Assembly of Human Small Nuclear RNA Gene-specific Transcription Factor IIIB Complex de Novo On and Off Promoter*

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In humans, transcription factor IIIB (TFIIB)-α governs basal transcription from small nuclear RNA genes by RNA polymerase III (pol III). One of the components of this complex, BRFU/TFIIB150, is specific for these promoters, whereas TATA-binding protein (TBP) and hB⁺ are required for pol III transcription from both gene external and internal promoters. We show that hB⁺ is specifically recruited to a promoter-bound TBP-BRFU complex, which we have previously demonstrated as forming on TATA-containing templates. The N-terminal region of BRFU, containing a zinc ribbon domain, acts as a damper of hB⁺ binding. TBP deactivates this negative mechanism through protein-protein contacts with both BRFU and hB⁺, which may then promote their cooperative binding to form TFIIB-α. In addition, we have identified a GC-rich sequence downstream from the TATA box (the BURE) which, depending on the strength of TATA box, can either enhance BRFU binding to the TBP-DNA complex or hB⁺ association with the BRFU-TBP-DNA complex, and subsequently stimulate pol III transcription. Moreover, mutation of the BURE reduces pol III transcription and induces transcription by RNA polymerase II from the U2 gene promoter carrying a minimal TATA box.

Accurate initiation of transcription of snRNA⁷ genes by RNA polymerase III (pol III) requires the formation of a large, multiprotein complex at the promoter (reviewed in Refs. 1–3). A typical type 3 pol III core promoter, commonly represented by 7SK and U6 genes, contains a proximal sequence element, covering −15 bp and located −55 bp upstream of the transcription start (4) and TATA box at around −25 (5–7). The proximal sequence element is a binding site for the multisubunit factor PBP/PTF/SNAP, (8–10). TBP binds at the TATA box, and this interaction is enhanced by PTF (11). Recently, we showed that TBP can also be recruited to the TATA box by interaction with BRFU/TFIIB150 (12, 13), which is a TFIIB- and BRF-related basal transcription factor, specifically required for U6/7SK transcription (12–14). BRFU/TFIIB50 can be purified from human cells as part of a multisubunit complex, and functional data suggest that as yet unidentified additional TFIIB50-associated subunits are also required for an in vitro 7SK transcription, reconstituted from partially purified components. However, recombinant BRFU alone is sufficient to support basal U6 transcription using a more defined and hence less complex system (15). BRFU/TFIIB50, together with TBP and hB⁺/TFIIB150, may constitute the minimal snRNA gene-specific TFIIB activity (TFIIB-α) that is required for transcription of U6 or 7SK genes in vertebrates (12, 13).

Human B⁺ is a large protein (1388 amino acid residues) that is similar to yeast scb⁺ over an ~300-amino acid residue stretch comprising the SANT domain (43% identity), which is essential for transcription in yeast, and N-terminal as well as C-terminal flanking segments (~20% identity). The C-terminal domain consists of nine long repeats of a 55-amino acid motif, each of which can be divided into two divergent 26–28-amino acid parts with regularly spaced serine and threonine residues that are potential phosphorylation sites. An antibody raised against recombinant hB⁺ identifies an ~160-kDa protein in HeLa cells, and hB⁺-immunodepleted HeLa nuclear extract is unable to support U6 and VA1 gene transcription, which can be both restored with bacteria-expressed hB⁺ (12). Thus, hB⁺/TFIIB150 is also involved, together with TBP and TFIIB90 (12, 16, 17), in the other form of human TFIIB complex, (TFIIB-β) (18). This complex functions instead on the tRNA-type Ad2 VA1 promoter (18, 19) that exemplifies a class of pol III promoters whose essential elements are all located within the gene coding region.

It is generally accepted that binding of TBP to the TATA box nucleates the formation of preinitiation complexes either through stepwise assembly of TFIIB and other basal factors or through recruitment of a holoenzyme (reviewed in Ref. 20). Here we report on the formation of a minimal TFIIB-α complex on TATA-containing snRNA promoters where hB⁺ can join a preassembled BRFU-TBP-DNA sub-complex. We find that the N-terminal region of BRFU, containing a zinc ribbon, inhibits the direct association of BRFU with hB⁺ in solution. This auto-inhibition is deactivated by TBP bridging, which promotes cooperative assembly of TFIIB-α from recombinant TBP, BRFU, and B⁺ subunits. Such a mechanism may ensure creation of the “proper” architecture of the TFIIB-α complex.

