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Evidence That Transcription Factor IIB Is Required for a Post-assembly Step in Transcription Initiation*

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Mutation of glutamate 62 to lysine in yeast transcription factor (TF) IIB (Sua7) causes a cold-sensitive phenotype. This mutant also leads to preferential transcription of downstream start sites on some promoters *in vivo*. To explore the molecular nature of these phenotypes, the TFIIB E62K mutant was characterized *in vitro*. The mutant interacts with TATA-binding protein normally. In three different assays, the mutant can also interact with RNA polymerase II and recruit it and the other basal transcription factors to a promoter. Despite the ability to assemble a transcription complex, the TFIIB E62K protein is severely defective in transcription *in vitro*. Therefore, the role of TFIIB must be more than simply bridging TATA-binding protein and polymerase at the promoter. We propose that the region around Glu-62 in yeast TFIIB plays a role in start site selection, perhaps mediating a conformational change in the polymerase or the DNA during the search for initiation sites. This step may be related to the yeast-specific spacing between TATA elements and start sites since mutations of the corresponding glutamate in mammalian TFIIB do not produce a similar effect.

Transcription initiation by RNA polymerase II (pol II)[1] in eukaryotes requires an assembly of general transcription factors on the promoter to form a preinitiation complex (PIC) (reviewed in Refs. 1–3). An initial committed complex is formed by TBP/TFIID binding to the TATA element of a promoter (4). Subsequent interaction with TFIIB bridges TFIID on the TATA element and RNA pol II/TFIIF, TFIIE, and TFIIH (5). Several steps occur after transcription complex assembly, including promoter melting, start site selection and initiation, promoter clearance, elongation, and reinitiation. During the initiation process, numerous protein-protein and protein-DNA interactions must be established, adjusted, and then disrupted as the transcription machinery moves away from the start site.

The mechanism of transcription start site selection is not well characterized. In higher eukaryotes, promoter melting and transcription initiation overlap at a fixed distance of ~25 nucleotides downstream from a TATA box (6, 7), suggesting that start site selection is simply due to the geometry of the transcription complex. In contrast, initiation in *Saccharomyces cerevisiae* generally occurs at multiple sites within a broad window of 30–120 nucleotides from TATA (8–11).

Genetic methods have been applied to identify factors that affect start site selection in yeast cells. Mutations in TBP (*spt15* alleles (12)), TFIIB (*sua7* (13)), and two polymerase subunits (*rpb1*/*sua8* (14) and *rpb9*/*shi*/*ssu73* (15–18)) can alter start site selection *in vivo*. In contrast to the *spt* mutations, which affect TATA element selection, the *sua* and *shi* mutations alter the spacing between the TATA element and initiation sites. Rather than causing completely novel initiation sites to be used, the mutations change the relative usage of normal initiation sites (*i.e.* strongly favoring upstream or downstream start sites). It is interesting to note that these mutations generally do not affect overall promoter strength and that many promoters are unaffected. In agreement with the genetic experiments, species-specific selection of transcription start sites in *S. cerevisiae* and *Schizosaccharomyces pombe* was specified *in vitro* by pairwise replacement of both TFIIB and pol II (19).

TFIIB has two domains that correlate with its two known interactions. At the N terminus is a zinc ribbon domain that is essential for the pol II/TFIIF recruiting activity (20, 21). A proteolytically resistant C-terminal domain of TFIIB is necessary and sufficient for the interaction with the TBP·DNA complex (20–24). The structures of these two domains have been characterized separately (25–27). However, a highly conserved region linking two domains does not appear in the structures and has been proposed to be a flexible "hinge" region. Although the N-terminal domain of TFIIB is absent from the x-ray structure of the DNA·TBP·TFIIBc complex, this domain is predicted to be close to the initiation site (27).

TFIIB physically links TBP/TFIID and pol II/TFIIF and may thereby define the spacing between them. Some mutations in the TFIIB hinge region can dramatically affect the spacing between the TATA element and the initiation sites *in vivo* (13, 28, 29). This phenotype allowed the original isolation of the yeast TFIIB gene (*SUA7*) as a suppressor of upstream ATG codons (13). Interestingly, these start site selection mutants also exhibit a cold-sensitive phenotype. In an attempt to characterize the role of TFIIB in transcription start site selection, we analyzed one of the Sua7 mutant proteins *in vitro*. Surprisingly, we found that the Sua7 E62K mutant can assemble transcription complexes normally, but is severely defective in transcription *in vitro*. These findings indicate that TFIIB is required not only for initial assembly of the transcription complex, but also for a later step in initiation such as promoter melting, start site selection, or promoter clearance.

MATERIALS AND METHODS

Native Protein Purification—The purification of RNA pol II and TFIIH was described (30). The Mono S (HR 5/5) (Amersham Pharmacia Biotech) fractions obtained during the purification of TFIIH (30) were

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[1] The abbreviations used are: pol II, polymerase II; PIC, preinitiation complex; TBP, TATA-binding protein; TF, transcription factor; DTT, dithiothreitol; CTD, C-terminal domain; bp, base pair(s).

used for the purification of TFIIF. The fractions were assayed by immunoblot analysis using anti-Tfg2 polyclonal antibody. The TFIIF fractions were pooled and applied directly onto a Mono Q (HR 5/5) column (Amersham Pharmacia Biotech) pre-equilibrated with buffer A (20 mM HEPES-KOH (pH 7.6), 1 mM EDTA, 1 mM DTT, and 20% (v/v) glycerol) containing 0.5 M KOAc and protease inhibitors (1 μg/ml antipain, 2 μg/ml aprotinin, 0.1 mM benzamidine HCl, 5 μg/ml chymostatin, 1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The column was developed with a 15-ml gradient of 0.5–1 M KOAc in the same buffer. Fractions containing TFIIF, which eluted at ~0.85 M KOAc, were pooled and dialyzed against storage buffer (20 mM HEPES-KOH (pH 7.6), 0.1 M KOAc, 1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol, and protease inhibitors).

