Minimal Promoter Systems Reveal the Importance of Conserved Residues in the B-finger of Human Transcription Factor IIIB*§

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The “B-finger” of transcription factor IIB (TFIIB) is highly conserved and believed to play a role in the initiation process. We performed alanine substitutions across the B-finger of human TFIIB, made change-of-charge mutations in selected residues, and substituted the B-finger sequence from other organisms. Mutant proteins were examined in two minimal promoter systems (containing only RNA polymerase II, TATA-binding protein, and TFIIB) and in a complex system, using TFIIB-immunodepleted HeLa cell nuclear extract (NE). Mutations in conserved residues located on the sides of the B-finger had the greatest effect on activity in both minimal promoter systems, with mutations in residues Glu-51 and Arg-66 eliminating activity. The double change-of-charge mutant (E51R:R66E) did not show activity in either minimal promoter system. Mutations in the nonconserved residues at the tip of the B-finger did not significantly affect activity. However, all of the mutations in the B-finger showed at least 25% activity in the HeLa cell NE. Chimeric proteins, containing B-finger sequences from species with conserved residues on the side of the B-finger, showed wild-type activity in a minimal promoter system and in the HeLa cell NE. However, chimeric proteins whose sequence showed divergence on the sides of the B-finger had reduced activity. Transcription factor IIF (TFIIF) partially restored activity of the inactive mutants in the minimal promoter system, suggesting that TFIIF in HeLa cell NE helps to rescue the inactive mutations by interacting with either the B-finger or another component of the initiation complex that is influenced by the B-finger.

The RNA polymerase II (RNAP II)2 initiation complex is extremely complicated and, thus, very difficult to meaningfully dissect. Genes that are transcribed into mRNA by RNAP II generally require five general transcription factors (TFs), designated TFIIB, TFIID, TFIIE, TFIIF, and TFIH (reviewed in Refs. 1–5). However, arguments have been made to include the “elongation” factor TFIIS (6) and the mediator complex (7) in the list of “general” transcription factors. RNAP II and most of the TFs are multimeric protein complexes. If all proteins contained in the yeast (Saccharomyces cerevisiae) RNAP II initiation complex were considered, the count of ~60 polypeptides would have a mass of ~3 MDa (8). The high resolution crystal structure of yeast RNAP II has provided invaluable insight into the topology of the RNAP II transcription initiation complex (9). Furthermore, the crystal structures of bacterial RNAP (10, 11) and RNAP from an archaeal organism (12) establish that RNAP from all three domains of life (Bacteria, Archaea, and Eukarya) show high conservation of overall structure. However, many mechanistic details of the transcription process have yet to be determined.

TFIIB functions as a single polypeptide with a two-domain structure. The C-terminal domain of TFIIB (cTFIIB) contains an imperfect direct repeat motif. The co-crystal structure of cTFIIB with the DNA-bound TATA-binding protein (TBP) of TFIID shows that the direct repeats of cTFIIB are responsible for interacting with TBP (13). In addition, cTFIIB interacts with DNA upstream and downstream of the TBP-bound TATA-box (reviewed in Ref. 14) and also interacts with the “wall” region of yeast RNAP II in pre-initiation complexes (15).

The N-terminal domain of TFIIB (nTFIIB) contains a zinc ribbon followed by a region that is highly conserved in TFIIB from many species. Early work showed that one function of nTFIIB is to bridge the RNAP II-TFIIF complex to the TBP-cTFIIB-DNA complex at the promoter (16, 17). However, Cho and Buratowski (18) later showed that the nTFIIB probably plays an additional role in post-preinitiation complex formation.

Yeast nTFIIB has been crystallized in a complex with yeast RNAP II (19). The crystal structure (at 4.5 Å) shows the zinc ribbon contacting the largest subunit of RNAP II (Rpb1) at the previously described “dock” region (9). The highly conserved region of nTFIIB threads into the “saddle” of the RNAP II and then returns by the same path, creating a loop, or “B-finger,” transcription factor; TFIIB, transcription factor IIIB; nTFIIB, N-terminal domain of TFIIB; cTFIIB, C-terminal domain of TFIIB; TFIIE, transcription factor IIE; TFIIF, transcription factor IIIF; TFIH, transcription factor IHI; TFIIS, transcription factor IIS; DTT, dithiothreitol; mAb, monoclonal antibody.
that approaches the active site of the enzyme and occupies the RNA exit channel (19). This location was supported by biochemical dissection of the yeast B-finger region, using residue-positioned hydroxyl radical cleavage (20).

Mutations in residues Glu-62 and Arg-78 of yeast TFIIB were studied before structural information was available because they affect start site selection on the yeast cyc1 gene (21). These two residues are within the B-finger region of yeast TFIIB as described from the co-crystal structure of yeast RNAPII and nTFIIB (19), and these charged residues are believed to form a salt bridge (21). The low resolution crystal structure suggests that these two residues are positioned to accommodate a salt bridge (19). Mutations in the homologous residues in human TFIIB (Glu-51 and Arg-66) have been studied and seem to affect start site selection in a promoter-specific manner (22, 23).

However, mechanisms for start site selection in yeast and human are different, with yeast employing a more elaborate “scanning” mechanism (reviewed in Ref. 5).

Given the presumed role of the B-finger in transcription initiation and the high conservation of amino acid sequence among species, it is surprising that other investigators have reported some in vitro transcriptional activity in HeLa cell NE using deletion mutants that lack part or all of the human TFIIB B-finger (24, 25) and in a defined transcription system that lacks all of the archaeal B-finger region (26). No systematic study has addressed the role of the individual residues of the B-finger in a defined transcription system.

Eukaryotic minimal promoter systems that require only RNAPII, TBP, and TFIIB were first described in 1993 (27, 28). These minimal promoter systems are similar to the archaeal transcription system (reviewed in Ref. 29). However, only a few eukaryotic promoters can be transcribed by a minimal set of factors and only if the promoter is contained in the context of a super-coiled template (27, 28). Alternatively, a minimal promoter complex can be studied on a linear template if the promoter contains a properly placed mismatch, creating a bubble over the initiation site (30). These template topologies eliminate the need for TFIIIE, TFIIF, and TFIIH. Although TFIIIF is not required, transcription on the mismatched promoter system can be stimulated by TFIIF (30), and transcription on supercoiled templates can be stimulated by the addition of the RNA polymerase-associated protein 30 (RAP30), a component of TFIIF (28, 31). We have found minimal promoter systems to be useful in studying the small group of transcription proteins: RNAPII, TBP, TFIIB, and RAP30 (31–34).

