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RNA Polymerase III Transcription from the Human U6 and Adenovirus Type 2 VAI Promoters Has Different Requirements for Human BRF, a Subunit of Human TFIIIB

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Mammalian TFIIIB can be separated into two fractions required for transcription of the adenovirus type 2 VAI gene, which have been designated 0.38M-TFIIIB and 0.48M-TFIIIB. While 0.48M-TFIIIB has not been characterized, 0.38M-TFIIIB corresponds to a TBP-containing complex. We describe here the purification of this complex, which consists of TBP and a closely associated polypeptide of 88 kDa, and the isolation of a cDNA corresponding to the 88-kDa polypeptide. The predicted protein sequence reveals that the 88-kDa polypeptide corresponds to a human homolog of the *Saccharomyces cerevisiae* BRF protein, a subunit of yeast TFIIIB. Human BRF (hBRF) probably corresponds to TFIIIB90, a protein previously cloned by Wang and Roeder (Proc. Natl. Acad. Sci. USA 92:7026–7030, 1995), although its predicted amino acid sequence differs from that reported for TFIIIB90 over a stretch of 67 amino acids as a result of frameshifts. Immunodepletion of more than 90 to 95% of the hBRF present in a transcription extract severely debilitates transcription from the tRNA-type VAI promoter but does not affect transcription from the TATA box-containing human U6 promoter, suggesting that the 0.38M-TFIIIB complex, and perhaps hBRF as well, is not required for U6 transcription.

The RNA polymerase III promoters can be divided into three classes that differ both in structure and in the nature of the basal transcription factors they recruit (see references 12, 21, and 53 for reviews). Class 1 and 2 promoters are intragenic and are exemplified by the 5S RNA and tRNA promoters, respectively. The class 1 promoters recruit the transcription factor TFIIIA, the founding member of the C_2H_2 zinc finger family of DNA-binding proteins (37; see references 1 and 8 for reviews). The binding of TFIIIA then allows the recruitment of TFIIIC, an activity that contains a large protein complex, of which two forms with slightly different subunit compositions have been purified (9, 26, 58, 59). Class 2 promoters consist of gene-internal A and B boxes which, unlike the 5S promoters, recruit TFIIIC directly, without prior binding of TFIIIA. In both classes of promoters, the recruitment of TFIIIC is followed by the recruitment of TFIIIB, which in *Saccharomyces cerevisiae* corresponds to a well-characterized TBP-containing complex (see references 12, 17, and 21 for reviews) that appears to contact RNA polymerase III directly. Indeed, once *S. cerevisiae* TFIIIB has been assembled onto the promoter with the help of TFIIIA and/or TFIIIC, TFIIIA and TFIIIC can be removed from the DNA by treatment with a high level of salt or heparin; TFIIIB remains associated with the DNA through an apparently nonionic interaction and is sufficient to direct several rounds of RNA polymerase III transcription (23).

Class 3 promoters are defined as entirely extragenic RNA polymerase III promoters and are exemplified by the human U6 small nuclear RNA (snRNA) promoter (see references 21 and 30 for reviews). The human U6 promoter is located entirely upstream of the U6 snRNA coding region. The basal promoter consists of a proximal sequence element (PSE) located between nucleotides −65 and −48 and a TATA box located between nucleotides −31 and −24 upstream of the transcription start site. The PSE recruits the snRNA-activating protein complex ($SNAP_c$) (16, 44), also referred to as the PSE-binding transcription factor (39, 56), to the promoter. The $SNAP_c$/PSE-binding transcription factor consists of at least four subunits we refer to as SNAP43, SNAP45, SNAP50, and SNAP190 and binds the TATA box-binding protein TBP (16, 43, 56, 57). The TATA box also binds TBP, and the binding of $SNAP_c$ to the PSE and of TBP to the TATA box is highly cooperative (14, 37a). Consistent with its structure, the human U6 promoter does not appear to recruit TFIIIA or TFIIIC (40, 51).

In contrast to the human U6 promoter, the *S. cerevisiae* U6 promoter contains both gene-external and gene-internal elements. Like the human U6 promoter, it contains a TATA box located upstream of the RNA-coding region, but unlike the human U6 promoter, it does not contain a PSE. Instead, it contains a B box similar to the gene-internal B box of class 2 promoters but located in the 3′ flanking region of the gene, as well as a gene-internal A box (2, 5, 10). The *S. cerevisiae* U6 gene requires the B box and TFIIIC for transcription in vivo, although in vitro TFIIIC is dispensable and the TATA box can recruit TFIIIB directly (2, 4, 13, 19, 34, 38, 54).

S. cerevisiae TFIIIB is well defined. It consists of three components: TBP; the 67-kDa TFIIB-related factor BRF, encoded by the *BRF1*/*TDS4*/*PCF4* gene (3, 7, 33); and the B″ fraction, which contains the 90-kDa TFC5 protein, encoded by the *TFC5* gene (24, 42). Both the TFIIIC-dependent and the TFIIIC-independent (but TATA box-dependent) pathways for assembly of TFIIIB on *S. cerevisiae* RNA polymerase III promoters have been characterized in detail and involve all three subunits of TFIIIB (18–20, 54). Thus, in *S. cerevisiae*, the same TFIIIB complex is recruited by the TATA box-containing U6 snRNA promoter and the TATA-less class 1 and 2 promoters.

The composition of mammalian TFIIIB is less clear, but there are indications that some TATA box-containing and TATA-less RNA polymerase III promoters may use different forms of TFIIIB. First, we observed that in a TBP-depleted nuclear extract, transcription from the human U6 snRNA promoter could be reconstituted by addition of only recombinant TBP, whereas transcription from the adenovirus type 2 (Ad2)

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VAI promoter, a class 2 RNA polymerase III promoter, was reconstituted only by addition of a partially purified TBP-containing complex (designated 0.38M-TFIIIB) present in the TFIIIB fraction (31). This observation suggested that the 0.38M-TFIIIB TBP-containing complex is required for VAI but not for U6 gene transcription. Second, Teichmann and Seifart (50) have separated two forms of TFIIIB, TFIIIB-α and TFIIIB-β, whose composition is not known but which are active for transcription of the U6 and VAI genes, respectively (50). Third, Wang and Roeder (52) have isolated a cDNA encoding a human homolog of yeast BRF, which they refer to as TFIIIB90 (52). They observe that immunodepletions of TFIIIB90 inhibit transcription from both the VAI and the U6 promoters but that only VAI transcription can be reconstituted by addition of recombinant TBP and TFIIIB90, suggesting that TFIIIB90 and/or an associated factor different from TBP is required for transcription of the U6 gene.

Here, we report the purification of a protein closely associated with TBP in the TFIIIB fraction and the cloning of corresponding cDNAs. The protein sequence is identical to that of TFIIIB90 except for a segment of 67 consecutive and a few scattered amino acid differences. To avoid confusion with the 90-kDa TFC5 subunit of yeast TFIIIB, we refer to this protein as human BRF (hBRF), in accordance with one of the original names of its yeast homolog (7). Immunodepletions with anti-hBRF antibodies, which remove more than 90 to 95% of the hBRF present in extracts, severely debilitate VAI transcription but have no effect on U6 transcription. Further, after immunodepletions with anti-TBP antibodies, which remove more than 95% of the hBRF present in extracts, U6 but not VAI transcription can be reconstituted by addition of only recombinant TBP. These results suggest that, unlike for *S. cerevisiae*, in which the same three TFIIIB subunits are required for transcription from TATA box-containing and TATA-less RNA polymerase III promoters (18–20, 54), the hBRF-TBP complex, and perhaps hBRF by itself, is not required for transcription from the human U6 promoter.

MATERIALS AND METHODS

Purification of hBRF. The phosphocellulose B fraction was prepared by fractionation of a cytoplasmic extract over a phosphocellulose P11 (Whatman) column (see, for example, reference 41) and was the generous gift of Danny Reinberg and colleagues. The phosphocellulose B fraction was dialyzed against buffer D (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]–KOH [pH 7.9] at room temperature, 0.2 mM EDTA, 20% glycerol, 100 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 3 mM dithiothreitol [DTT]). The Mono S fractions used in Fig. 2A, 2B, 5, 6A, and 8A were obtained as follows. One hundred twenty milliliters of the phosphocellulose B fraction (840 mg of protein) was loaded onto a HiLoad 26/10 Q Sepharose column (Pharmacia) equilibrated in buffer Q (20 mM HEPES–KOH [pH 7.9] at room temperature, 10 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 0.01% Tween 20, 0.5 mM PMSF, 1 mM benzamidine, 1 mM sodium bisulfite, 1 μM pepstatin, 3 mM DTT) containing 100 mM KCl. The column was washed with five column volumes of buffer Q with 100 mM KCl, and the bound material was eluted with a linear gradient of 100 to 600 mM KCl in buffer Q. The fractions that reconstituted VAI transcription (eluting between 310 and 360 mM KCl; 100 ml, 40 mg of protein) were pooled and dialyzed against buffer D containing 5 mM $MgCl_2$, and 7 ml was loaded onto a Mono S HR 5/5 column (Pharmacia) equilibrated in buffer Q containing 50 mM KCl. The column was washed with five column volumes of buffer Q with 50 mM KCl, and the bound material was eluted with a linear gradient of 50 to 600 mM KCl in buffer Q. The fractions were then tested for their ability to reconstitute VAI transcription (see Fig. 2A) and used for immunoprecipitations (see Fig. 2B, 2C, and 5). The 0.38M-TFIIIB activity eluted between 220 and 305 mM KCl from the Mono S column.