Recombinant Protein Production and Purification—The open reading frames of wild-type and E62K, G204D, and C45F mutant Sua7 genes were amplified by polymerase chain reaction; cloned into pET-11d; and transformed into *Escherichia coli* BL21(DE3)/pLysS. The C-terminal truncation expression construct (Met-119 to the end) was generously provided by Song Tan (Pennsylvania State University). Each strain containing wild-type or mutant plasmid was cultured in LB medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) at 30 °C. Cells were induced with isopropyl-β-D-thiogalactopyranoside (0.4 mM) when $A_{600\ nm}$ reached 0.6. The preparation of extract and the purification of each recombinant protein were performed essentially as described for human TFIIB by Buratowski and Zhou (20). In the case of E62K and G204D, the full-length recombinant protein fraction also contained a truncation product that arises from the internal translation initiation at methionine 119 (31). To separate the full-length protein from this product, the S-Sepharose (Amersham Pharmacia Biotech) eluate fraction was applied to a hydroxylapatite column (Bio-Rad) equilibrated with 10 mM potassium phosphate (pH 7.7), 100 mM KOAc, 1 mM DTT, 20% glycerol, and 1 mM phenylmethylsulfonyl fluoride. Bound proteins were eluted with a gradient to 240 mM potassium phosphate in the same buffer. A second round of hydroxylapatite chromatography was necessary for complete isolation of the full-length protein. The final hydroxylapatite eluate was further purified by Mono S (HR 5/5) fast protein liquid chromatography as described (20). Proteins were monitored by Coomassie Blue staining and confirmed by immunoblot analysis. Human wild-type and E51A and E51R mutant TFIIB proteins were purified as described (20). Yeast TBP was expressed and purified as described (20). The purification of Gal4-VP16 was described (30).

Co-immunoprecipitation of TFIIB and pol II—200 ng of recombinant yeast TFIIB (wild type or the indicated mutants) was incubated with 400 ng of purified pol II in 200 μl of buffer A containing 0.6 mg/ml bovine serum albumin and 0.03% Nonidet P-40 for 1 h at room temperature. The reaction mixture also contained 20 μl of protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech) to preclear the reaction. After centrifugation to remove the beads, either preimmune serum or anti-Sua7 serum (1 μl) and a fresh aliquot of protein A-Sepharose beads were added to the reaction mixture. Incubation continued for another hour at room temperature. The beads were collected and washed five times with ice-cold buffer A plus 0.03% Nonidet P-40 (1 ml each time). The precipitate was resuspended in 20 μl of SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. Mouse monoclonal antibody 8WG16 against the C-terminal domain (CTD) of pol II (32) was used to detect RNA pol II.

Gel Mobility Shift Assay—The 10–13-μl binding reaction mixture typically contained 20 mM HEPES-KOH (pH 7.6), 5 mM MgOAc, 30–60 μg/ml poly(dG·dC), 0.6 mg/ml bovine serum albumin, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 1–3 $\times$ 10^4 cpm of probe. The *Eco*RI/*Hin*dIII fragment of pRW, which contains the adenovirus major late promoter TATA element and initiator, was used as a probe (5). Protein components were added to the binding reaction as indicated. The reaction mixture was incubated at room temperature for 30–50 min and loaded onto a 4% polyacrylamide gel containing 25 mM Tris, 40 mM glycine, and 1 mM EDTA. Magnesium acetate (3 mM) was added to the gel in the experiment shown in Fig. 3*B*. A typical binding reaction contained 10 ng of TBP, 30 ng of TFIIB, 80 ng of pol II fraction, and 25 ng of TFIIF fraction.