The information obtained from the co-crystal structure of yeast RNAPII with the yeast nTFIIB (19), prompted us to test mutations in the human TFIIB B-finger in minimal promoter systems. We undertook an extensive, systematic mutagenic examination of the B-finger region of human TFIIB. We have characterized the mutant TFIIB proteins in two minimal promoter systems, using only calf thymus RNAPII, human TBP, and human TFIIB. We then examined the behavior of these mutants in the complex HeLa cell NE. These results yield insight into the biology of B-finger region of human TFIIB and indicate why transcriptional activity of TFIIB mutants can differ in the minimal promoter systems and complex promoter systems.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All general chemicals were at least reagent grade and obtained from major commercial suppliers. Where specific materials were required, we have noted the vendor.

**Buffers**—TE buffer contained 50 mM Tris-HCl and 0.1 mM EDTA, pH 7.9. TBE buffer contained 90 mM Tris base, 90 mM boric acid, and 2.5 mM EDTA, pH 8.0. Phosphate-buffered saline contained 2 mM KH2PO4, 10 mM Na2HPO4, 3 mM KCl, and 150 mM NaCl, pH 7.2. Gel-shift buffer contained 20 mM Hepes (pH 7.2), 25 mM KCl, 2 mM spermidine, 0.1 mM EDTA, 0.025% Nonidet P-40, 10% (v/v) glycerol (Fisher Scientific), 0.5 mM dithiothreitol (DTT), 100 μg/ml bovine serum albumin (BSA) and 2 mM MgCl2. Buffer D contained 20 mM Hepes (pH 7.9), 0.2 mM EDTA, 100 mM KCl, 0.5 mM DTT, and 20% (v/v) glycerol. The IgH promoter transcription buffer contained 20 mM Hepes (pH 7.9), 1 mM EDTA, 40 mM KCl, 4 mM MgCl2, 1 mM DTT, 150 μg/ml acetylated BSA (Promega, Madison, WI), and 20% (v/v) glycerol. The mismatched minimal promoter (MMP) assay buffer contained 20 mM Hepes (pH 7.9), 0.1 mM EDTA, 50 mM KCl, 8 mM MgCl2, 1 mM DTT, 100 μg/ml acetylated BSA, and 10% (v/v) glycerol. BLOTTO contained 1% non-fat dry milk in phosphate-buffered saline. All pH values were determined at 23 °C.

**Plasmids and Mutagenesis**—To create point mutations in human TFIIB, a cassette containing the N-terminal domain of the human TFIIB was constructed. The coding region of TFIIB (obtained from Danny Reinberg, Robert Woods Johnson Medical School, Piscataway, NJ) was subcloned into the NdeI and BamHI sites of pET33b (EMD/Novagen, Madison, WI), which contains a NcoI site in the multiple cloning site. Digestion of this plasmid with NcoI yielded a 444-bp fragment containing 72 bp from the vector and nucleotides 1–372 of human TFIIB due to an internal NcoI site in TFIIB. This fragment was cloned into the NcoI site of pGEM5zf (Promega) and used as a cassette for the mutagenesis of TFIIB. Oligonucleotides containing the specific point mutations were prepared by the University of Wisconsin Biotechnology Center, and mutations were generated by use of the QuikChange system (Stratagene, La Jolla, CA) that uses a PCR amplified with a high fidelity enzyme (pfu). Sequencing was performed by the McArthur Laboratory DNA Sequencing Facility, using an automatic ABI PRISM 373 DNA Sequencer (Applied Biosystems, Foster City, CA) to identify the mutated clones. The cassette containing the desired mutation was then cloned back into TFIIB contained in either pET11a, using the NdeI and NcoI sites (1–372), or into TFIIB contained in pET33b, using the NcoI site in the vector and the NcoI site at position 372 of TFIIB. Cloning into pET33b yielded a His Six tag (contained in a 25-amino acid tag) fused to the N terminus of TFIIB. All final constructs were sequenced by the automated method.

The construct that allowed the B-finger to be exchanged was prepared from the construct containing TFIIB cloned into the NdeI and BamHI sites of the pET33b vector. The BamHI site at the 3’-end of this construct was destroyed by cutting with BamHI, filling in the ends with Klenow, and ligation the blunt ends together. Point mutations in the human B-finger were prepared by site-directed mutagenesis, incorporating a new
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BamHI site and a KpnI site into the coding region at each end of the B-finger. The result was a silent mutation at residue 49 (Gly-49) and an amino acid change at residue 69 (D69T). Pairs of oligonucleotides were prepared that correspond to the B-finger sequence of the desired B-finger, containing sticky BamHI and KpnI sites at the ends. Oligonucleotides were phosphorylated with polynucleotide kinase, mixed in equimolar proportions, and heated to 95 °C for 20 min in a heating block. The heating block was then allowed to return to room temperature (~1 h), and the annealed oligonucleotides were purified from agarose gels with a gel-extraction kit (Qiagen, Valencia, CA) and ligated into the plasmid containing TFII B that had been cut with BamHI and KpnI. All constructs were sequenced.

Antibodies—mAbs IIB8 and IIB5 that react with human TFII B were prepared by standard hybridoma methods and have been described (32, 33). The epitope for mAb IIB8 is contained within residues 61–68 of human TFII B (34). The epitope for mAb IIB5 is not well defined, but is known to be contained within amino acids 106–316 of human TFII B (33). Western blots were prepared as described (33), except that the secondary antibody was conjugated to horseradish peroxidase (American Qualex, San Clemente, CA), and enhanced chemiluminescence reagents (ECL Plus, Amer sham Biosciences) were used for detection. For Western blots, the mAb contained in ascites fluid was diluted 1:1000. In experiments that used mAb IIB to inhibit transcription, the antibody was purified by affinity chromatography on staphylococcal protein A as described (32, 33).

