Revised Subunit Structure of Yeast Transcription Factor IIH (TFIIH) and Reconciliation with Human TFIIH

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Tfb4 is identified as a subunit of the core complex of yeast RNA polymerase II general transcription factor IIH (TFIIH) by affinity purification, by peptide sequence analysis, and by expression of the entire complex in insect cells. Tfb3, previously identified as a component of the core complex, is shown instead to form a complex with cdk and cyclin subunits of TFIIH. This realignment of subunits resolves a longstanding discrepancy between yeast and human TFIIH complexes.

TFIIH is remarkable among RNA polymerase II (pol II) transcription factors for its size, catalytic activities, and multiple functional roles (1). Consisting of nine subunits, with a total mass of about 500 kDa, TFIIH is comparable in size and complexity to pol II. The largest subunits of TFIIH, termed Ssl2 and Rad3 in yeast, are DNA-dependent ATPase/helicases and are essential for unwinding promoter DNA at the active center of pol II (2–9). Two smaller subunits form a cyclin-dependent protein kinase (cdk)-cyclin pair that phosphorylates the C-terminal domain of pol II during the transition from transcription initiation to elongation (10, 11). Beyond its role in transcription, six TFIIH subunits, including Ssl2 and Rad3, are components of a DNA "repairosome," responsible for nucleotide excision repair of DNA damage (12, 13). In human cells, the components of a DNA "repairosome," responsible for nucleotide excision repair (27), it was not definitively assigned to a subcomplex. Recent studies (28) have indicated an association of Tfb3 with the cdk-cyclin pair rather than with the yeast core complex. We now find that the component of the core complex originally identified as Tfb3 is, in fact, Tfb4. We confirm and extend the evidence for a Tfb3-cdk-cyclin trimer. The revised molecular description of yeast TFIIH is entirely coincident with that of the human factor.

MATERIALS AND METHODS

Construction of Baculoviruses Containing Genes for TFIIH Subunits and Expression in Insect Cells—Open reading frames (ORFs) of genes encoding, Rad3, Tfb1, Tfb2, Ssl1, Tfb3, and Tfb4, were amplified from yeast genomic DNA by polymerase chain reaction (PCR) and cloned into the BacPAK9 baculovirus expression vector (Clontech). A hexahistidine tag was added at the C terminus of the Tfb1 ORF. Recombinant viruses were produced in monolayer of Sf21 cells as described (Clontech).

For protein expression, Sf21 cells (~1.5 × 10^7) in a T75 flask were infected with various combinations of cloned virus stocks at a multiplicity of infection of 2–10. After 72 h, the cells were harvested and stored at −80 °C until use. Cells were lysed in 1 ml of buffer A (50 mM Heps-RKH (pH 7.6), 10% glycerol, and 5 mM β-mercaptoethanol) containing 600 mM potassium acetate, 0.5% Nonidet P-40 (Calbiochem), and protease inhibitor mix (final concentrations of 6 μM leupeptin, 20 μM pepstatin A, 20 μM benzamidine, and 10 μM phenylmethylsulfonyl fluoride). The cell lysate was clarified by centrifugation at 20,000 × g for 30 min and loaded on a 0.5 ml column of Ni-NTA resin (Qiagen) equilibrated with buffer A containing 600 mM potassium acetate and 0.01% Nonidet P-40. After washing with buffer A containing 2 M potassium acetate and 0.01% Nonidet P-40, and 5 ml of buffer A containing 150 mM potassium acetate, proteins were eluted with buffer A containing 150 mM potassium acetate and 300 mM imidazole (pH 8.0). Peak fractions (10 μl) were subjected to immunoblot analysis.

Construction of GST Fusion Proteins and Antibody Production—The ORFs of yeast Ssl1 and Tfb4 genes were amplified by PCR and cloned between BamHI and XhoI sites of pGEX6P-1 (Amersham Bioscience). GST-Ssl1 and GST-Tfb4 were overexpressed in Escherichia coli BL21 CodonPlus cells (Strategene), grown in 500 ml of Luria broth at 37 °C to an A600 of 0.6–0.8, and induced with 0.5 mM isopropyl-β-thio-galactopyranoside for 6 h at room temperature. The cells were harvested, frozen in liquid nitrogen, and ground in a mortar and pestle under liquid nitrogen to a fine powder (~5–10 min). After the cell powder was thawed, 50 ml of lysis buffer (phosphate-buffered saline containing 1 M NaCl, 10 mM dithiothreitol, and protease inhibitor mix) was added, activating the cdk’s that drive the cell cycle (14, 15).

