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Functional Dissection of the B″ Component of RNA Polymerase III Transcription Factor IIIB: a Scaffolding Protein with Multiple Roles in Assembly and Initiation of Transcription

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Transcription factor IIIB (TFIIIB), the central transcription factor of *Saccharomyces cerevisiae* RNA polymerase III, is composed of TATA-binding protein, the TFIIB-related protein Brf, and B″. B″, the last component to enter the TFIIIB-DNA complex, confers extremely tight DNA binding on TFIIIB. Terminally and internally deleted B″ derivatives were tested for competence to form TFIIIB-DNA complexes by TFIIIC-dependent and -independent pathways on the *SUP4* tRNA^{Tyr} and U6 snRNA (*SNR6*) genes, respectively, and for transcription. Selected TFIIIB-TFIIIC-DNA complexes assembled with truncated B″ were analyzed by DNase I footprinting, and the surface topography of B″ in the TFIIIB-DNA complex was also analyzed by hydroxyl radical protein footprinting. These analyses define functional domains of B″ and also reveal roles in start site selection by RNA polymerase III and in clearing TFIIIC from the transcriptional start. Although absolutely required for transcription, B″ can be extensively truncated. Core proteins retaining as few as 176 (of 594) amino acids remain competent to transcribe the *SNR6* gene in vitro. TFIIIC-dependent assembly on DNA and transcription requires a larger core of B″: two domains (I and II) that are required for *SNR6* transcription on an either-or basis are simultaneously required for TFIIIC-dependent assembly of DNA complexes and transcription. Domains I and II of B″ are buried upon assembly of the TFIIIB-DNA complex, as determined by protein footprinting. The picture of the TFIIIB-DNA complex that emerges is that B″ serves as its scaffold and is folded over in the complex so that domains I and II are near one another.

The yeast RNA polymerase III (pol III) transcription machinery is the least complex and, at this time, the most highly resolved of the eukaryotic nuclear transcription systems. As such, it comes closest to supporting a comprehensive and deeper analysis of interactions and mechanisms in the initiation of eukaryotic transcription. The components of its transcription factors, transcription factor IIIA (TFIIIA), TFIIIB, and TFIIIC, are now enumerated and known to be encoded by 10 essential genes (2, 9, 12, 16, 20, 24, 33, 36, 37, 40, 42, 44, 45a, 48). Three of these genes encode the three subunits of its central transcription factor TFIIIB: the TATA-binding protein (TBP), which is required for all nuclear transcription (reviewed in reference 17), Brf, a TFIIB-related 596-amino-acid subunit (also called TFIIIB70), and B″ (also called TFIIIB90), a 594-amino-acid subunit with no discernible homology to other transcription proteins.

The central role of TFIIIB is defined by its ability to recruit pol III to the transcriptional start site (21, 25, 27). TFIIIB binds very tightly to ~25 to 30 bp of greatly varying DNA sequence upstream of the transcriptional start site. It can be brought to these initiation-specifying sites by two mechanisms: through the action of the assembly factor TFIIIC (5, 27) and through direct DNA recognition by its TBP subunit (10, 38, 51). TFIIIC-dependent assembly of TFIIIB and pol III on the promoter is conveniently analyzed on the *SUP4* tRNA^{Tyr} gene; the TFIIIC-independent, TBP-directed assembly process is readily analyzed on the strong TATA box-containing *SNR6* U6 snRNA gene. Both assembly pathways require the participation of all three TFIIIB subunits (14, 20, 22, 50). Entry of B″ into the TFIIIB-DNA complex, the last step of either assembly pathway, greatly changes the physical properties of the DNA complex (22, 24–26).

Stepwise assembly of TFIIIB on DNA upstream of the start site of transcription of the *SUP4* tRNA gene in vitro (3, 25, 26) begins with binding of Brf to the TFIIIC-tRNA gene (tDNA) complex, through its interaction with the second-largest subunit of TFIIIC (τ_{120}; gene *TFC4*) (3, 11, 29, 37). Brf-dependent entry of TBP generates the relatively stable B′ (Brf plus TBP)-TFIIIC-tDNA complex with a DNase I footprint that covers the start site of transcription (+1) and extends further upstream. Entry of B′ into the DNA complex also greatly stabilizes binding of TFIIIC to *box A*. Although Brf interacts with the 34-kDa subunit (gene *RPC34*) of pol III (29, 49), the B′-TFIIIC-tDNA complex is unable to stably incorporate pol III into a transcription initiation complex. Drastic changes occur upon entry of B″ and assembly of the complete TFIIIB-TFIIIC-tDNA complex: (i) nuclease protection upstream of the transcriptional start is more complete and extends further upstream, but the transcriptional start site becomes fully exposed to DNase I; (ii) photochemical cross-linking of DNA to Tfc4/τ_{120} upstream of the transcriptional start is greatly diminished, as is cross-linking to TBP; (iii) the TFIIIB-DNA complex is resistant to stripping by 1 M NaCl and by polyanions such as heparin or poly(dC); and (iv) competence for stable assembly of pol III over the start site of transcription is acquired.

The preceding observations indicate the critical role of B″ in TFIIIB function and in pol III-mediated transcription and suggest the existence of interactions with DNA, pol III, Tfc4/τ_{120}, and TBP. An interaction with Brf is also expected, since B″ does not stably associate with the TBP-DNA complex at the

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SNR6 (U6) TATA box in the absence of Brf (22, 24, 42); B″ may generate a conformational change in Brf and/or TBP that makes the TFIIIB-DNA complex salt and polyanion resistant.

The recent cloning of the *TFC5* gene, encoding B″ (24, 42, 44), makes it possible to dissect the internal and external interactions of the components of TFIIIB along the assembly pathway of the pol III transcription initiation complex. In the experiments that are described below, we have carried out a deletion analysis of the B″ protein, assessing competence of truncated B″ according to the following functional criteria: ability to form a DNA complex with the *SUP4* tRNA gene under the direction of TFIIIC and with the *SNR6* U6 snRNA gene in the absence of TFIIIC, resistance of these DNA complexes to dissociation by the polyanion heparin and ability to direct pol III to transcribe the *SUP4* and *SNR6* genes. The deletion analysis defines segments of B″ that are required for tight binding of TFIIIB to DNA, for interaction with TFIIIC, for preventing occlusion of the start site of transcription by TFIIIC, and for start site selection by pol III. A comparison of the outcome of the deletion analysis with the surface topography of B″ determined by protein footprinting points to two regions of B″ that are important for TFIIIB function and that become buried in the assembly of the TFIIIB-DNA complex.

MATERIALS AND METHODS

DNA templates and probes. Plasmids pTZ1, containing the *SUP4* $tRNA^{Tyr}$ gene with a G62→C promoter-up mutation (28), pCS6, containing the wild-type *SNR6* (U6 snRNA) gene (8), and pU6LboxB and pU6R, containing altered *SNR6* genes constructs (51), have been described elsewhere. Labeled DNA probes for electrophoretic mobility shift assays (EMSA) were generated by PCR with one of two oligonucleotide primers 5′ end labeled with T4 polynucleotide kinase. The *SNR6* TATA box probe (134 bp) was produced by PCR with pCS6 as the template and oligonucleotides 5′-TCAACGGTACCTGGCATGAACAG T-3′ and 5′-TGGTTATGCATTGACCAAATGTCCAC-3′. The *SUP4* tRNA gene probe (240 bp) was PCR amplified from pTZ1 with 5′-TTCACTCGGTA CCCATCTTGGAAG-3′ and 5′-TTTCAACATGCATGTCTGGAAGTG-3′. DNA probes were purified on 4% polyacrylamide gels under native conditions, passively eluted overnight into 10 mM Tris-Cl (pH 8.0)–1 mM EDTA–250 mM NaCl, and chromatographed on NACS 52 (Bethesda Research Laboratories) to remove residual acrylamide.

Transcription and EMSA. Protein-DNA complexes were formed as specified in each figure legend in reaction mixtures of 20 μl (transcription) or 15 μl (EMSA) containing 40 mM Tris-Cl (pH 8.0), 7 mM $MgCl_2$, 3 mM dithiothreitol (DTT), and 100 μg of bovine serum albumin (BSA) per ml, with 80 to 90 mM NaCl for *SUP4* tRNA gene binding and transcription or 60 to 80 mM NaCl for *SNR6* U6 snRNA gene binding and transcription. The reaction mixture analyzed in the top panel of Fig. 3 contained 40 mM NaCl and 25 mM potassium acetate. Samples for EMSA also contained 6% glycerol and 100 ng of pGEM1 (*SUP4* probes) or poly(dG-dC)·poly(dG-dC) (*SNR6* probes) as nonspecific carrier DNA. Native gel electrophoresis was performed as previously described (7). Multiple-round transcription reactions were initiated by adding 5 μl of a nucleotide mixture in transcription buffer providing 200 μM ATP, 100 μM CTP, 100 μM GTP, and 25 μM [α-^{32}P]UTP (10,000 cpm/pmol) and analyzed as described previously (28).

Proteins. TFIIIC, pol III, recombinant TBP, Brf, and wild-type B″ (full length and C-terminally His_6 tagged) were purified and assayed as previously described or cited (24). Quantities of protein are specified as femtomoles of DNA-binding activity for TFIIIC, femtomoles of protein capable of forming heparin-resistant TFIIIB-DNA complexes for Brf and TBP, femtomoles of protein for wild-type and deletion mutant B″ preparations (estimated relative to a Coomassie blue-stained BSA standard curve on sodium dodecyl sulfate [SDS]-polyacrylamide gels), and femtomoles of pol III active for specific transcription (26, 28). DNA binding was determined by EMSA (Brf was 15% active in this assay; TBP and wild type B″ were each nearly 100% active).

N- and C-terminal deletions of B″ were generated by PCR amplification of the wild-type B″ expression plasmid (24) with a 1:1 mixture of *Pfu* and *Taq* DNA polymerases and oligonucleotides (sequences available upon request) that introduced *Nco*I and *Xho*I sites at the N and C termini, respectively, for insertion into the vector pET21d. PCR-generated C termini were located at B″ amino acid residue 378, 400, 418, 441, 464, 487, or 594 (full length), followed by the sequence LEHHHHHH derived from the vector. PCR-generated N termini were located at B″ amino acid residues 1 (with an S2→G substitution [24]); 40 and 224 (both preceded by the sequence MA); 116, 138, 158, 240, and 263 (each preceded by M); and 186 (preceded by MD), 276, and 298.

