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Multiple states of protein–DNA interaction in the assembly of transcription complexes on *Saccharomyces cerevisiae* 5S ribosomal RNA genes

(RNA polymerase III/transcription factors/protein–DNA complexes)

BURKHARD R. BRAUN, DANIEL L. RIGGS*, GEORGE A. KASSAVETIS, AND E. PETER GEIDUSCHEK

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

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ABSTRACT **Multiple stages of protein–DNA interaction in the assembly of RNA polymerase III transcription complexes on a *Saccharomyces cerevisiae* 5S rRNA gene have been distinguished by DNase I "footprinting" and gel retardation. Transcription factor IIIA interacts with approximately 35 base pairs of the internal promoter region. Transcription factors IIIC and IIIB incrementally extend the interaction along the 5S gene, if, and only if, transcription factor IIIA is also bound. Complexes assembled from the complete set of purified transcription factors or from a complete transcription system extend over the entire transcription unit together with almost 50 base pairs of 5′ flanking sequence.**

Transcription of tRNA and 5S rRNA genes by RNA polymerase III (pol III) requires the action of distinct transcription initiation factors (TFs): TFIIIA, which is specific for 5S rRNA genes, TFIIIB, and TFIIIC (1, 2). The agglomeration of multiple proteins, some of which bind to specific sites in DNA, is a common theme in eukaryotic gene regulation and is relatively well-defined in pol III transcription. Thus, the nature of the stable complexes formed on these genes by the TFs is interesting from structural and general gene-regulatory perspectives. In addition, the intragenic promoters of tRNA and 5S rRNA genes present the unique problem of how transcription proceeds through a stably bound protein–DNA complex without dispersing it (3–7).

Xenopus TFIIIA, the prototype Zn-finger DNA-binding protein (8–11), binds to approximately five helical turns of the 5S gene internal control region (ICR) (Fig. 1), most strongly within the element called *box C* [*ca.* base pairs (bp) 80–97; refs. 15–17]. Two other elements of the ICR determine promoter strength: the 5′-proximal *box A* (bp 49–60) with adjacent base pairs and the intermediate element (bp 67–72). The sequential assembly of transcription complexes on 5S genes involves the subsequent binding of TFIIIC; TFIIIB binding requires the prior assembly of TFIIIC and TFIIIA on the gene (3, 18, 19). [It is now known that HeLa TFIIIC is a mixture of two proteins (20), TFIIIC1 and C2. The order of their assembly into 5S gene transcription complexes has not yet been reported.] It is curious that this sequential assembly of proteins on vertebrate 5S genes generates little or no signature when probed by DNase I footprinting. (*i*) The footprints of *Xenopus* TFIIIA and of HeLa TFIIIC/*Xenopus* TFIIIA on a *Xenopus* somatic 5S gene are indistinguishable (18). (*ii*) The footprints, on *Xenopus* somatic and oocyte 5S genes, of *Xenopus* TFIIIA and of a complete crude *Xenopus* nuclear extract capable of converting a high fraction of added 5S genes to active transcription are nearly identical: only the ICR is protected from digestion by DNase I and only some enhancements distinguish the two kinds of footprints (7). We have found an entirely different situation for a *Saccharomyces cerevisiae* 5S gene. Under conditions that are consistent with active and specific transcription, we observed an interaction with protein that extended over the entire 5S gene and a large segment of contiguous 5′ DNA sequence. In contrast, a fraction containing the *S. cerevisiae* TFIIIA-equivalent activity gave a DNase I footprint that was confined to only a part of the presumptive ICR (Fig. 1). The very different properties of the yeast transcription system make it especially suitable for probing the assembly and internal structure of these multifactor pol III transcription complexes. Here we distinguish multiple stages of protein–DNA interaction in the assembly of transcription complexes on a *S. cerevisiae* 5S gene.

