Alignment of the B\textsuperscript{0} Subunit of RNA Polymerase III Transcription Factor IIIB in Its Promoter Complex\textsuperscript{*}

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Sheila M. A. Shah\textsuperscript{1}, Ashok Kumar, E. Peter Geiduschek, and George A. Kassavetis\textsuperscript{§}

From the Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92039-0634

TFIIB, the central transcription initiation factor of the eukaryotic nuclear RNA polymerase (pol) III is composed of three subunits: the TATA-binding protein; Brf, the TFIIB-related subunit; and B\textsuperscript{0}, the Saccharomyces cerevisiae, TFC5 gene product. The orientation of the B\textsuperscript{0} subunit within the TFIIB-DNA complex has been analyzed at two promoters by two approaches that involve site-specific photochemical protein-DNA cross-linking: a collection of B\textsuperscript{0} internal and external deletion proteins has been surveyed for those deletions that alter the interaction of B\textsuperscript{0} with DNA or change the orientation of B\textsuperscript{0} relative to DNA; a method for regionally mapping cross-links between specific DNA sites and \textsuperscript{32}P-end-labeled protein has also been applied. The results map an N-proximal segment of B\textsuperscript{0} to the upstream end of the TFIIB-DNA complex and amino acids 299–315 to the principal DNA-contact site, approximately 8 base pairs upstream of the TATA box. The analysis also indicates that a segment comprising amino acids 316–434 loops away from DNA, and locates the C-proximal 170 amino acids of B\textsuperscript{0} downstream of the TATA box. Examination of two-cross-link products formed by DNA with adjacent and nearby photolinkable nucleotides supports the conclusion that Brf and B\textsuperscript{0} share an extended interface along the length of the TFIIB-DNA complex.

The eukaryotic (nuclear) RNA polymerases are brought to their promoters by relatively complex core transcription apparatus. In the RNA polymerase (pol) III transcription system of Saccharomyces cerevisiae, which is the focus of this work, the polymerase recruitment function is executed by the core transcription factor (TF) IIIB. TFIIC and TFIIB, the other components of the core transcription apparatus, bind DNA and serve as assembly factors for TFIIB; the six-subunit TFIIC interacts directly with TFIIB, and TFIIB serves as a 5 S rRNA gene-specific platform for TFIIC. TFIIB is composed of three subunits: the TATA-binding protein (TBP), Brf, and B\textsuperscript{0} (Tc5). TBP co-directs binding of TFIIB to very strong TATA boxes. At promoters that lack these intrinsic TBP-binding sites, TFIIC functions as an assembly factor that deposits TFIIB on its upstream DNA site (reviewed in Ref. 1). In both kinds of situations, TBP is located close to DNA (2).

The 596-amino acid Brf is joined from two evolutionarily distinct parts. Its designation as the TFIIB-related factor derives from the homology of its N-proximal half to TFIIB (3–5). Just as TFIIB is able to recruit pol II to the transcriptional start site, so the principal polymerase recruitment capacity of TFIIB resides in the corresponding, N-proximal, half of Brf. The C-proximal half of Brf is pol III-specific (6), and has no sequence-homologous counterparts in the pol I and pol II transcription apparatus. The principal TBP and B\textsuperscript{0} affinities of Brf, and also its TFIIC affinity, reside in this C-proximal half (7). However, the N-proximal half of Brf alone has sufficient affinity for TBP and B\textsuperscript{0} to form a TFIIB-DNA complex at a strong TATA box that is able to recruit pol III to accurately initiated transcription (7).

Since TFIIB and the N-proximal half of Brf sit in corresponding locations in their respective DNA complexes (7, 8), and exercise similar functions, it is surprising that Brf and TBP are not by themselves competent to direct transcriptional initiation by pol III. In fact, the 594-amino acid B\textsuperscript{0} is absolutely required for transcription by pol III, in duplex DNA or chromatin, at TATA-containing and TATA-less promoters, in vivo as well as in vitro. It is also B\textsuperscript{0} that makes the TFIIB-DNA complex extraordinarily stable (9).

