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Functional interchangeability of TFIIIB components from yeast and human cells *in vitro*

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In eukaryotes, TFIIIB is required for proper initiation by RNA polymerase III. In the yeast *Saccharomyces cerevisiae* a single form of TFIIIB (yTFIIIB) is sufficient for transcription of all pol III genes, whereas in extracts derived from human cells two different hTFIIIB complexes exist which we have previously designated as hTFIIIB-α and hTFIIIB-β. Human TFIIIB-α is a TBP-free entity and must be complemented by TBP for transcription of pol III genes driven by gene external promoters, whereas hTFIIIB-β is a TBP–TAF complex which governs transcription from internal pol III promoters. We show that hTFIIIB-β cannot be replaced by yeast TFIIIB for transcription of tRNA genes, but that the B″ component of yTFIIIB can substitute for hTFIIIB-α activity in transcription of the human U6 gene. Moreover, hTFIIIB-α can be chromatographically divided into activities which are functionally related to yTFIIIE and recombinant yB″$_{90}$, suggesting that hTFIIIB-α is a human homolog of yeast TFIIIB″. In addition, we show that yeast TBP can only be exchanged against human TBP for *in vitro* transcription of the human and yeast U6 gene but virtually not for that of the yeast tRNA$_4^{Sup}$ gene. This deficiency can be counteracted by a mutant of human TBP (R231K) which is able to replace yeast TBP for transcription of yeast tRNA genes *in vitro*.
Keywords: human/pol III/TFIIIB/transcription/yeast

Introduction

In vertebrate cells, genes expressed by RNA polymerase III (pol III) are controlled either by gene internal class 1 (5S rRNA) or class 2 (e.g. tRNA) promoters or they are governed by class 3 sequence elements located upstream of the initiation site (e.g. U6 snRNA) comprising a TATA-Box, as well as a proximal sequence element (PSE) and a distal sequence element (DSE). In yeast cells, the U6 gene lacks both latter promoter elements. Its expression *in vivo* depends on a TATA-Box at –25 and a B-Box, which is located downstream of the transcriptional termination site, whereas transcription *in vitro* minimally requires the TATA-Box. Pol III transcription factors are best characterized in the yeast system, in which case yTFIIIB and yTFIIIC are required together with pol III for the expression of U6 and tRNA genes *in vivo* (Burnol *et al.*, 1993a,b; Huet *et al.*, 1994; Joazeiro *et al.*, 1994; Kaiser and Brow, 1995; for review, see Willis, 1993; Geiduschek and Kassavetis, 1995). All these yeast factors have been purified to homogeneity and some of them have been cloned. yTFIIIB comprises three polypeptides: TBP, TFIIIB70 and B″$_{90}$ which are necessary and sufficient for pol III recruitment and accurate initiation (Kassavetis *et al.*, 1995; Roberts *et al.*, 1996). Yet an additional still poorly characterized factor, TFIIIE, appears to be required for efficient transcription (Dieci *et al.*, 1993; Rüth *et al.*, 1996).

In human cells, the situation is more complex and class 1, 2 and 3 promoters have individual transcription factor requirements. Class 2 promoters (for example tRNA and VAI gene) depend on TFIIIC2, TFIIIC1, TFIIIB-β and pol III (Yoshinaga *et al.*, 1987; Teichmann and Seifart, 1995 and references therein; Wang and Roeder, 1995, 1996; Yoon *et al.*, 1995). In contrast, class 3 promoters (U6 and 7SK gene) are expressed by PBP (PTF; SNAPc), TFIIIC1, TFIIIB-α and pol III (Waldschmidt *et al.*, 1991; Henry *et al.*, 1995; Teichmann and Seifart, 1995; Wang and Roeder, 1995; Yoon *et al.*, 1995; Oettel *et al.*, 1997). Although a similar function for the yeast and human transcription factors TFIIIC (recognizing the B- and A-Box) and TFIIIB (mediating initiation) have been assumed, there is no sequence similarity between the two human TFIIIC2 subunits cloned thus far and yeast TFIIIC (Swanson *et al.*, 1991; Lefebvre *et al.*, 1992; Parsons and Weil, 1992; Marck *et al.*, 1993; Lagna *et al.*, 1994; L'Etoile *et al.*, 1994; Sinn *et al.*, 1995). Moreover, the human TFIIIB90 protein, although showing significant homology, is only 30% identical in amino acid sequence to its yeast homolog TFIIIB70 (Wang and Roeder, 1995; Mital *et al.*, 1996). In addition, no protein complex has been found which exhibits a PBP- (PTF-, SNAPc-) comparable function in yeast cells.

At present, relatively little is known about the functional conservation or divergence of pol III components during evolution. Most studies have concentrated on pol II transcription and many transcription factors have been found to possess sequence similarity and/or a comparable function in yeast and higher eukaryotes (Hoffmann *et al.*, 1990; Pinto *et al.*, 1992; Tschochner *et al.*, 1992; Feaver *et al.*, 1994; Humbert *et al.*, 1994; Verrijzer *et al.*, 1994; Wang *et al.*, 1994; Hisatake *et al.*, 1995; Sun and Hampsey, 1995). Some functional conservation between yeast and human proteins has been shown for the TATA-binding protein in pol II transcription (Cavalini *et al.*, 1988, 1989; Horikoshi *et al.*, 1989; Kelleher *et al.*, 1992). In addition,

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yeast TBP assembles with human TAFs into a functional TFIID complex when stably transfected into human cells (Zhou *et al.*, 1995) and yeast TBP also has the capacity to replace human TBP in the pol I factor SL1 (Rudloff *et al.*, 1994). Nevertheless, the human TBP gene, introduced into a yeast strain carrying a mutated endogenous TBP, failed to functionally substitute for the *Saccharomyces cerevisiae* TBP (Cormack *et al.*, 1991; Gill and Tjian, 1991). The region responsible for the species specificity has been mapped to the conserved C-terminus of TBP by these authors. Furthermore, it was shown that simultaneous reintroduction of human TBP and a yeast TBP which was defective for pol I and pol II transcription (Cormack and Struhl, 1993) into the above described mutant yeast strains restored viability. This suggested that the inability of the human TBP to rescue a knock-out yeast TBP strain is due to its inability to support pol III transcription in yeast (Cormack *et al.*, 1994).