MATERIALS AND METHODS

Transcription Factors. S-100 cell-free extract (17.9 g of protein) from 480 g of *S. cerevisiae* strain BJ926 (protease-deficient: pep4, prb1, prc1; from E. Jones, Carnegie Mellon University) was prepared according to ref. 21, with minor variations, and then fractionated on Bio-Rex 70 (14) with steps of elution at 250, 500, and 650 or 700 mM KCl in buffer D [20 mM Hepes, pH 7.7/10% (vol/vol) glycerol/5 mM $MgCl_2$/1 mM dithiothreitol/1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride]. The fraction eluting with 500 mM KCl (BRα; 930 mg of protein) contained pol III, TFIIIB, TFIIIC, and minor amounts of TFIIIA. The fraction eluting with 650 mM KCl (BRβ; 97 mg of protein) contained TFIIIA. Part of BRβ (37 mg of protein) was concentrated and subjected to gel filtration chromatography in buffer A/800 mM NaCl (buffer A = 20 mM Hepes, pH 7.7/10% glycerol/7 mM $MgCl_2$/5 mM 2-mercaptoethanol/1 mM EDTA/0.5 mM $ZnSO_4$/0.5 mM phenylmethylsulfonyl fluoride/0.05% Nonidet P-40) on Sephacryl S-200 (66 × 1.5 cm diameter column at 8 ml/hr), where its apparent molecular weight was 60,000. The peak TFIIIA-containing fractions (5.3 mg of protein) were dialyzed into buffer A/80 mM NaCl (final volume, 9.5 ml) and incubated with 2.2 ml of 5S rRNA-Sepharose in buffer A/110 mM NaCl for 1.5 hr at room temperature. [The RNA was isolated by phenol extraction of whole yeast cells and gel-purified (22). Fifty micrograms of RNA was coupled per ml of CNBr-activated Sepharose CL-4B (23) and stored at pH 5.0 to minimize RNA hydrolysis.] The slurry was poured into a syringe at 2°C and then washed successively with buffer A/110 mM and with buffer A/250 mM NaCl. TFIIIA was eluted with buffer A/500 mM NaCl (*ca.* 15 μg of protein in 0.5 ml). The purification of TFIIIB and -C is described elsewhere (24). TFIIIC was affinity-purified on *box*

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Abbreviations: pol, RNA polymerase; TFIII, transcription factor for RNA polymerase III; ICR, internal control (promoter) region.

*Present address: Department of Biological Chemistry, University of California at Irvine, Irvine, CA 92717.

