Assembly of transcription factor IIB at a promoter *in vivo* requires contact with RNA polymerase II

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The general transcription factor TFIIB has a central role in the assembly of the preinitiation complex at the promoter, providing a platform for the entry of RNA polymerase II/TFIIF. We used an RNA interference (RNAi)-based system in which TFIIB expression is ablated *in vivo* and replaced with a TFIIB derivative that contains a silent mutation and is refractory to the RNAi. Using this approach, we found that transcriptionally defective TFIIB amino-terminal mutants showed distinct effects on the basis of their ability to compete with wild-type TFIIB *in vivo*. Moreover, analysis of the TFIIB mutant derivatives by chromatin immunoprecipitation showed that promoter occupancy by TFIIB is dependent on the association with RNA polymerase II. Together, our results support a mode of preinitiation complex assembly in which TFIIB/RNA polymerase II recruitment to the promoter occurs *in vivo*.

Keywords: transcription; RNA polymerase II; TFIIB; promoter

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INTRODUCTION

The recruitment of RNA polymerase II (Pol II) to the promoter of a gene requires general transcription factors (GTFs; reviewed by Butler & Kadonaga, 2002; Hahn, 2004). TFIID binds to the TATA box (when present) within the promoter, which is then stabilized by transcription factor IIB (TFIIB). TFIIB can also make sequence-specific contact with the core promoter DNA (Lagrange *et al*, 1998; Qureshi & Jackson, 1998; Evans *et al*, 2001; Deng & Roberts, 2005). The assembly of TFIIB at the promoter is a prerequisite for the recruitment of Pol II, which enters the preinitiation complex (PIC) being formed along with TFIIF. TFIIE, TFIIH and other factors can then enter the complex. Several previous studies have suggested the existence of a preformed Pol II holoenzyme complex containing GTFs in addition to other components (reviewed by Lee & Young, 2000; Gill, 2001).

TFIIB has a crucial role in the PIC, showing absolute requirement at all promoters for the recruitment of Pol II.

TFIIB contains a zinc ribbon at the amino terminus and a carboxy-terminal core domain composed of α -helices that form two direct repeats (Bagby *et al*, 1995; Nikolov *et al*, 1995; Zhu *et al*, 1996). In between the zinc ribbon and core domain lies the most conserved region of TFIIB, termed the B-finger (Chen & Hahn, 2003, 2004; Bushnell *et al*, 2004). Recently, intact TFIIB has been crystallized as a complex with Pol II (Bushnell *et al*, 2004). In this structure, the B-finger lies within the Pol II catalytic centre, which is consistent with previous observations that mutations in this domain can cause alterations in the start site of transcription (Pinto *et al*, 1994; Fatar *et al*, 2001; Fairley *et al*, 2002).

So far, most analyses of mammalian TFIIB function have used *in vitro* transcription systems. In this study, we have used an RNA interference (RNAi)-based system to replace endogenous TFIIB in living cells. We uncover distinct mechanistic effects of TFIIB mutations that demonstrate the requirement for TFIIB to associate with RNA Pol II to form a stable PIC *in vivo*.

RESULTS AND DISCUSSION

Replacement of TFIIB in living cells using RNAi

We developed an RNAi-based approach to analyse TFIIB mutants in mammalian cells (see supplementary Fig S1 online). The vector pSUPER was used to drive expression of a short interfering RNA (siRNA) that targets the endogenous TFIIB transcript. Two silent point mutations were introduced into the human TFIIB complementary DNA (under the control of a cytomegalovirus (CMV) promoter) in the region targeted by the siRNA (smTFIIB). By simultaneous co-transfection of these plasmids, endogenous TFIIB expression can be ablated and replaced by a TFIIB derivative that is not targeted by the siRNA. In addition, the transfected smTFIIB encodes a T7 tag fused to the C terminus; this results in a slower migration in SDS–polyacrylamide gel electrophoresis (SDS–PAGE), which can easily be distinguished from endogenous TFIIB.

