

Studies of *Schizosaccharomyces pombe* TFIIE indicate conformational and functional changes in RNA polymerase II at transcription initiation

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The general transcription factor TFIIE plays essential roles in transcription by RNA polymerase II (PolII). Despite recent progress, the elucidation of its precise mechanisms including biological functions awaits further characterization. We report the isolation and characterization of *Schizosaccharomyces pombe* TFIIE (spTFIIE). Like human and other eukaryotic TFIIE proteins, spTFIIE consists of α and β subunits and the genes encoding both subunits are essential for viability. Chromatin immunoprecipitation assays demonstrated that spTFIIE localizes to promoters *in vivo*. Mutational analysis of the C-terminal basic helix-loop region of TFIIE β , which is involved in the transition from transcription initiation to elongation, revealed that transcription-defective mutants affected in this region are also cold sensitive. The spTFIIE β subunit binds both spTFIIE β and spTFIIE α but spTFIIE α binds only spTFIIE β . These results indicate that TFIIE forms an $\alpha_2\beta_2$ heterotetramer in which two $\alpha\beta$ heterodimers are connected via β subunits. Further analysis of binding specificities showed that spTFIIE β binds the Rpb2 and Rpb12 subunits of PolII, whereas spTFIIE α predominantly binds Rpb5, which is located at the clamp region and changes conformation upon transcription initiation.

Introduction

Recent progress in genome sequencing projects has made it possible to identify homologs of human proteins in other eukaryotes, in archaea, and in some cases in bacteria. Such studies have shown that the system that mediates transcription of protein-encoding genes is well conserved among eukaryotes and is catalyzed by RNA

polymerase II (PolII). Transcription initiation by PolII requires five general transcription factors (TFIIB, TFIID, TFIIE, TFIIIF, and TFIIH), which form a preinitiation complex (PIC) on promoters (see reviews, Ohkuma 1997; Hampsey 1998; Roeder 1998). The general transcription factor TFIIE, alone and in collaboration with TFIIH, plays roles both in transcription initiation and in the transition from initiation to elongation. TFIIE is recruited to a PIC precursor consisting of TFIIB, TFIID, TFIIIF, and PolII by binding to all of these components as well as to double-stranded DNA (dsDNA) at around position -9 (nine bases upstream from the transcription initiation site, designated as +1), where the DNA helicase subunit XPB of TFIIH begins to promote melting at transcription initiation (Douziech *et al.* 2000; Yamamoto *et al.* 2001).

Human TFIIE (hTFIIE) consists of two subunits, hTFIIE α and hTFIIE β , which form an $\alpha_2\beta_2$ heterotetramer

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(Ohkuma *et al.* 1990). The larger (57-kDa) subunit hTFIIE α consists of 439 amino acid residues and the smaller (34-kDa) subunit hTFIIE β consists of 291 amino acid residues (Ohkuma *et al.* 1991; Sumimoto *et al.* 1991). Recently, we have studied hTFIIE β and found that this subunit has two important regions, one located in the middle, which includes a winged-helix (or forkhead) motif and binds to dsDNA, and the other located in the C-terminus, which possesses a basic region-helix-loop (bHL) sequence and which binds to PolII, various general transcription factors and single-stranded DNA (ssDNA) (Okamoto *et al.* 1998; Okuda *et al.* 2000). In addition, our studies of point mutations affecting the bHL region of hTFIIE β revealed that this region is essential for transcription by mediating the transition from transcription initiation to elongation (Watanabe *et al.* 2003).

In contrast, less is known about TFIIE α , and extensive structural and functional studies of this subunit have been undertaken only recently. An *in vitro* reconstituted transcription study showed that the amino (N)-terminal half of hTFIIE α (residues 1–173) is sufficient for both basal and activated transcription (Ohkuma *et al.* 1995). This finding was genetically confirmed with the budding yeast *Saccharomyces cerevisiae* (Kuldell & Buratowski 1997). Although null mutation of *TFA1*, which encodes the *S. cerevisiae* TFIIE α homolog (scTFIIE α , 482 amino acids), confers lethality, mutations affecting the N-terminal half (residues 1–211) permit viability but confer cold-sensitivity. The N-terminal half shows good homology with recently identified archaeobacterial TFIIE α homologs (TFE), which lack a region corresponding to the carboxy (C)-terminal half of hTFIIE α (Bell *et al.* 2001; Hanzelka *et al.* 2001). The acidic region near the C-terminus is the only functional region identified so far in the C-terminal half of hTFIIE α that directly binds TFIIH and stimulates basal transcription and TFIIH-mediated phosphorylation of the C-terminal domain (CTD) of the largest subunit of PolII. The CTD consists of repeats of the heptapeptide YSPTSPS (Ohkuma *et al.* 1995). Recently, the most N-terminal region of archaeal TFE was reported to possess a novel extended winged helix (forkhead) motif that is well conserved among eukaryotic TFIIE α homologs, although the functional role of this region is still obscure (Meinhart *et al.* 2003).

Judging from previous data, TFIIE β may play major roles in regulating the PIC at promoter melting upon transcription initiation by binding to both PolII and the promoter at around –10, where melting begins, as well as to the exposed ssDNA region of the promoter. The TFIIE α subunit may modulate the PIC both structurally and functionally, but evidence is still limited; one pro-

posal is that TFIIE α recruits TFIIH and stimulates its CTD phosphorylation activity. Recently, it has become apparent that the phosphorylated residues Ser-2 and Ser-5 of the CTD heptapeptide of PolII function as a platform for the recruitment of factors involved in transcription-coupled events such as mRNA processing and nucleosome-histone modification (Orphanides & Reinberg 2002; Hampsey & Reinberg 2003). We demonstrated that TFIIE specifically stimulates CTD Ser-5 phosphorylation (Yamamoto *et al.* 2001), suggesting that TFIIE is a key regulator of not only transcription itself but also transcription-coupled events. To further investigate the functional roles of TFIIE both genetically and biochemically, we isolated two TFIIE subunit genes (*spTFIIEA* and *spTFIIEB*) from the fission yeast *Schizosaccharomyces pombe*. Both genes are essential for viability. The *in vivo* localization of spTFIIE on promoters was tested with chromatin immunoprecipitation (ChIP) assay, and spTFIIE was found to be associated with promoter regions that include the transcription initiation site, clearly confirming that TFIIE functions in transcription, as was suggested by *in vitro* analyses. We also studied mutations affecting the C-terminus of spTFIIE β , for which the corresponding mutations in hTFIIE β were demonstrated to prevent the transition from transcription initiation to elongation, and to confer cold sensitivity. For biochemical analyses, spTFIIE α and spTFIIE β were expressed in bacteria. Using these proteins, we investigated the subunit-subunit interactions and searched for the target PolII subunits of the spTFIIE subunits. Our results suggest that TFIIE α contributes to conformational changes in the active center of PolII at transcription initiation by pulling the clamp module to close its cleft and by supporting to maintain the opened state of the promoter region during transcription initiation.

Results

Isolation of *S. pombe* TFIIE cDNAs

We previously isolated human TFIIE homologs from *Xenopus laevis* and *Caenorhabditis elegans* to examine the relationship between TFIIE structure and its role in transcription (Ohkuma *et al.* 1992a, 1992b; Yamamoto *et al.* 2001). These homologs were useful in initial biochemical investigations, and we next extended our studies of the biological roles of TFIIE by isolating TFIIE cDNAs from *S. pombe* (spTFIIE α and spTFIIE β). A TBLASTN homology search of the translated *S. pombe* database (NCBI) was carried out to identify regions with significant homology to human TFIIE (hTFIIE) subunits. This

search yielded spTFIIIE α cDNA, about 1.5 kb in length, which encodes a highly acidic 434 amino acid protein (pI 4.5) with a calculated molecular weight of 49.1 kDa, and its gene maps to chromosome I. Inspection of spTFIIIE α revealed that it shares 19% identity and 45% similarity with human TFIIIE α (hTFIIIE α) and 26% identity and 50% similarity with *S. cerevisiae* TFIIIE α (scTFIIIE α) over the whole sequence, respectively (Fig. 1A) (Ohkuma *et al.* 1991; Feaver *et al.* 1994). The overall levels of sequence conservation were even higher for three N-terminal regions (Forkhead, ZF, and Hydrophobic) that are essential for transcription activity and are well conserved among eukaryotes and found even in archaea, and for a second acidic region near the C-terminus, which directly binds TFIIH. The spTFIIIE β cDNA, about 1 kb in length, encodes a highly basic 285-amino acid protein (pI 9.5) with a calculated molecular weight of 32.2 kDa, and its gene maps to chromosome III. The spTFIIIE β protein shares 27% identity and 52% similarity with hTFIIIE β and 30% identity and 49% similarity with scTFIIIE β , respectively (Fig. 1B) (Sumimoto *et al.* 1991; Feaver *et al.* 1994). In contrast to spTFIIIE α , the entire regions of spTFIIIE β had higher homology over the whole sequence except for two regions in hTFIIIE β , one between N-ter and Forkhead and the other between bHLH and bHL, and one region in scTFIIIE β between N-ter and Forkhead.

