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Genetic Analysis of the Large Subunit of Yeast Transcription Factor IIE Reveals Two Regions with Distinct Functions

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Biochemical analysis of proteins necessary for transcription initiation by eukaryotic RNA polymerase II (pol II) has identified transcription factor IIE (TFIIE) as an essential component of the reaction. To better understand the role of TFIIE in transcription, we isolated conditional alleles of *TFA1*, the gene encoding the large subunit of TFIIE in the yeast *Saccharomyces cerevisiae*. The mutant Tfa1 proteins fall into two classes. The first class causes thermosensitive growth due to single amino acid substitutions of the cysteines comprising the Zn-binding motif. The second mutant class is made up of proteins that are C-terminally truncated and that cause a cold-sensitive growth phenotype. The behavior of these mutants suggests that Tfa1p possesses at least two domains with genetically distinct functions. The mutations in the Zn-binding motif do not affect the mutant protein's stability at the nonpermissive temperature or its ability to associate with the small subunit of TFIIE. Our studies further determined that wild-type TFIIE can bind to single-stranded DNA in vitro. However, this property is unaffected in the mutant TFIIE complexes. Finally, we have demonstrated the biological importance of TFIIE in pol II-mediated transcription by depleting the Tfa1 protein from the cells and observing a concomitant decrease in total poly(A)$^+$ mRNA.

The events that govern mRNA synthesis in eukaryotes have been studied both biochemically and genetically. Fractionation studies have defined the minimal set of protein components required to reconstitute transcription initiation in vitro (reviewed in references 6 and 47), while genetic experiments have identified a number of additional gene products that are not required for the in vitro reaction but that appear to play a role in transcription in vivo (for example, the SRB proteins [reviewed in reference 21]). Both approaches have advanced our understanding of the transcription reaction, and the differences between the in vitro and in vivo results underscore both the complexity of the reaction and the importance of the bipartite approach.

The biochemically defined transcription components are highly conserved from yeasts to humans, and their interactions with each other and with the promoter DNA have been extensively studied (reviewed in reference 29). Transcription factors IIA (TFIIA), TFIIB, TFIID, TFIIE, TFIIF, and TFIIH all act during the initiation of the transcription reaction, helping RNA polymerase II (pol II) to recognize, bind, and melt the promoter DNA before mRNA synthesis begins. In vitro assembly of the transcription complex revealed an ordered pattern of binding of the transcription factors to promoter DNA (3, 4). TFIID (or TATA-binding protein) binds sequence specifically to the promoter, recruiting TFIIB and subsequently polII and TFIIF. Entry of TFIIE and TFIIH into the preinitiation complex occurs thereafter, with the binding of TFIIE preceding the binding of TFIIH.

Their late entry into the transcription complex suggests that TFIIE and TFIIH are required for a late step in the initiation reaction. Consistent with this idea, biochemical studies have implicated TFIIE and TFIIH in promoter melting. Several groups observed that the requirement for TFIIE and TFIIH for transcription in vitro can be alleviated under certain conditions, such as when transcription initiates from a supercoiled template (where presumably the supercoils provide the energy for promoter melting) (11, 35, 36, 45) or from a premelted promoter (16, 17, 34, 43). Furthermore, highly purified preparations of TFIIH exhibit DNA-dependent ATPase, DNA helicase, and pol II-directed kinase activities (reviewed in reference 8). Any of these activities might contribute to promoter melting. Purified TFIIE has not been found to possess any enzymatic activities, and its role in promoter melting could be indirect, arising from its ability to recruit TFIIH to the transcription complex (4, 10). In this report, we show that TFIIE has the ability to bind single-stranded DNA (ssDNA) and discuss possible roles for this activity in transcription initiation.

Many of the transcription factors are themselves made up of several polypeptides, increasing the complexity of the transcription machinery and the difficulty in dissecting the contribution of each component to the initiation reaction. Two genes, *TFA1* and *TFA2*, encode TFIIE in *Saccharomyces cerevisiae*, and each is essential for viability (9). Tfa1p is a highly acidic protein, especially at the C terminus, while the C terminus of Tfa2p is basic (20). The only significant sequence motif in Tfa1p is a zinc-binding motif of the C_2C_2 class (9), but the functional significance of this motif and of the protein's charged regions has not been explored. In this work, we present an in vivo analysis of *TFA1*. We performed a genetic screen to isolate mutations in *TFA1* that confer conditional growth. Changes in the zinc-binding motif inhibit growth at high temperature, and the protein's acidic region is shown to be necessary for growth at low temperature. Therefore, the large subunit of TFIIE apparently consists of at least two domains with genetically distinguishable functions.

MATERIALS AND METHODS

Yeast strains and plasmids. Table 1 lists the *S. cerevisiae* strains used in this study. All yeast strains are derived from CTY143, a gift from the laboratory of R. Young, Whitehead Institute, except strain YSB382, which is derived from ZY60, a gift from the laboratory of K. Struhl, Harvard Medical School. Yeast strains were manipulated with standard techniques (2, 14).

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TABLE 1. *S. cerevisiae* strains used

Strain	Genotype
CTY143	*MAT***a**/*MAT*α *ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3*Δ*200/his3*Δ*200*
ZY60	*MAT***a** *ura3-52 trp1*Δ*1 ade2-101* Ace1-*ROX1* Ace1-*UBR1*
YSB279	*MAT***a**/*MAT*α *ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3*Δ*200/his3*Δ*200 TFA1/tfa1*Δ*1::LEU2*
YSB280	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::LEU2* [pRS316-TFA1]
YSB281	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::LEU2* [pRS316-TFA1]
YSB290	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS316-TFA1]
YSB317	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C127R)]
YSB318	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(Q212Stop)]
YSB319	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(K222Stop)]
YSB320	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(Q273Stop)]
YSB324	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-TFA1]
YSB325	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(T218Stop)]
YSB326	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(T218Δ)]
YSB331	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C127F)]
YSB332	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C127L)]
YSB333	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C152Y)]
YSB335	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C124S)]
YSB358	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C127S)]
YSB359	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C127W)]
YSB382	*MAT***a** *ura3-52 trp1*Δ*1 ade2-101* Ace1-*ROX1* Ace1-*UBR1 tfa1::Anb1-TFA1::URA3*
YSB419	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(N263Stop)]
YSB488	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C127Y)]
YSB504	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C149S)]
YSB505	*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 tfa1*Δ*1::HIS3* [pRS315-tfa1(C149A)]

Plasmids and strains used in the genetic screen for conditional mutants. To construct the strain containing the *TFA1* disruption, the diploid strain CTY143 was transformed with *Spe*I-digested pRS305ΔSpe-3′5′TFA1, and one of the resulting Leu$^+$ colonies was designated YSB279. Disruption of *TFA1* in YSB279 was confirmed by Southern analysis (data not shown). Plasmid pRS305ΔSpe-3′5′TFA1 is a derivative of pRS305 which bears 390 bp from the 3′ end of *TFA1* cloned on a *Cla*I-*Bam*HI fragment followed by 430 bp of sequence from upstream of the *TFA1* start codon, cloned on a *Bam*HI-*Pst*I fragment. The *Bam*HI sites were introduced into the *TFA1* sequence by PCR amplification of yeast genomic DNA, using oligonucleotides TFA1-A (GGGATCCTTGCAATGCAC GATGCAATATTCA) and TFA1-C (GGGATCCTCAATATTCTCACTAGT AATGTT), and the resulting clone of *TFA1* was inserted into *Bam*HI-digested pBluescript II (KS+). Digestion of pRS305ΔSpe-3′5′TFA1 with *Spe*I linearizes the plasmid, cutting between the 3′ and 5′ fragments of *TFA1*. We confirmed that *TFA1* is an essential gene by sporulating and dissecting tetrads of YSB279. Viability segregated 2:2, and all viable cells were Leu$^-$. YSB279 was then transformed with pRS316-TFA1 and sporulated. Tetrad dissection generated Leu$^+$ Ura$^+$ haploid strains of each mating type bearing the chromosomal deletion of *TFA1* and the plasmid encoding the wild-type gene product (YSB280 and YSB281). For technical reasons, we changed the *LEU2* marker that disrupts the *TFA1* gene in YSB280 to the *HIS3* marker by transforming YSB280 with linearized pRS303, selecting for His$^+$ colonies, and then screening for Leu$^-$ cells. (45a). The resulting strain, YSB290, was used for the genetic screen described below.

