A stable complex of a novel transcription factor IIB-related factor, human TFIIIB50, and associated proteins mediate selective transcription by RNA polymerase III of genes with upstream promoter elements

Martin Teichmann, Zhengxin Wang, and Robert G. Roeder*

Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021

Contributed by Robert G. Roeder, October 31, 2000

Transcription factor IIIB (TFIIIB) is directly involved in transcription initiation by RNA polymerase III in eukaryotes. Yeast contain a single TFIIIB activity that is comprised of the TATA-binding protein (TBP), TFIIB-related factor 1 (BRF1), and TFIIIB″, whereas two distinct TFIIIB activities, TFIIIB-α and TFIIIB-β, have been described in human cells. Human TFIIIB-β is required for transcription of genes with internal promoter elements, and contains TBP, a TFIIIB″ homologue (TFIIIB150), and a BRF1 homologue (TFIIIB90), whereas TFIIIB-α is required for transcription of genes with promoter elements upstream of the initiation site. Here we describe the identification, cloning, and characterization of TFIIIB50, a novel homologue of TFIIB and TFIIIB90. TFIIIB50 and tightly associated factors, along with TBP and TFIIIB150, reconstitute human TFIIIB-α activity. Thus, higher eukaryotes, in contrast to the yeast *Saccharomyces cerevisiae*, have evolved two distinct TFIIB-related factors that mediate promoter selectivity by RNA polymerase III.

In the yeast *Saccharomyces cerevisiae*, all genes transcribed by RNA polymerase III depend on essential promoter elements that are localized downstream of the transcription initiation site. These promoter elements are recognized by the multisubunit transcription factor IIIC (TFIIIC) (or by TFIIIA and TFIIIC) and the resulting complex in turn recruits TFIIIB. Yeast TFIIIB activity has been assigned to the TATA-binding protein (TBP), TFIIB-related factor 1 (BRF1), and the TFIIIB″ component (1–3). These three components have been shown to be necessary and sufficient for reconstitution of yeast tRNA and U6 RNA gene transcription in appropriate cell-free systems (reviewed in refs. 4 and 5).

In human cells, two generally distinct types of promoters direct RNA polymerase III transcription. The promoters of tRNA, VA RNA, and 5S RNA genes are internal to the gene and consist of either A- and B-boxes (tRNA and VA RNA) or A- and C-boxes (5S RNA). In contrast, the promoters of U6 and 7SK RNA genes lie upstream of the transcription initiation site and consist of the distal sequence element, the proximal sequence element (PSE), and a TATA-box. Gene-internal promoters are recognized by TFIIIC (or by TFIIIA and TFIIIC in the case of the 5S RNA gene; reviewed in ref. 6), whereas the gene-external promoters are recognized by Oct-1 and the PSE transcription factor (reviewed in ref. 7). As a consequence, two distinct TFIIIB activities, designated hTFIIIB-α and hTFIIIB-β, function with TFIIIC-bound internal promoters or PSE transcription factor-bound external promoters, respectively (8). Human TFIIIB-β (hTFIIIB-β) has been reconstituted from recombinant human (rh)TBP, rhTFIIIB90 (related in sequence to TFIIB), and rhTFIIIB150 that is related to the yeast TFIIIB″ component (unpublished observations), whereas hTFIIIB-α is less well characterized. The hTFIIIB-α activity isolated by DEAE chromatography was shown to support U6, but not VA1, transcription in crude reconstituted systems (8). It then was demonstrated that this hTFIIIB-α activity could be replaced either by *S. cerevisiae* TFIIIB″ (9) or its human homologue rhTFIIIB150 (unpublished observations). These studies also showed that hTFIIIB-α activity is not stably associated with TBP (8) and that it does not contain hTFIIIB90 (9). In agreement, it was reported that hTFIIIB90 is dispensable for, or even represses, U6 or 7SK transcription (ref. 10; unpublished observations). Recently, McCulloch and colleagues (11) reported the identification of a differentially spliced variant of hTFIIIB90 (BRF2) that was reported, as part of a poorly characterized immunopurified complex, to reconstitute U6 transcription in a nuclear extract that had been depleted with anti-BRF2 antibodies. This result suggested that BRF2 might be the TFIIB-related protein that is required for RNA polymerase III transcription of genes with upstream promoter elements.

Here we report the identification, cloning, and characterization of TFIIIB50, a protein with sequence homology to TFIIB and TFIIIB90. A complex of TFIIIB50 and four tightly associated factors constitutes, together with TBP and TFIIIB150, the complete TFIIIB-α activity that is required for transcription of U6 or 7SK genes in vertebrates. During preparation of this manuscript, Hernandez and colleagues (12) reported the cloning of a factor, designated BRFU, that is identical to hTFIIIB50 (12).

Materials and Methods

Plasmids. ph7SK and ptRNA have been described (6, 13).