Internal deletions in B″ were generated by transferring an *Xba*I-*Xho*I fragment harboring *TFC5* from the wild-type B″ expression plasmid into an M13 mp18 strain altered to contain an *Xho*I site in place of *Hin*dIII. Oligonucleotide-mediated loop-out deletions (sequences available upon request) were generated as described by Kunkel et al. (32). Oligonucleotides were designed to introduce a restriction endonuclease cleavage site for initial screening. B″ internal deletion mutants were then transferred back into pET21d as an *Xba*I-*Xho*I fragment. The resulting internal deletions are shown in Fig. 1. Internal deletion Δ355-372 inserts an R residue at the deletion site. B″ mutants containing internal deletion Δ272-292 with Δ424-438 or Δ438-449 were constructed by replacing an *Nco*I-*Bsm*I restriction fragment from Δ424-438 or Δ438-449 (the *Bsm*I site is unique, overlapping amino acid residues 314 to 316) with an *Nco*I-*Bsm*I fragment from Δ272-292.

B″ deletion mutants in pET21d were transformed into *Escherichia coli* BL21(DE3) and grown at 37°C in L broth containing 80 μg of ampicillin per ml to an absorbance at 600 nm of 0.4, and 20 ml of each culture was induced with 1 mM isopropylthiogalactopyranoside (IPTG). Cells were harvested 2 h after induction, resuspended in 2 ml of 100 mM $Na_xH_xPO_4$ (pH 8.0) containing 8 M freshly deionized urea, sonicated five times for 20 s each on ice, and sedimented for 10 min at 16,000 × *g*. The supernatant fluids were placed into tubes containing 50 μl of packed Ni-nitrilotriacetic acid (NTA) agarose (Qiagen) in the same buffer and allowed to bind for 2 h at room temperature with constant rotation. The resin was harvested by centrifugation and washed three times with 500-μl portions of 100 mM $Na_xH_xPO_4$ (pH 6.4) containing 8 M urea. B″ was then eluted in 100 μl of 100 mM $Na_xH_xPO_4$ (pH 6.4)–100 mM EDTA with 8 M urea. B″ was renatured by rapid 100-fold dilution into BSA diluent (24).

B″(138-594) was purified from 4.3 g of induced cells under native conditions as follows. Cells were resuspended in 12.9 ml of 50 mM Tris-Cl (pH 8.0)–0.1 mM EDTA–5% glycerol–10 mM 2-mercaptoethanol–0.5 mM phenylmethylsulfonyl fluoride (PMSF)–1 μg of pepstatin per ml–1 μg of leupeptin per ml–300 μg of lysozyme per ml and incubated for 30 min at 0°C. Tween 20 was added to 0.1% (vol/vol), and the mixture was sonicated five times for 30 s each on ice. The lysate was then diluted twofold with 17.2 ml of the same buffer containing 1 M NaCl but lacking EDTA and lysozyme and again sonicated five times for 30 s each on ice. The lysate was sedimented at 4°C for 1 h at 40,000 × *g*, and the supernatant fluid (32 ml; adjusted to contain 7 mM $MgCl_2$ and 10 mM imidazole [pH 7]) was loaded onto a 2.5-ml Ni-NTA agarose column equilibrated in buffer D500 (buffer D [20 mM sodium HEPES, {pH 7.8}, 7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.01% Tween 20, 10% glycerol, 0.5 mM PMSF] with 500 mM NaCl). The column was washed with 13 ml of D500 plus 10 mM imidazole and 15 ml of D500 plus 30 mM imidazole, and B″(138-594) was eluted with 15 ml of D500 plus 60 mM imidazole. The B″ fraction was diluted twofold with buffer D (to 250 mM NaCl) and loaded on a 2-ml Bio-Rex 70 (Bio-Rad) column equilibrated in buffer D250. The column was washed with 8 ml of buffer D250 and 12 ml of buffer D300, and B″ was eluted with buffer D750. The concentrated B″ (2 ml) was then chromatographed in 220-μl aliquots on Superose 12 (HR 10/30; Pharmacia) in buffer D500.

Protein footprinting of B″(138-594). B″(138-594), 22.5 pmol, was phosphorylated at S164 in a 25-μl volume containing 20 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 2.5 mM DTT, 5 μM [γ-^{32}P]ATP (6,000 Ci/mmol), and 25 U of bovine heart muscle protein kinase catalytic subunit for 60 min at 25°C. Unincorporated ATP was removed on a 600-μl Sephacryl S200 column in buffer D500. The efficiency of labeling was estimated to be 14%. This material was then chromatographed on a 20-μl Ni-NTA agarose column to remove traces of contamination with other labeled proteins as follows. The Sephacryl fraction was loaded in the presence of 10 mM imidazole, washed with buffer D500 (without PMSF) containing, successively, 10 and 20 mM imidazole (10 and 5 column volumes, respectively), and eluted with the same buffer containing 100 mM imidazole (2 column volumes). Size markers for gel electrophoresis were provided by phosphorylated N- and C-terminally deleted B″(138-487), -(138-464), -(138-441), -(138-418), -(138-400), and -(138-378). Other markers were made by partial digestion of B″(138-594) with endoproteinase Lys-C or endoproteinase Glu-C in buffer D with 50 mM NH_4HCO_3 (pH 7.7) in place of sodium HEPES and containing 100 μg of BSA per ml and 100 mM NaCl, with endoproteinase Arg-C (Clostripain) in 40 mM Tris-Cl (pH 8.0)–7 mM $MgCl_2$–100 mM NaCl–1 mM $CaCl_2$–10% glycerol–2.5 mM DTT–100 μg of BSA per ml or with CNBr in either 70% formic acid or 50 mM HCl–1% SDS.

For hydroxyl radical protein footprinting (15, 19), TFIIIB-DNA complexes were formed with 200 fmol of pTZ1, 150 fmol of TFIIIC, 250 fmol of TBP, 150 fmol of Brf, and 68 fmol of phosphorylated B″(138-594) for 60 min in a 30-μl reaction volume. NaCl was then added to 400 mM to strip TFIIIC from the complex, and plasmid DNA bearing TFIIIB was purified by chromatography on a 200-μl Sepharose CL2B column in buffer C (50 mM NH_4HCO_3 [pH 7.7], 7 mM $MgCl_2$, 100 mM NaCl, 0.01% Tween 20, 0.1 mM 2-mercaptoethanol, 0.1% glycerol, 1 mM EDTA, 200 μg of BSA per ml). Free B″ was transferred to buffer C by chromatography through Sephadex G-75. Hydroxyl radicals were generated for 1 min at 20°C by the sequential addition of 2 μl each of 50 mM Fe(III)EDTA, 40 mM sodium ascorbate, and either 40, 20, or 10 mM H_2O_2 to B″ or TFIIIB-DNA complexes in 14 μl of buffer C. The extent of cleavage was always less than 23% (i.e., single hit). Reactions were terminated by adding 5 μl of 315 mM Tris-Cl (pH 6.8)–25% 2-mercaptoethanol–15% SDS–30% glycerol–0.5% bromophenol blue, and half of each reaction volume was loaded onto an SDS–10 or 14% polyacrylamide gel (the resolving gel was 19 cm long).

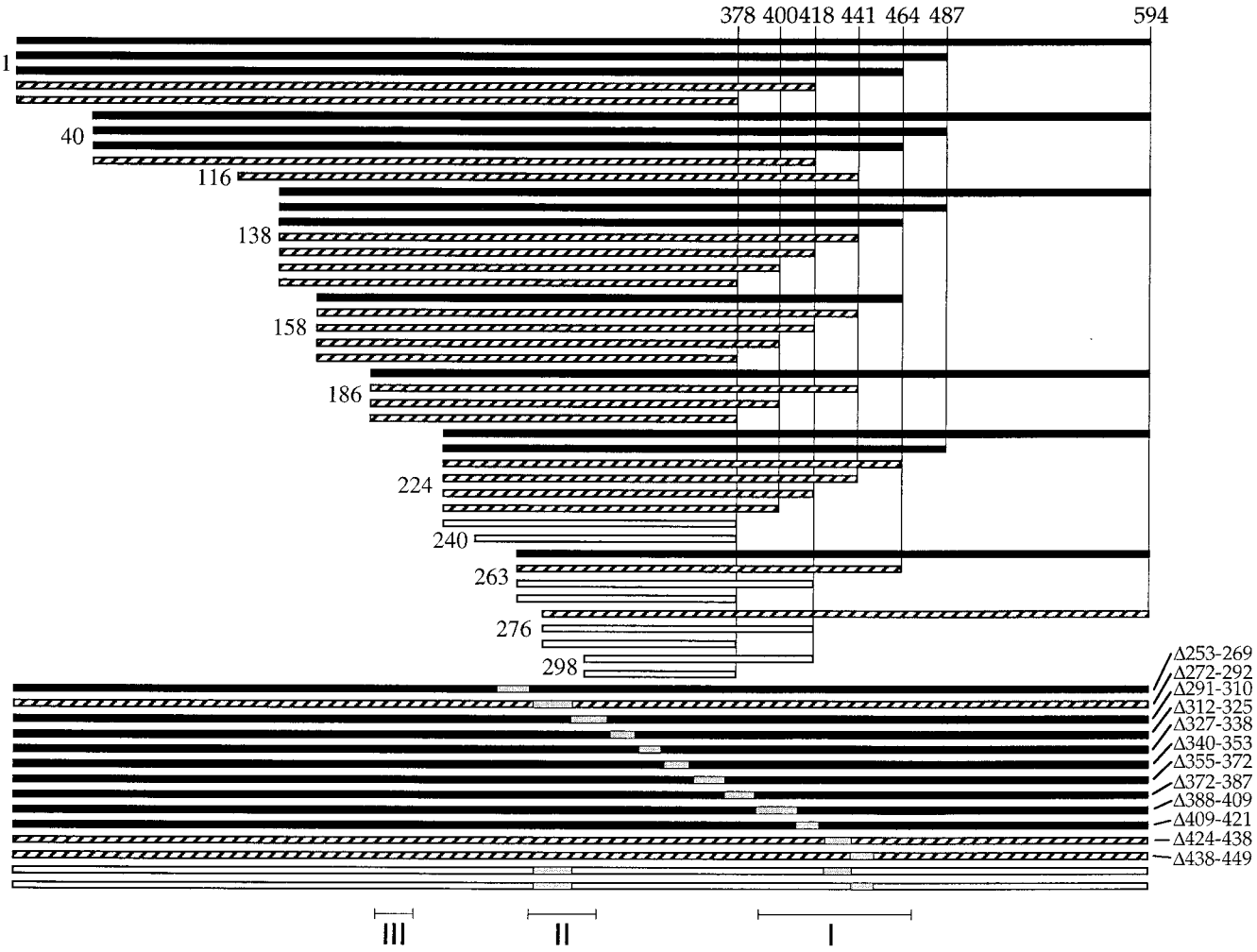

FIG. 1. Transcriptional competence of deletion variants of the B″ component of TFIIIB. N- and C-terminal deletions in *TFC5*, the gene encoding B″, were generated, and the encoded His_6-tagged proteins were purified, as described in Materials and Methods. The retained portions and endpoints of each B″ deletion mutant (N termini at the left) and competence for U6 snRNA and tRNA synthesis are indicated (■, active; □, inactive; ▨, active for U6 snRNA synthesis only). The lower portion specifies the transcriptional competence of internally deleted B″ (▩, extent of internal deletion). Transcriptional inactivity is defined as the failure to detect B″-dependent production of *SUP4* or *SNR6* RNA with at least 100 fmol of the specified B″ mutant. TFIIIC dependence was an additional requirement for assessing *SUP4* transcription since a very weak background of TFIIIC-independent transcription can be detected on this gene. Domains I, II, and III, defined by protein footprinting, are indicated.

