of the RS column contains TFIIIU activity. Individual fractions of the RS gradient were tested in U6 snRNA transcription *in vitro* for TFIIIU activity. All reactions contained 25 ng of pol III ($ESF_{0.55}$), 4 µg of TFIIIB-α ($ESF_{0.5}$), 1 µg of PBP ($MQ_{0.32}$) and 40 ng rhTBP. Reactions shown in lanes 2–9 additionally contained 0.4 µg of $TFIIIC_1$ ($ESF_{0.47}$). 10 and 20 µl of the flowthrough of the RS column (lanes 3 and 4) or 15 µl of the fractions 1–5 (lanes 5–9) eluted from the RS column were added and reactions were performed as described in Materials and Methods using 1 µg $pUmU6_{0.34}$. The position of the U6 transcript is indicated. (**C**) TFIIIU activity does not complement VAI transcription. To verify the specifity of TFIIIU for U6 transcription, the flowthrough of the RS column was analyzed for its ability to reconstitute or stimulate VAI transcription *in vitro* in the absence or presence of $TFIIIC_1$. *In vitro* transcription of 0.2 µg of pUVAI was performed in the presence of highly purified pol III, TFIIIB-β, $TFIIIC_2$ (lanes 1–11) as described for (A). Reactions shown in lanes 5–11 additionally contained 0.2 µg of $TFIIIC_1$ ($ESF_{0.47}$). Further components were added as indicated above each lane: 0.2 µg (lane 6), 0.4 µg (lanes 2 and 7) and 0.8 µg (lanes 3 and 8) of TFIIIU (flowthrough of the RS column); 0.25 µg (lane 9), 0.5 µg (lane 10) and 1 µg (lanes 4 and 11) of TBA (ESF-Grad. 430 mM KCl).

activity which resembles $TFIIIC_1$ ($TFIIIC_{1\text{-like}}$) and an activity stimulating U6 transcription (TFIIIU).

The termination site binding activity (TBA) is a distinct entity and can be completely separated from $TFIIIC_1$ and related activities

$TFIIIC_1$ can already be completely separated from TBA by MQ chromatography (19) and such fractions of $TFIIIC_1$ are transcriptionally fully active (Fig. 3) without showing any DNA footprint.

TBA co-elutes with a transcriptional activity upon MQ chromatography which can partially substitute for $TFIIIC_1$ and which we hence designated as $TFIIIC_{1\text{-like}}$. However, further purification by ESF (Fig. 1) or, alternatively, by RS chromatography (data not shown) completely separated these two activities from each other. Remarkably, this separation neither influenced the binding activity of TBA nor the transcriptional capacity of $TFIIIC_{1\text{-like}}$. TBA contained in the more purified ESF- or RS-fractions, still binds with high affinity to the termination region of the VAI gene but does not support transcription in the absence of $TFIIIC_1$, thus reinforcing that DNA-binding does not depend on $TFIIIC_1$ or related activities (Figs 1A and 4A). Moreover, $TFIIIC_1$ did not affect DNAse I protection of the termination region mediated by TBA (data not shown), although enhancing the binding of other primary DNA-binding pol III factors $TFIIIC_2$, TFIIIA and PBP (18,19).

In conclusion, our studies showed that TBA, the activity which binds to the termination region, is distinct from $TFIIIC_1$ and $TFIIIC_{1\text{-like}}$. Neither $TFIIIC_1$ nor $TFIIIC_{1\text{-like}}$ bind primarily to DNA and both reconstitute pol III transcription. Consequently, TBA does not represent an integral part of these transcriptional activities.

In agreement with our results, no termination binding activity was described for $TFIIIC_1$ or its related activity by Yoshinaga *et al.* (13). Hence we suppose that $TFIIIC_{1\text{-like}}$ reported here is related to the $TFIIIC_1$ related activity described by Yoshinaga *et al.* (13), because both activities stem from phosphocellulose fraction C, reveal a similar elution profile from MQ chromatography and complement VAI transcription to a much lesser extent than $TFIIIC_1$.