Mutagenic selection, in vitro transcription, and binding assays, in addition to cross-linking experiments using human factors, indicate that binding of TFIIB to mRNA pol II promoters is highly asymmetrical (21, 22) and dependent on a specific 7-bp TFIIB recognition element (BRE) immediately upstream of the TATA box (21). Our previous study (14) suggests that the TFIIB-like factor BRFU makes contacts with sequences flanking the TATA box, which may instead be more extensive downstream. In the 7SK gene the sequence just downstream from the TATA box is GC-rich and recognizably resembles a "back-

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The abbreviations used are: snRNA, small nuclear RNA; pol III, RNA polymerase III; TFIIB, transcription factor IIIB; TBP, TATA-binding protein; BRE, IIB recognition element; BURRE, BRFU recognition element; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay.
ward" BRE, suggesting it may play a role in BRFU-TBP-DNA interaction. We have investigated this possibility by studying the efficiency and accuracy of BRFU-TBP-DNA sub-complex formation and their consequences for assembling of the whole TFIIIB-α complex on snRNA promoters, transcribed by pol III, where this sequence has been mutated. The outcome of this analysis provides evidence for requirement of an intact GC-rich structure, designated as a BURE, for BRFU-TBP-DNA assembly at the first level. At the second level, the BURE influences the affinity of already assembled BRFU-TBP-DNA sub-complex for hB-α. Transcription studies confirm the importance of the BURE for efficient transcription both in vivo and in vitro and for maintenance of polymerase specificity.

Our data indicate that sequences located downstream from the TATA box can influence the assembly of TFIIIB-α in two unique ways to regulate the efficiency and specificity of basal pol III snRNA transcription.

**Experimental Procedures**

**Recombinant Protein Purifications**—The plasmids encoding C-terminal histidine-tagged human BRFU and B-α proteins for both in vitro transcription and translation and expression in *Escherichia coli* cells have been described previously (12). The plasmid encoding glutathione-S-transferase BRFU was derived from in vitro transcription-translation construct ptTfTBP (23) by PCR amplification of the TBP-coding region and placing into pGEX-2T (Amersham Biosciences) using BamHI and EcoRI sites. Polyhistidine-tagged hBRFU and hB-α were purified by nickel chelate affinity chromatography as described previously (14, 24). Immunoblot analysis of isolated B-α with the anti-His antibody (Roche Diagnostics) was performed by using the enhanced chemiluminescence system (Amersham Biosciences) as recommended by the manufacturers. Purification of GST-BRFU and its deletion derivatives, Δ1–37, Δ2–157, 1A69–266, and Δ72–266, GST-TBP, and GST on glutathione-agarose (Sigma) has been described previously (14).

**Transcription Templates and Corresponding EMSA Probes**—The 7SKwt and 7SK TATA–transcription constructs are identical to O′-P- (9) and O′-P′-T (5), respectively. In the 7SK BURE construct, the wild-type sequence between 19 and 14 was mutated to TTTTTT. The U2mTATA/7SK transcription construct contains U2 gene sequences 556 to 6 upstream from sequences 1 to 458 of the marked 7SK gene (9) between the EcoRI and PsiI sites of pGEM4. The U2 sequence between 32 and 27 was mutated to TTATTA, and thus a minimal TATA box was introduced. In U2mTATA/BURE/7SK the sequence between 23 and 14 was mutated to CTGGTGGCC. The probes for EMSA studies were made from the above characterized templates as described previously (9). In the U2 TATA probe (14) the sequence between 26 and 18 was mutated to TTATTA and as described by Lobo and Hernandez (7).

**Electrophoretic Gel Mobility Assays**—Probes for EMSA were prepared by end labeling double-stranded DNA with the Klenow enzyme and α-32P-deoxyadenosine triphosphates. Two binding and corresponding gel systems were used essentially as described previously (14).

The BRFU-TBP-DNA and B-α-BRFU-TBP-DNA complexes were analyzed using the "TGE gel system." Binding reactions contained 10 nl of probe, 0.2 μg of poly(dG-dC), 10 mM HEPES, pH 7.9, 30 mM Tris-HCl, pH 8.4, 60 mM KCl, 7.5 mM MgCl2, 10% glycerol, 20 mM dithiothreitol, and 0.1 mM of bovine serum albumin per ml. Reactions were initiated by the addition of proteins, and mixtures were incubated for 30 min at 30°C. The mixtures were electrophoresed on a 4% (37.5:1, acrylamide-bisacrylamide) polyacrylamide gel, with 0.5× Tris borate-EDTA (TBE) at 40 mA.

For detection of the TBP-DNA complex, the "TGEM gel system" was employed. The untagged TBP was incubated with various probes for 40 min at 30°C in buffer containing 0.2 μg of poly(dG-dC), 10 mM HEPES, pH 7.9, 10 mM Tris-HCl, pH 8.0, 60 mM KCl, 5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, 0.01% Nonidet P-40, and 0.1 mg of bovine serum albumin per ml. The binding reactions were resolved on 4% polyacrylamide gel in 1× TGEM running buffer (50 mM Tris base, 380 mM glycine, 2 mM EDTA, and 5 mM MgCl2) at 200 V.