DNase I footprinting. For footprinting the *SUP4* gene, a 294-bp *Eco*RI-*Xba*I (Fig. 7A) or a 315-bp *Eco*RI-*Hin*dIII (Fig. 7B and 8) fragment from pTZ1 was 3′ end labeled on the transcribed strand. For footprinting the *SNR6* gene, the 134-bp segment described above for EMSAs (5′ end labeled on the nontranscribed strand) was used. For Fig. 7A, 10 fmol of labeled DNA probe was mixed with 75 fmol of TFIIIC, approximately 150 fmol each of Brf and TBP, and 100 fmol of wild-type or internally deleted B″ for 60 min at room temperature. Digestion with 145 ng of DNase I per ml for 30 s was terminated by adding Na_3EDTA to 20 mM. For Fig. 7B and 8, 3.7 fmol of labeled DNA probe was incubated with 75 fmol of TFIIIC, 100 fmol of TBP, 60 fmol of Brf, and 100 to 200 fmol of wild-type or deleted B″ (Fig. 7B) or 400 fmol of B″(276-594) (Fig. 8). Digestion with 100 ng of DNase I per ml was terminated after 30 s by adding EDTA to 15 mM and, when specified, heparin to 200 μg/ml. TFIIIB-DNA, TFIIIB-TFIIIC-DNA, B′-TFIIIC-DNA, and TFIIIC-DNA complexes were purified by native polyacrylamide gel electrophoresis as described previously (7). The desired protein-DNA complexes were passively eluted from excised gel slices, and the contained partially digested DNA was resolved on 8 or 10% polyacrylamide sequencing gels as described previously (27).

RESULTS

Deletion analysis of B″. Domains that are responsible for the essential interactions of B″ have been defined by deletion analysis (Fig. 1). Effects of B″ deletions on the function of TFIIIB in the TFIIIC-dependent pathway of TFIIIB-DNA complex formation and transcriptional initiation have been examined on the *SUP4* tRNA gene. The *SNR6* (U6 snRNA) gene with its nearly symmetrical TATA box has been used to examine the TFIIIC-independent pathway of transcriptional initiation and TFIIIB-DNA complex formation, which is initiated by binding TBP to the TATA box, followed by Brf and B″ (20, 22, 42, 43).

It is remarkable that B″ can be extensively truncated yet remain functional for TFIIIC-independent U6 snRNA gene transcription. Although B″ is absolutely required for U6 snRNA synthesis, no region of B″ is absolutely essential (only the isolated amino acids 311, 326, 339, and 354 have not been tested). Results of the relevant experiments are summarized in Fig. 1, and examples of the transcription analysis are shown in Fig. 2A. (We used an *SNR6*-derived construct that allows one to display transcripts generated by TFIIIB binding to the U6 TATA box in either direction [51]. There were no changes of polarity selection due to B″ deletions [Fig. 2A].) Removal of

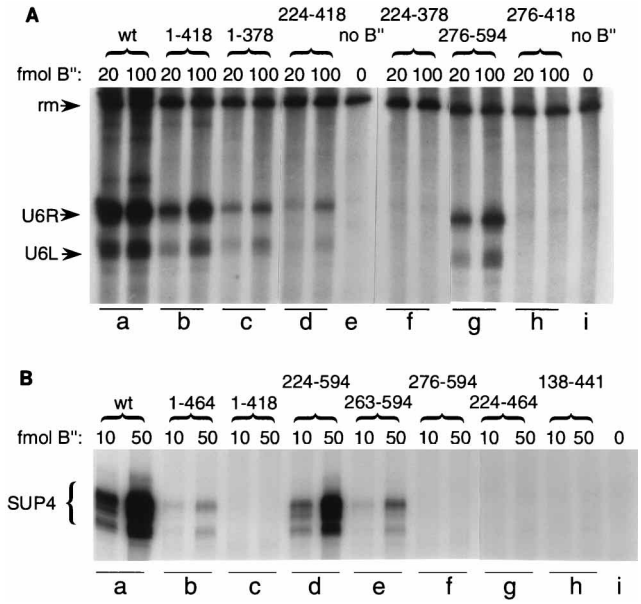

FIG. 2. Effects of N- and C-terminal truncations in B″ on TFIIIC-independent *SNR6* gene transcription (A) and TFIIIC-dependent *SUP4* gene transcription (B). (A) For *SNR6* transcription, preinitiation complexes were formed on plasmid pU6R (100 fmol) for 60 min with 100 fmol of TBP, 60 fmol of Brf, 16 fmol of pol III, and 20 or 100 fmol of the B″ designated at the top (the numbers specify the amino acids retained in each mutant protein; wt, full-length B″) followed by 30 min of transcription. The nearly symmetrical U6 TATA box generates rightward (R) and leftward (L) transcripts that are identified at the left. A labeled DNA recovery marker (rm) is also identified. (B) For *SUP4* transcription, preinitiation complexes were formed on pTZ1 (50 fmol) for 60 min with 100 fmol of TBP, 30 fmol of Brf, 37 fmol of TFIIIC, 16 fmol of pol III, and 10 or 50 fmol of the B″ preparations designated at the top. The *SUP4* transcripts are designated at the left. A labeled DNA recovery marker, not shown, testified to equivalent recoveries during processing of samples.

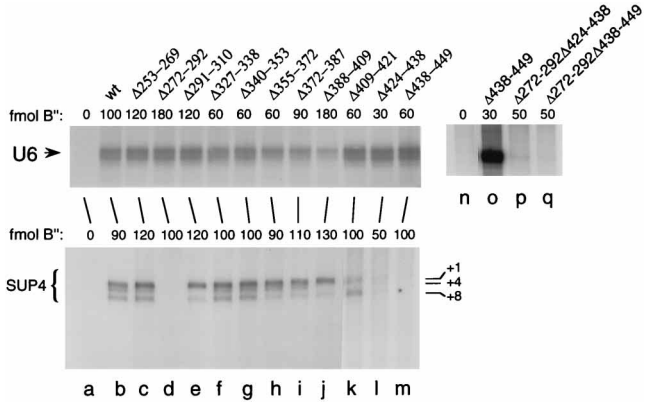

FIG. 3. Internal deletions of B″: effects of small internal deletions between amino acids 253 and 449 on TFIIIC-independent *SNR6* transcription and TFIIIC-dependent *SUP4* transcription. *SNR6* initiation complexes were formed for 60 min with 50 fmol of plasmid pCS6, 50 fmol of TBP, 34 fmol of Brf, 16 fmol of pol III, and the preparations and quantities of internally deleted B″ designated at the top. The reaction mixtures analyzed in lanes n to q were from a separate experiment. *SUP4* preinitiation complexes were formed for 60 min with 50 fmol of plasmid pTZ1, 19 fmol of TFIIIC, 29 fmol of Brf, 50 fmol of TBP, 8 fmol of pol III, and the quantities of internally deleted B″ shown. Transcription was started by adding ribonucleoside triphosphates and continued for 30 min. Lanes k to m of the *SUP4* panel were exposed to film nine times longer. Transcripts are identified at the left. Products from initiation at +1, +4, and +8 on the *SUP4* gene are further specified at the right. Labeled DNA markers demonstrating equivalent recoveries are not shown. wt, wild type.

the N-terminal 275 amino acids (Fig. 2A, lanes g) or the C-terminal 176 amino acids (lanes b) of B″ allowed relatively efficient transcription, and the C-terminal truncation to amino acid 378 also functioned, but less well (lanes c). B″ with small (12- to 22-amino-acid) internal deletions spanning amino acids 253 to 449 was also functional (Fig. 1 and 3). B″ becomes inactive for *SNR6* transcription when certain N- and C-terminal truncations or internal deletions are combined. Whereas B″(224-418) and B″(186-378) functioned (Fig. 2A, lanes d; Fig. 1), B″(224-378) (Fig. 2A, lanes f) and B″(263-418) (Fig. 1) did not. Combining B″ internal deletion Δ272-292 with Δ424-438 or Δ438-449 also led to loss of function (Fig. 3, lanes p and q). The principal conclusion of these experiments is that a minimal version of B″ containing only amino acids 224 to 400 (Fig. 1) is still competent for *SNR6* transcription. Deletions that approach this inner boundary generated quantitative defects but retained readily detectable transcription factor activity.

The demands on B″ for TFIIIC-dependent *SUP4* tRNA gene transcription are more stringent: additional segments of B″ at the N and C boundaries of the amino acid 224–400 core are essential (summarized in Fig. 1, with examples of the primary data in Fig. 2B and 3). N-terminally deleted B″(224-594) functioned well (Fig. 2B, lanes d), B″(263-594) functioned poorly (lanes e), and B″(276-594) failed to function (lanes f). C-terminally deleted B″(1-487) functioned well (Fig. 1) (24), B″(1-464) functioned poorly (lanes b), and B″(1-418) failed to function (lanes c). Internally deleted B″Δ272-292 (Fig. 3, lane d), Δ424-438 (lane 1), and Δ438-449 (lane m) were essentially inactive, and B″Δ409-421 was barely active (lane k). N- and C-terminal truncations of B″ that individually functioned for TFIIIC-dependent transcription failed in combination. For example, whereas B″(158-464) generated transcription in amounts indistinguishable from those generated by B″(1-464), and B″(224-487) allowed transcription at nearly full levels (Fig. 1), B″(224-464) was essentially inactive (Fig. 2B, lanes g).