Reconstituted *in Vitro* Transcription System. cEDF 0.2 and cEDF 1 M KCl fractions were obtained by chromatography of the phosphocellulose P11 0.6 fraction (fraction C) over EMD-DEAE-Fractogel (EDF; Merck) and elution with 200 mM and 1 M KCl, respectively, in BC buffer (20 mM Hepes, pH 7.9/10% glycerol/3mM DTT/0.2 mM PMSF). Of the known RNA polymerase III transcription factor activities, the cEDF 0.2 fraction contains TFIIIC0, TFIIIC1 (14, 15), and the TFIIIB50 complex described here, whereas the cEDF 1 M fraction contains the PSE transcription factor (16), TFIIIC2, and RNA polymerase III

Abbreviations: TBP, TATA-binding protein; TF, transcription factor; hTF, human TF; rh, recombinant human; EDF, EMD-DEAE-Fractogel; cEDF, EDF fraction C; HA, hemagglutinin; fHA, FLAG-HA.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF206673).

*To whom reprint requests should be addressed. E-mail: roeder@rockvax.rockefeller.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Fig. 1. Inhibition of 7SK transcription by anti-hTFIIIB90 antibodies. Transcription reactions in lanes 1–5 were reconstituted with 24 ng rhTFIIIB150 and 40 ng rhTBP. The following protein fractions were added to individual reactions: lanes 1 and 3–5, 1.5 μl cEDF 1 M; lanes 2 and 3, 20 μl cEDF 0.2; lane 4, 20 μl flow-through (FT) from anti-hTFIIIB90 antibodies that had been incubated with a cEDF 0.2 fraction; and lane 5, 20 μl flow-through from nonrelevant antibodies that had been incubated with a cEDF 0.2 fraction. Immunodepletion is described in *Materials and Methods*. Transcription reactions were performed as described (13).

itself (6). Expression and purification of rhTBP and TFIIIIB150 were as described (ref. 17; unpublished observations).

Purification of the FLAG/Hemagglutinin (HA)-hTFIIIB50 Complex. The generation of a HeLa cell line that stably expresses FLAG- and HA-tagged hTFIIIB50 was as described (13). The complex was purified essentially as described (13) except that 500 mM KCl was used for incubation of the extracts with M2 agarose and for subsequent wash steps. The complex was eluted from M2 agarose by incubation with 200 ng/μl FLAG peptide in BC buffer containing 60 mM KCl for 30 min at room temperature.

Peptide Sequences. Proteins were blotted onto poly(vinylidene difluoride) membrane after SDS/4–20% PAGE, excised, and digested with LysC. The following internal peptide sequences were obtained: p30 (calcyclin-binding protein), PAAVVAPIT-TGYTVK; p40, (S)YADSYYYEDGGMK and (D)LEQQDC-EIAQEIQEK; p50α (elongation factor 1α), XGDAAIVDM-VP(G)K; and p50β (hTFIIIB50), RPASPALLLPPCMLK.

Results

We showed previously that whereas anti-hTFIIIB90 antibodies deplete VA, tRNA, 5S, U6, and 7SK transcription activity from nuclear extracts, readdition of rhTFIIIB90 is able to restore transcription from VA, tRNA, and 5S genes but not U6 or 7SK genes (18). This finding led us to search for a protein that was depleted by anti-hTFIIIB90 antibodies and was required for U6/7SK transcription. For this purpose, we established a 7SK transcription system comprised of partially purified fractions (*Materials and Methods*) and determined by immuno-depletion which fraction contained such a factor.

Anti-hTFIIIB90 Antibodies Deplete an Activity from the cEDF 0.2 Fraction That Is Essential for 7SK Transcription. The cEDF 0.2 fraction was found to contain a factor that is essential for 7SK transcription and that could be specifically depleted by anti-hTFIIIB90 antibodies but not by control antibodies (Fig. 1, compare lanes 3–5). The corresponding Western blot showed that the cEDF 0.2 fraction does not contain any detectable hTFIIIB90 (Fig. 2*A*, lane 1), suggesting that a protein different from hTFIIIB90 was depleted from the cEDF 0.2 fraction. This surprising finding led us to search for the protein(s) that had