In Vitro Transcription—Whole cell extracts were prepared from wild-type TFIIB cells (YSB143 = *MAT***a**, *ura3-52*, *leu2-3,112*, *his3*Δ*200*, *sua7*Δ*::LEU2* (pRS313-*SUA7*)) or isogenic TFIIB E62K mutant cells (YSB176 = same genetic background as YSB143 but containing (pRS313-*sua7-35*)) as described by Woontner *et al.* (33). *In vitro* transcription was performed as described by Woontner and Jaehning (34) with slight modifications. Reaction mixtures (30 μl) contained 25 mM HEPES-KOH (pH 7.6), 10 mM MgOAc, 5 mM EGTA, 2.5 mM DTT, 100 mM KOAc, 8–10% (v/v) glycerol, 1 unit of creatine kinase (Sigma), 10 mM phosphocreatine (Sigma), 10 units of rRNasin (Promega), 4 mM phosphoenolpyruvate, 0.5 mM each CTP and ATP, 10 μM UTP, 5 μCi of [α-^{32}P]UTP (3000 Ci/mmol; NEN Life Science Products), 0.5 mM 3′-*O*-methyl-GTP (Amersham Pharmacia Biotech), 60–80 μg of whole cell extract protein, and 200 ng of pGal4CG$^-$ (35). After a 50–60-min incubation at room temperature, reactions were stopped and processed with RNase T1 and proteinase K as described (34). For single round transcription, extracts were incubated with template for 20 min in the transcription reaction buffer without NTPs to allow formation of PIC. For multiple round initiations, the NTPs were added, and the reaction mixture was further incubated for 20 min. For single round transcriptions, Sarkosyl (0.1 or 0.3% final concentration) was added 1.5 min after NTP addition and incubated for 10 min. The RNA was extracted with phenol/chloroform, precipitated, and analyzed on a 7 M urea and 5% polyacrylamide gel. Reactions were supplemented, where indicated, with 100 ng of Gal4-VP16. Mammalian *in vitro* transcription was performed in a reconstituted system as described by Buratowski and Zhou (20).

Immobilized Template Assay—Transcription template DNA was made by cutting pGal4CG$^-$ with *Ehe*I and *Afl*III. The *Afl*III site was filled with biotin-14-dATP (Life Technologies, Inc.), dGTP, dCTP, and dTTP using Klenow fragment. Template DNA was then gel-purified. The 1.1-kilobase pair fragment contains one Gal4-binding site, the *CYC1* promoter, and a G-less cassette. The biotinylated fragment was incubated with streptavidin-coupled M-280 Dynabeads (3–5 pmol of DNA/mg of streptavidin-coupled M-280 Dynabeads; Dynal Inc.) in buffer B (2 M NaCl, 10 mM Tris-Cl (pH 7.4), and 0.01% Nonidet P-40) at room temperature overnight. As a promoterless negative control, the *Ehe*I/*Afl*III fragment of p$(C_2AT)_{19}$ (36) was used. The DNA-bound beads were washed several times in buffer B and subsequently in Tris/EDTA buffer. The beads were preincubated with bovine serum albumin (0.05 mg/ml) and washed briefly right before use with simple transcription buffer (25 mM HEPES-KOH (pH 7.6), 10 mM MgOAc, 5 mM EGTA, 2.5 mM DTT, 100 mM KOAc, and 10% glycerol). Transcription initiation complexes were assembled on immobilized template DNA by incubation of 120 μg of whole cell extract with 3 μl of beads in 60 μl of simple transcription buffer. Hexokinase (2 units) and glucose (2 mM final concentration) were added to the mixture during the assembly reaction to deplete endogenous NTPs. After 40 min, the transcription complexes were magnetically purified and extensively washed with simple transcription buffer containing 0.003% Nonidet P-40. Beads were resuspended in either SDS-polyacrylamide gel electrophoresis sample loading buffer directly or kinase reaction buffer (37). The kinase reaction continued for 1 h at room temperature. The reaction was stopped by addition of sample loading buffer for SDS-polyacrylamide gel electrophoresis and electrophoresed on a 4~20% gradient polyacrylamide gel (Bio-Rad). The proteins were analyzed by immunoblotting with polyclonal antibodies against TFIIB (anti-Sua7), TFIIE (anti-Tfa1), TFIID (anti-TBP), and TFIIH (anti-Tfb1, provided by W. J. Feaver and R. D. Kornberg) and monoclonal antibody 8WG16. Autoradiography was used to detect phosphorylated pol II.

RESULTS

The TFIIB E62K Mutant Is Defective in Transcription in Vitro—The yeast TFIIB allele *sua7-1* contains a substitution of lysine for glutamate 62 (E62K) and was isolated as a suppressor of an aberrant ATG translation initiation site in the leader region of the *CYC1* gene (13). As a result of this single amino acid substitution, transcription initiation sites at some promoters are shifted downstream *in vivo*. In a search for conditional alleles of *SUA7*, we independently isolated E62K as a cold-sensitive variant.[2]

To characterize the mechanism of transcription start site selection *in vitro*, whole cell extract was prepared from yeast strains expressing either wild-type or E62K mutant TFIIB. The transcription template (pGal4CG$^-$) contains a single Gal4-binding site and the *CYC1* TATA element upstream of a 400-bp G-less cassette (35). In wild-type extracts, two major *in vitro* transcription initiation sites are apparent (Fig. 1, *lane 1*). In contrast to its *in vivo* behavior, E62K replacement greatly reduced *in vitro* transcription activity at the *CYC1* promoter

[2] C. Dollard, H. Zhou, and S. Buratowski, unpublished data.

