Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9786–9790, October 1995
Biochemistry

Cloning, expression, and function of *TFC5*, the gene encoding the B″ component of the *Saccharomyces cerevisiae* RNA polymerase III transcription factor TFIIIB

(promoter complexes/initiation factors/protein–DNA interactions/multiprotein complex assembly/DNA footprinting)

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ABSTRACT ***TFC5*, the unique and essential gene encoding the B″ component of the *Saccharomyces cerevisiae* RNA polymerase III transcription factor (TF)IIIB has been cloned. It encodes a 594-amino acid protein (67,688 Da). *Escherichia coli*-produced B″ has been used to reconstitute entirely recombinant TFIIIB that is fully functional for TFIIIC-directed, as well as TATA box-dependent, DNA binding and transcription. The DNase I footprints of entirely recombinant TFIIIB, composed of B″, the 67-kDa Brf, and TATA box-binding protein, and TFIIIB reconstituted with natural B″ are indistinguishable. A truncated form of B″ lacking 39 N-terminal and 107 C-terminal amino acids is also functional for transcription.**

The central transcription factor (TF) of RNA polymerase (pol) III is TFIIIB: TFIIIB alone suffices to recruit pol III for multiple rounds of accurately initiated transcription (1). Yeast TFIIIB binds avidly to greatly varying DNA sequence upstream of the transcriptional start sites of pol III-transcribed genes but requires an assembly factor, TFIIIC, for proper positioning (reviewed in refs. 2–4). An exception to this general rule is provided by the yeast U6 snRNA gene (*SNR6*), to whose TATA box TFIIIB can bind on its own. In fact, accurately initiating transcription of the *SNR6* gene can be executed *in vitro* by TFIIIB and pol III alone (5–7).

Yeast TFIIIB comprises three components (8): the TATA box-binding protein, TBP; the TBP-interacting 67-kDa TFIIB-related protein, Brf (gene *BRF1/PCF4/TDS4*) (9–11); and a chromatographically separable component named B″ (12). Active B″ was previously isolated as a 90-kDa "band" out of denaturing (SDS) gels, renatured, and shown to reconstitute active TFIIIB. In the experiments that are described below, we complete the proof of constitution of TFIIIB by cloning the gene encoding the B″ protein, expressing it in *Escherichia coli*, and using the resulting protein to reconstitute transcriptionally active TFIIIB entirely from its three recombinant constituents. Wholly recombinant TFIIIB suffices for precise binding to the upstream promoter of the yeast *SNR6* gene, for accurate recruitment by highly purified TFIIIC to a yeast tRNA gene promoter, and for accurate initiation of U6 snRNA and tRNA synthesis by yeast pol III.§

MATERIAL AND METHODS

DNA and Proteins. Plasmids pTZ2 (containing the *SUP4* $tRNA^{Tyr}$ G62 → C promoter-up mutation), pLNG56 (the same gene with the C56 → G promoter-down mutation), pLNWT (containing the wild-type *SUP4* gene; similar to pTZ1), and pCS6 (containing the *SNR6* gene coding for U6 snRNA) have been described (13, 14). The p539H6-derived $pU6_R$ is described elsewhere (ref. 14; S. K. Whitehall, G. A. K., and E.P.G., unpublished data). TFIIIC affinity-purified on *box B*-DNA, Mono Q-purified pol III, and recombinant yeast TBP were prepared as described (1, 7). His_6-tagged Brf (10) was purified on Ni^{2+}-chelating agarose as cited for the purification of recombinant B″ below. Greater purification was achieved by eliminating a previously used step of Mono S chromatography (7) and, instead, allowing Brf to precipitate upon direct dialysis of the Ni^{2+}-chelating agarose Brf fraction out of 7 M urea into 40 mM Tris·HCl, pH 8/100 mM NaCl/10% (vol/vol) glycerol and MPLP (10 mM 2-mercaptoethanol/0.5 mM phenylmethylsulfonyl fluoride/1 μg of leupeptin per ml/1 μg of pepstatin per ml). The Brf precipitate was resuspended in 6 M guanidine hydrochloride and renatured (15), using bovine serum albumin (BSA) diluent (16) with 500 mM NaCl as the dilution buffer. The preparation of Brf (Fig. 1, lane d), which contains the 67-kDa full-length protein and a 49-kDa fragment (resulting from proteolysis or internal translational initiation) that extends from amino acid V165 to the C-terminal His_6 tag, was 15% active in formation of heparin-resistant TFIIIB–DNA complexes (12).

Conditions for analysis of transcription, DNase I protection, and gel mobility shift with the *SUP4* and *SNR6* genes have been described (7, 16). Quantities of protein are specified as fmol of DNA-binding activity for TFIIIC, fmol of protein capable of forming heparin-resistant TFIIIB–DNA complexes for Brf, TBP, and B″, and fmol of pol III active in specific transcription (12, 13).