**Protein-Protein Interaction Assays**—The [35S]Met-labeled full-length B-α, BRFU, and TBP were produced using a Taq coupled transcription-translation system (Promega). GST-BRFU, GST-TBP, and GST proteins bound to glutathione beads were rotated with [32P]-labeled proteins in the binding buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 10% glycerol, 0.5 mM MgCl2, 0.5% Nonidet P-40, 0.2 mM EDTA, 0.4 mg of ethidium bromide per ml) at 4°C for 3 h. Where indicated, binding reactions were supplemented with 10 pmol of each *E. coli* expressed full-length TBP or core TBP. The beads were washed five times with binding buffer, boiled in SDS sample buffer, and then pelleted, and the supernatant was loaded onto an SDS-10% polyacrylamide gel. The TBP-labeled proteins were detected by autoradiography or analyzed on a PhosphorImager (Amersham Biosciences).

**Transfections and S1 Nuclease Analysis**—Human embryonic kidney 293 and cervical carcinoma HeLa cells were cotransfected with 5 μg of snRNA gene promoter reporter construct and 0.25 μg of VAI plasmid using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Cells were harvested 48 h after transfection. Total RNA was isolated using the TRI Reagent® (Sigma) and then analyzed by S1 assay (25). The sequences of S1 oligonucleotides for VAI, U2mTATA/7SK, and its BURE-deficient derivative were described previously (14).

**In Vitro Transcription**—The nuclear extract was prepared from HeLa cells as described previously (26). Twenty five-μl transcription reactions were carried out as described by Murphy et al. (27) with 1 μg/ml a-amanitin, 250 ng of each 7SK template, and 2 ng of VAI construct as an internal control. Following 1 h of incubation at 30°C, the transcripts were analyzed by S1 analysis as described for RNA collected from transfected cells. The S1 oligonucleotide sequence for 7SK and its derivatives has been published previously (25).

**RESULTS**

**Human B-α Loosely Associates with the DNA-bound TBP-BRFU Complex**—We have shown previously (14) that the snRNA-specific factor BRFU cooperates with TBP to create a relatively strong complex on TATA box containing pol III snRNA gene promoters. TBP and BRFU together with hB-α constitute the TATA-dependent snRNA-specific TFIIIB transcription activity, designated as TFIIIB-α (13). We therefore wondered whether hB-α could be selectively recruited to promoters by direct binding to DNA, DNA-TBP, or DNA-TBP-BRFU sub-complexes.

Human B-α protein expressed in and purified from *E. coli* (Fig. 1A) was first assayed for DNA binding alone and in the presence of recombinant TBP (Fig. 1B). In this electromobility shift assay, we have used a template derived from the TATA-containing, pol III-transcribed 7SK gene. TBP alone can be detected binding to this template only when a TGEM gel system is used but not in a TBE system (14). B-α is unable to form a stable complex with DNA in the TGEM system either alone or in the presence of TBP (data not shown). Incubation of TBP, B-α, and DNA did not result in the appearance of any B-α-specific complex in the TBE gel system (Fig. 1B, lanes 1 and 3). We therefore tested the ability of B-α to interact with a BRFU-TBP-DNA complex. Indeed, in the presence of TBP, BRFU, and template DNA, the addition of B-α results in the appearance of a slowly migrating complex on a TBE gel (Fig. 1B, compare lanes 5 and 4). To confirm this finding, we also used another probe, prepared from a pol III-transcribed snRNA gene construct containing U2 promoter sequences with an introduced TATA box. Again, in addition to the DNA-TBP-BRFU complex (Fig. 1B, compare lanes 7 and 6) an additional slow migrating complex appears in the presence of B-α (lane 7). Taken together, these results suggest that we detect formation of a quaternary DNATBP-BRFU-B-α complex. However, only a small amount of the input B-α appears to become associated with the DNA-TBP-BRFU complex (compare intensities of upper B-α-TBP-DNA band with the lower BRFU-TBP-DNA band in lanes 5 and 7; see also longer exposure in lanes 5′ and 7′). Thus, the quaternary complex may be relatively unstable to the gel conditions used or B-α may only be loosely associated within the complex. We observed another complex just above the DNA-TBP-BRFU complex (Fig. 1B, lanes 5 and 7, marked by asterisk). As shown in Fig. 1A, hB-α protein preparation contains an ~67-kDa fragment, which was identified as a C terminus comprising part of B-α. This B-α fragment can also associate with the BRFU, TBP, and DNA to create a faster migrating complex. Inspection of
the autoradiograms reveal 10% association of both B' protein forms with the BRFU-TBP-DNA complex, from which full-length B' is responsible for 30% binding.