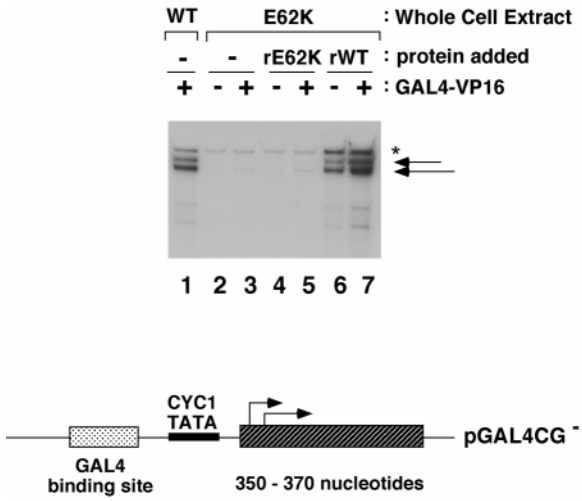

FIG. 1. **The TFIIB E62K mutant is defective in transcription *in vitro*.** *In vitro* transcription was performed with either wild-type (*WT*) or E62K mutant TFIIB whole cell extracts in the presence (+) or absence (−) of the activator Gal4-VP16. E62K transcription was supplemented with 100 ng of either recombinant E62K (*rE62K*) (*lanes 4 and 5*) or recombinant wild-type (*rWT*) (*lanes 6* and *7*) TFIIB. The two major transcripts are indicated by *arrows*. The *uppermost band* (indicated with an *asterisk*) is from read-through transcription of the G-less cassette. The template plasmid used for the transcription reaction (pGal4CG⁻) is shown at the bottom. This plasmid contains a single Gal4-binding site and a *CYC1* TATA element that controls expression of G-less transcripts 350–370 nucleotides long.

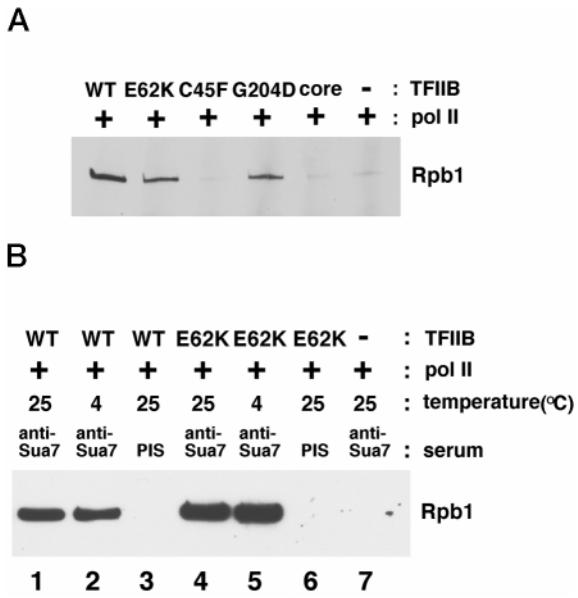

FIG. 2. **TFIIB E62K can interact with RNA polymerase II.** *A*, recombinant wild-type (*WT*) or several mutant TFIIB proteins were incubated with purified pol II for 1 h. The reactions were immunoprecipitated with anti-Sua7 (yeast TFIIB) antibodies. The precipitates were then immunoblotted with antibodies against the largest subunit of pol II (8WG16). *B*, interaction of wild-type and E62K mutant TFIIB proteins with pol II was examined at both 4 and 25 °C. The binding reaction and precipitation were performed as described for *A*. Reactions incubated at the indicated temperatures were precipitated with either antibodies against TFIIB (anti-Sua7) or preimmune serum (*PIS*).

(Fig. 1, *lanes 2* and *3*). The small amount of transcription supported by E62K was still responsive to the activator Gal4-VP16 (see below). PhosphorImager quantitation indicated that the mutant extracts produced ~10–15-fold less transcript than the wild-type extracts. Since the E62K mutant *in vivo* causes a downstream shift in start site selection, the ratio of the two transcripts was quantitated. Surprisingly, the ratio was roughly the same in both extracts: ~1.5 downstream transcripts to each upstream transcript.

Transcription activity could be restored to the mutant extract by addition of recombinant wild-type TFIIB (Fig. 1, *lanes 6* and *7*). However, addition of excess amounts of recombinant E62K protein to the mutant extract did not increase transcription levels, suggesting that the defect was not due to low levels of TFIIB protein (Fig. 1, *lanes 4* and *5*). The recombinant E62K protein also failed to support the transcription in a purified reconstituted transcription system (data not shown). We also tested several other promoters, including the adenovirus major late promoter (pMLΔ53(200) (38)), the IgH promoter (pμ(−47)-G⁻-IV (39)), the IgH-adenovirus major late hybrid promoter (pμ(-47)-ML-(G⁻) (40)), the *PYK1* promoter (pPYKC/G⁻; gift from R. D. Kornberg), and his4-912δ (pLG80; gift from F. Winston). None of them were transcribed efficiently in the E62K mutant extract (data not shown). Based on these findings, we conclude that the TFIIB E62K mutant protein is intrinsically defective in at least one function required for normal transcription.

TFIIB E62K Can Interact with TBP and pol II to Form a Stable DNA·TBP·TFIIB·pol II·TFIIF Complex—Since E62K extracts are defective in transcription, one or more essential interactions within the transcription complex must be affected. TFIIB is known to interact directly with TBP and with pol II. To characterize the biochemical defect of E62K, two protein interaction assays were used: co-immunoprecipitation with pol II and native gel electrophoresis of partial transcription complexes. We also tested three additional mutant proteins with well characterized defects. C45F mutant TFIIB is mutated at a conserved cysteine residue within the zinc finger motif, and the "core" TFIIB is a complete deletion of this N-terminal domain. These mutants do not support viability in yeast (data not shown), and similar substitutions in mammalian TFIIB block interaction with pol II (20). Another mutant, G204D, causes a severe growth defect in yeast and is severely defective in the interaction with TBP, consistent with predictions from the DNA·TBP·TFIIB structure (27).