Purification of the B″ Component of TFIIIB for Micro-Sequence Analysis. B″ was extracted with 6 M urea from S-100 pellets derived from 1600 g of *Saccharomyces cerevisiae* BJ926 and subjected to chromatography on Bio-Rex 70 as described (8). Ninety percent of this material (70 mg of protein) was loaded onto a 60-ml hydroxylapatite column (Bio-Gel HTP; Bio-Rad) equilibrated in 100 mM sodium phosphate, pH 7.8/20% (vol/vol) glycerol/0.01% (vol/vol) Tween 20 and MPLP, washed with 60 ml of the same buffer followed by sequential 120-ml washes of the same buffer with 135, 160, 185, 215, and 500 mM in place of 100 mM sodium phosphate (pH 7.8). B″ TF activity eluted at the end of the 160 mM and throughout the 185 mM sodium phosphate step. Eighty percent of this material (3.6 mg of protein) was concentrated 5-fold (Centriprep 30; Amicon), diluted with buffer D [40 mM

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Abbreviations: TF, transcription factor; pol, RNA polymerase; BSA, bovine serum albumin; RACE, rapid amplification of cDNA ends; TBP, TATA box-binding protein.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U31819).

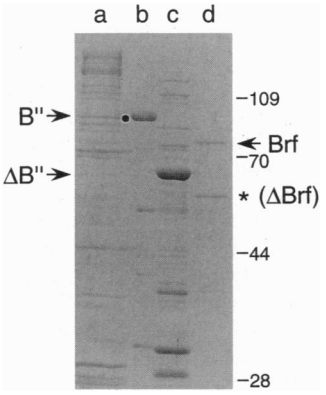

FIG. 1. Polypeptide composition of B″ fractions and of Brf. Eleven micrograms of Mono S-purified B″ (lane a; the dot to the right of the lane identifies the material subjected to microsequencing; the slower migrating minor shadow band is not resolved from the major B″ polypeptide on this gel), 0.9 μg of His_6-tagged recombinant B″ (lane b), 1.2 μg of His_6-tagged truncated B″ (ΔB″; lane c), and ≈0.5 μg of purified His_6-tagged Brf (lane d; full-length Brf and the fragment referred to in the text are identified at the right) were analyzed on a 10% polyacrylamide/SDS gel and stained with Coomassie brilliant blue R.

sodium Hepes, pH 7.8/7 mM $MgCl_2$/20% (vol/vol) glycerol/0.01% (vol/vol) Tween 20 and MPLP] to the conductivity of buffer D plus 150 mM NaCl, loaded onto Mono S (Pharmacia, HR 5/5 column) equilibrated in buffer D plus 150 mM NaCl, and eluted with a 25-ml 150–600 mM NaCl gradient in buffer D. B″ activity eluted between 290 and 350 mM NaCl; 2 mg of total protein contained ≈170 pmol of B″ measured by formation of heparin-resistant TFIIIB–DNA complexes with excess recombinant Brf and TBP in gel retardation assays (8).

Substantial purification of Mono S-purified B″ (Fig. 1, lane a) was achieved by chromatography on poly(dA-dT)·poly(dA-dT) coupled to CNBr-activated Sepharose (17) with only one polypeptide band in the 67- to 100-kDa range (data not shown but marked in Fig. 1, lane a) coeluting with B″ activity. The identity of this ≈90-kDa polypeptide as B″ was confirmed by subjecting 7 μg of protein from the Mono S-derived B″ fraction to SDS/PAGE. The material stained with Coomassie brilliant blue G was precisely excised, eluted, and renatured as described (8) along with slices of similar size taken above and below the presumptive band of B″ protein. B″ activity was observed in two gel slices: the presumptive B″ polypeptide and a weak (shadow) band directly above it. The quantity of protein in these two bands was estimated from the Coomassie stain density, using a dilution series of BSA on the same gel as a standard curve. Since 31 μg of 90-kDa polypeptide (340 pmol) yielded 170 pmol of active B″, we concluded that most of this material was the desired B″ polypeptide(s).

The protein in the major ≈90-kDa band and the weaker (shadow) band above it were separately isolated for microsequencing: 406 μg of protein from the Mono S B″ fraction was precipitated with acetone, resuspended in 1× sample buffer (18), separated by SDS/PAGE, and stained with 0.05% Coomassie brilliant blue G. Bands corresponding to the major and minor 90-kDa proteins were precisely excised, soaked in HPLC-grade water, and stored at −70°C. An in-gel digestion method (19) was used with modification to obtain internal amino acid sequence of B″. Individual gel slices were digested with *Achromobacter* protease I (lysylendopeptidase), and the resulting peptides were separated by reverse-phase HPLC (Vydac C_{18} column: 1.0 × 250 mm, 10 μm, 300 Å) for analysis of amino acid sequence (Applied Biosystems sequencer, models 470 and 477, with on-line HPLC analysis of phenylthiohydantoin-amino acids). The reverse-phase chromatographic profiles of peptides generated by digestion of the major and minor 90-kDa protein with lysylendopeptidase were nearly identical (one or two additional peptides were possibly present only in the minor 90-kDa protein).

