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Two Components of *Saccharomyces cerevisiae* Transcription Factor IIIB (TFIIIB) Are Stereospecifically Located Upstream of a tRNA Gene and Interact with the Second-Largest Subunit of TFIIIC

BLAINE BARTHOLOMEW, GEORGE A. KASSAVETIS, AND E. PETER GEIDUSCHEK*

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0634

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A novel photocrosslinking method has been used to identify the components of transcription factor IIIB (TFIIIB) and TFIIIC that associate with DNA upstream of the *Saccharomyces cerevisiae SUP4* $tRNA^{Tyr}$ gene and to map these components to specific positions in DNA. When TFIIIC binds to the tRNA gene, only its second-largest subunit (135 kDa) is accessible for reaction with a photoactive nucleotide, 5-[*N*-(*p*-azidobenzoyl)-3-aminoallyl]-dUMP, inserted into DNA upstream of the transcriptional start. Formation of TFIII(C+B)-tRNA gene complexes specifically brings two additional polypeptides (90 and 70 kDa) within reach of upstream photoprobes. A collection of 13 probes has been used to map the locations of these three proteins along a 45-bp segment of DNA upstream of the transcriptional start site. Evidence is presented that the 90- and 70-kDa polypeptides are separate and distinct components of yeast TFIIIB, that they are accessible to crosslinking on opposite sides of the DNA helix in a 6-bp segment centered 35 bp upstream of the $tRNA^{Tyr}$ gene transcriptional start, and that they interact with the second-largest subunit of TFIIIC.

Initiation of transcription by eukaryotic RNA polymerases involves the ordered assembly of proteins on DNA. For yeast RNA polymerase III, which is the focus of the work reported here, a transcription complex on a tRNA gene is assembled in three steps. First, transcription factor IIIC (TFIIIC) binds to the *box A* and *box B+* internal promoter elements. The TFIIIC-DNA complex next recruits TFIIIB to an upstream DNA-binding site. In the third step, RNA polymerase III is correctly positioned over the transcriptional start site (9, 10, 11, 14) and an internal structural change, suggestive of promoter opening, occurs (8; unpublished data). The final assembly in *Saccharomyces cerevisiae* contains 20 or more polypeptide chains: 14 contributed by RNA polymerase III (reviewed by Sentenac [18]), at least 4 and perhaps as many as 6 (2) contributed by TFIIIC, and as we show here, at least 2 contributed by TFIIIB. Determining the internal structure of this nucleoprotein complex and understanding how that structure determines its functions in transcriptional initiation is a relatively complex task.

We have recently applied a photocrosslinking method to one aspect of this problem, the identification of polypeptide chains that are part of transcription complexes and the exploration of their locations relative to DNA (2). In this method, a photoactive nucleotide, 5-[*N*-(*p*-azidobenzoyl)-3-aminoallyl]-dUMP (N_3RdUMP) (Fig. 1), is enzymatically incorporated into specific sites in the DNA probe, in close juxtaposition to one or more radioactive nucleotides. A protein assembly is built up on this probe and irradiated. The nitrene that is generated upon irradiation rapidly forms covalent bonds with amino acid side chains that are located in the immediate vicinity. N_3RdU places the photoactive substituent approximately 0.9 to 1.0 nm away from the pyrimidine ring on a relatively stiff tether. Thus, the azido group probes a segment of space outside the DNA helix, but in the vicinity of a specific nucleotide, for protein side chains.

In previous experiments along these lines, we have analyzed the subunits of *S. cerevisiae* TFIIIC that interact with the tRNA gene. We have shown that polypeptide chains with apparent molecular weights of approximately 145, 135, 95, and 55,000 are accessible to photocrosslinking from the *SUP4* $tRNA^{Tyr}$ gene, have mapped these subunits to different segments of this gene, and have demonstrated that different subunits are located on different sides of the DNA helix in the vicinity of the *box A* internal promoter element. In the experiments reported below, we show that one of the subunits of TFIIIC is accessible to photocrosslinking by N_3RdUMP incorporated into DNA upstream of the transcriptional start site. We have used the same approach to search for components of yeast TFIIIB that are positioned upstream of the transcriptional start site in a TFIIIC-dependent process. We show that two polypeptides, with apparent molecular weights of 90,000 and 70,000, are associated with the DNA-binding and transcription activities of TFIIIB and that they are distinct polypeptide chains. We also show that these two components of TFIIIB specifically occupy different parts of the space around the yeast tRNA gene.

MATERIALS AND METHODS

Materials. Plasmids containing a promoter-up and promoter-down mutation in the *SUP4* $tRNA^{Tyr}$ gene have been described elsewhere (2, 9). Plasmids p1A1B-4 and p1A1B-5 (promoter-down, C84→G in the numbering system of Fig. 2) and plasmids p2A2D and p2A2B (promoter-up, G90→C) have the tRNA gene inserted into M13mp18 or M13mp19. Growth and purification of M13 phage and of single-stranded DNA were carried out as described previously (16). The DNA of plasmids pLNG56 (promoter-down) and pTZ1 (promoter-up) containing the *SUP4* gene in pGEM1 was purified as described previously (9). N_3RdUTP was prepared as described elsewhere (2). The preparation of yeast TFIIIC

* Corresponding author.

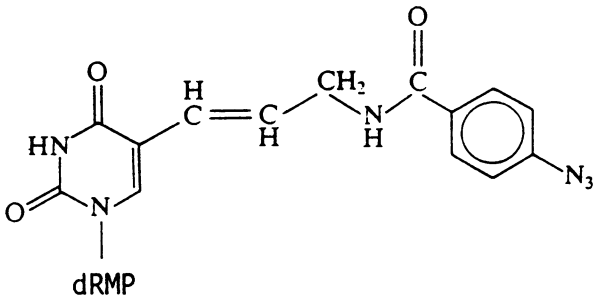

FIG. 1. Structure of N_3RdUMP.

has been described elsewhere (9), as has the composition of the material used in these experiments (2). Similar highly purified preparations have been described by others (6, 17). TFIIIB was purified as described previously (9). All proteins were used in buffers containing 10 mM β-mercaptoethanol in place of dithiothreitol. Quantities of TFIIIB and TFIIIC are specified in terms of femtomoles of footprinting activity, measured as described previously (9).