To obtain enough material for microsequencing, 200 ml of the phosphocellulose B fraction (1,400 mg of protein) was loaded onto a HiLoad 26/10 Q Sepharose column equilibrated in buffer Q containing 100 mM KCl, as described above. The column was washed with five column volumes of buffer Q with 100 mM KCl, and the bound material was eluted with a linear gradient of 100 to 600 mM KCl in buffer Q. This protocol was repeated six times. The fractions that reconstituted VAI transcription were pooled (720 ml, 367 mg of protein), dialyzed against buffer Q with 50 mM KCl, and loaded onto two Mono S HR 10/10 columns (360 ml of fraction per column). The Mono S fractions that reconstituted VAI transcription from the two columns were pooled (52 ml) and used for large-scale nondenaturing immunoprecipitations, which were performed as described below under Immunoprecipitations with the following modifications. Anti-TBP monoclonal antibody (MAb) SL30b (32) (1.4 ml of ascites fluid) was chemically cross-linked to protein G-agarose beads (3.6 ml; Boehringer Mannheim) as described by Harlow and Lane (15). The beads were then mixed with the Mono S pooled fractions. Binding was allowed to proceed for 2 h at 4°C with gentle rocking. The supernatant was then transferred to tubes containing a second batch of antibody beads. Binding was allowed to proceed as before. After extensive washing, the bound material from the two sets of beads was eluted by boiling in Laemmli buffer (27), concentrated in Centricon-10 concentrators (Amicon) as recommended by the manufacturer, and fractionated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. The 88-kDa doublet was excised and used for microsequencing as described below.

Protein sequencing and isolation of a cDNA encoding hBRF. The preparative SDS-polyacrylamide gel was stained with 0.05% Coomassie blue G for 15 min, destained, and soaked in water for 1 h. The 88-kDa doublet as well as the TBP band were excised from the gel and subjected to digestion in situ with lysylendopeptidase. The resulting peptides were separated by reverse-phase high-pressure liquid chromatography and sequenced with an Applied Biosystems, Inc. automated protein sequencer as described previously (55). Degenerate primers were designed from some of the obtained amino acid sequences (peptides 2 and 3 in Fig. 3) and used for PCR with cDNA generated from total RNA as a template. A 171-bp fragment (encoding amino acids 264 to 320) was obtained and used as a probe to screen a human λgt10 cDNA library from NTera2D1 cells (47), a human teratocarcinoma cell line. Of 900,000 plaques screened, we obtained 4 strong-positive plaques, which were analyzed further. The four recombinants from these plaques contained overlapping inserts of different sizes, which were subcloned into pUC118 for nucleic acid sequencing. The inserts were sequenced on both strands. None of them contained the sequences encoding the extreme amino terminus of the protein, and the screening of additional libraries to obtain the missing sequences was unsuccessful. We therefore performed rapid amplification of cDNA 5′ ends (5′ RACE) as described by Frohman (11), except that we first disrupted the secondary structure of the RNA with 10 mM methylmercuric hydroxide for 10 min at room temperature. The methylmercuric hydroxide was then dissociated from the RNA by treatment with 100 mM DTT for 4 min at room temperature. cDNA with a gene-specific primer was then generated with SuperScriptII RNase H^- reverse transcriptase (GIBCO BRL) and amplified by PCR. The longest resulting fragment gave us the sequence encoding the first 81 amino acids shown in Fig. 3, and a further 67 upstream nucleotides.

Immunoprecipitations. The anti-TBP MAbs indicated in the figure legends were chemically cross-linked to protein G-agarose beads as described previously (15). For nondenaturing immunoprecipitations, 20 μl of packed beads was mixed with 300 μl of Mono S column fractions (Fig. 2B, 2C, and 5) for 1 h at 4°C. The beads were then washed three times with buffer D containing 350 mM KCl and three times with buffer D containing 100 mM KCl. The bound material was then eluted by boiling in Laemmli buffer, the beads were pelleted by centrifugation in a microcentrifuge, and the supernatant was loaded onto an SDS-polyacrylamide gel. For denaturing immunoprecipitations, the protein fraction was first diluted with the same volume of buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.2% SDS, 50 mM Tris [pH 8.0]) and heated to 45°C for 15 min before addition of the anti-TBP antibody beads.

Generation of anti-peptide antibodies for hBRF. Synthetic peptides corresponding to various segments of the predicted amino acid sequence were coupled to keyhole limpet hemocyanin (Pierce) as described by Harlow and Lane (15) and injected into rabbits to generate polyclonal anti-peptide antibodies.

In vitro transcriptions. To test column fractions for 0.38M-TFIIIB activity (see Fig. 2A), we depleted a phosphocellulose B fraction of TBP by first adding the anti-TBP MAb SL27a (32) and then incubating it for 30 min at 4°C. The MAb was then removed by addition of protein G-agarose beads, incubation at 4°C for 30 min, and removal of the beads by centrifugation. Six microliters of this depleted B fraction [designated B(TBP^-)] was then combined with 3 μl of a phosphocellulose C fraction and 3 μl of column fractions. The resulting protein mixture was programmed with 0.25 μg of the plasmid pBSM13$^+$VAI (31), which contains the Ad2 VAI gene. The transcription reactions were performed in a total volume of 17 μl under the conditions described previously (31).

To test the role of hBRF in VAI transcription (see Fig. 6A), we depleted 50 μl of the phosphocellulose B fraction with either 10, 20, or 50 μl of α-CSH407 or preimmune antibody beads. Of these depleted B fractions 3.6, 4.2, or 6 μl was combined with 3 μl of the phosphocellulose C fraction and programmed with 0.25 μg of plasmid pBSM13$^+$VAI. The transcription reactions were performed in a total volume of 14 μl under the conditions described by Lobo et al. (31).

For all the other depletion experiments, a whole-cell extract was depleted by two successive incubations with antibody beads. The beads were mixed with the extract for 30 min at ambient temperature and pelleted gently, and the supernatant was removed to fresh tubes containing fresh antibody beads for a second incubation. For the experiment shown in Fig. 6B, we used α-CSH407 beads at a ratio of beads to extract of 1:1. For the experiment shown in Fig. 7, 40 μl of whole-cell extract was depleted by two successive incubations with either 10, 20, or 40 μl of α-CSH407 antibody or preimmune antibody beads. For the experi-

ment shown in Fig. 8, we depleted the whole-cell extract by two successive incubations with a mixture of polyclonal anti-TBP and SL30b MAb beads at a ratio of beads to extract of 1:1.5 as described above.

The doubly depleted extracts were then tested for VAI transcription (see Fig. 6B) or VAI and U6 transcription in parallel (see Fig. 7A and 8A). For the VAI gene, the transcription reactions were performed in a total volume of 20 μl containing 6 μl of doubly immunodepleted extract and 0.25 μg of supercoiled DNA template, under the conditions described previously (31). For the U6 gene, the transcription reactions were performed as previously described (31) in a total volume of 22 μl and contained 9 μl of the doubly depleted extract and 0.25 μg of the plasmid pU6/Hae/RA.2 (29). Transcripts derived from the U6 promoter were detected by RNase T_1 protection with the probe U6/Hae.2/143 as previously described (29).