The *TFA1* gene, PCR amplified from yeast genomic DNA by using primers TFA1-A and TFA1-C (described above), was inserted into the *Bam*HI site of pRS315 and pRS316 to yield plasmids pRS315-TFA1 and pRS316-TFA1, respectively. The polylinker of pRS315-TFA1 was modified by digesting it with *Sma*I and *Xho*I, filling in the *Xho*I overhang with Klenow fragment, and reclosing the backbone. This created a Tfa1 expression plasmid with a unique *Pst*I restriction site just upstream of the gene's start codon and a unique *Hin*dIII site just downstream of the stop codon. Site-directed mutagenesis (22) was used to introduce silent changes into the *TFA1* coding sequence with oligonucleotides TFA1-3′1220 (TAACCTGTCAGATCTTAGTC), which introduced a *Bgl*II site 183 bp from the ATG of *TFA1*, and TFA1-3′1540 (CATTAACCTATTAAGC TTATCTTGCTTC), which introduces a *Hin*dIII site 511 bp from the ATG of *TFA1*. Introduction of the *Bgl*II and *Hin*dIII restriction sites to pRS315-TFA1 yielded pNK6. The *Hin*dIII site downstream of the *TFA1* stop codon on pNK6 was destroyed by filling in the overhang with Klenow fragment and religating.

Plasmids and yeast strains for analysis of poly(A)$^+$ mRNA. The strain used for the *TFA1* double-shutoff experiment bears a chromosomal insertion that puts a ubiquitin-tagged version of *TFA1* under the control of a Rox1-repressible promoter and creates a nonfunctional, truncated allele of *TFA1* (amino acids 1 to 170) under the control of the gene's natural promoter. The strain further contains copper-inducible *UBR1* and *ROX1* alleles (26). The ubiquitin-Tfa1p fusion expressed from the Rox1-repressible promoter was created on a plasmid in two steps. First, a fragment of *TFA1* was PCR amplified from pNK6, using primers TFA1-B (GGGATCCCATATGGATAGACCTATAGATGATATTG) and TFA1-3′1540 (described above), and cloned into the *Bam*HI-*Hin*dIII backbone of pZM41, an integrating vector with a *URA3* selectable marker. The PCR-amplified *TFA1* fragment contains a *Bam*HI restriction site just upstream of the *TFA1* ATG, a *Bgl*II restriction site 183 bp downstream of the ATG, and a *Hin*dIII restriction site 511 bp downstream of the ATG. Next, a Rox1-repressible promoter and ubiquitin tag from pZM168 were cloned on a *Kpn*I-*Spe*I fragment into the *Kpn*I and *Bam*HI sites of the integrating vector bearing the *TFA1* fragment. The *Spe*I and *Bam*HI overhangs were filled in with Klenow fragment to create the proper reading frame for the ubiquitin-Tfa1p fusion, and the resulting plasmid was called pZM41/168-TFA1Δ. Plasmids pZM41 and pZM168 were kindly provided by the laboratory of K. Struhl. To construct the *TFA1* double-shutoff strain, ZY60 was transformed with *Bgl*II-digested pZM41/168-TFA1Δ, and the resulting Ura$^+$ colonies were tested for copper-sensitive growth on synthetic defined plates without uracil supplemented with 500 μM $CuSO_4$. One of the copper-sensitive integrants was designated YSB382.

Plasmids for overexpression of the Tfa1 and Tfa2 proteins. The histidine-tagged Tfa1 and Tfa2 proteins were expressed from pET11a and pET11d, respectively (9). These expression constructs were kindly provided by the laboratory of R. Kornberg, Stanford University School of Medicine. The plasmid that expresses the C-terminally truncated Tfa1 protein was derived from pET11a-TFA1 by digesting it with *Bst*B1 and *Bam*HI, filling in the overhangs with Klenow fragment, and religating. The resulting plasmid, pET11a-TFA1 (1-200), is expected to express histidine-tagged Tfa1p (amino acids 1 to 199) fused to four non-Tfa1 residues (Gly Ser Gly Cys). The plasmid that expresses histidine-tagged Tfa1p with the C127F substitution in the protein's Zn-binding motif was constructed in three steps. First, pNK6 expressing the C127F mutant protein was digested with *Cla*I and *Spe*I, the overhangs were filled in with Klenow fragment, and the backbone was religated. This was done to remove the *Nde*I restriction site from the 3′ end of *TFA1*. Second, the 5′ end of the histidine-tagged Tfa1p fusion was PCR amplified from pET11a-TFA1 by using the T7 promoter primer and TFA1-3′1220 (described above). The PCR product was digested with *Nde*I and *Bgl*II and inserted into the *Nde*I-*Bgl*II backbone of the modified pNK6 from step 1. This step joined the histidine tag to the C127F mutation in *TFA1*. Finally, an *Nde*I-*Nsi*I fragment from the plasmid created in step 2 was inserted into the *Nde*I-*Nsi*I backbone of pET11a-TFA1. This yields pET11a-tfa1 (C127F), which expresses the full-length Tfa1 protein bearing the N-terminal histidine tag and the C127F mutation. Analogous steps were used to create plasmids pET11a-tfa1 (C124F) and pET11a-tfa1 (C127S), which express histidine-tagged Tfa1p bearing the C124F and C127S mutations, respectively.

Isolation of *tfa1* mutants. The genetic screen was performed as follows. The entire *TFA1* coding region was mutagenized by using PCR-mediated random mutagenesis under the PCR conditions described elsewhere (48). Four independent reactions were performed with the −40 sequencing primer and the TFA1-A primer (described above) and with pRS315-TFA1 as a template. The PCR-mutagenized pools of *TFA1* were transformed into YSB290 in the presence of a gapped plasmid (pRS315-TFA1 digested with *Pst*I and *Hin*dIII and purified from agarose by using GeneClean [Bio 101]). The gapped plasmid and the PCR product had 440 bp of homology from the region upstream of the *TFA1* ATG

and 250 bp of homology from the region downstream of the 3′ end of *TFA1*, allowing for the ligation of the gapped plasmid and the PCR product through in vivo recombination (27). Cotransformation of YSB290 with the gapped plasmid and the PCR product gave rise to three times as many transformants as transformation with the gapped plasmid alone. We screened approximately 20,000 His^+ Leu^+ transformants (two-thirds of which were expected to carry the PCR-mutagenized copy of *TFA1*) for conditional growth. To select for loss of the unmutagenized copy of *TFA1*, the His^+ Leu^+ transformants were replica plated onto medium containing 5-fluoro-orotic acid (5-FOA; PCR, Inc.). Based on the frequency of 5-FOA sensitivity, we estimate the efficiency of the PCR-mediated mutagenesis to be approximately 6%. The 5-FOA-resistant cells were replica plated in triplicate and incubated at room temperature for 3 days, 37°C for 3 days, and 15°C for 14 days. All of the conditional mutants grew as well at 30°C as at room temperature (data not shown). Plasmid DNA was isolated from the mutant strains by passage through *Escherichia coli* and was retransformed into YSB290. The resulting His^+ Leu^+ transformants, after growth on 5-FOA, exhibited conditional growth, confirming that the mutations were plasmid linked. The plasmids were then sequenced to identify the mutations. In some cases, more than one change in the *TFA1* coding sequence was found, and site-directed mutagenesis was used to identify which mutation was responsible for the mutant phenotype.

Site-directed mutagenesis was performed by the procedure of Kunkel et al. (22). The substitution at the C124, C127, or C149 codon was introduced by using oligonucleotide TFA1-3′C124FLSY (ACAAATCGGSDACATGTAACCA), TFA1-3′C127FLSY (CTTGGTCAASDAAATCGGACTG), TFA1-3′C127W (CTTGGTCAACCAAATCGGAC), TFA1-3′C149A (ACATAGGGAAGCTA AAAATTCT), or TFA1-3′C149FLSY (ACATAGGGASDATAAAAATTCT). Each oligonucleotide was used in separate reactions to mutagenize pNK6, and mutants were identified by DNA sequencing. The *Bgl*II-*Hin*dIII fragment from each mutant plasmid was subcloned into an unmutagenized *Bgl*II-*Hin*dIII backbone of pNK6, and the resulting plasmids were transformed into YSB290. The His^+ Leu^+ transformants, after growth on 5-FOA, exhibited temperature-sensitive growth, confirming that the mutations conferred the conditional phenotype.

Site-directed mutagenesis was also used to introduce premature stop codons into *TFA1*. The stop codon at residue 218 was introduced into pNK6 by using oligonucleotide TFA1-3′T218Stop (TTGGATTGTACTAGTATGCTGC) to yield pNK6/T218Stop. Mutants were identified by the presence of a *Spe*I restriction site which is also introduced by the mutagenesis. The 3′ sequences of *TFA1* were deleted by digesting pNK6/T218Stop with *Spe*I and religating. The resulting plasmid bears the T218Δ allele and was called pRS315-tfa1(T218Δ). The stop codon at residue 263 was introduced into pRS315-TFA1 by using oligonucleotide TFA1-3′N263Stop (GTGGTAATCTAGATCTGTAATGTG) to yield pRS315-tfa1 (N263Stop). Mutagenesis with TFA1-3′N263Stop also introduces a *Bgl*II restriction site at residue 263. Mutants were identified by the presence of this site.