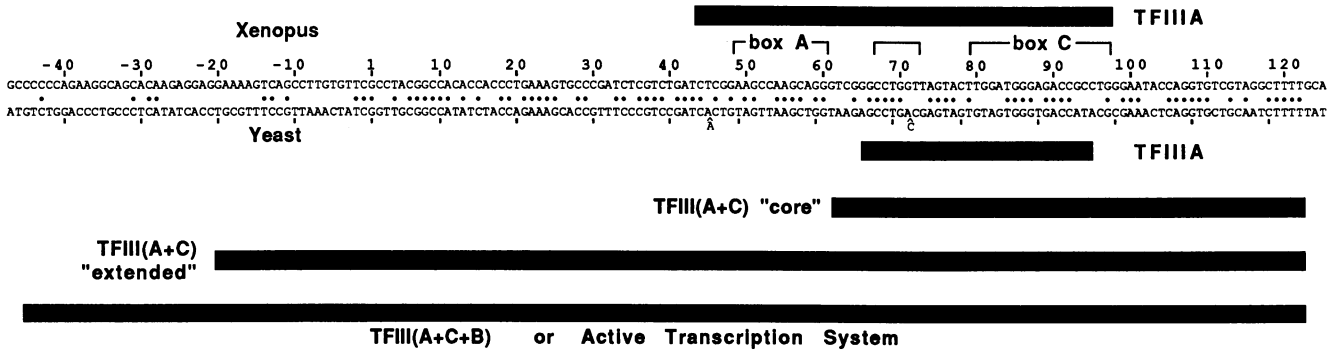

FIG. 1. Comparison of *Xenopus* and *S. cerevisiae* 5S genes. Sequences of the nontranscribed strands are presented. The *Xenopus borealis* somatic gene sequence is from ref. 12. The *S. cerevisiae* repeat 5S gene sequence is from ref. 13. Transcription starts at bp +1. Identities of sequence are noted by dots; the *S. cerevisiae* sequence has been folded out at bp 46 and bp 73 to improve the fit (14) to the *box A* (bp 49–60), *box C* (bp 80–97), and intermediate segments (bp 67–72) of the *X. borealis* 5S gene ICR (15), which are shown. A second sequence in the *S. cerevisiae* gene that is consensual with *box A* (bp 76–87) has been noted (14). The extents of DNase I footprints of these genes by their respective TFIIIA and of the yeast gene (transcribed DNA strand) by combinations with other *S. cerevisiae* proteins or fractions are schematically indicated by solid rectangles. The data for *Xenopus* TFIIIA are from refs. 8, 16, and 17. The data for *S. cerevisiae* are presented below. "Core" and "extended" TFIII(A+C) footprints are discussed in the text.

B DNA-Sepharose in Mg^{2+}-containing buffer or on *box B+* DNA-Sepharose in buffer without Mg^{2+}, as described (24). (*Box B+* is the *box B* segment of tRNA gene ICR plus 7 bp of 5′ flanking sequence in which point mutations affect promoter strength.) Each purified TF was free of the activities of the other factors.

Plasmids. The following plasmids containing *S. cerevisiae* 5S rRNA and tRNA genes were used: pB1, pBB111F, and pBB111R (3411, 3125, and 3125 bp, respectively) contain the 5S rRNA repeat gene in pUC12, pGEM-1, and pGEM-2, respectively. To make pBB111F and pBB111R, the 297-bp *Taq* I–*Ssp* I fragment containing the 5S rRNA repeat gene from pY1rG12 (13), containing an 8-bp *Eco*RI linker on the *Ssp* I end and a 7-bp *Cla* I–*Hin*dIII fragment from pBR322 on the *Taq* I end, was inserted between the *Eco*RI and *Hin*dIII sites of pGEM-1 and -2, respectively. To make pB1, a 731-bp *Msp* I fragment containing the 5S rRNA repeat was blunt-ended and ligated into the *Hin*cII site of pUC12 (25). pGE33 and PTZ1 (4632 and 3135 bp, respectively) contain the SUP4 $tRNA^{Tyr}$ gene with a G-62 → C promoter-up mutation (26) in pBR322 and pGEM-1, respectively. pAG56 and pLNG56 contain the SUP4 $tRNA^{Tyr}$ gene with a C-56 → G promoter-down mutation (26) in pBR322 and pGEM-1, respectively. pPC1 contains a $tRNA^{Leu_3}$ gene in pUC12 (3029 bp). pJD 5′ half and 3′ half (ref. 27; from J. D. Johnson, University of Wyoming) contain the 5′ and 3′ halves of the same $tRNA^{Leu_3}$ gene, severed at the *Hpa* I site in the intervening sequence, between *box A* and *B*, and with different extensions of flanking sequence, in pBR322 (*ca.* 4790 and 6340 bp, respectively). Details of construction are given in ref. 24.