Construction of plasmid pET11c-GST-hBRF. The full-length cDNA coding for hBRF was reconstructed by joining together the fragment obtained by 5′ RACE-PCR and the coding sequences present in the longest λgt10 insert. To construct the pET11c-GST-hBRF expression plasmid, the full-length hBRF-coding sequence was amplified with *Pfu* polymerase (Stratagene) and subcloned into pET11c ori$^+$ (−), a modified pET11c vector containing unique *Xba*I and *Bam*HI sites downstream of the glutathione *S*-transferase (GST)-coding sequences (28). The expression vector encoding GST-TBP (pET11c-GST-TBP) was a kind gift of W. P. Tansey (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Expression of GST-hBRF in *Escherichia coli*. BL21 (DE3) pLys S cells (500 ml) harboring the pET11c-GST-hBRF expression construct were grown at 37°C to an optical density at 600 nm of 0.6 and induced for 3 h at 37°C with 0.2 mM isopropyl-1-thio-β-galactopyranoside. Cells were harvested and lysed for 15 min at 30°C in 30 ml of lysis buffer (25 mM HEPES–KOH [pH 7.9] at 4°C, 20% glycerol, 12.5 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 0.2 mM PMSF, 2 μg each of aprotinin, leupeptin, and pepstatin per ml) containing 100 μg of lysozyme per ml. Nonidet P-40 was then added to a final concentration of 0.1%, and the cells were sonicated on ice. The lysate was cleared by centrifugation, and 300 μl of a 1:1 slurry of glutathione-agarose beads (Sigma) washed with a buffer containing 20% glycerol, 25 mM HEPES–KOH (pH 7.9), 150 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.2 mM DTT, and 2 μg each of aprotinin, leupeptin, and pepstatin A per ml was added. Binding was allowed to proceed for 2 h at 4°C. The bound material was eluted with 150 μl of 5 mM reduced glutathione in 50 mM Tris-HCl, pH 8.8, and GST-hBRF was detected by immunoblotting with the α-CSH407 antibody.

Nucleotide sequence accession number. The hBRF sequence has been deposited in GenBank (accession number U75276).

RESULTS

Purification of 0.38M-TFIIIB. In the first purification step of the basal RNA polymerase III transcription factors, a crude extract is divided into three fractions, a 100 mM KCl flowthrough or A fraction, a 100 to 300 mM KCl elution step or B fraction, and a 300 to 600 mM KCl elution step or C fraction, which contain TFIIIA, TFIIIB, and TFIIIC, respectively (45, 46). We previously showed that the B fraction contains two activities required for reconstitution of VAI transcription, 0.38M-TFIIIB and 0.48M-TFIIIB, which can be separated by chromatography on a Mono Q column (Fig. 1). Our initial characterization of one of these two activities, 0.38M-TFIIIB, identified it as a TBP-containing complex, and immunoprecipitations from the 0.38M-TFIIIB fraction with an anti-TBP antibody revealed several TBP-associated polypeptides (reference 31 and data not shown).

To determine which of these TBP-associated polypeptides is part of the 0.38M-TFIIIB activity, we purified the 0.38M-TFIIIB fraction further by chromatography over a Mono S column, as shown in Fig. 1. The bound material was eluted with a salt gradient, and every other resulting fraction (even-numbered fractions) was tested in a complementation assay for transcription from the tRNA-type Ad2 VAI promoter. We combined a phosphocellulose C fraction, which provides TFIIIC and RNA polymerase III, a phosphocellulose B fraction immunodepleted of TBP and associated proteins [designated B(TBP$^-$)], which provides RNA polymerase III and other components of the TFIIIB activity but should lack 0.38M-TFIIIB, and the Mono S fractions. As shown in Fig. 2A, lane 2, a combination of the B(TBP$^-$) and C fractions did not support VAI transcription, as expected. However, upon addition of Mono S fractions 26 to 34, which eluted from the Mono S column between 220 and 305 mM KCl, we could observe robust VAI transcription (lanes 5 to 9), with the transcriptional activity peaking in fractions 26 and 28.

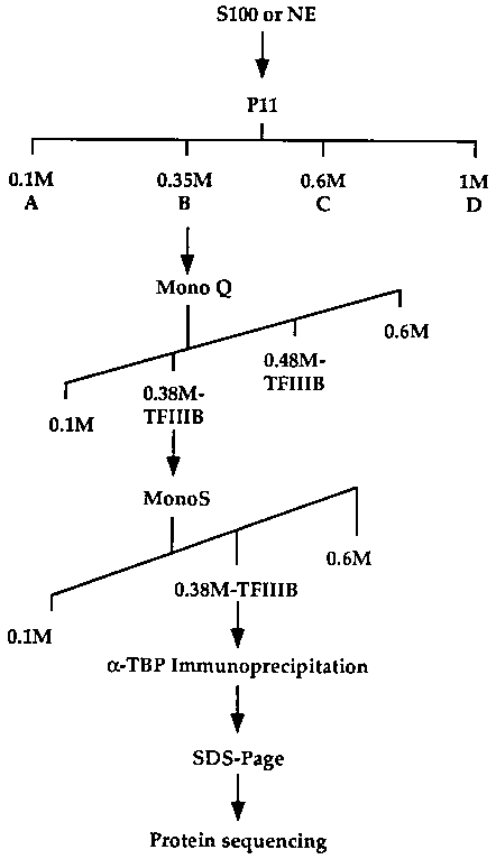

FIG. 1. Scheme for the purification of the 0.38M-TFIIIB TBP-containing complex. The 0.38M-IIIB complex eluted between 310 and 360 mM KCl from the Mono Q column and between 220 and 305 mM KCl from the Mono S column. S100, cytoplasmic extract; NE, nuclear extract; P11, phosphocellulose P11 column; Page, polyacrylamide gel electrophoresis.

We then used the same fractions to perform nondenaturing immunoprecipitations with the anti-TBP MAb SL30b (32). As shown in Fig. 2B, we obtained a TBP profile that corresponded exactly with the profile of transcription activity observed in Fig. 2A. In addition, a closely spaced doublet migrating with an apparent molecular mass of 88 kDa peaked in the same fractions as TBP. We also observed several minor bands, which likely contained substoichiometric amounts of protein relative to TBP; in particular, bands migrating with apparent molecular masses of 27, 45, 67, and 70 kDa (labeled with asterisks in Fig. 2A, lane 4) were seen. Unlike TBP, neither the 88-kDa polypeptide nor the minor polypeptides were observed in a denaturing immunoprecipitation (lane 1), suggesting that these proteins corresponded to TBP-associated polypeptides. To eliminate the remaining possibility that immunoprecipitation of the major 88-kDa polypeptide resulted from cross-reaction of the anti-TBP MAb SL30b with a denaturation-sensitive epitope, we tested whether this polypeptide could also be immunoprecipitated with other anti-TBP MAbs (32). As shown in Fig. 2C, SL39a, SL33a, SL2a, and SL30b all immunoprecipitated both TBP and the 88-kDa protein under nondenaturing conditions (lanes 2, 4, 6, and 8) and TBP but not the 88-kDa protein under denaturing conditions (lanes 3, 5, 7, and 9).

