Subunit Interactions in Yeast Transcription/Repair Factor TFIIH

REQUIREMENT FOR Tfb3 SUBUNIT IN NUCLEOTIDE EXCISION REPAIR

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A yeast strain harboring a temperature-sensitive allele of TFB3 (tfb3ts), the 38-kDa subunit of the RNA polymerase II transcription/nucleotide excision repair factor TFIIH, was found to be sensitive to ultraviolet (UV) radiation and defective for nucleotide excision repair in vitro. Interestingly, tfb3ts failed to grow on medium containing caffeine. A comprehensive pairwise two-hybrid analysis between yeast TFIIH subunits identified novel interactions between Rad3 and Tfb3, Tfb4 and Ssl1, as well as Ssl2 and Tfb2. These interactions have facilitated a more complete model of the structure of TFIIH and the nucleotide excision repairosome.

The yeast transcription/nucleotide excision repair (NER)1 factor TFIIH has been extensively purified and characterized (1). Comprised of a total of nine individual subunits, holotFIIH is unique among RNA polymerase II (RNAP II) initiation factors in that it possesses enzymatic activity (2, 3). The subunits Rad3 and Ssl2 endow holotFIIH with bi-directional DNA helicase activity (3). TFIIH also has a kinase activity that phosphorylates the C-terminal domain of Rpb1, the largest subunit of yeast RNA polymerase II. Under some conditions holotFIIH can dissociate into the seven-subunit coreTFIIH and the two subunit TFIIK subcomplexes (4). C-terminal domain kinase activity has been shown to reside in TFIIK (1, 5). The recent isolation of TFB2, TFB3, and TFB4 completed the cloning of genes encoding subunits of yeast TFIIH (5). All TFIIH subunits are encoded by essential genes and have highly conserved counterparts in humans (6).

A requirement for yeast TFIIH in NER was first suggested by the identification of the well characterized DNA helicase and NER protein Rad3 as a subunit of core TFIIH (3). This requirement was subsequently demonstrated directly using an in vitro NER assay (7). To date, viable or conditional mutants of all the subunits of coreTFIIH except Tfb3 have been used to demonstrate a role for these polypeptides in NER (6–9). In contrast to core TFIIH, a requirement for TFIIH in NER has not been demonstrated. In this study we report the generation of a yeast strain with a temperature-sensitive allele of TFB3 (tfb3ts). This strain is sensitive to ultraviolet (UV) radiation in vivo and defective for NER in vitro. We conclude that Tfb3, like all coreTFIIH subunits, is indispensable for NER.

A form of TFIIH has been identified that is associated with all of the other polypeptides known to be required for the early steps of NER in the absence of DNA damage. This large, preformed “super complex” is referred to as the nucleotide excision repairosome (4, 10). A number of earlier studies used a variety of approaches to reveal interactions between TFIIH and/or repairosome subunits (see references in Table II). The cloning of genes encoding the Tfb2, Tfb3, and Tfb4 polypeptides has facilitated the inclusion of these subunits as well. We report here two-hybrid interactions between Ssl2 and Tfb2, Rad3 and Tfb3, and Tfb4 and Ssl1. Based on these interactions together with those previously known, we propose a more refined model for the structure of coreTFIIH and the repairosome.

Experimental Procedures

Construction of TFB3 C-terminal Deletions—pRS315/TFB3Δ1 and pRS315/TFB3Δ2 were made as follows: TFB3Δ1 was amplified by high fidelity polymerase chain reaction (PCR) from 50 ng of SacI-digested pRS/TFB3/3500 (6) with primers 5′-TCACACAGGAAAAAGCTTATGAG-3′ (reverse primer) and 5′-TATAAACGTCTTATTAGCCACCG-3′ (underlined) on the 3′-end of the amplified fragment. TFB3Δ2 was amplified by PCR as described above with the reverse primer and 5′-TATAAACGTCTTATTAGCCACCG-3′, also introducing a HindIII restriction site (underlined) on the 3′-end of the amplified fragment. Digestion with XhoI and HindIII, the PCR products were cloned into the same sites of pRS315 (13). Plasmids pRS315/TFB3Δ1, pRS315/TFB3Δ2, pRS315/TFB3, and pRS315 were transformed into the haploid strain CRY3-TFB3::HIS3[pRS316/TFB3] (6). Plasmid pRS316/TFB3 was subsequently cured from transformants by growth on medium containing 1 mg/ml 5-fluoroorotic acid. pRS315/TFB3 was constructed by subcloning the 3.5-kilobase HindIII fragment from pRS316/TFB3 into pRS315.