Co-immunoprecipitation experiments (Fig. 2*A*) showed that pol II could bind to the wild-type and E62K and G204D mutant TFIIB proteins. In contrast, the zinc finger mutant (C45F) and the N-terminal truncation mutant (core) were not able to precipitate pol II, consistent with other reports that the zinc finger region of TFIIB is necessary for pol II interaction (20–24). The interaction between E62K and pol II was not affected by lowering the temperature (Fig. 2*B*). Therefore, the *in vitro* transcription defect and cold-sensitive phenotype of E62K cannot be attributed to a defect in pol II interaction.

We next used native gel electrophoresis to determine whether E62K and the other mutant proteins were able to interact with TBP and to recruit pol II and TFIIF to the promoter DNA. This system has previously been used to analyze mammalian transcription complexes (5). With yeast factors, complexes consisting of DNA·TBP·TFIIB (Fig. 3*A*) and DNA·TBP·TFIIB·pol II·TFIIF (Fig. 3*B*) can be visualized. All the TFIIB proteins except for G204D were able to form the DNA·TBP·TFIIB complex, indicating that E62K is not defective in this interaction (Fig. 3*A*). Formation of partial transcription complexes is visualized in Fig. 3*B*. In Fig. 3*B*, 3 mM magnesium acetate was included in the gel and running buffer to destabilize nonspecific polymerase complexes. The DNA·TBP·TFIIB complex was not seen under these conditions. Both the wild-type and E62K mutant TFIIF proteins were able to form the DNA·TBP·TFIIB·pol II·TFIIF complex (Fig. 3*B*). This complex was completely dependent upon the presence of TFIIF. The TFIIB mutant proteins defective in pol II interaction or TBP

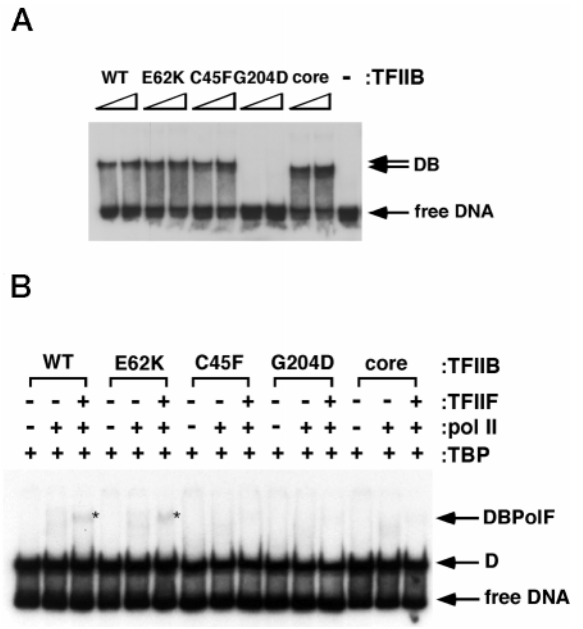

FIG. 3. **TFIIB E62K can interact with TBP and pol II/TFIIF to form a transcription complex on the promoter.** *A*, the ability of each TFIIB to interact with TBP bound to the promoter was tested by native gel electrophoresis. Two different amounts (20 and 40 ng) of each TFIIB or no TFIIB (designated −) was incubated with TBP (10 ng) and the adenovirus major late promoter DNA probe for 30 min at room temperature. The DNA·TBP·TFIIB (*DB*) complexes were resolved on the native gel. *B*, different combinations of general transcription factors, as indicated at the top, were incubated at room temperature for 40 min. The complexes were resolved on the native gel containing 3 mM MgOAc. The promoter complexes formed on the promoter DNA probe are denoted as follows: *D*, DNA·TBP; and *DBPolF*, DNA·TBP·TFIIB·pol II·TFIIF (also marked by an *asterisk)*.

interaction were unable to form a DNA·TBP·TFIIB·pol II·TFIIF complex on the promoter. Therefore, the E62K mutant appears to be functional for recruitment of pol II and TFIIF to the promoter DNA.

TFIIB E62K Effectively Assembles Transcription Initiation Complexes—The native gel electrophoresis assay allowed observation of only partial transcription complexes, and therefore, it was possible that the TFIIB mutant might block the association of other transcription factors such as TFIIE or TFIIH. To look at complete transcription complexes assembled from whole cell extracts, we took the advantage of an immobilized template assay (30). Wild-type or mutant extract was incubated with template DNA linked to magnetic beads. After incubation to allow transcription complexes to assemble, the beads were collected magnetically and washed extensively. The presence of basal transcription factors was assayed by immunoblotting, and phosphorylation of the pol II CTD by TFIIH was detected by autoradiography (Fig. 4*A*).

Assembly of the transcription complex was dependent on the presence of the promoter sequences (Fig. 4*A*, compare *lanes 1* and *2*) and was modestly stimulated by the presence of the activator Gal4-VP16. Interestingly, the transcriptionally inactive E62K extract assembled all the required transcription factors at the promoter. Upon addition of ATP, the transcription complexes containing the TFIIB E62K mutant also exhibited phosphorylation of the Rpb1 CTD. Therefore, the E62K mutant does not affect the ability of TFIIH to phosphorylate the CTD.

