The Role of Human TFIIB in Transcription Start Site Selection in Vitro and in Vivo

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The general transcription factor TFIIB plays a crucial role in selecting the transcription initiation site in yeast. We have analyzed the human homologs of TFIIB mutants that have previously been shown to affect transcription start site selection in the yeast Saccharomyces cerevisiae. Despite the distinct mechanisms of transcription start site selection observed in S. cerevisiae and humans, the role of TFIIB in this process is similar. However, unlike their yeast counterparts, the human mutants do not show a severe defect in supporting either basal transcription or transcription stimulated by an acidic activator in vitro. Transient transfection analysis revealed that, in addition to a role in transcription start site selection, human TFIIB residue Arg-66 performs a critical function in vivo that is bypassed in vitro. Furthermore, although correct transcription start site selection is dependent upon an arginine residue at position 66 in human TFIIB, innate function in vivo is determined by the charge of the residue alone. Our observations raise questions as to the evolutionary conservation of TFIIB and uncover an additional function for TFIIB that is required in vivo but can be bypassed in vitro.

Transcription of a gene by RNA polymerase II (pol II) requires the assembly of the general transcription factors (GTFs) at the promoter to form a preinitiation complex (PIC; reviewed in Ref. 1 and 2). The GTFs assemble in an ordered fashion, beginning with the binding of TFIID to the TATA element present at most promoters. TFIID itself is a multiprotein complex of which one component mediates binding to the TATA element (TATA-binding protein; TBP). TFIIF interacts with the N terminus of TFIIB and pol II with the PIC. Deletion analysis of human TFIIB demonstrated that TFIIF interacts with the N terminus of TFIIB and pol II with the core C-terminal region (4). Thus, a conformational change in TFIIB may facilitate the interaction between TFIIB and pol II.

Underlying the central role of TFIIB in PIC assembly, TFIIB has been proposed as a target of transcriptional activator proteins (reviewed in Ref. 1 and 2). At the adenovirus E4 promoter, TFIIB assembly is a limiting event that can be facilitated by an activator protein (13). Moreover, several activation domains can interact with TFIIB, and in vitro evidence suggests that this interaction is required for transcriptional activation (14). Significantly, the acidic activation domain of the herpes simplex virus VP16 protein can induce a conformational change in TFIIB that disrupts the intramolecular interaction (12). This provides a possible mechanism by which activators could stimulate further assembly of the PIC.

Once a PIC has assembled, the region around the transcription initiation site melts to provide a template strand for pol II. The actual site at which transcription initiation occurs is approximately 25–30 base pairs downstream of the TATA box in most eukaryotes (reviewed in Ref. 1). Where present, the TATA element is the sole determinant of the transcription initiation site, and initiation will occur at the distance set by TATA regardless of the sequence around the site of initiation (15). One well studied exception to this is the yeast S. cerevisiae, in
which the transcription initiation site can occur anywhere between 40 base pairs and 120 base pairs downstream of the TATA element (reviewed in Ref. 1). Even so, open complex formation occurs at a distance downstream of TATA similar to that seen in other organisms. A scanning polymerase model has been proposed in S. cerevisiae in which the PIC translocates downstream of the melted DNA and initiates transcription at distant sites (16). Schizosaccharomyces pombe exhibits a start site position akin to that seen in human cells, 25–30 base pairs downstream of the TATA element. Factor-swapping experiments between S. pombe and S. cerevisiae found that TFII B and pol II play a key role in determining the transcription initiation site (17). TFII B and pol II are not individually interchangeable between the two yeast species. However, when exchanged together, not only does this result in full transcriptional activity in both species, but the start site use reflects the species from which the TFII B and pol II were derived. Thus, TFII B and pol II in concert determine the differences in transcription initiation site between S. cerevisiae and other eukaryotes.