Effects on start site selection by pol III were also observed with three internally deleted B″ proteins (Fig. 3). In addition to the major initiation site for *SUP4* transcription at +1, less efficient initiation also occurred at +4 and +8 (23, 34); each initiation site generated several primary transcripts due to release at any of the last four T residues of the seven T's that constitute the *SUP4* termination signal (39). B″Δ291-310 (lane e), B″Δ388-409 (lane j), and B″Δ409-421 (lane k) suppressed initiation at +4, while the barely active B″Δ409-421 suppressed initiation at +1, making +8 the major start site. These relatively subtle effects imply that B″ serves a metric function in transcriptional initiation, either through a direct interaction with pol III or indirectly, through its scaffold of interactions with the other components of the promoter complex.

TFIIIB-DNA complex formation. Failure of a particular mutant B″ to promote transcription can be due to failure to bind to the B′(Brf plus TBP)-DNA complex or may arise at a subsequent step, such as recruitment of pol III to the TFIIIB-DNA complex. The ability of B″ deletion mutants to bind to the B′-DNA complex was examined by electrophoretic mobility shift measurements. Results for TFIIIC-independent TFIIIB-U6 snDNA complex formation by N- and C-terminally truncated B″ are summarized in Table 1. As predicted by their competence for transcription, B″ N-terminally truncated to amino acid 276 or C-terminally truncated to amino acid 378 retained competence to form TFIIIB-DNA complexes. C-terminal deletions extending beyond amino acid 464 generated TFIIIB-DNA complexes that were sensitive to stripping by heparin. B″(224-378) and B″(241-378) participated in TFIIIB-DNA complex formation but were inactive in transcription. Since B″(224-418) and B″(186-378) were active for transcrip-

TABLE 1. Effects of N- and C-terminal deletions of B″ on TFIIIB-DNA complex formation and stability

Mutant(s)	*SNR6*		*SUP4*	
	Formation	Susceptibility to heparin[a]	Formation	Susceptibility to heparin
1-594, 1-464	+	r	+	r
1-418, 1-378	+	s	−	
138-594, 138-464	+	r	+	r
138-441	ND[c]		−	
138-378	+	s	ND	
158-464	+	r	+	r
158-378	+	s	ND	
186-594	+	r	+	r
186-378	+	s	ND	
224-594	+	r	+	r
224-464	+	r	−	
224-441, 224-418, 224-400	+	s	ND	
224-378	±	s	−	
240-378	+	s	ND	
263-594	+	r	+	r
263-464	+	r	−	
263-418, 263-378	−[b]		ND	
276-594	+	r	+	s
276-418	−		−	

[a] r, resistant; s, susceptible.
[b] −, may form trace amounts of TFIIIB-DNA complexes that are not resolved from the B′-DNA complex.
[c] ND, not determined.

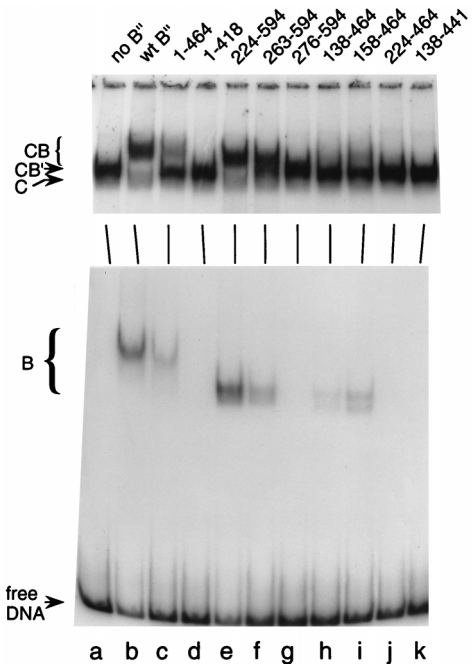

FIG. 4. Effects of N- and C-terminal truncations of B″ on TFIIIB complex formation with the *SUP4* gene. Shown is EMSA of TFIIIB-DNA complexes formed with 9 fmol of SUP4 DNA, 100 of fmol TBP, 29 fmol of Brf, 37 fmol of TFIIIC, and 100 fmol of the B″ preparation designated at the top. Samples for the bottom panel were subsequently treated with 200 μg of heparin per ml for ~7 min prior to electrophoresis. Only the top of the gel, showing protein-DNA complexes (there was very little free DNA in these samples), is presented in the upper panel. The mobilities of unbound DNA, TFIIIB-DNA (B), TFIIIC-TFIIIB-DNA (CB), TFIIIC-B′-DNA (CB′), and TFIIIC-DNA (C) complexes are identified at the left. wt, wild type.

tion, this finding suggests that the amino acid 378–418 and 186–224 segments are involved in the interplay between pol III and TFIIIB.

As expected, all B″ single internal deletion mutants were competent to form TFIIIB-U6 snDNA complexes (summarized in Table 2), but resistance to heparin was lost with Δ272-292, Δ409-421, Δ424-438, and Δ438-449. Combining internal deletion Δ272-292 with Δ424-438 or Δ438-449 produced B″ mutants that failed to generate TFIIIB-DNA complexes.

The abilities of B″ deletion variants to bind to B′-TFIIIC-*SUP4* tDNA complexes are summarized in Tables 1 and 2, with examples of the primary data shown in Fig. 4 and 5. The ability of N- and C-terminally truncated B″ to generate transcription closely followed the ability to be incorporated into a heparin-resistant TFIIIB-DNA complex (Fig. 4). For example, B″(224-594) and B″(1-464) allowed transcription and generated heparin-resistant TFIIIC-dependent TFIIIB-DNA complexes (lanes e and c, respectively), whereas B″(224-464) failed on both counts (lane j). The ability to determine whether B″ deletion mutants incapable of generating transcription or heparin-resistant TFIIIB-DNA complex formation might still be incorporated into a heparin-sensitive TFIIIB-TFIIIC-tDNA complex was compromised by the fact that as more of B″ is deleted, the resulting TFIIIB-TFIIIC-DNA complexes are not well separated from the B′-TFIIIC-DNA complex on gels. In this regard, B″(276-594) generated complexes that migrated slightly more slowly than the B′-TFIIIC-tDNA complex in the absence of heparin (lane g). In other experiments (not shown), the mobility of this complex decreased in a continuous and progressive manner with increasing concentration of B″(276-594), suggesting that B″(276-594) binds to the B′-TFIIIC-tDNA complex but dissociates and reassociates during electrophoresis [free B″(276-594) is expected to enter these gels]. Among the internally deleted B″, the transcriptionally inactive Δ424-438 and Δ438-449 failed to generate TFIIIB-DNA complexes (Fig. 5, lanes l and m); Δ272-292 and Δ409-421 formed heparin-sensitive TFIIIB-TFIIIC-DNA complexes (lanes d and k, respectively), with Δ409-421 transcriptionally competent and Δ272-292 transcriptionally inert on the *SUP4* gene.

TABLE 2. Internal deletions in B″ and TFIIIB-DNA complex formation[a]

Deletion(s)	*SNR6*		*SUP4*	
	Formation	Susceptibility to heparin[a]	Formation	Susceptibility to heparin
253-269	+	r	+	r
272-292	+	s	+	s
291-310, 312-325, 327-338, 340-355, 355-372, 372-387, 388-409	+	r	+	r
409-421	+	s	+	s
424-438, 438-449	+	s	—	
272-292/424-438, 272-292/438-449	−	ND	ND	

[a] For details, see the footnotes to Table 1.

Evidence that wild-type B″ resolves a potential interference by TFIIIC with pol III at the transcriptional start site. The ability of B″Δ272-292, -Δ424-438, and -Δ438-449 to function for TFIIIC-independent *SNR6* gene transcription but not for TFIIIC-dependent *SUP4* gene transcription is puzzling. The failure of *SUP4* transcription with B″Δ424-438 and -Δ438-449 is attributable to the inability to associate with the B′-TFIIIC-

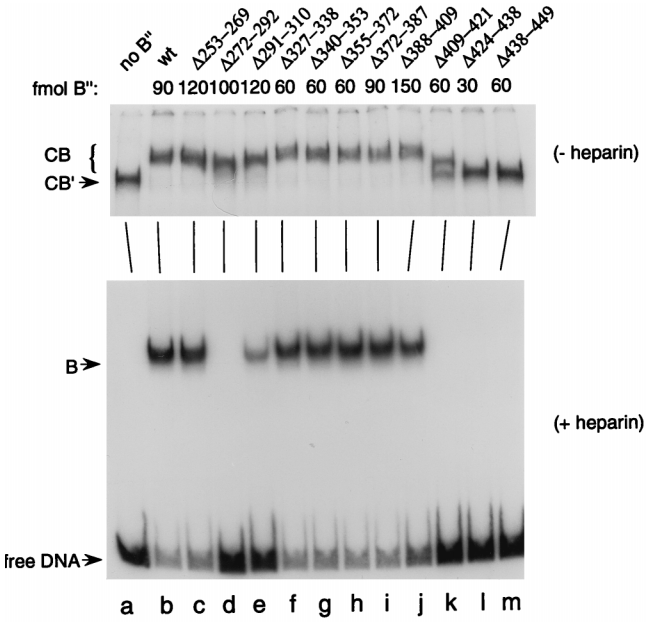

FIG. 5. Effects of small internal deletions in B″ on formation and stability of TFIIIB-DNA complexes with the *SUP4* gene. TFIIIB-DNA complexes were assembled with 5 fmol of *SUP4* tDNA, 37 fmol of TFIIIC, 100 fmol of TBP, 29 fmol of Brf, and the B″ preparations and amounts indicated above each pair of lanes. For samples shown in the bottom panel, heparin was added to 180 μg/ml subsequent to complex formation. Analysis and presentation of data are as for Fig. 4.