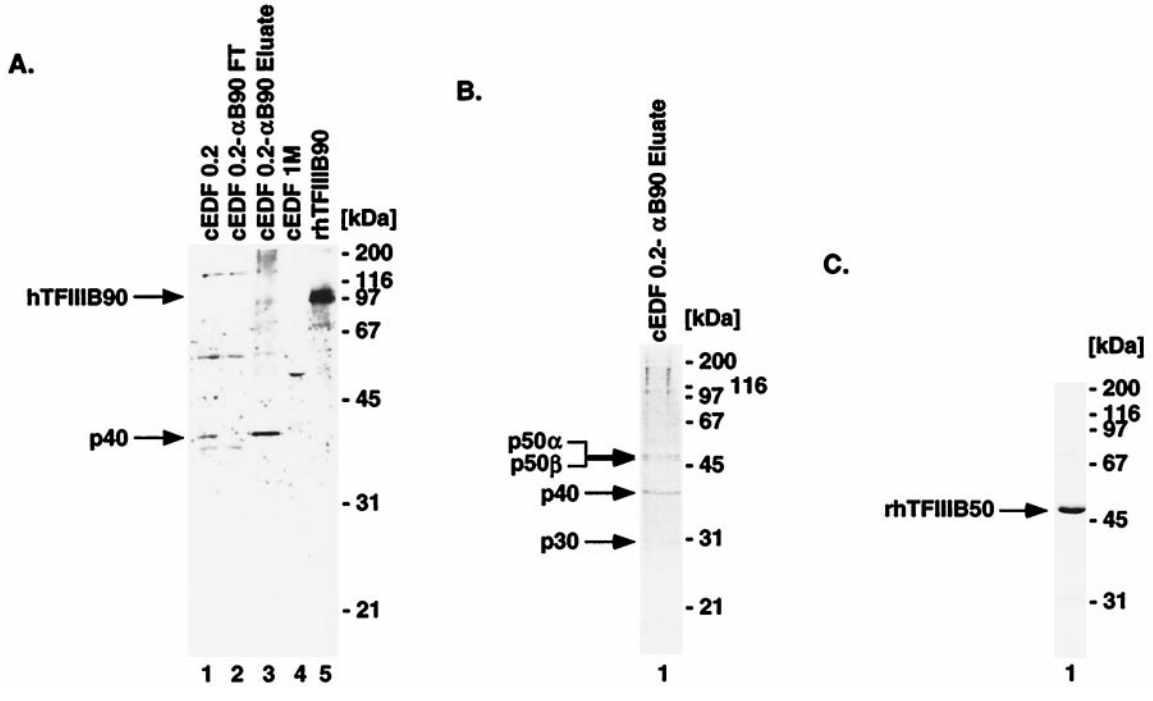

Fig. 2. Purification of hTFIIIB50 from HeLa cells and expression of the recombinant protein in *E. coli*. (*A*) Western blot with anti-hTFIIIB90 antibodies. The following fractions were separated by SDS/12.5% PAGE: lane 1, 10 μl cEDF 0.2; lane 2, 10 μl flow-through (FT) from the anti-hTFIIIB90 antibody column, onto which a cEDF 0.2 fraction had been loaded; lane 3, 10 μl eluate from the anti-hTFIIIB90 antibody depletion; lane 4, 1.5 μl cEDF 1 M; and lane 5, 2.5 ng rhTFIIIB90. cEDF fractions and rhTFIIIB90 were purified as described (*Materials and Methods*; ref. 18). SDS/PAGE and Western blot procedures were essentially as described (8). (*B*) SDS/12.5% PAGE. Ten microliters of protein fractions that were retained from a cEDF 0.2 fraction by the anti-hTFIIIB90 antibody column and eluted with 0.1 M glycine, pH 2.6 was neutralized and separated by SDS/PAGE and stained with Coomassie brilliant blue (8). (*C*) SDS/10% PAGE. Two micrograms of recombinant Ni-NTA agarose purified hTFIIIB50 was separated by SDS/PAGE. Purification of the recombinant protein and SDS/PAGE were as described (8).

BIOCHEMISTRY

been depleted by the anti-hTFIIIB90 antibodies and that were required for 7SK transcription. Antibodies against hTFIIIB90 predominantly and specifically retained proteins of 30 and 40 kDa as well as a doublet of 50-kDa proteins from the cEDF 0.2 fraction, and these proteins could be eluted with 0.1 M glycine, pH 2.6 (Fig. 2*B*; data not shown). Of these proteins, only a 40-kDa polypeptide was recognized by anti-hTFIIIB90 antibodies in a Western blot analysis (Fig. 2*A*, lane 3). Although this protein band appears irrelevant for 7SK/U6 transcription (below), its recognition by anti-hTFIIIB90 antibodies has been confirmed in experiments with the corresponding recombinant protein (data not shown). Moreover, although anti-hTFIIIB90 antibodies apparently recognize and coimmunoprecipitate a TFIIIB90-related 50-kDa protein in the cEDF 0.2 fraction (below), they do not appear to recognize this protein after denaturation and Western blot analysis (Fig. 2*A*).

Identification of hTFIIIB50. We obtained internal protein sequences of tryptic peptides from the four major protein bands that could be eluted from anti-hTFIIIB90 immunoprecipitates and that were identified on SDS/PAGE (*Materials and Methods*). Peptide sequences from the 30-kDa protein (p30) matched the sequence of the calcyclin-binding protein (CACYBP; GenBank accession no. GI7656952), those of the 40-kDa protein (p40) matched expressed sequence tags coding for a protein of unknown function (p40; GenBank accession nos. AA101892, AI111898, AI180527, and AA835673), and those from one of the 50-kDa protein bands matched translation elongation factor 1α (EF1α; GenBank accession no. GI1070665). However, neither the corresponding recombinant proteins (CACYBP, p40, and EF1α) nor the cognate antibodies showed any effect on, and appear to be irrelevant to, *in vitro* transcription of the 7SK/U6 genes (data not shown).