Synthesis of DNA photoaffinity probes. Single-stranded M13 DNA containing either strand of the promoter-up or promoter-down mutations of the *SUP4* gene was annealed to a *SUP4* gene site-specific oligonucleotide. A typical reaction mixture containing 1 pmol of M13 DNA and 3 pmol of oligonucleotide in 10 μl of buffer A (30 mM Tris-HCl [pH 8], 50 mM KCl, 7 mM $MgCl_2$, 1 mM β-mercaptoethanol) was incubated at 90°C for 3 min and then at 37°C for 30 min. After centrifugation to deposit the entire sample at the bottom of its tube, the volume was adjusted to 20 μl by the addition of [α-^{32}P]labelled deoxynucleoside triphosphate (dNTP; specific activity, 6,000 Ci/mmol), N_3RdUTP, unlabelled dNTP, and bovine serum albumin to final concentrations of 0.5 μM, 10 μM, 50 μM, and 100 μg/ml, respectively. One or two dNTPs were omitted to limit DNA primer extension and nonradioactive nucleotides were purified on an FPLC apparatus (Pharmacia). Exonuclease-free Klenow fragment DNA polymerase I (U.S. Biochemical) that had been passed through a Sephadex G-50 column to exchange dithiothreitol for β-mercaptoethanol was added at 1 U/20-μl reaction. After 15 min of DNA synthesis at 37°C, further reaction was stopped by the addition of sodium dodecyl sulfate (SDS) to 0.2% (wt/vol), and a 0.5-μl aliquot of the sample was removed for subsequent analysis of the reaction product. DNA polymerase and SDS were removed from the principal sample on a 200-μl Sephacryl S-400 spin column equilibrated in buffer A. A second primer (3 pmol), complementary to the M13 vector just upstream from the inserted *SUP4* gene, was added next, together with bovine serum albumin to 100 μg/ml, and the primer was allowed to anneal to its template during 30 min at 37°C. DNA synthesis was completed by the addition of all four dNTPs (to 500 μM) and 1 to 4 U of T4 DNA polymerase (GIBCO/BRL). After 15 min at 37°C, further DNA polymerization was blocked by adding SDS, and the T4 DNA polymerase was removed on a 200-μl Sephacryl S-400 spin column equilibrated in buffer B (6 mM Tris-HCl [pH 8], 6 mM $MgCl_2$, 1 mM β-mercaptoethanol, 150 mM NaCl). The DNA was cut with *Bam*HI, and the 274-bp photoactive probe extending from 106 bp upstream to 168 bp downstream of the start of transcription was purified from the digest as detailed previously (2). Probes were stored at 4°C in 100 mM NaCl–10 mM Tris-HCl (pH 8)–0.1 mM EDTA–0.02% (vol/vol) Tween 20.

Photoaffinity labelling of proteins. Protein-DNA complexes were formed in reaction mixtures containing 2 fmol of DNA photoaffinity probe, 18 fmol (footprinting activity) of TFIIIC, 50 fmol of TFIIIB, and 900 ng of pGEM1 cut with *Eco*RI in 25 μl of buffer C (7 mM $MgCl_2$, 90 mM NaCl, 200 μg of bovine serum albumin per ml, 50 mM Tris-HCl [pH 8.0], 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml). Reaction mixtures with TFIIIC only were incubated at 21°C for 15 min; those with TFIIIC and TFIIIB were incubated for 30 min. These reaction conditions were effective in bringing 90% or more of the DNA probe into specific TFIIIC-DNA or TFIII(B+C)-DNA complexes. DNA-protein complex formation was analyzed by electrophoresis in nondenaturing 4% polyacrylamide (35:1 acrylamide/bisacrylamide) gels (PAGE) as described previously (3).

Samples were photocrosslinked by setting the 1.5-ml polypropylene microcentrifuge tube with its top open at a distance of 60 cm from three 15-W germicidal lamps for 5 min. The light intensity at 254 nm at this distance from the light source was measured to be 380 μW/cm^2 with an IL 1500 research radiometer. This is a slightly greater (~2.5×) light intensity than previously obtained from a small hand-held UV lamp held directly over the sample (2); the new arrangement allowed for a more uniform irradiation of a large number of samples.

After irradiation, samples were digested either with DNase I and micrococcal nuclease as described previously (2) or with DNase I and S1 nuclease. In the latter procedure, DNase I and $CaCl_2$ were added to 50 μg/ml and 5 mM, respectively, and incubated for 10 min at 21°C. Protein-DNA complexes were then denatured by adding SDS to 0.5%, and some samples were heated for 3 min at 90°C. The pH was next adjusted to 4.5 with acetic acid; $ZnCl_2$ was added to 1 mM, and digestion with 20 U of S1 nuclease (GIBCO/BRL) was done for 10 min at 37°C. The pH was readjusted to approximately 6.8 with Tris-HCl (pH 8.8), and samples were denatured in 5% (vol/vol) β-mercaptoethanol–2% SDS–70 mM Tris-HCl (pH 6.8) (13) at 90°C for 3 min before loading on an SDS-polyacrylamide gel. Digestion with DNase I and S1 nuclease afforded a more extensive nuclease treatment, which proved necessary for analyzing TFIII(B+C)-DNA complexes with certain DNA probes. High-molecular-weight standards (GIBCO/BRL) and photoaffinity-labelled TFIIIC served as markers for estimating relative molecular weights.

Peptide mapping of photoaffinity-labelled 70- and 90-kDa subunits of TFIIIB. A 100-μl (4×) photocrosslinking reaction was prepared, and complexes were digested with DNase I and S1 nuclease and then loaded onto a 1-mm-thick 8% polyacrylamide gel in a 2-cm-long well. After electrophoresis, the 70- and 90-kDa photoaffinity-labelled proteins were visualized by autoradiography of the wet gel. Slices containing these proteins were cut out of the gel and soaked in 0.125 M Tris-HCl (pH 6.8)–0.1% SDS for 20 min, further cut into 0.5-cm-long pieces, and placed in the wells of a 1.5-mm-thick 15% polyacrylamide gel. Gel slices were covered with 10 μl of 0.125 M Tris-HCl (pH 6.8)–1 mM EDTA–0.5% β-mercaptoethanol–0.1% SDS containing 20% glycerol and then with 15 μl of the same buffer with 0.01% bromophenol blue, 10% glycerol, and either 1 or 500 μg of Glu-C endoproteinase (V8 protease from *Staphylococcus aureus*) per ml. Electric power (60 V) was applied until the dye had travelled halfway through the stacking gel and was then turned off for 30 min to permit protease digestion (4, 5). Electrophoresis (at 200 V)