All nine subunits of TFIIH have been conserved in amino acid sequence from yeast to humans (1), and structural studies have demonstrated conservation as well (16, 17). An intact nine-subunit “holo” TFIIH, capable of fulfilling the requirement for transcription, has been isolated from both yeast (18) and mammalian cells (19–21). Subcomplexes, apparently related to the distinct functional roles of TFIIH, have been reported (22, 23). In previous work from this laboratory, a 5-subunit “core” complex of the yeast proteins Rad3, Ssl1, Tfb1, Tfb2, and Tfb3 was described (18, 24), as was a separate complex of the cdk-cyclin pair, termed TFIIK (10).

While subcomplexes of TFIIH subunits were also obtained from human cells (20, 21, 25), with similar compositions to those from S. cerevisiae, a notable discrepancy arose in regard to the yeast human counterpart of Tfb3 (25). MAT1 was isolated in association with the cdk-cyclin pair, rather than as part of the core complex (14, 15, 25). Another protein, p34, replaced MAT1 in the human core complex (23, 25, 26). The yeast homolog of p34, termed Tfb4, was late to be identified (24), and although it was shown to be required for both transcription and nucleotide excision repair (27), it was not definitively assigned to a subcomplex. Recent studies (28) have indicated an association of Tfb3 with the cdk-cyclin pair rather than with the yeast core complex. We now find that the component of the core complex originally identified as Tfb3 is, in fact, Tfb4. We confirm and extend the evidence for a Tfb3-cdk-cyclin trimer. The revised molecular description of yeast TFIIH is entirely coincident with that of the human factor.
followed by stirring for 20 min at 4°C, brief sonication, and centrifugation at 100,000 × g for 60 min. The supernatant was loaded on a 3 ml column of GST-agarose (Sigma) equilibrated with lysis buffer. The column was washed with 10 volumes of lysis buffer, followed by 10 volumes of lysis buffer without NaCl. GST fusion proteins were eluted with lysis buffer containing 10 mM glutathione and no NaCl. GST-Tfb4 was dialyzed overnight against phosphate-buffered saline, followed by concentration to 2 mg/ml in a Vivaspin 6 ml concentrator, 30,000 molecular weight cutoff (Vivascience). About 1 mg of GST-Tfb4 was used to inoculate a rabbit (Covance, PA). GST-Ssl1 was fractionated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250. The GST-Ssl1 protein band was excised and used to inoculate rabbits (Covance, PA).

Affinity Purification of TFIIH Complexes—Yeast strain YTO62 or YTO63 was grown in 7.5 liter of 2% yeast extract, 1% peptone, 0.7% yeast, 0.09% tris acetate, 1.8 mM EDTA, 18% glycerol, 10 mM β-mercaptoethanol, protease inhibitor mix was added in the buffer.

The mixture was stirred at 4°C for 30 min and clarified by centrifugation in a Beckman JA14 rotor at 13,000 rpm for 20 min and then in a Beckman T45 rotor at 42,000 rpm for 90 min. Ammonium sulfate was added to 60% of saturation, followed by centrifugation in a Beckman JA14 rotor at 13,000 rpm for 45 min. The pellet was resuspended in 50 ml of buffer A (50 mM Heps-KOH (pH 7.6), 10% glycerol, 5 mM β-mercaptoethanol) containing protease inhibitor mix, clarified by centrifugation in a Beckman JA14 rotor at 13,000 rpm for 20 min, and loaded on a 0.5 ml IgG-agarose column (Sigma) equilibrated in buffer A containing 500 mM ammonium sulfate at 4°C. The column was washed with 10 ml of buffer A containing 500 mM ammonium sulfate and with 10 ml of the buffer A containing 100 mM ammonium sulfate. The column was equilibrated with 50 mM Heps-KOH (pH 8.0), 0.1 mM EDTA, 200 mM potassium acetate, 5 mM β-mercaptoethanol and eluted by incubation overnight in the same buffer containing TEV protease (40 μg/ml) at 4°C.

