A conformational change in TFIIB is required for activator-mediated assembly of the preinitiation complex

James A. Glossop, Tim R. Dafforn and Stefan G. E. Roberts*

School of Biological Sciences, The Michael Smith Building, University of Manchester, Oxford Road, Manchester M13 9PT, UK

Received January 26, 2004; Revised and Accepted March 4, 2004

ABSTRACT

TFIIB plays a pivotal role during assembly of the RNA polymerase II transcription preinitiation complex. TFIIB is composed of two domains that engage in an intramolecular interaction that can be disrupted by the VP16 activation domain. In this study, we describe a novel human TFIIB derivative harbouring two point mutations in the highly conserved N-terminal charged cluster domain. This mutant, TFIIB R53E:R66E, exhibits an enhanced affinity in its intramolecular interaction when compared with wild-type TFIIB. Consistent with this, the mutant displays a significantly reduced affinity for VP16. However, its ability to complex with TATA-binding protein at a model promoter is equivalent to that of wild-type TFIIB. Furthermore, this TFIIB derivative is able to support high order preinitiation complex assembly in the absence of an activator. Strikingly though, an activator fails to recruit the TFIIB mutant to the promoter. Taken together, our results show that a TFIIB conformational change is critical for the formation of activator-dependent transcription complexes.

INTRODUCTION

The initiation of class II gene transcription in eukaryotes requires the assembly of a preinitiation complex (PIC) in order to recruit RNA polymerase II (pol II) to the promoter (1). The general transcription factor TFIIB plays a central role during this process, providing a physical bridge between promoter-bound TFIID and pol II/TFIIF.

TFIIB contains two defined domains. The structure of the core C-terminal domain has been solved and comprises two imperfect direct α-helical repeats (2,3). The second of these repeats contains a helix–turn–helix motif that confers sequence-specific binding to the TFIIB recognition element (BRE) located immediately upstream of the TATA box in a subset of promoters (4–6). TFIIB can also make specific contacts with the DNA downstream of TATA (7). The extreme N-terminus of TFIIB contains a zinc ribbon, for which the structure has been determined (8). TFIIB has also been reported to possess acetyltransferase activity and can acetylate itself (9).

The N- and C-terminal domains of TFIIB engage in an intramolecular interaction that has been proposed to regulate transcription (10). The transcriptional activation domain of the herpes simplex virus 1 (HSV1) VP16 protein is able to disrupt the TFIIB intramolecular interaction, inducing a conformational change in TFIIB (11). Subsequent studies have demonstrated that TFIIB conformation can modulate transcription, but the underlying mechanism(s) has yet to be determined (12–14).

In addition to stabilizing and correctly orienting the TATA-binding protein (TBP)–promoter complex and recruiting pol II/TFIIF, other roles for TFIIB during pol II transcription have been established. For instance, TFIIB plays a critical role in selection of the transcription start site (15–17) and also has a function following PIC assembly (7,18–20). Both require a region within the N-terminus of TFIIB that lies between the zinc ribbon and the core domain. This domain, for which no structural information is available, contains a cluster of highly conserved charged residues (charged cluster domain, CCD) (Fig. 1A).

We have shown previously that a point mutation in the CCD of human TFIIB (R66E) enhances the affinity of the intramolecular interaction, driving the equilibrium towards a closed conformation (Fig. 1B) (14). TFIIB R66E was found to severely inhibit transcription in vivo but not in vitro, precluding a direct analysis of the alteration in TFIIB function that arises when the closed conformation is predominant.

In the present study, we have generated a novel TFIIB derivative that possesses an enhanced intramolecular interaction and, significantly, is defective in in vitro transcription assays. This has enabled us to analyse the effects of TFIIB conformation on PIC assembly and activator-mediated recruitment of TFIIB. Our results suggest that TFIIB conformational status does not regulate assembly of TFIIB into a complex with TBP at the promoter or the recruitment of pol II/TFIIF. However, we find that a change in TFIIB conformation is required for activator-mediated recruitment of TFIIB and thus to achieve high levels of transcription.