Identification and Cloning of the *TFC5* Gene. Degenerate inosine-containing oligodeoxynucleotides based on peptide sequence were synthesized (Operon Technologies, Alameda, CA) in sense and antisense orientation for amplification of genomic DNA fragments coding for portions of the B″ gene by PCR (Table 1). Sense and antisense primer pairs (100 pmol each) were combined with 10 ng of yeast genomic DNA and 20 μM dNTPs for touchdown PCR amplification (20). Single-primer control reactions (21) proved useful in eliminating incorrect products. Each sense and antisense primer pair capable of correctly amplifying a portion of the B″ gene did so. The resulting map of PCR products was confirmed by generating predicted internal PCR products from larger, gel-isolated PCR products (22) and by identifying additional already-determined peptide sequence in the DNA sequence.

Confirmed PCR products derived from primer combinations 2 + 7, 2 + 8, and 4 + 8 were labeled by nick-translation (23) (4–6 × 10^8 cpm/μg of DNA) and sequentially used to probe mapping membranes for *S. cerevisiae* (prime clone grid filters; ATCC 77284) as well as colony blots of an *S. cerevisiae* genomic plasmid library [ATCC 77162, kindly provided by J. L. Cereghino and S. Emr, University of California at San

Table 1. Oligonucleotides

No.	Location*	Peptide sequence	Dir.†	5′ → 3′ sequence‡
1	33–39	TPQLFIP	S	ACICCICARYTITTYATHCC
2	218–224	PPTAMTD	S	CCICCIACIGCNATGACNGA
3	218–224	PPTAMTD	A	TCIGTCATIGCIGTNGGNGG
4	396–403	KVDENPFA	S	AAIGTIGAIGARAAYCCNTTYGC
5	396–402	KVDENPF	A	AAIGGITTYTCRTCNACYTT
6	406–412	YNYGSYG	S	TAIAAYTAYGGITCNTAYGG
7	406–413	YNYGSYGR	A	CTICCRTAIGAICCRTARTTRTA
8	490–496	KNIGTVA	S	GCIACIGTDCCDATRAAYTT§
9	N	—	S	GGTATCCATGGGTAGTATTGTTAATAAAAGTG
10	C	—	A	AATGTCTCGAGTTGATCAATCTCAGG
11	N (40)	—	S	TTGTCCATGGCCGAAAGTAAAGAAATAGA
12	C (487)	—	A	TCTTCTCGAGTTCGCAGCAATACTCAT

*Amino acid positions in Fig. 2; oligonucleotides 9–12 were used to generate expression plasmids for the complete and truncated B″ proteins.
†(S)ense or (A)ntisense orientation of the oligonucleotide.
‡I: inosine; R: purine; Y: pyrimidine; H: A, C, or T; D: A, G, or T; N: A, G, C, or T.
§The two underlined A residues should have been T residues, an error of specification rather than peptide microsequencing.

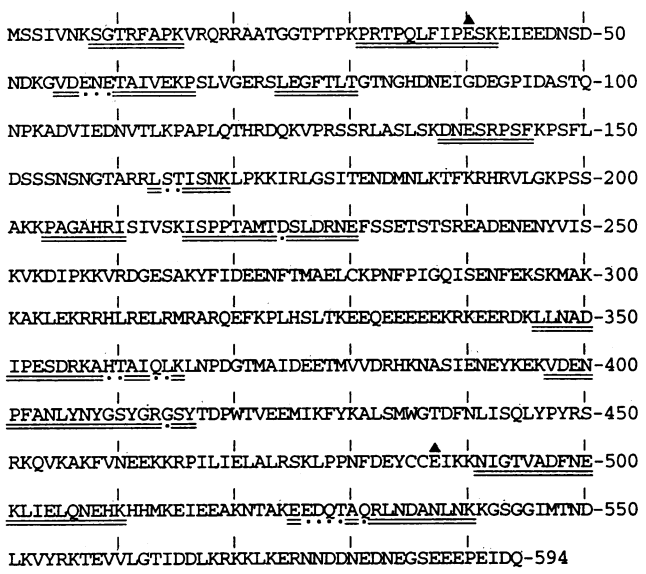

FIG. 2. Amino acid sequence of the B″ component of TFIIIB. Peptide sequences obtained by microsequencing Mono S-purified B″ are underlined (dots are placed under unassigned or uncertain and, as it turned out, incorrectly assigned residues). The N and C termini of B″ sequence remaining in the truncated form of B″ (residues 40 and 487) are designated with ▲ above the line.