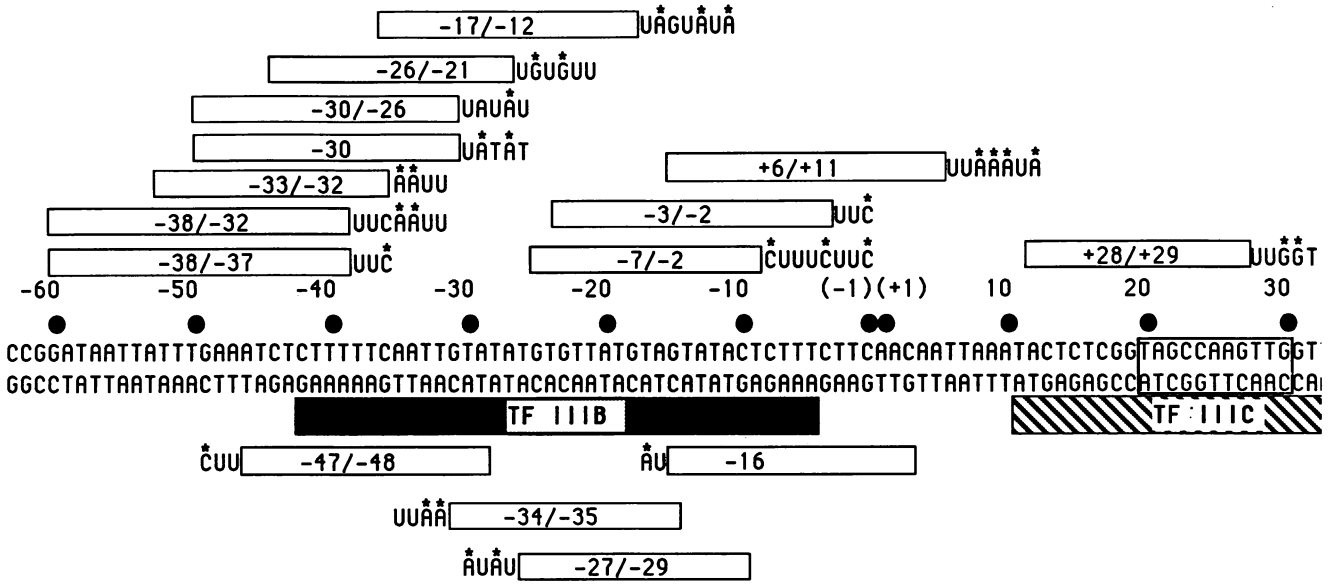

FIG. 2. Photoaffinity probes of the upstream region of the *SUP4* gene. The sequence of the *SUP4* $tRNA^{Tyr}$ gene extending from 63 bp upstream of the start site of transcription (designated as +1) to bp 32 is shown. The shaded boxes below the sequence indicate the DNase I footprint attributed to TFIIIB (bp −42 to −5) and the extreme 5′ end of DNase I protection afforded by TFIIIC alone (bp 11 to 32). The open boxes above and below the DNA sequence represent oligonucleotides that were used in the synthesis of DNA photoaffinity probes; the letters show nucleotides that were incorporated in the labelling step, U representing N_3RdUMP. Asterisks indicate α-^{32}P labelling. Each photoaffinity probe is referred to by a number or a pair of numbers indicating the location of a single N_3RdUMP residue or by the upstream and downstream ends of a cluster of N_3RdUMP residues, respectively.

proceeded until the bromophenol blue reached the bottom of the gel.

RESULTS

We have probed DNA upstream of the *S. cerevisiae SUP4* $tRNA^{Tyr}$ gene for proteins that are brought into close proximity as a specific consequence of the formation of transcription complexes. To carry out this search, we synthesized 13 constructs that place one to five photoactive nucleotides (N_3RdUMP) at various positions extending across the DNA segment that is protected from DNase I in complexes with TFIII(C+B) but not with TFIIIC (Fig. 2). A control probe placing five N_3RdUMP residues well upstream of the transcription complex was also available from prior work (probe −96/−88), as was a probe placing a single N_3RdUMP in the vicinity of *box B*+ (probe +75; not shown in Fig. 2).

In the first series of experiments, complexes of these probes with highly purified TFIIIC were examined. In this preparation, polypeptides with apparent molecular masses of 145, 135, 95, 62, and 55 kDa accounted for approximately 80% of the silver stain density of SDS-polyacrylamide gel electropherograms (2). A gel retardation analysis showed that TFIIIC bound normally to these probes. The specificity of TFIIIC binding was confirmed by failure to form complexes with a photoprobe synthesized on the promoter-down mutant *SUP4* gene (Fig. 3a; compare lanes 5 and 4); the standard promoter-up *SUP4* gene and the promoter-down mutant gene differ only at bp 84 and 90 (in the numbering system of Fig. 2). Specificity of interaction was also confirmed by competition with unlabelled plasmid DNA containing one copy of the promoter-up *SUP4* gene (pTZ1; lane 7) but not with the otherwise identical plasmid containing the promoter-down *SUP4* gene (pLNG56; lane 6).

These complexes were UV irradiated, digested with nucleases, and then subjected to SDS-PAGE to resolve polypeptide chains containing covalently linked small, radioactive DNA fragments (Fig. 3b). The previously analyzed +75 probe (lane 8) photo-cross-linked only to the largest subunit (145 kDa) of TFIIIC, as expected (2). The efficiency of photo-cross-linking with probe +75 relative to the other probes used in this experiment is indicated by the strength of signal due to TFIIIC-DNA complex formation in lane 2 relative to the signal due to photocrosslinking in lane 8 compared with other pairs of lanes (e.g., lane 3 relative to lane 9, lane 4 relative to lane 10, etc.). The control −96/−88 probe gave no background of nonspecific photocrosslinking to TFIIIC at this sensitivity of detection, despite its relatively high specific radioactivity and despite the presence of five N_3RdUMP residues (compare lanes 9 and 3). Longer exposure revealed two nonspecifically labelled proteins at ~145 and 120 kDa but no labelling of the 135-kDa protein. The −7/−2 probe photocrosslinked only to the 135-kDa subunit of TFIIIC (lane 10). We have previously shown that this subunit can also be crosslinked from the downstream side of the transcriptional start, but always together with other subunits of TFIIIC (2). The crosslinking of the −7/−2 probe to the 135-kDa subunit was shown to be promoter specific (lanes 10 to 13).