In the experiments that are reported here, we have mapped B\textsuperscript{0} along its DNA site in the TFIIB-DNA complex; B\textsuperscript{0} external and internal deletion proteins that form stable TFIIB-DNA complexes have been examined by photochemical protein-DNA cross-linking along the extended DNA site in order to find out which of these deletions alter the interaction of B\textsuperscript{0} with DNA or change the orientation of B\textsuperscript{0} relative to DNA. A relatively simple and highly sensitive method has also been developed to map cross-links from specific DNA sites to their targeted region on \textsuperscript{32}P-end-labeled protein.

MATERIALS AND METHODS

Synthesis of DNA Probes for Photochemical Cross-linking—The SUP4\textsuperscript{+} RNA gene was polymerase chain reaction-amplified from plasmid pTZ1 (10) with primers creating a BspE1 site beginning at bp –64 (+1 designating the start site of transcription) and an NsiI site beginning at bp +124. The DNA was purified on native 5% polyacrylamide gel, passively eluted (11), restricted with BspE1 and NsiI, and 5’ end-labeled with T4 polynucleotide kinase. The 191-nucleotide non-transcribed and 183-nucleotide transcribed strands were separated on 5% polyacrylamide, 8 m urea gel, excised, and recovered by passive elution (11). The transcribed strand was used to construct DNA with site-specifically placed photoactive nucleotides, as described (12). Cross-linking probes were synthesized by annealing specific oligonucleotides to the transcribed strand. Primer extension to add ABdUMP (5’-[N-(p-azidobenzyloxyl)-3-aminolyl]-deoxouridine monophosphate) and [\textsuperscript{32}P]dNMP utilized 1 unit of exonuclease-free Klenow fragment (incu-
bution for 5 min at 37 °C with 10 μM ABdUTP and 0.5 μM appropriate [α-32P]dNTP. Extension of the photoactive DNA strand was completed by an additional 10-min incubation with 500 μM unlabeled dNTPs. Probes were prepared with ABdUMP at bp 38–39/37,35/32–30, –26–24, –19/17, –14/12, –10/7, and –3/0 of the non-transcribed strand (Fig. 1A). For probes 19/17, 14/12, and 7/3, the probes were left upstream, 22-nucleotide 3’ overhang. An upstream oligonucleotide (the same as the primer for making the 38/37 probe) was annealed and ligated to these constructs as the final step in preparing the corresponding photoactive 183-bp DNA.

SNR6 photoprobes 38/39, 38–35, 28–32, 13/12, and 5/4 were made as just described, using the appropriate non-transcribed strand oligonucleotides annealed to an 88-mer transcribed strand spanning bp −56 to bp +32. SNR6 photoprobes 42 and 23/22 were made using the appropriate non-transcribed strand oligonucleotide primer annealed to a 60-mer transcribed strand template spanning bp −58 to bp +2. Proteins—Expression plasmids for B’-(1–370) and B’-(371–594) were constructed as described and referenced for other B’ deletions (11). The corresponding proteins were overproduced in E. coli BL21(DE3). TFIIIC, wild-type TBP, TBPm3, Bref, Brf3(1–282), Brf3(284–596), truncated and full-length B’ were purified as described (7, 13–15). Concentrations of TBP, full-length B’ and B’-(38–594) were determined by their predicted extinction coefficients. Concentrations of TBPm3, Brf, and other B’ deletion proteins were estimated by Coomasie blue 250 staining, SDS-polyacrylamide gel standardized to bovine serum albumin. TFIIIC concentration was measured as described (9).

**Photolabeling of Proteins**—Protein–SUP4 gene complexes were allowed to form for 60 min at 21 °C in 20 μl of pol III buffer (40 μM Tris–Cl (pH 8.0), 100 μM MgCl2, 100 mM NaCl, and 2.5 mM dithiothreitol (11)) for [32P]labeled of its natural phosphorylation site at Ser-164. Unincorporated [γ-32P]ATP was removed, and efficiency of phosphorylation determined as described (11).