Protein expression. The wild-type and mutant Tfa1 proteins were expressed and purified as described by Feaver et al. (9), except that the Ni^{2+}-nitrilotriacetic acid (NTA)-agarose fractions, which appeared to be approximately 90% pure by Coomassie staining after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), were not subjected to DE52 column chromatography. *E. coli* BL21(DE3) cells were transformed with the protein expression plasmids described above and grown to mid-log phase in LB with 50 μg of ampicillin per ml. Expression of the Tfa1 proteins was induced by the addition of 0.4 mM IPTG (isopropylthiogalactopyranoside) to the growth medium, and the cells were grown for an additional 3 h at 30°C. The cells were harvested by centrifugation and lysed by sonication. Lysates were cleared of cell debris by centrifugation, and the soluble material was incubated with Ni^{2+}-NTA-agarose (Qiagen) at 4°C for 1 to 2 h. The Ni^{2+}-NTA-agarose was washed several times with a solution containing 2 mM imidazole, and the Tfa1 proteins were eluted from the column with 250 mM imidazole.

The expression and purification of Tfa2 protein were performed identically, except that the cells were induced with higher concentrations of IPTG (0.5 to 1 mM) and at different temperatures (ranging from 20 to 37°C). These changes were made to improve the induction of the Tfa2 protein. Most of the Tfa2 preparations appeared approximately 30% pure after elution from the Ni^{2+}-NTA-agarose and ran as a doublet on Coomassie-stained SDS-polyacrylamide gels. The only exception was a preparation of Tfa2 protein isolated after the induced cells were lysed in 5 mM imidazole, the column was washed with 60 mM imidazole, and the protein was eluted with 1 M imidazole. All solutions also contained 0.5 M NaCl and 20 mM Tris-HCl (pH 8). Under these conditions, the preparation of Tfa2 protein appeared approximately 80% pure.

Western analysis and coimmunoprecipitation. Antisera were raised against the histidine-tagged Tfa1 and Tfa2 proteins at Charles River Pharmservices (Southbridge, Mass.) by immunizing rabbits with 250 μg of purified proteins conjugated with RIBI adjuvant. The animals were boosted with 100 μg of proteins every 3 weeks. For the experiment shown in Fig. 2, the antiserum against Tfa1p was affinity purified by using Affi-Gel 10 resin (Bio-Rad) according to the protocol of Harlow and Lane (15).

Whole-cell yeast extracts were prepared according to a protocol provided by the laboratory of F. Winston, Harvard Medical School. The cells were harvested by centrifugation, resuspended in 250 μl of ice-cold breaking buffer (0.1 M Tris-HCl [pH 8], 20% [vol/vol] glycerol, 1 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride), and transferred to a 13- by 100-mm glass tube. An equal volume of acid-washed glass beads (Sigma) was added to each sample, and the cells were lysed by being vortexed four times for 15 s, with resting on ice for 1 min between vortexes. An additional 250 μl of ice-cold breaking buffer was added, and each sample was vortexed again for 10 s. The supernatants were transferred to 1.5-ml microcentrifuge tubes and cleared of cell debris by centrifugation for 15 min in a microcentrifuge at 4°C. The supernatants were transferred to a fresh microcentrifuge tube, and their protein concentrations were determined by using the Bio-Rad protein assay.

For the Western analysis shown in Fig. 2, whole-cell extracts (15 μg) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and probed for Tfa1 protein by using affinity-purified antisera (diluted in 20 mM Tris-HCl [pH 7.6]–137 mM NaCl–0.1% [vol/vol] Tween 20 [TBS-T] to 1 μg/ml). The secondary antibody was horseradish peroxidase-conjugated goat antirabbit immunoglobulin G (Sigma), diluted 1:10,000 in TBS-T, and complexes were detected with the Amersham ECL Western detection kit. For the Western analysis shown in Fig. 3, whole-cell extracts (50 μg) were treated as described above except that the nitrocellulose filter was probed with whole antisera, diluted 1:1,000 in TBS-T, and the complexes were detected with the LumiGLO chemiluminescent reagents (Kirkegaard & Perry Laboratories).

To perform the coimmunoprecipitation experiments shown in Fig. 4, whole-cell extracts were prepared and subjected to BioRex70 column chromatography as described by Sayre et al. (42) except that the supernatant from the glass bead lysis was not cleared with potassium acetate before being applied to the BioRex70 resin (Bio-Rad). The extract on the BioRex70 column was washed with buffer A (20 mM HEPES [pH 7.6], 20% [vol/vol] glycerol, 1 mM EDTA, 1 mM DTT) containing 0.1 and then 0.3 M potassium acetate. The transcription factors were eluted from the BioRex70 column in buffer A containing 0.6 M potassium acetate and then dialyzed into Nonidet P-40 (NP-40) buffer (150 mM NaCl, 1% [vol/vol] NP-40, 50 mM Tris-HCl [pH 8]). Immunoprecipitations were performed with 200 μg of protein in a final volume of 250 μl of NP-40 buffer, using 50 μl of preswollen protein A-Sepharose beads (10% [vol/vol] in NP-40 buffer) to which the antisera had been cross-linked. Cross-linking of the antisera to the protein A-Sepharose beads (Sigma) was performed with dimethyl pimelimidate dihydrochloride (Sigma) as described by Harlow and Lane (15). The immunoprecipitation reaction mixtures were incubated at 4°C for 1 h on a rotator. The beads were harvested by centrifugation and washed five times with NP-40 buffer. The proteins were eluted with 40 μl of low-pH elution buffer (100 mM glycine [pH 2.5], 150 mM NaCl). The low-pH elution was necessary to reduce the amount of antisera eluting from the beads. The eluates were neutralized with 5 μl of 1 M Tris-HCl (pH 8) before the addition of SDS-PAGE loading buffer and electrophoresis. Western analysis of the immunoprecipitates was performed as described above, probing the nitrocellulose filter with whole antisera, diluted 1:1,000 in TBS-T, and detecting complexes with the ECL Western detection kit (Amersham). The histidine-tagged Tfa1 and Tfa2 proteins were coimmunoprecipitated by using 500 ng of each purified protein in reactions identical to those described for the yeast extracts.

Poly(A)$^+$ mRNA analysis. For the experiment shown in Fig. 3, total RNA was prepared from cells according to a protocol provided by the laboratory of K. Struhl. Samples were harvested by centrifugation, and the cell pellets were washed once with cold water and then resuspended in 400 μl of RNA lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.5% [wt/vol] SDS). Total RNA was isolated by extracting the cells with an equal volume of unequilibrated phenol and incubating the samples for 1 h at 65°C. Samples were placed on ice for 5 min and then centrifuged at 4°C for 5 min. The supernatants were extracted a second time with unequilibrated phenol, followed by one chloroform extraction. The RNA was ethanol precipitated twice and resuspended in diethylpyrocarbonate-treated water, and the concentration of each sample was determined by measuring the A_{260}.

Slot blot analysis (44) was performed according to a protocol provided by the laboratory of R. Young, Whitehead Institute. Total RNA (1 μg in a 1-μl volume) was incubated with 100 μl of denaturing solution (66% deionized formamide, 8% formaldehyde, 0.66× MOPS [morpholinepropanesulfonic acid] buffer) at 65°C for 15 min then placed on ice. Cold 20× SSC (1× SSC is 0.15 M NaCl plus 0.05 M sodium citrate) (200 μl) was added to each sample, and each was applied to a nitrocellulose filter in a slot blot manifold (Schleicher & Schuell). The samples were washed twice with 2× SSC, and the filter was baked under vacuum for 2 h at 80°C.

The poly(dT) probe was radiolabeled by incubating 6.25 μg of poly(dA), 1.25 μg of oligo$(dT)_{12–18}$, 8.25 μg of TTP, 150 μCi of [α-^{32}P]TTP (800 Ci/mmol), and 1× Moloney murine leukemia virus reverse transcriptase reaction buffer (Promega) in a 25-μl volume at 65°C for 5 min. After the reaction mixture cooled, 500 U of Moloney murine leukemia virus reverse transcriptase (Promega) was added, and the reaction mixture was incubated at 37°C for 90 min. To terminate the reaction, 1 μl of 0.5 M EDTA and 1.5 μl of 2 M NaOH were added, and the reaction mixture was incubated at 65°C for 30 min. The reaction mixture was neutralized with 3 μl of 1 M Tris-HCl (pH 7.5) and incubated at room temperature for 15 min, and then 70 μl of 4× SSC was added. The unincorporated nucleotides were removed with a G50 (Sigma) spin column.

Hybridization of the poly(dT) probe to the nitrocellulose filter was performed as follows. The filter was soaked in 4× SSC for 15 min, prehybridized at 37°C for

4 h in 4× SSC–10× Denhardt's solution–0.5% (wt/vol) SDS–100 μg of denatured salmon sperm DNA per ml, and hybridized overnight to the denatured poly(dT) probe in prehybridization solution at 37°C. The filter was washed once with prehybridization solution at 37°C for 15 min and then three times with 1× SSC–0.1% (wt/vol) SDS at room temperature for 25 min. The filter was air dried and autoradiographed.