TFIIIC complexes with the other probes diagrammed in Fig. 2 were also examined (Table 1). The important outcome of this survey was the demonstration that only the 135-kDa subunit is photocrosslinked from upstream of the transcriptional start. The efficiency of crosslinking was greatest for probes with N_3RdUMP located near the transcriptional start site (Table 1, column 2). The efficiency of labelling declined with probes that contain photoactive nucleotides further upstream, became very weak upstream of bp −30, and was barely detectable with the −47/−48 probe. Nevertheless,

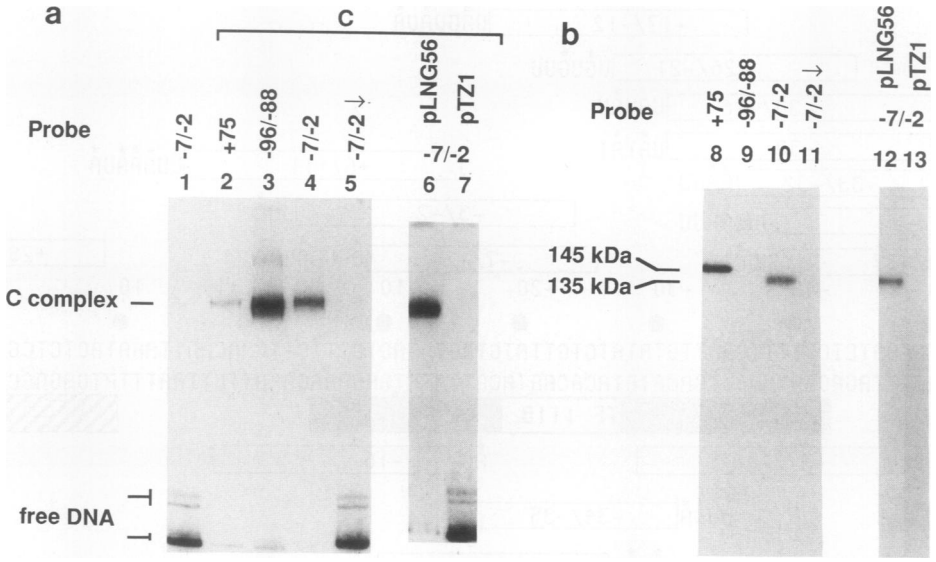

FIG. 3. Photoaffinity labelling of TFIIIC from the vicinity of the transcriptional start site. (a) Gel retardation analysis. The four DNA probes shown varied in their specific radioactivities, but equal quantities of DNA probe (2 fmol) and TFIIIC (18 fmol of DNA-binding activity) were mixed for each of the samples shown in lanes 2 to 7. The arrow in lane 5 (and in Fig. 4 and 6) indicates a probe constructed with the *box B* promoter-down mutant *SUP4* gene, whose two mutations at bp 84 and 90 result in a 3,000-fold-lower affinity for TFIIIC (1). Lane 1 is a no-protein control. Samples for lanes 1 to 5 also contained 450 fmol of pGEM1. The samples for lanes 6 and 7 contained 450 fmol of pLNG56 and pTZ1, respectively; these plasmids have one copy of the promoter-down and promoter-up *SUP4* genes, respectively, inserted in pGEM1. For lanes 4 and 5, the volumes of sample loaded on the gel were adjusted so that equal amounts of radioactivity were applied. (b) Photocrosslinking. Aliquots of samples for lanes 2 to 7 were photocrosslinked, processed, and analyzed by SDS-PAGE as described in Materials and Methods. The positions of the 145- and 135-kDa subunits of TFIIIC are shown at the left. Efficiencies of photocrosslinking can be examined by comparing corresponding samples in panels a and b (e.g., lanes 8 and 2).

competition assays similar to those in lanes 12 and 13 of Fig. 3b (see below) confirmed that the very weak signal with the −47/−48 probe was due to specific binding. Both the 135- and the 95-kDa subunits were photoaffinity labelled by DNA probe +6/+11, the former 3.5-fold more efficiently than the latter (data not shown).

In the next series of experiments, we examined TFIII (B+C) complexes formed on these probes. TFIII(B+C) complexes have DNase I footprints that extend to approximately 40 bp upstream of the transcriptional start site (9). TFIIIC can be stripped from these complexes by treatment with high concentrations of salt or heparin, leaving a TFIIIB-

TABLE 1. Efficiency of photoaffinity labelling

1 Location(s) of N_3RdUMP[a]	2 Relative efficiency of photoaffinity labelling of the 135-kDa subunit with TFIIIC alone	3 Relative efficiency of photoaffinity labelling of 70- and 90-kDa components[b]	4 Enhancement (fold) of photoaffinity labelling efficiency of the 135-kDa subunit of TFIIIC due to addition of TFIIIB[c]
−47, −48 (T)	0.02	0.1	10
−38, −37 (N)	0.06	0.2	4
−33, −32 (N)	ND[d]	1.5	3
−38, −37, −33, −32 (N)	0.06	1.2	9
−34, −35 (T)	0.02	0.2	ND
−30 (N)	0.05	0.5	5
−30, −28, −26 (N)	ND	5	3
−27, −29 (T)	ND	2	4
−26, −24, −22, −21 (N)	0.4	>10	1–2
−16 (T)	0.1	>20	1–2
−17, −14, −12 (N)	0.4	7	1–2
−7, −6, −5, −3, −2 (N)	1[e]	4	1[f]
−3, −2 (N)	1	2	1
47, 48, 51, 52, 53 (N)	0.4		

[a] (T), transcribed strand; (N), nontranscribed strand.
[b] Radioactivity in the 70-kDa band divided by radioactivity in the 90-kDa band; measurements made on TFIII(B+C)-DNA complexes.
[c] Determined for all complexes designated (C+B) together (see Fig. 4a, lane 1).
[d] ND, not done.
[e] Other values relative to this.
[f] No enhancement.

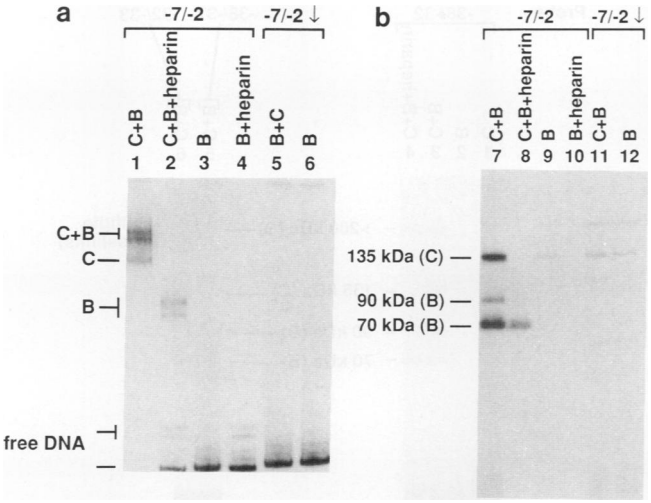

FIG. 4. Photoaffinity labelling of TFIIIB from the vicinity of the transcriptional start site. (a) Gel retardation analysis of complexes formed with TFIIIC and TFIIIB. The protein components mixed with the DNA probe are indicated above the lanes. In samples for lanes 2 and 4, protein-DNA complexes were first formed and heparin then was added to 100 μg/ml in order to strip off TFIIIC. The positions of TFIIIC-DNA, TFIIIB-DNA, and TFIII(C+B)-DNA complexes as well as of free DNA are shown. The DNA probe is identified at the top. (b) Photocrosslinking. Aliquots of samples for lanes 1 to 6 were photocrosslinked, processed, and analyzed as described in Materials and Methods and are shown in lanes 7 to 12, respectively. The photoaffinity-labelled proteins are identified at the left.