Peptide sequences from the other 50-kDa protein matched a human expressed sequence tag (EST; GenBank accession no. AI862404) but no known protein. Overlapping human ESTs (GenBank accession nos. AI862404, AI640304, AA442131, W68566, AA456203, Z19140, and T09256) encoded a protein of 419 aa that showed about 20% identity and 42% homology to both hTFIIB and TFIIIB90 within its N-terminal 231 aa. After cloning of the full-length cDNA, the full-length protein was expressed in *Escherichia coli*, purified (Fig. 2*C*), and used to raise polyclonal antibodies. As shown in Fig. 3, antibodies against this protein (designated hTFIIIB50 on the basis of subsequent analysis) specifically recognized a protein of 50 kDa in the cEDF 0.2 fraction (lane 1 in Fig. 3 *A* and *B*). As expected, hTFIIIB50 was specifically depleted from this fraction either with antibodies against hTFIIIB90 (Fig. 3*A*, lane 3) or antibodies against hTFIIIB50 (Fig. 3*B*, lane 2) but not with control antibodies (Fig. 3*A*, lane 2). Consistent with earlier results, hTFIIIB50 was found, by Western blot analyses with anti-hTFIIIB50 antibodies, in the 0.1 M glycine/pH 2.6 eluates from both anti-hTFIIIB90 and anti-hTFIIIB50 immunoprecipitates (Fig. 3 *A*, lane 4 and *B*, lane 3).

hTFIIIB50 Is Specifically Required for RNA Polymerase III Transcription of Genes with Upstream Promoter Elements. Because antibodies against hTFIIIB90 depleted hTFIIIB50 from the cEDF 0.2 fraction (Fig. 3*A*, lane 3) and at the same time depleted 7SK gene transcription activity from this fraction (Fig. 1, lane 4), we determined whether anti-hTFIIIB50 antibodies were similarly able to deplete 7SK gene transcription activity from the cEDF 0.2 fraction. As shown in Fig. 4, depletion of hTFIIIB50 from a cEDF 0.2 fraction severely inhibited transcription of the 7SK gene (*Upper*, compare lanes 2 and 3 and lanes 4 and 5). In contrast, depletion of hTFIIIB50 from the same cEDF 0.2 fraction did not inhibit, but instead slightly stimulated, tRNA transcription in a system reconstituted with partially purified

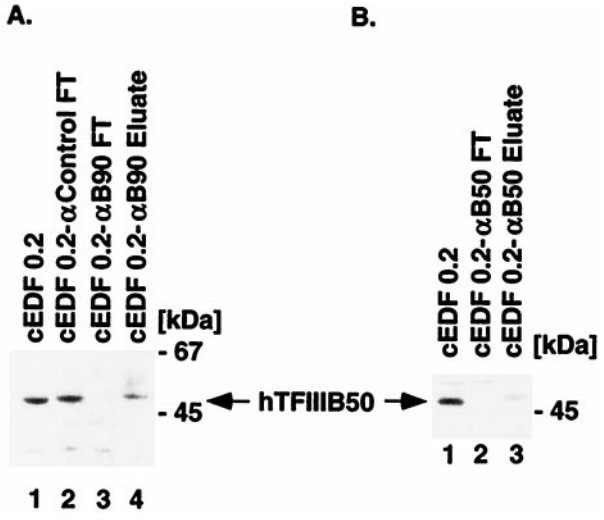

Fig. 3. Depletion of hTFIIIB50 from a partially purified fraction (cEDF 0.2) using antibodies against either hTFIIIB90 or hTFIIIB50. (*A*) Ten microliters of cEDF 0.2 (lane 1), preimmune control antibody flow-through (FT, lane 2), anti-hTFIIIB90 antibody flow-through (lane 3), or anti-hTFIIIB90 eluate (lane 4) was separated by SDS/12.5% PAGE and transferred onto nitrocellulose. A 1:10,000 dilution of anti-hTFIIIB50 antibody in PBS-0.05% Tween 20 and 5% skim milk powder was used for Western blot analyses that were performed as described (8). (*B*) Ten microliters of cEDF 0.2 (lane 1), anti-hTFIIIB50 antibody flow-through from cEDF 0.2 (lane 2) or anti-hTFIIIB50 eluate from cEDF 0.2 (lane 3) was separated by SDS/12.5% PAGE and transferred onto nitrocellulose. SDS/PAGE and Western blot procedures were described (*A*; ref. 8).

transcription factors (Fig. 4 *Lower*, compare lanes 2 and 3 and lanes 4 and 5). This result clearly indicates a specific requirement for hTFIIIB50 in transcription of genes with upstream promoter elements by RNA polymerase III.