To compare the immunoblot results with transcription activity, complexes assembled on immobilized template beads were washed extensively and then incubated in a transcription reaction (Fig. 4*B*). To test for the ability of transcription factors to exchange, a second template (pJJ460) was added along with the NTPs. Transcription from wild-type complexes was dependent upon an intact promoter and responded to the activator (Fig. 4*B*, *lanes 2–4*). Transcription was from pre-assembled factors on the immobilized template since no transcription was observed from the second template (pJJ460) added after PIC formation. As previously observed in the extracts, immobilized complexes assembled in the E62K whole cell extract did not produce transcripts (Fig. 4*B*, *lane 6*). Transcription from the mutant extract could be rescued by addition of wild-type TFIIB, but only if the protein was added before the beads were pelleted and washed (Fig. 4*B*, compare *lanes 7* and *8*). Therefore, once assembled into the complete initiation complex, TFIIB and the other basal factors cannot be exchanged freely.

E62K Transcription Is Defective in a Post-assembly Step—In some cases, multiple rounds of transcription initiation may occur from a single template without the necessity of completely re-assembling the PIC in each round. To determine whether TFIIB E62K might be normal for the first round of transcription but defective in reinitiation, we performed single round transcription reactions by blocking reinitiation with the detergent Sarkosyl. Complexes were formed in the wild-type and E62K extracts; NTPs were added to initiate transcription; and Sarkosyl (0.1 or 0.3%) was added after 1.5 min of incubation. In the absence of the Gal4-VP16 activator (Fig. 5, *odd-numbered lanes*), the E62K extract was 5–10-fold less active than the wild-type extract whether the reactions were single or multiple rounds. Therefore, the E62K defect is manifested even during the initial initiation event.

As shown in Fig. 5, Gal4-VP16 stimulated transcription under both multiple (without Sarkosyl) and single (with Sarkosyl) round initiation conditions. Under multiple round conditions, wild-type extracts showed ~6-fold activation, whereas E62K extracts showed ~2-fold activation. Interestingly, the relative activation of transcription was more strongly affected by Sarkosyl in wild-type extracts than in E62K extracts. Wild-type extracts exhibited 2-fold activation when limited to a single round of initiation, similar to E62K extracts under both single and multiple round conditions. Gal4-VP16 is known to increase reinitiation as well as PIC formation (41). Therefore, the E62K extracts appear to carry out less Gal4-VP16-directed reinitiation than the wild-type extracts. Although this observation might suggest that E62K has a specific defect in reinitiation, it can also be explained by the fact that E62K-containing complexes assembled in the first round remain bound at the promoter and thereby inhibit reinitiation. The second hypothesis is supported by the experiment in Fig. 4*B*. In this reaction, where multiple reinitiations can occur, wild-type TFIIB added back after PIC formation with E62K was not able to restore transcription.

Human Mutant Homologues Are Active for Transcription in Vitro—TFIIB is highly conserved over evolution, particularly in the region C-terminal to the zinc finger region. In fact, it has been shown that amino acids 52–140 of yeast TFIIB can be replaced by its corresponding human sequences *in vivo* (42). Since sequence conservation over evolution suggests that this region has an important functional role in TFIIB, we wanted to know whether mutation of the glutamate corresponding to Glu-62 would have similar effects on human TFIIB. We therefore mutagenized glutamate 51 (corresponding to yeast Glu-62) to alanine or arginine and expressed the mutants in *E. coli* along with human wild-type TFIIB. It has been shown that the E62R mutant is phenotypically similar to E62K in yeast (28).

The transcription activities of human wild-type TFIIB and the Glu-51 mutants were compared in a reconstituted *in vitro* transcription system (Fig. 6). In contrast to results obtained