**TBP Mediates the Assembly of Trimeric TFIIIB-α Complex Off the Promoter**—We next explored whether a TBP-BRFU-B' (TFIIIB-α) complex can form in the absence of DNA. Because recruitment of B' to the template is BRFU-dependent and BRFU recruitment to the template is TBP-dependent (14), the possibility of direct interactions between B' and BRFU and/or TBP were tested. For GST pull-down assays, BRFU and TBP were produced in *E. coli* as GST-tagged proteins and immobilized, together with GST alone, on glutathione-agarose beads (Fig. 2B). As no appreciable interaction was observed between in vitro translated [35S]methionine-labeled B' protein and GST-BRFU (Fig. 2A, lane 2), either conformational change of BRFU or an additional interaction with TBP may be necessary for B' recruitment. Indeed, when the assay was performed in the presence of recombinant TBP, B' was retained on GST-BRFU beads (Fig. 2A, lane 4). Ethidium bromide was added to preclude the possibility that DNA might mediate interactions among these proteins. To understand further the TBP-dependent mechanism through which BRFU recruits B', the 35S-labeled B' was incubated with the immobilized GST-TBP. As shown in Fig. 2A, lane 7, direct interaction between B' and GST-TBP was detected. BRFU and TBP have also been shown to associate directly in vitro (14). The interaction between TBP and BRFU was confirmed in a reciprocal experiment in which 35S-B' was selectively retained by immobilized GST-TBP (Fig. 2A, lane 10) but not GST alone (Fig. 2A, lane 11).

We have shown previously (14) that the conserved C-terminal core domain of human TBP is sufficient for both interaction with BRFU and recruitment of BRFU to the TATA box containing snRNA gene templates. Therefore, we were interested to determine whether the C-terminal half of TBP, like full-length TBP (δTBP), is capable of recruiting B' to the immobilized BRFU. For this purpose, equimolar amounts of δTBP and N-terminal truncated TBPΔ1-154 (cTBP) were included into binding reactions. As shown in Fig. 3, 35S-B' is brought to the GST-BRFU beads by cTBP as efficiently as by δTBP (Fig. 3, compare lanes 4 and 2).

Together these data suggest that TBP is central for the assembly BRFU with B', perhaps because TBP bridges, through its core domain, these two proteins by direct interactions.

**Deletion of the Zinc Ribbon Domain Reduces BRFU Binding to the TBP and Allows Direct Interaction between B' and TBP to Occur**—To gain a better understanding of the architecture of the TFIIIB-α complex, we focused our attention on the BRFU domain(s) responsible for TBP binding and B' recruitment. We used a set of GST-BRFU protein mutants where important TFIIIB-like domains (Fig. 4A) were deleted (14).

First, we compared the TBP binding abilities of equivalent amounts (as determined by quantitation of Coomassie Blue-stained SDS-polyacrylamide gel; data not shown) of GST-BRFUs that have been prebound to the beads (Fig. 4B). The outcome of this GST pull-down study confirms that 35S-TBP binds well to full-length BRFU (more than 3-fold of 10% input) (Fig. 4B, lane 2). Furthermore, deletion of repeat 1 (Fig. 4B, lane 4), repeat 2 (lane 5), and whole core (lane 6) resulted in an almost 50% reduction of BRFU-TBP binding, relative to full-length BRFU. Surprisingly, BRFU missing the first 37 amino acid residues interacts poorly with TBP (below 10% input) (Fig. 4B, lane 3), suggesting that zinc ribbon plays an important, if not decisive, role in BRFU-TBP interaction in solution.

Second, to address which of these BRFU mutants can recruit B', the same amounts of GST-BRFUs used in the previous assay were incubated with 35S-B' in the absence or presence of TBP (Fig. 4C). Strikingly, whereas full-length as well as repeat 1, repeat 2, and whole core BRFU deletion mutants are capable of recruiting B' only when TBP is present (Fig. 4C, compare lanes 6, 8, and 10 with lanes 3, 7, 9, and 11, respectively), the BRFU mutant lacking the zinc ribbon appears to have B' binding affinity on its own (lane 4). This suggests that BRFU contains a built-in mechanism to down-regulate its direct binding to B', which is counteracted by TBP bridging.
expressed and solved by SDS-PAGE gel and visualized by autoradiography. Proteins used in these protein-protein binding reactions.

proteins used for this pull-down experiment. Bound proteins were re-

... of GST-TBP (lane 1), GST-BRFU (lane 2), and GST (lane 3) proteins used in these protein-protein binding reactions. M indicates protein marker standard above the respective lane.