A Complex of hTFIIIB50 and Stably Associated Factors Is Required for 7SK/U6 Transcription. We next tested the ability of increasing amounts of bacterially expressed and affinity-purified rhTFIIIB50 to restore 7SK transcription activity to the reconstitution system that had been depleted of hTFIIIB50. Surpris-

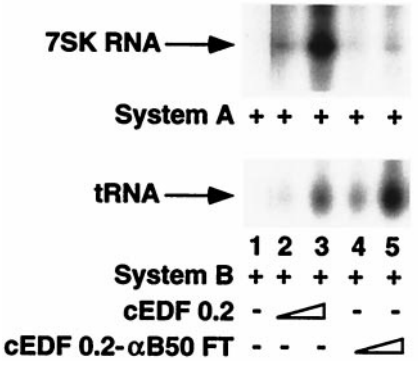

Fig. 4. hTFIIIB50 is specifically required for RNA polymerase III transcription of genes with upstream promoter elements. (*Upper*) Transcription of the 7SK gene. (*Lower*) Transcription of the human $tRNA^{MET}$ gene. System A was comprised of 1.5 μl cEDF 1 M fraction, 40 ng rhTBP, and 24 ng rhTFIIIB150. System B consisted of 1.5 μl cEDF 1 M fraction and 5 μl B-EDF 0.3 fraction (unpublished observations). The following fractions were added: lane 1, none; lanes 2 and 3, 5 and 10 μl cEDF 0.2, respectively; and lanes 4 and 5, 5 and 10 μl of flow-through (FT), respectively, of an anti-hTFIIIB50 antibody column onto which a cEDF 0.2 fraction had been loaded. Purification of the cEDF fractions and antibody depletion procedures are described in *Materials and Methods*.