The interchangeability of yeast and human pol III transcription factors *in vitro*, which has hitherto not been reported, is not only of fundamental interest from an evolutionary view, but could also help to understand the basic mechanisms underlying the formation of pol III transcription complexes. In this paper we show that human TFIIIB-α activity can be functionally replaced by yeast TFIIIB″ for *in vitro* transcription of the human U6 gene. All other recombinant or purified pol III-specific factors from both species could neither be exchanged singly nor could they be swapped in pairs. In addition, we present data pointing to a comparable composition of hTFIIIB-α and yeast TFIIIB″ consisting of B''_{90} and TFIIIE. Furthermore, we demonstrate that human and yeast TBP are completely exchangeable in the transcription of yeast and human U6 genes, whilst only the yeast protein is fully active in the transcription of yeast tRNA genes *in vitro*. These data provide new insights on the function of TBP in promoter selection and direction of transcription.

Results

Human TFIIIB90 co-elutes with hTFIIIB-β but not with hTFIIIB-α after chromatography over DEAE Fractogel

We could previously show that two different human TFIIIB complexes (hTFIIIB-α and -β) can be chromatographically separated (Teichmann and Seifart, 1995) which are either predominantly active in the transcription of the 5′-regulated human U6 gene (hTFIIIB-α; Figure 1A, lanes 5–7) or which exhibit most of their activity in the expression of RNA polymerase III (pol III) genes governed by internal promoters, exemplified here by transcription of tRNA genes from yeast and human (hTFIIIB-β; Figure 1B, lanes 12–15). As revealed by Western blot analysis, hTFIIIB-β co-elutes with the TATA-binding protein (TBP) and hTFIIIB90 upon chromatography over EMD-DEAE-Fractogel (EDF). In contrast, hTFIIIB-α is free of TBP and hTFIIIB90 (Figure 1C). This finding suggested the possibility that hTFIIIB-α represents a partial human TFIIIB activity. In order to address this question, we attempted to exchange one or both of the human TFIIIB activities against their yeast counterparts.

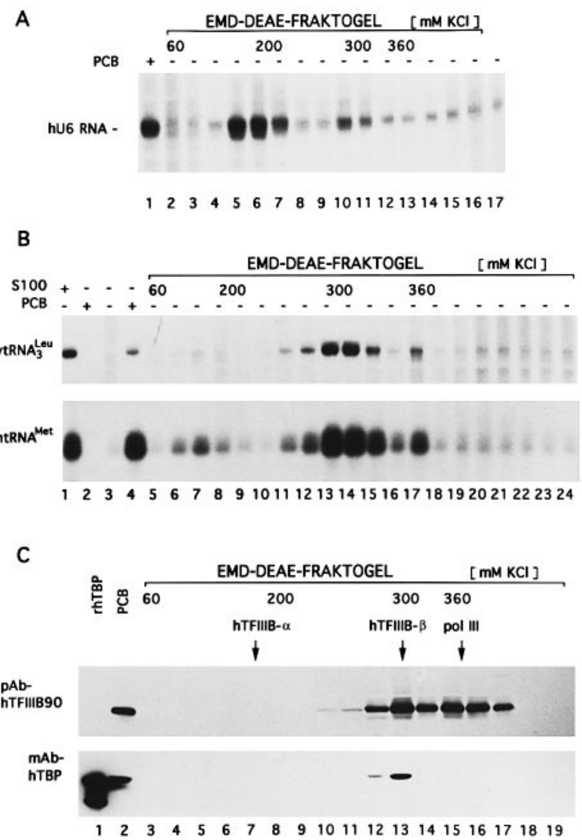

Fig. 1. Human TFIIIB-β, but not hTFIIIB-α co-elutes with hTFIIIB90 after chromatography over EMD-DEAE-FRACTOGEL (EDF). (**A**) Transcription of the human U6 gene (pUhU6wt). *In vitro* transcription was performed as described in Materials and methods. The position of the U6 transcript is indicated. Three micrograms of pUhU6wt were incubated with the indicated protein fractions. Lane 1: 100 µg PCB; lane 2: 50 µg of EDF flowthrough; lanes 3–17: 50 µg of fractions obtained by elution of the EDF column with a linear gradient from 60 to 450 mM KCl. The fractions assayed for TFIIIB activity were reconstituted with 25 µg TBP-depleted PCC, 10 µg PCA and 25 ng recombinant human TBP. (**B**) Comparative transcription of the ytRNA$_3^{Leu}$ (pUyt-LEU3-0.2) and the human htRNA^{Met} (pUht-MET) gene. *In vitro* transcription was performed separately for the two genes, as described in Materials and methods. The positions of ytRNA$_3^{Leu}$ and htRNA^{Met} transcripts are indicated. Two hundred nanograms of pUyt-LEU3-0.2 or 1 µg of pUht-MET were incubated with the indicated protein fractions. Lane 1: 25 µg HEK-S100; lanes 2 and 4: 20 µg PCB; lanes 3–24: 4.5 µg PCC; lane 5: 10 µg of EDF flowthrough; lanes 6–24: 10 µg of fractions obtained by elution of the EDF column with a linear gradient from 60 to 450 mM KCl. (**C**) Western-blot analysis using antibodies directed against hTFIIIB90 (polyclonal) or hTBP (monoclonal; Chatterjee *et al.*, 1993). One hundred nanograms of rhTBP (lane 1), 60 µg PCB (lane 2), 30 µg EDF flowthrough (lane 3) and 30 µg of fractions obtained by elution of an EDF column with a linear gradient from 60 to 450 mM KCl (lanes 4–19) were analyzed. SDS–PAGE and Western blot were performed as described in Materials and methods. The positions of hTFIIIB90 and hTBP are indicated.

hTFIIIB-α can functionally be replaced by yeast TFIIIB whereas the other pol III transcriptional components are species-specific

Yeast TFIIIB (yTFIIIB) is a multiprotein factor that comprises three components: TBP, TFIIIB70 and B''_{90}, which are sufficient to direct accurate transcription of yeast pol III genes *in vitro* (Kassavetis *et al.*, 1995; Roberts *et al.*, 1996; Rüth *et al.*, 1996). Efficient transcription further requires TFIIIE activity. A partially purified frac-

