Mutations in the RING Domain of TFB3, a Subunit of Yeast Transcription Factor IIH, Reveal a Role in Cell Cycle Progression*

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The RNA polymerase II general transcription factor TFIIH is composed of 9 known subunits and possesses DNA helicase and protein kinase activities. The kinase subunits of TFIIH in animal cells, Cdk7, cyclin H, and MAT1, were independently isolated as an activity termed CAK (Cdk-activating kinase), which phosphorylates and activates cell cycle kinases. However, CAK activity of TFIIH subunits could not be demonstrated in budding yeast. TFB3, the 38-kDa subunit of yeast TFIIH, is the homolog of mammalian MAT1. By random mutagenesis we have isolated a temperature-sensitive mutation in the conserved RING domain. The mutant Tfb3 protein associates less efficiently with the kinase moiety of TFIIH than the wild type protein. In contrast to lethal mutants in other subunits of TFIIH, this mutation does not impair general transcription. Transcription of CLB2, and possibly other genes, is reduced in the mutant. At the restrictive temperature, the cells display a defect in cell cycle progression, which is manifest at more than one phase of the cycle. To conclude, in the present study we bring another demonstration of the multifunctional nature of TFIIH.

The set of proteins required for regulated transcription of eukaryotic mRNAs includes in addition to RNA polymerase II the general transcription factors (TFIIFA, TFIIIB, TFIIID, TFIIIE, TFIIF, TFIIH) (Refs. 1–3 and references therein) as well as proteins involved in transducing regulatory influences on the general machinery (1, 2, 4) and modulating chromatin structure (4–6). Individual components have unique roles in the process of transcription. For example, TFIIH possesses DNA unwinding activity (3) and a protein kinase activity directed at the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II (3). A new perspective on the functions of the transcriptional machinery has emerged with the discovery that TFIIH is a necessary participant in a distinct cellular process: excision repair of DNA damage (reviewed in Refs. 7 and 8). Thus, components of the transcriptional machinery may be involved in other cellular processes, allowing a possibility of coordinating such processes with transcription.

TFIIH is composed of nine known subunits, all of which are conserved between yeast and animal cells. The subunits of yeast TFIIH are encoded by the genes: SSL2, RAD3, TFB1, SSL1, TFB2, TFB3, TFB4, CCL1, and KIN28 (3, 7). The Kin28p and Ccl1p subunits of TFIIH are, respectively, the catalytic and cyclin-like subunits of a protein kinase that phosphorylates the CTD of RNA polymerase II (3, 7, 9). The homologs of KIN28 and CCL1 in animal cells are cdk7 and cyclin H (cycH), respectively. Interestingly, cdk7 and cycH have been independently isolated as components of a protein kinase termed CAK (CDK-activating kinase) (3, 7, 9) that phosphorylates the cell cycle kinases on a threonine residue (Thr-161 of cdc2 or its equivalent) and activates them. A third subunit of CAK has been identified, termed MAT1. The MAT1 protein seems to act as an assembly factor, stimulating the activity of CAK by stabilizing the complex between the cdk7 and cycH subunits. MAT1 was subsequently found to be a component of TFIIH (3, 7, 9). MAT1 is the ortholog of yeast Tfb3 protein, suggesting that the triad of Tfb3-Kin28-Ccl1 may have similar functions in yeast and in animal cells. Mutations in TFB3 have been reported in a screen for mutants that are synthetic-lethal with a kin28-ts mutation (10); the gene is named RIG2. Two mutants described in that report are severely and generally defective in transcription; each mutant bears multiple amino acid substitutions spread over the coding sequence. Another recent report shows that the tfb3-2 mutant is moderately sensitive to UV radiation, and is defective in nucleotide excision repair in vitro (11). These data demonstrate that Tfb3p has multiple roles in transcription and DNA repair.

The N terminus of TFB3 contains a cysteine-rich motif known as the RING or C3HC4 zinc motif (12). The conserved RING motif folds into a compact domain in which two zinc ions are coordinated by the seven cysteines and one histidine (13, 14). Proteins with diverse cellular functions possess similar RING motifs, which are suggested to be important in the architecture of large complexes and in protein ubiquitination (12).