Immunodepletion of TFII B—Immunodepletion of TFII B from HeLa cell NE, using mAb IIB8, was performed by a modification of the method previously described (33). These modifications allowed us to more thoroughly immunodeplete the extract and gave a more robust, easily quantifiable signal with the addition of the purified bacterially expressed TFII B. The HeLa cell NE (200 μl) was heated to 47 °C for 15 min to inactivate the TFIIID (35). Five aliquots were processed, and the aliquots were pooled. Protein A-agarose beads (Repligen, Waltham, MA) were blocked with 1% nonfat, dry milk (1% BLOTTO) that had been centrifuged and filtered to remove insoluble material. The blocked beads (BLOTTO) that had been centrifuged and filtered to remove insoluble material. The blocked beads (BLOTTO) that had been centrifuged and filtered to remove the heating block was then allowed to return to room temperature (~1 h), and the annealed oligonucleotides were purified from agarose gels with a gel-extraction kit (Qiagen, Valencia, CA) and ligated into the plasmid containing TFII B that had been cut with BamHI and KpnI. All constructs were sequenced.

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Protein Purifications—All TFII B mutants were expressed in Escherichia coli BL21(DE3)pLysS by inducing log phase cells (A600 nm = 0.6) with 1 mM isopropyl-D-thiogalactopyranoside for 2.5 h. Many of the point mutations in the B-finger region of human TFII B reacted with the polyl-responsive mAb IIB8 (34). These mutants were expressed from pET11a and purified from the soluble fraction by chromatography on an Ni2+-nitrilotriacetic acid resin (Qiagen). The His6-tagged TFII B was eluted with 100–200 mM imidazole. Fractions were pooled, the proteins were dialyzed against Buffer D (without DTT) for 4 h at 4 °C, and then supplemented with 1 mM DTT and 0.1 mM ZnCl2. TFII B preparations were flash frozen in liquid N2 and stored at –80 °C. Purified yeast TFII B was a kind gift from David Bushnell and Roger Kornberg (Stanford University, Stanford, CA).

Calf thymus RNA polymerase II was purified either by the immunoaffinity chromatography procedure described previously (36) or by the standard chromatography method of Hodo and Blatti (37). Human TBP was purified by immunoaffinity chromatography as described (38). The RAP30 subunit of human TFII F was expressed and purified as described (31). TFII F was prepared by the method of Wang et al. (39).

Protein concentrations were determined by the Bradford assay (40), using a microplate assay and the Bradford reagent obtained from Pierce (Rockford, IL). BSA was used as a protein standard.

All protein preparations were stored in Buffer D, frozen in small volumes in liquid N2, and stored at –80 °C. Dilutions of each protein were prepared in Buffer D before being incorporated into the transcription assay.

Transcriptions—Transcriptions using the IgH minimal promoter system were performed as described by Parvin and Sharp (27) with slight modifications (33). Briefly, template DNA was incubated with TBP (10–20 ng) in 3 μl of gel-shift buffer for 3 min at 23 °C. Differing amounts (25–100 ng) of TFII B (or mutant TFII B) and calf thymus RNA polymerase II (2 μg) were added, and the complex was allowed to form by incubation at 23 °C for 5 min. The complex was diluted to 21 μl with the transcription buffer, and then initiated with a mixture of NTPs (400 μM ATP and CTP, 100 μM 3′-O-methyl-GTP, and 80 μM UTP containing 10 μCi of 32P-labeled α-UTP). After 30-min incubation at 30 °C, the reactions were stopped and extracted with a mixture of phenol/chloroform/isoamyl alcohol, and the RNA was precipitated with ethanol, using 10 μg of yeast tRNA as a carrier. Transcripts were run on a gel containing 6 M urea and 7% polyacrylamide.

Transcriptions using the MMP system used a double-stranded oligonucleotide (85 bp) containing the adenovirus-2 major late promoter (Ad2 MLP) with a mismatch over the sequences –9 to +3 with respect to the Ad2 MLP start site. These reactions were performed by a modification (34) of the method described by Pan and Greenblatt (30), except that a dinucleotide primer was not used in the reactions except where noted. Components of the reaction were added in the order described above for the IgH promoter, and the reactions processed identically to reactions containing the IgH promoter. Transcripts were run on a gel containing 6 M urea and 20% polyacrylamide. The transcription product from this promoter system was a 10-nt RNA with the sequence ACUCUCUCUC.

In experiments using the dinucleotide Cpa (Ribomed Biotechnologies, Inc., Carlsbad, CA) as a primer, the Cpa (0.5 mM) was added to the DNA template and incubated for 5 min at 23 °C before the other components of the reaction were added. The transcript product was then an 11-nt RNA with the sequence CACUCUCUCUC.
Transcriptions using the Ad2 MLP on a linearized template and a HeLa cell NE were performed by a modification of the procedure described previously (41). The HeLa cell NE was heat-treated to inactivate the TFIID activity (35) and immunodepleted of TFIIB as described above. For reconstitution assays, the template was incubated with purified human TBP and purified TFIIB in gel-shift buffer for 5 min at room temperature. The heat-treated, immunodepleted HeLa cell NE was then added to the TBP-DNA-TFIIB complex and incubated at room temperature for an additional 5 min. Transcriptions were initiated by the addition of a mixture of NTPs (200 μM ATP, CTP, UTP, and 20 μM of GTP containing 10 μCi of 32P-labeled α-GTP), incubated at 30 °C for 30 min, and processed as described previously (41).