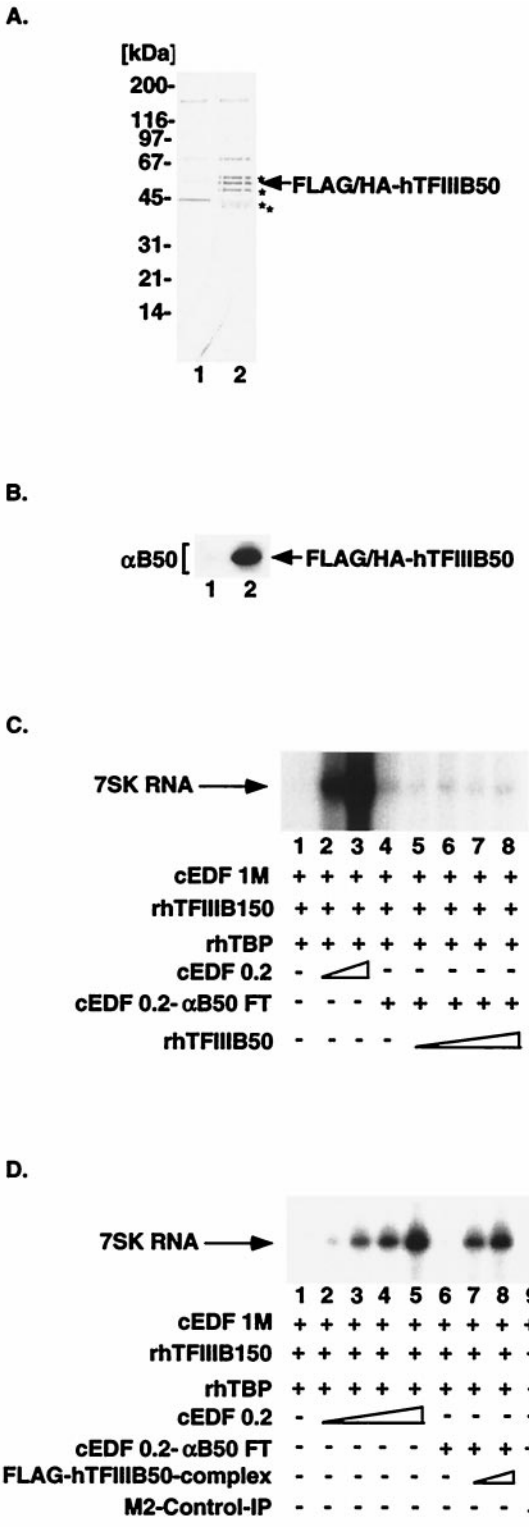

Fig. 5. A stable complex of hTFIIIB50 and associated factors of 54, 48, 42, and 40 kDa is required for transcription of the 7SK gene. (*A*) SDS/4–20% PAGE of 10 μl M2 eluates purified from nontransfected HeLa cells (lane 1) or HeLa cells that stably express hTFIIIB50 (lane 2). Construction of a HeLa cell line stably expressing fHA-tagged hTFIIIB50, as well as purification procedures on M2 agarose, were essentially as described (13). Purification of the hTFIIIB50 complex was performed in the presence of 500 mM KCl in BC buffer containing 0.05% NP-40. SDS/PAGE and silver stain were described (18). (*B*) Western blot with anti-hTFIIIB50 antibodies. Ten microliters M2 eluates purified from nontransfected HeLa cells (lane 1) or HeLa cells that stably express hTFIIIB50 (lane 2) was separated by SDS/5–20% PAGE and transferred onto nitrocellulose. SDS/PAGE and Western blot procedures were described (8). (*C*) Transcription of the 7SK gene. Transcription reactions were reconstituted with 1.5 μl cEDF 1 M, 24 ng rhTFIIIB150, and 40 ng rhTBP. The following fractions were added: lane 1, none; lanes 2 and 3, 10 and 20 μl cEDF 0.2, respectively; lanes 4–8, 20 μl flow-through (FT) of an anti-hTFIIIB50 antibody column onto which a cEDF 0.2 fraction had been loaded; and lanes 5–8, 5, 10, 20, and 40 ng of Ni-NTA agarose-purified rhTFIIIB50, respectively. Ni-NTA agarose-purified rhTFIIIB50 is shown in Fig. 2*C*. Transcription reactions were performed essentially as described (13). The film was heavily overexposed to analyze whether rhTFIIIB50 shows any residual activity in this assay system. (*D*) Transcription of the 7SK gene. Transcription reactions were reconstituted with 1.5 μl cEDF 1 M, 24 ng rhTFIIIB150, and 40 ng rhTBP. The following fractions were added to the reactions: lane 1, none; lanes 2–5, 2.5, 5, 10, and 20 μl cEDF 0.2, respectively; and lanes 6–10, 20 μl flow-through of an anti-hTFIIIB50 antibody column onto which a cEDF 0.2 fraction had been loaded. Reactions in lanes 7 and 8 or 9 and 10 contained in addition 10 or 20 μl M2 eluate, respectively, purified from HeLa cells that stably express fHA-hTFIIIB50 (lanes 7 and 8) or from control HeLa cells (lanes 9 and 10).

ingly, rhTFIIIB50 alone was not able to restore 7SK gene transcription activity to the immuno-depleted reconstitution system (Fig. 5*C*, lanes 4–8). Because modification of hTFIIIB50 or stable association with additional transcription factors were possible explanations for the inability of rhTFIIIB50 to restore transcription, we generated HeLa S cell lines that stably express epitope-tagged hTFIIIB50. We were able to purify a complex of hTFIIIB50 and associated factors from a cell line stably expressing N-terminally FLAG- and HA-tagged hTFIIIB50 (fHA-B50). fHA-B50 was retained by M2 agarose (containing anti-FLAG antibody) from extracts of HeLa cells stably expressing fHA-B50 and could be specifically eluted by FLAG peptide (Fig. 5 *A* and *B*, lane 2), but no hTFIIIB50 was retained by (or eluted from) M2 agarose with extracts from nontransfected HeLa cells (Fig. 5 *A* and *B*, lane 1). In addition to fHA-B50, proteins of 54, 48, 42, and 40 kDa were selectively retained by and eluted with FLAG peptide from M2 agarose that had been incubated with extracts from HeLa cells that stably express fHA-B50. These proteins were not present in M2 agarose-bound and eluted fractions from control cells not expressing fHA-B50 (Fig. 5*A*, compare lanes 1 and 2). The complex of fHA-B50 and associated factors then was analyzed for its ability to reconstitute transcription in a system of partially purified fractions that had been immuno-depleted of hTFIIIB50. The fHA-B50 complex, but not a preparation from a control cell line, was able to fully restore 7SK transcription in this reconstitution system (Fig. 5*D*, compare lanes 7 and 8 with lanes 9 and 10).

These results show that hTFIIIB50 forms a stable complex with associated proteins that, together with hTBP and hTFIIIB150, reconstitutes full hTFIIIB-α activity for transcription of U6/7SK genes in humans.

Discussion

Gene selectivity through structural variations in the general transcription machinery was first evident from identification of functionally distinct nuclear RNA polymerases with common, related, and distinct subunits (19), and subsequently extended through the demonstration of TBP-related factors (20) and TFIIB-related factors (21) with altered specificity within the same organism. The distinct hTFIIIB activities (hTFIIIB-α and hTFIIIB-β) that were described earlier (8) are now understood at the molecular level and reflect, minimally, the presence of distinct TFIIB-related proteins from distinct genes. Thus, it is clear that the evolution from yeast to humans of promoter elements within the genes transcribed by RNA polymerase III has been accompanied by the coevolutionary appearance of an additional TFIIB-related factor. The diversified set of promoter elements and the corresponding increase in complexity of DNA-binding transcription factors (Introduction) might allow or re-

BIOCHEMISTRY

quire the utilization of distinct TFIIB-related factors for achieving a more sophisticated regulation of RNA polymerase III transcription in vertebrates than in the unicellular eukaryote *S. cerevisiae*.