A Novel GC-rich Regulatory Region “BURE” Downstream of the TATA Box Is Responsible for Efficient DNA-TBP-BRFU Complex Formation—The snRNA promoters transcribed by RNA pol III (U6 and 7SK) contain the sequence TTTATATA located around −25 (27, 28), whereas the TATA box in mRNA promoters is often TATAAA. Thus, the pol III snRNA-type TATA box appears to be inverted with respect to mRNA promoters. A specific 7-bp TFIIIB recognition element (BRE) is located immediately upstream of “mRNA” TATA boxes in some cases and consists of a stretch of mostly G and C nucleotides (21). BRFU shares sequence similarities with TFIIIB (12) and also interacts with TBP (14). We have noticed that a G/C-rich region that fits closely to the BRE consensus is located downstream from the inverted TATA boxes in the 7SK gene and fortuitously in the U2 TATA constructs (7) that are transcribed efficiently by pol III. Thus, this sequence may play a role in either BRFU recruitment and/or assembly of whole snRNA-specific TFIIIB and, consequently, modulation of pol III transcription. As a potential BRFU-binding site, we have tentatively named this region the BRFU Recognition Element (BURE).

To investigate the role of this sequence, EMSA probes were constructed from two transcription templates. As depicted in Fig. 5A, the minimal TATA element, TTTATATA, was inserted into the U2/7SK hybrid construct (14) (Fig. 5A, wt), and the 10-bp U2 region downstream of TATA box was mutated from GCCCGAAGG to CTTGTGCGCC (Fig. 5A, BURE). The “wt” construct contains a region with remarkable similarity to an inverted ideal BRE: (G/C)(G/C)(G/A)CGCC (21) just downstream of the TATA box (Fig. 5A, wt). The resulting “wild-type” (wt) and mutant (BURE) probes were tested for their potential to recruit two different TBP-BRFU complexes where the BRFU is tagged with either GST or His. As shown in Fig. 5C, fewer BRFU-TBP complexes were detected using the BURE probe (lanes 4 and 6) than the “wt” probe (lanes 3 and 5). Most important, this effect was not due to a reduction of TBP binding to the BURE probe as verified in Fig. 5C, lanes 1 and 2. Interestingly, the BURE probe is able to retain even more TBP in comparison to “wt” (Fig. 5C, lane 2 versus lane 1). Because no contacts outside the 8-bp TATA element have been observed in either structural or biochemical studies of TBP-DNA interactions (29–31), we would not expect the BURE sequence to simply modulate TBP binding. Similarly to sequences flanking the TATA box on mRNA genes (32), the BURE may rather influence TBP stability by altering its off rate rather than the on rate.

Mutation of BURE Strongly Reduces pol III and Potentiates pol II Transcription—By having defined the BURE as an element required in addition to the TATA box for efficient recruitment of the pol III snRNA-specific TBP-BRFU sub-complex, we

P. Čabart and S. Murphy, unpublished results.
Assembly of hTFIIIB-α Complex

The designated S1 oligonucleotide can also detect another transcript 6 nucleotides longer which, when transcribed from a TATA-less U2/7SK template, originates at the U2 start site (Fig. 5A, labeled pol II). This transcript has been identified previously as α-amanitin-sensitive and hence pol II-dependent. Intriguingly, we find that appearance of this transcript is responsive to BURE mutation and is in the context of the intact minimal TATA box (Fig. 5B, labeled pol II, lanes 2 and 4). These results suggest that mutating BURE effectively reduces transcription by RNA polymerase III from the U2mTATA/7SK template and allows pol II-type transcription to occur.

Quantitation of the reduction in formation of BRFU-TBP complexes on the BURE mutant is shown in Fig. 5D. The data for GST- and His-BRFU-TBP-DNA complex formation on “wt” and BURE− U2mTATA/7SK probes (Fig. 5D, b) was normalized to the corresponding binding of TBP to the DNA (Fig. 5D, a). Mutation of the BURE reduces BRFU-TBP recruitment to ~28 and ~39%, respectively. Thus, the reduction in BRFU-TBP-DNA complex formation (Fig. 5D, b) correlates with the reduction in pol III-specific transcription (Fig. 5B, lanes 2 and 4).

