Different human TFIIIB activities direct RNA polymerase III transcription from TATA-containing and TATA-less promoters

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Transcription initiation at RNA polymerase III promoters requires transcription factor IIIB (TFIIIB), an activity that binds to RNA polymerase III promoters, generally through protein–protein contacts with DNA binding factors, and directly recruits RNA polymerase III. *Saccharomyces cerevisiae* TFIIIB is a complex of three subunits, TBP, the TFIIIB-related factor BRF, and the more loosely associated polypeptide β'. Although human homologs for two of the TFIIIB subunits, the TATA box–binding protein TBP and the TFIIIB-related factor BRF, have been characterized, a human homolog of yeast β' has not been described. Moreover, human BRF, unlike yeast BRF, is not universally required for RNA polymerase III transcription. In particular, it is not involved in transcription from the small nuclear RNA (snRNA)-type, TATA-containing, RNA polymerase III promoters. Here, we characterize two novel activities, a human homolog of yeast β', which is required for transcription of both TATA-less and snRNA-type RNA polymerase III promoters, and a factor equally related to human BRF and TFIIIB, designated BRFU, which is specifically required for transcription of snRNA-type RNA polymerase III promoters. Together, these results contribute to the definition of the basal RNA polymerase III transcription machinery and show that two types of TFIIIB activities, with specificities for different classes of RNA polymerase III promoters, have evolved in human cells.

[Key Words: human TFII; human B'; BRFU; U6 snRNA gene; RNA polymerase III]

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two new transcription factors, human BRF. Here, we report the characterization of two new factors we refer to as hBRFU. hBRFU is a protein with a calculated molecular mass of 156.129 kD, together with our previous results indicating that TFIIIB is required for transcription of the human RNA polymerase III snRNA genes (Kuhlman et al. 1999), these results suggest that the key event in the determination of RNA polymerase specificity in the human snRNA promoters is the recruitment of hBRFU versus TFIIIB.

Results

Characterization of a human homolog of S. cerevisiae B’’

Both the TBP and BRF subunits of yeast TFIIIB have human homologs, suggesting that a human homolog of yeast B’’ exists. S. cerevisiae B’’ contains a SANT domain. The SANT domain is related to a Myb repeat and was originally identified in a number of proteins, including the SWI3, ADA2, N-CoR, and yeast TFIIIB B’’ proteins (Asland et al. 1996). In yeast B’’, C-terminal deletions of B’’ that lack most of the SANT domain are inactive for in vitro transcription of a TATA-less tRNA gene, although they are still active for transcription of the TATA-containing U6 snRNA gene (Kumar et al. 1997). We used the S. cerevisiae sequence as a query to search the human and mouse expressed sequence tag (EST) databases and originally identified a short mouse sequence (GenBank accession number AA200560) encoding 21 amino acids with strong similarity to part of the yeast B’’ SANT domain. We used this short sequence to design primers, and through successive polymerase chain reactions (PCR) with various libraries and cDNA database searches, we assembled a sequence encoding a protein with a calculated molecular mass of 156.129 kD, shown in Figure 1A. We also obtained two variations in this sequence. In the first, nucleotides 414–578 [see GenBank accession number AF298151 for the nucleotide numbering], encoding amino acids 109–163, are missing (indicated by vertical brackets in Fig. 1A). Because the nucleotide sequence extending from nucleotides 414 to 578 starts with a GT and ends with an AG, the shorter sequence most probably corresponds to a splicing variant. In the second variation, an additional T residue is inserted at position 4192 [GenBank accession number AF298152]. As a result, the last 21 amino acids of the protein in Figure 1A [SEEINNSMIIILSPTTLKNL] are replaced by the shorter sequence FRGNK. The two sequences may correspond to different alleles. As detailed in Materials and Methods, several recent entries in the database can be aligned with part or most of the sequence shown in Figure 1A.

When the protein sequence shown in Figure 1A is used as the query to search the Swiss-Prot database, the best match identified is the S. cerevisiae B’’ protein. As detailed below, several other pieces of evidence suggest that this protein is indeed a functional human homolog of yeast B’’, and we therefore refer to it as hB’’.

Figure 1B shows the regions of similarity with yeast B’’ (ScB’’). hB’’ contains a region that is 43% identical with the yeast B’’ SANT domain [black box]. In addition,
Figure 1. (See facing page for legend.)
hB’ shows 21% identity over a 131-amino acid region upstream, and 17% identity over a 115-aa region downstream, of the SANT domain [hatched boxes].