All TFIIB mutations were tested using at least three levels of protein to correct for variability in the protein determination assays. Autoradiograms were prepared from all transcription assays; all transcriptions were quantified by the use of a PhosphorImager (Amersham Biosciences). In transcriptions using the Ad2 MLP in the mismatched promoter (MLP MMP), only the 10-nt transcript was quantified. In all experiments, background readings were taken in each lane above the quantified band; this background value was subtracted from value of the quantified band. The figures showing the actual transcription products Co., Clear Brook, VA) sizing column (8 × 300 mm) was used to separate transcription complexes. The MLP MMP reactions were assembled as above, except that NTPs were not added. A 200-μl volume (equivalent to eight reactions) was injected onto the Shodex column that had been equilibrated in MLP MMP buffer without acetylated BSA. The column was developed with same buffer at a flow rate of 1 ml/min, and 100-μl fractions were collected. Fractions were assayed for transcriptional activity by adding NTPs, incubating, and processing the samples as described above for the MLP MMP assay. The presence of TFIIB in each fraction was determined by Western blot analysis using mAb IIB5, which reacts with cTFIIB (33), as the primary antibody.

**RESULTS**

The schematic of the nTFIIB in Fig. 1A, shows the position of the B-finger in relation to the zinc ribbon and the relative positions of the conserved Glu-51 and Arg-66 residues, homologs of yeast Glu-62 and Arg-78 that have been suggested to form a salt bridge in yeast TFIIB (21). The activity of wild-type TFIIB purified by the immunoaffinity procedure and the His6-tagged wild-type TFIIB; elimination of any component results in loss of transcriptional activity (Fig. 2A, lanes 1–3). Wild-type TFIIB supports transcription in the IgH minimal promoter system (Fig. 2A, lanes 4 and 5), where the transcript is a G-less RNA of ~100 nt. When yeast residue Arg-78 is mutated to cysteine (R78C), the phenotype includes cold sensitivity and an alteration in start site selection (21). A mutation in this residue (R78L) also has been reported to inhibit in vitro transcriptional activity in a yeast extract (42). We constructed an alanine substitution (R66A) and a cysteine substitution (R66C) in the human B-finger patterns in yeast. Glu-51 and Arg-66 residues, homologs of yeast Glu-62 and Arg-78 that have been suggested to form a salt bridge in yeast TFIIB (21). The activity of wild-type TFIIB purified by the immunoaffinity procedure and the His6-tagged wild-type TFIIB; elimination of any component results in loss of transcriptional activity (Fig. 2A, lanes 1–3). Wild-type TFIIB supports transcription in the IgH minimal promoter system (Fig. 2A, lanes 4 and 5), where the transcript is a G-less RNA of ~100 nt. When yeast residue Arg-78 is mutated to cysteine (R78C), the phenotype includes cold sensitivity and an alteration in start site selection (21). A mutation in this residue (R78L) also has been reported to inhibit in vitro transcriptional activity in a yeast extract (42). We constructed an alanine substitution (R66A) and a cysteine substitution (R66C) in the human B-finger.
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FIGURE 2. Importance of Arg-66 in two minimal promoter systems. Reactions in lanes 1–3 lacked one of the required components. Reactions in lanes 4–9 contained the indicated amount of wild-type or mutant TFIIB. A, transcription assays using the supercoiled IgH promoter. The reactions were run on a 7% polyacrylamide gel containing 7 M urea, and an autoradiogram was prepared. The IgH transcript (100 nt) is indicated. B, transcription assays using the mismatched (−9 to +3) Ad2 MLP (MLP MMP). The reactions were run on a 20% polyacrylamide gel containing 7 M urea, and an autoradiogram was prepared. The primary transcript (10 nt) is indicated.

Neither of these mutants was active in this minimal promoter system (Fig. 2A, lanes 6–9).

The same experiment was performed with the Ad2 MLP mismatched minimal promoter (MLP MMP) system. Reactions in Fig. 2B (lanes 1–3) show that this minimal system is also dependent upon the presence of RNAP II, TBP, and TFIIB. Wild-type TFIIB supports transcription in the MLP MMP system (Fig. 2B, lanes 4 and 5). The major transcript from this promoter is a 10-nt G-less RNA. Longer and shorter minor transcripts are also present, indicating the “slippage” of the transcription complex that has been documented by others (43, 44) and is dependent upon the presence of TBP and TFIIB (44).

However, it is possible that some of the shorter, minor transcripts were generated by abortive mechanisms. Like the IgH minimal promoter system, the R66A and R66C mutants did not support transcription in the MLP MMP (Fig. 2B, lanes 6–9), and these mutants did not generate a longer or shorter transcript.

Change-of-Charge Mutants in Minimal Promoter Systems—Experiments performed in yeast (21) suggested that residues Glu-62 and Arg-78 might form a salt bridge, because a non-viable mutation in yeast (R78E) could be rescued by making a compensatory change-of-charge mutation in residue 62 (E62R: R78E). The location of these residues in the crystal structure positions them on the sides of the B-finger (19). Therefore, we constructed single change-of-charge mutants (E51R and R66E) and a double change-of-charge mutant (E51R:R66E) in the corresponding minimal promoter systems. The single change-of-charge mutants (E51R and R66E) were not active in the IgH minimal promoter system (Fig. 3A, lanes 5–8), and the double change-of-charge mutant (E51R:R66E) did not restore activity (Fig. 3A, lanes 9 and 10). The experiment was repeated with the MLP MMP system (Fig. 3B); neither of the single change-of-charge mutants (Fig. 3B, lanes 5–8) nor the double change-of-charge mutant (Fig. 3B, lanes 9 and 10) showed activity in the MLP MMP system. In addition, an alanine substitution (E51A) mutation in the human TFIIB was not active in either minimal promoter system (Fig. 3, A and B, lanes 3 and 4). Again, using the minimal promoter systems, a change in start site selection was never detected.

During the course of these experiments we found the MLP MMP system to be more sensitive and reproducible than the IgH system. Therefore, because the results were similar in the two minimal promoter systems (Figs. 2 and 3), we decided to use the MLP MMP for further studies. This assay had the added advantage that it can detect small RNA products that might be generated by abortive initiation. We attempted to perform single round transcription assays using the IgH minimal promoter system, but we could not detect single round products (data not shown), presumably because of the inefficiency of the system.