Construction of tfb3ts Strains—pRS315/TFB3 was mutagenized in vitro with hydroxylamine and transformed into a haploid derivative of YPH500 containing pRS316/TFB3 and a chromosomal TFBI3::LEU2 disruption. pRS313/3tfb3ts was identified by its inability to grow in the presence of 5-fluoroorotic acid at the nonpermissive temperature. Sequencing revealed that a cysteine residue in the RING finger had been changed to tyrosine (C16Y). A more detailed description of the isolation of the tfb3ts allele will be described elsewhere.2

pRS315/tfb3ts was made by subcloning the XhoI/SacI fragment from pRS313/tfb3ts into the same sites of pRS315. pRS315/tfb3ts was transformed into haploid derivative CRY3-TFB3::HIS3[pRS316/TFB3]. pRS316/TFB3 was subsequently cured from transformants by growth on medium containing 5-fluoroorotic acid.

Two-hybrid Analysis—Plasmids for two-hybrid analysis were constructed as follows: the N-terminal H fragment from pBT-11d/TFB2 (6) was subcloned into the same sites of pAS1-CYH2 and pACTII (14) to

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1 The abbreviations used are: NER, nucleotide excision repair; PCR, polymerase chain reaction.

2 O. Gileadi, manuscript in preparation.
give pAS1-CYH2/TFB2 and pACTII/TFB2, respectively. The TFB2Δ open reading frame was amplified by high fidelity polymerase chain reaction from 50 ng of XhoI-digested pRS315/TFB2A (6) with primers 5’-ATATCATGGAAGTGCATTCCCTGAA-3’ and 5’-TATAGGATTCCCTAACCCAC-3’ introducing NcoI and BamHI restriction sites (underlined) on the 5’ and 3’ ends of the open reading frame, respectively. The amplified fragment was digested with NcoI and BamHI and cloned into the same sites of pAS1-CYH2 and pACTII to give pAS1-CYH2/TFB2A and pACTII/TFB2Δ. For pAS1-CYH2/TFB4, the TFB4 open reading frame was amplified by high fidelity PCR from yeast genomic DNA with primers 5’-ATATCATGGAAGTGCATTCCCTGAA-3’ and 5’-ATATGAGATTCCCTAACCCAC-3’. The amplified fragment was digested with BamHI restriction sites (underlined) on the 5’ and 3’ ends of the open reading frame, respectively. The amplified fragment was digested with NcoI and BamHI and cloned into the same sites of pAS1-CYH2 and pACTII to give pAS1-CYH2/TFB2A and pACTII/TFB2Δ. For pAS1-CYH2/TFB4, the TFB4 open reading frame was amplified by high fidelity PCR from yeast genomic DNA with primers 5’-ATATCATGGAAGTGCATTCCCTGAA-3’ and 5’-ATATGAGATTCCCTAACCCAC-3’ introducing NdeI and BamHI sites (underlined) on the 5’ and 3’ ends of the amplified fragment, respectively. The PCR product was cloned directly into pCRII (Invitrogen) to give pCRII/TFB4. The NdeUBamHI fragment was subcloned into the same sites of pAS1-CYH2 to give pAS1-CYH2/TFB4. For pACTII/TFB4, the PCR product used to construct pFW668/TFB4 was digested with BamHI and cloned into the same site of pACTII to give pACTII/TFB4 (9). All other constructs for two-hybrid analysis listed in Table I have been previously described (6, 11, 12).