**Photophotoactivity**

Mapping B’ to the TFIIIB–Promoter Complex

for 30 min at 37 °C for cyanylation of cysteine residues. The pH was then adjusted to 9.0 with Tris base, and samples were incubated overnight at 37 °C for the subsequent proteolytic cleavage. The 32P-labeled fragments generated by NTCB or CNBr cleavage were resolved on 11–15% SDS-polyacrylamide gels. Comparisons of cleavage patterns of free and DNA-cross-linked 32P-labeled B’ were made on phosphomager profiles.

**RESULTS**

**Photochemical Cross-linking of B’ Deletion Mutants**—Previous examination of the internal structure of a TFIIIB–DNA complex by photochemical cross-linking revealed that B’ cross-links to DNA between 43 and 2 bp upstream of the transcription start site (16, 17). In order to map the locations of individual domains of B’ relative to DNA, we have analyzed the photochemical cross-linking patterns of B’ with internal and external deletions, using ABdUMP incorporated at specific sites along the TFIIIB–binding sites of the SUP4 and SNR6 genes (Fig. 1). Each DNA photoprobe also has a radioactive nucleotide incorporated next to, or in close vicinity to, its photoactive nucleotide(s). TFIIIB complexes containing either full-length or truncated B’ were assembled on each of these DNA photoprobes; reaction mixtures were then UV-irradiated, digested with nucleases, and analyzed by SDS-PAGE. The efficiency of TFIIIB–DNA complex formation was monitored in parallel by electrophoretic mobility-shift analysis of UV-irradiated, but not nuclease-treated, portion of each reaction mixture. If deletion of a specific segment of B’ results in the loss of cross-linking at a particular DNA site, this suggests localization of that peptide segment in the vicinity of that DNA site (so long as complex formation is not correspondingly diminished). In the case of the SNR6 gene promoter, unidirectional assembly of TFIIIB–DNA complexes is specified by a modified TATA-box (TGGAAATA) in conjunction with the mutant TBPm3 (15). At the SUP4 tRNA promoter, with its very weak TATA box, TFIIIB–DNA complex assembly requires the transcription factor TFIIIB as unidirectional (15).

**The SUP4 Promoter**—Efficiencies of forming heparin-stable TFIIIB–DNA complexes and photochemical cross-linking of B’ deletion mutant and wild type B’ were compared for the photoactive DNA probes shown in Fig. 1A. B’ lacking its N-terminal 262 amino acids or its C-terminal 130 amino acids remains competent to assemble into a heparin-resistant TFIIIB–DNA complex via the TFIIIC-dependent assembly pathway on the SUP4 gene (but does so relatively inefficiently;
cross-linking of B" between bp -26 and bp -2, resulted in cross-linking signals too close to background to be reliably quantified, but the observed levels were consistent with unimpaired cross-linking of B"-(263–594) at these positions (Table I, part A). C-terminally truncated B"-(1–464) and B"-(1–487) were not sufficiently well resolved from Brf in SDS-PAGE to quantify reliably. It was, however, possible to assess that B"-(1–487) was not significantly impaired in cross-linking to bp -38/–37, -33/–32, and -30, where B" cross-links efficiently (Table I, part A). N- and C-terminally truncated B"-(40–487) did resolve well from Brf, and its cross-linking to all probes tested was unimpaired (Table I, part A, and Fig. 2B). These results indicate that the cross-links of B" to the SUP4 gene (more specifically to the bp -38/–2 segment of this gene) primarily involve its amino acid 224–487 segment; this is also the active core of B" for SUP4 transcription (11). Internal deletion mutants of B", which span much of the region of B" not covered by the N- and C-terminal truncations, and which are capable of being assembled into heparin-resistant DNA complexes, were also examined. Only B" with amino acids 291–310 deleted displayed a defect in cross-linking, predominantly with probe -38/–37 for which cross-linking efficiency is reduced more than 10-fold (Fig. 2C, lanes 3 and 5) and, to a lesser extent, with probes -33/–32 and -30 (Table I, part A).

Fig. 2C shows two other aspects of this defect. 1) Heparin did not diminish cross-linking of intact B" (lanes 2 and 3), but substantially reduced cross-linking of B"Δ291–310 without substantially reducing the cross-linking of Brf (lanes 4 and 5). 2) Full-length B" displaced the 120-kDa subunit of TFIIIC from the vicinity of the photoactive nucleotide in the -39/–38 photoprobe (compare lanes 1 and 2), but B"Δ291–310 did not (lane 4).