Gel retardation assay. Gel retardation experiments were performed with a 35-nucleotide oligonucleotide synthesized by Integrated DNA Technologies, Inc., called AdMLPtop (TTCCTGAAGGGGGGCTATAAAAGGGGGTGG GGGCG), which bears a sequence from the top strand of the adenovirus major late promoter. The oligonucleotide (40 pmol) was radiolabeled with 4 pmol of [α-^{32}P]ATP (3,000 Ci/mmol; NEN-Dupont) by using T4 polynucleotide kinase (New England Biolabs). The unincorporated nucleotides were removed from the reaction mixture with a G50 spin column. The concentration of the radiolabeled ssDNA was approximately 400 nM. Binding reaction mixtures (10 μl) contained 20 nM TFIIE proteins (premixed and allowed to equilibrate at room temperature for 10 min), 20 nM ^{32}P-labeled ssDNA, 25 mM HEPES (pH 7.5), 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 μM $ZnSO_4$, 0.1 μM EDTA, 100 μg of bovine serum albumin per ml, 0.025% NP-40, and 10% (vol/vol) glycerol. The reactions shown in Fig. 5A also contained 2.5 μg of nonspecific double-stranded pBR322 DNA (dsDNA). Reaction mixtures were incubated at room temperature for 30 min. A 5% acrylamide gel, preelectrophoresed at 50 V for 1 h at 4°C, was used to resolve the bound complexes from the free probe. The gel was poured with a 3.4% cross-linking ratio and buffered with 0.5× Tris-borate-EDTA. Binding reaction mixtures were loaded and resolved with 180 V of current. Following electrophoresis, gels were dried and autoradiographed. The experiments shown in Fig. 5B and C were performed similarly except that the binding reaction mixtures did not contain any nonspecific DNA, and the complexes were resolved on 6% acrylamide gels run at room temperature. The dsDNA competitor used in Fig. 5B was prepared by annealing AdMLPtop with a complementary oligonucleotide, named AdMLPbtm. Equimolar amounts of both oligonucleotides (80 pmol) were annealed by incubating them at 95°C for 5 min in 250 mM Tris-HCl (pH 7.5) and then cooling the mix from 65°C to room temperature in a water bath.

RESULTS

Isolation of *tfa1* mutations. To identify the biologically important portions of the Tfa1 protein, we isolated *tfa1* mutations that confer conditional growth to cells expressing the mutant allele from a plasmid and harboring a deletion of the chromosomal copy of *tfa1*. Using PCR-mediated random mutagenesis followed by plasmid shuffling, we identified eight mutants that grew at 30°C but not at 37°C (temperature sensitive) and four mutants that grew at 30°C but not at 15°C (cold sensitive). In all cases, the mutant phenotype was plasmid linked and recessive to the wild-type allele.

Sequence analysis revealed that the majority of the mutant plasmids conferring temperature sensitivity harbored a substitution at the cluster of four cysteines that participate in the protein's Zn-binding motif (Table 2; Fig. 1). Changes at three of the four cysteines were isolated, in some cases with a second substitution elsewhere in the coding sequence. Subsequent introduction of the individual cysteine substitutions confirmed that the temperature-sensitive phenotype arose from these changes alone. Two of the eight temperature-sensitive mutants harbored plasmids with changes outside the protein's Zn-binding motif. The changes were found to destabilize the mutant proteins at the strain's nonpermissive temperature (data not shown), and these mutants were not studied further.

The role of the cysteines in Tfa1p function was further defined by using site-directed mutagenesis. We introduced a phenylalanine, leucine, serine, or tyrosine residue at amino acid position 124 or 127 of Tfa1p and examined the effect of each of these substitutions on cell growth (Table 2). This analysis revealed that dramatic changes in amino acid size and shape can be accommodated at position 127 but not at position 124. For example, a strain harboring the C127F substitution was temperature sensitive, whereas the C124F mutant was not viable, as judged by the inability to replace the wild-type copy of *TFA1* with the mutant copy in a plasmid shuffling experiment. Similarly, the C127S substitution had no effect on cell growth, whereas the C124S change conferred temperature sensitivity. We also mutagenized the codon for C149 and found that a change to serine or alanine resulted in a temperature-sensitive phenotype. These results suggest an asymmetry of function for the cysteines that form the Zn-binding motif of Tfa1p, and they reinforce the conclusion from the genetic screen that the Zn-binding structure of Tfa1p is important for TFIIE function in vivo.

TABLE 2. TFA1 mutants[a]

Substitution (aa, bp)	Phenotype	Mutagenesis method	No. of independent isolates
Zn-binding motif mutations			
C124F, TGT→TTC	Lethal[b]	Site directed	NA
C124L, TGT→TTG	Lethal	Site directed	NA
C124S, TGT→AGT	ts	PCR	1
C124Y, TGT→TAC	Lethal	Site directed	NA
C127F,[c] TGT→TTT	ts	PCR	1
C127L, TGT→TTG	ts	Site directed	NA
C127R,[c] TGT→CGT	ts	PCR	2
C127S, TGT→TCC	wt	Site directed	NA
C127W,[c] TGT→TGG	ts	PCR	1
C127Y, TGT→TAC	ts	Site directed	NA
C149A, TGT→GCT	ts	Site directed	NA
C149S, TGT→TCG	ts	Site directed	NA
C152Y, TGT→TAT	ts	PCR	1
Premature termination codons			
Q212^{ochre}, CAA→TAA	cs	PCR	2
T218^{amber}, ACG→TAG	cs	Site directed	NA
Q273^{ochre},[c] CAA→TAA	cs	PCR	1
K222^{ochre}, AAA→TAA	cs	PCR	1
N263^{amber}, AAC→TAG	cs	Site directed	NA

[a] Abbreviations: aa, amino acid; ts, temperature sensitive; wt, wild type; cs, cold sensitive; NA, not applicable.
[b] Lethality was indicated by an inability to grow on 5-FOA when the wild-type copy of *TFA1* was expressed from a plasmid marked with *URA3*.
[c] Originally isolated together with a second change in the *TFA1* coding region. The cysteine substitutions were subsequently introduced individually, and each was found to be sufficient to confer conditional growth.

Sequence analysis of the mutant plasmids from the cold-sensitive strains revealed that the *TFA1* gene bore premature stop codons (Table 2). The most 5′ stop codon occurred at the codon for residue 212, and the most 3′ was at the codon for residue 273. These substitutions remove the C-terminal 56 and 43%, respectively, of the protein (Fig. 1). To further define the biologically important regions of Tfa1p, we created other Tfa1p truncations. Cells harboring a more significantly truncated Tfa1 protein (to residue 172) were not viable, while a truncation beginning at residue 407 did not affect cell growth at

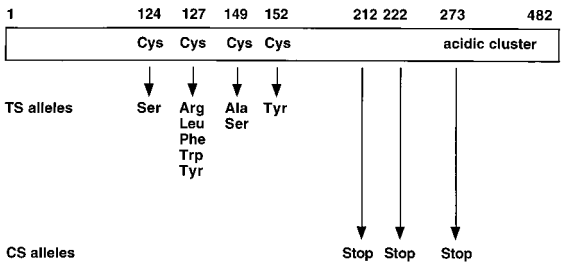

FIG. 1. Schematic diagram of the large subunit of TFIIE and the conditional mutant alleles. The substitutions isolated in the genetic screen or by site-directed mutagenesis and the phenotypes that they confer are indicated below the diagram. TS, temperature-sensitive; CS, cold-sensitive.

30 or 15°C (data not shown). Site-directed mutagenesis was used to change residues 218 and 263 to termination signals, creating two more C-terminally truncated mutants. These changes also conferred cold-sensitive growth. The results of our genetic analysis define the N-terminal half of the Tfa1 protein as sufficient for TFIIE's biological function at 30°C and identify a requirement for the C-terminal portion of the protein for growth at 15°C.

Effects of the *tfa1* mutations on cell growth, protein stability, and sensitivity to UV light. Figure 2 shows the growth of cells harboring plasmids encoding either wild-type Tfa1p or one of the mutant alleles. Representative samples of the temperature-sensitive and cold-sensitive strains are shown. The temperature-sensitive mutants grew normally on synthetic defined plates at 30°C (although the C152Y mutant strain grew as slightly smaller colonies) and failed to grow at 37°C. In liquid culture, however, all of the temperature-sensitive mutants grew less well than the wild-type strain at 30°C. Figure 2B shows the growth at 30°C in liquid media for the wild-type strain and the C127F mutant strain. The cell density of the mutant strain was less than that of the wild-type strain at every time point assayed, including the 24-h time point (not shown). Similar experiments carried out on the C124S and C152Y mutant strains showed slightly more severe growth defects at 30°C (data not shown).