Diego (UCSD)] as specified (24). Hybridization of all three probes to the prime clone grid filters identified cosmid c8487 (ATCC 71191) as containing the gene coding for B″. Adjacent and partially overlapping λ clones 7151 and 2713 failed to hybridize to the probes, thereby limiting the location of the B″ gene to between kbp 534.8 and 553.1 on the physical map of chromosome XIV. Three clones of the B″ gene were also obtained by screening colony blots of the genomic plasmid library. The presence of the gene in cosmid c8487 and in the three library-derived plasmids was confirmed by PCR amplification of B″ gene sequence from these sources. The gene coding for B″ was sequenced on both strands along with upstream (507 bp) and downstream (157 bp) flanking sequence, continuing further on one strand for 220 bp upstream and 450 bp downstream. Mapping of the 5′ and 3′ ends of mRNA coding for B″ utilized 5′ and 3′ RACE systems (RACE: rapid amplification of cDNA ends; GIBCO/BRL) and sequencing of the amplified products using total yeast RNA generously provided by G. Cereghino and I. E. Scheffler (UCSD).

Disruption of the B″ gene followed standard procedures (25) with a *Hin*dIII fragment (from pFL1; ref. 26) containing the *URA3* gene replacing a *Hin*dIII fragment of *TFC5* encoding amino acids 170–366 (Fig. 2) in the pET21d expression clone described below and introduction of a *Xba* I–*Xho* I fragment of this disrupted B″ gene into diploid *S. cerevisiae* strain MY4298 (*ura3, leu2, his3*) (27). MY4298 and pFL1 were kindly provided by M. Nickas and M. Yaffe (UCSD).

***TFC5* Gene Expression in *E. coli* and Purification of Recombinant B″.** The B″ coding sequence was PCR-amplified from cosmid c8487 with the high-fidelity Pfu DNA polymerase (Stratagene), using oligonucleotides 9 and 10 (Table 1) to generate an *Nco* I site at the N-terminal methionine (mutating the adjacent S to G) and an *Xho* I site at the ochre stop codon. The amplified product was cut with *Nco* I and *Xho* I and inserted into pET21d, extending the C-terminal amino acid sequence with LEHHHHHH. A similar strategy, with oligonucleotides 11 and 12 (Table 1), was used to clone a truncated version of B″, comprising amino acids 40–487, with MA at the N terminus and LEHHHHHH at the C terminus, into pET21d for expression in *E. coli* BL21(DE3)pLysS. The resulting His-tagged B″ proteins were purified on Ni^{2+}-chelating agarose (Qiagen) from 25 g of wet weight of cells under denaturing conditions, yielding ≈2.6 mg and ≈6 mg of full-length and truncated B″, respectively (Fig. 1, lanes b and c). B″ was renatured from the Ni^{2+}-chelating agarose elution buffer containing 7 M urea (adjusted to pH 8 with Tris base soon after elution) by rapid addition of 100 volumes of BSA diluent (16). The activity of recombinant, full-length B″ was determined by double-reciprocal plots of the formation of heparin-resistant TFIIIB–tDNA complexes with a fixed quantity of B″ (estimated relative to a BSA standard) and increasing concentrations of TFIIIC–Brf–TBP–tDNA complexes. Extrapolation of the least-squares best-fit linear plot (r^2 = 0.9996) to infinite DNA substrate indicated ≈100% active molecules. The specific activity of the truncated version of B″, although not quantified, appears comparable.

RESULTS

Identification and Isolation of the *TFC5* Gene. The B″ component of TFIIIB was purified from 6 M urea-extracted S-100 cellular debris on the basis of its ability to reconstitute active TFIIIB in combination with recombinant TBP and Brf. DNA–protein photocrosslinking (28) and renaturation of B″ TF activity from denaturing gels (8) had identified B″ as consisting of one or more ≈90-kDa polypeptide(s). The purification of B″ and precise identification of its corresponding polypeptide band (Fig. 1, lane a) are described in *Material and Methods*. In-gel digestion with lysylendopeptidase and microsequencing generated a subset of peptide sequences (underlined in Fig. 2) that were used to design degenerate oligonucleotides (Table 1, oligonucleotides 1–8) for PCR amplification of genomic DNA fragments encoding B″. Three confirmed amplification products (see *Material and Methods*) were sequentially used to probe phage λ and cosmid prime clone grid filters covering 95% of the *S. cerevisiae* genome. All three probes identified the presence of the gene encoding B″, *TFC5* [the fifth *t*ranscription *f*actor gene for class *C* (pol III) transcription], only on cosmid c8487, containing centromere-proximal sequence from chromosome XIV. The sequence of a 2445-bp region of cosmid c8487 contains an open reading frame whose deduced 594-amino acid sequence (67,688 Da) contains all 14 peptide sequences derived from B″.