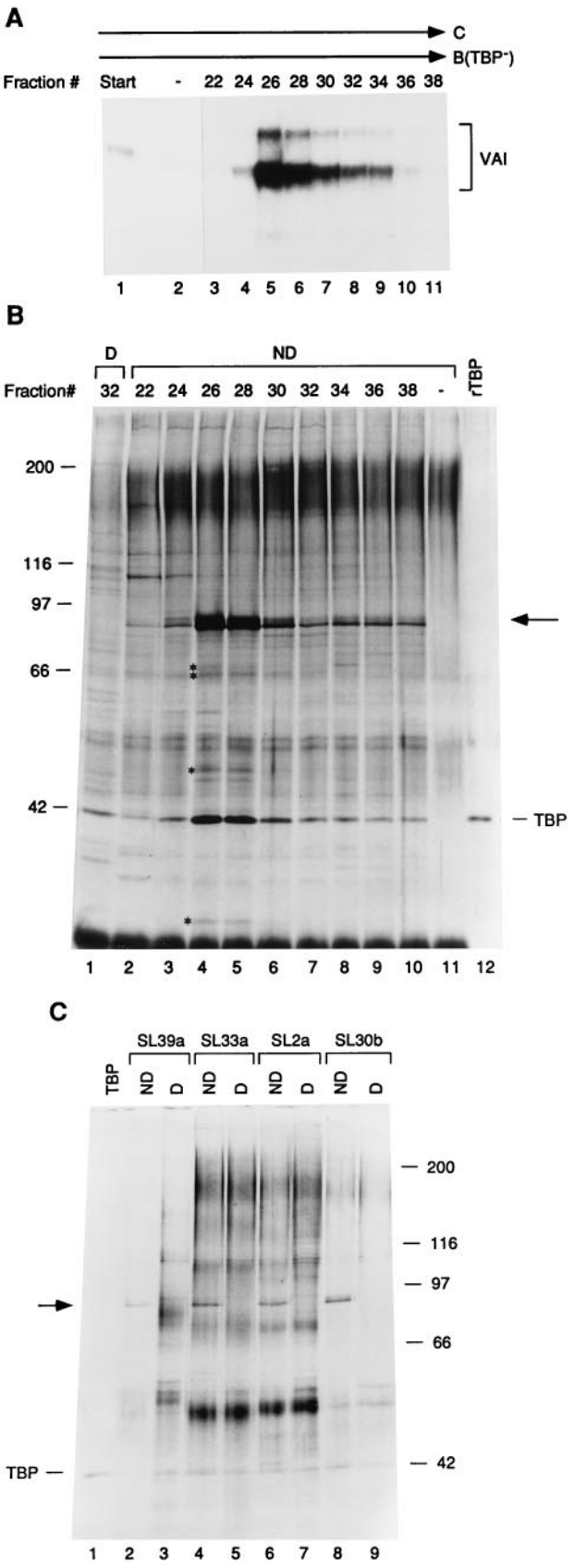

FIG. 2. 0.38M-TFIIIB transcriptional activity peaks with a complex containing TBP and an associated protein of 88 kDa. (A) VAI transcription peaks in Mono S fractions 26 to 28. The material loaded on the Mono S column (lane 1, labeled start) or the Mono S fractions (Fig. 1) indicated above the lanes (lanes 3 to 11) were tested for 0.38M-TFIIIB activity by combination with a phosphocellulose C fraction and a phosphocellulose B fraction that had been immunodepleted with the anti-TBP MAb SL27a attached to beads [B(TBP$^-$)] as described in Materials and Methods. Lane 2 shows the combination of the C and B(TBP$^-$) phosphocellulose fractions alone. The signal observed in lane 1 is weak because the 0.38M-TFIIIB present in the starting material of the Mono S column (a pool of Mono Q fractions) was very dilute. (B) A TBP-associated polypeptide with an apparent molecular mass of 88 kDa peaks in fractions 26 to 28. The Mono S fractions analyzed in panel A and indicated above the lanes were used in nondenaturing (ND; lanes 2 to 11) or denaturing (D; lane 1) immunoprecipitations with the anti-TBP MAb SL30b. The gel was stained with silver. Lane 12 contains 50 ng of human TBP. rTBP, recombinant TBP. The 88-kDa polypeptide is indicated with an arrow. Minor bands that also peak in fractions 26 to 28 are labeled with asterisks. (C) The 88-kDa polypeptide is associated with TBP. A 0.38M-TFIIIB-containing Mono S fraction was used for nondenaturing (ND; lanes 2, 4, 6, and 8) or denaturing (D; lanes 3, 5, 7, and 9) immunoprecipitations with the MAbs indicated above the lanes. The gel was stained with silver. The 88-kDa band is indicated by an arrow and is present in each nondenaturing immunoprecipitation. Lane 1 contains 50 ng of human TBP.

Because the precise epitopes recognized by SL39a and SL30b have been mapped and are known to be different (32), this result strongly suggests that immunoprecipitation of the 88-kDa protein was not due to cross-reaction with a denaturation-sensitive epitope.

Together, these results suggest that the TBP-containing complex required for VAI transcription consists, minimally, of TBP and an associated subunit of 88 kDa as well as, perhaps, additional, more loosely associated polypeptides that are underrepresented in the nondenaturing immunoprecipitation.

The 88-kDa polypeptide corresponds to a human homolog of yeast BRF. To obtain a cDNA encoding the 88-kDa polypeptide(s), we scaled up the purification shown in Fig. 1 to obtain enough material for protein microsequencing. The purified protein was digested with lysylendopeptidase, and we obtained amino acid sequences for 10 of the resulting peptides. On the basis of the protein sequence information, we then designed degenerate oligonucleotides for use in PCRs with cDNA from total RNA as a template (see Materials and Methods). We obtained a fragment of 171 bp, which was then used as a probe to screen a λgt10 cDNA library (47). The inserts of four positive recombinant λ phages were sequenced, and the longest one contained an open reading frame encoding the amino acids 82 to 677 shown in Fig. 3. We suspected that the longest cDNA did not encode the full-length protein because, despite strong similarity to the yeast BRF protein in the amino-terminal region (see below), the predicted amino acid sequence lacked homology to a putative zinc finger located at the amino terminus of the yeast BRF sequence. We therefore performed 5′ RACE-PCR (11) and obtained sequences encoding an additional 103 amino acids, including the zinc finger homology region. The open reading frame starting at the first methionine in the RACE fragment is shown in Fig. 3. It encodes a protein with a calculated molecular mass of 74 kDa and an isoelectric point of 5.1 and contains all 10 peptide sequences obtained from the purified protein. The sequence upstream of the first methionine does not contain any in-frame stop codons (data not shown), but the protein expressed from the open reading frame shown in Fig. 3 migrates with the expected mobility on an SDS-polyacrylamide gel, suggesting that it corresponds to the full-length protein.

The sequence is identical to that reported for TFIIIB90 (52), except for scattered nucleotide differences indicated in the figure legend and for five additional nucleotides between po-

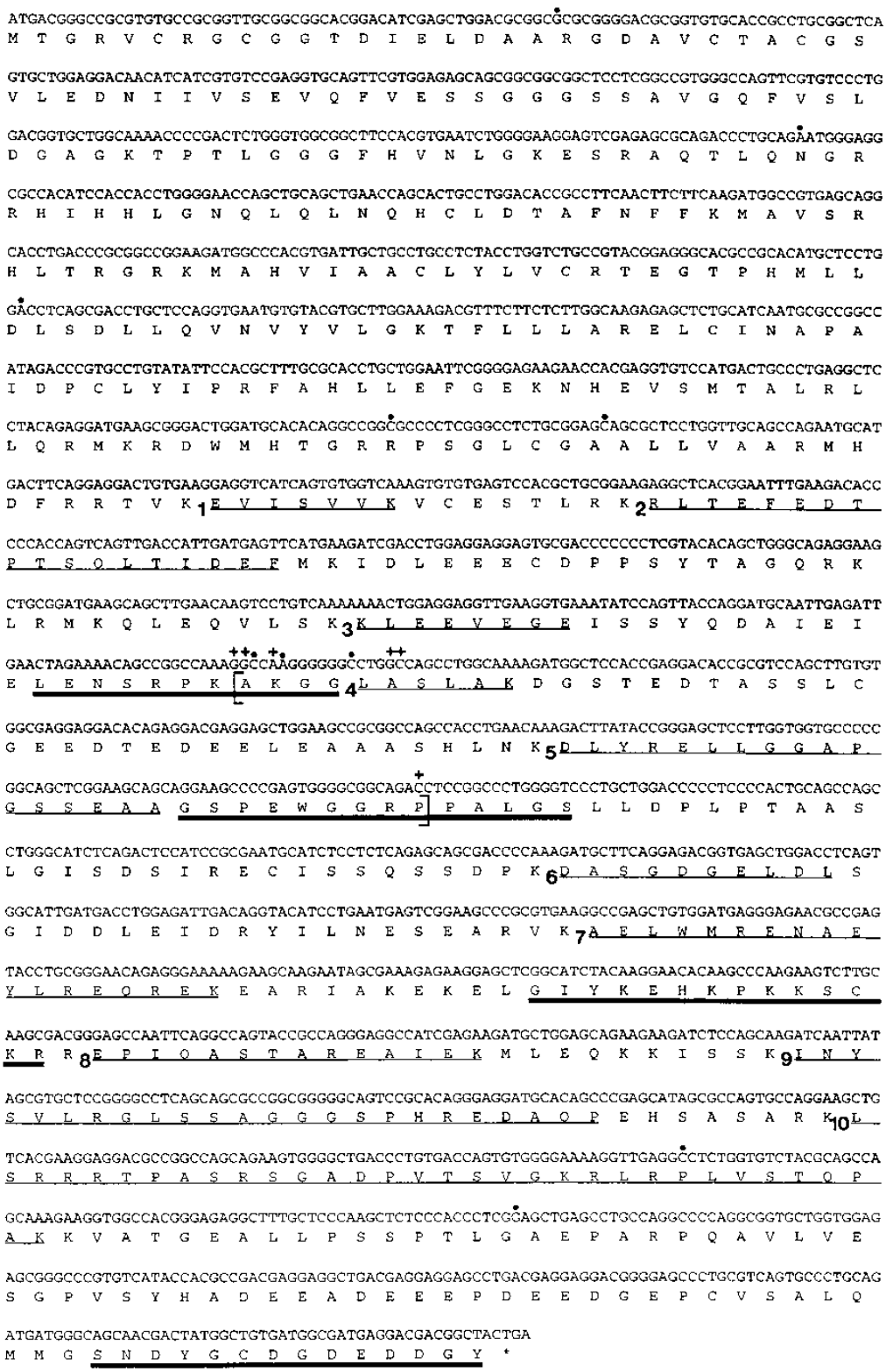

FIG. 3. Structure of hBRF. The nucleic acid sequence and predicted amino acid sequence of hBRF are shown. Nucleotides 1 to 243 were obtained by 5′ RACE-PCR. The peptide sequences obtained from the purified protein are lightly underlined and numbered. The sequences corresponding to the synthetic peptides used to raise antibodies are darkly underlined. The peptides corresponding to amino acids 332 to 342, 397 to 410, 499 to 512, and 664 to 677 are designated CSH408, CSH409, CSH406, and CSH407, respectively. The differences from the reported sequence of TFIIIB90 are as follows. The nucleotides labeled with a + sign are insertions relative to the TFIIIB90 sequence and result in a protein sequence that is 2 amino acids longer than the TFIIIB90 sequence. This sequence differs from that of TFIIIB90 in a stretch of 67 consecutive amino acids, which is indicated by brackets. The nucleotides labeled with a dot are mismatches relative to the TFIIIB90 sequence and result in the following amino acid changes: N-88 is a D in TFIIIB90, D-151 is a V, R-224 is a G, A-231 is a G, P-594 is a T, and G-618 is an E.