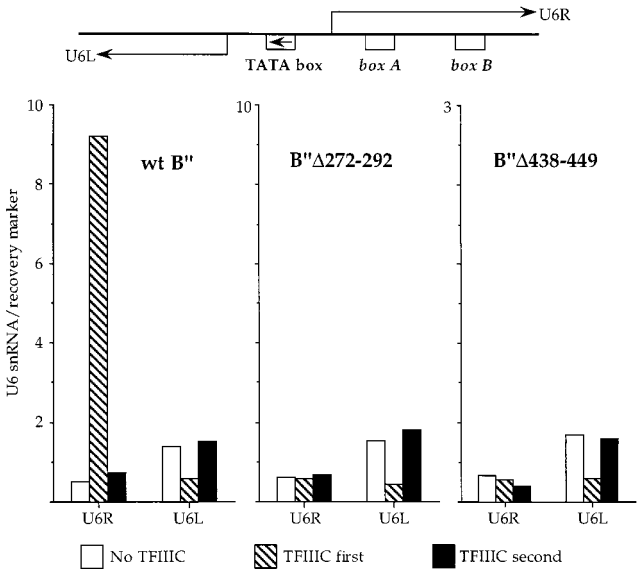

FIG. 6. Order-of-addition experiments with TFIIIC. Twenty-seven femtomoles of the U6 snRNA construct pU6LboxB (diagrammed at the top) was incubated with buffer (▭) or with 75 fmol of TFIIIC for 6 min either before ▨ or after ■ a 60 min-incubation with TFIIIB, constituted from 100 fmol of TBP, 67 fmol of Brf, and 50 fmol of wild-type (wt) B″ (left), B″Δ272-292 (middle), and B″Δ438-449 (right). Transcription was started by adding ribonucleoside triphosphates and 8 fmol of pol III and was allowed to proceed for 30 min. Samples were subsequently processed in the presence of a labeled DNA recovery marker; transcription products were separated by denaturing gel electrophoresis and quantified with a phosphorimager. The production of each transcript is normalized to the recovery marker.

DNA complex, but B″Δ272-292 fails despite incorporation into the B′-TFIIIC-DNA complex, indicating that the resulting complex is aberrant. We considered the following explanations for the dichotomy between *SNR6* and *SUP4* gene transcription: the *SNR6* consensus TATA box might contribute additional protein-DNA interactions that suppress a loss of B″ function in the context of a weaker TATA box; alternatively, TFIIIC might interfere with binding of these truncated B″ to the B′-DNA complex, or in the case of B″Δ272-292, TFIIIC might interfere with the recruitment of pol III by the transcription factor-DNA complex.

An order-of-addition experiment with plasmid pU6LboxB (51) was performed to distinguish between these alternative explanations. This plasmid contains a small artificial pol III transcription unit, U6L (Fig. 6, top), in opposite orientation to the natural U6 gene (U6R), with both transcriptional initiation sites correctly spaced relative to the nearly symmetrical U6 TATA box. The latter, and its flanking sequence, are turned around to favor leftward transcription of pU6LboxB: ~75% of bound TFIIIB directs pol III to transcription of U6L. The plasmid also has a *box B* inserted at the optimal distance from the *SNR6* gene's natural *box A*. This allows efficient binding of TFIIIC, which, in turn, assembles TFIIIB only for rightward transcription (U6R). If the TATA box ameliorates the defect in B″ internal deletion mutants, then binding TFIIIC prior to TFIIIB should stimulate rightward transcription. This did not happen (Fig. 6).

If TFIIIC generates aberrant binding of B″Δ272-292 and/or subsequently blocks productive association of pol III, then addition of TFIIIC prior to TFIIIB should reduce U6L (leftward) transcription by simple competition (since TFIIIC orients TFIIIB for rightward transcription) but not stimulate, and perhaps even inhibit, U6R (rightward) transcription. A similar result would be anticipated to occur if TFIIIC directly prevented the incorporation of B″Δ438-449 into the B′-DNA complex. The results of TFIIIC order-of-addition experiments with TFIIIB formed with full-length B″, B″Δ272-292, or B″Δ438-449 are compared in Fig. 6. The orientation of each form of TFIIIB at the TATA box was such as to direct approximately 75% of transcription to U6L in the absence of TFIIIC. Forming a TFIIIC-DNA complex prior to incubation with TFIIIB stimulated U6R transcription 18-fold with wild-type B″ but generated no stimulation with B″Δ272-292 or B″Δ438-449. Prior binding of TFIIIC diminished U6L transcription 2.5- to 3.5-fold with full-length B″, with B″Δ272-292, and with B″Δ438-449, while addition of TFIIIC after TFIIIB-DNA complex formation had no effect on the direction of transcription. (The ability of TFIIIC to nearly saturate preformed TFIIIB-DNA complexes was confirmed separately [data not shown].) Similar experiments with B″Δ424-438 yielded an essentially identical outcome (data not shown).

The inability of B″Δ272-292, -Δ424-438, and -Δ438-449 to function on the *SUP4* tRNA gene clearly is not due to the lack of a consensus 8-bp TATA box. For B″Δ424-438 and B″Δ438-449, TFIIIC prevented stable binding to the B′-DNA complex (Table 2), which accounts for their inability to support transcription. Incorporation of B″Δ272-292 into the B′-DNA complex apparently generated a TFIIIB-TFIIIC-DNA complex that was transcriptionally incompetent. The DNase I protection analysis that follows (Fig. 7) correlates this transcriptional inactivity with an aberrant structure around the start site of transcription.

Probing for structural alterations with DNase I footprinting. DNase I footprints of TFIIIC-TFIIIB-*SUP4* tDNA complexes assembled with wild-type B″ and B″ internally deleted between amino acids 253 and 409 are shown in Fig. 7A. As noted previously (26), addition of B′ to the TFIIIC-DNA complex increased protection attributable to TFIIIC around *box A*

between bp +41 and +4, generated new protection between bp +3 and −32, and enhanced DNase I cleavage at bp −36, −37, −38, and −44 (compare lane c with lane b [Fig. 7B, panel i]). Recruitment of wild-type B″ to the complex led to a substantial loss of protection around the start site of transcription (between bp +2 and −1), generated nearly complete protection between bp −14 and bp −39, and generated partial protection between bp −42 and −44 (Fig. 7A, lane d; Fig. 7B, panel ii). With the exception of B″Δ272-292 and B″Δ409-421, the B″ internal deletions that enter a TFIIIB′-DNA complex on the *SUP4* gene generated footprints closely matching that of wild-type B″ (Fig. 7A; compare lanes e and g to l with lane d), except for enhanced cleavage at bp −12 with B″Δ291-310 (lane g).

Incorporation of B″Δ272-292 (Fig. 7A, lane f; Fig. 7B, panel iii) or B″Δ409-421 (Fig. 7B, panel iv) into the B′-TFIIIC-DNA complex yielded complexes whose footprints were nearly identical hybrids of B′-TFIIIC-DNA and wild-type TFIIIB-TFIIIC-DNA complex footprints. Both deletion mutants resembled wild-type B″ in regard to the upstream segment of the footprint, with protection between bp −14 and −32 substantially more complete than for the B′-TFIIIC-DNA complex and new protection generated between bp −33 and −40 (although less complete than with wild-type B″, partly due to the faster migration of the heparin-sensitive TFIIIB-TFIIIC-DNA complexes containing these two internal deletion [Fig. 5], which led to increased cross-contamination with residual B′-TFIIIC-DNA complexes). However, both B″ internal deletions left the B′-TFIIIC-DNA complex unchanged downstream of bp −12 and, in particular, did not generate increased accessibility to DNase I around the start site of transcription. We conclude that both B″Δ409-421, which is barely active for *SUP4* transcription, and B″Δ272-292, which is inactive for *SUP4* transcription, generate an aberrant structure at the downstream end of the TFIIIB-DNA complex that leaves the transcriptional start site occluded. In contrast, the internally deleted forms of B″ covering amino acids 253 to 310 and 388 to 449 (i.e., including the two just referred-to mutants) generated TFIIIB-*SNR6* gene nontranscribed strand footprints that were essentially identical with the wild-type TFIIIB-*SNR6* footprint (data not shown), leaving the *SNR6* transcriptional start site fully accessible for DNase I cleavage. In particular, we detected no difference between these deletion mutants and the wild type and no differences in the footprint of heparin-sensitive and heparin-resistant TFIIIB-*SNR6* gene complexes (Table 2 and data not shown). We surmise that the aberrant DNase I footprints generated with B″Δ272-292 and B″Δ409-421 on the *SUP4* gene reflect an inability to fully displace the τ_{120} subunit of TFIIIC from start site-proximal DNA.

The analysis of DNase I footprints was extended to extreme N- and C-terminal truncations of B″ that were still competent in forming a TFIIIB complex on the *SUP4* gene. Although C-terminally truncated B″(1-464) was less active than wild-type B″ in *SUP4* transcription (Fig. 2B) and in the formation of heparin-resistant TFIIIB-DNA complexes (Fig. 3), the DNase I footprints generated by incorporation of B″(1-464) and wild-type B″ into the B′-TFIIIC-DNA complexes differed little (Fig. 8, panel i), except for increased accessibility of sequence surrounding the start site of transcription in the complex formed with B″(1-464). Incorporation of the N-terminally truncated B″(263-594) into the B′-TFIIIC-DNA complex produced a subtle but unexpected result (Fig. 8, panel ii). The footprint was superimposable with the TFIIIB footprint formed with wild-type B″ upstream of bp −14 (panel ii) but was superimposable with the TFIIIC-alone footprint downstream of bp −7 (panel iii). That is, the stabilization of TFIIIC interaction with the DNA region surrounding *box A* that is generated by formation of the B′-TFIIIC-DNA complex (Fig. 7B, panel i) and retained in the wild-type TFIIIB-TFIIIC-DNA complex (Fig. 7B, panel ii) was reversed in the TFIIIB [B″(263-594)]-TFIIIC-DNA complex. The protection between bp +6 and +3 and between bp −4 and −7 generated by formation of the B′-TFIIIC-DNA complex and retained in the wild-type TFIIIB-TFIIIC-DNA complex was also reversed in the complex formed with B″(263-594). Evidently, entry of B″(263-594) into the DNA complex destabilizes contacts between B′ and TFIIIC that are maintained in forming the wild-type TFIIIB-TFIIIC-DNA complex.