The evidence for CAK activity of the MAT1-cdk7-cycH complex in animal cells derives entirely from biochemical experiments that demonstrate such an activity in vitro. Experimental evidence that establishes such a role in vivo has been found in the fission yeast, Schizosaccharomyces pombe (15, 16). This is in contrast with budding yeast (Saccharomyces cerevisiae), where an exhaustive study using a mutant of KIN28 and immunodepletion of Kin28 protein from yeast extracts did not support a role for Kin28 protein in Cdc28p phosphorylation (17). On the other hand, mutants of KIN28 and CCL1 (as well

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1 The abbreviations used are: CAK, Cdk-activating kinase; HA, hemagglutinin; FOA, 5-fluoroorotic acid; ORF, open reading frame; HU, hydroxyurea; CDK, cyclin-dependent kinase; CTD, C-terminal domain.
as other subunits of TFIH) did establish a role in transcription and in the phosphorylation of the CTD.

Several publications identified an unusual protein kinase, termed Cak1/Civ1, as the major CAK in budding yeast (18–20). This identification is supported by both biochemical assays of the bacterially expressed protein, by observations in vivo, as well as from the finding of distantly related proteins in ANK/HK and fission yeast that could complement the yeast mutant (21, 22). Cak1p is only distantly related to Kin28p or cdk7. Cak1p/Civ1p is active as CAK and as CDK, and thus, does not require a cycin subunit or post-translational modification by another yeast protein for its activity. It is not clear whether there is a difference between budding yeast, fission yeast, and animal cells in the identity of Cak1, and whether complexes containing Kin28p/cdk7 have any role in Cdk activation in vivo.

In the present study, we have conducted a mutational analysis of the yeast TFB3 gene. Lethal mutations were found in metal-coordinating residues of the RING domain. The mutant phenotype links Tfb3p to cell cycle progression. We further show that the RING domain contributes to the stability of association of the kinase moiety of TFIH (known as TFIIK) with its core.

**EXPERIMENTAL PROCEDURES**

**Materials—**5-fluorouracilic acid (FOA) was from Diagnostic Chemicals. Radioactive nucleotides were from American Biosciences or PerkinElmer Life Sciences. Monoclonal antibody 12CA5 (purified IgG), directed at the influenza HA epitope, was a gift from J. Gerst. Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Jackson Immunoresearch. Antibodies against bacterially expressed Tfb1p (23) and Ssl2p were used in immunoblots as unfractionated sera from rabbits at 1:20,000 dilution. Affinity-purified polyclonal antibodies against Kin28p (24), a gift from R. Kornberg, were used at 1:1000. GST-kinase domain antibody against Rad1p, 3E3, was kindly supplied by A. E. Tomkinson and used at 1:1,000.

**Growth Media and Buffers—**Selective media for yeast were synthetic media grown at 30°C in SD plates (34) lacking the relevant nutrients: -Trp, -Leu, -His, -ura, or -can. YPD and YPAD media were as described in Kaiser (26). Buffer TA(x) consists of 20% glycerol, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40, protease inhibitors (27), and potassium acetate at the molar concentration given in parentheses.

**Plasmids—**Plasmids pRS313 (CEN, HIS3), pRS316 (CEN, URA3) and pRS426 [MATa, URA3 (28, 29)] were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

A genomic fragment containing the wild-type TFB3 gene (nucleotides 12141–16307 from GenBank™ accession U33050; the sequences was confirmed by sequencing with primers 16307 from GenBank™ accession U33050; the sequences was confirmed by sequencing with primers 5′–GAAATTAAATGTTAGTGTACAGTTGCTGCACTTACCGTAGTAGAATGTGCTGCG3′ and 5′–GGGTGT-TGACAGAGATTGTGGGTAGGTTGTTATATCCGAGGAGGTTCCGAGGCAGCTGAC3′. The template used for PCR (pYM3, Ref. 32) contains six repeats of the HA tag, followed by the TRP1 selectable marker from _K. lactis_ on a separate reading frame.