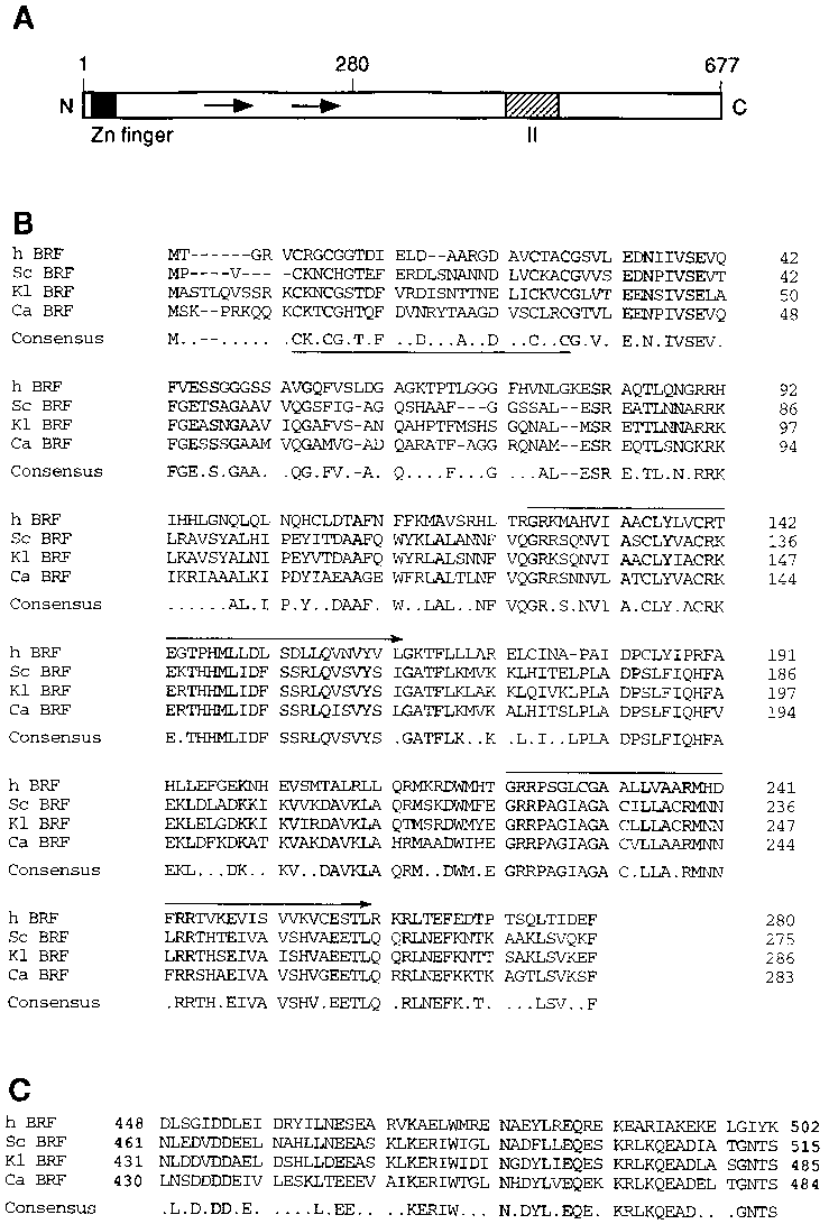

FIG. 4. (A) Schematic structure of hBRF. The conserved amino-terminal domain extends to amino acid 280 and contains a zinc finger motif (filled box) and two direct repeats (arrows). The carboxy-terminal domain (C) contains a region that is conserved with the three yeast BRF sequences (region II, stippled box). (B) Sequence alignment of the amino-terminal regions of hBRF, *S. cerevisiae* (Sc BRF), *K. lactis* (Kl BRF), and *C. albicans* (Ca BRF). The zinc finger is underlined. The imperfect direct repeats are indicated by arrows and extend from amino acids 125 to 163 and 222 to 260 in the human sequence. Amino acids that are identical in the four sequences are shaded. The consensus sequence indicates amino acids that are identical in at least three of the four sequences. (C) Conserved region in the BRF carboxy-terminal domain. The sequence alignment shows amino acids 448 to 502 of hBRF, 461 to 515 of *S. cerevisiae* BRF, 431 to 485 of *K. lactis* BRF, and 430 to 484 of *C. albicans* BRF. Amino acids that are identical in the four sequences are shaded. The consensus sequence indicates amino acids that are identical in at least three of the four sequences.

sitions 1013 and 1032 and another additional nucleotide at position 1213. These last differences change the reading frame and thus predict a different amino acid sequence over 67 residues. Our reading frame probably corresponds to the 88-kDa protein we purified, because the 67 residues that differ from TFIIIB90 match two of the peptide sequences we obtained from the purified protein.

The protein can be divided into two regions which differ in their similarities to yeast BRF: a conserved amino-terminal domain extending to amino acid 280 and a poorly conserved carboxy-terminal domain. As shown in Fig. 4B, the amino-terminal domains of hBRF and *S. cerevisiae* BRF are 41.4% identical, while the amino-terminal domains of all four known BRF sequences, hBRF, *S. cerevisiae* BRF, *Kluyveromyces lactis* BRF, and *Candida albicans* BRF (25), are 32.8% identical. All four BRF amino-terminal domains contain a zinc finger motif as well as two 39-amino-acid repeats (Fig. 4A and B). This amino-terminal region of hBRF is also similar to the transcription factor TFIIB (23.7% identical with the human TFIIB sequence; data not shown).

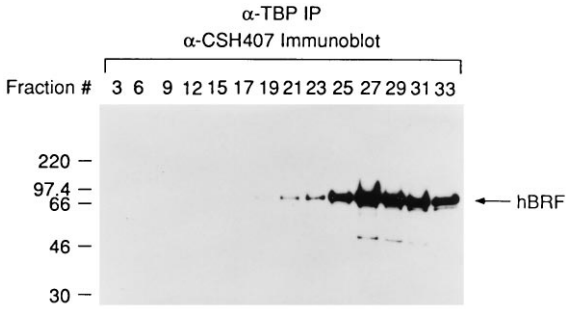

FIG. 5. hBRF is associated with TBP. The Mono S fractions indicated above the lanes were used for nondenaturing immunoprecipitations (IP) with the anti-TBP MAb SL30b covalently attached to protein G-agarose beads. The immunoprecipitates were fractionated on an SDS–12.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with the anti-hBRF antibody α-CSH407 by the chemiluminescence method (Amersham). Molecular size markers are noted at the left in kilodaltons.