*To whom correspondence should be addressed. Tel: +44 161 275 5758; Fax: +44 161 275 5600; Email: stefan.roberts@man.ac.uk
(GST), GST–VP16 and GST–TFIIB (1–124) and derivatives were purified as previously described (21). α-TFIIB and α-Gal4 antibodies have been described before (17). Highly purified pol II and recombinant TFIIF were purchased from ProteinOne. TFIIF CCD mutations were generated using the Quickchange kit (Stratagene) and were confirmed by DNA sequencing.

**In vitro transcription and electrophoretic mobility shift assays**

HeLa cell nuclear extracts [in buffer D (20 mM HEPES, pH 8, 0.2 mM EDTA, 20% v/v glycerol, 1 mM DTT, 0.2 mM PMSF)] were purchased from Computer Cell Culture Centre (Mons, Belgium). In vitro transcription assays were performed as described previously (17) using the amounts of TFIIF indicated in the figure legends. Electrophoretic mobility shift assays (EMSA) were performed as described previously (26) using the amounts of TFIIF indicated in the figure legends. Briefly, an AdML promoter fragment was end-labelled with Klenow and [α-32P]dATP and binding reactions were assembled in 10 mM HEPES, pH 8, 4 mM MgCl2, 5 mM ammonium sulfate, 8% (v/v) glycerol, 2% (w/v) PEG, 55 mM KCl, 5 mM β-mercaptoethanol, 0.2 mM EDTA and 0.2 mM PMSF. Reactions were incubated at 30°C for 1 h (except where a time course was used) and then loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed at 120 V for 3 h in 0.5× Tris–borate–EDTA (pH 8.3). Gels were dried and autoradiographed overnight.

**Tryptic proteolysis and circular dichroism**

Purified recombinant human TFIIF and derivatives (1 μg) were incubated with increasing amounts of trypsin (Sigma) (0, 1, 5, 10, 50 and 100 ng) for 15 min at room temperature in reactions which also included GST (5 μg) to moderate the rate of proteolysis. Reactions were assembled in 20 mM HEPES (pH 8), 0.2 mM EDTA, 10% (v/v) glycerol, 150 mM KCl, 2.5 mM CaCl2 and 1 mM DTT. The digestion products were subsequently resolved by SDS–PAGE and western blotting performed with α-T7 antibody (Novagen) to detect TFIIF fragments via a C-terminal T7 epitope tag.

Circular dichroism (CD) measurements were made using a JASCO J-810 spectropolarimeter using a 0.02 cm path length demountable cuvette. A 0.25 mg/ml solution of each protein was dialysed against 50 mM phosphate buffer (pH 8) overnight at 4°C to remove chloride ions. Spectra were measured between 195 and 240 nm at a scan speed of 100 nm/min. Each measurement was repeated 10 times and the average used. The spectra were deconvolved to provide estimations of secondary structure content using CDSSTR (27) as part of the CDPRO software package.

**Affinity chromatography and immobilized DNA template assays**

Purified recombinant human TFIIF and derivatives were applied to minicolumns in buffer D containing KCl at the concentrations indicated in the figure legends. SDS–PAGE loading buffer was added to the fractions and the proteins resolved by SDS–PAGE and immunoblotted onto Immobilon P membrane (Millipore). α-T7 antibody was used to detect the TFIIF derivatives via a C-terminal T7 epitope tag.
RESULTS

Our previous studies showed that the arginine residue at position 66 in human TFIIB is critical in modulating the affinity of the TFIIB intramolecular interaction (Fig. 1A and B) (14). Subsequent studies revealed that the similarly conserved arginine at position 53 is also involved (data not shown and see below). We therefore hypothesized that the simultaneous mutation of R53 and R66 to acidic residues (Fig. 1A) might sufficiently enhance the TFIIB intramolecular interaction so as to manifest a transcriptional defect in vitro that could then be analysed further. Wild-type human TFIIB and the derivatives R53E, R66E and R53E:R66E (Fig. 1A) were prepared from Escherichia coli to equivalent purities, as revealed by SDS–PAGE and Coomassie staining (Fig. 1C).