A genetic screen in S. cerevisiae recovered mutations in TFII B that confer a bias toward the use of downstream transcription initiation sites (18). Two critical residues in yeast TFII B were analyzed; Glu-62 and Arg-78. Substitution of residue Glu-62 with an amino acid of opposing charge (Lys) resulted in a yeast strain that exhibited cold sensitivity and a slow growth phenotype at 30 °C (19). Transcription at the CYC1 and ADH1 genes in this strain showed a preference for transcription start site selection that is involved in transcription start site selection (19). The mutants (E51R, R66E, R66A, R66K, and E51R/R66E) were cloned into the vector pCDNA3 for expression in 293 cells. The mutants (E51R, R66E, R66A, R66K, and E51R/R66E) were cloned downstream of 5 GAL4 sites in the vector pGEM3.

TFII B and Transcription Start Site Selection

Polymerase chain reaction-mediated site-directed mutagenesis was performed to produce the E51R, E51D, E51A, R66E, R66K, R66A, and E51R/R66E mutants. All clones were sequenced to ensure only the intended mutations were present. cDNAs encoding wild type TFII B and the mutants (E51R, R66E, R66A, R66K, and E51R/R66E) were cloned into the vector pcDNA3 for expression in 293 cells. The in vivo vector expressing GAL4-R11 has been described previously (24).

EXPERIMENTAL PROCEDURES

Plasmids—The promoter DNA template G5E4T has been described previously (23). G5ML contains nucleotides 50 to +22 from the adenovirus major late promoter cloned downstream of 5 GAL4 sites in the vector pGEM3.

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Protein Purification—TFII B and TFII B mutants and GAL4-AH were purified as described (4, 29). Polystyrene-tagged TBP was purified by nickel chelate affinity chromatography as described by the manufacturer (Qiagen). The HeLa fraction containing RNA polymerase II, TFII F, TFII E, and TFII H was purified as described previously (25).

RESULTS

Human TFII B Mutants E51R, R66E, and E51R/R66E Support Transcription in Vitro—The region of the TATA element of the SV40 promoter, and transcripts were detected by primer extension. We discuss these observations in light of current models of the role of TFII B in both PIC formation and transcription start site selection.

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Moreover, all of the mutants utilized the same transcription initiation site as that observed with wild type TFIIB.

In view of our finding that E51R, R66E, and E51R/R66E support transcription in vitro at a level equivalent to wild type TFIIB, we next tested the possibility that the mutants may be defective in supporting the levels of transcription required during transcriptional activation. To monitor the high levels of transcription in the presence of the activator GAL4-AH, we prepared a nuclear extract depleted of TFIIB by chromatography.
The three major groups of transcripts are indicated (A, B, and C). The sequence of this region of the AdE4 promoter is shown in Fig. 3B, wild type (wt) TFIIB or the mutants shown in panel A were tested in a TFIIB-depleted nuclear extract at the AdE4 promoter in the presence of GAL4-AH as described in Fig. 3A.

**Fig. 3.** The human TFIIB mutants cause a downstream shift in the transcription initiation site at the adenovirus E4 promoter. A, wild type (wt) TFIIB or the indicated mutants were tested as in Fig. 2B but with the adenovirus E4 promoter (shown below the autoradiogram). The three major groups of transcripts are indicated (A, B, and C). B, transcripts produced at the AdE4 promoter with wild type TFIIB and E51R as shown in panel A were resolved on a 10% sequencing gel alongside a sequencing ladder of the same promoter. C, sequence of the AdE4 transcription initiation region with arrows above and below, indicating the relative intensity of each transcript produced in the presence of wild type TFIIB and the mutant E51R, respectively.