Figure 1C shows an alignment including a 58-aa region directly upstream of the SANT domain and the SANT domain itself of hB’, ScB’, as well as putative B’ homologs from various organisms [see the legend to Fig. 1C]. In all of these sequences, the SANT domain is remarkably conserved, and the mouse and human sequences are identical over this region. The resulting consensus for B’ SANT domains differs from the SANT domain consensus [Aasland et al. 1996] at several positions, marked by arrowheads in Figure 1C. In addition, the B’ SANT sequences show absolute conservation of eleven amino acids [indicated in boldface], most of which are not absolutely conserved in all SANT domains. Thus, B’ SANT domains form a subfamily of SANT domains. The 58-aa region upstream of the SANT domain is also shown, because in ScB’, small deletions in this region severely debilitate transcription from linear [but not supercoiled] templates in vitro [Kassavetis et al. 1998]. Although this region is less conserved, it shows clusters of conserved amino acids, especially around the invariant glycine. Thus, this region may perform a similar function in all B’ homologs.

Starting at amino acid 822, human B’ consists of a series of nine long repeats of 53–55 amino acids, each of which can be further divided into two shorter but more degenerate repeats of 26–28 amino acids, followed by one additional short repeat. In a BLAST search of the Swiss-Prot database, regions of hB’ comprising the repeats and extending as far upstream of the SANT domain align with proteins belonging to the myosin and intermediate filament families and characterized by long, α-helical domains that can form coiled-coil structures. However, programs that predict coiled-coil regions such as COIL [Lupas et al. 1991] or PAIRCOIL [Berger et al. 1995], and that give very high scores to the proteins that align with hB’, do not predict similar extended rod-like structures in hB’, although hB’ does show short regions with high scores. Thus, the significance of these similarities is not clear. Each of the 19 short repeats contains serine and threonine residues [indicated in boldface in Fig. 1D] that are potential phosphorylation sites for various kinases, including PKC, PKA, CAMII, CDC2, and CKII kinases, suggesting that this part of the molecule may have a regulatory function. This very striking repeated sequence is not present in the yeast protein.

To determine whether the protein shown in Figure 1A corresponds to an endogenous protein, we raised antibodies against several peptides derived from the amino acid sequence shown in Figure 1A [see Materials and Methods]. We then performed an immunoblot of the following: hB’ translated in vitro in a rabbit reticulocyte lysate; whole-cell extract [WCE]; a mixture of in vitro–translated hB’ and whole-cell extract; and unprogrammed reticulocyte lysate [control]. As shown in Figure 2, the anti-hB’ antibody recognized a prominent polypeptide of ∼160 kD in HeLa-cell extracts [lane 3], which comigrated with in vitro–translated hB’ [lanes 1 and 2]. Another polypeptide of ∼250 kD that is prominently detected in the HeLa-cell extract [lane 3] may correspond to a cross-reacting protein or, perhaps, a hyperphosphorylated form of hB’. No major signal was obtained in the unprogrammed lysate control lane [lane 4]. Thus, cells contain a protein that is recognized by anti-hB’ antibodies and that comigrates with recombinant hB’ [rhB’].

**Figure 1.** Structure of a hB’. (A) Predicted hB’ protein sequence [GenBank accession number AF298151]. The SANT domain is indicated by a large horizontal bracket above the sequence. The repeated sequences are indicated by arrows. The sequence between the vertical brackets (amino acids 109–163) is lacking in some cDNAs that probably correspond to splicing variants. (B) Regions of similarity in the *Homo sapiens* and *S. cerevisiae* B’ sequences [hSb’ and Scb’, respectively]. The black boxes correspond to the SANT domain; the hatched boxes indicate regions of lower but still significant similarity on either side of the SANT domain. The percentages indicate identities between the two proteins in the regions delimited by the dotted lines. The numbers above the boxes indicate aa numbering. The small arrows indicate the repeats in hB’.