The pairs of plasmids listed in Table I were transformed into either strain Y190 (MATa gaa4 gaa8 cyh2 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 URA3::GAL χ2LYS2::GAL χHIS3) or GY::171 (14, 15, 16). Transformants were patched onto minimal selective plates overlaid with Hybond-N filters (Amer sham Pharmacia Biotech) and grown at 30 °C for 24 h. The filters were lifted, and the cells were lysed by one cycle of freeze/thawing in liquid nitrogen. The filters were incubated at 30 °C with 2.5 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 40 mM β-mercaptoethanol) until color development was apparent. An interaction between fusion proteins was scored positive when β-galactosidase activity was significantly increased over controls lacking either one of the fusions.

Other Methods—Growth of cells, preparation of whole cell extracts, measurement of in vitro NER activity, and quantification were as previously reported (8). Determination of UV radiation sensitivity has been described (9). Yeast transformations were performed by a standard lithium acetate protocol. YPD media contained 1% (w/v) yeast extract, 2% (w/v) bacto peptone, and 2% (w/v) dextrose. HolofTFIIH was prepared as previously reported (1).

RESULTS

The C-terminal Region of Tfb3 Is Essential for Viability—To investigate a possible role of Tfb3 protein in the NER, we generated a yeast strain containing a viable mutant allele of TFB3. Previous work showed that deletion of a small region of the C terminus of either Ssl2, Tfb1, or Tfb2 resulted in UV radiation sensitivity and defective NER in vitro (6–8, 16, 17). Unfortunately, deletion of either 22 (tfb3Δ) or 46 (tfb3Δ2) amino acids from the C terminus of Tfb3 was lethal, indicating that this region is essential for Tfb3 function (data not shown).

We previously showed that when fused to the DNA-binding domain of Gal4, Tfb3 activates expression of a β-galactosidase reporter plasmid containing Gal4 binding sites (6). The mutant alleles tfb3Δ and tfb3Δ2 also activated transcription in this assay, indicating that the C terminus of Tfb3 protein is not required for this activity (data not shown). We also previously showed that Tfb3 interacts with the Kin28 subunit of TFIIH, leading to the speculation that Tfb3 activates transcription by direct recruitment of TFIIH (6). The observation that tfb3Δ can still interact with Kin28 is consistent with this model (data not shown); however, other possible models of Tfb3 transcriptional activation cannot be excluded.

A Conditional Mutation in TFB3 Renders Cells Sensitive to UV Radiation—In view of the inviability of TFB3 deletion mutants, we generated a conditional, temperature-sensitive (ts) allele of the gene. The tfb3ts allele was isolated by standard yeast genetic techniques as described under “Experimental Procedures.” As shown in Fig. 1A, the tfb3ts strain failed to grow at 37 °C but grew normally at 30 °C. The isogenic wild-type strain grew at both temperatures (Fig. 1A). An increase in doubling time at the permissive temperature for the mutant strain (TFB3, 105 min; tfb3ts, 180 min) is consistent with partial impairment of the essential function. When tested for UV radiation sensitivity, a hallmark of defective NER, the mutant was found to be more sensitive than the isogenic wild-type at 30 °C (Fig. 1B). The magnitude of this effect was more pronounced at the semipermissive temperature of 33 °C (Fig. 1B), supporting our conclusion that UV radiation sensitivity is the result of thermolability of the tfb3ts protein.