**RNA Analysis—**Yeast cells grown at the conditions specified for each experiment were harvested by centrifugation, and RNA was prepared from 50–200 mL cultures by phenol extraction with glass beads (26). The quantity and quality of the RNA was assessed by absorption measurements and by ethidium-stained gel electrophoresis. Formaldehyde agarose gel electrophoresis and blot hybridization of 20 μg of each RNA sample was performed using Hybond membranes (Amersham Biosciences) or Genescreen (PerkinElmer Life Sciences) according to the manufacturer’s instructions.

**DNA Array Experiments—**For genome-wide expression experiments HA-tagged TFB3+ and _tfb3-2_ strains were grown to early exponential phase in YPD plates at 30°C. Then, half of each culture was shifted to 37°C for 90 min. Preparation of RNA, generation of amino-allyl labeled cDNA, labeling with Cy3 and Cy5, and hybridization was essentially done as described at The Microarray Center (www.uhnres.utoronto.ca/services/microarray/protocols/). Hybridizations were performed on yeast micro slides (CMT-GAPS-coated slides containing all yeast ORFs (27) × 2, lot 24301000, batch code 23020323) purchased from The Microarray Center MBRC, Toronto, Canada. Slides were scanned with ScanArray 4000 (Packard BioScience) and quantified with the QuanArray software (Packard BioScience). Then the intensities of the spots were normalized by subtracting the mean of the background (negative control). In the final step, we calculated log 2(ratio) of each spot, from which we subtracted the median of log 2(ratio) of all spots on the array. Differences of over 2-fold were considered to be significant. The full data set is available upon request.

**Cell Cycle Analysis—**Yeast cells were grown in YPD according to the scheme described in the legend to Fig. 3. Hydroxyurea was added, when indicated, as a solid to a concentration of 0.2 μM; the solid rapidly dissolved in the medium. To remove the hydroxyurea, cells were centrifuged and washed three times in fresh, prewarmed medium. After the appropriate incubations, 1 mL of each culture was centrifuged and the cells were suspended in 0.3 mL of ice-cold water and fixed by addition of 0.7 mL of ice-cold ethanol. For microscopic examination, the fixed cells were washed once in water and twice in phosphate-buffered saline. For flow cytometry, the cells were treated and stained with propidium iodide as described (33).

**Fractionation of TFB3 Complexes—**Yeast cells, TFB3+ HA and _tfb3-2_ HA, were grown at 30°C to early exponential phase. Then half of each culture was shifted and further grown at 37°C for 90 min. The cells were harvested, and whole cell extracts were prepared as described in Materials and Methods. To which the extracts with TFIH subunits, proteins were precipitated by adding 1.04 volumes of saturated ammonium sulfate (4°C, pH 7.5). Pellets were resuspended in TA(0) buffer and dialyzed overnight with two changes against TA(0.1). Extracts were then loaded on HiTrap Q Sepharose (1 mL, Amersham Biosciences) and eluted using a linear gradient of potassium acetate in TA buffer. Collected fractions were analyzed by Western blot using a set of antibodies against different subunits of TFIH. Two of the peaks that contained subunits of TFIH were further pooled (peak I and II), concentrated using Centricon-10 (Amicon), and were run in a size exclusion column (Superdex 200 HR10/30; Amersham Biosciences), using TA(0.2) as running buffer. Complex compositions were determined by Western blot, and sizes were estimated by comparing to a run with gel filtration molecular weight standards (Sigma). Analysis of proteins in cells was done using a trichloroacetic acid extraction procedure, followed by Western blot analysis of aliquots containing equal amounts of total protein (25).
RESULTS

Mutagenesis of TFB3—A plasmid-borne copy of TFB3 was mutagenized at random using hydroxylamine, and the pool of mutagenized plasmids was introduced into yeast cells. A plasmid shuffling protocol was used to eliminate the wild-type copy of the gene, testing the ability of mutant tfb3 to support growth and viability. Three recessive mutants with a severe phenotype were isolated: one conditional mutant (temperature-sensitive, tfb3-2 or tfb3-ts), and two absolutely lethal mutants (tfb3-5 and tfb3-35). Interestingly, sequencing of the mutant genes (Fig. 1A) revealed that all three mutations were alterations of conserved cysteines of the RING motif, which are involved in zinc binding and protein-protein interaction (12, 13). An initial characterization of the tfb3-2 strain revealed that this mutant exhibits an increased sensitivity to UV radiation and is impaired in nucleotide excision repair in vitro (11). Furthermore, the mutant is unable to grow in the presence of caffeine, hinting to a defect in the response to DNA damage.