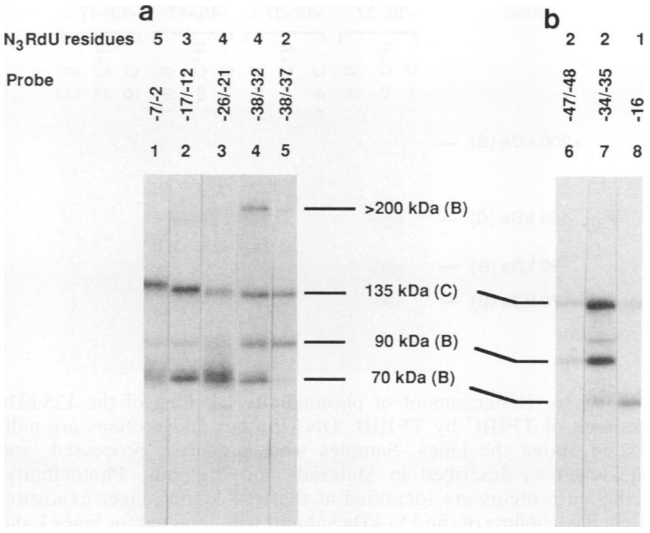

FIG. 5. Distinctive patterns of photocrosslinking of two components associated with TFIIIB. Five probes of the nontranscribed DNA strand (a) and three probes of the transcribed strand (b) were incubated with TFIIIC and TFIIIB and then photocrosslinked, processed, and analyzed as described in Materials and Methods. Each panel is a montaged rearrangement of samples that were run on the same gel but not in adjacent lanes. The location and number of N_3RdUMP residues in each probe are indicated above each lane. Radioactively labelled proteins are identified in the center. The high-molecular-mass (>200-kDa) material was formed as the result of multiple photochemical events probably linking two or more polypeptides associated with TFIIIB to one small segment of DNA.

DNA complex that is competent to correctly position RNA polymerase III and to direct the precise initiation of repeated rounds of transcription of the *SUP4* gene (8). In contrast to the TFIIIC that was used for these experiments, our best preparations of TFIIIB were still quite impure after numerous steps of chromatographic separation (9). Photocrosslinking analysis of TFIII(C+B)-*SUP4* gene complexes (probe −7/−2) that were made with these materials is shown in Fig. 4. TFIII(C+B) complexes were formed only with the promoter-up probe (compare lanes 1 and 5) and only in the presence of TFIIIC (compare lanes 1, 3, and 6). The TFIII (C+B) complexes were heterogeneous as judged by their electrophoretic mobilities. Subsequent experiments that are reported elsewhere show that this heterogeneity resides at least partly in TFIIIB (7). The DNA binding of the TFIIIB component of these complexes resisted challenge with heparin, albeit incompletely (lane 2). Only three polypeptides of these TFIII(C+B) complexes were specifically crosslinked to DNA: the 135-kDa subunit of TFIIIC and two new polypeptides with apparent molecular masses of 90 and 70 kDa. The specificity of TFIIIB crosslinking, against a minor background of nonspecific crosslinking, was demonstrated by its promoter dependence (compare lanes 7 and 11), heparin resistance (lane 8), and TFIIIC dependence (lanes 9 and 12).

In the TFIII(C+B) complex, the 70-kDa component was approximately four times more efficiently labelled by the −7/−2 probe than was the 90-kDa component (Fig. 4, lane 7; Table 1, column 3). Addition of TFIIIB did not substantially affect the efficiency of photocrosslinking of the −7/−2 probe to the 135-kDa subunit of TFIIIC (Table 1, column 4). Treatment with heparin eliminated the photocrosslinking of this protein, consistent with the stripping of TFIIIC that is known to occur under these conditions. Treatment with heparin also almost, but not completely, eliminated photocrosslinking of the 90-kDa component, suggesting either that heparin stripped this component away or that its local conformation in the vicinity of bp −7 to −2 was so changed by removal of TFIIIC that it was no longer within effective reach of the five N_3RdUMP residues in the −7/−2 probe. Further information that is presented below eliminates the first alternative.

TFIII(C+B) complexes with the other probes were also analyzed in the same way (Table 1). The photocrosslinking properties of complexes on seven of these probes are surveyed in Fig. 5. The principal point of this survey is to show that only the 90- and 70-kDa TFIIIB components were specifically photocrosslinked by these probes and that their relative efficiencies of photocrosslinking varied widely and systematically according to the locations of N_3RdUMP residues (Table 1, column 3). Specificity of labelling was assessed by TFIIIC-dependent binding and heparin resistance, by DNA competition assays (data not shown), or in some instances with promoter-down photoprobes (e.g., Fig. 6). Photoaffinity labelling of both the 70- and 90-kDa proteins was resistant to heparin treatment for most of the probes shown in Fig. 5, the exception being probe −7/−2 (Fig. 4b, lanes 7 and 8). On the other side of the transcriptional start, TFIII(C+B) complexes assembled on probe +28/+29 crosslinked only to the 95-kDa subunit of TFIIIC, which was clearly separated from the labelled 90-kDa protein on an 8% polyacrylamide gel, and did not photoaffinity label the 90- or 70-kDa protein (data not shown). The −38/−32 probe generated one or more high-molecular-weight products upon photocrosslinking. By analyzing the accumulation of this

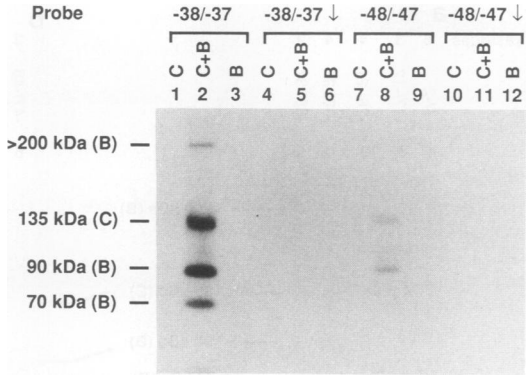

FIG. 6. Enhancement of photoaffinity labelling of the 135-kDa subunit of TFIIIC by TFIIIB. DNA probes and proteins are indicated above the lanes. Samples were prepared, processed, and analyzed as described in Materials and Methods. Photoaffinity-labelled proteins are identified at the left. Upon longer exposure, specific labelling of the 135-kDa subunit was detected for lanes 1 and 7 but not for lanes 4 and 10. The minor labelling of protein considerably smaller than 70 kDa that can be seen in lane 2 was not consistently observed.