Peptide Sequence Analysis and Protein Identification—A phenol column fraction of highly purified core TFIIH (60 μl), prepared as described previously (17), was precipitated with 20% acetone in the cold and subjected to 10% SDS-PAGE. The lowest molecular weight band, visualized with Coomassie Brilliant Blue R-250, was excised. The gel slice was dried in a SpeedVac. The protein was digested with trypsin, peptides were fractionated on a Poros 50 R2 RP micro-tip, and the resulting peptide pools were analyzed by matrix-assisted laser-desorption/ionization reflectron time-of-flight (MALDI-reTOF) MS using a Bruker UltraFlex TOF/TOF instrument (Bruker Daltonics; Bremen, Germany), as described previously (31, 32). Selected experimental masses (m/z) were then taken to search a non-redundant protein data base (1.46 x 106 entries; National Center for Biotechnology Information, Bethesda, MD), utilizing the PeptideSearch (Matthews Mann, Southern Denmark University, Odense, Denmark) algorithm. A molecular weight range twice the predicted weight was covered, with a mass accuracy restriction better than 40 ppm and maximum one missed cleavage site allowed per peptide.

Mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples, using the UltraFlex instrument in “LIFT” mode. Fragment ion spectra were then taken to search the non-redundant protein data base using the Mascot MS/MS Ion Search program (Matrix Science Ltd., London, UK). Any identification thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data.

Immunoblot Analysis—Eluates (10 μl) of Ni-NTA or IgG columns were subjected to 10% SDS-PAGE, transferred to Protran membranes (Schleicher & Schuell), and probed with anti-Rad3, anti-Ss1, anti-Tfb1, anti-Tfb2, anti-Tfb3, and anti-Tfb4 antibodies on the left. Controls on the right: expression of Tfb3 alone (lanes 7–9), Tfb4 alone (lanes 10–12), no expressed proteins (uninfected cells, lanes 13–15). Load; FT, flow-through; E, peak fraction of eluate.

RESULTS AND DISCUSSION

Tfb4, but Not Tfb3, Supports Expression of Core TFIIH in Insect Cells—We set out to express yeast TFIIH in insect cells, beginning with the previously defined core complex of Rad3, Ss1, Tfb1, Tfb2, and Tfb3. A monolayer of SF21 cells was infected with a mixture of baculoviruses, each harboring a gene

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**FIG. 1.** Assembly of core TFIIH in insect cells. Rad3, hexahistidine-tagged Tfb1, Tfb2, and Ss1 were expressed in insect cells along with either Tfb3 (lanes 1–3) or Tfb4 (lanes 4–6). Expressed proteins were purified on Ni-NTA columns as described. The Ni-NTA fractions were analyzed by 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-Rad3, anti-Ss1, anti-Tfb1, anti-Tfb2, anti-Tfb3, and anti-Tfb4 antibodies on the left. Controls on the right: expression of Tfb3 alone (lanes 7–9), Tfb4 alone (lanes 10–12), no expressed proteins (uninfected cells, lanes 13–15). Load; FT, flow-through; E, peak fraction of eluate.

**FIG. 2.** Sequence analysis of the smallest subunit of core TFIIH. A. SDS-PAGE of highly purified core TFIIH from yeast. A phenyl 5-WP fraction (60 μl) was precipitated, separated by 10% SDS-PAGE, and visualized by staining with Coomassie Blue. The smallest subunit, ~37 kDa, was subjected to peptide sequence analysis. Molecular masses of protein standards (Bio-Rad) are indicated in kilodaltons at the left. B. tryptic peptides from the smallest TFIIH subunit, identified as described, are underlined in the deduced amino acid sequence of the yeast Tfb4 ORF.
for one of the five proteins, with that for Tfb1 bearing a hexahistidine tag. Clarified cell extracts were applied to Ni-NTA resin and eluted with imidazole, and eluted proteins were detected by immunoblotting with antibodies against the five proteins. Only Rad3, Tfb1, and Ssl1 were detected in the eluate (Fig. 1, lane 3). When the experiment was repeated, substituting a virus expressing Tfb4 for that expressing Tfb3, a five-subunit complex was obtained, as shown by the detection of all expressed proteins in the eluate (Fig. 1, lane 6). In control experiments, none of the antibodies showed significant cross-reactivity with proteins in an extract from uninfected cells, and neither Tfb3 nor Tfb4, expressed individually, was retained nonspecifically on Ni-NTA resin (Fig. 1, lanes 9 and 12). We conclude that Tfb4 is required for the assembly of a five-protein core complex, which does not include Tfb3.