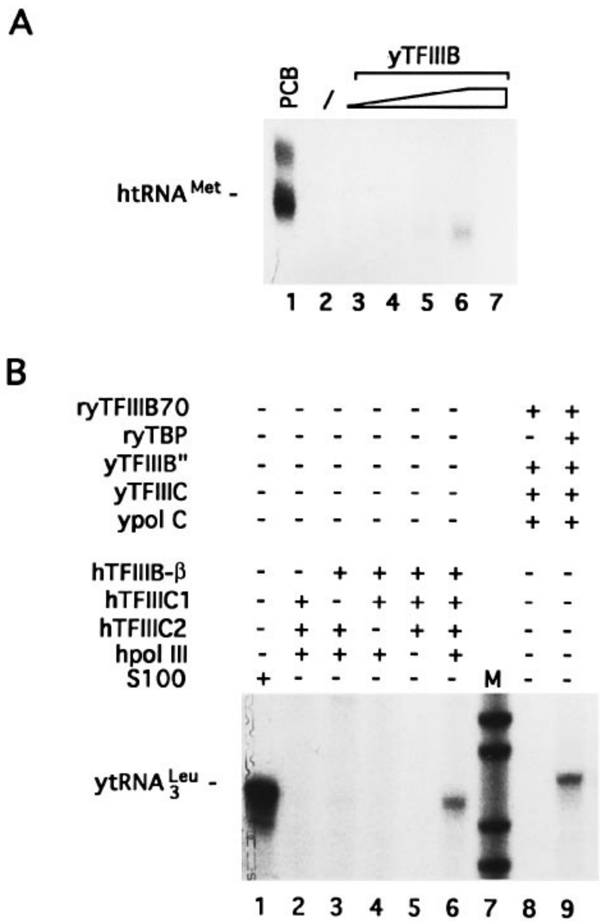

Fig. 2. Human TFIIIB-β cannot be functionally replaced by *Saccharomyces cerevisiae* TFIIIB (yTFIIIB). (**A**) Comparative analysis of hTFIIIB-β or yTFIIIB-containing fractions for transcription of an htRNA^{Met} (pUht-MET) gene. All reactions except lane 7 contained 4.5 μg of PCC from HEK (human embryonal kidney) cells as a minimal reconstitution system to assay TFIIIB activity. Fractions which were assayed for TFIIIB activity were: lane 1: 20 μg PCB; lane 2: none; lanes 3–6: 0.36, 0.72, 1.45 and 2.9 μg of yTFIIIB (Heparin ultrogel 0.26 M ammonium sulfate). Lane 7 had 2.9 μg of yTFIIIB alone. *In vitro* transcription was performed as described in Materials and methods. The position of htRNA^{Met} transcripts is indicated. (**B**) *In vitro* transcription of the ytRNA$_3^{Leu}$ gene either with purified human or purified and recombinant yeast transcription factors. Two hundred nanograms of pUyt-Leu3-0.2 were incubated with the indicated protein fractions. Lane 1: 25 μg HEK-S100; lane 6: complete reaction containing 100 ng hTFIIIB-β (mAb-TBP-5M), 900 ng hTFIIIC1 (SO_3^- 0.6), 300 ng hTFIIIC2 (B-Box-1M), 50 ng hpol III (SO_3^- 0.55). In lanes 2–5 individual components were deleted as appropriately indicated in Figure 2B. Lane 7: pBR322 DNA-*Msp*I; lane 8: 100 ng ryTFIIIB70, 400 ng yTFIIIB″, 50 ng yTFIIIC, 50 ng ypolC (III); lane 9: the reaction contained the same proteins assayed in lane 8 and was supplemented with 25 ng ryTBP. The position of the ytRNA$_3^{Leu}$ transcripts is indicated. *In vitro* transcription was performed as described in Materials and methods.

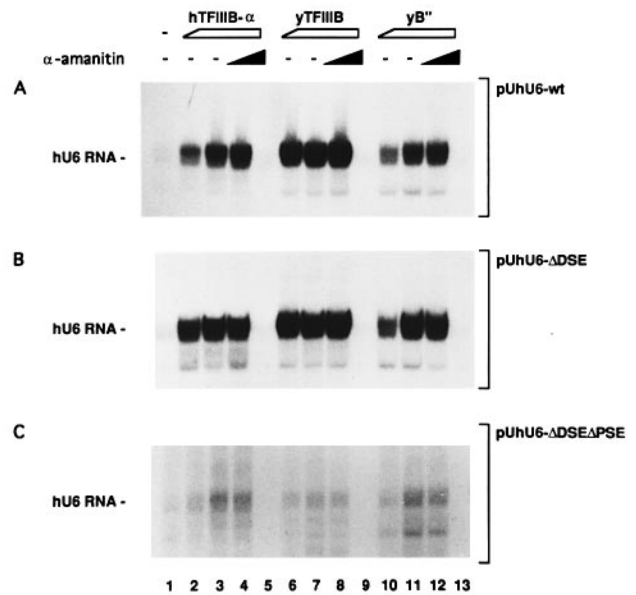

Fig. 3. The B″ component of yeast TFIIIB can functionally replace human TFIIIB-α. (**A**) Three micrograms of pUhU6wt was incubated with the following protein fractions: lanes 1–13: 25 μg TBP-depleted PCC, 10 μg PCA and 25 ng recombinant human TBP; lane 2: 7.5 μg hTFIIIB-α (SO_3^- 0.5); lanes 3–5: 15 μg hTFIIIB-α (SO_3^- 0.5); lane 6: 1.45 μg yeast TFIIIB (Heparin ultrogel 0.26 M ammonium sulfate); lanes 7–9: 2.9 μg yeast TFIIIB; lane 10: 1.3 μg yTFIIIB″ (FT250); lanes 11–13: 2.6 μg yTFIIIB″; lanes 4, 8 and 12: 2 μg/ml α-amanitin; lanes 5, 9 and 13: 300 μg/ml α-amanitin. (**B**) Three micrograms of pUhU6-0.26 (ΔDSE) were incubated with identical amounts of the protein fractions, depicted in (A). (**C**) Three micrograms of pUhU6-0.25 (ΔDSEΔPSE) were incubated with identical amounts of the protein fractions, depicted in (A). *In vitro* transcription was performed as described in Materials and methods. The positions of the U6 transcripts are appropriately indicated in (A), (B) and (C).