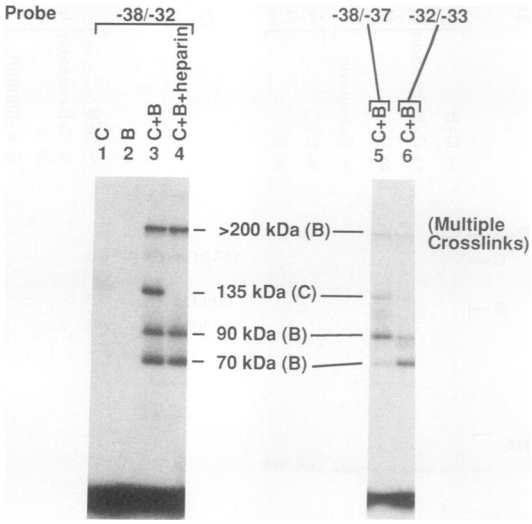

FIG. 7. Indications that the 70- and 90-kDa proteins associated with TFIIIB lie on opposite sides of the DNA helix. Probe −38/−32 (four N_3RdUMP residues) and probes −38/−37 and −33/−32 (two N_3RdUMP residues each) were mixed with TFIIIC, TFIIIB, or both and treated with heparin (final concentration, 100 μg/ml) to selectively strip TFIIIC from DNA, as indicated above each lane. Complexes were UV irradiated, processed, and analyzed by SDS-PAGE. The moderately light, diffuse band in lane 5 with relative molecular mass ~120 kDa was separately shown to be due to less than adequate digestion of the photoaffinity-labelled 70- and 90-kDa subunits with nucleases.

high-molecular-weight photoproduct as a function of the radiation dose, it was possible to show that it was generated by multiphoton events. We have previously proposed that high-molecular-weight products of this kind consist of multiple polypeptide chains connected by two or more cross-links (2). The weakly labelled ~110-kDa polypeptide in lane 7 of Fig. 5 was due to nonspecific photocrosslinking of a component in the TFIIIB preparation that did not require TFIIIC for binding and was readily stripped from DNA with heparin.

We also noted that the cross-linking efficiency of several probes for the 135-kDa subunit of TFIIIC increased upon formation of TFIII(C+B) complexes. The competition of other proteins for reaction with the nitrene that is generated upon irradiation might have further diminished the already low photocrosslinking by probes with N_3RdUMP residues upstream of bp −25. The opposite proved to be the case (Table 1, column 4). Particularly striking enhancements of photoaffinity labelling of the 135-kDa protein were noted with the −38/−37, −47/−48, and −38/−32 probes (compare lanes 1 and 2 and lanes 7 and 8 in Fig. 6; compare lanes 1 and 3 in Fig. 7). DNA photoaffinity probes −3/−2, −7/−2, −17/−12, −16, and −26/−21 only negligibly enhanced their efficiency of labelling the 135-kDa protein upon the addition of TFIIIB. Thus, the TFIIIB-dependent enhancement of photoaffinity labelling of the TFIIIC 135-kDa subunit was specific to the 5′ end of the upstream binding region and required a functional *box B+* (Fig. 6; compare lanes 1, 2, 4, 5, 7, 8, 10, and 11).

The preceding experiments convincingly associate 70- and 90-kDa polypeptides with TFIIIB, based on the observations that (i) the association of these two components with DNA is detected only upon the addition of a TFIIIB fraction that has been purified through five chromatographic steps to ensure complete removal of TFIIIC and RNA polymerase III; (ii) these two components cannot associate with DNA in the absence of highly purified TFIIIC; (iii) they share the unusual and TFIIIB-specific property of remaining bound to DNA in the presence of heparin; (iv) they are photoaffinity labelled to regions of the *SUP4* tRNA gene that were previously shown to be protected from DNase I cleavage by TFIIIB (8, 9); (v) photoaffinity labelling of these components requires a competent *box B+* promoter element approximately 100 bp downstream of the photoreactive nucleotide; and (vi) photoaffinity labelling of these components is eliminated by competition from promoter-up mutant *SUP4* DNA but not by promoter-down mutant *SUP4* DNA. We now provide two lines of evidence indicating that the 70- and 90-kDa polypeptides are independent components of TFIIIB and arguing against the alternative possibility that one of these proteins is a modification product of the other. The first experiments examined the spatial distribution of the 90- and 70-kDa proteins around the segment of the *SUP4* gene lying between bp −38 and −32 (Fig. 7). TFIII(C+B)-DNA complexes of the −38/−32 probe photoaffinity labelled 90- and 70-kDa components almost equally upon irradiation. Stripping these complexes with heparin to remove TFIIIC before photo-cross-linking eliminated labelling of the 135-kDa subunit of TFIIIC but left the 70- and 90-kDa components bound to the *SUP4* gene and therefore capable of being photocrosslinked (Fig. 7, lanes 3 and 4). The multiply crosslinked high-molecular-weight component was also formed when the heparin-stripped complex was irradiated, indicating that it was composed of components of TFIIIB (lane 4). Two probes were synthesized to contain only the two upstream or two downstream N_3RdUMP residues of this DNA segment and were examined in the same way. Since they are 5 bp apart, these two pairs of photoactive nucleotides lie on opposite sides of the B-DNA helix. The upstream −38/−37 probe preferentially photocrosslinked the 90-kDa component, while the downstream −33/−32 probe more efficiently photo-cross-linked the 70-kDa component. A probe containing N_3RdUMP at −35 and −34 on the complementary strand also preferentially labelled the 90-kDa component (Fig. 5, lane 7). Thus, the 90- and 70-kDa components

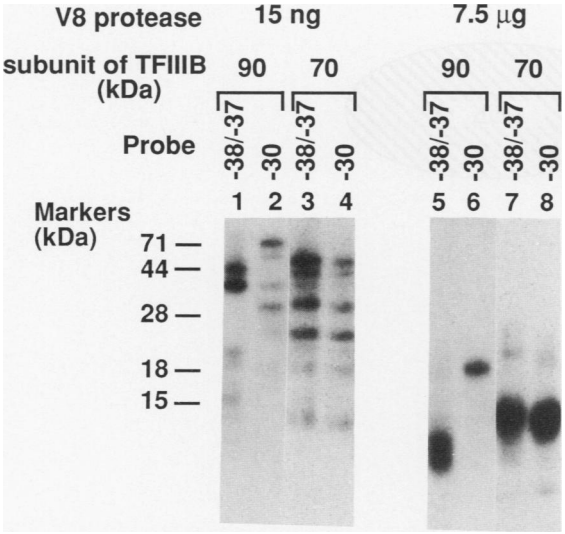

FIG. 8. Peptide mapping of photoaffinity-labelled 70- and 90-kDa proteins. TFIII(C+B)-DNA complexes on probes −38/−37 and −30 were formed, photocrosslinked, processed, and separated by SDS-PAGE. The locations of labelled proteins were determined by autoradiography of the wet gel, and gel segments containing the 90- and 70-kDa proteins were cut out for peptide mapping. Gel slices were placed in the wells of an SDS–15% polyacrylamide gel and treated with various amounts of V8 protease as outlined in Materials and Methods. The resulting proteolytic fragments were separated on a second polyacrylamide gel and detected by autoradiography as shown. Lanes 3, 4, 7, and 8 are from a longer exposure of the same gel as lanes 1, 2, 5, and 6. The probe, the labelled TFIIIB subunit, and the amount of V8 protease used for digestion are indicated above each lane. The approximate apparent relative molecular mass scale at the left was determined with prestained molecular weight markers.