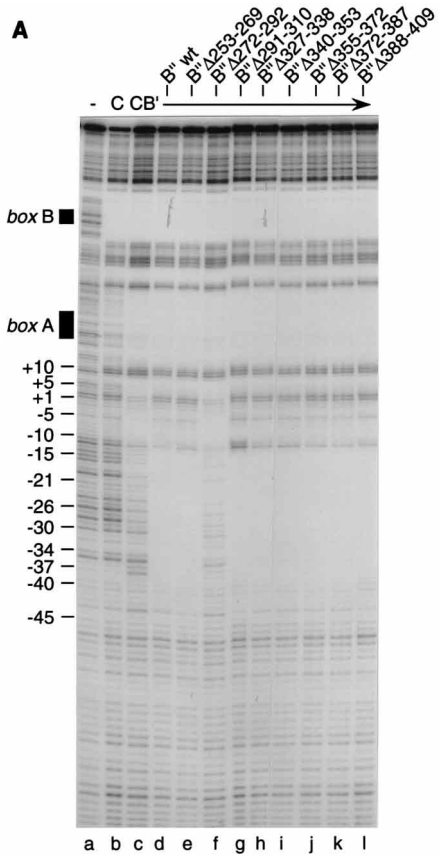

FIG. 7. DNase I protection analysis of TFIIIB-TFIIIC-*SUP4* gene complexes containing internally deleted variants of B″. (A) Ten femtomoles of a 294-bp *Eco*RI-*Xba*I fragment containing the *SUP4* gene, 3′ end labeled on the transcribed strand, was incubated as specified in Materials and Methods with TFIIIC (lanes b to l), Brf and TBP (lanes c to l), and 100 fmol of wild-type (wt) or internally deleted B″, as specified above lanes d to l. Complexes were treated with DNase I, and samples were processed as described in Materials and Methods. The locations of the *box A*- and *box B*-binding sites of TFIIIC and further upstream sequence are indicated at the left. (B) B″Δ272-292 and B″Δ409-421 are incorporated into a TFIIIB-TFIIIC-*SUP4* gene complex but fail to free DNA surrounding the start site of transcription for access by pol III. DNase I footprinting was performed as described in Materials and Methods, and phosphorimage density profiles were normalized for the total radioactivity loaded in each gel lane and subjected to pairwise comparison. i, comparison of the TFIIIC complex (fine black line) and the B′-TFIIIC complex (heavy grey line) footprints. ii, comparison of the B′-TFIIIC complex (fine black line) and the wild-type TFIIIB-TFIIIC complex (heavy grey line), showing in the latter exposure of the transcriptional start site (+1) and protection between bp −12 and −44. iii, footprint of the TFIIIB(B″Δ272-292)-TFIIIC complex (heavy grey line). iv, footprint of the TFIIIB(B″Δ409-421)-TFIIIC complex (heavy grey line). Comparisons in panels iii and iv with the B′-TFIIIC complex (fine black line) show that these TFIIIB-TFIIIC complexes do not expose the vicinity of the transcriptional start site (+1). Locations in the DNA sequence are indexed (up- and down-pointing arrows), and *box A* is indicated (filled box).

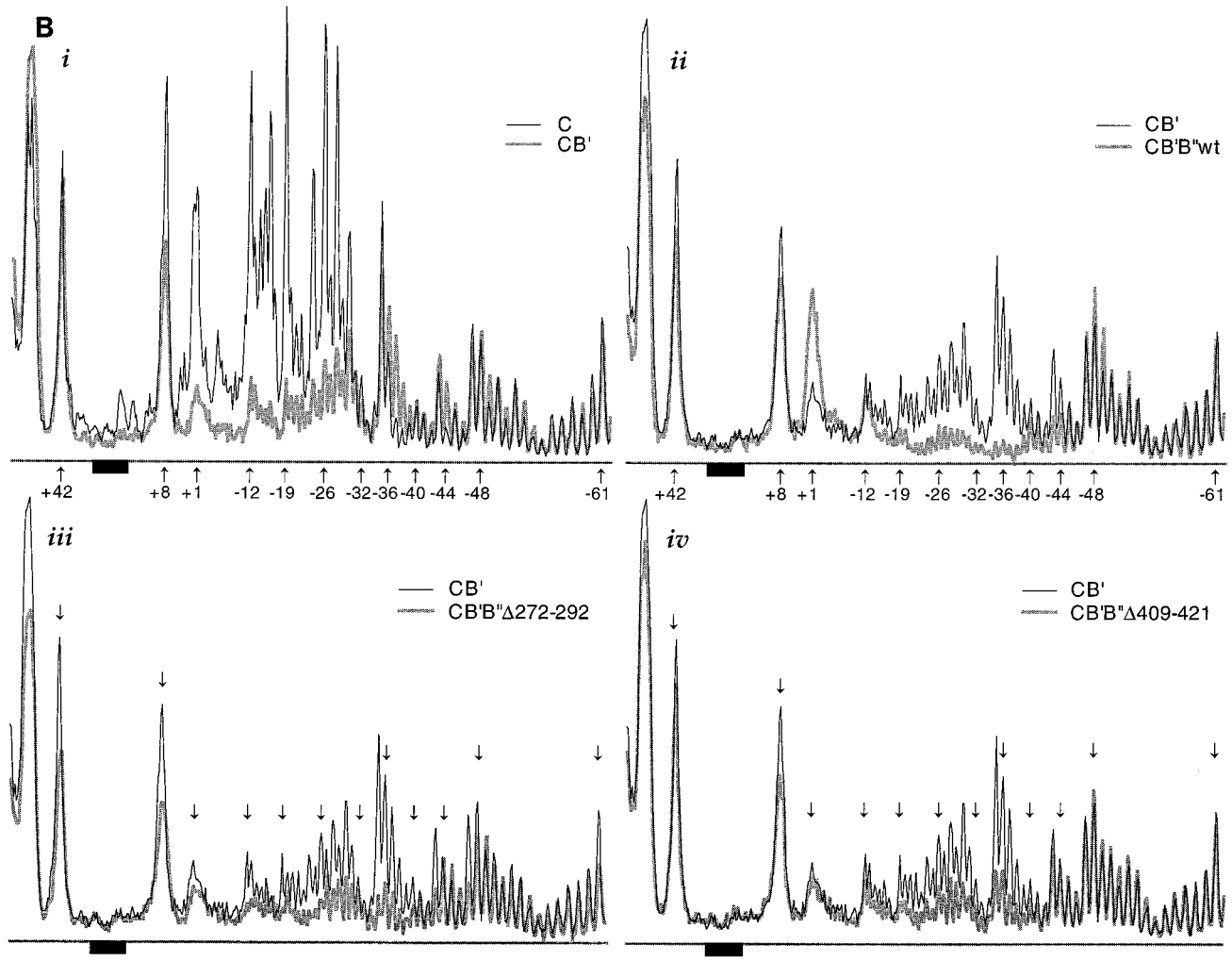

FIG. 7—*Continued.*

The DNase I footprint of the transcriptionally inactive TFIIIB [B″(276-594)]-TFIIIC-*SUP4* DNA complex (Fig. 2B and Table 1) was also examined. Unlike TFIIIB complexes formed with internally deleted B″Δ272-292 and N-terminally deleted B″(263-594), B″(276-594) failed to generate significant protection upstream of bp −32 and for the most part closely conformed to the B′-TFIIIC-DNA complex footprint (Fig. 8, panel iv).

In summary, the analysis of these three external deletions shows that removal of 262 N-terminal or 131 C-terminal amino acids of B″ generates only relatively subtly changed DNase I footprints and that such changes as occur are confined to the same (downstream) side of the DNA. Removal of only 13 more N-terminal amino acids from B″ generates a protein that is grossly defective in generating the DNA contacts that are characteristic of the TFIIIC-dependent TFIIIB complex with the *SUP4* gene. These findings prompt two speculations that are discussed further below: (i) the amino and carboxy ends of B″ may be located near each other in the TFIIIB-DNA complex, and (ii) B″ serves as a scaffold that remolds the B′-DNA complex.

Extensive structure changes of B″ during assembly of TFIIIB-DNA complexes. The loss of function that is generated by certain combinations of deletions that are widely separated on the B″ polypeptide (Fig. 3 and 5) suggests that B″ might be a partner in multiple, reinforcing interactions within the TFIIIB-DNA complex. A concrete and independent view of these protein-protein and protein-DNA interactions is provided by hydroxyl radical protein footprinting (15, 18, 19) of B″ within TFIIIB-DNA complexes. B″ contains a consensus site, RRLS, for phosphorylation by cyclic AMP-dependent protein kinase at amino acids 161 to 164 (with S164 getting phosphorylated). B″(138-594) is fully functional (Fig. 2 and data not shown) and places this natural phosphorylation site sufficiently close to the N terminus to serve as an effective ^{32}P end label. We ascertained that S164 is the only site in B″(138-594) that is labeled by cyclic AMP-dependent protein kinase by showing that only full-length C-terminally His-tagged and ^{32}P-labeled B″(138-594) is retained on Ni-NTA agarose following extensive digestion with endoproteinase Glu-C (data not shown). Gel mobility shift assays also demonstrated the ability of pol III to assemble onto TFIIIB-DNA complexes containing S164-phosphorylated B″ (data not shown).

End-labeled B″(138-594) was used to form TFIIIB-TFIIIC-*SUP4* DNA complexes. These were then treated with 400 mM NaCl to strip away TFIIIC and nonspecifically bound proteins and purified away from unbound proteins on Sepharose CL2B (27). Figure 9A displays the size distribution of fragments generated by hydroxyl radical cleavage of B″(138-594) free in solution (lanes f to h) and in a TFIIIB-*SUP4* DNA complex (lanes b to d). Since cleavage was single hit, the sizes of the