Complete hTFIIIB-α Activity Is Composed of TBP, hTFIIIB150, and a Complex of hTFIIIB50 and Associated Proteins. In this report, we describe the molecular composition of hTFIIIB-α activity, as it appears to be required for *in vitro* transcription of 7SK and U6 genes. The data confirm, at the molecular level, the existence of two distinct hTFIIIB activities as previously shown (8). According to data presented here, the complete hTFIIIB-α activity is composed of TBP, hTFIIIB150 (related to the *S. cerevisiae* TFIIIB′′), and a complex containing both a novel TFIIB-related factor (hTFIIIB50) and at least four stably associated proteins. The nature of the reconstitution system used in earlier studies of the hTFIIIB-α activity (8, 9) only monitored the hTFIIIB150 activity, because all of the other components of complete hTFIIIB-α also were provided by the reconstituted transcription system. Nevertheless, the originally purified hTFIIIB-α fractions also contain hTFIIIB50 in addition to hTFIIIB150 (data not shown). Whether they also contain the hTFIIIB50-associated factors described here is not yet known. However, the association of TBP with hTFIIIB-α activity appears to be weak, and it has not been clarified which source of TBP is required for transcription of U6/7SK genes *in vivo*. Recently, a complex of TBP and TFIIA has been described that could have such a function (22).

TFIIIB50 Forms a Stable Complex with Associated Factors in HeLa Cells. hTFIIIB-α and TFIIIB-β activities contain TBP, hTFIIIB150, and a specific TFIIB-related factor, and are thus generally conserved in structure from yeast to human. The individual TFIIB-related factors (hTFIIIB90 and hTFIIIB50) specific to hTFIIIB-β versus hTFIIIB-α are likely involved in conferring promoter specificity to RNA polymerase III, but also might depend on associated factors for doing so. In HeLa cells, hTFIIIB90 forms an extremely stable complex with TBP, whereas other proteins (e.g. TFIIIB150) seem to be either transiently or loosely associated with hTFIIIB90 (data not shown). In sharp contrast, hTFIIIB50 forms a highly stable complex with four associated proteins, and this complex is stable to immuno-purification in the presence of high salt (500 mM KCl; Fig. 5*A*, lane 2). Furthermore, immuno-depletion of hTFIIIB50 from the cEDF 0.2 fraction in the presence of 2 M urea with polyclonal anti-hTFIIIB50 antibodies abolishes transcription of the 7SK gene, and restoration of transcription activity is not mediated by rhTFIIIB50 but requires the complex of hTFIIIB50 and associated factors (data not shown). One explanation for these observations is that the hTFIIIB50 complex is very stable and resists dissociation by incubation with high salt or 2 M urea. Alternatively, it might be that antibody treatment disrupts the complex of hTFIIIB50 and associated factors under certain circumstances and that the complex is unable to reform upon subsequent addition of rhTFIIIB50. Whatever the explanation, rhTFIIIB50 alone is unable to restore transcription of the 7SK/U6 gene under our assay conditions.

Surprisingly, however, these results are in sharp contrast to those of Hernandez and coworkers (12). They reported depletion of hTFIIIB50 (BRFU) from whole-cell extracts with a peptide antibody (CS1043) directed against amino acids 229–248 of hTFIIIB90 (BRF). As expected, this antibody was able to deplete VA1 and U6 transcription activity from the extracts but, in contrast to the results presented here, those authors reported an ability to restore U6 transcription by addition of rhBRFU (TFIIIB50) alone. A possible explanation might be that the peptide antibody used by Hernandez and coworkers is disruptive to the hTFIIIB50 complex whereas our polyclonal anti-hTFIIIB90 and anti-hTFIIIB50 antibodies, even under high-salt or partially denaturing (2 M urea) conditions, are not. Also surprisingly and in contrast to our results, the antipeptide hTFIIIB90 antibody (CS1043) of Hernandez and coworkers was reported to recognize rhTFIIIB50 in Western blot analyses whereas our polyclonal antibody, even though raised against a part of hTFIIIB90 containing the region (amino acids 229–248) used for the production of the peptide antibody of Hernandez and coworkers, failed to do so. These discrepancies need to be addressed in further experiments to clarify why similar reagents show different results in comparable experiments.