tion containing yeast TFIIIB was assayed for its ability to replace human TFIIIB-β for *in vitro* transcription of a human initiator tRNA^{Met} gene and it was found to be insufficient to provide detectable TFIIIB activity (Figure 2A, lanes 3–6). To analyze whether this inability was a consequence of the use of a human instead of a yeast tRNA gene, we expressed the yeast tRNA$_3^{Leu}$ gene with purified transcription factors from yeast or human cells, appropriately indicated in Figure 2B. This gene was expressed at approximately equal rates by a complete set of either highly purified human or yeast pol III transcription factors (Figure 2B; compare lanes 6 and 9). However, none of the factors could be exchanged alone, nor could they be swapped in pairs (data not shown). It must be emphasized that the same TFIIIB fraction from yeast cells, which was unable to replace human TFIIIB-β for transcription of the human tRNA^{Met} gene (Figure 2A, lanes 3–6), was capable of efficiently replacing human TFIIIB-α activity, when reconstituted with human transcription factors for human U6 gene transcription (Figure 3A, compare lanes 2–3 and 6–7). The data also show that *in vitro* transcription of the human U6 gene was not significantly influenced by the DSE, but strongly depended on the presence of the PSE, the removal of which led to a dramatic reduction of transcription, regardless of whether human TFIIIB-α or yeast TFIIIB was used (compare lanes 2–3 and 6–7 of Figure 3A, B and C).

The B″ component of TFIIIB from Saccharomyces cerevisiae is a functional homolog of human TFIIIB-α

In order to further differentiate which of the yeast TFIIIB components could functionally replace human TFIIIB-α for transcription of the human U6 gene we analyzed a partially purified yeast TFIIIB″ (yB″) fraction (Huet *et al.*, 1994) for *in vitro* exchange experiments. We initially concentrated on this fraction because hTFIIIB-α did not contain hTBP or hTFIIIB90 (Figure 1C) and it was hence

expected to functionally resemble yTFIIIB″. As shown in Figure 3A (lanes 10–11), the yB″ fraction alone was sufficient to completely replace hTFIIIB-α activity (Figure 3A, lanes 2–3) and addition of recombinant yeast TFIIIB70 did not further enhance this activity (data not shown). Deletion of upstream promoter elements (DSE and PSE) did not influence the capacity of yB″ to functionally replace human TFIIIB-α for transcription of the human U6 gene, although the promoter strength was dramatically reduced after deletion of the PSE (Figure 3A, B and C; compare lanes 2–3 with lanes 10–11). Transcription of the human U6 gene and its promoter deletion constructs was catalyzed by human RNA polymerase III, independently of whether human TFIIIB-α or yeast TFIIIB components were used, since transcription was insensitive to 2 μg/ml but completely abolished by 300 μg/ml α-amanitin (compare lanes 4–5, 8–9 and 12–13 of Figure 3A, B and C).

Human TFIIIB-α is minimally composed of two activities functionally related to TFIIIE and B''_{90} from yeast cells

Since optimal yeast TFIIIB″ (yB″) activity is composed of yTFIIIE and cloned yB''_{90} (Rüth *et al.*, 1996) and given that yB″ can replace hTFIIIB-α for *in vitro* transcription of the human U6 gene, we wanted to know whether hTFIIIB-α shows a comparable composition to that of the yeast B″ activity. For this purpose we raised polyclonal antibodies against recombinant yeast B''_{90} and coupled them covalently to protein A–Sepharose (pAb-B''_{90}–Sepharose). An EMD-SO_3^--Fractogel 0.5 fraction containing human TFIIIB-α activity was purified over such a column. Neither the flowthrough nor the fraction eluted with 5 M urea alone were able to fully restore a TFIIIB-α activity comparable to that of the load (Figure 4A, compare lanes 4 and 5–7 respectively, with lane 3). Only the combination of these two fractions yielded an activity comparable with that of loaded hTFIIIB-α (Figure 4A, lanes 8–10). Similarly, recombinant yeast B''_{90} alone was not able to completely replace hTFIIIB-α in a reconstituted *in vitro* transcription assay with human transcription factors (Figure 4A, lanes 11–16), but when complemented with the flowthrough of the pAb-B''_{90}–Sepharose a significant hTFIIIB-α-like activity was restored (Figure 4A, lanes 17–22). In addition, partially purified yeast TFIIIE, complemented with the human pAb-B''_{90}–Sepharose 5 M urea fraction, yielded a hybrid TFIIIB activity which to some extent replaced hTFIIIB-α for transcription of the human U6 gene *in vitro* (Figure 4B, lanes 6–7).

The proteins retained on the pAb-B''_{90}–Sepharose were analyzed by SDS–12.5%-PAGE. The bulk of protein did not bind to the column and hence eluted with the flowthrough (Figure 4C, lane 3) whereas proteins of 90, 67 and 65 kDa were retained by and were eluted with 5 M urea from this column (Figure 4C, lane 4). This procedure led to a significant degree of purification and 1–4 ng of the protein depicted in Figure 4C, lane 4 were highly active in reconstitution of human U6 gene transcription (Figure 4A, lanes 8–10). The polypeptide of ~90 kDa was not identical to hTFIIIB90 since the latter component was not detectable by Western blot analysis in hTFIIIB-α fractions (Figure 1C).