We generated plasmids driving the expression of smTFIIB that also harbour either the C34A or the R66E mutation. These TFIIB mutations, which perturb the zinc ribbon and B-finger, respectively, have previously been shown to have transcriptional defects (Buratowski & Zhou, 1993; Hawkes *et al*, 2000). pcDNA-smTFIIB_{T7}, or derivatives containing either the C34A or the R66E mutation, were transfected as above with pSUPER RNAi and a reporter containing the AdML promoter linked to chloramphenicol

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Fig 1 | Analysis of TFIIB function *in vivo* by replacement of endogenous TFIIB. (A) Cells were transfected with pSUPER RNAi and the indicated smTFIIB derivatives. CAT activity was measured and is presented graphically: each bar is the mean of three independent experiments with standard deviation. Below is an immunoblot of the transfected cells performed with anti-TFIIB antibodies. (B) As in (A), except that empty pSUPER vector was used instead of pSUPER RNAi. CAT, chloramphenicol acetyltransferase; RNAi, RNA interference; TFIIB, general transcription factor IIB; wtIIB, wild-type TFIIB.

acetyltransferase (CAT) and controlled by nine GAL4 DNA-binding sites. Where indicated, a plasmid driving the expression of GAL4(1–94)-VP16 was included. After the transfection procedure, CAT activity was measured (Fig 1A). Below the graph is an immunoblot of each transfection sample with anti-TFIIB antibodies. The results show that when the expression of endogenous TFIIB is ablated by RNAi, neither TFIIB R66E nor TFIIB C34A is able to restore transcription to a level observed with wild-type smTFIIB₇. The immunoblot with anti-TFIIB antibodies demonstrates the replacement of endogenous TFIIB with the smTFIIB derivatives. We also performed the same experiment, but using empty pSUPER vector (Fig 1B). As reported previously, TFIIB R66E is a potent inhibitor of transcription in living cells in the presence of endogenous TFIIB (Hawkes *et al*, 2000). By contrast, TFIIB C34A does not inhibit transcription under the same conditions. Together, the results in Fig 1 show distinct mechanistic defects caused by the C34A and R66E mutations in TFIIB. Although an

undefined amount of endogenous TFIIB will remain in cells treated with RNAi, our results indicate that TFIIB has become sufficiently limiting to discriminate between the effects of the co-transfected mutant TFIIB derivatives. Thus, TFIIB R66E fails to support transcription when it is the sole source of TFIIB and also inhibits transcription in the presence of endogenous TFIIB. By contrast, TFIIB C34A fails to support transcription when it is the only source of TFIIB, but does not inhibit transcription in the presence of endogenous TFIIB.

Distinct effects of TFIIB N-terminal mutations

We considered next whether the differential abilities of TFIIB C34A and TFIIB R66E to compete with endogenous TFIIB were promoter specific. Transfection experiments were performed in which wild-type TFIIB, TFIIB C34A or TFIIB R66E were ectopically expressed along with GAL4-VP16 and either the AdE4 promoter or the human immunodeficiency virus long terminal repeat (HIV LTR). In each case transcripts were detected by primer extension (Fig 2A). The results show that at both the AdE4 and HIV LTR promoters, TFIIB R66E is a potent inhibitor of transcription, whereas TFIIB C34A has no effect. We also found that the HSV thymidine kinase and human IGF II core promoters show similar effects with the TFIIB derivatives (data not shown). Thus, the inert and inhibitory effects of TFIIB C34A and TFIIB R66E, respectively, do not seem to be promoter specific.

We determined next whether the different effects that we observed with TFIIB R66E and TFIIB C34A could be recapitulated when we monitored expression of a stably integrated promoter rather than an episomal transfected reporter. A 293 cell line containing an integrated G9AdML-Luciferase cassette was transfected with a plasmid driving the expression of GAL4(1–94)-VP16 along with wild-type TFIIB, TFIIB R66E or TFIIB C34A. After 48 h, luciferase activity was measured (Fig 2B). We note that expression of wild-type TFIIB caused a reduction in the activity of the endogenous reporter. However, as we observed with the episomal reporter, overexpression of TFIIB R66E was an inhibitor of transcription but overexpression of TFIIB C34A was without significant effect when compared with wild-type TFIIB. Thus, the distinct effects of TFIIB C34A and TFIIB R66E are generally observed at different promoters in either an episomal or an integrated context.