A TFB3 Mutant with Cell Cycle Defects—The mutant tfb3-2 in which the second cysteine of the RING motif is mutated to tyrosine, supports growth at temperatures up to 34°C. However, a tfb3-2 culture fails to grow or to form colonies at 37°C or higher temperatures, even when the mutant protein is overexpressed (not shown). To test whether the mutant is defective in RNA polymerase II transcription, RNA was extracted from mutant cells at various times following a shift to a restrictive temperature. The levels of several mRNA sequences were followed by Northern blot hybridization. For comparison, wild type cells and cells harboring a temperature-sensitive mutation in KIN28, another TFIIH subunit, were similarly analyzed.

Fig. 2 shows the results of a representative experiment. The transcripts of SSM1/2, HIS3, ADH1, and CLB2 decay rapidly upon shift of the transcriptionally defective kin28-ts16 mutant cells to the restrictive temperature. In contrast, shift of the tfb3-2 mutant to 37°C did not affect mRNA levels of most genes tested. The only exception we found was the CLB2 gene, encoding a G2 cyclin; results obtained with this gene were variable, with transcript levels declining by 30–80% relative to those seen at the permissive temperature. To extend our results to the genome at large, we analyzed the samples on DNA arrays and got similar results in which general transcription is almost not affected in tfb3-2 mutant, since over 96% of the genes are similarly transcribed (Fig. 2C). Interestingly, the

![Fig. 1. TFB3 mutations. A, the RING motif of TFB3 (single letter amino acid code). Numbers in parentheses denote the number of amino acids between the indicated cysteines. The binding of amino acids to the relevant zinc ion (Zn1 & Zn2) is depicted below the sequence (14). B, sequence alterations that occur, separately, in three mutant alleles of TFB3. Top, amino acid alterations; bottom, nucleotide alterations.](image)

![Fig. 2. The tfb3-2 temperature-sensitive mutant is not defective in general transcription. A, wild type cells, tfb3-2 mutant cells, and kin28-ts mutant cells were shifted from 26 to 37°C at time 0. RNA was extracted from the cells at the times indicated on the top, and 20 μg of each sample were subjected to blot hybridization with the gene probes indicated on the left. Lanes marked with C, for control, contain RNA from cells incubated for 6 h at 26°C. B, quantification of the samples presented in A. C, whole genome analysis of genes affected by the mutation in TFB3 gene, presented as a Venn diagram. 1, 1.75% of the genes are exclusively affected in the tfb3-2 mutant relative to the wild type strain at the permissive temperature; 2, 0.87% are affected in the mutant relative to the wild type strain at both the permissive and restrictive temperatures; 3, the expression of 1.24% of the genes is exclusively affected in the mutant strain at the restrictive temperature.](image)
major groups of genes that are affected by the mutation belong to metabolic enzymes and ribosomal genes, which suggest that the observed change may be the consequence of the reduced growth rate and the accompanying physiological changes. Finally, we have performed an exhaustive screen for possible multicopy suppressors of the tfb3-2 mutation, but failed to pick any gene except for 13 clones of the TFB3 gene (not shown). Thus, although the lethality of the tfb3-2 mutation may be due to impaired transcription of a subset of essential genes, the defect is unlikely to be due to deficiency in a single gene.