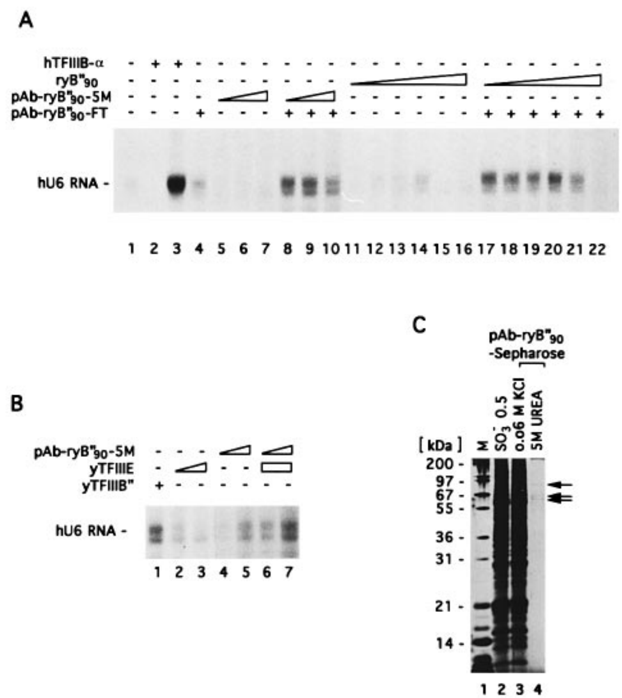

Fig. 4. Human TFIIIB-α is composed of at least two activities, functionally related to ryB''_{90} and yTFIIIE. (**A**) All reactions except in lane 2 were reconstituted with 25 μg TBP-depleted PCC, 10 μg PCA and 25 ng recombinant human TBP. The following fractions were analyzed for their TFIIIB activity: lanes 2 and 3: 15 μg SO_3^- 0.5 (hTFIIIB-α); lanes 4, 8–10 and 17–22: 15 μg flowthrough of the pAb-B''_{90}–Sepharose; lanes 5–7 and 8–10: 1, 2 and 4 ng of the fraction eluted with 5 M urea from the pAb-B''_{90}–Sepharose; lanes 11–16 and 17–22: 0.5, 1, 2, 4, 8 and 16 ng of Heparin-Fractogel 0.45 M KCl (ryB''_{90}). (**B**) All reactions were reconstituted with 25 μg TBP-depleted PCC, 10 μg PCA and 25 ng recombinant human TBP. The following fractions were analyzed for their TFIIIB activity: lane 1: 400 ng B''_{90}; lane 2: 2 μg yTFIIIE; lanes 3 and 6–7: 4 μg yTFIIIE; lanes 4–5 and 6–7: 1 and 2 ng of the fraction eluted with 5 M urea from the pAb-B''_{90}–Sepharose. *In vitro* transcription was performed as described in Materials and methods. The position of the hU6 transcript is indicated. (**C**) SDS–12.5% PAGE of hTFIIIB-α purified over pAb-B''_{90}–Sepharose. Lane 1: the migration of 0.5 μg of marker proteins is appropriately indicated; lane 2: 50 μg SO_3^- 0.5 (hTFIIIB-α); lane 3: 50 μg of flowthrough from the pAb-B''_{90}–Sepharose eluting with 60 mM KCl; lane 4: 25 ng of the fraction eluting with 5 M urea from the pAb-B''_{90}–Sepharose. SDS–PAGE was performed as described in Materials and methods.

Human and yeast TBP are interchangeable for transcription of human and yeast U6 genes in vitro

The TATA-binding protein (TBP) is a component of human as well as yeast TFIIIB. hTFIIIB-α and yeast B″ can be easily separated from TBP employing native chromatographic procedures (Huet *et al.*, 1994; Teichmann and Seifart, 1995). We used TFIIIB activities from both species which were TBP-free and which could be complemented by addition of either human or yeast TBP. As shown in Figure 5A, recombinant TBP from both species was comparably active in the transcription of the human U6 gene *in vitro*.

Transcription of the yeast U6 gene reconstituted with yB″ activity, highly purified ypol III and recombinant yTFIIIB70 was completely dependent upon TBP (Figure 5B, lane 4) but could likewise be restored equally well by the addition of 25 ng recombinant TBP from yeast (Figure 5B, lane 3) or human (Figure 5B, lane 5).

Fig. 5. Recombinant TBP from yeast and human cells is functionally exchangeable for transcription of yeast and human U6 genes but not for transcription of yeast tRNA genes *in vitro*. (**A**) Twenty-five micrograms of TBP-depleted PCC, 10 μg PCA and 100 μg TBP-depleted PCB in lanes 1–12 were supplemented with 2.5, 5, 10, 20, 40 and 80 ng of recombinant TBP from human cells (lanes 1–6) or yeast cells (lanes 7–12). *In vitro* transcription was performed as described in Materials and methods using 3 μg of pUhU6wt as template. (**B**) Transcription of the yU6 gene. The following protein fractions were incubated with 200 ng pTaq6: Lanes 1 and 3: 100 ng ryTFIIIB70, 25 ng ryTBP and 400 ng yTFIIIB″. Lane 3 additionally contained 50 ng ypolC (III) which was also used to reconstitute the reactions in lanes 2, 4 and 5. The reactions in lanes 4 and 5 contained 100 ng ryTFIIIB70 and 400 ng yTFIIIB″, but ryTBP was omitted. Lanes 2 and 5 contained 25 ng recombinant human TBP. *In vitro* transcription was performed as described in Materials and methods and the position of the yeast U6 transcript is indicated. (**C**) Two hundred nanograms of pRS316 (SUP4 ytRNA gene) were incubated with the following protein fractions: 100 ng ryTFIIIB70, 400 ng yTFIIIB″, 50 ng yTFIIIC and 50 ng ypolC (III) in all reactions. Lane 1: 25 ng ryTBP; lane 3: 25 ng rhTBP.

Experiments in which yeast and human TBP were titrated between 12.5 and 50 ng confirmed this result (data not shown).

Mutant human TBP (R231K) but not wild-type hTBP can replace yeast TBP in the transcription of yeast tRNA genes in vitro

Identical concentrations (25 ng) of the same recombinant TBP preparations from yeast and human, used above for transcription of human and yeast U6 genes (Figure 5A and B), were used for transcription of either the yeast Leu3 tRNA gene (data not shown) or the yeast SUP4 tRNA gene in a system which was entirely dependent on TBP (Figure 5C, lane 2). It was found that only yeast TBP showed full transcription activity but that human TBP resulted in very weak, if any transcription of both yeast tRNA genes examined (Figure 5C, compare lanes 1 and 3). It could be argued that this finding is the result of a sub-optimal concentration of human TBP in the transcription reaction. This is, however, not the case since reconstitution of SUP4 tRNA transcription by comparative titration of yeast and human TBP from 12.5 to 50 ng (Figure 6, compare lanes 2–4 and 5–7) confirmed the result of Figure 5C.