**Fig. 4.** Determinants in TFIIB required for correct transcription start site selection. A, recombinant human TFIIB or the indicated mutants were purified from E. coli and analyzed by SDS-polyacrylamide gel electrophoresis/Coomassie staining. B, wild type (wt) TFIIB or the mutants shown in panel A were tested in a TFIIB-depleted nuclear extract at the AdE4 promoter. Consistent with the results we obtained with the AdML promoter, E51R, R66E, and E51R/R66E supported a level of transcriptional activation equivalent to wild type TFIIB. However, there was a clear shift toward the use of downstream initiation sites by all three of the mutants compared with wild type TFIIB. Specifically, there was an increase in the use of distal transcription initiation sites (indicated B and C) compared with proximal (A). The same effect was observed when we used GAL4-SP1 as the transcriptional activator (data not shown). To examine this further, we resolved the different transcripts produced by wild type TFIIB and E51R alongside a sequencing ladder of the AdE4 promoter produced using the same radiolabeled primer (Fig. 3B). Comparing the transcripts produced by wild type TFIIB with those produced by E51R shows a clear shift toward the use of downstream initiation sites.

**Determinants of the Transcription Start Site Shift**—The TFIIB start site mutations used above involve the substitution of charged amino acids with residues of opposite charge. We next constructed substitutions of Glu-51 and Arg-66 to either alanine (E51A and R66A) or different amino acids of similar charge (E51D and R66K). The recombinant proteins (shown in Fig. 4A) were tested in a TFIIB-depleted nuclear extract at the AdE4 promoter (Fig. 4B). As seen with the original mutants, E51A, E51D, R66A, and R66K all supported transcription in vitro to a level equivalent to that observed with wild type TFIIB. Furthermore, they all caused a downstream shift in the transcription start sites. Thus, the charge per se of these residues is not the critical determinant in correct transcription start site selection in vitro.

**Analysis of the Human TFIIB Mutants in Vivo**—The human TFIIB start site mutants E51R, R66E, and E51R/R66E support levels of basal and activated transcription in vitro similar to that seen with wild type TFIIB. It would be predicted therefore that the addition of an excess of one of the mutant TFIIB proteins to a native nuclear extract would titrate the HeLa TFIIB and result in a downstream shift in the transcription initiation site. We tested this possibility...
and found that this was indeed the case (data not shown and Fig. 5A). We reasoned that this effect would also occur in a living cell and therefore used a transient transfection assay to determine whether the start site shift effects we observe in vitro could be recapitulated in vivo. Transfection of G5E4T into living cells failed to produce a stable message. We therefore used the same G5E4 core promoter but fused to CAT, which allowed us to detect stable transcripts. Thus, it was first necessary to test G5E4CAT in vitro to ensure that we could make a direct comparison with the in vivo effects. Fig. 5A shows a transcription assay using G5E4CAT and native nuclear extract to which either wild type TFIIB, E51R, R66E, or E51/R66E had been added. Results comparable with those seen in a TFIIB-depleted extract with G5E4T were obtained. Specifically, the mutants caused a shift toward the use of distal transcription initiation sites (B and C) with a concomitant reduction in the proximal initiation sites (A). Next, wild type TFIIB or the mutants (E51R, R66E, and E51/R66E) under a cytomegalovirus promoter were transfected into human embryonic kidney 293 cells along with the activator Gal4-R11 and G5E4CAT. The cells were harvested 48 h after transfection, total RNA was prepared, then primer extension was performed to analyze the transcripts. A representative assay is shown in Fig. 5B. There are several striking features in this result. First, there is a clear difference in the transcription start site pattern at the AdE4 promoter in vivo compared with that seen in vitro. Essentially, the most distal initiation sites seen in vitro (C) are not used in vivo. This cannot be explained by the difference in cell type as nuclear extracts made from 293 cells produce the same pattern of transcripts at the AdE4 promoter as HeLa cell nuclear extracts (data not shown). Second, in contrast to our in vitro observations, R66E significantly inhibited transcription in the transient transfection assay, but E51R and E51/R66E did not. This was not because of a difference in the levels of the various TFIIB constructs, as immunoblotting showed that wild type TFIIB and the mutants were of similar abundance (Fig. 5C). Finally, overexpression of E51R and E51R/R66E caused a shift toward the use of the downstream transcription initiation sites. The intensities of the two sets of transcripts (A and B) were quantified and are expressed as a ratio (B/A) below each lane. Thus, although there are greater constraints on the pattern of transcription initiation at the AdE4 promoter in vivo, there is a clear shift toward the use of the downstream transcription initiation sites in vivo by the TFIIB mutants. Moreover, the low level of E4 transcription remaining in cells transfected with R66E also exhibited the downstream shift. We next performed a set of transfections identical to those above but using G5MLCAT as a reporter (Fig. 5D). As seen with the E4 promoter, R66E significantly inhibited transcription, but E51R and E51/R66E did not. Consistent with our in vitro data, only a single transcript was produced from the AdML promoter, and the mutants did not cause the use of an alternative site.