(C) Alignment of the SANT domain and region immediately upstream of hB’ [Hs] and Scb’ [Sc], as well as putative mouse (Mm), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), *Schizosaccharomyces pombe* (Sp), and *Arabidopsis thaliana* (At) B’ homologs. The SANT domain is indicated by the horizontal bracket above the sequence. The region N-terminal of the SANT domain was shown, in Scb’, to be required for efficient transcription of linear yeast U6 templates in vitro [Kassavetis et al. 1998]. The amino acids in boldface are identical in all sequences. The amino acids indicated in the consensus are identical in at least four of the seven sequences. [*] similar amino acids in all sequences; [+] similar amino acids in at least five of the seven sequences. The following amino acids were considered similar: V, I, L, and M; D and E; N and Q; R, K, and H; S and T; and W, F, and Y. The SANT consensus sequence [Aasland et al. 1996] is also shown. In the SANT consensus: [%] semiconserved hydrophobicity; [b] strongly conserved hydrophobicity; [a] conserved acidic residues, and [b] conserved basic residues. The positions marked with arrowheads indicate positions not conserved in the B’ and SANT consensus sequences. The GenBank accession numbers are as follows: Mm ESTs, AI1315861, AI1315862, and AI787462; Ce genomic sequences U97016 [B02611] [nucleotides 25059–25185 and 25518–25780]; Dm protein, AAF55291.1 [CG3905 gene product]; Sp protein, T41239; and At protein T08564 [hypothetical protein T22F8.60]. The numberings correspond to the sequence shown in (A) for hSb’, to the sequences published [Kassavetis et al. 1995; Roberts et al. 1996; Rüth et al. 1996] for Scb’, and to the sequences corresponding to the protein accession numbers indicated for other sequences. For the Mm sequence, the numbering corresponds to a putative mouse B’ N-terminal region (amino acids 1–377) assembled from an alignment of various ESTs [GenBank accession numbers AI527964, AI317698, AI787358, AI526613, AI316225, AI639984, AA168265, AW456745, AW049390, AI429566, AI430369, AI409297, W91010, AI787462, AI1315861, AI1315862, AI892036, and AI1315863], and for the Ce sequence, the numbering is arbitrary. (D) Alignment of the repeats within the C-terminal half of hB’. As indicated by the arrows, each repeat can itself be subdivided into two short imperfect repeats. Residues in boldface indicate potential phosphorylation sites for various kinases, including PKC, PKA, CAMII, and CKII kinases.
hB is required for transcription from both the VAI and the U6 promoters

To determine whether hB plays any role in transcription by RNA polymerase III, we covalently attached anti-hB antibodies to beads and used these beads to deplete extracts of endogenous hB. The extracts were then tested for their ability to support transcription from four types of promoters: the Ad2 VAI gene–internal RNA polymerase III promoter, the RNA polymerase III U6 and RNA polymerase II U1 snRNA promoters, and the Ad2 major late (Ad2 ML) promoter, a typical mRNA-type RNA polymerase II promoter. The results are shown in Figure 3. In all cases, treatment of the extracts with preimmune antibody beads had little effect (cf. lane 10 with lanes 1 and 5). In contrast, treatment of the extracts with anti-B antibodies directed against two different peptides (antibodies 913 and 843) severely debilitated transcription from both the VAI and U6 RNA polymerase III promoters but had little effect on transcription from the RNA polymerase II U1 and Ad2 ML promoters (cf. lane 2 with lane 1, and lane 6 with lane 5). The effect was specific, because incubation of each antibody with the peptide against which it was raised, but not with a nonspecific peptide, abolished the inhibitory effect (lanes 3, 4, 7, and 8). As a control, we also treated the extracts with an antibody directed against the SNAP43 subunit of SNAPc (Henry et al. 1995). We have shown before that SNAPc is specifically required for transcription from both RNA polymerase II and III snRNA promoters (Sadowski et al. 1993; Henry et al. 1998b). As expected, treatment with the anti-SNAP43 antibody debilitated RNA polymerase III transcription from the U6 snRNA but not the Ad2 VAI promoter (lane 9). Note that in the U1 panel, the lower band [U1] corresponds to correctly initiated U1 RNA; the upper band [5’ RT] corresponds to a read-through RNA initiating at cryptic promoters within vector sequence (Sadowski et al. 1993).

To ensure that the debilitating effect on VAI and U6 transcription observed above upon treatment of transcription extracts with anti-hB antibodies resulted from depletion of hB, we tested whether rhB could reconstitute transcription in the depleted extracts. As shown in Figure 4, addition of increasing amounts of recombinant hB [rhB] produced in Escherichia coli to...
depleted extracts restored both VAI [Fig. 4A] and U6 [Fig. 4B] transcription to levels higher than, or comparable to, those observed in the starting extract (cf. lanes 3–5 with lanes 1, 2, and 5 in Fig. 4B; note that in Fig. 4B, lane 5 was overloaded as evidenced by the intensity of the internal control signal [IC]). These results indicate that hB’’ is an RNA polymerase III transcription factor that, unlike hBRF (Mital et al. 1996), is required for transcription from both TATA-less and TATA-containing promoters.