Fig. 6. Mutant human TBP-R231K is able to replace yeast TBP for SUP4 ytRNA and yU6 transcription *in vitro*. In lanes 1–10 the following protein fractions were incubated with 200 ng of pRS316 (SUP4 ytRNA gene): 100 ng ryTFIIIB70, 400 ng yTFIIIB″, 50 ng yTFIIIC and 50 ng ypolC (III) in all reactions. Lane 1 contains no TBP; lanes 2–4 contain 12.5, 25 and 50 ng recombinant yeast TBP; lanes 5–7 contain 12.5, 25 and 50 ng recombinant human wild-type TBP; lanes 8–10 contain 12.5, 25 and 50 ng recombinant mutant human TBP-R231K. Lane 11: labeled marker DNA [pBR322 DNA-*Msp*I; sizes of fragments in nucleotides (nt) are appropriately indicated]; lanes 12–15: transcription of the yU6 gene. All reactions contained 100 ng ryTFIIIB70, 400 ng yTFIIIB″ and 50 ng ypol III. Lane 12 contains no TBP, lanes 13–15 contain 25 ng ryTBP, rhTBP and rhTBP-R231K respectively.

It has previously been shown that human TBP, mutated at position 231 from Arg to Lys, is able to complement the defects of yeast TBP mutants *in vivo* (Cormack *et al.*, 1994). Since our *in vitro* results pointed to a specific inability of human TBP to support tRNA transcription together with the heterologous yeast transcription factors, we analyzed the recombinant human mutant TBP-R231K for its ability to replace yeast TBP in pol III transcription. Indeed, we found that human TBP-R231K was able to replace yeast TBP for *in vitro* transcription of the yeast SUP4 tRNA gene (Figure 6, lanes 8–10), as well as the yeast U6 gene (Figure 6, lane 15).

Discussion

The B″ component of TFIIIB from S.cerevisiae can replace human TFIIIB for transcription of the human U6 gene

For reconstituted *in vitro* transcription of the human U6 RNA gene, yeast and human TBP molecules are functionally interchangeable and hTFIIIB-α can be replaced by yB″. These data imply that yeast TBP and proteins in the yeast B″ fraction are able to productively interact with basal components of the evolutionary divergent human pol III transcriptional machinery. Inter-

actions of yB″ with components of human TFIIIB are a prerequisite to establish a hybrid TFIIIB activity, which is required to correctly express the human U6 gene by RNA polymerase III. It is hence likely that essential domains of yeast B″ and hTFIIIB-α have been structurally conserved during the evolution of these highly diverged eukaryotes.

In contrast to hTFIIIB-α, hTFIIIB-β cannot be functionally replaced by yeast TFIIIB or any of its components. Hence we did not find a functional conservation between yeast and human of such TFIIIB proteins, which are required for the transcription of classical pol III genes. We cannot exclude, however, that a human component, which is homologous to or fulfils the function of yB″ in the transcription of these pol III genes could also be present in the partially purified fractions used for reconstitution of tRNA transcription. Accordingly, it has been reported that B″ activity, sufficient for *in vitro* transcription of a yeast tRNA gene, can also be found in partially purified TFIIIC and pol III fractions (Kassavetis *et al.*, 1991).

Is hTFIIIB90 involved in transcription of the human U6 gene?

Human TFIIIB90, which shows sequence homology to yeast TFIIIB70 (Wang and Roeder, 1995; Mital *et al.*, 1996), is required for transcription of human pol III genes regulated by internal promoters. A more complex picture has emerged concerning the function of this protein in transcription of human U6 and 7SK genes. (i) By using antibodies raised against full-length hTFIIIB90, Wang and Roeder (1995) could show that hTFIIIB90 as well as hTBP are removed by immunodepletion from cytoplasmic extracts. As a result, transcription of U6 and 7SK genes was dramatically reduced. Surprisingly, readdition of recombinant human TFIIIB90 and recombinant human TBP to these immunodepleted extracts only reconstituted VAI, tRNA and 5S rRNA transcription, but did not restore expression of U6 and 7SK genes. This implies that another protein(s), cross-reactive with anti-hTFIIIB90 antibodies and possibly corresponding to or representing a part of hTFIIIB-α, was also removed by this depletion of the extracts. Alternatively, recombinant hTFIIIB90 could lack a specific modification (e.g. phosphorylation?) required for expression of U6 and 7SK genes; (ii) Mital *et al.* (1996) were unable to deplete hTFIIIB activity required for transcription of the U6 gene from whole-cell extracts using an antibody directed against a peptide comprising 14 amino acids close to the C-terminus of hTFIIIB90. This antibody depleted >95% of hTFIIIB90 from the extracts and abolished VAI transcription. These results suggested either that a different protein was required for transcription of the U6 gene or that the form of hTFIIIB90 that was active in U6 transcription carried a modification which was not recognized by this peptide antibody; (iii) here we present evidence that human TFIIIB-α, required for U6 transcription, does not contain hTFIIIB90, as analyzed by Western blot. In addition, we show that yeast B″, which does not contain yTFIIIB70, is sufficient to functionally replace hTFIIIB-α in the transcription of the human U6 gene. These data suggest that hTFIIIB-α is not tightly associated with hTFIIIB90, which could however be introduced into the *in vitro* transcription reaction by the fractions used for reconstitution of U6 transcription. Alternatively, the function of a yeast TFIIIB70 homolog in human U6 transcription could be exerted by a protein, which is different from or represents a modified form of hTFIIIB90.

In accordance with our results, the antibody-depletion data presented by Wang and Roeder (1995) as well as those of Mital *et al.* (1996) comprise the possibility that a modification of hTFIIIB90 or a different activity, which is however cross-reactive with anti-full-length hTFIIIB90 antibodies, is required for transcription of human U6 and 7SK genes.