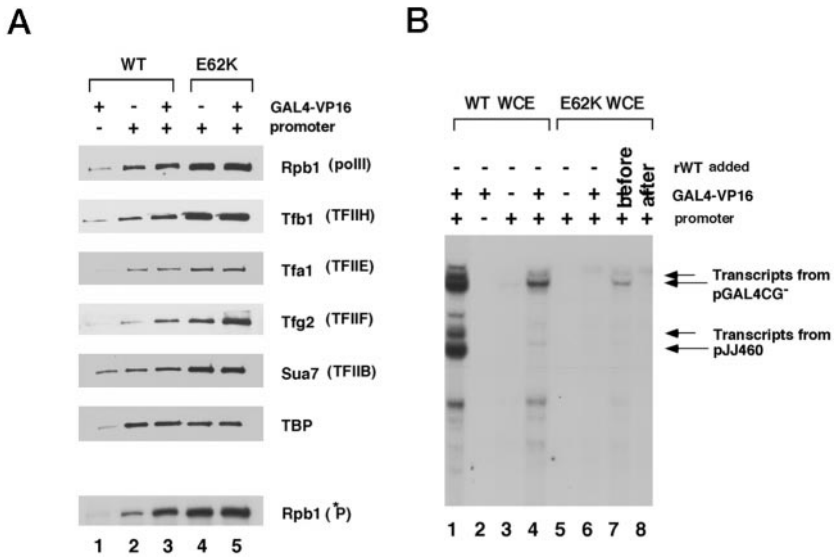

FIG. 4. **TFIIB E62K can recruit the basal transcription factors to form a transcription complex on the promoter.** *A*, transcription complexes were assembled on beads carrying either a DNA fragment containing the *CYC1* promoter (*lanes 2–5*) or a control fragment without a promoter (*lane 1*). Extracts were from either wild-type (*WT*) or E62K mutant TFIIB cells. The activator Gal4-VP16 was added as indicated. The immobilized templates were washed extensively, collected, and tested for the presence of various transcription factors by immunoblotting. Rpb1 is the largest subunit of pol II; Tfb1 is a subunit of TFIIH; Tfa1 is a subunit of TFIIE; Tfg2 is a subunit of TFIIF; Sua7 is TFIIB; and TBP is the TATA-binding protein subunit of TFIID. Before gel electrophoresis, the beads were incubated with [γ-^{32}P]ATP, and the phosphorylated CTD of Rpb1 (*Rpb1 (*P)*) was detected by autoradiography (*bottom panel*). *B*, E62K is stably incorporated into the transcription complex. The yeast whole cell extracts were incubated with immobilized template beads carrying a promoterless (*lane 2*) or *CYC1* promoter-containing (*lanes 3–8*) template. Transcription complexes were purified as described for *A*, except that 80 ng of recombinant wild-type (*rWT*) TFIIB was added before (*lane 7*) or after (*lane 8*) complex purification. The washed beads were resuspended in transcription buffer that also contained 300 ng of pJJ460. The plasmid pJJ460 contains the *CYC1* promoter, but gives shorter transcript (250–270 nucleotides), and was added to determine whether the factors associated with the immobilized template could exchange onto a second promoter. *Lane 1* shows a positive control containing both immobilized and free templates transcribed in wild-type whole cell extract (*WCE*).

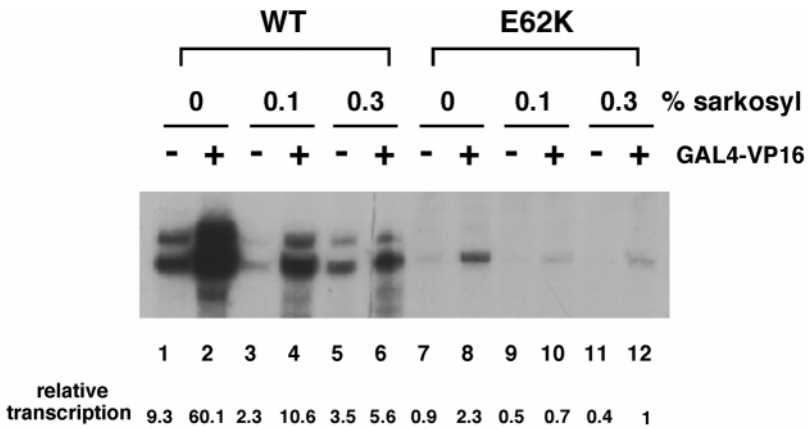

FIG. 5. **TFIIB E62K is defective in single round transcription.** Transcription reactions under multiple (no Sarkosyl) and single (0.1 and 0.3%) round initiation conditions were carried out. Preinitiation complexes were formed by incubating template for 20 min in wild-type (*WT*) or E62K extracts. The template used in this experiment was p5×Gal4CG$^-$, the same construct as pGal4CG$^-$, but carrying five copies of the Gal4-binding site. NTPs were added to initiation reactions, and Sarkosyl was added 1.5 min later to a final concentration of 0.1% (*lanes 3*, *4*, *9*, and *10*) or 0.3% (*lanes 5*, *6*, *11*, and *12*). Reactions were further incubated for 10 min. Transcription reactions were carried out both in the presence (+) and absence (−) of the Gal4-VP16 activator. Transcripts were quantitated by PhosphorImager, and relative levels are shown as numbers under each lane.

with yeast TFIIB, both E51A and E51R were unaffected in their ability to support *in vitro* transcription. We also tested the proteins by native gel electrophoresis and found that they were able to form a DNA·TBP·TFIIB·pol II·TFIIF complex at levels similar to wild-type TFIIB (data not shown). Therefore, the essential function abrogated by the Glu-62 mutation in yeast TFIIB either is not affected by the corresponding human Glu-51 mutation or is not rate-limiting for mammalian transcription.

DISCUSSION

Although many aspects of pol II transcription are conserved over eukaryotic evolution, spacing between the TATA element and the initiation site is different in yeast. To understand start site selection, we have begun to biochemically characterize a yeast TFIIB mutant (E62K) that causes initiation to shift downstream relative to wild-type transcription *in vivo*. In contrast to its *in vivo* behavior, we find that the E62K mutation causes a profound loss of transcription activity *in vitro*. This defect is not due to defects in TBP interaction since the E62K mutant TFIIB protein forms complexes as assayed by native gel electrophoresis. It is also not due to a defect in pol II interaction as tested by three independent assays: co-immunoprecipitation of purified TFIIB and pol II, native gel electrophoresis of a DNA·TBP·TFIIB·pol II·TFIIF complex, and assembly of initiation complexes on immobilized templates in crude extracts. The immobilized template experiment further dem-

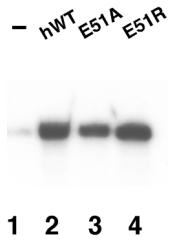

FIG. 6. **Mammalian transcription of the adenovirus major late promoter is not affected by mutation of glutamate 51 in TFIIB.** The human residue (Glu-51) corresponding to Glu-62 in yeast was mutated to alanine or arginine. Transcription activity was tested using a reconstituted mammalian transcription system and the adenovirus major late promoter as template (pMLΔ53(200) (38)). Each reaction received no TFIIB (−; *lane 1*), human wild-type TFIIB (*hWT*; *lane 2*), or mutant TFIIB with glutamate 51 changed to alanine (E51A) (*lane 3*) or arginine (E51R) (*lane 4*).

onstrated that each of the basal transcription factors is present in the E62K mutant complexes. Therefore, we conclude that the E62K mutation is likely to affect a step in transcription that occurs after initiation complex assembly. We suspect that the step that is blocked *in vitro* is also important for determining start site selection *in vivo*.