The Proteins Associated with hTFIIIB50. The cDNAs encoding the proteins that form a complex with hTFIIIB50 have not yet been cloned. Hence we do not know whether these proteins are completely distinct or potentially related. The fact that the smaller (42 and 40 kDa) polypeptides appear to be substoichiometric may reflect derivitization (e.g., by proteolysis) from larger components, differential staining properties, or differential cellular levels of these proteins. We originally had identified hTFIIIB50 by an immuno-purification procedure with anti-hTFIIIB90 antibodies that also yielded proteins of 30 kDa (calcyclin-binding protein), 40 kDa, and 50 kDa (elongation factor 1α). However, these proteins were not detected in the fHA-hTFIIIB50-complex by Western blot analyses (data not shown), consistent with their failure to show any effects on 7SK gene transcription.

Finally, we have not been able to ascertain whether the hTFIIIB50 complex described here also contains the 23-kDa hTFIIIB90 splice variant (BRF2) reported by McCulloch *et al.* (11) to function in U6 transcription. A circa 27-kDa protein has been observed in some preparations of the hTFIIIB50 complex, but a possible relationship to BRF2 remains to be determined. It is also not clear from the data of McCulloch *et al.* (11) whether the active U6 transcription component in the low stringency anti-BRF2 immuno-precipitate is BRF2 or another associated component such as hTFIIIB50. In this regard it should be noted that BRF2 does not contain all of the structural elements that are indicative of TFIIB-related proteins (zinc finger, repeat 1 and repeat 2), being comprised only of part of the second direct repeat. Thus, BRF2 might possibly be involved in hTFIIIB50 complex assembly without itself representing the TFIIB-related transcriptional activity.

This work has been supported by National Institutes of Health Grant CA42567 to R.G.R. and postdoctoral fellowships from the Deutsche Forschungsgemeinschaft (DFG) and the Leukemia Research Foundation to M.T.

1. Kassavetis, G. A., Nguyen, S. T., Kobayashi, R., Kumar, A., Geiduschek, E. P. & Pisano, M. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 9786–9790.
2. Ruth, J., Conesa, C., Dieci, G., Lefebvre, O., Dusterhoft, A., Ottonello, S. & Sentenac, A. (1996) *EMBO J.* **15,** 1941–1949.
3. Roberts, S., Miller, S. J., Lane, W. S., Lee, S. & Hahn, S. (1996) *J. Biol. Chem.* **271,** 14903–14909.
4. Kumar, A., Grove, A., Kassavetis, G. A. & Geiduschek, E. P. (1998) *Cold Spring Harb. Symp. Quant. Biol.* **63,** 121–129.
5. Chedin, S., Ferri, M. L., Peyroche, G., Andrau, J. C., Jourdain, S., Lefebvre, O., Werner, M., Carles, C. & Sentenac, A. (1998) *Cold Spring Harb. Symp. Quant. Biol.* **63,** 381–389.
6. Wang, Z., Luo, T. & Roeder, R. G. (1997) *Genes Dev.* **11,** 2371–2382.
7. Paule, M. R. & White, R. J. (2000) *Nucleic Acids Res.* **28,** 1283–1298.
8. Teichmann, M. & Seifart, K. H. (1995) *EMBO J.* **14,** 5974–5983.
9. Teichmann, M., Dieci, G., Huet, J., Ruth, J., Sentenac, A. & Seifart, K. H. (1997) *EMBO J.* **16,** 4708–4716.
10. Mital, R., Kobayashi, R. & Hernandez, N. (1996) *Mol. Cell. Biol.* **16,** 7031–7042.
11. McCulloch, V., Hardin, P., Peng, W., Ruppert, J. M. & Lobo-Ruppert, S. M. (2000) *EMBO J.* **19,** 4134–4143.
12. Schramm, L., Pendergrast, P. S., Sun, Y. & Hernandez, N. (2000) *Genes Dev.* **14,** 2650–2663.

13. Teichmann, M., Wang, Z., Martinez, E., Tjernberg, A., Zhang, D., Vollmer, F., Chait, B. T. & Roeder, R. G. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 13720–13725.
14. Oettel, S., Hartel, F., Kober, I., Iben, S. & Seifart, K. H. (1997) *Nucleic Acids Res.* **25,** 2440–2447.
15. Yoshinaga, S. K., Boulanger, P. A. & Berk, A. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3585–3589.
16. Yoon, J. B. & Roeder, R. G. (1996) *Mol. Cell. Biol.* **16,** 1–9.
17. Ge, H., Martinez, E., Chiang, C. M. & Roeder, R. G. (1996) *Methods Enzymol.* **274,** 57–71.
18. Wang, Z. & Roeder, R. G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7026–7030.
19. Sentenac, A. (1985) *CRC Crit. Rev. Biochem.* **18,** 31–90.
20. Dantonel, J. C., Wurtz, J. M., Poch, O., Moras, D. & Tora, L. (1999) *Trends Biochem. Sci.* **24,** 335–339.
21. Colbert, T. & Hahn, S. (1992) *Genes Dev.* **6,** 1940–1949.
22. Mitsiou, D. J. & Stunnenberg, H. G. (2000) *Mol. Cell* **3,** 527–537.