The Intact BURE in the 7SK Gene Promoter Contributes to Efficient pol III Transcription—To evaluate the importance of the BURE in an authentic snRNA promoter recognized by RNA polymerase III, the 6-bp GTGCGC block located downstream of the TATA box (Fig. 6A, wt) was replaced by TTTTTT (Fig. 6A, BURE−). Fig. 6B demonstrates that this mutation decreases 7SK transcription in both 293 cells and HeLa nuclear extract (Fig. 6B, compare lanes 1 and 3 with lanes 2 and 4, respectively). Addition of a low level of α-amanitin to reconstituted transcription reactions (33) ensures that only polymerase III-specific transcription is detected. It appears that for in vitro transcription, mutation of the BURE has a smaller effect than the TATA box mutation (5) (Fig. 6B, compare lanes 4 and 5). We should emphasize that the complete 7SK TATA box, TTTTATA, is able to recruit TBP much more potently than the minimal 3bp TATA box (Fig. 6A, BURE−). Fig. 6B demonstrates that mutation decreases 7SK transcription in both 293 cells and HeLa nuclear extract (Fig. 6B, compare lanes 1 and 3 with lanes 2 and 4, respectively). Addition of a low level of α-amanitin to reconstituted transcription reactions (33) ensures that only polymerase III-specific transcription is detected. It appears that for in vitro transcription, mutation of the BURE has a smaller effect than the TATA box mutation (5) (Fig. 6B, compare lanes 4 and 5). We should emphasize that the complete 7SK TATA box, TTTTATA, is able to recruit TBP much more potently than the minimal TTTTATA introduced into U2/7SK (see short and long exposures of Figs. 6C and 5C, lanes 1 and 2, respectively), and the strength of binding of TBP on its own may well modulate the TBP-BRFU sub-complex binding affinity to this class of promoter. Thus, the exact sequence of the TATA box itself and the adjacent BURE may independently modulate the level of transcription.

The BURE Is Required for Efficient Formation of the Trimeric TFIIIB-α Complex at the Promoter—Our previous observation that mutating the BURE strongly reduces pol III transcription from two different snRNA-specific templates and the results on impaired recruitment of TBP-BRFU sub-complexes suggest that BURE can play a role in the assembly of TFIIIB-α on the promoter. Does it always function at the first step characterized by TBP-BRFU recruitment? A demonstration of an additional molecular mechanism is shown in Fig. 6D. EMSA probes were derived from 7SK wt and 7SK BURE− transcription constructs and incubated with TBP, BRFU, and B* Strikingly, in the absence of B*, TBP-BRFU complex binding affinity to BURE− probe is only slightly reduced and parallels the binding of TBP alone (compare lanes 1 and 7 in Fig. 6D with lanes 1 and 2 in Fig. 6C). However, increasing amounts of B* reveal that a larger complex is formed only on the wt probe but not on the BURE− probe (Fig. 6D, see lanes 3 and 4). The highest B* concentration used does not contribute to the enrichment of specific DNA-TBP-BRFU-B* complex but, considering loss of lower DNA-TBP-BRFU signal (Fig. 6D, lane 4), we assumed larger than quaternary complexes are assembled using wt probe, and these are incapable of entering the 3.5% non-denaturing gel. In contrast, the ternary DNA-TBP-BRFU complex is prevalent on the BURE− probe with little appear-

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Fig. 4. Deletion of the zinc ribbon domain reduces BRFU binding to the TBP and allows direct interaction between BRFU and B* to occur. A, scheme showing the structural organization of BRFU. The locations of zinc ribbon and core domains encompassing repeats 1 and 2 are indicated. In addition to full-length protein (fl), mutated GST-tagged BRFU derivatives were prepared: Zn−, deletion of amino acids 1–37; Rep1−, deletion of amino acids 72–157; Rep2−, deletion of amino acids 169–266; Core−, deletion of amino acids 72–266. B, 35S-labeled TBP was synthesized by in vitro translation, captured by GST-BRFU, GST-BRFU derivatives, or GST alone (shown above the lanes), and analyzed by SDS-PAGE. Lane 1, 10% of the amount of 35S-TBP used in the pull-down assay. The binding signals (lanes 2–7) were normalized against 10% protein input (10% IN, lane 1), and the mean values ± S.D. from three independent experiments are represented graphically below the autoradiogram. C, GST-BRFU or its derivatives, shown above the lanes, were mixed with 35S-labeled B* (lanes 2, 4, 6, 8, and 10). The corresponding binding reactions were each supplemented with 10 pmol of TBP (lanes 3, 5, 7, 9, and 11). After being washed, bound proteins were separated by SDS-PAGE. Lane 1 shows 10% of input 35S-B* used in the interaction assay.

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3 S. Murphy, unpublished observations.
A novel GC-rich regulatory region “BURE” downstream of the TATA box is responsible for efficient DNA/TBP-BRFU complex formation and ensures efficient transcription by pol III. Mutation of BURE strongly reduces pol III and potentiates pol II transcription. The start sites and directions of the pol III and pol II snRNA-specific transcriptions and the relative positions of the transcription.