Human TFIIIB-α activity is composed of at least two activities which are functionally analogous to yB''_{90} and yTFIIIE

The yeast TFIIIB″ (yB″) activity is composed of B''_{90} and the less well characterized yTFIIIE component (Dieci *et al.*, 1993; Rüth *et al.*, 1996). Both activities are necessary for efficient transcription of yeast tRNA and U6 RNA genes and we present data implying that the principal composition of yB″ and hTFIIIB-α could likewise have been conserved. Human TFIIIB-α activity can also be split into two fractions by chromatography over a yB''_{90}-antibody affinity column and both activities obtained from the column (flowthrough and 5 M urea eluate) were necessary to regain significant hTFIIIB-α activity. In addition, both activities could be replaced to some extent by their homologs from yeast cells for transcription of the human U6 gene (the flowthrough by yTFIIIE and the 5 M urea eluate by recombinant yB''_{90}), pointing to some conservation of the functional entities of yB″ and hTFIIIB-α. Since the flowthrough obtained after chromatography over a yB''_{90}-antibody affinity column showed more activity than yTFIIIE in reconstitution of human U6 transcription, we cannot exclude the possible existence of additional protein(s) within hTFIIIB-α fractions which might influence the efficiency of human U6 transcription.

Essential components of the pol III transcriptional apparatus have diverged during evolution from yeast to human

In contrast to the findings reported for human TFIIIB-α, it was found that the exchangeability of hTFIIIB-β and yTFIIIB is poor and only faintly detectable in less purified reconstitution systems (Figure 2A and data not shown). These results point to an evolutionary divergence from yeast to human of such TFIIIB components required to transcribe pol III genes governed by gene internal promoters. It should be pointed out that, in contrast to yeast cells, human TFIIIB-β is a stable TBP–TAF complex. This implies that an intricate interplay of protein–protein interactions may have evolved relatively late during evolution to establish the TBP–TAF complex and that these interactions may limit the interspecies compatibility of these components.

Simultaneously, TFIIIC composition has significantly changed from yeast to higher eukaryotes. The function of TFIIIC, which in yeast cells is exerted by a well characterized single complex consisting of six subunits, is carried out in human cells by two distinct complexes, hTFIIIC1 (A-Box-binding) and hTFIIIC2 (B-Box-binding; Yoshinaga *et al.*, 1987). None of the hTFIIIC2 subunits cloned until now shows sequence similarity to any yTFIIIC protein.

However, recently published data (Wang and Roeder, 1996) suggest that there is a human homolog of the τ131 subunit of yeast TFIIIC. yτ131 is responsible for yTFIIIB assembly and has been shown to contact yB''_{90} (Rüth *et al.*, 1996). The co-evolution of hTFIIIB-β and hTFIIIC components has possibly led to a species-specific restriction of hTFIIIB-β and yTFIIIB to utilize the heterologous form of TFIIIC. Probably due to the evolutionary divergence of TFIIIC, the B''_{90} component of yeast TFIIIB may be unable to recognize the τ131 homolog of human TFIIIC and it may hence be unable to generate interactions with the human proteins necessary for the assembly of pre-initiation complexes on classical pol III promoters.

Exchangeability of yeast and human TBP in pol III transcription

The TATA-binding protein is the second component of the pol III transcriptional system which can be functionally exchanged *in vitro*, within certain limits, between yeast and human. TBP from both species can exert its function in the TATA-box-dependent transcription of either human or yeast U6 snRNA genes (Figure 5A and B). In this context the results of Mittal and Hernandez (1997) are interesting; they reported that the N-terminal region of human TBP down-regulated its binding to the U6 TATA-Box, unless this terminus contacts SNAPc (PBP; PTF), thereby mediating co-operative binding of the complex and enhancement of U6 transcription. Given that the sequences of the N-terminus of yeast and human TBP are unrelated and since no yeast homolog of SNAPc (PBP; PTF) has hitherto been found, it seems unlikely that a direct interaction of the N-terminus of yeast TBP and human SNAPc is required for human U6 transcription. Instead, yeast TBP must be able to interact with hTFIIIB-α at the human U6 promoter to generate a functional pre-initiation complex and these interactions might be stabilized through protein–protein interactions of human TFIIIB-α and SNAPc (PBP; PTF). To this extent, yeast and human TBP might have comparable functions, which could also involve the participation of the N-termini of these proteins.

When yeast tRNA genes are transcribed *in vitro*, the exchangeability of yeast and human TBP is limited and only yTBP shows full activity. In contrast to transcription of U6 genes, TBP is incorporated into the tRNA gene initiation complex through protein–protein interactions which could limit the compatibility between human TBP and the reconstitution system from yeast cells. In support of this observation, it has been reported that human TBP is unable to functionally replace yeast TBP for cell viability (Cormack *et al.*, 1991; Gill and Tjian, 1991). This failure has been mapped to the highly conserved C-terminal 180 amino acids (80% identity between yeast and human) and does not involve the variable N-terminus. Furthermore, it has been suggested that these strains are not viable because they are impaired in transcription of pol I, pol III and TATA-less pol II genes. A point mutation in hTBP (R231K) allows the human protein to support cell growth (Cormack *et al.*, 1994). This is consistent with our observation that the mutant human TBP-R231K, but not the wild-type protein, is able to replace yeast TBP for *in vitro* transcription of the SUP4 tRNA gene. Our *in vitro* exchange experiments could help to explain the inability of human TBP to rescue the mutant yeast strains as a consequence of impaired tRNA transcription.

Materials and methods

HEK (human embryonal kidney) cells and extracts

Cytoplasmic extracts (S100) from HEK 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 $pUht^{Met}$, pUhU6wt, pTaq6 and pRS316-SUP4 were as previously described (Schneider *et al.*, 1989; Simmen *et al.*, 1992; Dieci Sentenac, 1996) and contained a single copy of the genes coding for $htRNA^{Met}$, human U6-snRNA, yeast U6-snRNA and yeast SUP4 tRNA respectively. The plasmid pUytLEU3-0.2 contains a single copy of a 174 bp fragment comprising the bases –40 to +134 of the $ytRNA_3^{Leu}$ gene (Johnson and Raymond, 1984), amplified by PCR and cloned into pUC18 *Bam*HI–*Pst*I. The plasmid pUhU6–0.26 contains a *Nde*I–*Msp*I fragment, comprising the sequence from –70 to +183 of the human U6 gene, cloned into pUC19/*Sma*1. The plasmid pUhU6-0.25 contains a *Taq*I–*Sno*I fragment, comprising the sequence from –43 to +188 of the human U6 gene, cloned into pUC18/*Hinc*II.