**hB’’ is found on the U6 promoter region in vivo**

These experiments strongly suggest that hB’’ functions in transcription of the human U6 gene. To determine whether hB’’ can be found in the human U6 promoter region in vivo, we used the chromatin immunoprecipitation assay, in which intact cells are first treated with formaldehyde to cross-link proteins to DNA and then chromatin is extracted, extensively sonicated, and used as starting material for immunoprecipitations, with antibodies directed against the protein of interest (see Boyd et al. 1998; Kuo and Allis 1999, and references therein). The presence of specific DNA sequences in the immunoprecipitates can then be ascertained by PCR. We performed immunoprecipitations of cross-linked chromatin with anti-hB’’ and, as a control, preimmune antibodies and anti-TFIIB antibodies [Kuhlman et al. 1999]. We then checked the immunoprecipitates for the presence of U6 and, as a control, U1, promoter sequences.

The organization of the human U6 genes is not well understood, but U6 genes and pseudogenes appear dispersed in the genome. A BLAST search with the U6 gene we work with [Kunkel et al. 1986] indicated that the promoter region is probably unique; we therefore designed a pair of test primers within the U6 promoter. The control primers hybridized to a region 4 kb upstream of the U6 transcription start site. The U1 snRNA genes number an estimated 30–100 copies per haploid genome, each carried on a tandemly repeated 45-kb fragment [Bernstein et al. 1985]. In addition, the human genome contains a large number of U1 pseudogenes. Most have different flanking sequences than the true genes, but the U1 class I pseudogenes have 5’ and 3’ flanking regions very similar to those of the true genes [Denison and Weiner 1982]. We designed test primers capable of hybridizing to the true U1 promoters but not to flanking regions of U1 pseudogenes. The control primers hybridized to a region within the 45-kb repeat [but not in repeated sequences such as Alu sequences] at 7 kb upstream of the U1 start site, to ensure that any difference in signal between test and control primers did not result from a difference in copy number of the target DNA sequence. For both the U6 and the U1 control primers, the size of the expected fragment is close to that expected from the test primers, to ensure comparable PCR efficiency.

As shown in Figure 5, both the test and control primers gave the expected fragments when used to amplify the starting material for immunoprecipitation (lanes 9–12). As expected, no fragments were obtained with material precipitated by preimmune antibodies or in the input.
absence of antibodies [lanes 1, 2, 7, and 8]. However, the material immunoprecipitated by anti-B’’ antibodies contained U6 promoter sequences but not sequences located 4 kb upstream of the U6 transcription start site. U1 promoter sequences were not present above the background obtained with the test primers in this immunoprecipitation. In sharp contrast, U1, but not U6, promoter sequences were present at levels well above the background in material immunoprecipitated with an anti-TFIIB antibody, consistent with our previous observations that TFIIB is required for RNA polymerase II but not RNA polymerase III transcription from snRNA promoters (Kuhlman et al. 1999). Together, these data strongly argue that hBRF is indeed part of the U6 initiation complex.

**Isolation of a cDNA encoding a factor related to both hBRF and TFIIB**

We recently generated antibodies directed against various peptides within the TFIIB-related N-terminal domain of hBRF and observed that depletion of extracts with one of these antibodies [1043], unlike depletion with all other anti-hBRF antibodies we have tested, debilitated U6 transcription [see below]. This result was similar to the results obtained by Wang and Roeder (1995) and suggested that a protein different from, but related to hBRF was required for U6 transcription. We therefore, searched the database for human proteins with similarity to the N-terminal domain of hBRF. The highest scoring hit after TFIIB was an unnamed protein with a calculated molecular mass of 46.42 kD (GenBank accession number BAA91975). We used the corresponding DNA sequence to design primers and amplify by PCR a sequence from total HeLa-cell cDNA, which can code for the protein shown in Figure 6A (GenBank accession number AF298153). This protein is identical to that encoded by the BAA91975 entry except that a cysteine at position 157 in BAA91975 is replaced by a tyrosine in the protein shown in Figure 6A (GenBank accession number BAA91975). We used the corresponding antibody to hBRFU functions for U6 but not VAI transcription in vitro

To determine whether cells indeed contain a protein corresponding to hBRFU, we fractionated on an SDS–polyacrylamide gel purified recombinant hBRFU (rhBRFU) expressed in *E. coli*, a P11 B fraction from HeLa-cell extracts, and a mixture of both. The proteins were transferred to nitrocellulose and probed with the antibody capable of debilitating U6 transcription in vitro (antibody 1043). As shown in Figure 7, the antibody recognized rhBRFU as well as a comigrating HeLa-cell protein. Thus, the protein we identified in the database is indeed recognized by the 1043 antibody and is probably expressed in HeLa cells.