What might this step be? Since a single TATA element can specify multiple start sites in yeast, there must be some flexibility of components between these two elements, *i.e.* in the intervening DNA and/or the intervening proteins. One plausible model is that after the transcription complex is assembled and DNA is unwound, the polymerase active site must be positioned within the newly formed single-stranded region. Initiation sites would be recognized by "scanning" the unwound DNA for the appropriate sequences (43, 44). We suspect that movement in the TFIIB hinge region is required for this step and is inhibited by the E62K mutation. A conformational change in TFIIB would presumably require thermal energy, and the cold-sensitive phenotype of the E62K strain might reflect an increase in the energetic barrier in the mutant.

Several lines of evidence support a model in which TFIIB undergoes a conformational change. 1) Regions of DNA melting on the yeast *GAL1* and *GAL10* promoters were mapped *in vivo* 20–80 bp downstream of the TATA element, well upstream of the initiation sites (43). In higher eukaryotes, promoter melting and transcription initiation overlap at ~25–30 bp from the TATA element (45). Therefore, DNA melting and the start site selection step may be closely coupled in higher eukaryotes, but in yeast, the polymerase may be required to scan a larger region of unwound DNA before an initiation site is chosen.

2) As estimated from two-dimensional crystallographic studies of yeast pol II (44), the minimal distance from TFIIB (and presumably TBP/TATA) to the active site of pol II is ~30 bp, consistent with the position of the melted region mapped *in vivo*. A straight DNA path of 30 bp could explain the minimum distance from the TATA box to the start site, but longer distances to the start site suggest alternative pathways.

3) In the presence of a transcriptional activator, mammalian TFIIB was found to exhibit increased protease sensitivity. This sensitivity has been interpreted as an indication of a conformational change. Interestingly, the protease sensitivity maps near Glu-62 in the region between the zinc finger and the repeats (46).

4) The cold-sensitive phenotype of a TFIIB E62G mutant is suppressed by overexpression of the yeast PC4 homologue Sub1 (47). Furthermore, Sub1 becomes essential in the presence of this TFIIB mutant. The molecular nature of these genetic interactions is not yet known. However, the PC4 protein is a transcriptional coactivator that binds melted regions of DNA (48, 49). We speculate that increased Sub1 concentrations stabilize an open transcription complex, allowing sufficient time for the TFIIB conformation to occur in the mutant protein.

We note that several TFIIB mutants in residues close to Glu-62 have been described (22, 29, 50) and exhibit several similarities to E62K. Those tested are severely reduced for *in vitro* transcription and cause start site alterations and/or cold sensitivity *in vivo*. The R64E mutant is reported to stabilize binding of a DNA·TBP·TFIIB complex, leading to the suggestion that this region of TFIIB affects an interaction with DNA (29). Several of these mutants (W63P, R64A, R64E, F66D, and H71E) have been shown to interact with pol II by co-immunoprecipitation, although in the same set of experiments, E62K did not (29, 50). We do not understand this discrepancy with our results using E62K since our experiments suggest that E62K behaves very similarly to other mutants at positions 63–66 (50). We also note that while this paper was being reviewed, findings similar to those in Fig. 4 were published for the TFIIB E62G mutant (51).

The proposed conformational change in yeast TFIIB may also be relevant to the RNA polymerase III system, in which factor TFIIIB contains both TBP and a TFIIB homologue. Kassavetis *et al.* (52) have characterized certain mutant TFIIIB subunits (Brf or B″) that can recruit pol III, but that are inactive in promoter opening. We are attempting to determine whether pol II transcription complexes with TFIIB E62K are competent for promoter melting.

It is surprising that the human homologue of the E62K mutant appears to function normally in transcription despite the high level of conservation in this region. This indicates that the mutation probably affects a step that is either specific to or rate-limiting only in the *S. cerevisiae* transcription system. We presume that this step is important for start site selection since yeast transcription exhibits unusual and variable TATA-to-initiation site spacing and the E62K mutation affects start site selection *in vivo*. While this paper was being reviewed, another group also reported that human Glu-51 mutant TFIIB transcribed the adenovirus major late promoter normally (53). Interestingly, they also observed that the TFIIB mutant showed an increase in downstream initiation sites on the adenovirus E4 promoter, which has multiple start sites. The downstream initiation sites observed *in vivo* with the E62K mutant may represent initiation events that are less dependent upon a TFIIB conformational change, perhaps promoted by chromatin structure or some other aspect of transcription that is not reproduced in the *in vitro* systems. Further mechanistic and structural studies of TFIIB will help to further define this step and help us to understand how the transcription machinery determines initiation sites.

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