We were concerned that the effect of the inactive mutants might be due to the inability to correctly identify the start site. This concern was especially important because we used a G-less transcript in our assays, and a change in start site might not be detected. To examine this possibility, we added the dinucleotide CpA (500 μM) to the reaction. This dinucleotide hybridizes to the −1/+1 site on the mismatched promoter, and this “priming” establishes the start site. The major transcript is then an 11-nt RNA. In the presence of the primer, but in the absence of TFIIB, a small amount of the 11-nt RNA is produced (Fig. 3C, lane 2); this is in agreement with a previous report that RNAP II alone can transcribe inefficiently from a primed start site (45). In addition, a nonspecific transcript (NS) of ~13 nt was also produced. We do not know the identity of this nonspecific transcript, but it is independent of TFIIB and dependent upon the addition of CpA (Fig. 3C, lanes 1 and 2). When wild-type TFIIB was used in the reaction, the expected 10-nt transcript was produced (lane 3), but the addition of CpA shifted most of the transcript to 11 nt (lane 4), as expected. When CpA was added...
the experiments were the same, regardless of the form of RNAP polymerase molecules with the CTD intact (36). The results of the CTD and isolates a homogeneous preparation containing the heptapeptide repeat on the C-terminal domain of the largest subunit (CTD). The RNAP II that was used in the original MLP MMP studies (30) was purified by this method. To establish that activity of the inactive mutants was not dependent upon the presence of the CTD, we examined the effect of priming the reaction with the CpA dinucleotide. Reactions in lanes 2, 4, 6, 8, and 10 contained the CpA primer. TFIIB was omitted from lanes 1 and 2. The correctly primed reaction is an 11-nt transcript. A nonspecific product (NS), which is present in lanes 2 (that contained CpA but no TFIIB) is noted. The gel conditions were as described in Fig. 2.

Previous reports from the yeast transcription system established that mutations in Glu-62 and Arg-78 of yeast TFIIIB did not affect the ability of yeast TFIIIB to form a pre-initiation complex (18, 20). These studies used electrophoretic mobility shift assays but did not use minimal promoter systems, where the complex might be less stable. We were unable to generate a discrete band in electrophoretic mobility shift assay using only RNAP II, TBP, and TFIIA (data not shown).

To establish that inactive mutants were able to enter the transcription complex, we separated the transcription complexes by high-pressure liquid chromatography size-exclusion chromatography as described under “Experimental Procedures.” The chromatogram, using the wild-type His-tagged TFIIA(WT) is shown in supplemental Fig. S3A along with the chromatogram using the His6-tagged E51R:R66E(DM). Two peaks were discernible (supplemental Fig. S3B). Silver staining across both peaks indicated that the largest peak (peak B) contained mainly the BSA that was in the original transcription buffer, and also a small amount of free TBP. The earlier eluting Peak A contained RNAP II, TBP, and TFIIA (data not shown).

Fractions were assayed for activity in the MLP MMP, and the activity, using the wild-type TFIIA was contained primarily in fractions C3–C6 (supplemental Fig. S3B). Fractions C3–C6 from the experiment using the double mutant E51R:R66E showed no transcriptional activity. Western blots performed across the entire chromatogram using mAb IB5, a mAb that reacts with the C-terminal domain of TFIIA (33), showed that TFIIA was contained primarily in fractions C2–C6 regardless of whether the wild-type TFIIA or the E51R:R66E mutant was used in the assay (supplemental Fig. S3C). We conclude that both wild-type and mutant TFIIA support stable complex formation, although the mutant-containing complex is inactive in the transcription assay.

**Mutational Analysis across the B-finger: Effects in Minimal Promoter Systems**—A systematic mutational study of the residues in the B-finger has not been reported. Because residues Glu-51 and Arg-66 were essential in the minimal promoter system, we reasoned that the MLP MMP system would be useful to assess effects of point mutations in each of the residues of the B-finger. Each residue in amino acids 50–70 of human TFIIA was replaced with alanine, and the mutant was assayed in the MLP MMP system. The activity of each of the B-finger mutants is shown in the histogram in Fig. 4A, where activity is expressed as the percent activity of the wild-type TFIIA in the same experiment. Surprisingly, all of the mutations, with the exception of E51A and R66A, showed some activity. Substitutions with the most deleterious effects on transcription were conserved residues located on the sides of the B-finger, clustered around residues Glu-51 and R66. An example of these activities, using the T54A and F55A mutants, is shown in Fig. 4B. All mutants that showed reduced activity maintained the ratios of the minor transcripts to the 10-nt transcript in the MLP MMP, but for simplicity, only the 10-nt transcript was quantified. The same pattern of activity was observed when all of the B-finger
FIGURE 4. Effect of individual alanine replacements throughout the human B-finger in the MLP MMP system. A, activity of individual alanine substitutions on transcription from the MLP MMP. The transcripts were quantified by the use of a PhosphorImager and expressed as the percent activity of the wild-type TFIIB run in the same experiment. Each histogram bar represents the average of two experiments using three different concentrations of the mutant, run against the same three concentrations of wild-type TFIIB. The error bars represent the range of the values. B, a representative example of the reactions seen with the single alanine substitutions T54A (lanes 4–6) and F55A (lanes 7–9). C, activities of two different amino acid substitutions, D58A (lanes 4–6) and D58K (lanes 7–9), for the wild-type aspartic acid residue. D, activity of the D63A mutant in the MLP MMP system. E, activity of the double mutant (D58A:D63A) in the MLP MMP system. F, effect of priming the D58A, D63A, and D58A:D63A reactions with the CpA dinucleotide. Reactions in lanes 1, 3, 5, 7, and 9 contained the CpA primer. TFIIB was omitted from lane 1. The correctly primed reaction is an 11-nt transcript. A nonspecific product (NS), which is present in lane 1 (that contained CpA but no TFIIB) is noted. The autoradiograms were prepared from a 20% denaturing gel. The percentages given in B, C, D, and E represent the percent activity of the same concentration of wild-type protein run in the experiment presented.
The priming did not increase the amount of transcript produced by the mutants, although, as with wild-type TFIIB, the major 10-nt transcript was shifted to 11 nt. In addition, there was no increase in the lower transcripts that would indicate an increase in abortive initiation.