**Discussion**

Unlike the TFIIB activity in yeast cells, which has been well defined and is apparently unique, the TFIIB activity in other organisms has not been completely defined. Here, we have characterized a human homolog of yeast...
Figure 6. Structure of hBRFU. (A) Predicted hBRFU protein sequence (GenBank accession number AF298153). (B) Regions of similarities in the *Pyrococcus furiosus* TFIIH homolog (PfTFIIB) and the hTFIIB, hBRF, and hBRFU sequences. The location of the structured zinc ribbon as determined in PfTFIIB by nuclear magnetic resonance (Zhu et al. 1996), and as modeled in BRF and TFIIB (Hahn and Roberts 2000), and the location of the corresponding region in hBRFU are indicated in blue. The location of the structured core domain of TFIIB (Bagby et al. 1995; Nikolov et al. 1995) and that of the corresponding regions in the other proteins are indicated in purple. The region conserved between the proteins is indicated by the vertical striped lines. The percentages below the sequences indicate percentage of identity between hBRFU and PfTFIIB, hTFIIB, or hBRF within the region bracketed by the vertical striped lines as calculated from the alignment shown in C. (C) Alignment of PfTFIIB, hTFIIB, and the N-terminal regions of hBRF and hBRFU. The alignment was performed with the ClustalW program and the default parameters. The amino acids in boldface are identical in all sequences. The amino acids indicated in the consensus are identical in three of the four sequences. (+) similar amino acids in all sequences; (*) similar amino acids in three of the four sequences. The following amino acids were considered similar: V, I, L, and M; D and E; N and Q; R, K, and H; S and T; and W, F, and Y. The horizontal arrows above the sequences indicate the location of the structural repeats (Nikolov et al. 1995). The location of the structural zinc ribbon is indicated in blue, and that of the core [hTFIIB amino acids 113–316] in purple.
B’’, and we have shown that this protein is required for transcription of both TATA-less, tRNA-type promoters and TATA-containing, snRNA-type RNA polymerase III promoters. In addition, we have identified a factor related to both BRF and TFIIIB, which is specifically required for transcription from the U6 snRNA-type promoter. These results characterize TFIIIB, the central RNA polymerase III transcription factor, in human cells.

Identification of a functional human homolog of yeast B’’

hB’’ is a functional homolog of yeast B” by several criteria. First, a BLAST search of the protein database with hB’’ as the query returns yeast B” as the highest scoring match; second, hB’’ is functional for RNA polymerase III transcription from both the VAI and the U6 promoters in vitro; and third, it can be localized to the U6 promoter in vivo. We have shown in Figure 1C putative B” homologs from a number of species, including Mus musculus, D. melanogaster, C. elegans, S. pombe, and A. thaliana. Although there are no functional data concerning these proteins, the observations that [1] they show sequence conservation upstream of the SANT domain, in a region known to be functionally important in yeast B” [Kumar et al. 1997], and that [2] their SANT domains are more similar to each other than to SANT domains from other proteins, suggest that they indeed correspond to functional B’’ proteins.

The C-terminal region of hB’’ consists of 19 repeats of 26–28 amino acids, each of which contains a number of potential serine and threonine phosphorylation sites. This is reminiscent of the C-terminal domain of the largest subunit of RNA polymerase II, which for human RNA polymerase II consists of 52 heptad repeats with serine, threonine, and tyrosine phosphorylation sites. In the case of the largest RNA polymerase II subunit, the C-terminal domain is involved in a number of functions, including release of the transcription complex from the promoter [promoter clearance], recruitment of mRNA processing factors, and perhaps inactivation of transcription at mitosis (see Orphanides et al. 1996; Parsons and Spencer 1997; McCracken et al. 1998; and references therein). RNA polymerases III and I do not contain domains similar to the RNA polymerase II CTD, and the RNA polymerase III initiation complex, unlike the RNA polymerase II initiation complex, is not known to contain any kinase activity. The hB’’ C terminus may, however, be involved in regulatory functions. For example, inactivation of RNA polymerase III transcription at mitosis is known to occur through inactivation of TFIIIB, but the TFIIIB components affected have not been defined [Gottesfeld et al. 1994; White et al. 1995; Leresche et al. 1996]. Perhaps this inactivation involves phosphorylation of the repetitive C-terminal domain of hB’’.