Recombinant *S. pombe* TFIIIE subunits are identical to the native form

To confirm that we had isolated *bona fide* spTFIIIE cDNAs, spTFIIIE α and spTFIIIE β were expressed independently in *Escherichia coli* with an N-terminal six histidine-tag (6H-spTFIIIE α and 6H-spTFIIIE β) and purified on Ni-NTA agarose columns (Fig. 1C, lanes 1 and 2). 6H-spTFIIIE α proved to have low solubility (less than 10%), and it was therefore first solubilized in 4 M guanidine before passage through Ni-NTA agarose. However, both subunits were soluble when they were co-expressed in *E. coli* as a polycistronic construct which encodes an N-terminally His-tagged spTFIIIE α subunit (Fig. 1C, lane 3). Native spTFIIIE subunits were detected in *S. pombe* nuclear extracts on Western blots using specific antibodies (Fig. 1D). The calculated molecular weight of the band detected with the anti-spTFIIIE α antibody was 56 kDa and that detected with the anti-spTFIIIE β antibody was 34 kDa (Fig. 1D, lanes 1 and 2), both of which match well the estimated molecular weights of the recombinant spTFIIIE α and spTFIIIE β bands determined on the SDS-PAGE gel shown in Fig. 1C.

The spTFIIIE genes are essential in *S. pombe*

The genes for the two spTFIIIE subunits (spTF2EA and spTF2EB) were isolated by PCR and disrupted by replacing each open reading frame (ORF) with the *URA4* gene, as shown in Fig. 2A. We constructed diploid strains (FKH10 and FKH11) carrying a disrupted copy of each spTFIIIE gene as described in Experimental procedures. Replacement of each ORF by the *URA4* sequence was confirmed by Southern blotting with [³²P]-labeled probes as indicated in Fig. 2A, which clearly demonstrated that the disrupted strains were heterozygous mutants, spTF2EA/spTF2ea::URA4 and spTF2EB/spTF2eb::URA4 (Fig. 2B). These FKH10 and FKH11 cells were sporulated and subjected to tetrad analysis (Fig. 2C). Of nine tetrads dissected for each subunit gene disruption, all contained two viable and two inviable spores. Moreover, all of the viable spore colonies were *ura4*⁻, indicating that spTF2e::URA4 spores were inviable. These results demonstrate that both spTFIIIE genes, like their *S. cerevisiae* counterparts (Feaver *et al.* 1994), are essential for cell viability.

spTFIIIE localizes to promoter regions *in vivo*

To examine the *in vivo* distribution of spTFIIIE on PolII-transcribed genes we carried out chromatin immunoprecipitation (ChIP) assays after cross-linking nuclear proteins to DNA using formaldehyde (Komarnitsky *et al.* 2000). Two genes, *adh1* and *tef3*, which encode alcohol dehydrogenase and translation elongation factor 3, respectively, were chosen since they possess a TATA box and are strongly and constitutively transcribed. As shown in Fig. 3B, four regions were monitored for each gene and relative amounts of PCR products were determined. Although the second largest spRpb2 subunit of spPol II was almost equally distributed in all regions, both spTFIIIE subunits were observed predominantly at the promoter and promoter-proximal region of both *adh1* and *tef3*, as also seen for spTBP (Fig. 3A). These results confirm functional data from human and *S. cerevisiae* indicating that TFIIIE is involved in two sequential stages, transcription initiation and the transition from initiation to elongation.

Mutations in the C-terminal essential residues of the spTFIIIE β subunit confer cold-sensitivity

In a previous study of the C-terminal point mutations affecting hTFIIIE β , we identified residues essential for binding to PolII, to the general transcription factors TFIIIB, hTFIIIE α , and hTFIIIF β , and to ssDNA in both

the bHLH and bHL regions, which are essential for transcription initiation (Watanabe *et al.* 2003). We also showed that the C-terminal helix of the bHL region is involved in the transition from transcription initiation to elongation. To assess the biological relevance of these residues, we created point mutations affecting the corresponding residues of spTFIIE β and determined their effects on cell viability by over-expressing the mutated spTFIIE β subunits (Fig. 4). Since all the mutants possess the hexa-histidine tag at the N-terminus, we confirmed that the expression level of each mutant at 30 °C was almost the same by Western blotting detecting with anti-histidine tag antibody (Qiagen) (data not shown). The residues mutated in the C-terminal bHLH and bHL regions are summarized in Fig. 4A. Six mutants showed cold-sensitivity, one possesses mutation in the bHLH region (W218A) and five others possess mutations in the bHL region (K258E, K260E K261E, R265A R266A, R265E R266E, and Y284A) (Fig. 4B). The mutant W218A corresponds to the human mutant W220A, which confers defects in both hTFIIE α binding and transcription initiation (Watanabe *et al.* 2003). The mutants K258E and K260E K261E correspond to the human mutants R258E R259E and K260E K261E, respectively, which confer defects in binding to PolII, TFIIB, TFIIF β , and ssDNA as well as in transcription initiation. The mutants R265A R266A and R265E R266E correspond to the human mutants R268A R269A and R268E R269E, which confer only modest defects in transcription initiation and binding to PolII and TFIIB. However, hTFIIE β has an additional basic residue, K267, which does not exist in spTFIIE β , and therefore the effects of the spTFIIE β mutants R265A R266A and R265E R266E on transcription must be stronger than that of the human mutants R268A R269A and R268E R269E. The most notable mutant is Y280A, which corresponds to the human mutant Y284A and, in contrast to other mutants, all of which were associated with defects in transcription initiation, conferred a severe defect in the transition from transcription initiation to elongation (Watanabe *et al.* 2003). It is significant that none of the mutants tested were impaired at any defects at 37 °C (not shown), but six of them were cold sensitive despite their transcriptional defects, whether at initiation or in the transition to elongation.

spTFIIE forms a heterotetramer

Gel filtration analysis of both native and recombinant hTFIIE proteins indicated that hTFIIE forms an $\alpha_2\beta_2$ heterotetramer (Ohkuma *et al.* 1990; Peterson *et al.* 1991). It was thought that both subunits can bind themselves and each other but conclusive analyses had not

been performed. Here we studied the binding specificities of both subunits by Far Western blotting analysis (Fig. 5). For probes, [³⁵S]-labeled 6H-spTFIIE α and 6H-spTFIIE β were expressed in *E. coli* BL21(DE3) pLysS by the addition of [³⁵S]-methionine into the minimal essential media and purified as shown in Fig. 5A. The left panel of Fig. 5B shows that [³⁵S]-labeled 6H-spTFIIE α bound only to 6H-spTFIIE β . In contrast, [³⁵S]-labeled 6H-spTFIIE β bound to both the 6H-spTFIIE α and 6H-spTFIIE β subunits (Fig. 5B, right panel). Gel filtration analysis of recombinant spTFIIE protein was also carried out and the native molecular mass was around 180 kDa, similar to the case of human protein (data not shown). These results confirm that TFIIE forms a heterotetramer, and they also show that spTFIIE forms a heterotetramer in which the two α - β heterodimers are connected via a β - β interaction.

The spTFIIE β subunit binds to intact spPol II

As shown in Fig. 6A,B, the binding specificities of intact PolII with respect to general transcription factors were determined in a human system by using GST-pull down assays. These clearly demonstrated that hPol II binds to hTFIIE β , hTFIIF β (hRap30), and the XPB subunit of TFIIEH (Fig. 6A, upper panel, lanes 5 and 7; lower panel, lane 3). To determine whether spPol II also binds to spTFIIE β , Flag-tagged PolII was purified from nuclear extracts of a *S. pombe* JY741/*f-rpb3* cell line that express an N-terminally Flag-tagged spRpb3 through anti-Flag antibody (M2)-agarose (Sigma) (Kimura *et al.* 2002) (Fig. 6B). GST-pull down assays were carried out with purified Flag-PolII and GST-tagged spTFIIE subunits (Fig. 6C). Intact spPol II bound to spTFIIE β in crude bacterial lysate or in purified fractions (Fig. 6C, lanes 4 and 5, respectively), and it was also confirmed that intact spPol II cannot bind to spTFIIE α (Fig. 6C, lane 3).

spPol II subunits specifically bind to general transcription factors *in vitro*

To further describe interactions between spTFIIE and spPol II at the molecular level we examined the binding specificities of spTFIIE to spPol II subunits (Fig. 6D,E). Binding to the two larger subunits, spRpb1 and spRpb2, was monitored by Far Western blotting analysis with [³⁵S]-methionine labeled 6H-spTFIIE α and 6H-spTFIIE β (Fig. 6D). As a positive control, 6H-spTFIIE β was run in parallel with purified Flag-PolII. As shown, spTFIIE β bound to spRpb2 and weakly to spRpb1 (Fig. 6D, lane 3) and spTFIIE α bound weakly to both spRpb1 and spRpb2 (Fig. 6D, lane 1). GST-pull down assays were