Mechanism of inhibition by TFIIB B-finger mutation

We sought next to determine the mechanistic basis for the effects that we observed with the TFIIB derivatives C34A and R66E. We first assessed, by band shift, their ability to assemble with TATA-binding protein (TBP) at the promoter and also to recruit Pol II/TFIIF (Fig 3A). Both TFIIB C34A and TFIIB R66E were able to form a TFIIB–TBP–AdML promoter complex that was indistinguishable from that observed with wild-type TFIIB. However, TFIIB C34A showed a clear defect in the formation of a complex containing Pol II/TFIIF, which is consistent with previous reports using other zinc ribbon mutations in TFIIB (Buratowski & Zhou, 1993; Tubon *et al*, 2004). In addition, as we have reported previously, TFIIB R66E does not show a defect in the recruitment of Pol II/TFIIF to the promoter (Glossop *et al*, 2004). Thus, both TFIIB C34A and TFIIB R66E can form a complex with TBP at the promoter. However, TFIIB R66E, but not TFIIB C34A, is able to extend PIC formation to incorporate Pol II and TFIIF.

A

wtIIIB C34A R66E

+ + + + + + + + + + + + -

- - - - - - - - - - - - -

Pol/F
TBP

TBPolIF

TB

FP

B

GST GST-RAP30 GST-RAP74

wtIIIB

C34A

R66E

C

wtIIIB C34A R66E

Inputs

+ + + + + +

Anti-ctrl
Anti-pol II

TFIIB

wtIIIB C34A R66E

Fig 3 | Analysis of preinitiation complex formation by TFIIB derivatives. (A) Electrophoretic mobility shift assay to analyse the formation of a Pol II/TFIIF-TFIIB-TBP-AdML promoter complex. Where indicated, the reactions contained 4 ng TBP, 50 ng Pol II, 10 ng TFIIF and 25 or 50 ng TFIIB (or derivatives). Complexes were resolved by non-denaturing electrophoresis and detected by autoradiography. FP is free probe, TB is TFIIB-TBP promoter complex and TBPoF is Pol II-TFIIF-TFIIB-TBP promoter complex. (B) GST, GST-RAP30 or GST-RAP74 (each at 1 µg) was used as an affinity matrix in binding assays with recombinant wild-type TFIIB (wtIIB; 500 ng) or the indicated TFIIB derivatives. Stably bound proteins were resolved by SDS-PAGE, immunoblotted with anti-TFIIB antibodies and detected by enhanced chemiluminescence. (C) Highly purified Pol II (50 ng) was incubated with wild-type TFIIB (wtIIB) or the indicated TFIIB mutant derivative (100 ng of each protein; input shown as a Coomassie-stained gel at left). Immunoprecipitation was performed with either anti-Pol II or anti-promyelocytic leukaemia protein (anti-ctrl) antibodies and the products were resolved by SDS-PAGE, immunoblotted with anti-T7 antibodies and then detected by enhanced chemiluminescence. GST, glutathione *S*-transferase; Pol II, polymerase II; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBP, TATA-binding protein; TFIIB, general transcription factor IIB.

Our interaction data suggest that both TFIIB C34A and TFIIB R66E can form a complex with TBP at the promoter and that TFIIB R66E, but not TFIIB C34A, can extend PIC assembly further to incorporate Pol II/TFIIF. Thus, the inhibitory effect of TFIIB R66E may be due to its ability to interact with Pol II. To test this possibility, we generated a TFIIB derivative that contained both C34A and R66E mutations. Plasmids driving the expression of wild-type TFIIB, TFIIB R66E, TFIIB C34A or the compound mutant TFIIB C34A:R66E were transfected in 293 cells with the G9MLCAT reporter and GAL4(1–94)-VP16 expression plasmid. CAT transcripts were analysed by primer extension and are presented in Fig 4. The results show that TFIIB R66E inhibits

Fig 4 | Analysis of the mode of inhibition by TFIIB R66E *in vivo*. Plasmids driving the expression of wild-type TFIIB (wtIIB) or the indicated mutants (2 μ g) were introduced into embryonic kidney 293 cells with 2 μ g of the reporter G9MLCAT and a plasmid driving the expression of GAL4(1–94)-VP16 (0.2 μ g). After 48 h, RNA was prepared and CAT transcripts were detected by primer extension. An autoradiograph detecting the transcripts is shown. CAT, chloramphenicol acetyltransferase; TFIIB, general transcription factor IIB.

transcription as before, but the compound mutant TFIIB C34A:R66E does not inhibit transcription. Thus, the C34A mutation renders TFIIB R66E incapable of competing with endogenous TFIIB to inhibit transcription, which suggests that inhibition of transcription requires an intact TFIIB–Pol II interaction. Analysis of TFIIB zinc ribbon mutant G30A further demonstrated that a TFIIB derivative that is defective in Pol II recruitment cannot compete with wild-type TFIIB to inhibit transcription (see supplementary Fig S2 online).