The SNR6 Promoter—On the SNR6 gene (18, 19), B" truncated by N-terminal deletion to amino acid 186 or by C-terminal deletion to amino acid 487 was not deficient in cross-linking (Table I, part B). N-terminal truncation to amino acid 224 or 263 somewhat lowered cross-linking at bp -13/–12 and -5 (20–40% of that obtained with intact B"; Table I, part B), but a comparable depression of cross-linking was not observed when TFIIIB complexes with the SUP4 gene were probed at the close-by -14/–12 and -3/–2 sites (Table I, part A). Since no single region of B" is required for TFIIIB-SNR6 DNA complex formation, a more complete set of internal deletions, spanning regions not covered by N- and C-terminal truncations, could be examined at this promoter. B" cross-links most efficiently to bp -39/–38 on the SNR6 gene (7); only at bp -33 and -13/–12 do cross-linking efficiencies exceed 10% of cross-linking to bp -39/–38. Only B"Δ272–292 was deficient in cross-linking to probe -39/–38 (20% of the efficiency of cross-linking to intact B"; Table I, part B, and Fig. 2D). Curiously, B"Δ291–310, which was deficient for cross-linking at a similar location on the SUP4 gene, was not significantly impaired for cross-linking on the SNR6 gene (Table I and Fig. 2; B"Δ272–292 was not examined on the SUP4 gene because it does not assemble into a stable, heparin-resistant complex; Ref. 11). B"Δ424–438 displayed somewhat lower cross-linking to bp -28 and -13/–12 (20–40% of the cross-linking efficiency with intact B") and B"Δ409–421 was somewhat impaired in cross-linking to bp -5 (40% efficiency relative to intact B")

Comparisons of cross-linking to the SUP4 and SNR6 genes are complicated by the fact that TFIIIC positions TFIIIB somewhat heterogeneously onto the AT-rich upstream sequence of the SUP4 gene (20), whereas assembly on the SNR6 gene directed by TBPm3 is fixed at the TGTA box. One would therefore expect a “blurring” in the B" cross-linking pattern to the SUP4 gene relative to SNR6 (i.e. efficient cross-linking be-
between bp −38 and −30 on the SUP4 gene, but predominantly at bp −39/−38 on the SNR6 gene). Conversely, even if as few as 2% of TFIIIB complexes on the SNR6 gene were bound in the reverse orientation (cf. Ref. 15), they might make a significant contribution to cross-linking at bp −13/−12 (through the B′ segment that cross-links efficiently to bp −39/−38 in the opposite orientation of TFIIIB).

Split B′−B′ assembly into a TFIIIB-DNA complex buries two regions that are more surface-exposed in the free protein (as judged by hydroxyl radical footprinting): region I, covering amino acids −390−470; and region II, covering amino acids −270−305 (11). The above experiments clearly suggest that region II lies close to the major sites of B′ cross-linking on the SNR6 and SUP4 genes (since B′Δ291−310 depressed cross-linking to the SUP4 gene and B′Δ272−292 to the SNR6 gene). In order to gain further information about the location of region II relative to DNA, B′ was split at amino acid 370 (which is highly accessible to hydroxyl radical cleavage in TFIIIB-DNA complexes; Ref. 11). Heparin-resistant TFIIIB-DNA complexes can be formed on both the SUP4 and SNR6 genes only when both parts of this split B′ (amino acids 1−370 and 371−594) are provided together. The transcriptional activity of these TFIIIB-DNA complexes is, however, diminished (data not shown). Fig. 3 compares the cross-linking profiles of wild type and split B′ on the SUP4 and SNR6 genes. Cross-linking to the SUP4 gene at bp −38/−37, −33/−32, and −30 is contributed almost entirely by amino acids 1−370 of B′, but there is some cross-linking by the C-terminal amino acids 371−594 to bp −33/−32 (Fig. 3A). The weak cross-linking of intact B′ to sites downstream of bp −30 on the SUP4 gene appears to involve solely its C-terminal half. Similarly, B′(1−370) dominates cross-linking at bp −39/−38 of the SNR6 gene (Fig. 3B), but some cross-linking of the C-terminal segment is also apparent at this site. In contrast to the cross-linking pattern at bp −33/−32 of the SUP4 promoter, most of the cross-linking of B′ at bp −33 of SNR6 is contributed by its C-terminal segment. As on the SUP4 gene, most of the C-terminal B′ segment cross-links also to bp −28, −13/−12, and −5, but low level cross-linking of the N-terminal segment at these sites also persists.