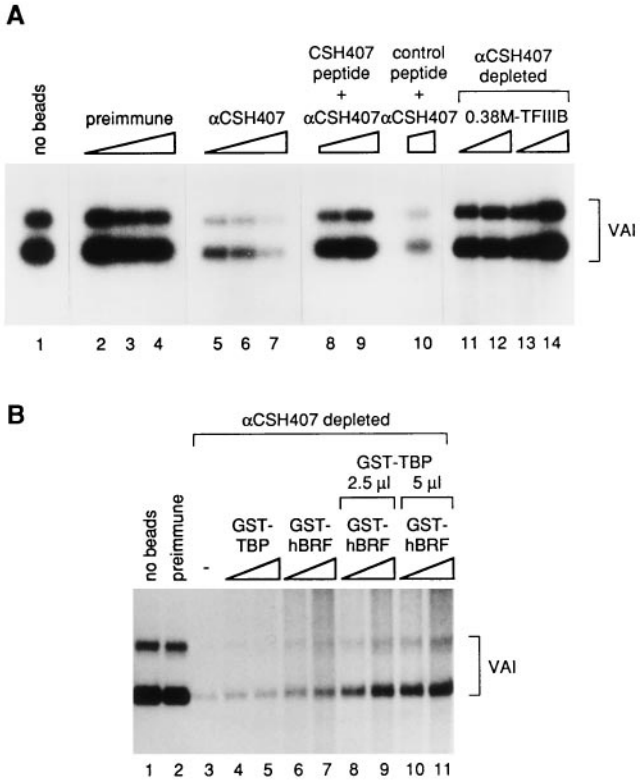

FIG. 6. hBRF is required for in vitro transcription of the VAI gene. (A) A phosphocellulose B fraction was either left untreated (lane 1) or mock immunodepleted with a 1:5 (lane 2), 1:2.5 (lane 3), or 1:1 (lane 4) ratio of preimmune antibody beads to fraction or immunodepleted with a 1:5 (lane 5), 1:2.5 (lanes 6, 8, 9, 11, and 12), or 1:1 (lanes 7, 10, 13, and 14) ratio of anti-hBRF (α-CSH407) antibody beads to fraction. Combinations of 3 μl of phosphocellulose C fraction and either 3 μl (lane 1), 3.6 μl (lanes 2 and 5), 4.2 μl (lanes 3, 6, 8, 9, 11, and 12), or 6 μl (lanes 4, 7, 10, 13, and 14) of the phosphocellulose B fractions treated as indicated above were tested for their ability to support VAI transcription as described in Materials and Methods. In lanes 8 and 9, 0.8 μg of the specific CSH407 peptide per μl and in lane 10, 0.8 μg of an irrelevant peptide per μl were added to the fractions before addition of the antibody beads. In lanes 11, 12, 13, and 14, increasing amounts of a 0.38M-TFIIIB-containing fraction (Mono S fraction 25) were added, as schematically indicated above the lanes. (B) A whole-cell extract was either left untreated (lane 1) or depleted with the same volume of preimmune antibody beads (lane 2) or depleted with the same volume of α-CSH407 antibody beads (lanes 3 to 11). The depleted extract was then supplemented with either nothing (lanes 2 and 3) or 2.5 μl (lane 4) and 5 μl (lane 5) of recombinant GST-TBP, or 1 μl (lane 6) and 2 μl (lane 7) of recombinant GST-hBRF, or 1 μl (lane 8) and 2 μl (lane 9) of GST-hBRF together with 2.5 μl of GST-TBP, or 1 μl (lane 10) and 2 μl (lane 11) of GST-hBRF together with 5 μl of GST-TBP and tested for VAI transcription as described in Materials and Methods. The signals in lanes 4 to 11 were 1.14-, 1.23-, 1.75-, 2.41-, 2.81-, 3.58-, 4.12-, and 4.72-fold higher, respectively, than the signal in lane 3, and the highest signal obtained with added recombinant proteins (GST-TBP and GST-BRF, lane 11) was 54% of that obtained in extract treated with preimmune beads (lane 2), as measured with a PhosphorImager.

The carboxy-terminal domain of the protein contains weak similarity (20% identity) to HMG boxes 1 and 2 of chicken high-mobility-group protein 2 (HMG-2) (48), as noted by Wang and Roeder (52) for TFIIIB90, but shows little sequence conservation with either TFIIB or the yeast BRFs. One notable exception, however, is the small region labeled region II in Fig. 4A and shown in Fig. 4C. This region extends from amino acids 448 to 502 in hBRF and shows 29% identity with the *S. cerevisiae* sequence and 21.8% identity with the sequences of all three yeast BRFs. This conserved region corresponds to a large portion of the HMG box 1 similarity and to yeast BRF region II, the longest of three regions that are conserved among the carboxy-terminal domains of the three yeast BRF sequences (25).

hBRF is associated with TBP. To confirm that hBRF corresponds to the TBP-associated protein we purified, we raised polyclonal antibodies against synthetic peptides corresponding to the sequences darkly underlined in Fig. 3. We performed nondenaturing immunoprecipitations with the Mono S fractions, alternating those used in Fig. 2 (odd-numbered fractions) with an anti-TBP antibody, and this was followed by immunoblotting with the anti-hBRF antibody directed against the carboxy terminus of the protein (α-CSH407). As shown in Fig. 5, the profile of hBRF in these fractions is consistent with the profile of the TBP-associated protein observed in Fig. 2B, confirming that hBRF indeed corresponds to the 88-kDa TBP-associated protein.

hBRF is required for VAI transcription. The copurification of hBRF with VAI transcription activity (Fig. 2A) suggested that hBRF is required for VAI transcription. To test this directly, we depleted a phosphocellulose B fraction with either preimmune antibodies or the anti-hBRF antibody α-CSH407 covalently attached to beads. The mock-depleted or depleted B fractions were then combined with a phosphocellulose C fraction and tested for VAI transcription. As shown in Fig. 6A, VAI transcription was severely reduced by depletion with increasing amounts of α-CSH407 beads (lanes 5 to 7) but not preimmune beads (lanes 2 to 4). The inhibitory effect was specific, because it could be suppressed by preincubation of the α-CSH407 antibody with its cognate peptide (compare lanes 8 and 9 with lanes 6 and 7) but not by preincubation with a nonspecific peptide (compare lane 10 with lane 7). Transcription was efficiently restored by addition of a 0.38M-TFIIIB-containing Mono S fraction (compare lanes 11 and 12 with lane 6 and lanes 13 and 14 with lane 7). Together, these data suggest that hBRF and/or an associated factor is required for transcription from the VAI promoter.

To distinguish whether VAI transcription was inhibited because of depletion of hBRF or an associated factor or both, we then tested whether VAI transcription could be reconstituted by addition of recombinant TBP and/or hBRF as GST fusion proteins. As shown in Fig. 6B, addition of increasing amounts of a recombinant GST-TBP fusion protein to an extract depleted with α-CSH407 antibody beads had little effect on transcription (compare lanes 4 and 5 with lane 3). Addition of increasing amounts of recombinant GST-hBRF restored a low level of transcription (compare lanes 6 and 7 with lane 3).

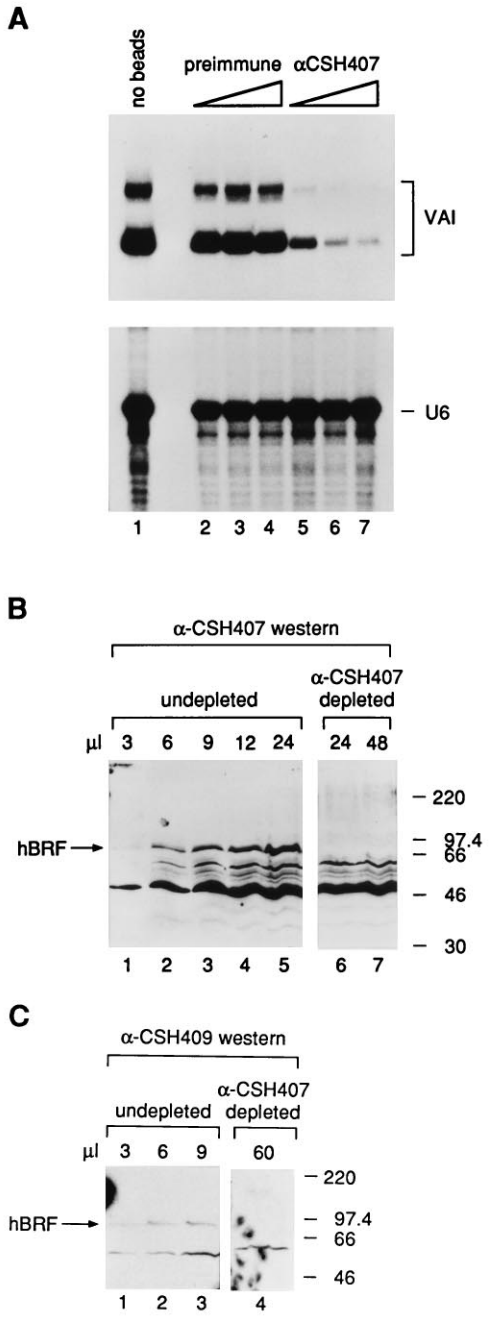

FIG. 7. Depletion of hBRF with the α-CSH407 antibodies inhibits VAI but not U6 transcription in vitro. (A) A whole-cell extract (40 μl) was either left untreated (lane 1) or mock immunodepleted twice with 10 μl (lane 2), 20 μl (lane 3), and 40 μl (lane 4) of preimmune antibody beads or immunodepleted twice with 10 μl (lane 5), 20 μl (lane 6), and 40 μl (lane 7) of anti-hBRF (α-CSH407) antibody beads and then tested in parallel for its ability to support VAI (upper panel) or U6 (lower panel) transcription as described in Materials and Methods. (B) Various amounts (as indicated above the lanes) of either the undepleted extract (lanes 1 to 5) or the α-CSH407-depleted extract (lanes 6 and 7) used for the experiment shown in lane 7 of panel A were analyzed by immunoblotting with the α-CSH407 antibody for the presence of hBRF by the chemiluminescence method (Amersham). The band corresponding to hBRF is indicated with an arrow. The other bands were not specifically recognized by the α-CSH407 antibody, because unlike the hBRF signal, they were not suppressed by preincubation of the antibody with its cognate peptide (data not shown). (C) Various amounts (as indicated above the lanes) of either the undepleted extract (lanes 1 to 3) or the α-CSH407-depleted extract (lane 4) were analyzed by immunoblotting as described for panel B, except that the α-CSH409 antibody was used. The band below the hBRF signal was not specifically recognized by the α-CSH409 antibody, because unlike the hBRF signal, it was not suppressed by preincubation of the antibody with its cognate peptide (data not shown). Molecular size markers are noted at the right in kilodaltons.