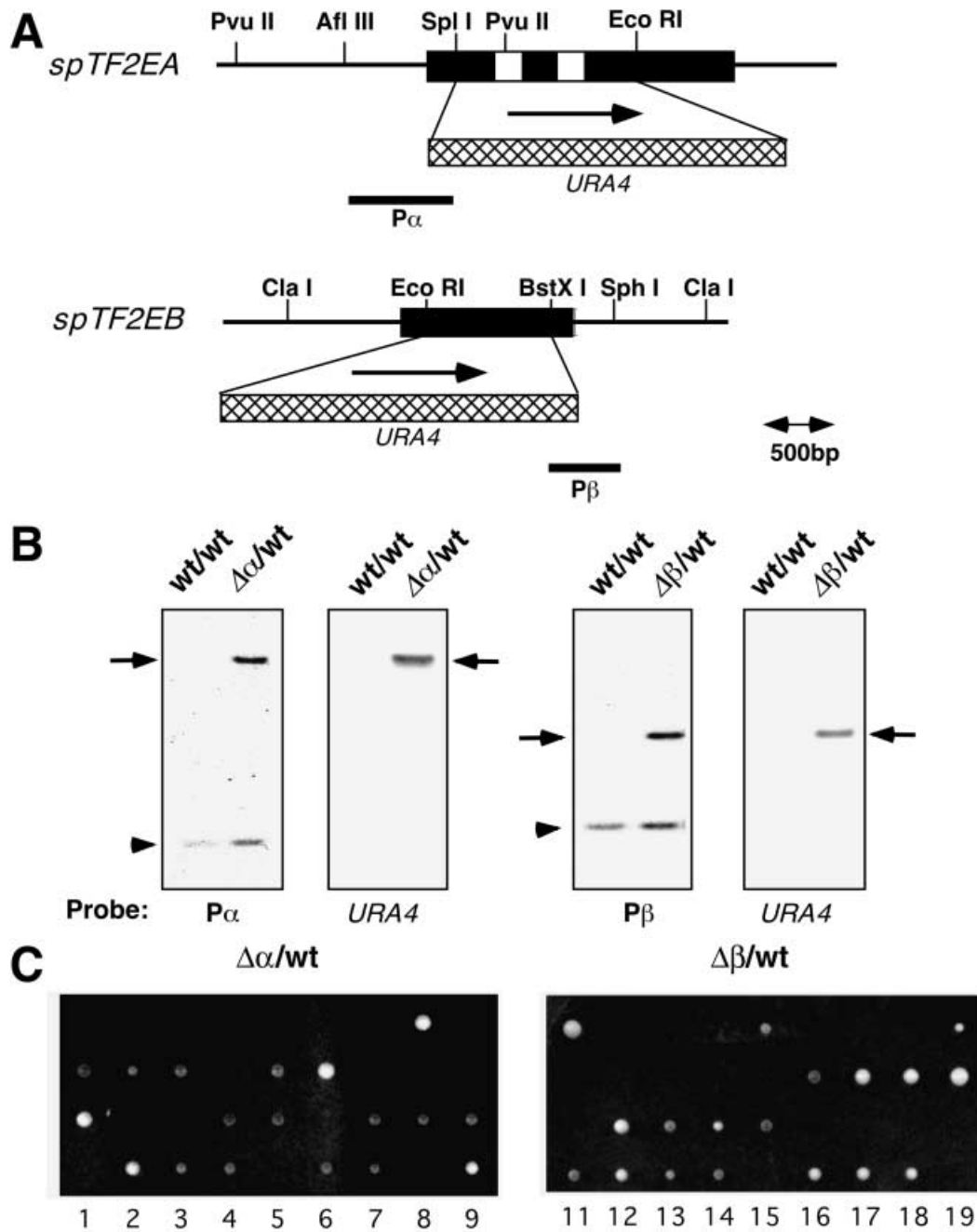


Figure 2 Tetrad analysis of *spTF2EA* and *spTF2EB* heterozygous mutants. (A) Replacement of the coding regions of the *spTF2EA* and *spTF2EB* genes with the *URA4* gene. The restriction map of each subunit gene is shown. The position of each gene is boxed and the exons are indicated by black boxes. The *URA4* gene is indicated by boxes with cross-hatching. The positions of gene-specific probes are indicated by the bars. (B) Confirmation of gene disruption. Chromosomal DNA was isolated from heterozygous mutant cells FKH10 ($\Delta\alpha$ /wt) and FKH11 ($\Delta\beta$ /wt) as well as wild-type cells (wt/wt), and digested with the restriction enzyme *Pvu*II to confirm *spTF2EA* disruption, and with *Cla*I to confirm *spTF2EB* disruption. As a control, the same treatments were performed with wild-type DNA. The digested DNA was subjected to Southern analysis with [³²P]-labeled probes (P_{α} and *URA4* for *spTF2EA*, and P_{β} and *URA4* for *spTF2EB*) and detected by autoradiography. Arrows indicate bands specific for the disrupted locus, and arrowheads indicate bands specific for the intact locus. (C) Tetrad results of both heterozygous mutants. The segregants from nine dissected asci were grown on YPD plates at 30 °C for 3 days and photographed.

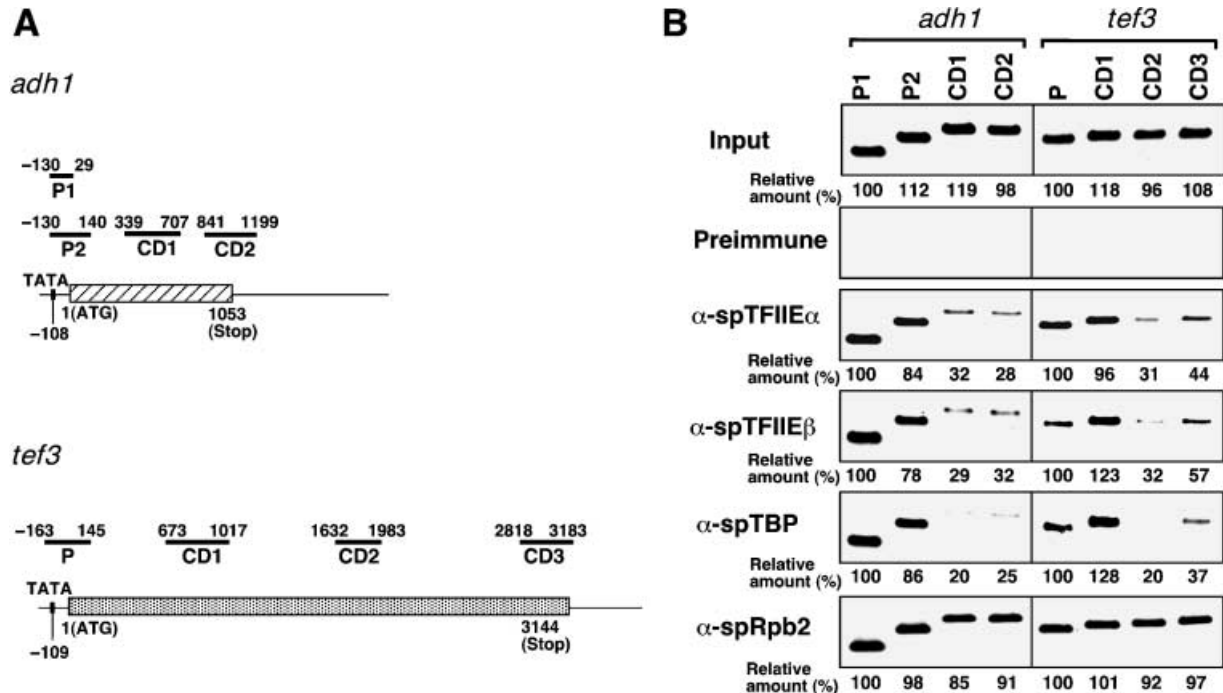


Figure 3 Localization of spTFIIE subunits on actively transcribed genes as determined by chromatin immunoprecipitation. (A) Schematic of the actively transcribed genes *adh1* and *tef3*. Open reading frames are represented by open boxes and the TATA boxes by black boxes. All numbers are relative to the first nucleotide of the initiation codon (+ 1). Bars above the two genes show the positions of the PCR products used in the ChIP analysis; the name of each PCR fragment is indicated. (B) ChIP assays. Samples were immunoprecipitated with rabbit polyclonal antisera specific to spTFIIE α , spTFIIE β , spTBP or the spRpb2 subunit of spPol II. Preimmune sera was used as a negative control. The relative amount of each PCR product was determined on agarose gels with a Fuji LAS-1000 lumino image analyzer (Fujifilm); the amount of PCR product specific to the promoter region (P1 for *adh1* and P for *tef3*) was normalized to 100%. Each lane of the agarose gel is labeled with the PCR product name indicated in (A). PCR products and combinations of primer oligonucleotides are as follows. *adh1*: P1, adh1pro-1T and adh1pro-0B; P2, adh1pro-1T and adh1pro-1B; CD1, adh1-2T and adh1-2B; CD2, adh1-3T and adh1-3B. *tef3*: P, tef3-1T and tef3-1B; CD1, tef3-2T and tef3-2B; CD2, tef3-3T and tef3-3B; CD3, tef3-4T and tef3-4B. These primer sequences are presented in Supplementary Table S2 at <http://www.blackwellpublishing.com/products/journals/suppmat/GTC/GTC833/GTC833.htm>.

carried out to detect interactions with the smaller spPol II subunits, spRpb3 to spRpb12, each of which was N-terminally tagged with GST (Fig. 6E). HA-tagged spTFIIB, spTFIIE α , spTFIIE β , and spTFIIF β were used for these binding assays. Since it was reported that hTFIIF β (hRap30) binds to the hRpb5 subunit and that *S. cerevisiae* (sc)TFIIB-related scPol III factor Brf binds to the scRpb4 subunit, we used spTFIIF β and spTFIIB as positive controls (Ferri *et al.* 2000; Wei *et al.* 2001). As a result, we confirm the binding specificities for the *S. pombe* counterparts. spTFIIF β also bound to spRpb5 and, additionally, to spRpb12 and weakly to spRpb3 and spRpb4 (Fig. 6E, bottom panel, lanes 5, 12, 3, and 4, respectively). spTFIIB bound to spRpb4 and, additionally, to spRpb12, and weakly to spRpb3 and spRpb11 (Fig. 6E, third panel from the top, lanes 4, 12, 3, and 11, respectively). When both subunits of spTFIIE were

analyzed, spTFIIE β was found to bind to spRpb12 in addition to spRpb2 (Fig. 6E, top panel, lane 12), and intriguingly, spTFIIE α was found to bind solely to spRpb5 (Fig. 6E, second panel from the top, lane 5). It is noteworthy that TFIIB and TFIIE α can bind to individually expressed PolII subunits but not to intact PolII (Fig. 6A, lanes 3 and 4 and Fig. 6C, lane 3).