Single-Round Transcription: Counting Active Molecules of TFs. The assay procedure has been described (24). Transcription proteins or fractions were incubated with 40 fmol of pBB111R for 40 min at 20°C in 20 μl of 40 mM Tris·HCl/7 mM $MgCl_2$/5 mM 2-mercaptoethanol/1 mM Na_2EDTA/0.5 mM $ZnCl_2$/8% glycerol/70 mM NaCl/150 mM potassium glutamate. Transcription complexes precisely paused at nucleotide 10 (separately confirmed) were generated in the presence of GTP, CTP, and UTP [FPLC-purified (Pharmacia); [α-^{32}P]UTP at 20–40,000 cpm/pmol (NEN DuPont)] at 400, 100, and 25 μM, respectively. Heparin and ATP were then added to 300 μg/ml and 100 μM, respectively, allowing transcripts to be extended and terminated, but prohibiting reinitiation. The number of transcripts synthesized in the single cycle of transcription, quantified as described (24), was taken to equal the number of active transcription complexes formed during preincubation. The number of molecules of active transcription complexes formed by affinity-purified TFIIIA was assayed in combination with fraction BRα, containing TFIIIB, TFIIIC, and pol III, which had been rechromatographed on Bio-Rex 70 to remove trace TFIIIA. Affinity-purified TFIIIC and TFIIIB were assayed with a tRNA gene template, as described (24).

DNase I Footprinting. Footprinting was done and quantified as described (24).

Electrophoretic Analysis of DNA–Protein Complexes (26). Binding was done for 1 hr at room temperature (*ca.* 22°C) in 20 μl of 40 mM Tris·HCl, pH 8.0/5% glycerol/1 mM EDTA/0.5 mM $ZnSO_4$/7 mM $MgCl_2$/0.5% polyvinyl alcohol/3 mM dithiothreitol/100 mM NaCl/bovine serum albumin (300 μg/ml) containing pGEM-1 DNA (3 μg/ml) linearized at the *Eco*RI site, 1.2 fmol of TFIIIA, 1.7 fmol of TFIIIC, 30 fmol of the indicated DNA competitor (as supercoiled plasmid), and, as probe, 1 fmol of gel-purified 308-bp *Eco*RI–*Hin*dIII fragment of pBB111F, ^{32}P-labeled at the *Eco*RI end with the Klenow fragment of DNA polymerase. Protein–DNA complexes were resolved on 4% polyacrylamide gels [acrylamide/*N,N′*-methylene bisacrylamide, 100:1 (wt/wt)] in 20 mM Tris·HCl, pH 8.0/2 mM EDTA/5% glycerol (glycerol was omitted from the electrode buffer). The gels were prepared several hours before use, prerun 1 hr with one change of buffer, and run at 150 V, all at room temperature, with recirculation of electrode buffer.

RESULTS

The initial observations motivating these experiments were made with a crude transcription extract, prepared from *S. cerevisiae* according to Klekamp and Weil (21), in which active transcription complexes could be assembled on a high proportion of *S. cerevisiae* 5S genes. Under such conditions, we detected an interaction with protein covering the entire 5S gene and a segment of contiguous 5′ DNA sequence. This result impressed us in the context of what had been previously reported regarding other 5S gene transcrption complexes, whose specific binding signal, the DNase I footprint, is confined to the ICR (7, 18), as mentioned above. In contrast, extended incubation of a crude fraction containing only the 5S gene-specific transcription activity of TFIIIA (the BRβ fraction) with the 5S gene probe gave a DNase I footprint that was confined to a limited segment of the presumptive ICR. [The *S. cerevisiae* 5S gene ICR has been only roughly delimited by a deletion analysis (14). bp 56–97 are required for promoter activity, but not sufficient for full promoter strength, since certain substitutions in the 5′ flanking sequence to this ICR are capable of substantially

changing transcription activity *in vitro*.] The contrast between the two kinds of footprints indicated that it would be possible to directly analyze additional interactions involving the assembly of 5S gene transcription complexes.