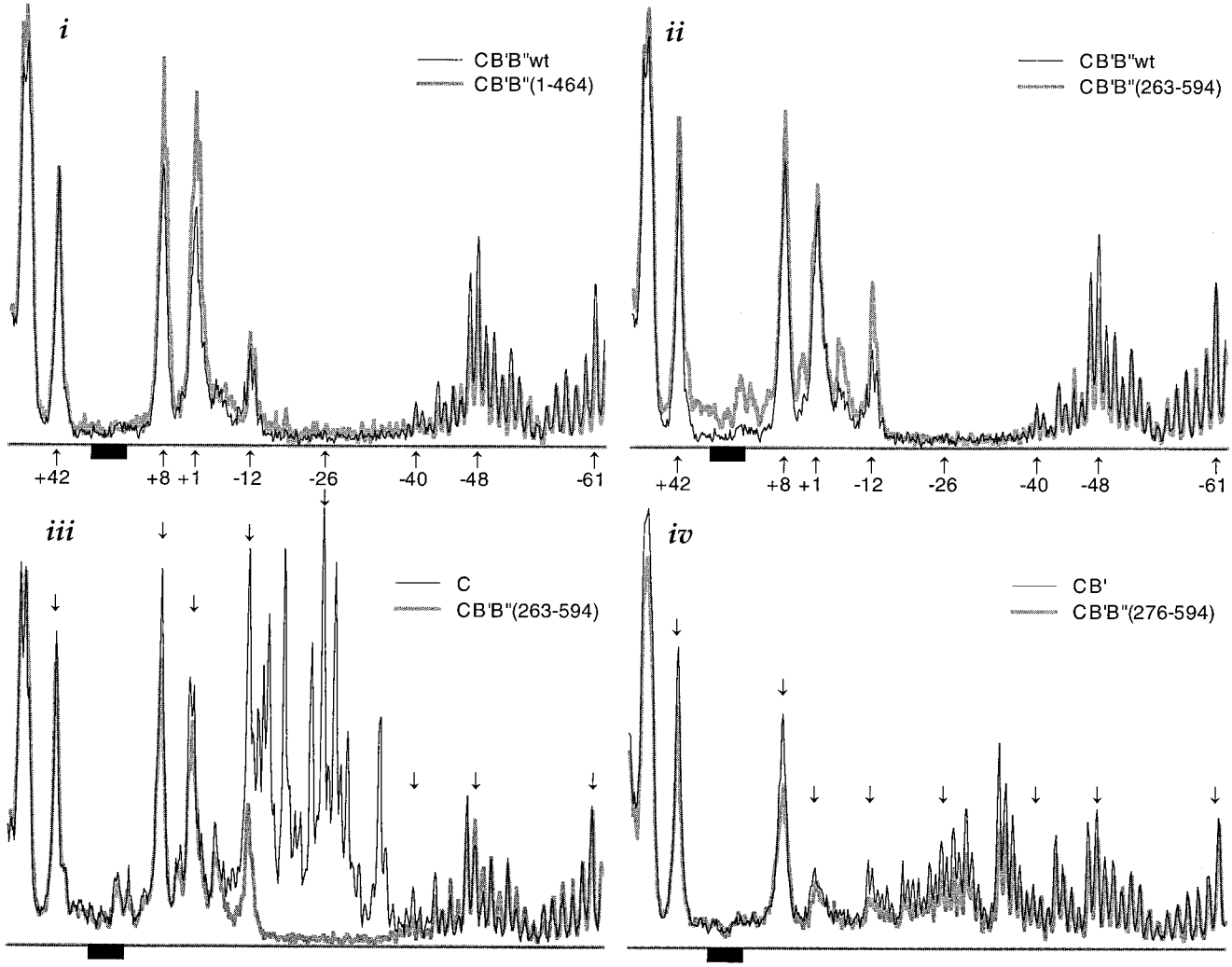

FIG. 8. DNase I footprints of TFIIIB-TFIIIC-*SUP4* gene complexes containing terminally deleted B″. DNase I footprinting was performed as described in Materials and Methods. TFIIIC was stripped from TFIIIB-DNA complexes containing wild-type (wt) B″, B″(1-464), and B″(263-594) after DNase I treatment by addition of heparin. i, comparison of the wild-type and B″(1-464)-TFIIIB-TFIIIC footprints (fine and heavy lines, respectively). ii and iii, comparison of the TFIIIB[B″(263-594)]-TFIIIC footprint (heavy line) with the wild-type TFIIIB-TFIIIC footprint (panel ii, fine line) and the TFIIIC footprint (panel iii, fine line). iv, binding of B″(276-594) (heavy line) barely changes the B′-TFIIIC footprint (fine line). Locations in the DNA sequence are indicated as in Fig. 7B.

products correspond to the distance between the site of B″ cleavage and the N terminus. B″ migrates anomalously in SDS-polyacrylamide gel electrophoresis (as a 90-kDa protein [24]). It turns out that no single region of B″ generates this effect; all of the B″ deletion mutants made thus far migrate anomalously. To accurately locate sites of hydroxyl radical-mediated cleavage, size standards were generated based on the predicted partial CNBr (lane i), endoproteinase Glu-C (lane k), and Lys-C and Arg-C (data not shown) digestion products of B″(138-594). Phosphorylation of double-deletion B″(138-*x*) proteins provided additional markers (lane j). A standard curve generated from data in lanes i to k of Fig. 9A shows that the dependence of electrophoretic mobility on B″ polypeptide length is irregular (Fig. 9B), the most notable deviation occurring between amino acids 434 and 399.

An alignment of the hydroxyl radical cleavage profiles generated by the reactions analyzed in lanes b (TFIIIB-DNA complex) and f (free B″) of Fig. 9A is shown in Fig. 9C. Two major regions of protection from hydroxyl radical cleavage upon formation of a TFIIIB-*SUP4* DNA complex involve B″ amino acids ~470 to 390 (region I in Fig. 9C and 1) and ~305 to 270 (region II). We also reproducibly noted enhanced accessibility of amino acids ~210 to 190 (region III) to hydroxyl radicals. Smaller changes of susceptibility to hydroxyl radical cleavage elsewhere (e.g. at amino acid ~350 to 305 and ~245 to 255 were reproducibly seen but are more difficult to interpret because alignment and normalization of patterns with as many differences as are seen here is subject to some judgment and alternative adjustment.

However, the principal insight that is provided by hydroxyl radical footprinting of B″ is unencumbered by ambiguity. The two regions of B″ that are most strongly protected from cleavage by hydroxyl radical upon incorporation into a TFIIIB-DNA complex cover the locations of deletions that individually generate susceptibility of TFIIIB-*SNR6* DNA complexes to heparin (Fig. 9C). Certain combinations of deletions from regions I and II lead to a failure to form TFIIIB-DNA complexes and to support transcription. Deletions in regions I and II individually make B″ incompetent for TFIIIC-dependent transcription.

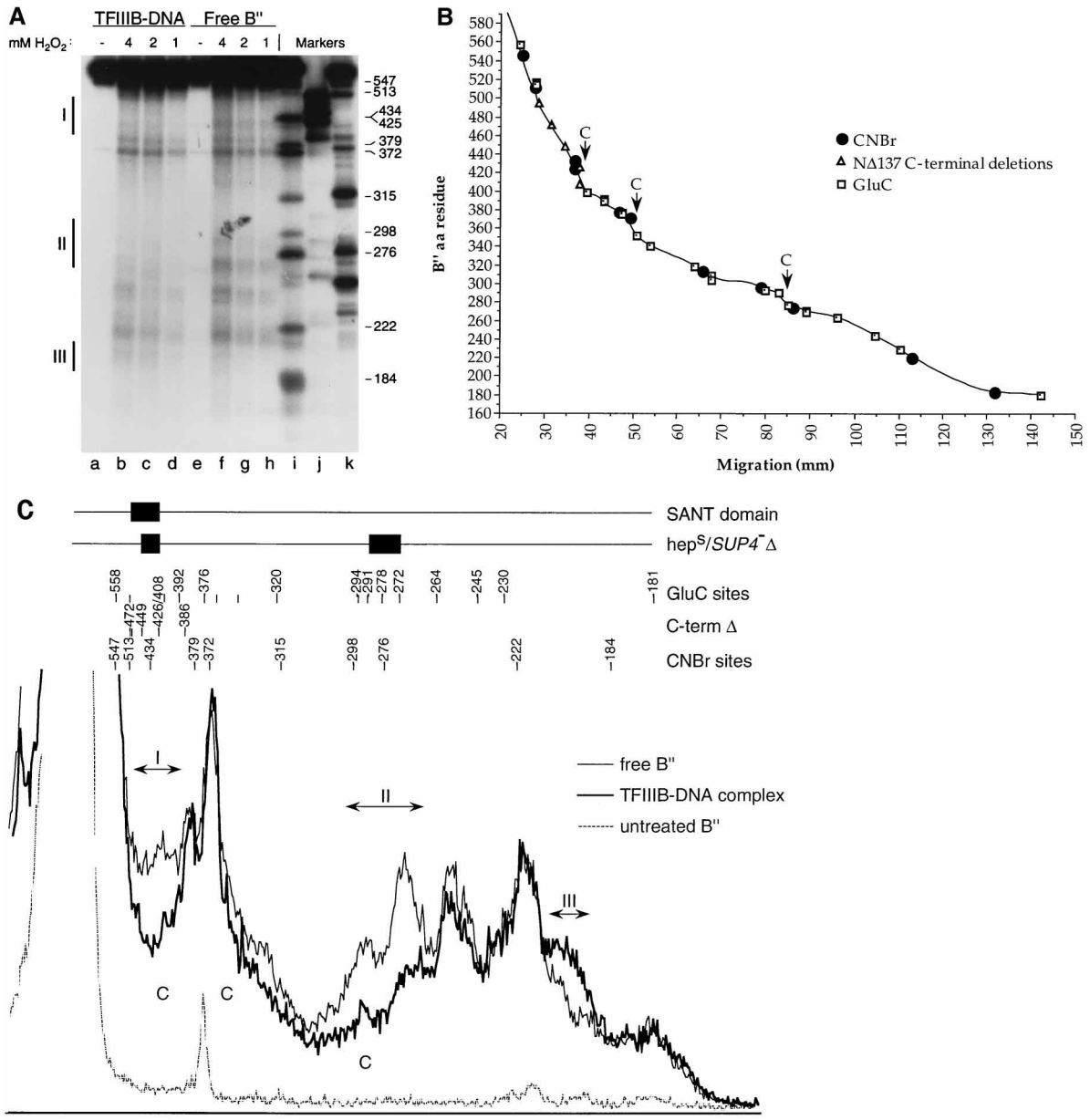

FIG. 9. Hydroxyl radical protein footprints of B″ in the TFIIIB-*SUP4* gene complex: mapping the topographical changes accompanying assembly. (A) Comparison of the hydroxyl radical cleavage patterns of B″(138-594) ^{32}P-labeled at S164 in a TFIIIB-DNA complex (lanes a to d) and free in solution (lanes e to h). Partial CNBr (lane i) and endoproteinase Glu-C (lane k) digestions of B″(138-594) and C-terminal deletions of B″(138-594) (lane j) serve as size standards (see Materials and Methods). The C termini of the CNBr partial digestion products [i.e., B″(138-*x*)] are identified at the right. Domains I, II, and III are indicated at the left. (B) Standard curve of size markers shown in panel A. Not every endoproteinase Glu-C fragment was unambiguously identified due to anomalous electrophoretic migration. Only those cleavage products identified by further partial cleavage with endoproteinase Lys-C, endoproteinase Arg-C, and CNBr are plotted. (C) Phosphorimage profile of the cleavage reactions shown in panel A, lane a (untreated B″; thin mottled line, bottom), lane b (TFIIIB-DNA complex; thick line, middle), and lane f (free B″; thin line, top). The splotches in lanes e to g of panel A were autoradiographic (caused by static electrical charge) and not part of the further analyzed phosphorimager output. Profiles were initially normalized to total phosphorimager signal in each gel lane. The profiles for free B″ and B″ in the TFIIIB-DNA complex were renormalized to total phosphorimager signal of cleavage products with C termini between amino acids ~164 and 190 and between amino acids 210 and 241. Migration of marker proteins is noted at the top, as an aid to mapping distance along the horizontal axis to location in the B″ peptide chain. C indicates compressions of the gel (panel B) near amino acids 420, 360, and 285. These compressions involve only small segments of domains I and II that are buried upon TFIIIB-DNA complex formation and are absent from the region of domain III, which is brought more to the surface upon complex formation. The locations of the putative SANT domain (44) and of internal deletions conferring heparin sensitivity (hep^s) on TFIIIB-DNA complexes as well as defective *SUP4* transcription are noted at the top. C-term Δ, C-terminal deletion.