To assess the severity of the growth defect at the mutant's nonpermissive temperature, we compared growth rates in liquid culture at 30 and 37°C. Cells were grown at 30°C until early log phase, and then a portion of the cells were grown at 37°C. Cell densities of the 30 and 37°C cultures were compared every hour. Data for the C127F mutant are shown in Fig. 2B. A difference in cell density at the two temperatures was noticeable after 3 h. Similar data were obtained for the C124S and C152Y mutants.

The cessation of growth for the temperature-sensitive strains does not arise from a rapid degradation of the mutant Tfa1p at the nonpermissive temperature. Western analysis of cell extracts from the wild-type and C127F mutant strains revealed that the C127F mutant protein remains as abundant as the wild-type Tfa1p after 5 h at 37°C (Fig. 2C). Similar data were obtained for the C124S and C152Y proteins (not shown). The stability of the mutant proteins at the nonpermissive temperature suggests that the mutations do not cause gross perturbations in Tfa1p folding, as such changes might be expected to cause rapid protein degradation. Indeed, two mutations in *TFA1* which did not map to the Zn-binding motif were identified in the genetic screen for temperature-sensitive mutants. Western analysis of extracts from strains expressing these mutant proteins revealed low concentrations of the protein after 3 h at 37°C (data not shown).

The growth of the cold-sensitive strains was also examined in liquid culture. Data for one cold-sensitive mutant, T218Δ, are shown (Fig. 2B). Unlike the temperature-sensitive strains, the cold-sensitive mutants grew as well as the wild-type strain in liquid culture at 30°C. At 15°C, a difference in cell density between the wild-type and mutant strain was observed after 5 h, not an unexpectedly long time given the slow growth of both strains at the low temperature. Western analysis of cell extracts from the cold-sensitive strains was performed; however, the truncated Tfa1 proteins could not be detected even in extracts from cells grown at the strain's permissive temperature. Since *TFA1* is an essential gene, we believe that the truncated proteins must be present at 30°C and hypothesized that the failure to detect them by Western analysis arose from deletion of the epitope(s) recognized by our anti-Tfa1p antibody. The inability of the antisera to recognize truncated Tfa1p was confirmed by Western analysis using recombinant truncated Tfa1p (data not shown).

Given that TFIIE interacts with TFIIH in vitro (4, 10) and that TFIIH is involved in nucleotide excision repair of DNA (reviewed in reference 8), we examined the effect of UV light on the TFIIE mutants. The wild-type, temperature-sensitive, and cold-sensitive strains were irradiated with UV light, and the number of surviving cells was determined as described previously (13). Data for one temperature-sensitive and one cold-sensitive mutant are shown in Fig. 2D. We observed no differences in the UV sensitivity of the mutant strains relative to the wild-type strain. In contrast, a mutant strain bearing a C-terminal deletion of *TFB1*, one of the subunits of TFIIH, is severely affected by exposure to UV light (24). These results suggest that the Zn-binding motif and the C-terminal half of TFIIE do not play a direct role in the repair of DNA damage.

Effect of Tfa1p depletion on poly(A)$^+$ mRNA production. The requirement for TFIIE for transcription in vitro can be alleviated under certain conditions, such as transcription of a supercoiled template (11, 35, 36, 45) or from a premelted promoter (16, 17, 34, 43). Given the relatively slow cessation of growth observed when the temperature-sensitive strains were grown at 37°C (Fig. 2B) and the concomitant slow decrease in total poly(A)$^+$ mRNA production (data not shown), we wondered if TFIIE was generally required for pol II-mediated transcription in vivo or if it was required for transcription of only a subset of genes. To distinguish between these possibilities, we took advantage of a double-shutoff strategy (26) in which the addition of copper to the growth medium leads to the cessation of *TFA1* mRNA synthesis and to the degradation of the preexisting pool of Tfa1 protein. We assessed the effect of Tfa1p depletion on transcription by monitoring the strain's growth rate, its Tfa1 protein concentration, and its production of poly(A)$^+$ mRNA at several time points after the addition of copper. We reasoned that if TFIIE was required for transcription from only a subset of genes in vivo, then poly(A)$^+$ mRNA production should continue even as the concentration of Tfa1p decreased. Conversely, if there was a general requirement for TFIIE in vivo, then we should detect a simultaneous decrease in Tfa1 protein and poly(A)$^+$ mRNA. Similar experiments have been used to demonstrate the general requirement for σ^{70}-containing RNA polymerase in *E. coli* (12) and for SRBs in pol II-mediated transcription in *S. cerevisiae* (44) and to demonstrate that certain TATA-binding protein-associated factors are not generally required for transcriptional activation (26).

Figure 3 shows the effect of copper on the growth rate, protein level, and mRNA production of a *TFA1* double-shutoff strain. A difference in growth rate between the wild-type and Tfa1p depletion strains can be observed 3 h after the addition of copper. Extracts made from cells grown for 2, 4, 6, and 9 h in copper-containing medium were subjected to Western analysis, which revealed a rapid decline in the concentration of Tfa1p. Analysis of poly(A)$^+$ mRNA in the cells at these times showed a similar decline in mRNA levels. The simultaneous decrease in cell growth, concentration of Tfa1p, and production of poly(A)$^+$ mRNA suggests a general requirement for TFIIE for transcription in vivo, although the existence of a small group of TFIIE-independent promoters cannot be ruled out by this experiment.

Effects of *tfa1* mutations on the interaction of the large and small subunits of TFIIE. To determine if mutations in the Zn-binding motif of Tfa1p affect its interaction with the small subunit of TFIIE (Tfa2p), we performed coimmunoprecipitation experiments using either cell extracts or purified recombinant proteins (Fig. 4).