A High Molecular Weight Photoproduct—TFIIIB-DNA complexes with the −38/−37 SUP4 probe, which contains two AbDUMP residues, yielded a high molecular weight material cross-linking adduct, whose mobility depended on the B′ external deletion mutant used (Fig. 4, lanes 1 and 3). Such high molecular weight bands have been characterized as the products of multiple cross-linking events (21). That this particular multiply cross-linked product contains both B′ and Brf was demonstrated in the following way. The mobility of the high molecular weight band increased when B′ was truncated, indicating that this DNA-protein adduct contains B′ (Fig. 4, lane 3). That it also contains Brf was shown by using split Brf. The two Brf fragments, Brf(1−282) and Brf(284−596), together form a stable and transcriptionally fully competent TFIIIB complex on the SUP4 gene (7). The cross-linked products of the TFIIIB-SUP4 gene complex formed with intact and split Brf also yielded high molecular weight bands with different electrophoretic mobilities (Fig. 4, lane 3). Since the high molecular weight Brf-B′-DNA photochemical adduct has also been observed in UV-irradiated TFIIIB complexes formed on SUP4 photoprobe −33/−32, −22/−21, −19/−17, −14/−12, and −3/−2 (data not shown), we conclude that Brf and B′ share an extended interface with DNA.

Partial CNBr Cleavage of B′-DNA Photoproducts—Use of internal deletions to map the orientation of a protein along its DNA site is subject to the restriction that those deletions should not drastically change the protein-DNA alignment. A second approach, which is free of that restriction, was developed for mapping specific B′ segments to the vicinity of specific sites on the SUP4 and SNR6 genes. An example of the mapping procedure is shown in Fig. 5. N-proximally 32P-labeled B′-(138−594) was used to form TFIIIB-DNA complexes with unlabeled −39/−38 SNR6 photoprobe and subjected to UV-irra-
diation (Fig. 5A). The $[^{32}P]B'$-DNA adduct was recovered from a 6% polyacrylamide-SDS gel (Fig. 5B, lane 3) and subjected to partial CNBr cleavage. The cleavage products were then resolved on a 13% polyacrylamide-SDS gel (Fig. 5C). The partial cleavage patterns of free $B'_{\text{wt}}$-(138–594) is shown in lane 2 of panel C, with the methionine C-terminal to each cleavage site identified at the left of the panel. The partial digestion pattern of the $B'_{\text{wt}}$-(138–594)-DNA adduct and free $B'_{\text{(1–370)}}$ were identical to Met-298, but products of cleavage at Met-315, -372, -379, and -425/434 were shifted to lower mobility due to their attached DNA (lane 3). This specifies that the major site of cross-linking is to bp 39/38 is situated between amino acids 299 and 315. An identical site between Met-298 and Met-315 was identified for the multiply cross-linked products identified in Fig. 5B (data not shown).

Figs. 6 and 7 summarize the results of this method of mapping $B'$ cross-links to the SNR6 gene at bp −42, −39/38, −33, −23/22, and −13/12 by partial CNBr and NTCB (cysteine-specific) cleavage, respectively. Panel A of Fig. 6 shows that, when $B'_{\text{(138–594)}}$ is cross-linked to the SNR6 DNA probe, products of CNBr cleavage C-terminal of Met-222 are shifted to lower mobility, implying that the $B'$ DNA link is located between amino acids 223 and 276. Panel B displays the profile of the experiment with probe −39/38 shown in Fig. 5. The Met-298 peak was consistently somewhat reduced relative to the Met-276 peak (in seven out of seven experiments), suggesting that a subsidiary cross-linking site of $B'$ is located between amino acids 277 and 297. Panel A of Fig. 7 confirms that the bulk of the cross-linking to the −39/38 probe occurs C-terminal to Cys-280.