DISCUSSION

The 594-amino-acid B″ subunit of yeast TFIIIB is essential for all transcription by pol III, and it is encoded by an essential yeast gene. It is remarkable, therefore, that our deletion analysis has not identified a single absolutely required amino acid for B″ function in *SNR6* transcription (only four isolated amino acids remaining unexplored). With regard to *SNR6* transcription, B″ is an indispensable protein constructed from dispensable domains! Nevertheless, pairwise combinations of deletions identify two regions of B″ that are essential for TFIIIC-independent U6 snRNA synthesis on an either/or basis. The

absence of amino acid sequence that is essential for *SNR6* transcription is consistent with the existence of multiple sites of interaction, with DNA, pol III, Brf, TBP, and the Tfc4/τ_{120}, subunit of TFIIIC (3, 22, 24–26, 42, 44). It is conceivable that loss of one DNA, Brf, or TBP contact or one internal contact within B″ would generate a TFIIIB-DNA complex that is stable enough for transcription but no longer resistant to displacement by polyanions.

Topography of B″. Footprinting with hydroxyl radical maps accessibility of the surface of a protein to a very small, reactive molecule (18), potentially to single-peptide bond resolution. The hydroxyl radical protein footprint of B″ yields a second remarkable outcome. The B″-alone footprint is dominated by four well-separated peaks of susceptibility to hydroxyl radical cleavage (Fig. 9A and C). These are likely to be protrusions in the structure of B″ in solution or less ordered segments. More important for our immediate concerns, there is an extensive burial of surface in two extended regions of B″ (I and II), and unfolding in a third region (III), when B″ is incorporated into a TFIIIB-DNA complex. In region II, a peak of susceptibility to cleavage by hydroxyl radical is almost completely eliminated when B″ enters the TFIIIB-DNA complex. Region I (amino acids 390 to 460) includes the previously identified SANT domain (1, 44). SANT domains are implicated in protein-protein and protein-nucleic acid interactions (both of which, of course, contribute to the burial of protein surface [e.g., reference 46]). Regions I and II of B″, which get buried in the TFIIIB-DNA complex, are also identified as important by the deletion analysis. Clearly, these are sites at which interactions of B″ with the other components of TFIIIB (or internal interactions between separate domains of B″) are required for function. Region III, which becomes more exposed to cleavage by hydroxyl radical upon entry into a TFIIIB-DNA complex, is not essential for transcription in vitro.

This analysis of the topography of TFIIIB by protein footprinting gives a clear indication of just how useful the method will be in probing the interior architecture of the pol III promoter complex, particularly in combination with other approaches, such as protein-DNA and protein-protein photochemical cross-linking and site-directed mutagenesis.

B″ exerts a profound influence on the structure of the fully assembled TFIIIB-DNA complex: it confers heparin insensitivity and very tight binding and extends the DNase I footprint ~10 bp further upstream, while at the same time making the start site of transcription accessible to cleavage by DNase I (and presumably to binding by pol III) by shifting TFIIIC out of the way. It is known that B″ is within photochemical cross-linking range (~9 Å) of DNA at multiple sites throughout the span of the TFIIIB-DNA complex (3, 5). One might therefore anticipate seeing B″ aligned with and extended along DNA. To our surprise, we have not found DNase I footprints retracting progressively from either end of the DNA-binding site of TFIIIB as B″ is truncated or internally deleted. Extensive deletions from the N and C ends of B″ (more than 390 amino acids altogether) do not change the footprint of the corresponding TFIIIB-DNA complex. Smaller (11- to 22-amino-acid) internal deletions between amino acids 253 and 310 and between amino acids 388 and 449 also leave the TFIIIB footprint on the *SNR6* gene essentially unchanged. At this point, only amino acids 311 to 387 remain untested in the search for a segment of B″ that directly generates the upstream-extending end of the TFIIIB footprint on the *SNR6* gene. (The search is likely to be fruitless since smaller internal deletions of B″ between amino acids 327 and 387 generated TFIIIB-tDNA footprints that were indistinguishable from the wild-type footprint [Fig. 7A].) These findings leave open the possibility that B″ principally generates the upstream footprint extension of the TFIIIB-TFIIIC-DNA complex (relative to the B′-TFIIIC-DNA complex) indirectly, through its action on Brf.

B″ as the scaffold of the TFIIIB-DNA complex. The outcome of the DNase I footprinting analysis of B″ deletion mutants (Fig. 7 and 8), interpreted in the light of prior information from photochemical DNA-protein cross-linking analyses (3, 5), prompts the speculation that B″ serves as a kind of scaffold for the TFIIIB-DNA complex. B″ may remold the B′-DNA complex as it binds to it and generate the heparin resistance of the TFIIIB-DNA complex indirectly by means of protein-protein interactions that "lock down" the TFIIIB-DNA complex rather than exclusively by a simple, separate and discrete B″-DNA interaction. Perhaps that is why internal deletions in the SANT domain of B″ (in region I) and in region II (Fig. 9), which make the corresponding TFIIIB (B″Δ)-*SNR6* gene complex heparin sensitive, show no characteristically different DNase I footprint. Detailed mapping of the B″ polypeptide chain to DNA should allow one to test this suggestion, by determining whether segments of domains I and II that are associated with heparin resistance (Fig. 9C) are in direct contact with, or close to, DNA. We have begun to map such close approaches between B″ and DNA at the respective protein as well as DNA ends by a photochemical method.

TFIIIC-dependent tRNA gene transcription demands more of B″ than does TFIIIC-independent U6 gene transcription. Domains I and II, which serve on an either/or basis for the latter, are separately required for the former. Moreover, B″(263-464), which includes regions I and II, is inadequate for *SUP4* transcription. Transcriptional competence for the *SUP4* gene is conferred either by additional C-terminal or by N-proximal amino acids, since B″(263-594) and B″(158-464) are transcriptionally active (Fig. 1). We attribute these extra requirements to an interaction of B″ with the Tfc4/τ_{120} subunit of TFIIIC, which lies near B″ on upstream DNA sequence. The existence of such an interaction is supported by a dominant mutation in the second tetratricopeptide repeat of Tfc4/τ_{120} that increases B″ activity in fractionated yeast extracts 11-fold (41) and also by the strong activation levels achieved with a two-hybrid construct demanding an interaction between B″ and mutant Tfc4/τ_{120} deleted for its second tetratricopeptide repeat (44).

Our findings (Fig. 1 and 2) implicate two domains of B″ that are widely separated on the polypeptide chain in interaction with TFIIIC, which clearly sits to one side of TFIIIB in the DNA complex (3–5, 45). Two small internal deletions (B″Δ272-292 in domain II and B″Δ409-421 in domain I) generate essentially identical effects on the TFIIIB-TFIIIC-DNA complex footprint at its downstream end (Fig. 7), suggestive of a defect in displacing the τ_{120} subunit of TFIIIC from the transcriptional start (Fig. 6). The two mutants are, respectively, severely and completely defective in *SUP4* transcription. This finding is readily rationalized if B″ is folded over in the preinitiation complex (TFIIIB-TFIIIC-DNA) so that domains I and II are near one another. Since DNA is very sharply bent in the TFIIIB complex (6, 35), more sharply than by TBP alone (30, 31, 47), we suggest that B″ folds DNA along with it. A folded structure of the TFIIIB-DNA complex would allow segments of the B″ polypeptide chain that are associated with the two flanks of the DNA contact site (3) to be also located near each other, consistent with the observation that transcriptional activity on the *SUP4* gene can be restored to the U6 transcription-competent cores of B″ with additions of N-proximal amino acids or C-proximal amino acids (Fig. 1). If B″ were assembled in the TFIIIB-DNA complex as a head-to-tail dimer, one could also imagine domain I-II interaction between B″ subunits. We

believe that TFIIIB contains only a single molecule of B″ for the following reasons. (i) Photochemical cross-linking experiments with DNA probes containing multiple photoactive nucleotides clearly show co-cross-linking of Brf and B″ to a single molecule of DNA but no evidence for two copies of B″ (3). (ii) Attempts to regenerate B″ active for tRNA gene transcription, by complementing B″(1-418) with B″(276-594) to provide additional N- and C-terminal sequence, failed. In contrast, tRNA gene transcription was partially rescued by complementing B″(1-418) with an essentially nonoverlapping C-terminal peptide containing amino acids 418 to 594 (31a). (iii) We saw neither stimulation nor inhibition of tRNA gene transcription in attempts to drive B″ heterodimer formation with dilute wild type B″ (1 to 20 fmol, in titration) and excess B″(276-594), B″(1-418), or B″(224-464) (150 fmol of each [data not shown]).

A close examination of *SUP4* gene transcription yields evidence that B″ plays a role in determining the spacing of the TFIIIB-binding site relative to the site of transcriptional initiation (Fig. 3). This could be due to a direct interaction between B″ and pol III. (In fact, a direct, specific interaction has been detected in B″ affinity chromatography experiments [27a]). However, seeing the effect in *SUP4* transcription, but not *SNR6* transcription, and realizing that the *SNR6* gene harbors alternative start sites for transcription in vivo (13), we suggest that B″ exerts its effect on pol III docking indirectly, probably through TFIIIC.

Other evidence for aberrant preinitiation complex assembly by B″ deletion mutants can also be discerned. B″(276-594), which has a deletion extending into domain II, forms a heparin-resistant TFIIIB complex with the *SNR6* gene that is transcriptionally active (Table 1 and Fig. 2A) but forms a heparin-sensitive TFIIIB-TFIIIC complex with the *SUP4* gene (Fig. 4 and data not shown) that is transcriptionally inert (Fig. 1, Fig. 2B, and Table 1). Although B″(276-594) clearly binds to the B′-TFIIIC-*SUP4* DNA complex (Fig. 4 and data not shown), it leaves the DNase I footprint of that complex only barely changed from the B′ form (Fig. 8, panel iv). The interesting implication of this finding is that a failure of interaction with TFIIIC leads to gross misassembly of B″(276-594) into a TFIIIB-DNA complex.

To this point, the analysis of B″ has focused on transcription complex assembly and transcriptional initiation on DNA templates. It will be interesting to know whether extensively trimmed down versions of B″ also confer transcriptional competence in the context of chromatin, and whether they sustain viability in vivo.

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