For the immunoprecipitation of cell extracts, we chose three

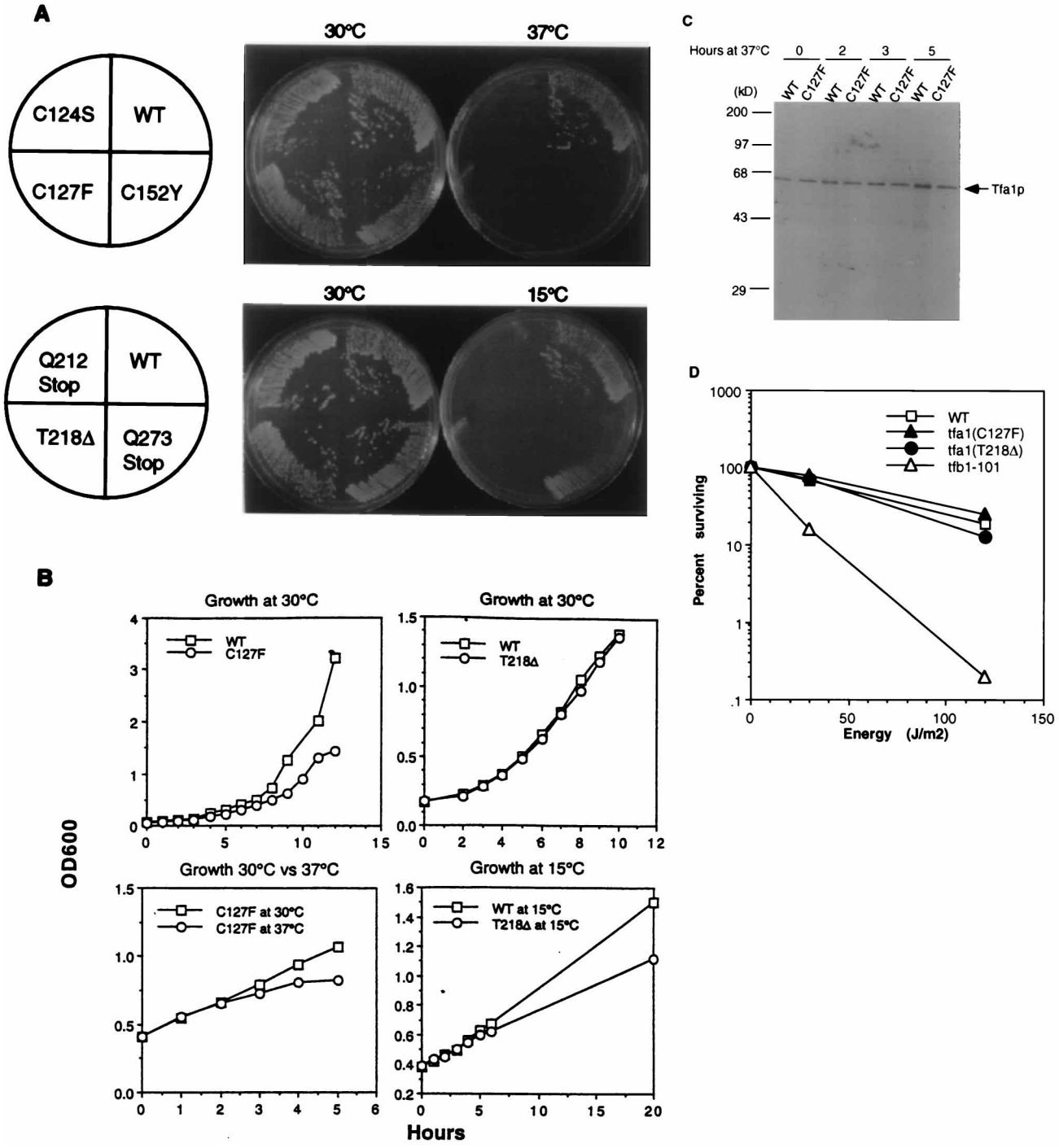

FIG. 2. Characterization of *tfa1* mutant strains. (A) Conditional phenotypes of *tfa1* mutants. Representative samples of the temperature-sensitive strains (YSB331, YSB333, and YSB335), the cold-sensitive strains (YSB318, YSB320, and YSB326), and the wild-type (WT) parent (YSB324) were streaked on synthetic defined plates lacking histidine and leucine and incubated at the indicated temperatures. Photographs were taken after 3 days of growth at 30 and 37°C and after 6 days at 15°C. (B) Growth curves of the wild-type and mutant strains were generated by diluting overnight cultures into fresh dropout medium lacking histidine and leucine and periodically measuring cell density. To assess the growth of the mutant strains at the nonpermissive temperature, a sample of cells growing at the permissive temperature was moved to the nonpermissive temperature at time zero. Cell density was measured at the indicated times after the temperature shift. When the optical density at 600 nm (OD600) of the cultures exceeded 1, measurements were made by using a 1:10 dilution of the culture. (C) Western analysis of extracts from the C127F mutant strain and its wild-type parent strain. Whole-cell extracts were prepared from cells grown at 37°C for the times indicated. Western analysis was performed by resolving 15 μg of each extract by SDS-PAGE (10% gel), transferring the proteins to a nitrocellulose filter, and probing the filter with affinity-purified antibody to Tfa1p. The mobilities of protein standards are indicated. (D) TFIIE mutant strains are not UV sensitive. Saturated cultures of wild-type cells (YSB324), TFIIE mutant cells (YSB326 and YSB331), and TFIIH mutant cells (YSB260) were diluted in water and plated onto YPD. The plates were immediately irradiated with the indicated dose of UV light from a germicidal lamp; the cells were grown in the dark at room temperature for 24 h and shifted to 30°C for 2 days. The percent surviving was determined by comparing the number of cells growing on the irradiated and unirradiated plates (13).

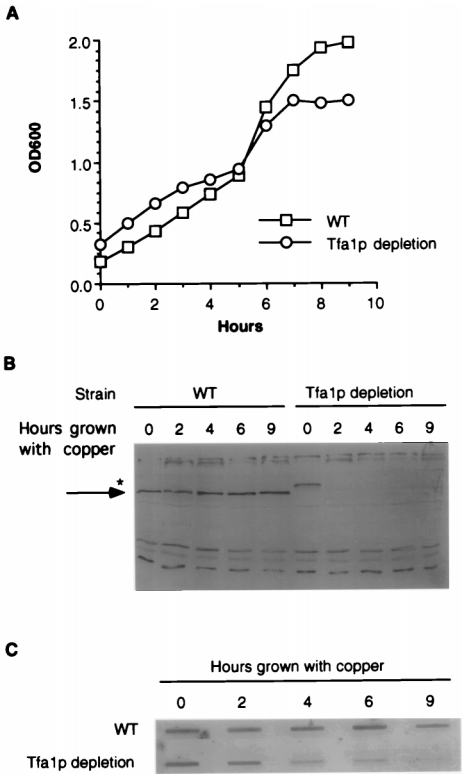

FIG. 3. Effect of Tfa1p depletion on growth and poly(A)$^+$ mRNA production. (A) Growth curves of the Tfa1p depletion strain (YSB382) and its parent strain (ZY60) transformed with pRS316. Saturated cultures were diluted into fresh dropout medium lacking uracil and grown at 30°C until early log phase. At time zero 500 μM $CuSO_4$ was added to each, and cell density was periodically measured. When the optical density at 600 nm (OD600) exceeded 1, measurements were made by using a 1:10 dilution of the culture. WT, wild type. (B) Western analysis of extracts from cells grown in the presence of copper for the indicated times. Whole-cell extracts (50 μg) were resolved by SDS-PAGE (10% gel) and transferred to a nitrocellulose filter. The filter was probed with antibody to Tfa1p. The arrow indicates the mobility of the wild-type Tfa1p, and the asterisk indicates the mobility of the ubiquitin-tagged Tfa1p. (C) The concentration of poly(A)$^+$ mRNA declines as Tfa1p is depleted. Total mRNA was prepared from cells grown in the presence of copper for the indicated times. Equivalent amounts of total mRNA (1 μg) were applied to a nitrocellulose filter in a slot blot manifold and probed for poly(A)$^+$ mRNA with [^{32}P]poly(T).

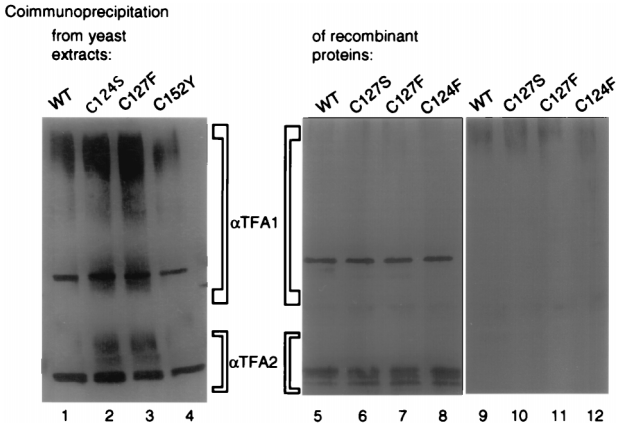

FIG. 4. Mutations in the Zn-binding motif of Tfa1p do not affect its interaction with Tfa2p. For the data in the panel on the left, whole-cell extracts were prepared from wild-type (WT; YSB324) and mutant (YSB331, YSB333, and YSB335) strains and were further purified by using BioRex70 resin. For the data in the panel on the right, wild-type and mutant proteins were expressed in bacteria and purified by using Ni^{2+}-NTA-agarose. Immunoprecipitations were performed with 200 μg of each yeast extract or 500 ng of each recombinant protein. Proteins were precipitated with antibody to Tfa2p (lanes 1 to 8) or preimmune serum (lanes 9 to 12) which had been cross-linked to protein A-Sepharose beads. The precipitated proteins were eluted from the beads, separated by SDS-PAGE (10% gel), and transferred to nitrocellulose filters. The filters were probed with antibodies to Tfa1p (top portion of each panel) or Tfa2p (bottom portion of each panel). Coimmunoprecipitation reactions using antibody to Tfa1p gave results similar to those shown in lanes 1 to 8 (data not shown).

mutant strains for analysis. Each strain harbored a substitution at a different cysteine residue in the Zn-binding motif of Tfa1p. Cell extracts from the wild-type and mutant strains were fractionated on a BioRex70 column to enrich the extracts for transcription factors (42) and then were immunoprecipitated with either anti-Tfa1p, anti-Tfa2p, or preimmune serum. Immunoprecipitates were subjected to SDS-PAGE and analyzed for Tfa1p and Tfa2p by Western blotting. No differences were observed in the ratios of these proteins in the various extracts, suggesting that the mutations in the Zn-binding motif of Tfa1p do not affect its ability to associate with Tfa2p in vivo.

Coimmunoprecipitation experiments were also performed with bacterially expressed recombinant proteins. We chose to study three mutant Tfa1 proteins that conferred three distinct phenotypes when expressed in yeast. One mutant protein (C127S) is phenotypically silent, i.e., a yeast strain expressing the C127S-substituted Tfa1p grows normally at both 30 and 37°C. The second mutant protein that we chose (C127F) confers temperature sensitivity, and the third mutant (C124F) is lethal to the cells even at 30°C (Table 2). If the Zn-binding motif of Tfa1p was important for its interaction with the small subunit of TFIIE, then we might expect the severity of the yeast growth phenotype and the extent of Tfa1p-Tfa2p coimmunoprecipitation to correlate directly. Specifically, the C124F-substituted Tfa1 protein, which confers the most severe phenotype, should associate least well with Tfa2p. Each mutant protein was purified from bacteria, mixed with purified Tfa2p, and immunoprecipitated as described above. Again, no differences were observed in the ratios of Tfa2p and the wild-type or mutant Tfa1 proteins. These results reinforce the conclusion that the association of Tfa1p and Tfa2p is not affected by mutations in the Zn-binding motif of Tfa1p.