B-finger Substitutions: Effects in the MLP MMP System—A survey of B-finger sequences in the data base showed that the B-finger is highly conserved in primary sequence among many species. As shown in Fig. 1B, divergence in primary sequence between yeast and human TFIIB is found only at the tip of the B-finger. We tested yeast TFIIB in the MLP MMP system with human TBP and calf thymus RNAP II, and yeast TFIIB could not function in this assay (supplemental Fig. S5). Other investigators have reported activity with yeast-human chimeric TFIIB proteins in complex systems (24, 46). To determine if the nature of the nonconserved residues at the tip of the B-finger plays a role in the activity of TFIIB in the MLP MMP system, we made a chimeric protein and tested it in the MLP MMP system.

A cassette to genetically replace the B-finger from recombinant human TFIIB with the B-finger sequences from yeast TFIIB was prepared as described under “Experimental Procedures.” This construction resulted in one amino acid change (D69T), on the edge of the human B-finger (Fig. 5A). The D69T mutation did not affect the activity of human TFIIB in the MLP MMP system (Fig. 5B, lanes 2 and 3). The chimeric protein (human TFIIB containing the yeast B-finger sequence) was at least as active as the wild-type TFIIB in the MLP MMP system (Fig. 5B, lanes 4 and 5). We also tested the B-finger sequence from the fission yeast Schizosaccharomyces pombe (which is also conserved on the sides of the finger but divergent at the tip) in human TFIIB; this chimeric TFIIB was at least as active as the human B-finger sequence (Fig. 5A). A B-finger that showed divergence in both the sides and tip was difficult to find; we selected the sequence from a TFIIB-like protein encoded by mimivirus, the “giant” virus isolated from the protozoan Acanthamoeba polyphaga (47). The experiment, using this chimeric TFIIB, showed only 22% activity (Fig. 5C) in the MLP MMP assay. The activity obtained with each of the chimeric proteins tested in the MLP MMP system (compared with the D69T mutant) is shown in the first column of Fig. 5A. The results with the B-finger substitutions indicate that, like the point mutations (Fig. 4 and supplemental Fig. S4), as the residues on the side of the B-finger (adjacent to Glu-51 and Arg-66) become more divergent from the human sequence, the activity in the MLP MMP system was reduced. A “control” B-finger, constructed by using correctly placed Glu-51 and Arg-66 residues...
and divergent residues throughout the rest of the B-finger, did not show activity in the MLP MMP system.

**Effect of B-finger Mutations in Reconstituted HeLa Cell NE**—Previous studies examined selected mutations in the human B-finger in a reconstituted HeLa cell NE (18, 22, 25), and in all cases, these mutants showed some activity in this more complex transcriptional system. However, a systematic study of the activities of all of the residues across the B-finger has not been reported. Therefore, we examined our set of B-finger mutants in HeLa cell NE.

A linear template (1846 bp) containing the Ad2 MLP (with no mismatched nucleotides) was used in this assay. A correctly initiated transcript yields a 196-nt run-off transcript (41). The HeLa cell NE was heat-treated to inactivate the TFIID activity and immunodepleted of endogenous TFIIB as described under “Experimental Procedures,” and the activity was restored by the addition of human recombinant TBP and TFIIB (Fig. 6A). This protocol was used for three reasons. First, conditions could be reproduced that show that TFIIB enters the transcription complex (supplemental Fig. S3). Second, these conditions gave a consistently robust signal that was easily quantifiable (Fig. 6A: compare lanes 1 and 6). Third, this protocol gave less batch-to-batch variation in transcriptional activity between preparations of HeLa cell NE (data not shown). A Western blot performed on the immunodepleted HeLa cell NE is shown in supplemental Fig. S6. A small amount of endogenous TFIIB remained after the second round of immunodepletion (supplemental Fig. S6), and further rounds of immunodepletion did not reduce this activity.

**FIGURE 6.** Reconstitution of activity on the Ad2 MLP in TFIIB-depleted HeLa cell NE with purified mutant TFIIB proteins. A, addition of wild-type TFIIB restored activity to the HeLa cell NE. Transcription (196-nt run-off transcript) of the Ad2 MLP in untreated HeLa cell NE (lane 1), after heat-treatment (lane 2), and when TBP was added back to the heat-treated NE (lane 3). The heat-treated NE was then immunodepleted with mAb IIB8 (lane 4), and this NE was then supplemented with TBP (lane 5) or TBP and 12.5–50 ng of TFIIB (lanes 6–8), B, summary of the activities of all of the alanine substitutions in the HeLa cell NE. C, representative example of the activities of three alanine substitutions (E51A, W52A, and R53A) assayed in the HeLa cell NE. D, effect of the change-of-charge mutants in residues Glu-51 and Arg-66 (lanes 4–7) and the double change-of-charge (E51R:R66E) mutant (lanes 8 and 9) in the reconstituted HeLa cell NE. E, activity of the double mutant in which the aspartic acid residues at the edge of the conserved region where changed to alanine residues. The autoradiograms were prepared from a 7% denaturing gel. The percentages given in C, D, and E represent the percent activity of the same concentration of wild-type protein run in the experiment presented.

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residual amount (data not shown). As shown in the transcription assay in Fig. 6A (lane 5) the addition of only TBP to the heat-treated, immunodepleted HeLa NE resulted in a very low background activity. To correct for this residual TFIIB activity, we added only TBP to the heat-inactivated and immunodepleted HeLa cell NE, and this value was subtracted from all of the quantifications (Fig. 6B). This control can be seen in lane 1 of Fig. 6 (C–E). The addition of 50 ng of wild-type TFIIB completely restored activity to the reaction (Fig. 6A, lanes 6–8); this is consistent with the estimation of the amount of TFIIB in our HeLa cell NE reactions (~60 ng in 6 μl) that we had reported previously (32). Therefore, the mutant proteins were tested at 25, 50, and 100 ng of TFIIB added back.