Site-specific mutagenesis of human TBP

The mutant human TBP-R231K was generated using a PCR-based protocol (Perrin Gililand, 1990). The primer used (GAACAGTCCAA-GCTTGCAGCAAGAAAATATGC) anneals to bases 670–701 of the human wild-type TBP gene and introduces a single amino acid change at position 231 of the protein.

In vitro transcription

The *in vitro* transcription reactions were performed as previously described (Teichmann and Seifart, 1995; Dieci and Sentenac, 1996). *In vitro* synthesized RNA products were electrophoretically separated on 6% denaturating urea sequencing gels and autoradiographed for at least 12 h at –80°C with an intensifying screen.

Purification of hTFIIIB-α and hTFIIIB-β

The following buffers were used: buffer 1: 20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 3 mM DTT, 0.2 mM 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. The human TFIIIB activities were purified as described (Teichmann and Seifart, 1995) with the following modifications: hTFIIIB-α activity eluting with 200 mM KCl in buffer 2 from an EMD-DEAE-Fractogel (EDF) column was directly applied to either a Mono S or an EMD-SO_3^- Fractogel (ESF; supplied by E.Merck, Darmstadt) column and eluted stepwise with 200 mM KCl, 500 mM KCl and 1 M KCl. Human TFIIIB-α activity eluted with 500 mM KCl. Human TFIIIB-β activity, eluting with 300 mM KCl from an EDF column was diluted to 220 mM KCl in buffer 2, applied to an ESF column and eluted with a linear gradient from 220–450 mM KCl. The hTFIIIB-β activity eluting with 350 mM KCl from the ESF was then applied to a mAb-TBP column (Teichmann and Seifart, 1995), washed with 350 mM KCl and the bound hTFIIIB-β activity was eluted with 5 M urea. All protein fractions eluting with >100 mM KCl from the individual columns were dialyzed against an at least 100-fold excess of buffer 2 containing 60 mM KCl.

Purification of human TFIIIC1, TFIIIC2 and RNA polymerase III

RNA polymerase III (pol III) from human cells eluted with 350 (PCB) and with 600 mM KCl (PCC) after phosphocellulose chromatography (PC), as analyzed by non-specific pol test and sensitivity to α-amanitin. Pol III which co-purified with hTFIIIB-α and hTFIIIB-β after PC (PCB) was subjected to chromatography over EDF as described for the human TFIIIB complexes. From this column it eluted with 360 mM KCl. These fractions were pooled, dialyzed against a 100-fold excess of buffer 2 containing 60 mM KCl and applied to a single-stranded DNA-cellulose column (SIGMA) which was subsequently eluted with a linear salt gradient from 60 to 450 mM KCl. Fractions eluting with 300 mM KCl from this column were directly applied to an ESF column. From this column, a linear salt gradient from 300 to 700 mM KCl eluted pol III with 550 mM KCl. Human TFIIIC1 and TFIIIC2 were purified as

described (Yoshinaga *et al.*, 1987) with the following modifications: hTFIIIC1-containing fractions, eluting with 270 mM KCl from Mono Q, were directly applied to a single-stranded DNA-cellulose column (SIGMA) to remove trace amounts of RNA polymerase III. From this column hTFIIIC1 eluted with the flowthrough and was directly applied to an ESF from which it eluted with 600 mM KCl; human TFIIIC2-containing fractions, eluting with 350 mM KCl from Mono Q, were pooled and directly applied to an ESF from which it eluted with 500 mM KCl. These fractions were pooled, dialyzed against a 300-fold excess of buffer 2 containing 60 mM KCl and applied to a B-Box affinity column. This column was performed stepwise with buffer 2 containing 60 mM, 200 mM and 1 M KCl. hTFIIIC2 activity eluted with 1 M KCl and was dialyzed against a 100-fold excess of buffer 2 containing 60 mM KCl.

Purification of RNA polymerase III and pol III transcription factors from yeast cells

Yeast RNA polymerase III was purified as described (Huet *et al.*, 1985). Purification of TFIIIB was carried out as described (Huet *et al.*, 1994). Purification of single yTFIIIB components was as follows: ryTBP was purified by the procedure of Burton *et al.* (1991); ryTFIIIB70 was expressed and purified as described (Colbert and Hahn, 1992; Dieci and Sentenac, 1996); fraction B″ was derived from the chromatin pellet and purified according to Kassavetis *et al.* (1992). In Figure 3, a yTFIIIB″ fraction (FT250) was used, which had been depleted from epitope-tagged ryTFIIIB70 by 12CA5 protein A–Sepharose (Huet *et al.*, 1994). Recombinant yeast B''_{90} was expressed and purified over Ni-NTA–Agarose as described (Rüth *et al.*, 1996). The eluate with 250 mM imidazole was dialyzed against a 100-fold excess of buffer 2 containing 60 mM KCl and applied to a Heparin-Fractogel column (E.Merck, Darmstadt). Bound proteins were recovered by a linear KCl gradient from 60 to 500 mM. ryB''_{90} eluted with 450 mM KCl from this column. yTFIIIC was purified as described (Gabrielsen *et al.*, 1989). The fraction eluting with 0.11–0.16 M ammonium sulfate from the DEAE–Sephadex column was used for the experiments. yTFIIIE was purified as described (Dieci *et al.*, 1993).

SDS–PAGE

SDS–PAGE and silver stain were carried out as described (Teichmann and Seifart, 1995).

Western blot analysis

Buffer 3 contained 50 mM Tris–HCl, 50 mM borate, pH 8.3, 0.01% SDS, 10% methanol.

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 (Teichmann and Seifart, 1995). Detection of antigen–antibody complexes was achieved by employing ECL (Amersham). Antibodies which had bound to the membrane were removed from it by incubation in 2% SDS, 100 mM DTT and 70°C for 2 h. The membrane was subsequently washed several times with PBS/0.05% Tween 20 and re-blocked with PBS/0.05% Tween 20/10% skimmed milk powder. The membrane could subsequently be reused for incubation with another antibody.

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