TFIIE binds ssDNA. A three-dimensional model for the Zn-binding motif of the large subunit of TFIIE has been proposed based on the Zn ribbon structure found in the transcriptional elongation factor TFIIS (38). The TFIIS structure, in which four cysteine residues coordinate a single zinc ion with tetrahedral geometry and the surrounding residues fold into three antiparallel β-sheets (39), was applied, using homology modeling to the TFIIE Zn-binding motif with no obvious biophysical violations. The role of the TFIIS Zn ribbon in transcriptional elongation is not clear, but the structure contributes to binding in vitro to both duplex DNA and ssDNA, as well as to RNA and an RNA-DNA hybrid (1). Given the proposed similarity of the TFIIE and TFIIS Zn-binding structures and the oligonucleotide binding ability of TFIIS, we examined the ability of the yeast TFIIE to bind oligonucleotides and determined if mutations in the Zn-binding motif of Tfa1p affected this function.

The ability of bacterially expressed recombinant Tfa1p and Tfa2p to bind ssDNA was examined in a gel retardation assay (Fig. 5). The oligonucleotide used in these studies, chosen at random, is a 35-base sequence found in the top strand of the adenovirus major late promoter. When purified Tfa1p and Tfa2p were mixed with the radiolabeled ssDNA and subjected to nondenaturing gel electrophoresis, a complex with altered

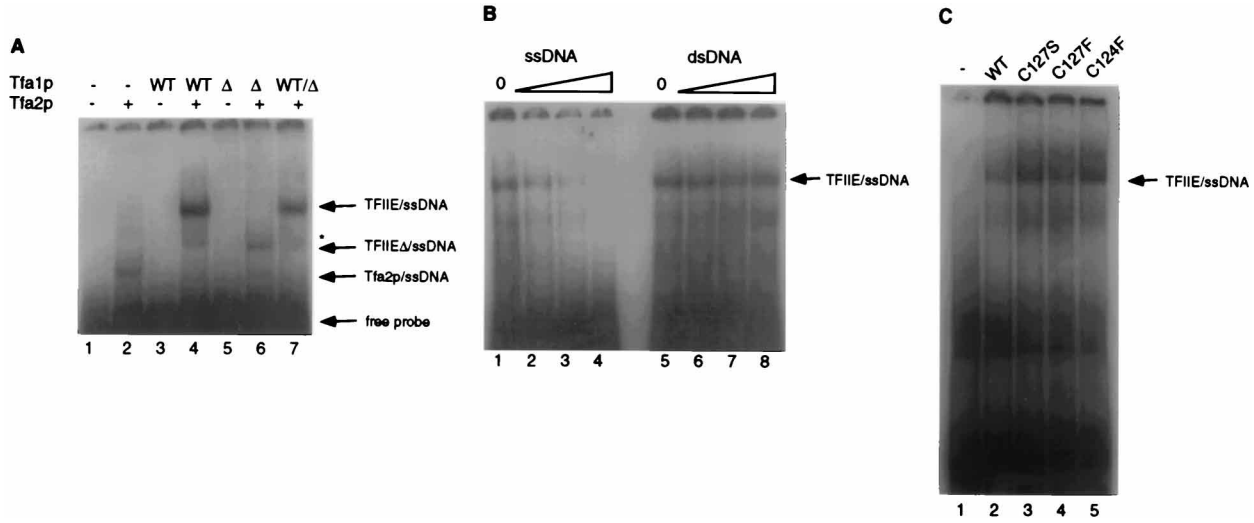

FIG. 5. Binding of TFIIE to ssDNA. (A) Wild-type TFIIE binds ssDNA. A gel retardation assay was performed with recombinant proteins and radiolabeled ssDNA. WT indicates reactions with wild-type, full-length Tfa1p; Δ indicates reactions with a truncated form of Tfa1p bearing residues 1 to 199. The mobilities of free probe and of the protein-ssDNA complexes are indicated at the right. The complex indicated by the asterisk likely was formed by a Tfa1p truncation product in the Tfa1p preparation. The complexes were resolved on a 5% native acrylamide gel at 4°C. (B) TFIIE binding is sensitive to competition by unlabeled ssDNA and not dsDNA. Increasing amounts of unlabeled competitors (one-, two-, and fourfold molar excesses) were added to reaction mixtures containing recombinant Tfa1p and Tfa2p and radiolabeled ssDNA. The complexes were resolved on a 6% native acrylamide gel at room temperature. (C) Mutations in the Zn-binding motif do not affect complex formation. Binding reactions were performed with radiolabeled ssDNA, recombinant Tfa2p, and recombinant Tfa1 proteins bearing the indicated mutations. The reactions were incubated at 37°C prior to loading on a 6% native acrylamide gel at room temperature.

mobility was detected (Fig. 5A, lane 4). Formation of this complex required both Tfa1p and Tfa2p in the binding reaction. In some experiments, binding reactions with Tfa2p alone gave rise to a complex of greater mobility than the complex formed with both Tfa1p and Tfa2p (Fig. 5A, lane 2). In no experiments were we able to detect a change in oligonucleotide mobility from binding reactions with Tfa1p alone. To confirm that the complex detected in the presence of Tfa1p and Tfa2p is, in fact, these proteins and not a contaminating protein bound to the ssDNA, we purified a truncated version of Tfa1p called Tfa1pΔ. This derivative bears only the first 199 amino acids of Tfa1p plus four additional non-Tfa1 residues, resulting in a 203-amino-acid protein. This protein is expected to bind to Tfa2p normally, given the viability of cells expressing C-terminally truncated Tfa1p. Gel retardation analysis using purified Tfa1pΔ revealed that it does not affect the mobility of the radiolabeled ssDNA unless the binding reaction also contains purified Tfa2p. The complex formed in the presence of Tfa1pΔ and Tfa2p migrates faster than the complex formed in the presence of the full-length proteins, supporting the proposal that the complexes contain the Tfa1 and Tfa2 products and represent binding of TFIIE to ssDNA.

Experiments using gel filtration to determine the native size of TFIIE have suggested that the complex might exist as a tetramer made of two large and two small subunits (19, 33, 37, 41). However, other types of analysis produce data that are more consistent with TFIIE existing as a dimer (5, 23, 41). In an effort to determine the stoichiometry of the bound protein complexes in our gel retardation assay, we performed mixing experiments, akin to those originally described by Hope and Struhl (18), in which the binding reaction mixtures contained the TFIIE small subunit and both the full-length and truncated large subunits. A complex that is a tetramer of two large and two small subunits should generate a heterotetramer when the binding reaction mixtures include the three proteins. The heterotetramer is predicted to migrate with intermediate mobility, and the ratio of the complexes should be 1:2:1 (1 tetramer with full-length Tfa1p and Tfa2p:2 heterotetramers each with Tfa2p plus one full-length Tfa1p and one truncated Tfa1p:1 tetramer with truncated Tfa1p and Tfa2p). As shown in Fig. 5A, lane 7, we were unable to detect any complex of intermediate mobility when the binding reactions contained Tfa2p and both Tfa1p and Tfa1pΔ, but formation of the original complexes decreased. The absence of a complex of intermediate mobility is consistent with a stoichiometry of one subunit of Tfa1p per TFIIE molecule. However, we cannot rule out that Tfa1p forms stable dimers which fail to exchange subunits with Tfa1pΔ dimers before associating with Tfa2p dimers.

To determine if the complex that we detected by gel retardation analysis is specific for ssDNA, we performed competition experiments with nonradiolabeled ssDNA or duplex DNA (Fig. 5B). The same 35-base sequence from the top strand of the adenovirus major late promoter was used as the ssDNA competitor or was annealed to its complementary sequence to generate the duplex DNA competitor. A titration of these oligonucleotides into the gel retardation binding reactions revealed that the complex formed by Tfa1p and Tfa2p is sensitive to competition by ssDNA but not duplex DNA, suggesting a specificity of TFIIE for the former. The biological implications of this observation are discussed below. The sequence of the ssDNA used as the competitor appears to be critical, as other single-stranded oligonucleotides failed to compete effectively for TFIIE binding (data not shown).

We wanted to determine if mutations in the Zn-binding motif of Tfa1p affect the ssDNA-TFIIE complex seen in our gel retardation assay. The three mutant proteins that we assayed confer three distinct phenotypes when expressed in yeast. If the Zn-binding motif of Tfa1p were important for the formation of the gel retardation complex, then we might expect a direct correlation between the severity of the yeast growth phenotype and the extent of complex formation by these mutants. However, Tfa2p and wild-type or mutant Tfa1 proteins all formed complexes with ssDNA that were detectable by gel retardation analysis. These complexes formed whether the re-

action mixtures were incubated at room temperature (data not shown) or at 37°C (Fig. 5C), suggesting that the Zn-binding motif of Tfa1p is not directly involved in their formation.