Panel C of Fig. 6 shows that shifting the site of DNA cross-linking downstream by only 5 bp, to bp −33, shifts the protein side of the cross-link far toward the C-end of $B'$; only CNBr cleavage products to the carboxyl side of Met-435 are shifted to lower electrophoretic mobility (the products of cleavage at Met-425 and Met-434 fail to separate on these gels). The corresponding NTCB cleavage profiles (Fig. 7, panel B) shows that the point of DNA attachment is to the amino side of Cys-485. Thus, the cross-link to bp −33 is located between amino acids 425 and 485 of $B'$. The result supports the evidence presented in Fig. 3B that the amino acid 371–594 segment of $B'$ cross-links to bp −33 of the SNR6 gene.

Cross-linking to the SNR6 gene at bp −23/22 and −13/12 has also been examined in this manner. In these cases, the level of cross-linking was low enough that the background of free $B'$ (see Fig. 5B, lane 2) makes the outcome of the analysis more tentative. Panels D and E of Fig. 6 display the CNBr cleavage profiles of probes −23/22 and −13/12, respectively. Both profiles imply that the cross-linking occurs at the C-terminal side of Met-425 and/or Met-434. Partial cleavage at cysteine indicates that at least part of the cross-linking to bp −13/12 occurs within the amino acid 426–485 segment (Fig. 7, panel C).

The same method was used to map segments of $B'$ that are located in vicinity to bp −38/37, −33/32, −30, and −26 of the $SUP4$ gene (in TFIIB-DNA complexes assembled with TFIIC and subsequently stripped of the latter with heparin). The three upstream-lying sites of cross-linking generated nearly identical partial CNBr digestion profiles (Fig. 8, panels A–C) indicating that the segment of $B'$ that cross-links to these three sites lies between amino acids 299 and 314. (There is an indication, at or near the limits of reliability, that the amino acid 277–297 segment makes a small contribution to DNA cross-linking at bp −38/37, but not at bp −30.) It is feasible for the amino acid 299–314 segment to span 8 base pairs. However, as proposed above, a more likely explanation is that TFIIC positions TFIIB heterogeneously over the AT-rich upstream region of the $SUP4$ gene promoter (20), and that the
DISCUSSION

The Alignment of B’ in the TFIIIB-DNA Complex—B’ is, to varying degree, accessible to cross-linking by ABdUMP placed site-specifically along the length of the TFIIIB-DNA complex (16). However, it is most efficiently cross-linked to DNA upstream of the TATA box (7). B’ binding to the TBP-Brf-DNA complex extends the DNA footprint ~10 bp upstream of the TATA box, and is stabilized by an additional 15–20 bp DNA stretch upstream of the TATA box (see Ref. 22 and Footnote 2). Thus, the DNA segment that is additionally covered when B’ adds to the TBP-Brf-DNA complex harbors a site of efficient B’ cross-linking and contributes to the stability of the TFIIIB-DNA complex.

A central ~225-amino acid segment of B’ appears to encompass its functional core. Two domains, one at each end of this core region (amino acids 272–292 and 424–449), are required for TFIIIC-dependent transcription in vitro, and one or the other of these segments is required for TFIIIC-independent transcription (11). An additional and partly overlapping ~65-amino acid segment (amino acids 355–421) is required for transcription of linear DNA (17).

The principal target for DNA cross-linking of B’ lies between amino acids 277 and 315, i.e. it encompasses the N-proximal “essential” amino acid 272–292 segment. This segment of B’ faces the upstream part of the DNA site occupied by TFIIIB. Thus, the DNase I footprint extension that is generated when B’ enters the B’-DNA complex is at least partly due to a direct B’-DNA interaction.