Moreover, TFIIIB activity is regulated during interphase and has been shown to associate with the retinoblastoma protein [Rb] and the p107 and p130 pocket proteins, as well as with p53, all of which can downregulate RNA polymerase III transcription [Larminie et al. 1997; Cairns and White 1998, Sutcliffe et al. 1999]. Perhaps some of these proteins associate with hB’’, and the association may be regulated in part by phosphorylation of the hB’’ C-terminal domain.

Identification of hBRFU

The generation of the anti-peptide antibody 1043, which is capable of depleting U6 transcription when used to deplete transcription extracts, allowed us to identify...
hBRFU. The N-terminal domain of hBRFU is related to the N-terminal domains of both TFIIIB and BRF, and thus hBRFU is a new member of the TFIIIB family of proteins, which includes TFIIIB and BRF of various species. Very strikingly, unlike TFIIIB and hBRF, which do not function for U6 transcription [Mital et al. 1996; Henry et al. 1998a; Kuhlman et al. 1999], hBRFU efficiently restores transcription from the U6 promoter in a depleted extract. Thus, TFIIIB activities in human cells contain TBP, a factor required very generally for initiation by all three RNA polymerases, hB′′, a more specialized factor required for initiation by RNA polymerase III at all or many types of RNA polymerase III promoters, and hBRF or hBRFU, very specialized factors required for initiation by RNA polymerase III at subclasses of RNA polymerase III promoters. Figure 9 illustrates the composition TFIIIB on TATA-less and TATA-containing RNA polymerase III promoters.

At TATA-less promoters of the tRNA type, the internal A and B boxes recruit the transcription factor IIIC (upper panel). This allows the subsequent recruitment of TFIIIB, which in this case consists of TBP, hBRF, and hB′′. TBP and hBRF are tightly associated to each other in HeLa-cell extracts [Wang and Roeder 1995; Mital et al. 1996], as indicated by the dotted line separating the two factors. In contrast, hB′′ appears only weakly associated with the TBP/hBRF complex, if at all, as indicated by the solid line. Indeed, depletion of extract with anti-hB′′ antibodies did not remove TBP or hBRF [Fig. 4], and in immunoprecipitations of endogenous hB′, we could detect only low levels of TBP and no hBRF [data not shown]. This is similar to the yeast TFIIIB complex, which consists of strongly associated TBP and BRF, and much more loosely associated B′′ [Kassavetis et al. 1991; Huet et al. 1994].

At a TATA-containing promoter such as the human U6 promoter, the gene external core promoter PSE and TATA elements recruit SNAP and TBP, respectively [lower panel]. This presumably allows the subsequent binding of hBRFU and hB′′. In this case, the three TFIIIB components TBP, hBRFU, and hB′′ do not appear to associate strongly with each other in solution, as indicated by the solid lines separating the three factors in Figure 9. Indeed, depletion of extracts with anti-TBP, anti-hBRFU, or anti-hB′′ antibodies does not, in each case, remove significant amounts of the two other factors [Figs. 4 and 8; see also Mital et al. 1996; Henry et al. 1998a]. Recently, Takada et al. [2000] showed that in Drosophila cells, the TFIIIB activity contains a hBRF homolog and a TBP-related factor called TRF1. Together with our results, these results suggest that there is a wide range of TFIIIB activities in different species, which contain various TBP-like and BRF-like subunits.

Determination of RNA polymerase specificity at the human snRNA promoters

In mRNA promoters, TFIIIB is the factor that directly recruits RNA polymerase II [Orphanides et al. 1996]. TFIIIB also functions on RNA polymerase II snRNA promoters, as evidenced by the observations that depletion of extracts with anti-TFIIIB antibodies debilitates RNA polymerase II transcription from the U1 promoter but not RNA polymerase III transcription from the U6 promoter, and that U1 transcription can be restored by addition of recombinant TFIIIB [Kuhlman et al. 1999]. Moreover, TFIIIB can be localized to U1 promoter regions in vivo by chromatin immunoprecipitation [Fig. 5]. Thus, although we have not excluded the possibility that hBRFU might also be functional at RNA polymerase II snRNA promoters, it seems likely that on the human snRNA promoters, RNA polymerase specificity is determined by the recruitment of TFIIIB or hBRFU.