occupy different parts of the space around the DNA helix, suggesting that they are independent and separate subunits of TFIIIB. The different efficiencies of photocrosslinking to the 70- and 90-kDa proteins from the other probes listed in Table 1 (column 3) also argue against the 70-kDa component being a proteolysis product of the 90-kDa component. That a TFIIIB-DNA complex contains 90-kDa as well as 70-kDa protein (rather than one or the other) was also suggested by the fact that the formation of the >200-kDa photocrosslinked product was greatly reduced with probes −38/−37 and −33/−32 relative to probe −38/−32, implying that this high-molecular-weight, multiply crosslinked product contains 90- and 70-kDa polypeptides.

Independent evidence for the separate nature of the 70- and 90-kDa components was provided by examining their patterns of digestion by V8 protease. For this experiment, TFIII(C+B) complexes were formed with the −38/−37 and −30 probes and irradiated. After nuclease digestion and denaturation, the gel-purified 90- and 70-kDa components were partly or completely digested with V8 protease, separated on an SDS-gel, and autoradiographed so that proteolytic fragments containing a photocrosslink could be examined (Fig. 8). The digestion patterns of the 70- and 90-kDa components were clearly different, regardless of whether they had been tagged with probe −30 (lanes 2 and 4) or with probe −38/−37 (lanes 1 and 3). The limit digestion products of the 90-kDa component were both larger and smaller than the single limit digestion product of the 70-kDa component (compare lanes 5 and 6 with lanes 7 or 8). This finding argues against a simple chemical modification of the 70-kDa component giving rise to a product that migrates as 90 kDa on SDS-gels. The implication of finding different limit digestion products of the 90-kDa component with different probes is that if more were known about the structure of this component, it should be possible to map its alignment with DNA at least roughly by photocrosslinking. (The result of the analysis in Fig. 8 does not exclude the possibility that TFIIIB-DNA complexes contain two separate 90-kDa components.)

DISCUSSION

The 135-kDa subunit of TFIIIC. Although three subunits of TFIIIC (135, 95, and 55 kDa) lie sufficiently close to *box A* to be photocrosslinkable from its immediate vicinity, only the 135-kDa subunit extends past the transcriptional start site and far into the upstream space that is also occupied by TFIIIB. Thus, this subunit is the clear-cut candidate for direct interaction with TFIIIB and for a role in positioning TFIIIB on the tRNA gene.

The 135-kDa subunit is within reach of a very long stretch of DNA: it is photo-cross-linked with comparable efficiency from the small intron between *boxes A* and *B* and from 20 bp upstream of the transcriptional start, and it is weakly cross-linked from 20 bp further upstream (2) (Fig. 9 and Table 1). That finding indicates at least one, and perhaps both, of the following two possibilities: (i) the 135-kDa subunit is anisometric, and (ii) DNA is bent around the 135-kDa subunit (15). The DNase I footprint of purified TFIIIC does not extend to or upstream of the transcriptional start site on either DNA strand of four different tRNA genes (9). Thus, the upstream-projecting part of the 135-kDa subunit is probably not stably and firmly bound to DNA. We suspect that the particularly low efficiency at which TFIIIC alone is labelled from bp −30 to −48 (Table 1; Fig. 6 and 7) reflects the ability of protein and DNA to undergo independent local motions. The four- to ninefold increase in the efficiency of labelling the 135-kDa subunit from these locations when TFIIIB enters into DNA complexes is remarkable, because it occurs despite the presence of additional protein that is positioned to compete for reaction with the photolytically generated nitrene. Evidently the addition of TFIIIB to the TFIIIC-DNA complex fixes the 135-kDa subunit in close proximity to upstream DNA. In part, this might be due to the DNA bending that is induced by TFIIIB (15). We suspect that it also reflects an interaction of some part of TFIIIB with this subunit of TFIIIC.

Two components of TFIIIB. Two polypeptide chains, with apparent molecular weights of 90,000 and 70,000, that become photocrosslinkable when TFIIIB binds to the *SUP4* tRNA gene have properties that are expected for TFIIIB: (i) their positioning in close proximity to upstream DNA is TFIIIC and *box B* dependent; (ii) the upstream segment of DNA that they closely approach is also closely protected from DNase I in TFIII(B+C) complexes but not in TFIIIC complexes; (iii) once the M_r-90,000 and -70,000 polypeptides have been positioned by TFIIIC in the vicinity of upstream DNA, they remain bound even when TFIIIC is subsequently stripped off; and (iv) although the preparation of TFIIIB that is used for these experiments is quite impure, even after multiple steps of chromatography, only these two polypeptide chains specifically associate with the *SUP4* gene.

We have provided two lines of evidence that the 90- and 70-kDa polypeptides are distinct and that they are not modification or cleavage products of each other. (i) They