VP16 and the N-terminus of TFIIB compete to interact with the TFIIB core domain (11,14). Thus, VP16 preferentially interacts with the open form of TFIIB. We exploited this fact, using VP16-binding capacity as an indicator of the affinities of the intramolecular interactions of our TFIIB CCD mutants. A salt elution profile was used to determine the relative affinities of wild-type TFIIB, TFIIB core domain (Δ4–85) and the CCD mutants for the VP16 activation domain immobilized as a GST fusion protein on a glutathione–agarose column (Fig. 2A). A GST column was used as a negative control. TFIIB Δ4–85 (which lacks a competing N-terminus) showed an enhanced affinity for VP16 (salt resistance) when compared to wild-type TFIIB. As shown before, TFIIB R66E (which favours the closed conformation) displayed a reduced affinity for VP16 (14). A similar effect was observed with TFIIB R53E. Significantly, and as anticipated, TFIIB R53E:R66E showed a further decrease in its affinity for VP16. These data strongly indicate that, when compared with the single amino acid CCD mutants, the R53E:R66E double point mutation drives the equilibrium further towards the closed conformation of TFIIB.

We next performed an affinity chromatography experiment to assess the avidity of the intramolecular interactions of the TFIIB derivatives in trans (Fig. 2B). Residues 1–124 of the wild-type and mutant human TFIIB derivatives were expressed as fusions to GST, immobilized onto beads and assembled into separate chromatography columns, along with GST alone as a control. TFIIB core domain (Δ4–85) was then applied to each of the TFIIB N-terminal derivative columns and a salt gradient used to determine the elution profile.

TFIIB core domain eluted from the wild-type TFIIB N-terminus column at a salt concentration of 100–120 mM. When the immobilized TFIIB N-terminus contained either the R53E or R66E single point mutations, 120–140 mM potassium chloride was required to elute all of the bound core domain, demonstrating an enhanced interaction. Combining these mutations within the CCD of TFIIB also caused an increase in the affinity of the TFIIB N-terminus for the core domain, but no further enhancement was observed when compared with the single point mutations. However, the VP16-binding data (Fig. 2A) strongly suggest that combining the R33E and R66E mutations further enhances the formation of closed form TFIIB. This disparity perhaps suggests a more complex or subtle alteration in the association of the N- and C-terminal domains in TFIIB R53E:R66E that occurs in intact TFIIB but may not be detectable using the separated domains.

The N-terminus of TFIIB has previously been shown to be sensitive to attack by trypsin (29,30). We therefore reasoned that the TFIIB CCD mutants, which show a greater propensity to adopt the closed form (Fig. 2), might exhibit a greater resistance to degradation by trypsin. Figure 3A shows the results of titrating trypsin into reactions containing either wild-type TFIIB or each of the CCD mutant derivatives. Intact TFIIB and the proteolytically derived core domain were detected by western blotting with an antibody directed against a C-terminal epitope tag. We consistently found that, as the
concentration of trypsin was increased, all of the TFIIB CCD mutants retained a higher proportion of intact full-length TFIIB when compared to the wild-type protein. Importantly, the amount of trypsin required to initiate the proteolysis of wild-type TFIIB and each derivative was the same. These data suggest that in all TFIIB populations there is a mix of the open and closed forms, but that the CCD mutations cause a shift in the equilibrium towards the closed conformation.