**Peptide Sequence Determination Identifies the Smallest Subunit of Highly Purified Core TFIIH as TFB4**—The assembly of a core complex in insect cells with Tfb4 but not Tfb3 led us to question the previous assignment of the lowest molecular weight band in SDS gels of yeast core TFIIH preparations to Tfb3. As this assignment was based on mass spectroscopy of tryptic peptides derived from the gel band (24), we repeated the analysis. We used a more recent, improved yeast core TFIIH preparation of sufficient purity for crystallization (17). Following SDS-PAGE (Fig. 2A), the smallest band, of about 37 kDa, was excised, dried, and subjected to MALDI analysis. All peptide fragments detected had sequences derived from Tfb4 (Fig. 2B). We conclude that the smallest subunit of core TFIIH is Tfb4 and that the previous results arose from contamination by Tfb3, nearly identical in size to Tfb4.

**Affinity Purification of Tfb4 from Yeast Yields Core TFIIH, whereas Affinity Purification of Tfb3 Yields a cdk-cyclin-Tfb3 Complex**—Despite the requirement for Tfb4 for assembly of a core TFIIH complex in insect cells, and despite the presence of Tfb4 along with four other proteins in a core TFIIH preparation from yeast, it still remains to be shown that all components of the core preparation are physically associated with one another. To this end, we expressed Tfb4 bearing a TAP tag in yeast. A crude extract was applied to an IgG column for binding the protein A moiety of the TAP tag, and specifically bound proteins were eluted by cleavage of the tag with TEV protease. SDS-PAGE and Coomassie Blue staining showed (Fig. 3A) the enrichment of Rad3, Tfb2, Ssl1, Tfb1, and Tfb2, together with Tfb4 (bearing the residual CBP component of the TAP tag). After this single step of affinity purification, only a few impurities remained.

When the alternative experiment was performed of expressing TAP-tagged Tfb3 rather than Tfb4, the eluate from the IgG column contained no core TFIIH subunits (Fig. 3). Rather, what emerged were apparently stoichiometric amounts of Tfb3 and the cdk-cyclin pair (Kin28 and Ccl1). Similar evidence for a Tfb3-cdk-cyclin complex has been reported elsewhere (28).

The results of affinity purification therefore support those from peptide sequence analysis and expression in insect cells, showing that Tfb4 is a subunit of core TFIIH, whereas Tfb3 is not. The results further demonstrate the association of Tfb3 with the cdk-cyclin pair, previously denoted TFIIK (10). With this reassignment of subunits between core TFIIH and TFIIK, the compositions of the yeast complexes now correspond perfectly with their counterparts in human cells (Table I).

**Table I**

| TFIIH Subunits | Yeast | Human |
|----------------|-------|-------|
| Ssl2           | XPB   |       |
| Rad3           | XPD   |       |
| Tfb1           |       |       |
| Tfb2           | p55   |       |
| Ssl1           | p44   |       |
| Tfb4           | p34   |       |
| Ccl1           | Cyclin|       |
| Tfb3           | Mat1  |       |
| Kin28          | MO15  |       |

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**REFERENCES**

1. Svejstrup, J. Q., Vichi, P., and Egly, J. M. (1996) *Trends Biochem. Sci.* **21**, 346–350.
2. Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoijmakers, J. H., Chambon, P., and Egly, J. M. (1993) *Science* **260**, 58–63.
3. Guzder, S. N., Qiu, H., Sammers, C. H., Sung, P., Prakash, L., and Prakash, S. (1994) *Nature* **367**, 81–84.
4. Guzder, S. N., Sung, P., Bailly, V., Prakash, L., and Prakash, S. (1994) *Nature* **369**, 578–581.