The tfb3ts Strain Is Defective for NER in Vitro—To rule out the possibility that tfb3ts is indirectly sensitive to UV radiation as a result of defective transcription of DNA repair genes, we tested the ability of extracts from wild-type and tfb3ts cells to perform NER in vitro (18, 19). Whole cell extracts were prepared from each strain and incubated with two plasmid DNA substrates, one of which contained base damage. NER activity was measured as the amount of repair synthesis specifically occurring on the damaged substrate as described previously (18, 19). In this assay tfb3ts was almost completely defective for NER (Fig. 2A, lanes 4–5), whereas the wild-type extract possessed considerable activity (Fig. 2A, lanes 1–3). To confirm that defective NER in tfb3ts was specifically due to the mutation in the TFB3 gene, we tested the ability of purified holofTFIIH (1) to complement the mutant extract. Core and holofTFIIH have been shown to be equally active for NER in vitro (4). As is shown in Fig. 2B, the addition of purified holofTFIIH did indeed restore NER to the mutant extract. We conclude from these experiments that the Tfb3 subunit of yeast TFIIH is required for NER.
Complementation between NER-defective whole cell extracts from various NER mutant strains has previously been used to gain insights into protein-protein interactions (8). For example, tfb2 and ssl1 extracts fail to complement one another for defective NER activity in vitro, presumably due to the low rate of exchange of these TFIIH subunits (Fig. 3, lanes 1–3). In contrast, the tfb3ts extract could be complemented by either rad14 or rad23 extracts (Fig. 3, lanes 4–8), consistent with the notion that these proteins are not tightly associated. These results predict that tfb3ts cannot be complemented by extracts from either ssl1 or tfb2 mutant cells. However, surprisingly, the tfb3ts extract could be complemented by either ssl1 or tfb2 mutant extracts (Fig. 3, lanes 9–12). These results suggest that under the conditions of our in vitro NER assay, exchange of Tfb3 can occur.

The tfb3ts Strain Exhibits Caffeine Sensitivity—In eukaryotic cells, caffeine is known to adversely affect genomic stability, at least in part by inhibiting DNA repair and/or overriding DNA damage checkpoint controls (20, 21). In an effort to determine whether UV radiation sensitivity could be increased by inclusion of caffeine in the growth media, we were surprised to observe that tfb3ts failed to grow in the presence of the drug, whereas the isogenic wild-type strain grew normally (Fig. 4). In contrast to tfb3ts, an NER-defective C-terminal tfb2 deletion mutant (6) did not exhibit abnormal sensitivity to caffeine (Fig. 4). Thus caffeine sensitivity of tfb3ts does not appear to be a direct or indirect result of defective NER. It also seems unlikely that caffeine sensitivity is the result of defective transcription of genes required for caffeine detoxification, since there is no evidence of a significant transcription defect in tfb3ts at the permissive temperature. Consistently, tfb3ts did not exhibit inositol auxotrophy at 30 °C (data not shown), a phenotype commonly associated with transcription-defective mutants (22). Conceivably caffeine sensitivity is related to an as-yet unidentified function of Tfb3 and/or TFIIH.

Two-hybrid Interactions between Ssl2 and Tfb2, Rad3 and Tfb3, and Tfb4 and Ssl1—As mentioned above, a number of studies have revealed pair-wise interactions (23) between yeast TFIIH subunits (see references given in Table I). In an effort to determine whether UV radiation sensitivity could be increased by inclusion of caffeine in the growth media, we were surprised to observe that tfb3ts failed to grow in the presence of the drug, whereas the isogenic wild-type strain grew normally (Fig. 4). In contrast to tfb3ts, an NER-defective C-terminal tfb2 deletion mutant (6) did not exhibit abnormal sensitivity to caffeine (Fig. 4). Thus caffeine sensitivity of tfb3ts does not appear to be a direct or indirect result of defective NER. It also seems unlikely that caffeine sensitivity is the result of defective transcription of genes required for caffeine detoxification, since there is no evidence of a significant transcription defect in tfb3ts at the permissive temperature. Consistently, tfb3ts did not exhibit inositol auxotrophy at 30 °C (data not shown), a phenotype commonly associated with transcription-defective mutants (22). Conceivably caffeine sensitivity is related to an as-yet unidentified function of Tfb3 and/or TFIIH.
of fusion proteins tested is given in Table I. Of all the interactions and controls tested, three gave significantly higher levels of β-galactosidase activity than either of the fusion proteins alone; Tfb3 and Rad3 (Fig. 5A), Ssl2 and Tfb2 (Fig. 5B), Tfb4 and Ssl1 (Fig. 5B). None of the fusion constructs giving these interactions behaved as “promiscuous interactors” (Fig. 5 and Table I). In the latter two cases an interaction was not observed with fusion proteins in the opposite orientation, a result not uncommon with this technique.