Microscopic examination of the mutant cells revealed that the cells held at the restrictive temperature were much larger than their wild-type counterparts (compare Fig. 3, A and B). The cell morphology was not uniform, indicating that the cells were not arrested at a unique point in the cell cycle. However, the abnormal size suggested to us some defect in cell cycle progression that may manifest itself at more than one point in the cycle. To uncover such defects we have used an experimental protocol used previously (33) to reveal the G2/M role of yeast Cdc28p (see scheme in Fig. 3E). Wild-type or mutant cells grown at 26 °C (a permissive temperature) were accumulated in S phase by hydroxyurea (HU), an inhibitor of DNA synthesis. Aliquots of the cells were then transferred to 37 °C for 1.5 h to inactivate the mutant Tfb3 protein. Finally, the cells were washed to remove hydroxyurea, and incubation was continued for an additional 6 h at 37 °C (Fig. 3C and D). Control cultures were subjected to the same treatment but either omitting the hydroxyurea (Fig. 3, A and B) or maintaining the temperature at 26 °C (not shown).

Wild type cells subjected to this treatment resumed replication after removal of the HU, leading to growing, asynchronous cultures after 6 h (Fig. 3C). In contrast, the tfb3-2 mutant cells kept at the restrictive temperature accumulated predominantly as enlarged budded cells (Fig. 3D). Flow cytometry (Fig. 4) confirmed that the wild-type cells were distributed along the cell cycle 6 h after release from the HU block (panel F), whereas in the mutant cells most of the population accumulated at postreplicative DNA content (panel K).

Reduced Levels of the Tfb3 Mutant Protein—To investigate the biochemical basis of the mutant phenotype we first analyzed the level of the wild-type and mutant Tfb3 proteins. To this end, we have introduced the tfb3-2 mutation into the chromosomal TFB3 gene in strain W303-1a (see "Experimental Procedures"). Then, we tagged the TFB3 gene in both the wild type and the mutant strains, on their 3′-end, with a 6×HA repeat tag, using the pYM vector system (32). Thus, Tfb3p and associated proteins could be identified using an αHA antibody. The strains bearing the tagged TFB3 (wild type or mutant) gene were indistinguishable from the corresponding untagged strains by growth rate, temperature sensitivity, UV sensitivity, and cell cycle distribution; in particular, the tagged tfb3-2 mutant strain had cell cycle defects similar to the untagged strain at the restrictive temperature.

We then prepared trichloroacetic acid protein extracts from early exponential phase cultures, and tested them by Western blot analysis. The level of Tfb3p is severely reduced in the mutant already at the permissive temperature (Fig. 5). When we incubate the mutant at the restrictive temperature, a further reduction in the Tfb3 protein level is observed as seen in Fig. 5. Although other explanations cannot be ruled out, it is...
likely that the reduction in the Tfb3p level is a result of the reduced stability of the mutant protein.

Altered TFIIH Complexes in tfb3-2 Mutant Cells —The procedures used to get highly purified TFIIH complex and subcomplexes include lengthy fractionation processes, yielding poor quantities, with high chances of causing alterations in the complexes (27, 34). We sought, therefore, to develop a fast biochemical method, suitable both for small- and large-scale studies, for the resolution of different forms of TFIIH from yeast. This method is based on the preparation of a whole cell extract, followed by salt fractionation (ammonium sulfate), and separation on anion exchange column. The different peak fractions from the anion exchange can then be further characterized on size exclusion columns or by immunoprecipitation. To further facilitate the biochemical examination process of the complexes, we have used the 6×HA-Tfb3p-tagged strains described in the previous section. Extracts of the wild type and mutated strains, grown at permissive and restrictive temperatures, were loaded on HiTrap Q Sepharose (Amersham Biosciences) and eluted using a linear gradient of potassium acetate. The resulting fractions were run on SDS-PAGE, blotted, and tested for the different protein complexes as shown in Fig. 6. We examined the distribution of Tbf1p, a core subunit of TFIIH; Ssl2p, a subunit of holo-TFIIH that is less stably associated with the core; epitope-tagged Tfb3p; Kin28p, the kinase subunit of TFIIH; and Rad1p, a protein involved in DNA repair that may be associated with TFIIH in repair complexes.