The N-proximal 223 amino acids of B’ are not essential for transcription of bare DNA (11). This segment of B’ diminishes DNA-protein cross-linking upstream of the TATA box at least 2-fold (Table I), as though it formed an obstruction of the corresponding DNA-interacting segments of B’. Deleting B’ amino acids 1–185 has the same effect on cross-linking (Table I). A segment of B’ extending approximately from amino acid 190 to amino acid 210 becomes more accessible to cleavage by hydroxyl radical upon entry into the TFIIIB-DNA complex (11). This is consistent with the notion that DNA access of B’ might be blocked by its internal folding, and uncovered upon formation of the TFIIIB-DNA complex. The 137 N-terminal amino acids of B’ are also removed in making 32P-end-labeled B’. This may inadvertently facilitate the mapping of protein segments cross-linking to specific DNA sites upstream of the TATA box by generating higher efficiencies of cross-linking. However, if such an effect exists, it is quantitative rather than qualitative.

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2 A. Kumar and G. A. Kassavetis, unpublished observations.
since cross-linking of $^{32}$P-labeled full-length B$^0$ to the SNR6/$^{24}$2 probe and the SUP4/$^{23}$3 probe likewise mapped to amino acids 299–315 (data not shown).

The disposition of B$^0$ relative to DNA has been most clearly mapped in the SNR6 gene-TFIIIB complex, as detailed below.

1) The amino acid 223–275 segment cross-links to bp $^{24}$2.  

2) The bp $^{23}$39/$^{22}$38 site principally cross-links to the amino acid 299–315 segment, but some cross-linking to amino acids 277–297 is consistently observed (Fig. 6). Deleting amino acids 272–292 also diminishes cross-linking of B$^0$ to the bp $^{23}$39/$^{22}$38 site (Table I).

3) Moving just 5 (or 6) bp toward the transcriptional start, to bp $^{22}$33, shifts the site of the B$^0$-DNA cross-link to the amino acid 435–485 segment. Thus, the intervening amino acids 316–434 must be positioned away from DNA. This part of B$^0$ includes the amino acid 355–421 segment, which is required for transcription of linear DNA, and the amino acid 424–449 segment, which is required for TFIIIC-dependent transcription; it extends into the amino acid 390–470 “region I” segment, which is protected from hydroxyl radical cleavage upon forming the TFIIIB-DNA complex (11). Since the amino acid 316–434 segment appears not to lie close to DNA, protection of region I from hydroxyl radical cleavage must be due to protein-protein interaction. A recently sequenced S. pombe open reading frame (NCBI accession CAA22645) considered to be a candidate homologue of S. cerevisiae B$^0$ has 31% identical (65% homologous) sequence in the region I segment.

4) Cross-linking to B$^0$ downstream of the SNR6 TATA box principally involves the C-proximal amino acid 371–594 segment (Fig. 3B); the cross-link appears to be located C-terminal to amino acid 425 (Fig. 6) and N-terminal to amino acid 487 (Table I), but more precise mapping has proved inconclusive (Fig. 7). This part of B$^0$ may not be uniquely positioned relative to DNA.

5) When B$^0$ enters into the TFIIIC-B$'$-DNA complex, it en-
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This inquiry was partly motivated by the observation that $B^\prime$-(263–594), $B^\prime$$\Delta$272–292, and $B^\prime$$\Delta$409–421 generates start site-proximal aberrations in the TFIIIB-TFIIIC-DNA complex footprint, with $B^\prime$272–292 and $B^\prime$409–421 generating identical effects (11). One interpretation of this observation is that regions I and II of $B^\prime$ are situated in close proximity to each other (11). The cross-linking of these two regions of $B^\prime$ to DNA sites that are separated by only 5 or 6 bp (Fig. 6) supports this contention. Additional cross-linking of the amino acid 426–487 segment of $B^\prime$ to sites downstream of the TATA box suggests that TFIIIB bends DNA (23) so as to bring the two flanks of the TATA box closer together. Somewhat depressed cross-linking downstream of the TATA box is seen for $B^\prime$263–594, $B^\prime$$\Delta$424–438, and $B^\prime$409–421 (Table I). This may reflect the proximity of these two parts of $B^\prime$ within the TFIIIB-DNA complex and subtle alterations in the path of DNA when these regions of $B^\prime$ are deleted.

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