**DISCUSSION**

In this report we present evidence that Tfb3, like the other subunits of yeast transcription/repair factor TFIIH, is directly involved in NER. We base this conclusion on UV radiation...
The interactions between Rad3/Tfb3, Ssl2/Tfb2, and Tfb4/Ssl1 reported here prompted us to propose a more detailed model of the subunit organization of yeast TFIIH and the repairosome (Fig. 6). A summary of the pairwise interactions among these subunits is given in Table II. It should be noted that additional interactions not listed in Table II have been inferred based on extensive co-purification of various proteins. For example, replication protein A has been shown to be associated with several TFIIH subunits, including Tfb3 (27), overexpressed Rad2 can be purified as a component of TFIIH (28), and Rad1/Rad10/Rad14 can be isolated as a discrete complex (29) as can Rad7/Rad16/Abf1. Additionally, there are necessarily additional protein-protein interactions that have yet to be identified. For example, Rad4 has been shown to co-immunoprecipitate with core TFIIH (12). However, it is not known through which TFIIH subunit(s) this interaction occurs. By two-hybrid analysis we failed to identify an interaction between Rad4 and any TFIIH subunit (Table I). Additional work will be required to elucidate the remaining determinants of TFIIH/repairosome structure.

**TABLE II**

| Interaction | Method | Reference No. |
|------------|--------|---------------|
| Tfb3/Ssl1  | TH     | 3, 11         |
| Rad3/Ssl1 | TH, CIP| 11            |
| Rad3/Ssl2 | CIP    | 11            |
| Rad2/Tfb1 | CIP    | 12            |
| Rad2/Ssl2 | CIP    | 12            |
| Tfb4/Ssl1 | TH     | This study    |
| Rad3/Tfb3 | TH     | This study    |
| Tfb2/Ssl2 | TH     | This study    |
| Tfb3/Kin28| TH     | 6             |
| Kin28/Cc11| TH     | 6, 30         |
| Rad4/Rad23| CP, TH | 31, 32        |
| Rad7/Rad4 | TH     | 32            |
| Rad1/Rad10| CIP, TH| 33, 34        |
| Rad7/Rad16| TH, GST, CP | 32, 35, 36 |

sensitivity of cells and defective NER in extracts, using a strain harboring a temperature-sensitive allele of TFB3 (tfb3ts). We have previously shown that polyclonal antisera against Tfb3 can partially inhibit RNA polymerase II transcription in vitro (6). A different temperature-sensitive allele of TFB3 has been shown to be defective for transcription at the nonpermissive temperature (24). Taken together, these results indicate that the Tfb3 protein is required for both NER and RNA polymerase II transcription.

The moderate level of UV radiation sensitivity of the tfb3ts mutant does not correlate quantitatively with the severe defect in NER in vitro. Similar results were observed for a TFB2 C-terminal deletion mutant (6). It would appear that in these cases the partial NER defect in vivo is amplified in the in vitro assay. In contrast to our results, another group has reported that yeast cells carrying different mutant alleles of TFB3 (ts) are not sensitive to UV radiation, leading to the conclusion that Tfb3 protein is not required for NER (24). However, these mutants were not directly tested for NER activity in vitro. These investigators isolated TFB3 alleles in a screen designed to identify mutations synthetically lethal with a temperature-sensitive allele of KIN28. Perhaps the different way in which our mutant was isolated can explain our apparently contradictory results. Given the relatively tight association of Tfb3 with core TFIIH based on co-purification, we find it implausible that all of the other subunits of core TFIIH play a role in NER but not Tfb3.

Our observation that under the conditions of in vitro NER employed in these experiments some exchange of Tfb3 between TFIIH complexes can occur is intriguing. Under similar conditions, no exchange of Tfb1, Ssl1, or Tfb2 was observed (6, 8). However, similar observations have been made with Ssl2 (7), and the selectiveСтатистика доступна для зарегистрированных пользователей. Войдите, чтобы получить полную информацию.
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