In contrast, $TFIIIC_1$ and a functionally fully equivalent isoform $TFIIIC_1'$ were described by Wang and Roeder (18), both of which correlated with strong binding activity to the termination region on VAI and tRNA genes and it is hence conceivable that these fractions contained TBA in addition to $TFIIIC_1$.

TBA stimulates VAI transcription only in the presence of $TFIIIC_1$ but not $TFIIIC_{1\text{-like}}$

TBA by itself cannot complement a $TFIIIC_1$ deficient transcription system (Fig. 1) but significantly stimulates VAI (Fig. 2) or 5S rRNA (data not shown) synthesis in the presence of $TFIIIC_1$ which documents that a particular relation of the two components is required for their optimal interaction (Fig. 2B). Importantly, TBA exerts its function to enhance VAI transcription only in the presence of $TFIIIC_1$ and not $TFIIIC_{1\text{-like}}$ (Fig. 2C), suggesting a functional interaction between TBA and $TFIIIC_1$. Moreover, these data show that $TFIIIC_1$ and $TFIIIC_{1\text{-like}}$ are not identical. Both $TFIIIC_1$ and $TFIIIC_{1\text{-like}}$ stem from phosphocellulose C fractions but could be separated from each other by MQ chromatography. Since it is conceivable that the multiprotein complex $TFIIIC_1$ can be partially disrupted in the course of the

fractionation procedure, we conclude from these findings that TFIIIC$_{1\text{-like}}$ could represent either a modified form or a subcomplex of TFIIIC$_1$ lacking at least that domain or subunit of TFIIIC$_1$ which is necessary to mediate the stimulatory activity of TBA.

Since TFIIIC$_1$ alone is able to fully restore transcription of type 1 and 2 pol III genes in the presence of TFIIIC$_2$, TFIIIB-β and pol III (Fig. 2B), TBA seems to be an activity which is not essentially required for pol III transcription *in vitro*, but which enhances transcription by an as yet unclarified mechanism involving the termination region.

However, template commitment experiments which we performed have not yet elucidated whether the binding of TBA to the termination region of the VAI gene is strong enough to strictly commit enhanced transcription to the first template (data not shown). Since these experiments revealed that DNA-binding of TBA *per se* is not sufficient, it is possible that protein–protein contacts between TBA and (an)other component(s) of the transcription complex are required and that such interactions must precede the event of DNA-binding to establish an activated transcription complex. Alternatively, it is possible that the dissociation rate of the binding of TBA is too high for template commitment.

Chu *et al*. (36) recently reported, for the human system, that palindromic sequences immediately preceding the termination region increased pol III transcription activity. Possibly these palindromic sequences are involved in stimulation of VAI transcription which we observed upon addition of the termination region binding factor TBA. Additionally, Dieci and Sentenac (37) found that the termination element of yeast pol III genes appears to be required to enable polymerase III to enter the facilitated reinitiation pathway. It is possible that the enhanced transcription level supported by TBA is related to these findings. Likewise, a specific binding activity for U-terminated nascent RNA was described for the human autoimmune antigen La (38) which was also discussed to clear the template for subsequent rounds of transcription (39). However, western blot analysis revealed that La did not co-chromatograph with TBA activity (data not shown).

A novel activity, TFIIIU, stimulates U6 snRNA transcription

We have described recently that transcription of the U6 snRNA gene is more effectively reconstituted by MQ TFIIIC$_0$ fractions than by MQ fractions containing TFIIIC$_1$ (19). Using an *in vitro* U6 transcription system comprising purified PBP, TFIIIB-α, pol III and rTBP (2,12), addition of highly purified TFIIIC$_1$ only resulted in a low level of U6 snRNA synthesis (Fig. 3). In agreement with this observation, Yoon *et al*. (3) reported for the 7SK gene that TFIIIC$_1$ could complement a heat-treated nuclear extract lacking TFIIIC activity but not a reconstituted system, possibly implying an additional component in the nuclear extract.