To examine further the basis for the differential effects of the TFIIB derivatives C34A and R66E, we tested their ability to assemble at a promoter in living cells. 293 cells were transfected with a plasmid driving the expression of wild-type TFIIB, TFIIB C34A or TFIIB R66E. After 48 h, chromatin immunoprecipitation was performed with T7 antibodies to specifically concentrate chromatin associated with each TFIIB derivative. Real-time PCR was then performed to monitor precipitation of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (Fig 5A). Both wild-type TFIIB and the TFIIB mutant derivative R66E assembled at the GAPDH promoter; by contrast, TFIIB C34A did not. We observed similar results when we examined assembly of these same TFIIB derivatives at the β -tubulin promoter (supplementary Fig S3 online). These data suggest that TFIIB R66E inhibits transcription by virtue of its ability to compete with wild-type TFIIB to assemble at the promoter. Furthermore, the results show a direct correlation between TFIIB–Pol II contact and not TFIIB–TBP contact in the recruitment of TFIIB to a promoter *in vivo*. It is not clear why TFIIB R66E should fail to form a functional PIC, but it is likely that TFIIB R66E is specifically defective in a crucial step in initiation. Interestingly, previous structural analysis locates TFIIB residue R66 close to the active site of Pol II in a PIC (Bushnell *et al*, 2004). Thus, a direct effect on Pol II may underlie the defect of TFIIB R66E.

Together, our results indicate that TFIIB cannot assemble at the promoter simply by virtue of its ability to interact with TBP and promoter DNA. Instead, the assembly of TFIIB at the promoter is determined by its interaction with Pol II (see model in Fig 5B). Studies in yeast also suggest that the inhibitory effect of some TFIIB mutants correlates with an intact TFIIB N terminus and also the core domain that associates with TBP (Gonzalez-Couto *et al*,

Fig 5 | Assembly of TFIIB amino-terminal mutants at the promoter *in vivo*. (A) 293 cells were transfected with 2 μ g of a plasmid driving the expression of wild-type TFIIB or the indicated derivative. After 24 h, chromatin immunoprecipitation was performed with anti-T7 antibodies to specifically isolate chromatin associated with the transfected TFIIB derivatives. Real-time quantitative PCR was performed with primers to amplify the GAPDH promoter region (left) or a control non-promoter region (right). Results shown are the average of three independent experiments, each normalized to input. (B) A model of TFIIB–Pol II association with the promoter is shown (discussed in the text). Assembly of wild-type TFIIB (wtIIB) and the TFIIB mutants (IIB_{R66E} and IIB_{C34A}) is depicted. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Pol II, polymerase II; TFIIB, general transcription factor IIB.

1997). Our present data agree with this idea and further suggest that stable assembly with TFIID at a promoter *in vivo* requires the association of TFIIB with Pol II. Previous studies in yeast demonstrated that recruitment of TBP to the promoter requires intact components of the holoenzyme (Kuras & Struhl, 1999; Li *et al*, 1999). Similarly, yeast TFIIB does not assemble at the promoter if Pol II or mediator is defective (Ranish *et al*, 1999). Thus, the interdependence of the GTFs in recruitment to the promoter probably occurs in both yeast and mammalian cells. Such a mode of assembly is consistent with the existence of a Pol II holoenzyme or transcription factories in living cells (Lee & Young, 2000; Pombo *et al*, 2000). A sequential recruitment mechanism is equally plausible (see for example Hatzis & Talianidis, 2002), but this would require that stable complexes form only at specific intermediates in the assembly process, and specifically a dependence on Pol II for the stable association of TFIIB.

METHODS

Plasmids and proteins. G9ML, pET TFIIB, pET6His-TBP, pcDNA3TFIIB and pCMV Gal4(1–94)-VP16 have been described previously (Ha *et al*, 1993; Hawkes & Roberts, 1999). The TFIIB derivatives were produced by site-directed mutagenesis using the Stratagene Quikchange kit (Stratagene, La Jolla, CA, USA).