The above U2mTATA/7SK template derivatives transfected into each cell line are indicated above the lanes. The positions of the pol III and pol II-specific transcripts and the internal VA1 transcripts are indicated above the promoter structure. Below, in the sequence between –32 and –13, an introduced minimal TATA box is shaded and the sequence that was then mutated is boxed (“wt”). The 3rd line shows the reverse complement of this sequence with the BURE element highlighted, and the TATA box shaded (inv). Mutated nucleotides within the region enclosed by dotted lines are shown in the 4th line. The bottom line shows the resulting (BURE”) mutant in the original orientation. B, S1 nuclease analysis of transcripts from U2mTATA/7SK and U2 BURE”/mTATA/7SK hybrid constructs (depicted in A) that were transfected into 293 (lanes 1 and 2) and HeLa cells (lanes 3 and 4). The U2mTATA/7SK template derivatives transfected into each cell line are indicated above the lanes. The positions of the pol III and pol II-specific transcripts and the internal VA1 transcripts are indicated at the left. An asterisk marks the undigested VA1 probe. C, EMSA was conducted in the TBE gel system in the absence of DNA and His-BRFU TBP complexes. In the left panel, EMSA was conducted in the TGEM gel system in the absence of DNA complexes (left panel of C) as a bar graph is shown (mean ± S.D., n = 3). D, b, the values shown for GST-BRFU-TBP-DNA and His-BRFU-TBP-DNA complexes have been normalized to the mean level of TBP-DNA complexes and are expressed relative to the values obtained for the “wt” probe (arbitrarily designated 1.0). Each bar represents the mean ± S.D. (n = 3).

DISCUSSION

Human TFIIIB can be chromatographically separated into two distinct activities, α and β (17, 18), required for transcription of snRNA genes and tRNA-type genes, respectively. Both types of promoter require TFIIIB components TBP and hB1/TFIIB150 for activity, whereas specificity for snRNA promoters is given by the TFIIB-α component BRFU/TFIIB50 (12, 13). Functional analysis of the BRFU/TFIIB50 and hB1/TFIIB150 requirements in a reconstituted U6/7SK transcription system (12, 13), together with the demonstration that TBP and BRFU are recruited coordinately to TATA box-containing promoters (14), suggests that TFIIB-α is minimally a three-subunit complex. We have therefore addressed the requirements for the assembly of this complex in solution and for recruitment to snRNA promoters.

DNA binding by TBP or TBP-containing complexes appears to be a rate-limiting step for transcription initiation at various pol II (34) and pol III promoters (35–37). Accordingly, many regulatory factors have been described that interact directly...
Figs. 6 and 7. The intact BURE in the 7SK gene contributes both to efficient pol III transcription and formation of the trimeric TFIIIB-α complex at the promoter. A, the top line shows 7SK promoter sequences from -31 to -13; the natural TATA box is shaded, and the nucleotides that were then mutated are boxed (wt). Below, the reverse complement of the same sequence is shown with the BURE highlighted and the TATA box shaded (inv). The 3rd lane shows the nucleotides within BURE that were mutated delimited by dotted lines. The TTTTTT stretch that replaces these in the BURE deficient probe is shown boxed (bottom line, marked BURE”). B, pol III transcription in 293 cells and HeLa nuclear extract, monitored by S1 analysis. 7SK wild-type and 7SK-BURE mutant templates (depicted in A) transfected into cells (lanes 1 and 2) or present in each reaction (lanes 3 and 4) and 7SK TATA box-deficient template (lane 5) are indicated above the lanes. The positions of the 7SK and internal VA1 transcripts are indicated at the right. C, EMSA conducted in the TGEM gel system with a 7SK wild-type probe (lane 1) or a 7SK probe carrying BURE mutation (lane 2), in the presence of TBP. D, EMSA performed in the TBE gel system with the 7SK wt (wt) or the 7SK BURE mutant (BURE”) probe, and constant amounts of TBP and BRFU, and either no B” (lanes 1 and 1’) or increasing amounts of B” (lanes 2–4 and 2”–4”), starting from 0.05 to 1 pmol per reaction. The positions of BRFU-TBP-DNA and B”-BRFU-TBP-DNA complexes are indicated at the left.

Fig. 7. A model for two-step modulation of TFIIIB-α complex assembly by the BURE. Wild-type, BRFU contacts TBP and the BURE to form a strong ternary complex which is joined by B’; pol III snRNA gene transcription is then favored. For BURE mutants, I, in the context of minimal TATA box, BRFU is unable to recognize the debilitated BURE and therefore to associate strongly with TBP-DNA complex. Pol III transcription is then greatly diminished, and pol II transcription can occur, indicating that TFIIIB and the remaining pol II transcription machinery can now assemble. For mutant II, in the context of complete TATA box, TBP and BRFU form a relatively strong but altered complex, which is not readily recognized by B’. Unavailability of functional TFIIIB-α results in a reduction of pol III transcription.

with TBP or its associated components to regulate transcription (11, 38, 39). In vitro, interactions that affect the initial steps in preinitiation complex formation can be readily monitored by electrophoretic mobility shift and protein-protein pull-down assays. By using these approaches, we show that a TBP-BRFU complex can recruit hB” both on and off DNA. However, despite formation of a relatively strong DNA-TBP-BRFU complex, association of hB” does not appear to be very stable to the gel retardation conditions used. This resembles yeast TFIIIB, where TBP and BR also form a very stable complex (designated B”) which is less tightly associated with hBRFU and either no B” (lanes 1 and 1’) or increasing amounts of B” (lanes 2–4 and 2”–4”), starting from 0.05 to 1 pmol per reaction. The positions of BRFU-TBP-DNA and B”-BRFU-TBP-DNA complexes are indicated at the left.