Discussion

Rapid progress in biochemical and structural techniques has made it possible to observe PolII and many other transcription factors at the atomic level. Recently, the X-ray structures of several different forms of scPol II (the 10-subunit core structure and its elongation form, and the 12-subunit holo structure) and the cocrystal structure of the general transcription factor scTFIIB and

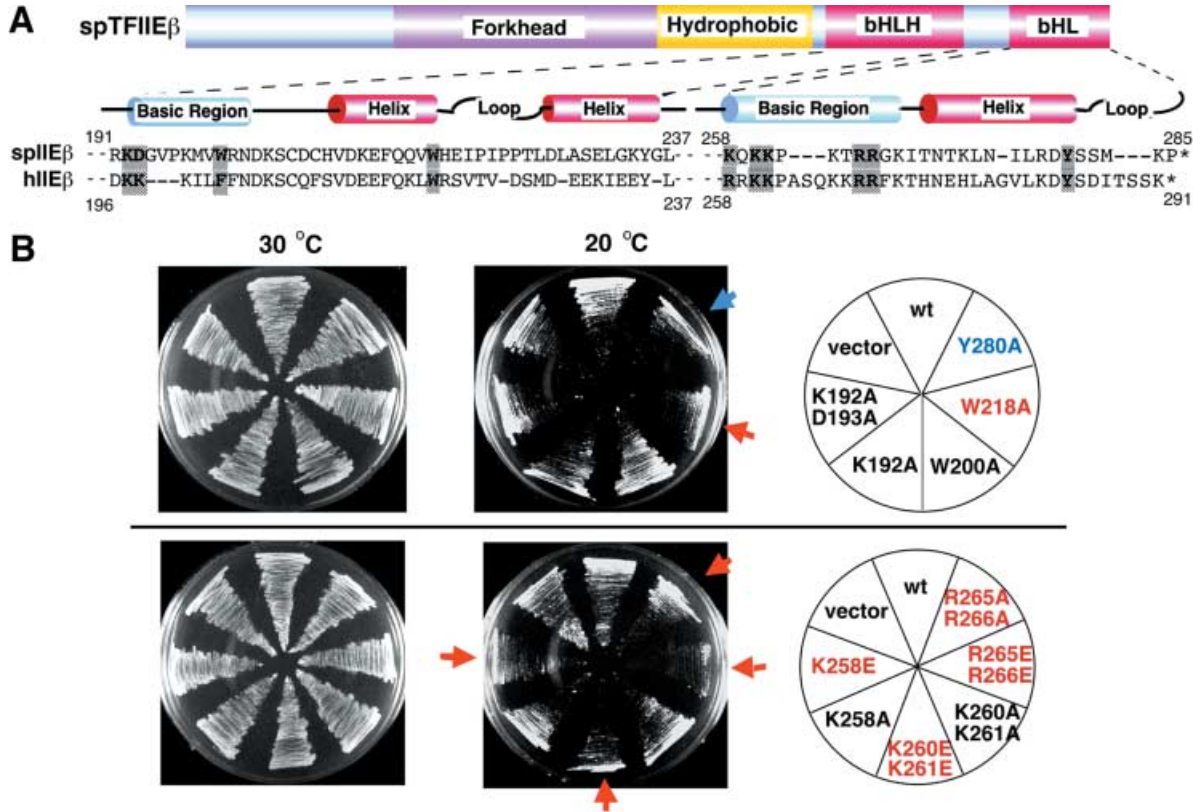


Figure 4 Effects of point mutations in the C-terminal bHLH and bHL regions of spTFIIIE β on cell viability. (A) Schematic of mutated residues in spTFIIIE β , indicated by shadowing. The hTFIIIE β sequence is aligned below. Residue numbers are provided above the spTFIIIE β sequence and below the hTFIIIE β sequence. The secondary structures of the C-terminal bHLH and bHL regions of spTFIIIE β are indicated above the sequence. (B) Growth characteristics of the point mutants. The positions of the mutants are indicated on the panels at right. The effects of point mutations on cell growth were determined by over-expression. The left panels show plates incubated under normal conditions (30 °C) and the middle panels show plates incubated under cold conditions (20 °C). Arrows indicate six mutants with growth defects. Five of them with defects at transcription initiation were indicated in red and one with a defect in the transition from initiation to elongation was indicated in cyan. Names of these mutants are designated in the same colors in the panels at right.

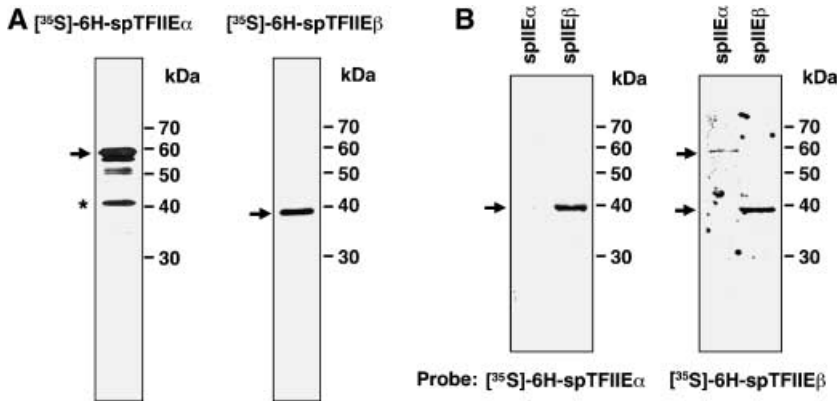


Figure 5 Subunit binding analysis of spTFIIIE. (A) [35 S]-labeled spTFIIIE subunits. After purification, labeled proteins were subjected to 12.5% SDS-PAGE, and bands were detected by autoradiography. Arrows indicate full length [35 S]-labeled spTFIIIE subunits. An asterisk indicates a major contaminant in the [35 S]-spTFIIIE α fraction. (B) Far Western blotting analysis of spTFIIIE subunits. Arrows indicate the positions of either spTFIIIE α (about 60 kDa) or spTFIIIE β (about 40 kDa). The positions of 10-kDa ladder marker proteins are indicated on the right side (in kDa).