Kin28 protein is clearly distributed in at least two regions in the elution profile: an early region, encompassing the peaks denoted 0 and I (fractions 4–14) and a late region, denoted as peak II (fractions 17–24). The behavior of Kin28 protein is strongly affected by the tfb3 mutation. In TFB3 wild type cells grown at 30°C (Fig. 6A, top panel), Kin28p fractionates mostly in peak II, with lesser amounts eluting at the earlier peaks. This distribution is reversed in tfb3-2 mutant cells, where only a small fraction of Kin28 protein is eluted in peak II. The differences are even more marked after the cells are shifted to 37°C, a restrictive temperature for tfb3-2 mutant cells (Fig. 6B). Now, Kin28p, which elutes almost exclusively in peak II in TFB3 wild type cells, is nearly absent from peak II in tfb3-2 mutant cells.

Close examination of the proteins detected by anti-Kin28 antibodies reveals two bands with slightly different electrophoretic mobilities in SDS-PAGE. These have been shown to represent phosphorylated (lower band) and unphosphorylated (upper band) forms of the protein (24). In most fractions derived from TFB3-wt cells, the phosphorylated form of Kin28p predominates (see top panels in Fig. 6, A and B). In contrast, the unphosphorylated form of Kin28p is the major form present in most fractions derived from the tfb3-2 mutant cells; a significant exception is the Kin28p found in peak II fractions from tfb3-2 mutant cells grown at the permissive temperature, which is mostly phosphorylated (see bottom panels of Fig. 6, A–C).
Thus, the \( tfb3-2 \) mutation affects both the chromatographic behavior and the phosphorylation state of Kin28 protein. We then examined the nature of the complexes present in the different column fractions. This is partly revealed by observing other TFIIH subunits. Tfb1 and Ssl2 proteins, which are indicators of core- and holo-TFIIH, are present mostly in peak II in all experiments; this distribution is not significantly affected by the \( tfb3-2 \) mutation or by the temperature shift. These data suggest that peak II contains holo-TFIIH; this is confirmed by analyses of pools of peak II by gel filtration, where all four
subunits co-migrate in high molecular mass complexes with a size compatible with holo-TFIIH (∼500 kDa; data not shown). Similar analysis of fractions derived from the tfb3-2 mutant strain show a slight reduction in molecular size, compatible with the loss of Kin28p and, possibly, additional subunits (not shown). Gel filtration analysis of the early peak fractions reveals that Kin28p is associated with complexes of ∼100 kDa, compatible with suggested sizes for TFIIK subcomplexes. Significantly, although Tfb3 protein is present in peak I fractions of the anion exchange column, it segregates from Kin28p in subsequent gel filtration (not shown). Gel filtration also reveals that Rad1 protein is not stably associated with any of the complexes described.

Finally, Tfb3 protein itself is associated mainly with TFIIH core subunits (peak II); a small amount of the protein present in peak I does not co-fractionate with Kin28 protein. Significantly, the mutant tfb3-3-2 protein does not dissociate from TFIIH, even at the restrictive temperature; furthermore, mutant tfb3p is absent from the fractions containing most of the Kin28p.

**DISCUSSION**

Transcription factor IIH was originally purified by virtue of its activity in transcription and has been since proven, by extensive studies in vitro as well as in vivo, to be an essential component of the RNA polymerase II transcription machinery. Several studies show that TFIIH is involved in two steps of transcription initiation: unwinding of DNA by an ATP-driven helicase activity (carried out by Ssl2p and its mammalian homolog XPB) (35–37) and phosphorylation of RNA polymerase II (carried out by Kin28p and cdkt7) (2, 38, 39). It is now well established that TFIIH is a participant in nucleotide excision repair of DNA as reviewed in Refs. 7, 8. Remarkably, individual subunits of TFIIH, such as Rad3p and Ssl2p (and their mammalian homologs XPD and XBP, respectively), have dual roles in both processes; this was revealed by the existence of mutations that affect separately repair and transcription, and by in vitro reconstituted DNA repair assays (7, 9). A rationale for the involvement of a basal transcription factor in DNA repair has been suggested on the basis of transcription-coupled repair, which allows for more efficient repair of regions in the DNA that serve as templates for mRNA synthesis (7, 8).