Mutants E51A and R66A, which were inactive in both minimal promoter systems, showed 41% to 67% activity of the wild-type TFIIB in the HeLa cell NE (supplemental Fig. S7). The addition of 1 μl of α-amanitin inhibited this activity (supplemental Fig. S7, lanes 4, 7, and 10). mAb IIB8 binds to an epitope in the B-finger region (Fig. 1B) and inhibits transcription in HeLa cell NE (33). Arg-66 is contained within the epitope for mAb IIB8, and R66A does not react with mAb IIB8 while mutant E51A reacts with this antibody (35). When mAb IIB8 was included in the reconstituted HeLa cell NE reactions, mAb IIB8 inhibited the activity of wild-type and the E51A mutant (supplemental Fig. S8, lanes 3 and 5), but did not inhibit the activity of the R66A mutant (supplemental Fig. S8, lane 7). Thus the results presented in supplemental Figs. S7 and S8 confirm that the transcriptional activity was indeed dependent on the TFIIB that was added to the reconstituted reaction.

The activities of the alanine substitutions throughout the B-finger were tested in the HeLa cell NE assay (Fig. 6B); each mutant showed at least 25% activity. A typical example of the activities of mutant E51A, W52A, and R53A is shown in Fig. 6C. Mutant W52A (which showed <10% activity in both minimal promoter systems) showed the lowest activity of all of the mutants in the HeLa cell NE assay (Fig. 6C, lanes 6 and 7). Importantly, the overall pattern of activity of the mutants in HeLa cell NE is quite similar to the results obtained in the minimal promoter systems, with the residues at the tip of the B-finger having little effect on activity, and the residues on the sides of the finger showing reduced activity.

The change-of-charge mutants (E51R and R66E) showed activity similar to the alanine substitutions at these residues, (30–45%), indicating that the result was not specific to alanine substitutions (Fig. 6D, lanes 4–7). The double change-of-charge mutant (E51R:R66E) showed slightly enhanced activity over the single change-of-charge mutants but was also reduced in activity compared with the wild type (Fig. 6D, lanes 8 and 9). Mutants, D58A and R63A showed wild-type activity in the HeLa cell NE, and the double mutant (D58A:D63A) showed ~80% of the activity of the wild-type (Fig. 6E).

The chimeric proteins described in Fig. 5 were also tested for transcriptional activity in the reconstituted HeLa cell NE. The data are presented in the second column of Fig. 5A. Chimeric proteins containing the B-finger from the two yeast species were at least as active as the D69T mutant, and the other chimeric proteins showed lower activity than the D69T mutant in the reconstituted HeLa cell NE. These data further support the observations made with the point mutations, that the conserved residues on the sides of the B-finger have a more deleterious effect on activity than those at the B-finger tip.

Effect of TFIIF on Mutants in the MLP MMP System—The observation that point mutants lacking activity in the minimal promoter systems had some activity in the reconstituted HeLa cell NE suggested that some factor(s) in the HeLa cell NE could rescue the activity of the mutants. Previously, it had been shown that the RAP30 subunit of TFIIF enhanced the activity in minimal promoter systems that use supercoiled DNA (28, 31). TFIIF (containing both RAP30 and RAP74) can enhance the activity of the mismatched minimal promoter system (30). TFIIF (containing only RAP30 and RAP74) can enhance the activity of the mismatched minimal promoter system (30). However, in the archaeal transcription system, the archaeal TFIIE stimulates transcription (26). Therefore we tested human TFIIF and TFIIE for the ability to complement the Glu-51 and Arg-66 mutations in the MLP MMP system.

Unlike the observation in the minimal promoter systems containing supercoiled DNA (28, 31), addition of RAP30 to the MLP MMP system did not stimulate transcriptional activity and did not restore activity to the E51A and R66A mutants (data not shown). However, the addition of the TFIIF complex slightly stimulated wild-type TFIIF in the MLP MMP system (Fig. 7A, lanes 5 and 6). When TFIIF was added to the MLP

![Figure 7](image)
Mutations in the TFIIB Finger

MMP reaction, in the absence of TFIIB, no activity was observed (Fig. 7A, lanes 3 and 4), but when TFIIF was added to reactions containing the double charge-of-charge mutant (E51R:R66E), some activity was observed (Fig. 7A, lanes 7 and 8). In addition, when the inactive point mutants E51A and R66A were supplemented with TFIIF, some activity was observed (Fig. 7B, lanes 5–9). In all cases, the start site was the same as with wild-type TFIIB.

We also tested the effect of the addition of TFIIE in the MLP MMP. Like TFIIF, TFIIE did not have activity on its own in the absence of TFIIB, but the addition of TFIIE to the MLP MMP system actually reduced the activity of the wild-type TFIIB by ~45% (data not shown). TFIIE did not restore activity to the inactive E51R:R66E mutant, nor did the addition of both TFIIE and TFIIF enhance the activity over that seen with the addition of TFIIF alone (data not shown).

DISCUSSION

To investigate the properties of the B-finger of human TFIIB at the most fundamental level, we chose to examine mutations in the B-finger in two minimal promoter systems that use only calf-thymus RNAP II, recombinant human TBP, and recombinant human TFIIB. Most of the structural and genetic studies on TFIIB have focused on the yeast transcription system. However, yeast RNAP II, TBP, and TFIIB have not been shown to work in a promoter system using only those three components. In addition, yeast TFIIB could not substitute for human TFIIB in the MLP MMP system (supplemental Fig. S5). Therefore, this mutational study could not be performed with yeast TFIIB. The major advantage of a minimal promoter system is that it allows the examination of the smallest set of protein–protein and protein–DNA interactions that are necessary to achieve accurate transcription initiation. As shown by the experiments presented here, the use of the minimal promoter systems provides a means to investigate effects of mutations in TFIIB that cannot be observed easily in a more complex system because of complementation by other transcription factors.

We found that in the minimal promoter systems mutations in the B-finger of human TFIIB fall into three distinct groups: 1) transcriptionally inactive mutations in residues Glu-51 and Arg-66, 2) transcriptionally compromised mutations in the conserved residues that constitute the “sides” of the B-finger, and 3) fully active mutants in the nonconserved residues that constitute the “tip” of the B-finger (Figs. 2, 3, 4, and S4).