The disparity between the apparent and sequence-deduced size of B″ (90 vs. 68 kDa) led us to examine the possibility that the gene coding for B″ contains an intron. However, analysis of the 3′ and 5′ ends of mRNA coding for B″ by RACE and sequencing of the products placed poly(A) sites ≈52 and ≈106 nt downstream of the ochre stop codon of the *TFC5* open reading frame and the 5′ end ≈39 nt upstream of the initiation codon, with no evidence for splice junctions (data not shown). Comparison of the amino acid sequence of B″ with the nonredundant combined data base at the National Center for Biotechnology Information (BLASTP, May 1995) (29) revealed no significant homologies outside of the glutamic acid-rich, highly charged region of residues 329–357, where homology with nucleolin and with ORF YKL202W on *S. cerevisiae* chromosome XIV was noted. We did not discern known sequence motifs in B″ (PROSITE, May 1995) (30) with the exception of potential signals for glycosylation, phosphorylation, and nuclear localization. *TFC5* is essential for cell viability: replacement of B″ amino acid residues 170–366 with the *URA3* gene and transformation into ura^- diploid yeast, followed by sporulation, produced no asci, out of 110 dissected, containing more than two viable spores.

Reconstitution of Entirely Recombinant TFIIIB. To confirm *TFC5* as the gene coding for B″, and to show that B″ consists of a single 68-kDa polypeptide that migrates anomalously on denaturing electrophoresis (apparent size, ≈90 kDa), we made His_6-tagged B″ in *E. coli* and purified it on Ni^{2+}-chelating agarose under denaturing conditions (see *Material and Meth-*

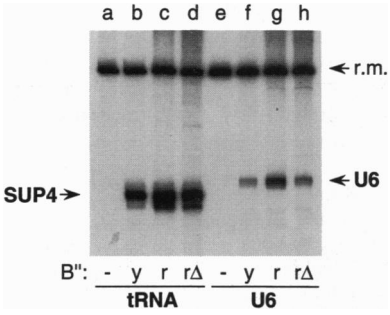

FIG. 3. Full-length and truncated recombinant B″ are competent for *in vitro* transcription of the *SUP4* tRNA and *SNR6* U6 small nuclear RNA (snRNA) genes. Two hundred nanograms of pTZ2 encoding the *SUP4* $tRNA^{Tyr}$ gene (lanes a–d) or pCS6 encoding the *SNR6* U6 snRNA gene (lanes e–h) was incubated with 100 fmol of recombinant TBP, 27 fmol of recombinant Brf, 10 fmol of purified pol III, 29 fmol of purified TFIIIC (lanes a–d only), and 5 fmol of Mono S-purified yeast B″ (lanes b and f; designated "y" below the figure), 46 fmol of recombinant His_6-tagged B″ (lanes c and g; designated "r"), or 4 ng of truncated B″ (lanes d and h; designated "rΔ") for 60 min at 20°C. Labeled ribonucleotides were then added for 30 min, generating multiple rounds of transcription. The *SUP4* and U6 transcripts are identified; r.m., labeled DNA recovery marker.

ods). Recombinant B″ migrated anomalously as an ≈90-kDa protein on SDS/PAGE (Fig. 1, lane b), indistinguishable in mobility from natural B″ (Fig. 1, lane a). A truncated variant of B″, lacking 39 N-terminal and 107 C-terminal amino acids (ΔB″; Fig. 2; molecular mass, 52 kDa), purified from *E. coli* as a C-terminally His_6-tagged protein, also migrated anomalously as an ≈61-kDa protein on SDS/PAGE (Fig. 1, lane c).

Recombinant full-length B″ and the truncated ΔB″ were able to replace natural yeast B″ for TFIIIC-dependent tRNA gene transcription (Fig. 3, compare lanes c and d with lane b) and for U6 snRNA gene transcription (Fig. 3, compare lanes g and h with lane f); the only nonrecombinant protein in the U6 assay was highly purified pol III (31).

The stability and structure of TFIIIB–DNA complexes formed with recombinant and natural B″ were also compared to determine whether some missing modification of the recombinant protein or the lack of an as yet uncharacterized protein in