Although the N-terminal half of BRF is very similar to TFIIIB, the functional domain arrangement of the two factors is different. In TFIIIB, the zinc ribbon domain (indicated in blue in Fig. 6) is absolutely required for recruitment of RNA polymerase II and/or TFIIIB, and the core region (indicated in purple in Fig. 6) is required for association with the TBP/DNA complex [Orphanides et al. 1996]. BRF, like TFIIIB, contacts directly the RNA polymerase, in this case RNA polymerase III [Kho et al. 1994; Wang and Roeder 1995; Brun et al. 1997; Ferri et al. 2000], but the zinc ribbon domain of BRF is not required for RNA polymerase III recruitment [Kassavetis et al. 1997, 1998; Hahn and Roberts 2000]. Instead, the BRF zinc ribbon domain, which is essential for both viability...
Materials and methods

Cloning of cDNAs encoding hBR’ and hBRFU

At the time we started isolating a human homolog of yeast B’, the only entry in the database that showed some similarity to the yeast factor was a mouse EST (GenBank accession number AA200560) encoding 21 amino acids with strong similarity to part of the yeast B’ SANT domain. We used this sequence information to design primers and then used these primers in combination with primers hybridizing to the arms of λ to screen various human cDNA libraries. Several rounds of screening, each time with new primers located near the end of the largest fragment obtained, resulted in the isolation of a partial cDNA encoding amino acids 1–1020 of hB’. The sequence contained a stop codon upstream of the first ATG (see GenBank accession number AF298151), indicating that it encoded the very N terminus of the protein. At this point, a new entry was deposited in the database (GenBank accession number AB033067). Nucleotides 822–2243 of the AB033067 sequence were, with a few exceptions, identical to the last 1422 nucleotides (encoding amino acids 547–1020) of our sequence, but then the AB033067 sequence extended a further 3.5 kb downstream, the first 1104 nucleotides of which extended the open reading frame another 368 amino acids in the C-terminal direction. The 5’ end of the AB033067 sequence was divergent. We used the AB033067 sequence to design primers hybridizing to a region downstream of the stop codon and to a region within the sequence we had obtained, and performed PCR reactions from cDNA prepared from total HeLa-cell RNA. The resulting fragment was cloned and sequenced, and was nearly identical (except for a few amino acid changes) to entry AB033067. The sequence of some portions of hB’ was then further confirmed by the generation, with the high fidelity Pfu polymerase (Stratagene), of PCR fragments from cDNA made from total HeLa-cell RNA, followed by sequencing of the resulting fragments. A very recent entry in the database (GenBank accession number AJ238520) matches our sequence over its entire length almost perfectly. An examination of genomic sequences in the database indicates that the entire human B’ DNA sequence is found on chromosome 5 (GenBank accession number AC008851) and that other entries in the database (accession numbers AJ238520 and AB033067) most likely correspond to alternatively spliced products from the same region. A large region corresponding to the C-terminal half of the human B’ sequence can also be found in genomic DNA from chromosome 2 (GenBank accession number AC025985).

hBRFU was identified in the database as the human protein most related to hBRF after TFIB (GenBank accession number BA914975). The sequence information was used to design primers for PCRs with cDNA made from total HeLa-cell RNA and the high fidelity Pfu polymerase (Stratagene). The amplified sequence (GenBank accession number AF298153) was identical to the entry in the database except for one silent nucleotide change and one nucleotide change that changed the codon encoding cysteine 157 in BAA91795 to a codon encoding tyrosine.