Using CD we next sought to determine if the mutations that we introduced into TFIIB had affected the secondary structure (Fig. 3B). Ellipticity measurements for each TFIIB protein were gathered between 195 and 240 nm and these plots were compared with the standard curves representing each class of protein secondary structure. The CD profile of wild-type human TFIIB reflected that of a protein population comprised mostly of α-helices (50%), consistent with the published structure of the TFIIB core domain (2,3). Each of the TFIIB mutants produced CD profiles that were equivalent to that observed with wild-type TFIIB. Thus, the CCD mutations analysed in this study do not cause significant changes to TFIIB secondary structure, but do augment the formation of closed form TFIIB (Figs 2 and 3A).

Figure 3. Analysis of the human TFIIB CCD mutants by proteolysis and CD. (A) Wild-type (wt) TFIIB or the indicated mutants were incubated with increasing amounts of trypsin (0, 1, 5, 10, 50 and 100 ng) for 15 min at room temperature. Following SDS–PAGE, western blotting was performed with α-T7 antibody to detect TFIIB fragments via a C-terminal T7 epitope tag. Data from one representative experiment of three independent repeats are shown. (B) A CD spectrum of each TFIIB protein was measured between 195 and 240 nm using a JASCO J-810 spectropolarimeter. Both experimental (exp) data and a fit (calc) obtained using CDSSTR are shown. mdeg, millidegrees. The table shows the secondary structure content parameters deconvolved from the spectra using CDSSTR. A representative result from three independent experiments is shown.

Figure 4. Human TFIIB R53E/R66E is defective for both basal and activated transcription in vitro. (A) Wild-type (wt) TFIIB or the indicated mutants (2 and 10 ng) were added to HeLa nuclear extract depleted of endogenous TFIIB by immunoaffinity chromatography. In vitro transcription from the AdML promoter was subsequently assayed by primer extension. NE, nuclear extract. Transcription was quantified using a phosphorimager as the level relative to basal transcription at the AdML promoter in standard NE. A representative result from three independent experiments is shown. A schematic representation of the AdML promoter construct used is shown above the autoradiogram with the Gal4 DNA-binding sites (Gal4 sites), TATA-box (TATA), initiator (INR) and chloramphenicol acetyltransferase (CAT) gene indicated. (B) Wild-type (wt) TFIIB or the indicated mutants (2 and 10 ng) were tested for their ability to support transcription in vitro as in (A), except that reactions also included the acidic activator Gal4–VP16 (upper panel), the synthetic acidic activator Gal4–AH (middle panel) or the glutamine-rich mammalian activator Gal4–Sp1 (lower panel) as indicated. NE, nuclear extract. Transcription was quantified using a phosphorimager as the level relative to basal transcription at the AdML promoter in standard NE. A representative result from at least three independent experiments is shown for each assay.
both the R53E and R66E TFIIB derivatives showed a modest impairment in supporting basal transcription when compared to wild-type TFIIB, the double point mutant R53E:R66E showed a significantly reduced activity.

TFIIB has been proposed as a target of transcriptional activator proteins (31). We therefore tested the ability of the TFIIB CCD mutants to support transcriptional activation mediated by Gal4–VP16, Gal4–AH and Gal4–SP1 (Fig. 4B). In each case the TFIIB R53E:R66E double substitution mutant showed a significant defect in supporting transcriptional activation that was not observed with the single CCD mutants. We therefore conclude that the TFIIB mutant R53E:R66E exhibits reduced function in supporting basal transcription, but is more defective in supporting activator-dependent transcription when compared to the single point mutations.

Having generated a TFIIB derivative that is sufficiently altered in conformation so as to manifest a transcription defect in vitro, we proceeded to determine the role of TFIIB conformation in PIC assembly. A central function of TFIIB is to form a complex with TBP at the promoter. Therefore, we assayed the abilities of each of the TFIIB CCD mutants to form a TFIIB–TBP–AdML promoter complex using EMSA. Increasing amounts of wild-type TFIIB (and the CCD mutants) were incubated with recombinant TBP and radio-labelled AdML promoter and the resulting complexes were resolved by electrophoresis (Fig. 5A). The results show that each of the CCD mutants forms a complex in a concentration-dependent manner equivalent to that observed with wild-type TFIIB. To investigate the possibility of more subtle effects, we also monitored the rate of assembly of the TFIIB derivatives into TFIIB–TBP–promoter complexes (Fig. 5B). The TFIIB CCD mutants were indistinguishable from wild-type TFIIB in their rate of promoter association.