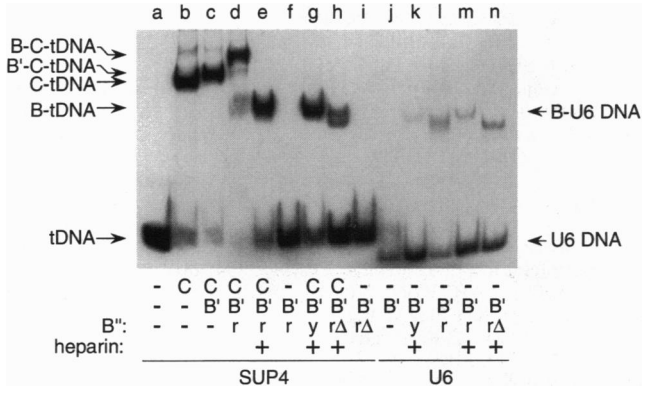

FIG. 4. Fully recombinant TFIIIB (B″ + Brf + TBP) forms heparin-resistant complexes with the *SUP4* and *SNR6* genes. The components incubated for 60 min at 21°C in 15-μl volume included 5.5 fmol of an ≈300 bp end-labeled fragment of pTZ2 containing the *SUP4* $tRNA^{tyr}$ gene (lanes a–i) or 5.8 fmol of a 201-bp end-labeled fragment of $pU6_R$ containing the *SNR6* gene (lanes j–n) and the following proteins as designated: TFIIIC (C; 58 fmol), recombinant yeast Brf and TBP (B′; 42 and 100 fmol, respectively), Mono S-purified yeast B″ (y; 5 fmol), recombinant His_6-tagged B″ (r; 46 fmol), and truncated, recombinant ΔB″ (rΔ; 4 ng). Heparin was added to 125 μg/ml for 5 min prior to loading on a 4% native polyacrylamide gel, as indicated. DNA complexes containing TFIIIC, TFIIIB, and the B′ component of TFIIIB (i.e., Brf + TBP) are identified at the sides.

relatively impure yeast B″ [such as TFIIIE (32)] might affect these properties. Fig. 4 compares the electrophoretic mobilities of TFIIIB–DNA complexes formed with recombinant B″, recombinant TBP, and recombinant Brf via TFIIIC-dependent assembly on the *SUP4* tRNA gene (lanes a–i) and TFIIIC-independent assembly on the *SNR6* gene (lanes j–n). On the *SUP4* gene, addition of Brf and TBP to TFIIIC–DNA complexes (lane b) generated the slightly slower migrating B″–TFIIIC–DNA complex (lane c). Inclusion of recombinant B″ generated the further retardation expected for TFIIIB–TFIIIC–DNA complexes (lane d). Treatment with heparin stripped TFIIIC and generated a TFIIIB–DNA complex (lane e) with the mobility of the corresponding complexes formed with Mono S-purified B″ (lane g). Formation of complexes with the *SUP4* gene was completely TFIIIC-dependent (lane f). Truncated B″ generated a slightly faster migrating TFIIIB complex that was also stable to heparin (lane h; the appearance of two discrete heparin-resistant bands was not reproducible and may have resulted from adventitious proteolysis). Recombinant and natural B″ likewise generated heparin-resistant complexes on the *SNR6* gene with identical mobilities (lanes m and k). The absence of any component derived from yeast in this reaction proves that the heparin resistance of this TFIIIB–DNA complex is solely a property of its three constituent polypeptides. The presence of heparin routinely diminishes the electrophoretic mobility of the TFIIIB–DNA complex (compare lanes l and m), possibly due to interaction with TFIIIB (see also ref. 7, Fig. 3).

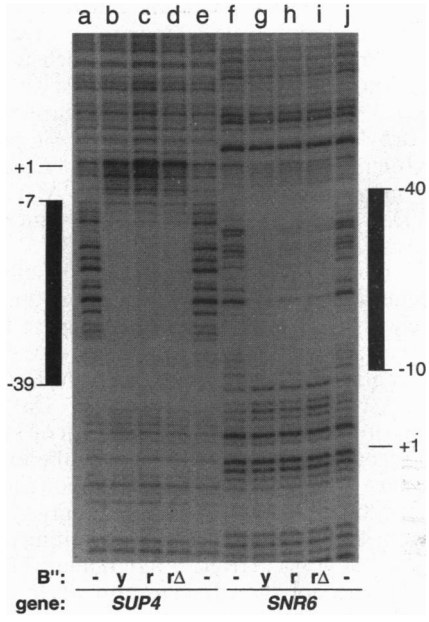

FIG. 5. TFIIIB reconstituted with natural and recombinant B″ generates identical DNase I footprints on the *SUP4* $tRNA^{tyr}$ and *SNR6* (U6 snRNA) genes. Six femtomoles of a 298-bp fragment of pLNWT containing the wild-type *SUP4* gene, 5′ end-labeled at the *Xba* I site (in the nontranscribed strand), was incubated with nothing (lanes a and e) or with TFIIIC (57 fmol), TBP (100 fmol), Brf (34 fmol), and either Mono S-purified yeast B″ (5 fmol, lane b), recombinant B″ (36 fmol, lane c), or truncated, recombinant ΔB″ (6 ng, lane d) in 15 μl of buffer for 60 min at 20°C. Heparin was then added (to 125 μg/ml) for 8 min, followed by DNase I digestion for 30 sec, at 21°C, and the reactions were terminated by adding EDTA to 20 mM. Free DNA and heparin-resistant TFIIIB–DNA complexes were separated on a 4% native polyacrylamide gel and their partial DNase I digestion patterns were analyzed on a 10% polyacrylamide/8 M urea gel, as shown. The samples displayed in lanes f–j are identical to those in lanes a–e, respectively, except that TFIIIC was omitted, and 4.4 fmol of the *SNR6* gene probe (3′ end-labeled on the transcribed strand) noted for Fig. 4 replaced the *SUP4* tRNA gene probe. The start sites of transcription (+1) and the extent of footprints (determined on a PhosphorImager scan) are indicated at the sides.