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**Fig. 3. Affinity-purified TFIIH subcomplexes.** *A*, TFIIH subcomplexes were purified from Tfb3-tagged or Tfb4-tagged yeast strains as described. Peak fractions of IgG eluate (20 μl) were analyzed by 10% SDS-PAGE and staining with Coomassie Blue. A crude extract was applied to an IgG column for binding the protein A moiety of the TAP tag. Ccl appears in two bands, designated Ccl1a and Ccl1b, as noted previously (33). Tfb3-CBP overlaps with Ccl1b. *, contaminant from the TEV preparation. **, unknown contaminant. B, immunoblot analysis of the IgG elutes. Each fraction (10 μl) was analyzed by 10% SDS-PAGE, transfer to nitrocellulose, and probing with anti-Rad3, anti-Ssl1, anti-Tfb1, anti-Tfb2, and anti-Tfb4 (left) or anti-Tfb3, anti-Ccl1, and anti-Kin28 antibodies (right). *, cross-reacting species with anti-Tfb1 antibodies.
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5. Sung, P., Prakash, L., Matson, S. W., and Prakash, S. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8951–8955
6. Sung, P., Prakash, L., Weber, S., and Prakash, S. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6045–6049
7. Sung, P., Bailly, V., Weber, C., Thompson, L. H., Prakash, L., and Prakash, S. (1993) Nature 365, 852–855
8. Bootsma, D., and Hoeijmakers, J. H. (1993) Nature 363, 114–115
9. Schaeffer, L., Moncollin, V., Roy, R., Staub, A., Mezzina, M., Sarasin, A., Weeda, G., Hoeijmakers, J. H., and Egly, J. M. (1994) EMBO J. 13, 2388–2392
10. Feaver, W. J., Svejstrup, J. Q., Henry, N. L., and Kornberg, R. D. (1994) Cell 79, 1103–1109
11. Lu, H., Zawel, L., Fisher, L., Egly, J. M., and Reinberg, D. (1994) Cell 78, 1103–1109
12. Svejstrup, J. Q., Wang, Z., Feaver, W. J., Wu, X., Bushnell, D. A., Denahue, T. F., Friedberg, E. C., and Kornberg, R. D. (1995) Cell 80, 21–28
13. Feaver, W. J., Huang, W., Gileadi, O., Myers, L., Gustafsson, C. M., Kornberg, R. D., and Friedberg, E. C. (2000) J. Biol. Chem. 275, 5941–5946
14. Devault, A., Martinez, A. M., Fesquet, D., Labbe, J. C., Morin, N., Tassan, J. P., Nigg, E. A., Cavadore, J. C., and Doree, M. (1995) EMBO J. 14, 5027–5036
15. Tassan, J. P., Schultz, S. J., Bartek, J., and Nigg, E. A. (1994) J. Cell Biol. 127, 467–478
16. Schultz, P., Fribourg, S., Peterszam, A., Malloub, V., Moras, D., and Egly, J. M. (2000) Cell 102, 599–607
17. Chang, W. H., and Kornberg, R. D. (2000) Cell 102, 609–613
18. Svejstrup, J. Q., Feaver, W. J., LaPointe, J., and Kornberg, R. D. (1994) J. Biol. Chem. 269, 28044–28048
19. Conaway, R. C., and Conaway, J. W. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7356–7360
20. Gerard, M., Fischer, L., Moncollin, V., Chipoulet, J. M., Chambon, P., and Egly, J. M. (1991) J. Biol. Chem. 266, 20940–20945
21. Flores, O., Lu, H., and Reinberg, D. (1996) J. Biol. Chem. 271, 2786–2793
22. Sung, P., Guzder, S. N., Prakash, L., and Prakash, S. (1996) J. Biol. Chem. 271, 10921–10926
23. Tirode, F., Busso, D., Coin, F., and Egly, J. M. (1999) Mol. Cell 3, 87–95
24. Feaver, W. J., Henry, N. L., Wang, Z., Wu, X., Svejstrup, J. Q., Bushnell, D. A., Friedberg, E. C., and Kornberg, R. D. (1997) J. Biol. Chem. 272, 19319–19327
25. Adamczewski, J. P., Rossignol, M., Tassan, J. P., Nigg, E. A., Moncollin, V., and Egly, J. M. (1996) EMBO J. 15, 1877–1884
26. Humbert, N., van Vuurens, H., Lutz, Y., Hoeijmakers, J. H., Egly, J. M., and Moncollin, V. (1994) EMBO J. 13, 2393–2398
27. Feaver, W. J., Huang, W., and Friedberg, E. C. (1999) J. Biol. Chem. 274, 29564–29567
28. Keogh, M. C., Cho, R. J., Podolny, V., and Buratowski, S. (2002) Mol. Cell. Biol. 22, 1288–1297
29. Rigaud, G., Shvchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999) Nat. Biotechnol. 17, 1030–1032
30. Boeger, H., Griesenbeck, J., Struttan, J. S., and Kornberg, R. D. (2003) Mol. Cell 11, 1587–1598
31. Erdjument-Bromage, H., Lui, M., Lacomis, L., Grewal, A., Annan, R. S., McNulty, D. E., Carr, S. A., and Tempst, P. (1998) J. Chromatogr. Sect. A 826, 167–181
32. Sebastiaan Winkler, G., Lacomis, L., Philip, J., Erdjument-Bromage, H., Svejstrup, J. Q., and Tempst, P. (2002) Methods 26, 260–269
33. Svejstrup, J. Q., Feaver, W. J., and Kornberg, R. D. (1996) J. Biol. Chem. 271, 643–645