Recombinant wild-type TFIIB, TFIIB derivatives and His-tagged TBP were purified as described previously (Reece *et al*, 1993; Hawkes & Roberts, 1999). Recombinant Rap30, Rap74 and purified Pol II were obtained from Protein One Inc (Bethesda, MD, USA).

Cell culture. Human embryonic kidney 293 (HEK 293) cells were maintained and transfected as described previously (Hawkes & Roberts, 1999). For production of stable cell lines, AdMLuc and pcDNA3 were transfected into 293 cells followed by selection with 1 mg/ml G418. After 7 days, stably transfected clones were selected, expanded and tested for reporter construct activity. For ablation of expression of endogenous TFIIB and replacement by smTFIIB, in conjunction with a reporter assay, HEK 293 cells were transfected twice in the following way: initially, cells were transfected with 2 µg of pSUPER-RNAi, 2 µg of each smTFIIB derivative and 0.2 µg of GAL4-VP16. After 48 h, cells were transfected with 2 µg of pSUPER-RNAi, 2 µg of each smTFIIB derivative and 2 µg of G9MLCAT. Cells were then collected at 96 h in PBS. CAT assays were performed and quantified by phosphorimager analysis. Total RNA preparation and detection of CAT RNA were as described (Hawkes & Roberts, 1999). Luciferase activity was measured with a kit (Promega, Madison, WI, USA).

Complex assembly and protein interaction assays. Band-shift assays were performed as described previously (Maldonado *et al*, 1990). GST-Rap30 and GST-Rap74 preparation, binding assays and immunoprecipitation were performed as described previously (Hawkes & Roberts, 1999). Immunoprecipitation was performed with anti-RNA Pol II antibody (Santa Cruz, CA, USA) or anti-promyelocytic leukaemia (PML) antibody (Santa Cruz), and the incubation was continued overnight. A 50 µl portion of a 50% slurry of Protein A agarose was then added to the samples and the samples were incubated at 4 °C with rocking for 2 h. The beads were then washed four times in immunoprecipitation buffer and bound proteins eluted with SDS-PAGE loading dye.

Chromatin immunoprecipitation. 293 cells were treated with 1% formaldehyde for 10 min at 20 °C before quenching with 0.125 M glycine for 5 min. Cells were collected in ice-cold PBS with complete protease inhibitors (Roche, Basel, Switzerland) and washed sequentially with Buffer I (10 mM HEPES pH 6.5, 0.5 mM EGTA, 10 mM EDTA, 0.25% Triton X-100) and Buffer II (10 mM HEPES pH 6.5, 0.5 mM EGTA, 1 mM EDTA, 200 mM NaCl) and then resuspended in SDS lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA, 1% SDS). Lysates were sonicated on ice to yield 200–800 bp DNA fragments. One half of a 6 cm dish was used per immunoprecipitation, diluted 1/10 in immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl) and incubated overnight at 4 °C with either 1 µg of T7 antibody (Novagen, Charlottesville, VA, USA) or 1 µg of nonspecific IgG (Upstate, Darmstadt, Germany). Immunocomplexes were precipitated by incubation for 30 min with protein G-conjugated magnetic beads (Dyna, Carlsbad, CA, USA) that had been pre-blocked by incubation with 10 µg of salmon sperm DNA. Immunoprecipitates were washed sequentially with TSEI (20 mM Tris pH 8.1, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), TSEII (20 mM Tris pH 8.1, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), Buffer III (10 mM Tris pH 8.1, 0.25 M LiCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate) and Tris-EDTA before eluting in 1% SDS/0.1 M NaHCO₃. Crosslinks were reversed by

heating to 65 °C overnight and then treating with proteinase K for 1 h at 45 °C. Chromatin was cleaned using QiaQuick PCR clean-up columns (Qiagen, Hilden, Germany). Quantitative PCR was performed with Quantitect SYBR green reagent (Qiagen) using primers specific to human *GAPDH* promoter (forward, AAAAGCGGGGAGAAAGTAGG; reverse, GCTGCGGGCTCAATT TATAG) or a non-promoter region in intron 3 of the *SRF* gene (forward, GCCACAGGGCAGTAGATGTT; reverse, TCAGGCC CAAGTATCCACTC).

Supplementary information is available at *EMBO reports* online (<http://www.emboareports.org>).

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