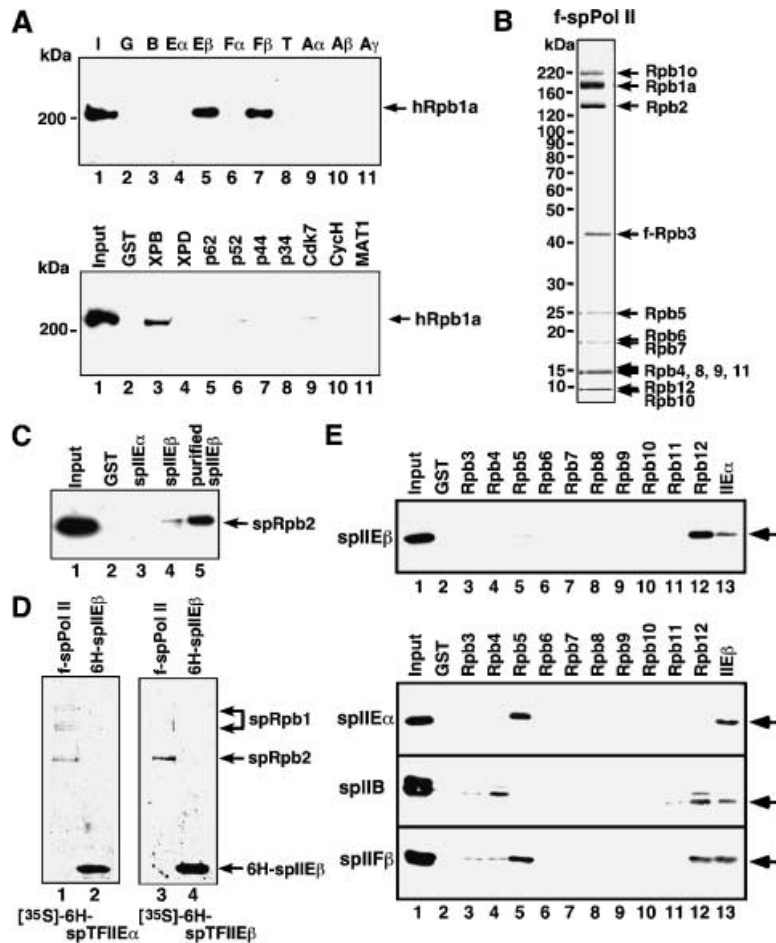


Figure 6 Binding assays of TFIIE to PolII. (A) GST pull-down assay of human general transcription factor subunits with intact human PolII. All of the GST-tagged human general transcription factors (300 ng each) except for the TAF subunits of TFIID were studied. The assay was carried out by addition of 1 μ g hPol II. After SDS-PAGE on a 5.5% polyacrylamide gel and Western blotting, bound hPol II was detected with an antibody to the CTD of the hRpb1 subunit. Upper panel: lane 1, 10% input of hPol II (I); lane 2, GST alone (G); lane 3, GST-TFIIB (B); lane 4, GST-TFIIE α (E α); lane 5, GST-TFIIE β (E β); lane 6, GST-TFIIF α (F α); lane 7, GST-TFIIF β (F β); lane 8, GST-TBP (T); lane 9, GST-TFIIA α (A α); lane 10, GST-TFIIA β (A β); and lane 11, GST-TFIIA γ (A γ). Lower panel: lane 1, 10% input of hPol II (I); lane 2, GST alone (GST); lane 3, GST-XPB (XPB); lane 4, GST-XPD (XPD); lane 5, GST-p62 (p62); lane 6, GST-p52 (p52); lane 7, GST-p44 (p44); lane 8, GST-p34 (p34); lane 9, GST-Cdk7 (Cdk7); lane 10, GST-CyclinH (CycH); and lane 11, GST-MAT1 (MAT1). The position of a 200-kDa molecular weight marker, myosin heavy chain, is indicated at left on each panel. Arrows indicate the position of the unphosphorylated form of the hRpb1 subunit of hPol II. (B) SDS-PAGE of purified spPol II. Flag-tagged spPol II (f-spPol II) was purified on an M2-agarose column from nuclear extracts of the *S. pombe* strain JY741/f-rpb3 (Kimura *et al.* 2002). Five hundred μ g of purified f-spPol II was subjected to SDS-PAGE and the gel was silver-stained. Arrows indicate the positions of spPol II subunits. The positions of 10-kDa ladder marker proteins are shown at left. (C) GST-pull down assay of spTFIIE subunits with spPol II. Bacterial lysates containing 300 ng GST-tagged spTFIIE subunit or 300 ng purified GST-spTFIIE β was mixed with f-spPol II and precipitated with glutathione-Sepharose. Bound spPol II was detected by Western blotting with a rabbit antibody against the spRpb2 subunit. Lane 1, 10% input (Input); lane 2, GST alone (GST); lane 3, GST-spTFIIE α (spIIE α); lane 4, GST-spTFIIE β (spIIE β); and lane 5, purified GST-spTFIIE α (purified spIIE α). (D) Far Western blotting analysis of binding of spTFIIE subunits to the two largest subunits of spPol II. Arrows indicate the positions of spRpb2 and 6H-spTFIIE β . (E) GST-pull down assay of spPol II subunits. Bacterial lysates containing 300 ng GST-tagged spPol II subunit (spRpb3-spRpb12) were mixed with HA-tagged general transcription factor and precipitated with glutathione-Sepharose. Bound factor was detected by Western blotting with a mouse anti-HA monoclonal antibody 12CA5. Top panel, HA-spTFIIE β (spIIE β); second panel, HA-spTFIIE α (spIIE α); third panel, HA-spTFIIB (spIIB); bottom panel, HA-spTFIIF β (spIIF β). Lane 1, 10% input (Input); lane 2, GST alone (GST); lanes 3-12, GST-spRpb3 to GST-spRpb12, respectively (Rpb3-Rpb12); lane 13, GST-spTFIIE α (IIE α) for top panel, and GST-spTFIIE β (IIE β) for second, third and bottom panels. Arrows indicate bound input proteins.

10-subunit core scPol II were reported (Cramer *et al.* 2001; Gnatt *et al.* 2001; Amarche *et al.* 2003; Bushnell & Kornberg 2003; Bushnell *et al.* 2004). Thus, it is now known how the active center of PolII is guided by the N-terminal half of TFIIB to the transcription initiation site and how the clamp module of PolII closes when transcription commences. However, we still do not know how PolII changes in conformation when it binds to TFIIF before interacting with the PIC or when transcription is initiated by the addition of TFIIE and TFIIF to the PIC. In the present study, we isolated the spTFIIE genes and cDNA, and confirmed previous *S. cerevisiae* data that TFIIE is essential for cell viability (Feaver *et al.* 1994). To further elucidate the TFIIE functions both biochemically and genetically, we carried out chromatin immunoprecipitation (ChIP) assays and point mutation studies to examine the effects on cell viability of mutations affecting the C-terminal bHLH and bHL regions which correspond to point mutations in hTFIIE β that confer defects in transcription, either at initiation or at the transition from initiation to elongation (Watanabe *et al.* 2003). We also biochemically analyzed the TFIIE subunits by determining their binding specificities, both to themselves as well as to spPol II subunits.

spTFIIE forms a preinitiation complex on the promoters of transcriptionally active genes

Each spTFIIE subunit had several highly conserved regions when compared with human and *S. cerevisiae*, and both were essential for cell viability (Figs 1 and 2). It is intriguing that both subunits possess the forkhead domain but only that in TFIIE β showed a dsDNA binding activity. The situation is similar for TFIIF since both subunits of TFIIF (TFIIF α and β) possess the forkhead domain but only that of TFIIF β shows a dsDNA binding activity. The functions of the forkhead domain other than DNA binding might be quite important because this region is essential for transcription. As written in the paper which reported the crystallographic structure of the forkhead domain of TFA, the spTFIIE α homolog in archaea, this domain might be essential for the protein-protein interactions (Amarche *et al.* 2003). We therefore examined, for the first time, whether both subunits were in the PIC *in vivo* by using the ChIP assay (Fig. 3). As a result, they were actually co-localized with spTBP but their localization profiles were completely different from spPol II. The previous paper reported that scTFIIE β located predominantly at the promoter in the budding yeast (Komarnitsky *et al.* 2000). Our data confirmed their result that spTFIIE β located at the promoter proximal regions with spTBP and, furthermore, demonstrated for

the first time that the spTFIIE α subunit also had overlapped localization with spTFIIE β and spTBP. Taken together with the tetrad result for cell viability, we conclude that spTFIIE is actually functional in *S. pombe*.

Cold sensitive growth correlated with transcriptional defects of spTFIIE β mutants

We recently identified the transcriptionally essential residues in the C-terminal bHLH and bHL regions of hTFIIE β , classified them in which transcriptional step they were involved, and found that the evolutionally conserved residues at the C-terminal helix region of bHL were essential for the transition from initiation to elongation and the rest of transcriptionally essential residues were basically involved in transcription initiation (Watanabe *et al.* 2003). To study the biological relevance of those residues, we mutated the residues in spTFIIE β corresponding to the transcriptionally defective residues in hTFIIE β and observed their phenotypes by over-expression (Fig. 4). Intriguingly, all defective mutants (W218A, K258E, K260E K261E, R265E R266E, R265A R266A, and Y280A) in the C-terminus of spTFIIE β showed cold sensitivities and their phenotypes were similar to the phenotypes of the previously reported C-terminally truncated mutants of scTFIIE α which may fail to bind properly to scTFIIF (Kuldell & Buratowski 1997). A common feature of those mutants is that all those are transcriptionally active to some extent (more than 25% of the wild-type) (Ohkuma *et al.* 1995; Watanabe *et al.* 2003). This is in clear contrast to the features of the zinc finger mutants which showed lethal or temperature sensitive phenotypes in *S. cerevisiae* and possessed much lower transcription activities (less than 20% of the wild-type) in human by using human *in vitro* transcription system (Kuldell & Buratowski 1997; A. Tanaka & Y. Ohkuma, unpublished observation).

TFIIE is a heterotetramer

As expected from the gel filtration column profiles of both native and recombinant hTFIIE proteins (Ohkuma *et al.* 1990; Peterson *et al.* 1991), our Far Western studies demonstrated that spTFIIE formed a heterotetramer *in vitro* by interactions between α and β , and β and β subunits (Fig. 5). Judging from the protein-DNA photocrosslinking data of the PIC components and the promoter DNA, both subunits of TFIIE bound to the wide range of promoter regions from -50 to +35 (Douziech *et al.* 2000; Forget *et al.* 2004) and, thus, this factor might be big enough to reach all sites. Our data that TFIIE is a heterotetramer with the molecular weight of 180 kDa