The association of the TFIIH subunits cdkt7, cyclin H, and MAT1 with CAK activity in animal cells suggested a role in cell cycle progression (40–44). Observations in budding yeast have cast a doubt on this role of cdkt7 complexes. First, Kin28p does not seem to contribute to CAK activity in yeast extracts. Second, temperature-sensitive alleles of KIN28 do not affect the phosphorylation of Cdc28p in vivo (17). Third, the bulk of CAK activity in yeast extracts as well as in vivo is associated with a different kinase, the product of a gene termed CAK1 or CIVI, analogs of which were recently discovered in fission yeast, *Arabidopsis*, and mammals (18–20, 22, 45, 46). Thus, the mechanism of CDK activation in vivo is not fully resolved.

Tfb3-2-mutated Strain Is Altered in a Cysteine That Binds Zinc Ion—I—We describe here a genetic study of the role of Tfb3p, the yeast TFIIH subunit related to MAT1 of animal cells. Random mutagenesis of the gene has produced three point mutants with a lethal phenotype. Remarkably, all mutants are alteration of cysteines in the RING domain, which are predicted to interfere with zinc binding and possibly disrupt the structure of this domain. A recent study of the structure of the RING domain of mammalian MAT1 (14) reveals the pattern of binding of the cysteines and the single histidine to the two zinc ions (Fig. 1A). According to their study the first cysteine pair binds zinc ion-I, the third cysteine and the histidine that follows bind zinc ion-II, and the remaining two pairs bind zinc ion I and II respectively (14). Examination of the mutants we have generated reveals, that the two mutants that are unconditionally lethal are mutated in cysteines that bind the second zinc ion. In contrast, the altered cysteine in the less severe, thermosensitive mutant tfb3-2 binds zinc ion-I (Fig. 1 and Ref. 14). It seems that disruption of binding of Zn-II is more detrimental to the structure of the RING domain, whereas the structure around Zn-I may be stabilized by other interactions.

The Mutation Within the RING Finger of Tfb3p Weakens the Interaction of Kin28p with Core TFIIH—Analysis of the native state of macromolecular complexes is prone to artifacts that derive from fractionation and purification procedures; abnormal dilution, salt concentrations, and surface effects may lead to unpredictable changes in subunit associations. Purification of TFIIH from yeast extracts, even by affinity chromatography, requires five chromatographic steps and results in substoichiometric amounts of some subunits.

To gain better insights into the biochemical properties of TFIIH subcomplexes, we devised a rapid fractionation scheme that allows us to follow the subunit composition and the approximate size of TFIIH complexes by immunoblotting. The procedure, based on ammonium sulfate fractionation followed by anion exchange chromatography, can be performed on a small scale and allows processing of several samples with reproducible results.

The most significant results from the chromatographic analyses concern the state of the kinase subunit of TFIIH. Kin28p is mostly associated with TFIIH and is predominantly in the phosphorylated form, in extracts from TFB3 wild type cells. In contrast, Kin28p is mostly dissociated from TFIIH in tfb3-2 mutant cells, and this is exacerbated when the cells are shifted to a restrictive temperature. Furthermore, most of the Kin28 protein in tfb3-2 mutant cells is unphosphorylated, except for the small amount associated with TFIIH. These results, which were consistently obtained in several fractionation experiments and with several variations on the chromatographic conditions (data not shown), indicate that the weakened association of Kin28p with core TFIIH in tfb3-2 mutant cells is not entirely an artifact of chromatography. Rather, it likely reflects the central role of Tfb3p in connecting the kinase subunit(s) to the core TFIIH or in stabilizing the interaction. The fractionation pattern of Tfb3p seen in our experiments clearly indicates that Tfb3p remains associated with the core TFIIH and not with smaller complexes containing Kin28p. This is in accordance with previous observations by Feaver et al. (24). A recent study by Buratowski and colleagues (47) reported the finding of a free yeast TFIIK complex (containing Tfb3p–Kin28p–Ccl1p, similar to mammalian TFIIK). We have not detected such a complex, though we cannot rule out its existence within the smaller complexes of peak II.