However, when we added both recombinant GST-TBP and GST-hBRF, we obtained high levels of transcription (lanes 8 to 11; see the figure legend for quantitations). These results show that hBRF as well as TBP are required for transcription from the VAI promoter. They also show that depletions with the anti-hBRF antibody α-CSH407 quantitatively remove only hBRF and TBP. This in turn suggests that other components of the TFIIIB activity required for VAI transcription are loosely or not associated with the hBRF-TBP complex.

Depletion of hBRF inhibits VAI but not U6 transcription. Our previous results suggest that U6 and VAI use different components in the TFIIIB fraction (31). We therefore tested whether hBRF is required for transcription of the U6 gene. We depleted an extract with either preimmune or α-CSH407 beads and tested the same depleted extract for transcription from the VAI and U6 promoters. As shown in Fig. 7A, VAI transcription was again severely inhibited by depletion with α-CSH407 antibody beads but not with preimmune antibody beads (compare lanes 5 to 7 with lanes 2 to 4). In sharp contrast, U6 transcription was unaffected (lanes 2 to 7). We then performed immunoblots with the same depleted extract to estimate the extent of the hBRF depletion. As shown in Fig. 7B, no hBRF was detectable with the α-CSH407 antibody when 48 μl of depleted extract was loaded on the gel, whereas hBRF was easily detected in as little as 3 μl of undepleted extract. To exclude the possibility that another form of hBRF not recognized by the α-CSH407 antibody was left in the extract, we probed a similar immunoblot with the α-CSH409 antibody, which was raised against amino acids 397 to 410. As shown in Fig. 7C, we could detect hBRF in 3 μl of undepleted extract (lane 1) but not in 60 μl of depleted extract (lane 4). Thus, at least 95% of the hBRF present in the extract was depleted, resulting in a severe effect on VAI transcription but no effect on U6 transcription.

The α-CSH407 antibody is directed against the carboxy terminus of hBRF. To determine whether depletions with antibodies directed against other portions of the protein would also leave U6 transcription unaffected, we tested the antibody α-CSH409, as well as the antibodies α-CSH406 and α-CSH408, which were raised against hBRF amino acids 499 to 512 and 332 to 342, respectively. Depletions with α-CSH406, α-CSH408, and α-CSH409 antibody beads inhibited VAI transcription, as expected, but to a much lesser degree (1.5- to 3.5-fold) than depletions with α-CSH407 antibody beads (at least 16-fold). Consistent with this observation, these antibodies only partially depleted hBRF from the extract as judged from immunoblots. Importantly, like the α-CSH407 beads, the α-CSH406 and α-CSH408 antibody beads had no specific effect on U6 transcription (data not shown). Together, these data suggest that hBRF is not required for U6 transcription.

Because all anti-peptide antibodies directed against hBRF except for α-CSH407 (Fig. 7) resulted in only partial depletions as judged both from immunoblots and from the VAI transcriptional activity remaining even in extracts depleted with the highest amounts of beads (data not shown), we sought another method to deplete the hBRF present in the transcription extracts. We reasoned that we might obtain extensive depletion of hBRF by using anti-TBP antibodies. Figure 8A shows the effects of anti-TBP antibody bead depletions on VAI and U6 transcription, and the quantitations are indicated in

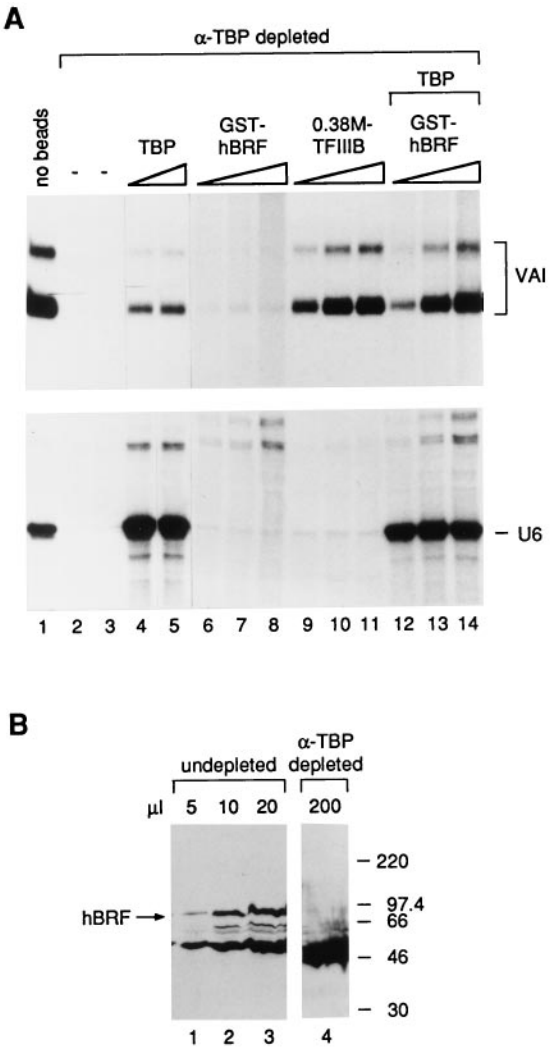

FIG. 8. U6 but not VAI transcription can be reconstituted by the addition of only recombinant TBP in a TBP-depleted extract. (A) A whole-cell extract was either left untreated (lane 1) or depleted with a mixture of beads (at a ratio of beads to extract of 1:1.5) coated with anti-TBP polyclonal antibodies and the anti-TBP MAb SL30b (lanes 2 to 14). The depleted extract was then supplemented with either nothing (lanes 2 and 3, which are duplicates), or 1 μl (lane 4) and 2 μl (lane 5) of recombinant TBP, or 0.5 μl (lane 6), 1 μl (lane 7), and 2 μl (lane 8) of GST-hBRF, or 1 μl (lane 9), 2.5 μl (lane 10), and 5 μl (lane 11) of a 0.38M-TFIIIB-containing fraction (Mono S fraction 25), or 0.5 μl (lane 12), 1 μl (lane 13), and 2 μl (lane 14) of GST-hBRF together with 1 μl (2 ng) of recombinant TBP and tested for its ability to support VAI (upper panel) or U6 (lower panel) transcription. For VAI, the signals in lanes 4 to 14 were 3.41-, 4.09-, 1.01-, 1.22-, 1.82-, 4.59-, 8.68-, 9.26-, 2.87-, 7.42-, and 10.24-fold the mean signal obtained in lanes 2 and 3, and the highest signal obtained by addition of recombinant proteins (TBP and GST-BRF, lane 14) was 41.2% of that obtained in untreated extract (lane 1), as measured with a PhosphorImager. For U6, the signals in lanes 4 to 14 were 30.51-, 24.61-, 0.86-, 0.86-, 0.88-, 0.79-, 0.81-, 1.02-, 12.81-, 14.86-, and 13.05-fold greater than the mean signal obtained in lanes 2 and 3, and the highest signal obtained by addition of recombinant protein (TBP, lane 4) was 313.9% of that obtained in untreated extract (lane 1), as measured with a PhosphorImager. (B) Various amounts (as indicated above the lanes) of either the undepleted extract (lanes 1 to 3) or the extract depleted with the anti-TBP antibody beads (lane 4) used for the experiment shown in panel A were analyzed by immunoblotting with the α-CSH407 antibody for the presence of hBRF by the chemiluminescence method. The band corresponding to hBRF is indicated with an arrow. The other bands were not specifically recognized by the α-CSH407 antibody, because unlike the hBRF signal, they were not suppressed by preincubation of the antibody with its cognate peptide (data not shown).

the figure legend. Depletion with anti-TBP antibody beads severely inhibited both VAI (upper panel) and U6 (lower panel) transcription (compare lanes 2 and 3 with lane 1). VAI transcription was not restored by addition of recombinant hBRF (lanes 6 to 8) and was restored only to low levels by addition of recombinant TBP (lanes 4 and 5). However, it was restored to high levels by addition of either the 0.38M-TFIIIB-containing Mono S fraction (lanes 9 to 11) or a combination of recombinant TBP and recombinant GST-hBRF (lanes 12 to 14). In sharp contrast, U6 transcription was restored to levels higher than those observed in the untreated extract by addition of only recombinant TBP (compare lanes 4 and 5 with lane 1), consistent with our previous results (31), and further addition of recombinant hBRF had no positive effect; in fact, it slightly inhibited U6 transcription (lanes 12 to 14; see the figure legend for quantitations). In addition, the Mono S fraction, which was very active for VAI transcription, had no effect on U6 transcription (lanes 9 to 11), suggesting that the TBP present in this fraction cannot function for U6 transcription. We then monitored the extent of hBRF depletion in the TBP-depleted extract by immunoblotting with the α-CSH407 antibody. As shown in Fig. 8B, hBRF was not detectable in 200 μl of TBP-depleted extract whereas it was clearly detectable in 5 μl of undepleted extract. Thus, in this case, more than 97% of hBRF had been depleted from the extract, and yet U6 transcription could be restored to levels higher than starting levels by addition of recombinant TBP alone. These results are consistent with the possibility that hBRF is not involved in U6 transcription.