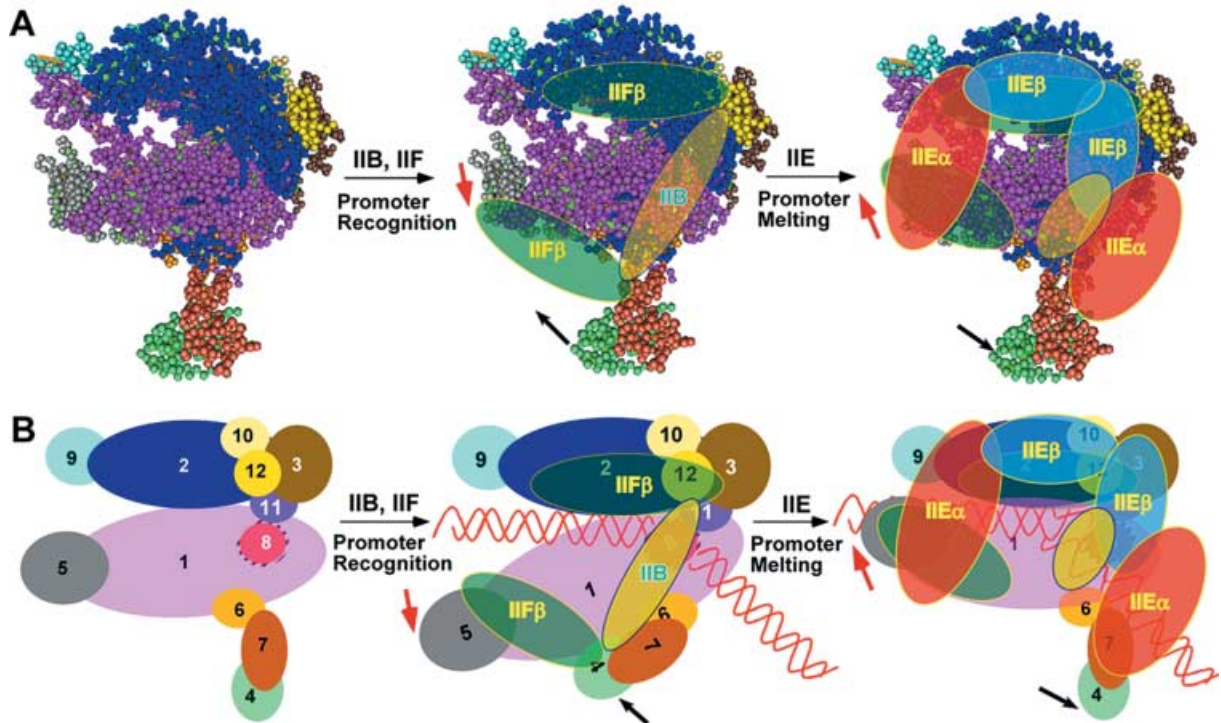


Figure 7 Schematic models of PolII conformational changes upon the binding of general transcription factors. (A) A model of the space-filled structure of the whole PolII (PDB accession number: 1NT9) from Cn3D. According to the factor addition, the estimated bound profiles of each factor were drawn. Red arrows indicate the movement of the clamp module and black arrows indicate the movement of the Rpb4-Rpb7 module. (B) Schematic model of PolII conformational changes upon binding of promoter DNA and general transcription factors (TFIIB, TFIIF β , and TFIIE). The positions of PolII subunits were drawn based on the PolII structures presented in (A). Red arrows indicate the movement of the clamp module and black arrows indicate the movement of the Rpb4-Rpb7 module as shown in (A).

and binds to all of the general transcription factors as well as PolII support these results and speculations (Ohkuma *et al.* 1990; Yamamoto *et al.* 2001).

PolII might change its conformation several times upon binding to various general transcription factors before transcription initiation

As shown in Fig. 6A,B, intact (no activation) hPol II bound predominantly to hTFIIE β , hTFIIF β and the XPB subunit of hTFIIH. The specific binding of PolII to TFIIE β was also confirmed in *S. pombe* (Fig. 6D). When the binding of the spTFIIE subunits to the individual spPol II subunit was tested, spTFIIE β mainly bound to spRpb2 and spRpb12 and quite faintly to spRpb1. The binding of spTFIIF β to spRpb5 was also confirmed but, additionally, the binding to spRpb12 was observed (Wei *et al.* 2001). The intriguing observations were that spTFIIE α , though no binding was observed with intact spPol II as shown, bound well to spRpb5 and faintly

to spRpb1 and spRpb2 as well and that spTFIIB, as suggested previously, bound to spRpb4 and was newly found to bind to spRpb12, in addition (Ferri *et al.* 2000). These results of spTFIIE α and spTFIIB that they bound to the individual spPol II subunits but could not bind to intact spPol II (Fig. 6C and data not shown) immediately indicate a strong possibility of the intramolecular conformational changes of PolII upon binding of the general transcription factors. From those results together with the reported X-ray cocystal structure of PolII-TFIIB in *S. cerevisiae*, spTFIIB becomes available to bind to spPol II when spPol II possibly changes its conformation by prebinding to spTFIIF (Bushnell *et al.* 2004). However, the opposite possibility may also be true since it was reported that spTFIIB changed its conformation to the open form upon binding to the transcriptional activator VP16, and possibly (we presume) upon formation of PIC with spTBP on the promoter (Glossop *et al.* 2004). This TFIIB open form is exactly the conformation of scTFIIB whose N-terminal half is inserted into the

active center of scPol II in the cocrystal with scPol II (Bushnell *et al.* 2004).

Figure 7 represents our models of PolII conformational changes during PIC formation based on our results in addition to the reported structures of PolII and the general transcription factors under various conditions (Gnatt *et al.* 2001; Amarche *et al.* 2003; Chung *et al.* 2003; Bushnell *et al.* 2004). In Fig. 7A, the general transcription factor binding steps and accompanying PolII conformational changes are drawn step-wise (left, middle and right panels) on the space fill model of scPol II according to the order of PIC formation. The corresponding schematic models with conformational changes on the promoter DNA are shown in Fig. 7B. As described above, changes in PolII are first provoked by binding of TFIIF and TFIIB to PolII. TFIIF β will stimulate the clamp module to open by binding to the Rpb2 and Rpb5 subunits (corresponding to the clamp module) of PolII, and the latter binding will cause the kink at the joint of the Rpb4–Rpb7 module inward to the PolII clamp module as shown with a black arrow (Fig. 7A,B, middle panel) since the counterpart of TFIIF β , TFIIF α , was demonstrated to bind extensively to the Rpb4–Rpb7 module (Chung *et al.* 2003). As a result, TFIIB and DNA can easily bind PolII; TFIIB binds to the PolII dock module at its N-terminal Zn ribbon motif and to the PolII cleft at its B finger region, maneuvering the promoter DNA around the transcription initiation site to the active center of PolII (Bushnell *et al.* 2004). TFIIB also binds to TBP and to the TATA box proximal region at its C-terminal half. Consequently, TFIIB shrinks in size on the surface of PolII because its N-terminal half is inserted into the active center and only the C-terminal half remains near the dock domain via interactions with the Rpb4–Rpb7 module. Therefore, TFIIB is drawn smaller in the panels at right of Fig. 7A,B than in the middle panels. As shown in the right panels of both Fig. 7A,B, the next PolII conformational change might be caused by the binding of TFIIE. Since TFIIE β can bind to intact PolII, two TFIIE β subunits will bind to the Rpb2 and Rpb12 subunits, without assistance from other factors, and at the same time to TBP, TFIIB, and TFIIF β as well as the promoter just upstream of the transcription initiation site. The PolII conformational changes induced by TFIIB and TFIIF will make TFIIE α accessible to PolII at the Rpb5 subunit, which positions the edge of the clamp module at the time when TFIIE β binds to the Rpb2 and Rpb12 subunits. The closing of the clamp module of PolII upon transcription initiation will be triggered by TFIIE α , since it binds to the clamp and bridges the clamp and jaw modules of PolII in a complex with TFIIE β , which binds to Rpb2 to form the jaw module of PolII.

This model also fits protein–DNA photo-crosslinking results which show that TFIIE α binds both upstream and downstream of the core promoter region and that TFIIE β binds just inside of the promoter region at approximately three regions, a region around the TATA box, a region thought to melt upon transcription initiation, and a region downstream of the initiation site (Douziech *et al.* 2000). This second PolII conformational change also enables TFIIE α to bind to TFIIE β near the PolII clamp module and to approach the CTD of Rpb1 for its phosphorylation at transcription initiation. Since the holo scPol II structure has been determined by X-ray crystallography and its cocrystal structures with other components have started to be determined (Amarche *et al.* 2003; Bushnell & Kornberg 2003; Kettenberger *et al.* 2003; Bushnell *et al.* 2004), the dynamic conformational changes in PolII during transcription will be elucidated in the near future. A further understanding of the effects of TFIIE binding to PolII on transcription from both structural and genetical viewpoints is now in progress.

Experimental procedures

Yeast strains, media and transformation

The *S. pombe* strains used in this study are shown in Supplementary Table S1 at <http://www.blackwellpublishing.com/products/journals/suppmat/GTC/GTC833/GTC833.htm>. NP1–6A and NP1–6D are parental strains. YE, EMM and ME media were prepared as previously described (Moreno *et al.* 1991). *S. pombe* was transformed by the lithium acetate method (Okazaki *et al.* 1990).

Isolation of *S. pombe* TFIIE cDNA clones

The putative spTFIIE α coding sequence was identified in a TBLASTN homology search of the *S. pombe* translated expressed sequence tag (EST) databank (Sanger Centre, Cambridge, UK) to locate regions with significant homology to the hTFIIE α amino acid sequence. For amplification of the full coding region of spTFIIE α cDNA, the primer SPEAR6T, which has an *Nde*I site at the initiation codon, was used in conjunction with the primer SPEAR2B, which has a *Bam*HI site behind the termination codon (see Supplementary Table S1 at <http://www.blackwellpublishing.com/products/journals/suppmat/GTC/GTC833/GTC833.htm>). PCR was performed using a *S. pombe* cDNA library as a template and a combination of the oligo cap-specific primer, Adaptor primer 1 (5′-CCATCCTAATACGACTCAC-TATAGGGC-3′; Clontech), and the primer SPEAR2B

with Advantage cDNA polymerase (Clontech). A *S. pombe* cDNA library was prepared with total mRNA from TP4-5 A and each cDNAs have the oligo capping at the top of themselves. The spTFIIE α cDNA was obtained using the first PCR product and the primers SPEAR6T and SPEAR2B with *Pyrobest* DNA polymerase (Takara). The PCR products were subcloned into the *Sma*I site of pBluescript II SK(-) (Stratagene). The nucleotide sequences of the cloned PCR products were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

The same strategy was used to identify several regions with high homology to the hTFIIE β amino acid sequence. Since the extent of homology was higher than the case of TFIIE α and the putative N and C termini were identified, it was easier to identify the entire putative coding region of spTFIIE β from *S. pombe* genomic sequences. The oligonucleotide SPEB1T (5'-GATTCCATATGAGTTCACTAAGCGATC-3') contains an *Nde*I site (underlined) at the first methionine codon, and the oligonucleotide SPEB3B (5'-CGTTGACTCGAGAGGTTTCATGGAGCTATAATCACG-3') contains an *Xho*I site (underlined) after the stop codon. The PCR products were subcloned into the *Sma*I site of pBluescript II SK(-) (Stratagene). The nucleotide sequences of the cloned PCR products were confirmed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Construction of spTFIIE expression vectors

The plasmid containing the spTFIIE α cDNA was digested with *Nde*I and *Bam*HI, and the relevant fragment was subcloned into the vectors 6HisT-pET11d and HA-pET11d to construct plasmids expressing six histidine-tagged spTFIIE α (6H-spTFIIE α) and hemagglutinin-tagged spTFIIE α (HA-spTFIIE α), respectively (Hoffmann & Roeder 1991; Okamoto *et al.* 1998). Similarly, the plasmid containing spTFIIE β cDNA was digested with *Nde*I and *Xho*I, and the relevant fragment was subcloned into the vectors 6HisT-pET11d and HA-pET11d to construct the expression plasmids 6H-spTFIIE β and HA-spTFIIE β , respectively.