Our observations on the effect of the tfb3-2 mutation on the phosphorylation of Kin28p are in accord with previous reports on experiments with Kin28 complexes produced in baculovirus-infected cells, where Tfb3p increased the efficiency of phosphorylation of Kin28p by Cak1p (48). These results suggest that the phosphorylation of Kin28p may be required either for assembly or for stabilization of the association between TFIIH core and the kinase subunits.

Busso et al. (49) reported the effects of mutations in human MAT1 protein on the properties of recombinant TFIIH produced in baculovirus-infected cells. In their reconstituted experiments, MAT1 bearing the cysteine substitutions homologous to the tfb3-2 and tfb3-5 mutations, as well as deletion of the entire RING domain abolished entirely in vitro transcription. The discrepancy between the consequences of the same mutation in MAT1 (complete elimination of transcription) and Tfb3 (conditional phenotype, mild transcriptional defect) may be due to the different properties of the human and yeast
proteins or to the very different experimental design. Nevertheless, the results of the reconstitution experiments of Busso et al. (49) indicate that the aberrations in TFIIH in the yeast mutant are not entirely due to the decrease in the amount of Tfb3p, but to some extent on the different properties of the mutant protein.

Linking Tfb3p, and TFIIH, to Cell Cycle Progression—Conditional-lethal mutants of many of the genes encoding general transcription factors are severely defective in overall mRNA synthesis (3). It is, therefore, surprising to find that the tfb3-2 mutant transcribes most genes at normal rates. The lethality of this mutant at high temperatures must derive from more specialized defects.

There are examples for selective transcriptional defects resulting from mutations of components of the general transcription machinery, e.g. TAF1145 (51–53) and SRB10 (54, 55). However, the subset of affected genes and the magnitude of the effects are much larger than those seen in the tfb3-2 mutant. We cannot currently resolve whether the phenotype of the tfb3-2 mutant cells is due to a primary defect in transcription.

The distinctive features of tfb3-2 mutant cells are presented in the following. (a) Cells kept at a restrictive temperature for 5–6 h or longer display an aberrant morphology: the cells are very large, but are heterogeneous in their budding state and in cell cycle position. We interpret this as a delay in cell division relative to the growth in cell mass. (b) Mutant cells at restrictive temperatures exhibit a cell cycle defect that is manifested at more than one point in the cycle, which can be uncovered by special treatment of the cells. For example, cells synchronized in S-phase prior to the shift to the restrictive temperature accumulate predominantly at G2/M DNA content. (c) At permissive temperatures, growth of single colonies of tfb3-2 mutant cells is delayed for 2–5 days relative to wild type cells following low level UV irradiation or incubation for 4–7 h in HU (data not shown). These doses are insufficient to cause significant mortality; the data seem to indicate that the mutant cells experience delays in emerging from checkpoint arrest. (d) The mutant cells are sensitive to caffeine and moderately sensitive to UV irradiation.

Interestingly, the tfb3-2 mutant phenotype bears some similarity to the phenotype of the cak1–22 mutant allele (19); the cells are enlarged, are arrested at more than one point in the cell cycle but are generally shifted to G2/M, and the arrest is established only several hours after the temperature shift. Another mutant allele, cak1–4, shares another set of characteristics with tfb3-2, including abnormally large after several hours at the restrictive temperature (20). Similar to the phenotypes of the CAK1/CIV1 mutants alleles, we observe a reduced level of Cdc28p phosphorylation (data not shown). Yet, we observe this effect only after prolonged incubation of the cells at restrictive temperature, which may be due to an indirect effect.

Another interesting link between Cak1p and TFIIH is that there is a synthetic effect when kin28-3 mutant is combined with either of the three independent cak1 mutants (48). Cak1p is required for the activating phosphorylation of Kin28p (48), although the two proteins are mostly located at different cellular compartments (50). Taken together these facts suggest that though these two proteins are known to have separate functions, both proteins can act in the same pathway, which may also depend on fully functional Tfb3 protein.

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Diverse Roles of Yeast TFB3