In view of the difference we observed in the properties of hTFIIB R66E in vivo versus in vitro, we next compared R66E with the mutants R66A and R66K in a transient transfection assay (Fig. 6A). As before, R66E inhibited transcription, with residual activity showing a downstream shift in transcription initiation site. The mutant R66A was also inhibitory, although we consistently found this mutant to be less inhibitory than R66E. In agreement with our in vitro observations, R66A also caused a downstream shift in the site of transcription initiation. Interestingly, R66K did not significantly inhibit transcription transcription in vivo, although it did cause a downstream shift in the transcription initiation site. All of the mutants were expressed at similar levels in the transfection assay, as assessed by immunoblotting (Fig. 6B). Thus, although the charge of hTFIIB residue 66 is critical in determining the innate transcription function of TFIIB in vivo, it is not sufficient for correct transcription start site utilization either in vivo or in vitro.

DISCUSSION

In this study we have demonstrated that, despite the distinct mechanisms in transcription start site selection observed in S. cerevisiae and mammals, the role of TFIIB in this process is highly conserved. The mutation of two highly conserved charged residues in the N terminus of human TFIIB, homologous to those previously described in the S. cerevisiae, caused a downstream shift in transcription start site selection both in vitro and in vivo. The transcription start site shift caused by
mutants used in this study was the same. Presumably, any alteration in the positioning of pol II at the promoter would be constrained relative to TATA and, therefore, provide a limited number of potential transcription start sites. We also note that the TFIIB mutants are still able to direct transcription initiation at all of the upstream sites seen with wild type TFIIB, albeit to a lesser degree. It is therefore likely that the role of TFIIB in the selection of the transcription initiation site may be restricted to modulating the 3′ parameter, whereas other factors determine the 5′ parameter. Indeed, mutants of the S. cerevisiae RBP9 subunit of pol II cause an upstream shift in transcription start site selection (28, 29).

The conservative TFIIB substitution mutants E51D and R66K also caused a downstream shift in transcription start site selection. Thus, charge per se within this region is not the sole determinant of correct transcription start site selection. Alterations in the size of the side chains of key amino acids may also have effects on the alignment of a protein-protein interaction. Significantly, the residues corresponding to human TFIIB Glu-51 and Arg-66 are totally conserved in all species sequenced to date. Although other charged residues are also totally conserved, some are substituted by residues of similar charge, and still others, where the charge is removed altogether. It is important to note that TFIIB exhibits a high degree of species specificity. Unlike TBP, S. cerevisiae and human TFIIB are not functionally interchangeable, even in basal transcription (30). In addition, it has been reported that human TFIIB can substitute for Drosophila TFIIB only at certain promoters (31). It is possible that the differences in the charge cluster domain of TFIIB contribute to this species specificity.