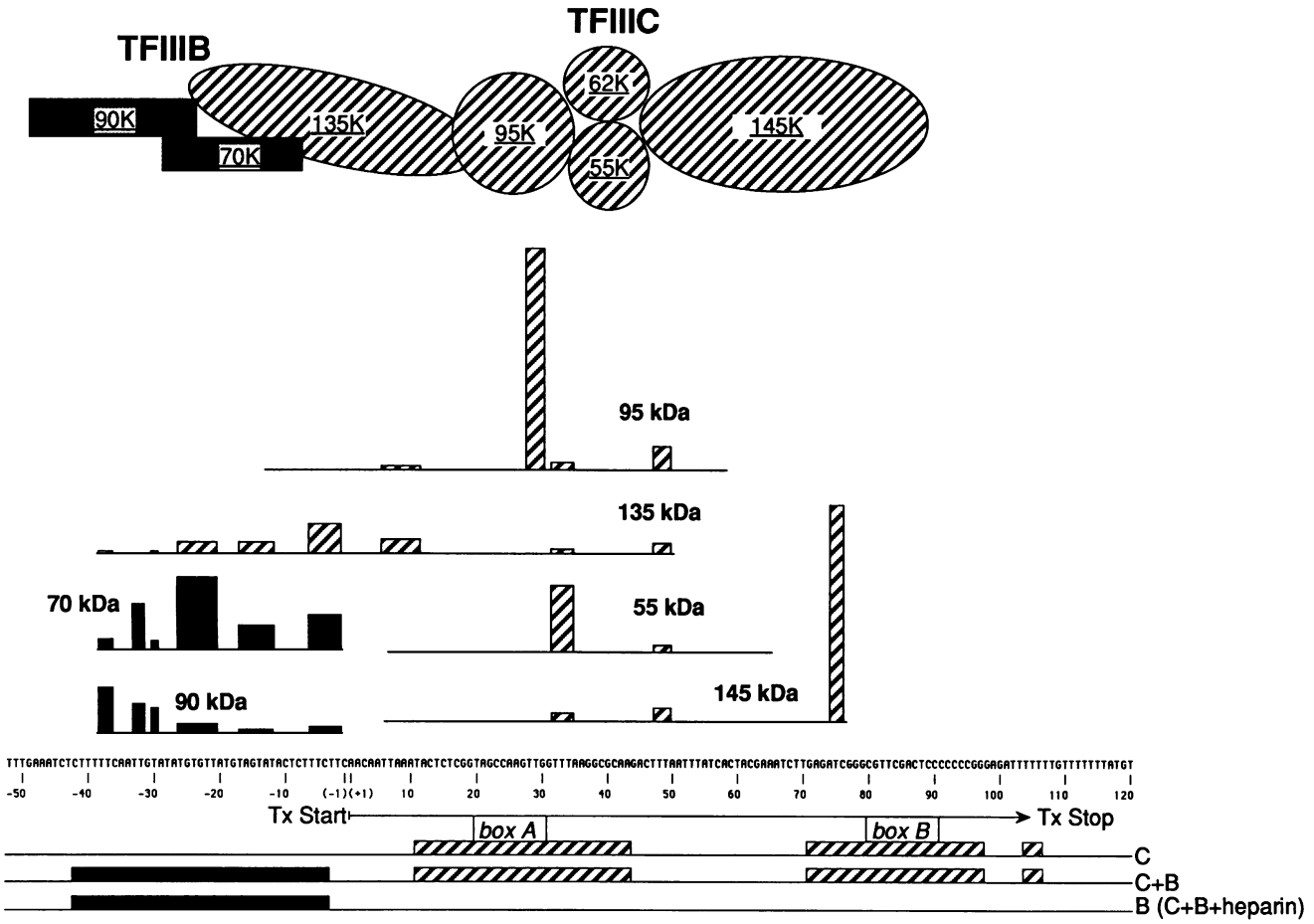

FIG. 9. Summary of information on the internal structure of transcription complexes on the *SUP4* tRNA^{Tyr} gene. Oval shapes designate subunits of TFIIIC, and boxes designate components of TFIIIB. The extent of the unprocessed transcript, the *box A* and *box B* promoter elements, and the extent of DNase I protection of the nontranscribed strand in TFIIIC-DNA, TFIII(C+B)-DNA, and specifically positioned TFIIIB-DNA complexes are shown below the sequence of the promoter-up gene. TFIIIB- and TFIIIC-associated components and footprints are shown as black and striped areas, respectively. Locations on DNA that are near different subunits of TFIIIC and components of TFIIIB (as judged by photocrosslinking) are indicated above the sequence. Relative efficiencies of crosslinking are schematically indicated by the height of bars on each line. The corresponding probes cover DNA segments that vary in extent (1 to 6 bp), as indicated by the width of each bar. Primary data are from references 1, 8, and 9 and this work; other information on components of TFIIIC is from references 7 and 17.

occupy different parts of the space around the *SUP4* gene. For example, at bp −38/−37 (nontranscribed strand) and −34/−35 (transcribed strand), the 90-kDa component is preferentially crosslinked from one side of the DNA helix, whereas probe −33/−32 (nontranscribed strand) on the opposite side labels the 70-kDa subunit predominantly (Fig. 7). Some small amount of photoaffinity labelling of the 70-kDa component from bp −38 and/or −37 is not unexpected because the reactive nitrene that is formed upon photolysis of N_3RdUMP is connected to its pyrimidine ring by a 0.9- to 1.0-nm tether. The same consideration applies to the low level of photocrosslinking of the 90-kDa component from bp −33 and/or −32. The 70-kDa component is also preferentially crosslinked from around bp −16 to −26, while the 90-kDa component is preferentially crosslinked from further upstream, poorly crosslinked from around bp −16 to −26, and again more effectively crosslinked from positions closer to the transcriptional start (Table 1 and Fig. 5). (ii) The V8 protease cleavage patterns of the 90- and 70-kDa components can be analyzed because of the high sensitivity of detection conferred by photocrosslinked ^{32}P-labelled nucleotide. The digestion pattern of the 90-kDa polypeptide is probe dependent, consistent with different parts of the polypeptide being associated with different segments of the DNA. The digestion patterns are also different for the 70- and 90-kDa proteins.

In closing, we briefly examine the accumulated information on the internal structure of transcription factor complexes on the *SUP4* tRNA^{Tyr} gene (Fig. 9), including the results of DNase I footprinting (8) and photocrosslinking of TFIIIC (2). Previous analyses and the work presented here show that six different proteins associated with TFIIIC and TFIIIB lie in close proximity to 125 bp of the *SUP4* tRNA^{Tyr} gene. (The vicinity of the transcriptional termination site has not yet been examined.) The upstream DNase I footprint that was previously associated with TFIIIB is now more concretely assigned to an interaction of the 90- and 70-kDa components with DNA. Because our photocrosslinking method probes the close vicinity of the DNA helix rather than just one or another groove, these experiments do not specify where the direct protein-DNA contacts of TFIIIB occur, or whether both components are so involved. How-

ever, it seems likely that both of these large proteins contribute to the DNase I footprint, which extends over ~40 bp, by being able to block access to either side of the DNA helix. With regard to the mechanism of transcriptional initiation (8), we propose that the 70- and 90-kDa components are separate and essential components of *S. cerevisiae* TFIIIB and that both components together position RNA polymerase III over the transcriptional start site. Yeast TFIIIB was previously purified as a protein containing a single 60-kDa polypeptide chain (11, 12). As has already been discussed (8, 9), the activity of the purified material was, however, anomalously low. We suspect either that the previously identified protein is (substantially) identical with our 70-kDa component or that it is a degradation product of the 90-kDa protein.

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