Expression of recombinant proteins in E. coli

hBRF was expressed as an N-terminal glutathione S-transferase fusion protein from a modified pSBET vector (Schenk et al. 1995). Induction with IPTG was for 1 h at 37°C, and the glutathione S-transferase moiety was cleaved with thrombin. Both hB’ and hBRFU were expressed in E. coli as N-terminal Histagged and C-terminal FLAG-tagged proteins with the T7 RNA polymerase expression system (Studier et al. 1990), from modified pSBET (Schenk et al. 1995) vectors. Briefly, the constructs were transformed into BL21 cells and single colonies grown in M9ZB [Studier et al. 1990] at 37°C to an OD600 of 0.8. Induction was with 0.4 mM IPTG for 4 h at room temperature. The cells were pelleted and resuspended in 1:10 of the culture volume in lysis buffer: 10 mM Tris-HCl [pH 8], 250 mM NaCl, 1% Triton X-100, 50 mM NaH2PO4, 20 mM imidazole, and protease inhibitors (1 mM PMSF, 0.5 mM sodium bisulfate, and 0.5 mM benzamidine). The cells were frozen in liquid nitrogen and thawed three times, and homogenized with a dounce homogenizer on ice. The cell debris was pelleted by centrifugation at 15,000 rpm for 15 min at 4°C in a SS34 rotor (Sorvall). The supernatant was then incubated with nickel agarose beads (Qiagen) overnight at 4°C. The beads were washed three times with five-bead volume of wash buffer 1 (20 mM Tris-HCl [pH 8], 50 mM NaCl, 20% glycerol, 0.1% Tween 20, 20 mM imidazole, and protease inhibitors), twice with five-bead volume of wash buffer 2 (10 mM Tris-HCl [pH 8], 100 mM NaCl, 20% glycerol, 0.01% Nonidet P-40, and protease inhibitors), and the bound proteins were eluted with wash buffer 2 containing 300 mM imidazole. The eluate was dialyzed against TBS [20 mM Tris-HCl [pH 8], 150 mM NaCl, and protease inhibitors] and then incubated in the same buffer with flag agarose beads (Sigma). The beads were then washed with TBS containing protease inhibitors and eluted with the same buffer containing 200 µg/mL flag peptide. The eluate was dialyzed against buffer D100: 20% glycerol, 20 mM HEPES-KOH [pH 7.9], 0.5 mM EDTA, and 100 mM KCl.

Antibodies and immunoblots

The rabbit polyclonal anti-hB’ antibodies were directed against peptides corresponding to amino acids 139–153 (peptide hB’-3, CS913) and 392–410 (peptide hB’-3, CS848) of hB’. The rabbit polyclonal anti-hBRF antibody that cross-reacts with hBRFU (rabbit CS1043) was raised against a peptide (R2A) corresponding to amino acids 229–248 of hBRF (Mital et al. 1996). The anti-TFIB antibody was from rabbit CS396 (anti-TFIB-4) (Kuhlman et al. 1999).

The protein samples were fractionated on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with TBS-T (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween 20) containing 5% BSA and 5% nonfat milk, washed twice for 10 min in TBS-T, and probed with anti-hB’ (CS913) or anti-hBRF/hBRFU (CS1043) antibody at a 1:1000 dilution in TBS-T containing 5% BSA overnight at 4°C. The membranes were washed twice for 10 min in TBS-T and probed with the secondary antibody (sheep anti-rabbit) at a 1:4000 dilution in TBS-T containing 5% BSA and 2.5% nonfat milk for 1 h at room temperature. The blots were developed with ECL (Amersham Life Science) and exposed to film.
In vitro transcriptions

In vitro transcriptions were performed as described by Kuhlman et al. [1999] with the following modifications. The U6 transcription reactions were performed in a total volume of 40 µL containing 75 ng of the pU6/Hae/RA.2 template, 500 ng of poly (dG-dC) · poly (dG-dC), and 8 µL of whole-cell extract diluted 1:1, and were complemented with 4 µL of a 10-fold ATP mix (0.5 M ATP, 10 µg/mL phosphocreatine kinase, and 0.5 M creatine phosphate) and 5–10 ng of recombinant TBP. The VAI transcription reactions were performed in a total volume of 20 µL, contained 250 ng of the pSBM13+VAI template and 8 µL of whole-cell extract diluted 1:1, and were incubated for 1 h at 37°C. The Ad2 major late (p19MLP[C2A]) transcription reactions were performed in a total volume of 30 µL and contained 10 µL of whole-cell extract diluted 1:1. The U1 (pU1*G1) transcription reactions were performed in a total volume of 40 µL containing 15 µL of whole-cell extract diluted 1:1. The whole-cell extract contained 28 mg of protein per mL.

For depletions, 200 µL of extract was mixed with 200 µL of protein A agarose beads covalently linked to the antibody of interest overnight at 4°C. The beads were then pelleted by centrifugation, and the depleted extract (diluted 1:1 relative to the starting extract) was used directly for transcriptions.

Chromatin immunoprecipitations

The formaldehyde cross-linking and immunoprecipitations were performed as described by P. Farnham and colleagues [Boyd et al. 1998], except that the NaCl concentration during immunoprecipitation was raised to 150 mM and the immunoprecipitations were performed overnight at 4°C. To design the control primers for the U6 promoter, we used the sequence in a 22-kb contig (GenBank accession number AC009292, contig extending from 140423 to 162578) that contains the U6 gene we work with. The control primers (U6C1UP and U6C1L) hybridized to a sequence located 4 kb upstream of the U6 transcription start site, and the test U6 primers (U6U4UP and U6U2L) bracketed the core U6 promoter. The PCR reaction consisted of 29 cycles.

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