The TFIIB–TBP–promoter complex provides a platform for the entry of pol II and TFIIF into the PIC. Indeed, it was originally proposed that the TFIIB intramolecular interaction might mask the binding sites for TFIIF and pol II and hence block PIC assembly (11). Therefore, we tested the ability of the TFIIB CCD mutants to form a TFIIB–TFIIF–Pol II–TBP–AdML promoter complex (Fig. 5C). Each of the TFIIB CCD mutants was able to mediate assembly of pol II at the promoter, although we note that TFIIB R53E showed a reduced ability. We have consistently observed that this is not the case with the TFIIB R53E:R66E mutant.

We finally considered if activator-mediated recruitment of TFIIB may underlie the defect that we observe in transcriptional activation with the R53E:R66E mutant. To investigate this, we used an immobilized DNA template to purify functional PICs from TFIIB-depleted nuclear extract that had been supplemented with either wild-type TFIIB or the R53E:R66E mutant. Figure 6A shows the results of a transcription assay performed using PICs purified in this way. As we observed before (Fig. 4B), the R53E:R66E TFIIB mutant is defective in supporting transcriptional activation. Figure 6B shows complexes that were purified as above but were instead immunoblotted to monitor the levels of GAL4–AH, TBP and TFIIB (wild-type and R53E:R66E mutant) that had assembled at the promoter. As observed before, TBP (TFIID) assembled at the promoter independently of the activator, whereas wild-type TFIIB showed activator-enhanced assembly at the promoter (28). In contrast, the TFIIB mutant R53E:R66E failed to undergo activator-mediated recruitment. Thus, the compromised transcriptional activation observed with TFIIB R53E:R66E can be explained, at least in part, by a defect in activator-mediated recruitment.
C-terminal domains of TFIIB. TFIIB that interfaces with the activator, these mutations activate. Rather than directly disrupting the surface within interaction surface for activator proteins, represent a different between an activator and TFIIB. Such TFIIB mutants are within the core domain that directly disrupt the interaction during transactivation (31). These studies used mutations activator protein and specifically for activator-mediated in the conformation of TFIIB is required in the response to an subsequent recruitment of pol II and TFIIF. Rather, a change TFIIB±TBP±promoter complex or play a significant role in the interaction does not modulate the assembly of TFIIB into a DISCUSSION

Previous studies using mutations that alter the affinity of the TFIIB intramolecular interaction have shown that a conformational change in TFIIB is vital for transcription (13,14). However, the underlying mechanism(s) was not established. The data presented here suggest that the TFIIB intramolecular interaction have shown that a conformational change in the mechanism of action of providing a threshold level of response. In summary, our may better compete with the TFIIB intramolecular interaction, for transcriptional synergy in that multiple activation domains transcriptional activators. It may also present a focal point the formation of PICs in response to transcriptional activators could provide a mechanism to control the potency of transcriptional activators. It may also present a focal point for transcriptional synergy in that multiple activation domains may better compete with the TFIIB intramolecular interaction, providing a threshold level of response. In summary, our present data have defined a critical function of the TFIIB conformational change in the mechanism of action of transcriptional activators.

ACKNOWLEDGEMENTS

We would like to thank John Glossop, Neil Perkins, Andy Sharrocks and members of the Roberts laboratory for helpful discussions and comments on the manuscript. We also thank Thomas Oelgeschläger for reagents. J.A.G. is supported by a research studentship from the BBSRC (UK). This work was funded by the Wellcome Trust (061207/Z/000/Z/CH/TG/dr). T.R.D. is an MRC Career Development Fellow. S.G.E.R. is a Wellcome Trust Senior Research Fellow.

REFERENCES