The DNase I footprints of *SUP4* (Fig. 5, lanes b–d) and *SNR6* complexes (lanes g–i) formed by TFIIIB reconstituted with natural (Mono S-purified) B″ (lanes b and g) and recombinant full-length B″ (lanes c and h) were indistinguishable. A subtle diminution of enhancement at and near the start site-proximal border was noted for the complex of recombinant truncated ΔB″ (lanes d and i) with *SUP4* (lane d). The absence of any yeast-derived component in the TFIIIB–*SNR6* complex shown in lane h proves that the extent of the TFIIIB footprint is solely due to B″, Brf, and TBP.

DISCUSSION

These experiments complete the proof of constitution of TFIIIB: three and only three proteins specify accurate initiation of transcription by yeast pol III. As expected, *TFC5* is an essential, unique gene. Screening of *S. cerevisiae* prime clone grid filters at low stringency failed to identify any other yeast gene related to the *TFC5* segment coding for amino acids 218–496.

TFIIIB that has been reconstituted from its separate *E. coli*-produced components is active for promoter binding on the *SNR6* and the *SUP4* genes. The precise correspondence of footprints generated on these genes by TFIIIB reconstituted with recombinant and natural B″ also strongly implies a comparable precision of placement. Thus, entirely recombinant TFIIIB is fully competent for its DNA-binding, TFIIIC-binding, and pol III-binding functions, as is the truncated ΔB″. The high activity and the simple, though chemically rigorous, purification scheme for reconstituting TFIIIB contrasts with what has been found recently to be required for reconstituting the pol I core transcription factor SL1, which is assembled from TBP and three TAF_I proteins with modest yield and some difficulty (33). The ability of B″ to acquire nearly full binding activity to Brf–TBP–DNA complexes after simple dilution from urea is reminiscent of the similar ability of *E. coli* σ^{70} (15). Like B″, σ^{70} migrates anomalously in SDS/PAGE (apparent mass, ≈90 kDa), possibly due to an SDS-resistant interaction of helices in its region 1.1 with its region 2.3/2.4 junction. Certain point mutations in these regions of σ^{70} eliminate the electrophoretic anomaly (34). The ready renaturability and anomalous electrophoretic mobility of B″ suggest that it may likewise contain a highly stable core structure. We note that, had it not been for its anomalous migration, B″, which has almost the same molecular mass as Brf (66,907 Da), would not have been recognized as a separate component of TFIIIB (28).

Although recombinant B″ is fully active in the formation of heparin-resistant TFIIIB–DNA complexes upon renaturation, we have noted that the recombinant B″-containing TFIIIB complexes were somewhat less active in recruiting pol III than similar amounts of yeast-derived B″-containing TFIIIB complexes (Fig. 3; data not shown). It is also worth noting that TFIIIE has recently been proposed as an additional core yeast pol III TF that is present in the B″ fraction of TFIIIB and acts subsequent to the binding of TFIIIB (32). Although our experiments appear to eliminate an essential role for TFIIIE in pol III transcription, it remains entirely plausible that TFIIIE contributes to transcription *in vitro*, perhaps due to an ability to protect one or more of the essential components from inactivation or to aid reactivation.

A truncated form of B″ lacking 39 N-terminal and 107 C-terminal amino acids is transcriptionally active and capable of forming very tightly bound, heparin-resistant transcription complexes. That surprising finding makes the further dissection of functional domains and interactions in B″ particularly interesting. It also makes it more important to determine whether the full-length *TFC5* gene has been cloned. Our principal reason for believing this to be the case is that there is no satisfactory acceptor splice site–TACTAAC box combination in the sequence. The sequences of these combinations in *S. cerevisiae* are highly constrained; only a few combinations deviating by a single nucleotide are known (35). We only find combinations of candidate sites deviating by two or more nucleotides. The sequence of the 5′ end of the 5′ RACE product also shows no indication of splicing at the N-proximal end of the gene, while 3′ RACE indicates poly(A) addition sites relatively close to the downstream end of the translational open reading frame.

We are grateful to C. Bowman and S. Beardsley for experimental assistance, P. Scott for most generous help with tetrad dissections, S. Emr for most generous hospitality, C. A. P. Joazeiro, S. K. Whitehall, G. Cereghino, and J. Kadonaga for discussions and advice, and J. Kadonaga and S. K. Whitehall for a critical reading of the manuscript. M. P. was a graduate fellow from the University of Sassari. Our research has been supported by the National Institute of General Medical Sciences (at UCSD) and the National Cancer Institute (at the Cold Spring Harbor Laboratory).