Mapping of protein-protein contacts within TFIIIB-α has shed some light on the requirements for complex formation. The evidence that the N terminus/zinc ribbon domain is primarily required for BRFU-TBP association is surprising in the light of data on DNA-TBP-BRFU complex formation, where instead repeat 2 of the core was necessary (14). However, it is conceivable that the conformation of BRFU changes when bound to DNA. In addition, radical deletions may alter the overall conformation of the protein being analyzed. TBP has also been shown here to bind independently to B’. Importantly, these interactions allow association of both BRFU and
hB, which does not occur in the absence of TBP. Therefore, TBP is essential for mediating the assembly of TFIIIB-A de novo both on and off DNA. Moreover, the C-terminal half of TBP, which binds BRFU, is sufficient to bridge BRFU and hB. It was unexpected that deletion of the zinc ribbon domain enables the direct association between BRFU and hB. How does this region down-regulate BRFU binding to the hB? It could perhaps mask potential binding site(s) within BRFU. Because the N terminus is a major contributor to the BRFU/TBP interaction, the rest of the molecule is likely to be exposed for contact(s) with hB. An intriguing possibility is that TBP, as well as interacting directly with hB, recruits hB indirectly through changes in the conformation of BRFU and thus exposing its surface for binding to hB. Taken together, these data indicate that the N-terminal region of BRFU acts as a damper of hB binding that is relieved by BRFU-TBP contacts. This would ensure stable interactions in solution only when all three components are present.

An alternative factor to BRFU has also been implicated in transcription of snRNA genes by pol III (41). This is a splice variant hBRF1/TFIIIB90, designated hBRF2, that is required for U6 gene transcription in vitro but not for tRNA-type VA1 gene transcription. It is a small protein (134 amino acid residues) that shares only one exon (encoding the second TFIIIB-related repeat and 32 adjoining amino acid residues) with hBRF. Similarly to BRFU and BRF, BRF2 binds to TBP, albeit much more weakly than BRF (41). Its possible interaction with hB remains to be determined. Also, it is not clear whether TFIIIB50/BRFU-associated proteins, required for 7SK gene transcription (13), participate in hB/TFIIIB150 recruitment.

In mRNA gene promoters, the GC-rich BRE is originally identified as an element that had a positive effect on transcription in vitro (21, 42). This effect correlates with an increase in stable TFIIIB-TBP-DNA complex formation mediated by direct contact of the BRE by TFIIB. Our functional studies on pol III-snRNA promoters indicate that the ~7-bp BRE-like segment contiguous with the downstream boundary of the TATA box is important for efficient pol III transcription both in vivo and in vitro. Moreover, mutating this sequence causes a switch in polymerase specificity when the TATA box is short, indicating that pol II-specific preinitiation complexes are generated. The last finding is particularly interesting and contributes to a model (Fig. 7) in which the composition and architecture of the preinitiation complexes formed on snRNA gene TATA promoters determine the selective recruitment of RNA pol III or II. Our results therefore establish the existence of a second core promoter element which, in addition to the TATA-element (5, 7), can modulate the polymerase specificity for snRNA transcription. Gel retardation assays suggest that this sequence, which is only modestly conserved, can provide a preferred, albeit somewhat variable, binding surface for BRFU. The observation that the GC-rich sequence, present downstream of the adenovirus major late promoter “typical mRNA gene” TATA box, still facilitates the DNA-TBP-BRFU complex assembly and allows pol III machinery to initiate transcription (14) illustrates the flexibility of this sequence. In the context of a “weak” TATA box, it is clear that BRFU-BURE interaction is important for the creation of DNA-TBP-BRFU sub-complex itself. It appears that a “strong” TATA box instead can overcome the BURE deficiency and direct DNA-TBP-BRFU sub-complex formation, but its presumably altered conformation does not allow hB to efficiently join such a complex. In both cases, BRFU-BURE interaction seems to play a substantial role in TFIIIB-a complex formation as a whole and subsequently in determining the overall strength of the pol III promoter. In agreement with the results of our gel retardation analysis, hB can be detected at the U6 promoter in vitro by chromatin immunoprecipitation analysis indicating close contact with the promoter (12). However, direct interaction between hB and DNA remains to be demonstrated.

The existence of such an additional promoter element would enable snRNA promoters to encode a broad range of promoter strength and specificity and increases the potential for regulation by transcriptional activators and repressors.

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