DISCUSSION

We have purified and cloned a TBP-associated protein present in the 0.38M-TFIIIB fraction which corresponds to a human homolog of yeast BRF. The deduced amino acid sequence of this protein is similar but not identical to that reported for TFIIIB90 (52). In addition to previously noted similarities to yeast BRF in the amino-terminal portion of the protein, there is a 55-amino-acid block of similarity with yeast BRF within the carboxy-terminal portion of hBRF. This block is 21.8% identical with all three BRF sequences available, the *S. cerevisiae*, *C. albicans*, and *K. lactis* BRF sequences (25). It corresponds to region II, one of three regions of similarity noted previously in the carboxy-terminal domains of the three yeast BRF sequences (25). Significantly, in both yeast BRF (25) and human TFIIIB90 (52), the nonconserved carboxy-terminal region of the protein has been shown to interact strongly with TBP. Furthermore, deletion of the carboxy-terminal 165 amino acid residues of yeast BRF, which include conserved region II, abolishes binding of the carboxy-terminal half of BRF to TBP (25). This 55-amino-acid region of similarity may, therefore, correspond to a TBP-interacting domain.

Together, recombinant TBP and hBRF restore the function of the 0.38M-TFIIIB activity (Fig. 6B and 8A), indicating that 0.38M-TFIIIB corresponds to the TBP-hBRF complex. hBRF is clearly required for transcription of the VAI gene, but we could not demonstrate a role of hBRF in transcription from the human U6 promoter. Our results differ from those of Wang and Roeder (52), who showed that depletion of TFIIIB90 from extracts inhibited VAI as well as U6 transcription, although only VAI transcription could be restored by addition of recombinant TBP and TFIIIB90. In our experiments, depletion of at least 90 to 95% of hBRF with an anti-hBRF antibody, resulting in a severe inhibition of VAI transcription, had no effect on U6 transcription. In addition, in extracts in which at least 95% of hBRF was depleted with anti-TBP antibody beads, U6 transcription could be restored to

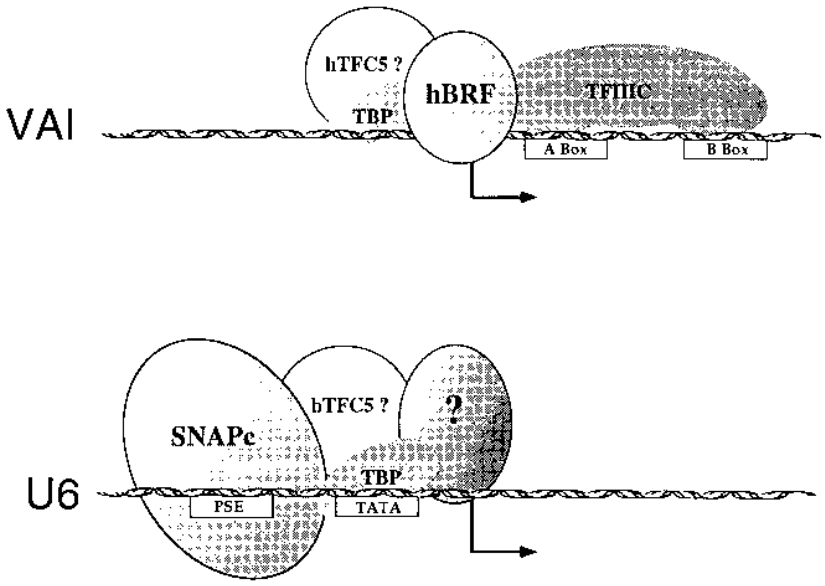

FIG. 9. Putative transcription initiation complexes assembled on the VAI and U6 promoters. The Ad2 VAI promoter recruits TFIIIC as well as the hBRF and TBP components of TFIIIB activity. By analogy to the yeast system, we imagine that hTFC5 is also required for VAI transcription. The human U6 snRNA promoter recruits $SNAP_c$ to the PSE and TBP to the TATA box. It may not recruit hBRF but perhaps recruits hTFC5 or hTFC5 and an hBRF-related polypeptide (indicated by an interrogation mark).

levels higher than starting levels by addition of only recombinant TBP. Together, these results strongly suggest that, consistent with our early observations (31), the TBP-hBRF complex is not involved in U6 transcription.

Our results also suggest that hBRF itself is not required. However, because only one of the anti-peptide antibodies (α-CSH407) we raised was capable of quantitatively depleting hBRF from extracts, we cannot exclude the possibility that a form of hBRF not recognized by this particular antibody and not associated with TBP in a complex recognized by the anti-TBP antibodies we used in the depletion experiment shown in Fig. 8 (a mixture of anti-TBP polyclonal antibodies and the MAb SL30b) is involved in U6 transcription. For example, perhaps the U6 promoter but not the VAI promoter can use a truncated form of hBRF that lacks the extreme carboxy-terminal sequences against which the α-CSH407 antibody was raised and that is not associated with TBP. Or perhaps hBRF is required for U6 transcription as part of a complex that does not contain TBP and in which the extreme carboxy terminus of the protein is inaccessible to the α-CSH407 antibody. This form of hBRF, however, would have to represent a very small percentage of the total hBRF present in extracts, since we cannot detect hBRF in the α-CSH407-depleted extracts even by immunoblotting with an antibody directed against another part of hBRF (Fig. 7C). Another possibility is that transcription from the human U6 promoter requires a factor related, but not identical, to hBRF. Such a factor might contain epitopes that cross-react with the anti-TFIIIB90 antibodies used by Wang and Roeder (52) but not with the anti-hBRF antibodies used in this study.

Which components of the mammalian phosphocellulose B fraction are required for VAI and U6 transcription? Yeast TFIIIB consists of three components, TBP, BRF, and the B″ fraction that contains TFC5. B″ can be easily separated from the yeast TBP-BRF complex (18, 22). In mammalian systems, the activity required for RNA polymerase III transcription from class 2 promoters (e.g., VAI) in the B fraction can also be separated into two components (which we refer to as 0.38M-TFIIIB and 0.48M-TFIIIB), only one of which contains TBP (6, 31, 36, 49). It is now clear that the TBP-containing component 0.38M-TFIIIB is the TBP-hBRF complex, but little is known about the other component, although it seems likely that it contains hTFC5. Indeed, in their purest TFIIIB-β fraction, a TFIIIB fraction required for VAI but not U6 transcription, Teichmann and Seifart (50) detected several polypeptides in addition to TBP, among which were polypeptides of 90 and 60 kDa. In addition, recent results suggest that *Xenopus laevis* TFIIIB consists of TBP and two associated factors with apparent molecular masses of 92 and 75 kDa (35). In both these cases, the associated factors may correspond to vertebrate BRF and TFC5. Like yeast TFC5, hTFC5 may be loosely associated with TBP and, upon chromatography on a Mono Q column, may fractionate largely in the 0.48M-TFIIIB fraction (although substoichiometric amounts may remain associated with TBP and may in fact correspond to one of the minor bands we observe in our purest 0.38M-TFIIIB fractions). Thus, the VAI initiation complex probably contains TFIIIC bound to the gene-internal A and B boxes, the 0.38M-TFIIIB complex consisting of TBP and hBRF, and perhaps hTFC5, as illustrated in the hypothetical model shown in Fig. 9. In contrast, perhaps transcription from the human U6 promoter requires only the TBP and TFC5 components of human TFIIIB, or, alternatively, TBP, hTFC5, and another factor related to hBRF. As depicted in Fig. 9, the human U6 initiation complex may then contain $SNAP_c$ bound to the PSE, TBP bound to the TATA box, and perhaps hTFC5 or hTFC5 together with an hBRF-related factor.

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