Residues Glu-51 and Arg-66 of human TFIIB have been the focus of many studies, because they are homologous to residues Glu-62 and Arg-78 in yeast TFIIB. These two residues are conserved among all of the TFIIB proteins in the data base, and, based on the yeast protein, they likely form a salt bridge (19, 21). All mutations that we created in human TFIIB resides Glu-51 and Arg-66 were inactive in the minimal promoter systems, and a change-of-charge mutant (E51R:R66E), which based on the yeast system, should have restored the salt bridge (21), did not show activity (Figs. 2 and 3). It is not clear if a salt bridge failed to form with the double mutant in our minimal promoter system experiments, or if residues Glu-51 and Arg-66 play another role in transcription initiation.

Of particular interest are the conserved residues on the sides of the B-finger. Mutations in these residues reduced, but did not eliminate, activity in the minimal promoter systems. Mutations W52A and F55A were severely affected in both minimal promoter systems (Figs. 4 and S4), and W52A was the most severely affected of all the mutants in the complex transcription system (Fig. 6). Because of the proximity of the B-finger to the active site of RNAP II (19), it is not without precedent to suggest that these aromatic residues might help to maintain the open complex, as has been suggested for the aromatic residues in region 2.3 of the bacterial transcription factor, sigma70 (48).

Surprisingly, mutations in the nonconserved residues at the tip of the finger, which approach the active site in the yeast crystal structure (19), did not have a significant effect on activity in either minimal promoter system (Figs. 4 and S4) or in the HeLa cell NE assay (Fig. 6). This is in agreement with the observation recently made by Tran and Gralla (25) that a seven-amino acid deletion (27NDKATKD63) reduced activity by ~65% in HeLa cell NE, whereas a deletion of only three-amino acids (29KAT41) showed nearly wild-type activity. We did not examine any deletion mutants in this study. However, the activity of the chimeric proteins (Fig. 5) reflected the results obtained with the point mutations. The tip of the B-finger can withstand considerable sequence differences and still function in the MLP MMP system. However, as the residues on the sides of the B-finger diverged from the human sequence, the activity was reduced.

Because of the results that they obtained with the deletion mutants, Tran and Gralla (25) also examined the double point mutant (D58A:D63A), which showed a loss of activity of ~65% in HeLa cell NE. By our interpretation, residues Asp-58 and Asp-63 are highly conserved, and we consider them to be contained within the conserved sides of the B-finger, rather than the nonconserved tip of the B-finger (Fig. 5). In our study, single point mutations in Asp-58 and Asp-63 showed a reduction in activity of ~50% in the MLP MMP system, and the double mutation reduced activity by ~85% (Fig. 4). Thus, we can conclude that these two acidic residues are important for activity, but other conserved residues are equally important.

Tran and Gralla (25) also reported the generation of abortive transcripts with their double mutant in HeLa cell NE. We did not see an increase in lower molecular weight transcripts with any of the mutants in the MLP MMP system (Fig. 4); this would indicate an increase in abortive products. Single round transcription assays with the IgH minimal promoter system were not successful, presumably because of the inefficiency of the system (data not shown). Tran and Gralla examined their B-finger mutants only in HeLa cell NE, using a modified, activated adenovirus E4 promoter. Differences in experimental conditions, particularly the use of different promoters, make it difficult to directly compare data. The generation of abortive products can be affected by the sequence of the initially transcribed product (45).

All point mutations showed some activity in HeLa cell NE (Fig. 6), even the mutants that were inactive in the minimal promoter systems. These results verify results reported previ-
ously using mutations in human Glu-51, Arg-66, and the double mutant E51R:D66E in the HeLa NE (18, 22). Although quantification was not performed in those experiments, visual inspection of the results suggests that there was a reduction in activity with these mutants in those studies. The compilation of the activities of all of the B-finger mutants, as determined in this study, gives a more complete picture of the effect of the B-finger mutants in the HeLa cell NE (Fig. 6B). This composite mimics the pattern seen with the mutants in the minimal promoter systems (Figs. 4 and S4), except that all mutants had some activity in the HeLa cell NE. It is important to note that, the D58A:D63A mutant examined by Tran and Gralla on the adenovirus E4 promoter (25) showed ∼30% activity in HeLa cell NE, comparable to the activity of some of our mutants on the sides of the B-finger in HeLa cell NE (Fig. 6).

In an effort to understand the different behaviors of the Glu-51 and Arg-66 mutants in the minimal promoter systems and in the complex HeLa cell NE, we investigated the effect of supplementing the MLP MMP reactions with some of the other general transcription factors. The addition of TFIIF to MLP MMP reactions containing mutations in residues Glu-51 and Arg-66 restored some activity (Fig. 7). The W52A and V67A mutants (adjacent to Glu-51 and Arg-66), which showed compromised activity in the MLP MMP, were also enhanced by the addition of TFIIF (data not shown). The effect of TFIIF on the TFIIB mutations is consistent with previous information about the role of TFIIF in transcription initiation/promoter clearance. Evidence drawn from a variety of structural (49, 50), biochemical (15), and genetic (51, 52) studies indicate that the interface of the TFIIF subunits likely interacts with the B-finger, and that RNA Pol II appears to change conformation when TFIIF is present (50). The effect of TFIIF on TFIIB might be an allosteric effect that stabilizes the structure of the human B-finger, but other factors in the HeLa cell NE might play a role in stabilizing either the pre-initiation complex or the early ternary complex. However, this observation helps to explain why the effect of mutations in residues Glu-51 and Arg-66 of human TFIIB in HeLa cell NE have been obscured in other studies (18, 22).

We have not been able to directly examine TFIIF in the HeLa cell NE because our mAbs to RAP30 do not immunodeplete TFIIF from HeLa cell NE, presumably because of inaccessibility of the epitopes (31). These epitopes are contained in the N-terminal domain of RAP30 and are likely masked by interaction with RAP74. Addition of excess TFIIF to the HeLa cell NE did not restore the activity of the R66A mutant to wild-type levels (data not shown), suggesting that TFIIF is not a limiting factor.

Investigation of the mechanism by which TFIIF can rescue the inactive mutants in the minimal promoter system will be the subject of a future study. What seems indisputable is that the function of the B-finger is important enough that other factors in HeLa cell NE, including TFIIF, complement defects in this important region.

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