A co-expression plasmid containing two spTFIIE subunit cDNAs was constructed essentially as previously described (Yamamoto *et al.* 2001). The spTFIIE β cDNA fragment was isolated by digestion with *Nde*I and *Xho*I and used to construct an untagged spTFIIE β -expressing plasmid (in pET3a). This plasmid was digested with *Xba*I and blunt-ended, and the resulting fragment was treated with calf intestine phosphatase. Finally, the 6H-spTFIIE α expression plasmid was digested with *Xba*I and *Bam*HI, and the ends of the fragment containing spTFIIE α

cDNA were blunt-ended. Finally, this 6H-spTFIIE α cDNA fragment was subcloned into the *Xba*I (blunt) sites of the spTFIIE β expression plasmid such that both cDNAs were tandemly oriented to create the expression plasmid 6H-spTFIIE.

Glutathione S-transferase (GST) fusion constructs of both spTFIIE subunits were made in pGEX-2TL(+) as previously described by digestion with the same combinations of restriction enzymes (*Nde*I-*Bam*HI for spTFIIE α and *Nde*I-*Xho*I for spTFIIE β) (Okamoto *et al.* 1998). Fusions of GST constructs to spPol II subunits (spRpb3-spRpb12) were made as follows. The coding region of each subunit was excised from 6His-tagged subunit constructs in pET21d with appropriate restriction enzymes (*Nde*I-*Xho*I for 6H-spRpb3 and *Nde*I-*Bam*HI for all others) and subcloned into pGEX-2TL(+). All subunits were GST-tagged at the N-terminus except that spRpb3 contains a 6His-tag immediately after the GST-tag at the N-terminus.

Expression and purification of recombinant proteins

Recombinant proteins were expressed in *E. coli* BL21(DE3)pLysS by induction with isopropyl- β -D-thiogalactopyranoside (IPTG) (Okamoto *et al.* 1998). Soluble bacterial lysates were used for general purification. For miniscale preparations, lysates (1 mL) representing 50–100 mL culture were mixed directly with 1 mL buffer B (20 mM Tris-HCl (pH 7.9 at 4 °C), 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μ g/mL anti-pain, 2 μ g/mL aprotinin, 1 μ g/mL leupeptin, 0.8 μ g/mL pepstatin, 10 mM 2-mercaptoethanol) containing 500 mM NaCl (BB500), and 100 μ L Ni²⁺-nitrilotriacetic acid (NTA) agarose (Qiagen) and incubated for 4 h at 4 °C. The resin samples were washed twice with 1 mL BB500, twice with 1 mL buffer D (20 mM Tris-HCl (pH 7.9 at 4 °C), 20% (v/v) glycerol, 1 mM PMSF, 10 mM 2-mercaptoethanol) containing 500 mM KCl (BD500), and twice with 500 μ L BD500 containing 40 mM imidazole-HCl (pH 7.9). Bound proteins were eluted twice with 300 μ L BD500 containing 100 mM imidazole-HCl (pH 7.9). Typical preparations were > 80% pure as judged by Coomassie Brilliant Blue staining of a sodium dodecyl sulfate (SDS)-polyacrylamide gel. For purification of 6H-spTFIIE α , the protein was first precipitated with 33% (v/v) saturated ammonium sulfate in 20 mM Tris-HCl (pH 7.9 at 4 °C) by ultracentrifugation at 20 000 *g* for 20 min at 4 °C using the 45 Ti rotor (Coulter-Beckman), resuspended with BB500 containing 10 mM imidazole-HCl (pH 7.9), and purified with Ni²⁺-NTA agarose as described above.

HA-tagged and GST fusion derivatives of spTFIIE subunits were expressed in *E. coli* BL21(DE3)pLysS by induction with IPTG. Cells were harvested from 50 mL culture, resuspended in 1 mL BB500, and sonicated. Soluble lysates were separated from insoluble debris by ultracentrifugation at 20 000 g for 20 min at 4 °C using the 50.2 Ti rotor (Coulter-Beckman) and stored at -80 °C until use for the GST pull-down assay. For purification of GST fusion proteins, soluble lysates were mixed with glutathione-Sepharose resin (Amersham Pharmacia) equilibrated with buffer C (20 mM Tris-HCl (pH 7.9 at 4 °C), 20% (v/v) glycerol, 0.5 mM EDTA, 1 mM PMSF, 10 mM 2-mercaptoethanol) containing 500 mM KCl (BC500) and then eluted with BC500 containing 20 mM glutathione.

Generation of antibodies against spTFIIE subunits

Both 6H-spTFIIE subunits were expressed independently in *E. coli*, solubilized by sonication and purified on a Ni-NTA agarose column. Since 6H-spTFIIE β was mostly soluble (> 80% in soluble lysate) and 6H-spTFIIE α was mostly insoluble (> 90% in pellet), 6H-spTFIIE β was purified from bacterial lysates and 6H-spTFIIE α from bacterial pellets after solubilization with 4 M guanidine-HCl (pH 7.5). Two mg of each purified protein was subjected to SDS-PAGE and the appropriate bands were excised from the gel after Coomassie Blue staining.

To raise rabbit polyclonal antibodies against spTFIIE subunits, 200 μ g of each 6H-TFIIE subunit was mixed with complete Freund's adjuvant (Difco) and injected intramuscularly into a separate rabbit. Two weeks after the first injection, a second injection of 100 μ g of each 6H-ceTFIIE subunit mixed with incomplete Freund's adjuvant (Difco) was given both intramuscularly and subcutaneously. The third and fourth injections, identical to the second, were given after a further two weeks. Blood was collected 8 days after the fourth injection. Each raised antibody recognized its corresponding spTFIIE subunit, whether natural or recombinant, in solution and on Western blots.

Preparation of yeast nuclei

S. pombe strain NP1-6A was grown to OD₆₀₀ 1.0 in YEA medium. The cells were harvested and the pellet was washed twice with chilled Sorbitol buffer (1.4 M sorbitol, 40 mM HEPES (pH 7.5), 0.5 mM MgCl₂) containing 1 mM PMSF and 10 mM β -ME by centrifugation at 5000 r.p.m. for 5 min at 4 °C with a HB4 swinging bucket rotor. The pellet was resuspended in a 4-fold volume of Sorbitol buffer containing 1 mM PMSF and

2 mM β -ME and lysed with zymolyase 100T (Seikagaku Kogyo). Spheroblasts were collected by centrifugation, washed twice with chilled sorbitol buffer containing 1 mM PMSF and suspended in Ficoll buffer (18% Ficoll 400, 20 mM PIPES (pH 6.5), 0.5 mM MgCl₂). The suspension was homogenized with a glass pestle, laid over Glycerol-Ficoll buffer (7% Ficoll 400, 20% (v/v) glycerol, 20 mM PIPES (pH 6.5), 0.5 mM MgCl₂ containing 1 mM PMSF) and centrifuged at 24 000 g for 30 min at 4 °C using a SW 28 swinging bucket rotor (Coulter-Beckman). The pellet was resuspended in Ficoll buffer and the supernatant was collected by centrifugation as above. The supernatant was washed with Ficoll buffer and the nuclear pellet was prepared.

Disruption of the spTFIIE genes

A 2.8 kb genomic DNA fragment containing the *spTFIIE α* gene (*spTF2EA*) from position -632 to +2196 (where +1 is the first nucleotide of the translation start codon) was amplified with *Pyrobest* DNA polymerase (Takara) and primers SPEAG1 and SPEAG2 (see Supplementary Table S2 at <http://www.blackwellpublishing.com/products/journals/suppmat/GTC/GTC833/GTC833.htm>) with *S. pombe* genomic DNA as a template. After treatment with T4 polynucleotide kinase (Roche), the fragment was subcloned into pBluescript II SK (-) at the *Sma*I site to construct pBS-*spTF2EA*. For disruption of *spTF2EA*, pBS-*spTF2EA* was digested with *Sp*II and *Eco*RV, in which the entire coding region and the 1.7 kb *URA4* fragment (blunt) were included, to construct pBS-*spf2ea::URA4*. A 2.8 kb *spf2ea::URA4* fragment was produced by PCR using the same primer sets as above and pBS-*spf2ea::URA4* as the template. For disruption of *spTFIIE β* gene (*spTF2EB*), the same strategy was performed as above. A 2.3 kb genomic DNA segment including *spTF2EB* from positions -618 to +1705 was amplified using primers SPEBG1 and SPEBG2 (see Supplementary Table S2 at <http://www.blackwellpublishing.com/products/journals/suppmat/GTC/GTC833/GTC833.htm>) with *S. pombe* genomic DNA as a template, phosphorylated and subcloned into pBluescript II SK (-) at the *Sma*I site to construct pBS-*spTF2EB*. For disruption of *spTF2EB*, pBS-*spTF2EB* was digested with *Eco*RI and *Bst*XI and blunt-ended, and 1.7 kb blunt-ending *URA4* fragment was inserted to construct pBS-*spf2eb::URA4*. A 2.8 kb *spf2eb::URA4* fragment was produced by PCR by using the same primer sets as above and pBS-*spf2eb::URA4* as the template. To generate the *spTF2EA/spf2ea::URA4* (FKH10) and the *spTF2EB/spf2eb::URA4* (FKH11) diploid strains, these fragments were used to transform a diploid by mating NP1-6A

and NP1–6D. Disruption was confirmed by Southern blot analysis performed with genomic DNA extracted from the diploid transformants, FKH10 and FKH11. Genomic DNA from FKH10 was digested with *PvuII* and gene disruption was confirmed by hybridization with a [³²P]-labeled specific *AflIII*–*SpII* fragment (P α in Fig. 1B) and the *URA4* fragment as probes. Genomic DNA from FKH11 was digested with *Clal* and hybridized with a [³²P]-labeled *BstXI*–*SpHI* fragment (P β in Fig. 1B) and the *URA4* fragment as probes.