By analyzing individual fractions of the ESF gradient derived from MQ TFIIIC$_0$ fractions for their ability to further reconstitute U6 transcription in the presence of TFIIIC$_1$, we identified an activity eluting with 365–415 mM KCl which strongly stimulated U6 transcription (Fig. 3A). We designated this activity as TFIIIU (stimulating the U6 gene; exemplified here for pol III genes with an upstream located promoter). TFIIIU was unable to complement htRNA (data not shown) or VAI transcription and was shown to be chromatographically and functionally different from TBA (Fig. 4). Remarkably, U6 transcription could not be supplemented with high amounts of either TFIIIC$_1$ or TFIIIU alone (Fig. 3B and C), whereas a combination of both led to high levels of U6 RNA synthesis, indicating that both activities are essentially required. These studies confirm that TFIIIC$_1$ functions as an essential basal pol III factor but they also show that TFIIIC$_1$ *per se* is not sufficient for transcription of mammalian U6 snRNA gene in the presence of all hitherto known U6 transcription factors.

The mechanism by which TFIIIU acts is as yet unknown, but we demonstrate that this activity is probably not related to any of the other established proteins required for formation of the U6 transcription complex (Fig. 3C), two of which, PBP and TFIIIB-α, are specific for pol III genes with upstream promoter elements (2,12,40). The proximal sequence element of the U6 gene is specifically recognized by the PSE-binding protein PBP, also designated as PTF (3–5) or SNAPc (6–8). As shown in Figure 3C, no U6 snRNA synthesis could be observed in the absence of PBP, indicating that TFIIIU cannot substitute for and thus is distinct from PBP. Moreover, neither TFIIIU nor TFIIIC$_1$ alone showed any DNAse I protection of upstream located promoter or gene specific sequences (data not shown). No PSE protection could likewise be observed when both activities were present, confirming that TFIIIU does not merely reconstitute residual PBP subcomplexes, possibly contained in the TFIIIC$_1$ fraction (data not shown).

We recently found (27) that human TFIIIB-α is composed of at least two activities which could be functionally analogous to yeast TFIIIB″$_{90}$ and yeast TFIIIE (41,42). It is, however, very unlikely that yTFIIIE and hTFIIIU are functionally related, because the fractions of TFIIIB-α which we employed for our experiments were not depleted from the TFIIIE analogous activity described by Teichmann *et al*. (27). It is furthermore unlikely that TFIIIU is related to the *Xenopus* zinc finger protein Staf, a transcription activator of snRNA and snRNA-type genes of various species by RNA polymerases II and III (43,44). The potential Staf binding sites are located in the DSEs of these genes. Moreover, these authors reported a synergistic activating effect of octamer and staf motifs on the transcription by pol III. U6 *in vitro* transcription reported here was performed with sequences of the mouse U6 gene from –150 to +190, thus containing the PSE and TATA promoter sequences but lacking the DSE element (2). Therefore, TFIIIU acts in a way which is independent of a potential Staf binding sequence.

Importantly, TFIIIU is also distinct from TBA. Although the elution profiles of these components overlapped when ESF chromatography of MQ TFIIIC$_0$ fractions was employed (compare Figs 1A and 3A), the results depicted in Figure 4 demonstrate that the TBA activity was completely separated from TFIIIU by chromatography on Resource S and that such TFIIIU containing fractions had no effect on VAI transcription (Fig. 4C). Whereas TBA activity functions as an activator of basal transcription of pol III genes governed by internal promoters, TFIIIU seems to be essentially involved in U6 transcription. Upon further purification of both, TBA and TFIIIU, it will become possible to dissect the protein–protein interactions and the different mechanisms by which these factors exert their functions. This will help to elucidate differences in function and composition of the transcription complexes on pol III genes formed with upstream or intragenic promoters.

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