In contrast to our results with human TFIIB, homologous yeast TFIIB mutants are defective for transcription in vitro (10, 20–22). This defect of the S. cerevisiae mutants correlates with their inability to stably interact with RNA polymerase II in several different assays. Deletion mapping studies of human TFIIB have shown that the N terminus (including the region studied in this work) is dispensable for interaction with RNA polymerase II (4). However, deletion of the N terminus of S. cerevisiae TFIIB abolishes the interaction with pol II (22). Furthermore, evidence has been presented that the region of S. cerevisiae TFIIB responsible for transcription start site selection and direct interaction with pol II may constitute distinct, but overlapping, domains within the N terminus (22). Thus, it is possible that the nature of the contacts between TFIIB and pol II (and/or other components of the general transcription machinery) have diverged from S. cerevisiae to humans. Perhaps yeast TFIIB requires a more intricate interaction with pol II as part of the scanning mechanism responsible for transcription start site selection in S. cerevisiae. Because S. pombe exhibits a transcription start site pattern similar to that seen in human cells, analysis of the TFIIB-pol II interaction in this species should help resolve this issue.

The human TFIIB R66E was indistinguishable from E51R or E51R/R66E in vitro transcription assays. However, in transfection analysis R66E was a potent inhibitor of transcription, whereas E51R and E51R/R66E caused effects similar to those seen in vitro. Importantly, the low level of transcription observed in the presence of R66E in vivo exhibited a downstream shift in the transcription initiation site. Thus, TFIIB R66E is severely compromised in its ability to support transcription in vivo in a manner that is bypassed in vitro. Furthermore, this defect can be suppressed by the additional mutation E51R. This compensation effect in vitro is consistent with the previous observation that the S. cerevisiae homolog of human R66E is lethal but can be rescued by a second mutation.

The transcription start site shift induced by the various

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corresponding to human E51R (19). The conclusion of the observations in *S. cerevisiae* was that these two residues of opposite charge form a salt bridge. Our data are consistent with this proposal but also suggest that the salt bridge has a function that is not critical for the innate ability of TFIIB to support transcription of a naked DNA template in *vitro*. Substitution of Arg-66 with lysine also results in a TFIIB mutant that causes a downstream shift in transcription initiation site both in *vitro* and *in vivo*. However, this conservative substitution mutant does not significantly inhibit transcription in *vivo*. Taken together, these data suggest that Arg-66 plays a role in two functions; first, transcription start site selection that is determined by the specific amino acid side chain, and second, a role that is required in *vivo* but not in *vitro* and is dependent upon the charge of the residue only.

A function that is required of TFIIB in *vivo*, but is bypassed in *vitro*, has several implications for our functional PIC in *vitro* as compared with those observed in *vitro*. Several GTFs are dispensable for transcription in *vitro* using supercoiled DNA templates (reviewed in Ref. 2). TFIIF is one such GTF and is highly relevant in this case, because both subunits (RAP30 and RAP74) can interact with TFIIB and more specifically the N terminus of human TFIIB (4, 32). Furthermore, suppressor mutants of the large subunit of TFIIF have been isolated that can reverse the slow growth phenotype and transcription start site defects of the yeast TFIIB mutant E62K (human Glu-51 (33)). However, our preliminary data suggest that neither TFIIB E51R or R66E are defective for interaction with RAP30 or RAP74 in *vitro*. As discussed above, these mutations in TFIIB may alter the specificity rather than abolish interactions with other factors. It is possible that the RAP74 suppressor mutants restore the alignment of the yTFIIB(E62R)-pol II/TFIIF interaction, leading to normal transcription start site selection.

DNA topology may affect the ability of R66E to substitute the function of wild type TFIIB in *vivo*. For example, R66E may be nonfunctional at a nucleosome-assembled promoter. Experiments in *vitro* using nucleosomal DNA templates could be used to test this hypothesis. It is also possible that the in *vivo*-specific defect of R66E involves the response to upstream transcriptional activators. Indeed, a *S. cerevisiae* protein (SUB1) similar to the mammalian coactivator PC4 was isolated as a suppressor of the yTFIIB R78H cold-sensitive phenotype (34). The prospect of an additional function for TFIIB underscores the pivotal role that this GTF plays in transcription. Further studies are currently in progress to determine the function of TFIIB that is specifically required in a living cell.

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