Tetrad analysis

The diploid strains *spTF2EA/spf2ea::URA4* (FKH10) and *spTF2EB/spf2eb::URA4* (FKH11) were grown on ME plates at 28 °C for 2 days and then streaked to a YPD plate and incubated at 30 °C for several days. The asci formed were isolated with a manipulator on a YE plate containing 100 μ g/mL adenine, spores were dissected, and the plate was incubated at 30 °C for 3 days. All viable spore colonies were found to be *ura4⁻* by replica plating to EMM2 plates containing uracil, adenine, leucine and histidine.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed essentially as described (Komarnitsky *et al.* 2000) with minor modifications. A 50 mL-volume of *S. pombe* strain NP1–6A was grown to an optical density of 0.9 at 600 nm in YEA medium. Formaldehyde was added to a final concentration of 1% for 30 min at room temperature, and incubation was continued with 120 mM glycine at room temperature for 5 min. Cells were harvested by centrifugation, washed twice with chilled Tris-buffered saline, and lysed with glass beads in lysis buffer (50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 1 mM PMSE, 1 μ g/mL leupeptine, 1 μ g/mL pepstatin A). Chromatin was treated by sonication to yield fragments ranging from 200 to 800 bp. This cell lysate was allowed to react with antibody at 4 °C for 12 h and was incubated with protein G-Sepharose 4 Fast Flow (Amersham Pharmacia). The immunoprecipitate-bound resins were collected by centrifugation and washed successively with lysis buffer containing 140, 250 and 500 mM NaCl. After washing with LiCl/detergent wash buffer (1 mM LiCl, 10 mM Tris-HCl (pH 7.9 at 4 °C), 1 mM EDTA, 0.5% Na-deoxycholate, 0.5% Nonidet P-40) and then TE buffer (pH 8.0), the resins were incubated with elution buffer (50 mM Tris-HCl (pH 7.9 at 4 °C), 10 mM EDTA, 1% SDS) at 65 °C for 10 min. ChIP fractions were collected by centrifugation, treated with protease, and

cross-linking was reversed. PCR was performed with 25 cycles using AmpliTaq Gold (Applied Biosystems). The relative amount of each PCR product was measured on an agarose gel with a Fuji LAS-1000 lumino image analyzer (Fujifilm), and normalized to the amount of PCR product of each precipitate at the promoter region, which was defined as 100%.

Preparation of spTFIIE β point mutants

Plasmids harboring mutated spTFIIE β cDNA were constructed with a site-directed mutagenesis system as previously described (Watanabe *et al.* 2003). A cDNA of full length spTFIIE β with hexa histidine-tag at the N-terminus (6H-spTFIIE β) was subcloned into the plasmid pREP1 (Maundrell 1990). A site-directed mutagenesis kit Mutan-K (Takara) was used to create various oligonucleotide-mediated point mutations with the plasmid pREP1–6H-spTFIIE β as a template (see Supplementary Table S2 at <http://www.blackwellpublishing.com/products/journals/suppmat/GTC/GTC833/GTC833.htm>). The mutants were then checked by sequencing as above. The mutant plasmids were transformed into the *S. pombe* strain TP4–5 A and cells over-expressing mutated spTFIIE β derivatives were constructed (see Supplementary Table S1 at <http://www.blackwellpublishing.com/products/journals/suppmat/GTC/GTC833/GTC833.htm>). The transformed strains were cultured in liquid EMM2 medium containing 100 μ g of uracil and adenine, and 2 μ M thiamine to 1×10^7 cells/mL at 30 °C and were streaked on thiamine-free EMM2 plates containing 100 μ g of uracil and adenine. Plates were incubated at 20 °C, 30 °C and 37 °C, and cold-sensitive mutants were identified after several days. The expression level of each mutated protein at 30 °C was checked by Western blotting of cell lysate with mouse anti-Penta-His monoclonal antibody (cat. no. 34660, Qiagen).

Labeling of spTFIIE subunits

Both 6H-spTFIIE α and 6H-spTFIIE β were [³⁵S]-labeled by IPTG-induction in *E. coli* BL21(DE3)pLysS in the presence of 0.15 mCi/mL [³⁵S]-methionine (Amersham Pharmacia) and 200 μ g/mL rifampicin. [³⁵S]-6H-spTFIIE β was purified through Ni²⁺-NTA agarose as described above. Because the recovery of [³⁵S]-6H-spTFIIE α by Ni²⁺-NTA affinity chromatography was low, the labeled protein was concentrated by 33% (v/v) saturated ammonium sulfate in 20 mM Tris-HCl (pH 7.9 at 4 °C) by ultracentrifugation at 20 000 g for 20 min at 4 °C using the 50.2 Ti rotor (Coulter-Beckman). The precipitate was resuspended in buffer B without KCl (BB0) and

adjusted to 100 mM KCl before use in Far Western blotting experiments.

Far Western blotting analysis

Proteins were electrotransferred from an SDS-polyacrylamide gel to a PVDF membrane (Immobilon P, Millipore) and denatured twice with 6 M guanidine-HCl in BC100 for 30 min at 4 °C, followed by successive 10-min treatments with 3.0, 1.5, 0.75, and 0.375 M guanidine-HCl in BC100. The membrane was washed twice with BC100 and treated with BC100 containing 5% skim milk for 2 h at 4 °C. The membrane was then soaked in BC100 containing 200 µg/mL BSA and [³⁵S]-labeled probe protein (6H-spTFIIE α or 6H-spTFIIE β) for 6 h at room temperature. The membrane was washed with BC100 and the bound [³⁵S]-labeled proteins were detected by autoradiography using Fuji RX-U X-ray film.

Purification of f-Pol II

This purification method was based on that previously described (Kimura *et al.* 2002). The strain JY741/f-rpb3 was cultured in YE medium containing 75 µg/mL of adenine and uracil at 30 °C. Cells were harvested at the exponential phase, suspended in four times the cell weight of buffer E (62.5 mM Tris-HCl (pH 7.9 at 4 °C), 12.5% (v/v) glycerol, 0.125 mM EDTA, 1.25 mM DTT, 0.625 mM PMSF, 2 µg/mL anti-pain, 2 µg/mL aprotinin, 1 µg/mL leupeptin, 0.8 µg/mL pepstatin) containing 125 mM (NH₄)₂SO₄ (pH 8.0) and disrupted with a French Press (Otake, Tokyo, Japan). The cell suspension was sonicated and centrifuged at 18 000 g for 20 min at 4 °C. The supernatant was diluted 4-fold with buffer F (50 mM Tris-HCl (pH 7.9 at 4 °C), 10% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) containing 100 mM (NH₄)₂SO₄ (pH 8.0) (BF100) and 0.1% poly-ethylenimine was added. After incubation for 1 h, the precipitate was collected by centrifugation at 18 000 g for 20 min and extracted by 2-fold cell weight of buffer F containing 200 mM (NH₄)₂SO₄ (pH 8.0) (BF200). After centrifugation at 18 000 g for 20 min, 20 microliters of M2-agarose (Sigma) equilibrated with BF200 was added and gently mixed for 2 h at 4 °C. The M2-agarose was washed four times with 1 mL of BF200, and FLAG-PolIII (f-PolII) was eluted with BF200 containing 100 µg/mL FLAG-peptide (Sigma).

GST pull-down assay

GST-fusion proteins were used for protein interaction assays. Two hundred ng of each protein to be tested (or

500 ng in the case of f-spPol II) was incubated with lysates containing 300 ng GST-fusion proteins together with 10 µL (packed volume) glutathione-Sepharose (Amersham Pharmacia) in a 500-µL reaction volume in BC100 containing 200 µg/mL BSA for 4 h at 4 °C with rotation. The glutathione-Sepharose resin was then washed twice with 500 µL BC200 and once with 500 µL BC100, and boiled in SDS sample buffer. The proteins released from the resin were separated by SDS-PAGE and detected by Western blotting.

DDBJ accession numbers

The DDBJ accession numbers for the spTFIIE α and spTFIIE β cDNA sequences are AB176672 and AB176673, respectively.

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Supplementary material

The following supplementary material is available at <http://www.blackwellpublishing.com/products/journals/suppmat/GTC/GTC833/GTC833.htm>.

Supplementary Table S1 Strains and their genotypes used in this study.

Supplementary Table S2 Oligonucleotides used in this study.

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