1. Kassavetis, G. A., Braun, B. R., Nguyen, L. H. & Geiduschek, E. P. (1990) *Cell* **60,** 235–245.
2. White, R. J. (1994) *RNA Polymerase III Transcription* (Landes, Austin, TX).
3. Willis, I. M. (1993) *Eur. J. Biochem.* **212,** 1–11.
4. Geiduschek, E. P. & Kassavetis, G. A. (1995) *Curr. Opin. Cell Biol.* **7,** 344–351.
5. Margottin, F., Dujardin, G., Gérard, M., Egly, J.-M., Huet, J. & Sentenac, A. (1991) *Science* **251,** 424–426.
6. Burnol, A. F., Margottin, F., Schultz, P., Marsolier, M. C., Oudet, P. & Sentenac, A. (1993) *J. Mol. Biol.* **233,** 644–658.
7. Joazeiro, C. A. P., Kassavetis, G. A. & Geiduschek, E. P. (1994) *Mol. Cell. Biol.* **14,** 2798–2808.
8. Kassavetis, G. A., Joazeiro, C. A. P., Pisano, M., Geiduschek, E. P., Colbert, T., Hahn, S. & Blanco, J. A. (1992) *Cell* **71,** 1055–1064.
9. Buratowski, S. & Zhou, H. (1992) *Cell* **71,** 221–230.
10. Colbert, T. & Hahn, S. (1992) *Genes Dev.* **6,** 940–1949.
11. López-de-León, A., Librizzi, M., Puglia, K. & Willis, I. (1992) *Cell* **71,** 211–220.
12. Kassavetis, G. A., Bartholomew, B., Blanco, J. A., Johnson, T. E. & Geiduschek, E. P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7308–7312.
13. Kassavetis, G. A., Riggs, D. L., Negri, R., Nguyen, L. H. & Geiduschek, E. P. (1989) *Mol. Cell. Biol.* **9,** 2551–2566.
14. Brow, D. A. & Guthrie, C. (1990) *Genes Dev.* **4,** 1345–1356.
15. Hager, D. A. & Burgess, R. R. (1980) *Anal. Biochem.* **109,** 76–86.
16. Kassavetis, G., Blanco, J. A., Johnson, T. E. & Geiduschek, E. P. (1992) *J. Mol. Biol.* **226,** 47–58.
17. Kadonaga, J. T. (1991) *Methods Enzymol.* **208,** 10–23.
18. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
19. Rosenfeld, J., Capdevielle, J., Guillemot, J. C. & Ferrara, P. (1992) *Anal. Biochem.* **203,** 173–179.
20. Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K. & Mattick, J. S. (1991) *Nucleic Acids Res.* **19,** 4008.
21. Hiltunen, T., Raja-Honkala, M., Nikkari, T. & Ylä-Herttuala, S. (1994) *BioTechniques* **17,** 240–242.
22. Peterson, M. G. & Tjian, R. (1993) *Methods Enzymol.* **218,** 493–507.
23. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
24. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1992) *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology* (Wiley, New York), 2nd Ed.
25. Rothstein, R. (1991) *Methods Enzymol.* **194,** 281–301.
26. Chevallier, M.-R., Bloch, J.-C. & Lacroute, F. (1980) *Gene* **11,** 11–19.
27. Smith, B. J. & Yaffe, M. P. (1991) *Mol. Cell. Biol.* **11,** 2647–2655.
28. Bartholomew, B., Kassavetis, G. A. & Geiduschek, E. P. (1991) *Mol. Cell. Biol.* **11,** 5181–5189.
29. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
30. Bairoch, A. & Bucher, P. (1994) *Nucleic Acids Res.* **22,** 3583–3589.
31. Bartholomew, B., Durkovich, D., Kassavetis, G. A. & Geiduschek, E. P. (1993) *Mol. Cell. Biol.* **13,** 942–952.
32. Dieci, G., Duimio, L., Coda-Zabetta, F., Sprague, K. U. & Ottonello, S. (1993) *J. Biol. Chem.* **268,** 11199–11207.
33. Zomerdijk, M. C. B. M., Beckmann, H., Comai, L. & Tjian, R. (1994) *Science* **266,** 2015–2018.
34. Gopal, V., Ma, H.-W., Kumaran, M. K. & Chatterji, D. (1994) *J. Mol. Biol.* **242,** 9–22.
35. Rymond, B. C. & Rosbash, M. (1992) in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression,* eds. Broach, J. R., Pringle, J. R. & Jones, E. W. (Cold Spring Harbor Lab. Press, Plainview, NY), Vol. 2, pp. 143–192.