DISCUSSION

Our investigations into the role of TFIIE in transcription initiation have identified two regions of the large subunit that are critical for its function in vivo. The protein's Zn-binding motif can accommodate some unexpectedly dramatic substitutions without complete loss of function, while the C-terminal half of the protein appears to be dispensable except at low temperature. During the course of these studies, we identified a previously unknown property of TFIIE, its ability to bind ssDNA in vitro. This property is unaffected by the mutations in TFA1 that confer conditional growth. Further investigations into the biochemical consequences of the *TFA1* mutations as well as genetic analysis of their suppression should provide further insight into the mechanism of transcription initiation and its regulation.

Changes in the Zn-binding motif. The temperature-sensitive mutants that we identified genetically bear amino acid substitutions of the cysteines that participate in TFIIE's Zn-binding structure. Somewhat surprisingly, we found that two of the cysteines could be replaced by amino acids with bulky, aromatic side chains. These dramatic substitutions only moderately affect cell growth, and presumably TFIIE function, at 30°C. It is possible that the mutant proteins fail to chelate zinc entirely, indicating that zinc is not essential for TFIIE function. However, the Zn-binding motif is conserved in all species from which TFIIE has been cloned, suggesting that it is an important structural feature (9, 31, 37, 46).

How do such bulky substitutions in the Zn-binding structure of Tfa1p function? One means by which a residue with an aromatic side chain could coordinate a zinc ion is through cation-π interactions (7). These interactions, in which a cation is attracted to the negative electrostatic potential of the π face of an aromatic side chain, can be sufficiently strong to draw cationic ligands from solution into the hydrophobic environment of a protein. While some of the substitutions that were isolated in Tfa1p cannot participate in cation-π interactions, it is possible that the aromatic residue substitutions can create an environment within the protein that is sufficiently polar to bind a zinc ion while remaining hydrophobic enough to suit the protein's folded structure.

Using site-directed mutagenesis, we discovered an asymmetry in the role of the cysteines in TFIIE's Zn-binding structure. The observation that the same amino acid substitution at different cysteine residues results in different growth phenotypes can be explained in several ways. Perhaps the folded structure of Tfa1p constrains the cysteines unequally, allowing only conservative changes at one position and more dramatic changes at another. Alternatively, the cysteines may not play identical roles in zinc coordination. It is noteworthy in this context that only three of the four cysteines in a Zn-binding structure the bacterial UvrA protein are required for function (27), although UvrA differs from Tfa1p in that it bears two Zn-binding motifs.

In vitro analysis of human TFIIE has previously demonstrated the importance of the Zn-binding structure for transcriptional activity, revealing that substitution of the cysteine at residue 154 with an alanine diminishes the protein's binding of zinc from one atom/protein molecule to the background level of zinc detection and abolishes the entry of TFIIE into the transcription complex in vitro (25). Surprisingly, the corresponding yeast mutant (C149A) is viable albeit temperature sensitive (Table 2). Consistent with data presented here, the C154A substitution in the human protein did not affect its association with the small subunit of TFIIE. It remains unclear, however, whether the mutations in the Zn-binding structure of TFIIE descirbed here and elsewhere affect the association of TFIIE with other proteins in the transcription complex or affect its recognition of a protein-nucleic acid structure.

TFIIE's Zn-binding motif is thought to fold into a Zn ribbon structure similar to that of TFIIS, a transcriptional elongation factor (38), and TFIIB, a transcription initiation factor (49). The motif's sequence is also similar to the sequences of the putative Zn-binding motifs found in a yeast pol II subunit (RPB9), the DNA primases from the bacterial T3, T4, and T7 phages, and a bacterial DNA repair protein (UvrA) (38). All of these proteins are part of multiprotein-nucleic acid complexes, but the role of the (putative) Zn-binding structures in these complexes is unknown.

Truncations of the Tfa1p C terminus. The gene for the large subunit of TFIIE has been identified in *S. cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*, and HeLa cells, and in all cases the protein's C terminus is predicted to be highly acidic (9, 31, 37, 46). We have demonstrated that in yeast, growth at low temperature requires this acidic region. One possible explanation for the mutant strain's cold sensitivity is an instability of the truncated proteins at low temperatures. Since our antisera against Tfa1p apparently recognize only epitopes in the deleted region, we were unable to detect the truncated proteins immunochemically, and so we cannot rule out this possibility.

A more likely explanation is that the C-terminal domain of the TFIIE large subunit has a function that is particularly crucial at low temperatures. For example, the C-terminal domain might contribute to establishment or stabilization of ssDNA near the transcription initiation start site (see below). It is noteworthy that modifications to the in vitro transcription reaction which increase the helical stability of promoter DNA, i.e., higher ionic strength and lower reaction temperatures, result in an increased dependence on TFIIE (16). Alternatively, the C-terminal domain could stabilize an interaction with another component of the initiation complex. Ohkuma et al. reported that human TFIIE bearing a C-terminally truncated large subunit could no longer interact with TFIIH in vitro (32). Perhaps one of the enzymatic activities associated with TFIIH is required for transcription in cells grown at low temperatures, and the truncated TFIIE proteins, being defective for interaction with TFIIH, are unable to recruit or activate this function in the preinitiation complex. Purified TFIIH possesses ATPase, DNA helicase, and pol II-directed kinase activities (reviewed in reference 8). While any of these activities might be critical for growth at low temperature, the DNA helicase activity is a particularly likely candidate given the number of helicase-like proteins that are mutable to the cold-sensitive phenotype (30). Perhaps helicases are readily trapped in an inactive conformation by low temperature or are critical for a cold-sensitive process such as DNA or RNA unwinding. Finally, it is possible that the acidic region of Tfa1p is important for the interaction of TFIIE with chromatin, as was suggested previously (20). At lower temperatures, the interaction of TFIIE with chromatin might be important for loosening the condensed chromatin fibers and exposing the DNA to the action of other proteins.

Strains expressing the truncated Tfa1 proteins grow indistinguishably from wild-type strains at 30°C, indicating that the protein's N-terminal half is sufficient for TFIIE function in vivo. This finding is perfectly consistent with the in vitro analysis of the human TFIIE by Ohkuma et al. (32). They systematically deleted portions of the large subunit of the human

TFIIE and assayed the activity of the mutated proteins in a reconstituted transcription system. They observed that C-terminal deletions extending as far as residue 174 in the human protein, which corresponds to residue 168 in the yeast protein, could support basal transcription activity. The viability of our *TFA1* deletion mutants establishes the in vivo relevance of this somewhat surprising finding.

TFIIE is an ssDNA-binding protein. Using a gel retardation assay, we have detected an affinity of TFIIE for ssDNA. When we mixed the TFIIE small subunit with an artificially truncated large subunit, we found that the mobility of the protein-ssDNA complex was affected, confirming that the complex detected in our assay is, in fact, TFIIE bound to ssDNA. Neither the Zn-binding motif nor the acidic cluster at the C terminus of the large TFIIE subunit appears to be critical for the binding of TFIIE to ssDNA in our assay. We have observed some weak binding of the small subunit of TFIIE to ssDNA in the absence of the large subunit, and so the large subunit, rather than contacting the DNA directly, might increase the affinity of the small TFIIE subunit by positioning it, inducing a conformational change in it, or oligomerizing it. This proposal is supported by photo-cross-linking data which showed that the small subunit of human TFIIE can be specifically cross-linked to a region between positions −14 and −2 of the adenovirus major late promoter, but only in the presence of the large TFIIE subunit (40).

The gel retardation assay further revealed a significantly higher affinity of TFIIE for ssDNA than for dsDNA. This difference in affinity suggests a function for TFIIE in transcription initiation. The transition of the preinitiation complex from the closed to open state involves melting some of the DNA duplex at the promoter. TFIIE could function in one of two ways. First, it might stabilize a melted region once it is unwound by the TFIIH DNA helicase activity. Alternatively, TFIIE might nucleate a short stretch of ssDNA that acts as a starting point for the TFIIH helicase.

Either of these proposals is consistent with the observation that the requirement for TFIIE for the reconstitution of transcription in vitro can be alleviated by premelting the promoter (16, 17, 34, 43). In vitro analysis of transcription from the adenovirus major late promoter suggests a role for TFIIE in destabilizing the promoter's +1 and +2 positions (17). Also consistent with these models are the cross-linking data mentioned above. Furthermore, the two-dimensional crystal structure of the polII-TFIIE complex places TFIIE near the active site of polII, presumably the region where the duplex DNA is melted to allow for transcription of the template to RNA (23).

Further biochemical and genetic experiments should help us to better understand the role of TFIIE in promoter melting and transcription initiation. The isolation of conditional TFIIE mutants provides a starting point for some of these experiments.

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ADDENDUM IN PROOF

While our paper was in press, Sakurai et al. published a paper describing truncated cold-sensitive alleles of *TFA1* that are similar to our alleles (H. Sakurai, T. Ohishi, and T. Fukasawa, J. Biol. Chem. **272:**15936-15942, 1997).

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