To define the participation of individual transcription factors in the formation and structures of such complexes, TFIIIA, TFIIIB, and TFIIIC were further purified. The combination of gel filtration and *S. cerevisiae* 5S RNA affinity chromatography served to purify TFIIIA from the BRβ fraction another *ca.* 2200-fold by protein content and 440-fold by activity. The transcription factor activity of this material was assayed by a single-round transcription method (24), and a footprint titration was also done. Comparison of the results showed that the affinity-purified TFIIIA had quantitatively equivalent transcription and DNA-binding activities (Fig. 2): The affinity-purified TFIIIA (Fig. 3*A*) protected the 5S gene transcribed strand from DNase I digestion between bp 66 and 95 and enhanced cleavage at sites between bp 50 and 65 (lane 2 and arrows). On the nontranscribed strand (Fig. 3*A*, lane 5) protection extended from bp 63 to bp 96 (with an unprotected band near bp 75) and less enhancement was seen. Specificity of binding was shown by competition with 5S rRNA (Fig. 3*B*, lane 5), with 5S rDNA (lane 4), and with a synthetic *box C* oligonucleotide (bp 69–98 with 4-bp 3′ overhangs, lane 6). $tRNA^{Tyr}$ gene DNA (lane 3), a synthetic *box B+* oligonucleotide (lane 7), and the vector (lane 2) did not compete for TFIIIA binding.

TFIIIC was purified from the BRα fraction by chromatography on DEAE-Sephadex A-15, followed by affinity chromatography on oligonucleotide (*box B+*) polymerized and coupled to Sepharose (23), as described (24). Combining TFIIIC with TFIIIA (Figure 3*A*) increased protection from DNase I on both strands in the segment between bp 66 and 95, extended protection of the transcribed strand downstream to bp 124, and enhanced cleavage at bp 69 and near bp 120, with slightly increased protection at bp 61 and 62. On the nontranscribed strand, protection also increased and extended to an enhanced band around bp 114, with another enhanced band near bp 124.

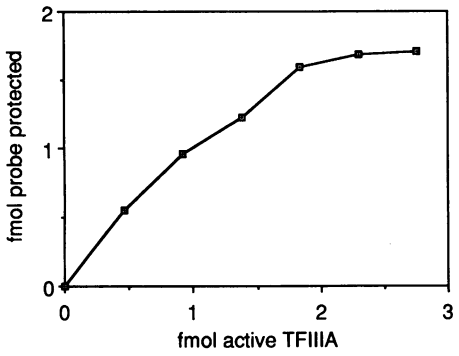

FIG. 2. Quantitative analysis of TFIIIA by single-round transcription and by DNase I footprinting. To determine transcriptional activity, 0, 0.5, 1, or 3 μl of affinity-purified TFIIIA and 4 μl of rechromatographed BRα were preincubated with 50 fmol of pBB111F (5S gene template). A single round of transcription was allowed to take place, and the resulting transcripts were quantified to yield the number of active transcription complexes formed: <0.1 (background), 1.2, 2.3, and 2.3 fmol, respectively. The same amount of rechromatographed BRα, with no TFIIIA added, incubated with 50 fmol of $tRNA^{Tyr}$ gene template, yielded 2.4 fmol of active transcription complexes. Thus, the plateau of 2.3 fmol of active TFIIIA-containing transcription complex reflected a limiting component in fraction BRα; this component was separately determined to be TFIIIB. According to the linear range of its activity, there were, therefore, 2.3 fmol of transcriptionally active TFIIIA per μl of this material. Increasing amounts of this TFIIIA (abscissa) were incubated with 2 fmol of transcribed-strand 5S gene probe (3′-end-labeled with ^{32}P) in the presence of 50 fmol of nonspecific pKD1 and subjected to DNase I digestion. Autoradiograms of these footprints were made without intensifying screens and scanned; the fractional decrease of film density over the protected segment of the ICR is represented as fmol of probe protected (on the assumption that occupancy of the binding site generates complete protection from DNase I).

The TFIIIC-specific part of this footprint could be competed by DNA containing a SUP4 $tRNA^{Tyr}$ gene with G-62 → C mutation that generates a stronger TFIIIC binding site but not by DNA changed at C-56 → G to inactivate that site (26) (Fig. 3*B*, compare lanes 9 and 10). The TFIIIA specificity of the complex was shown by competition for the entire footprint with 5S gene DNA and 5S rRNA (Fig. 3*B*, lanes 11 and 12).

A different, but also TFIIIA- and TFIIIC-specific complex was generated by a preparation of TFIIIC that had been affinity-purified in a slightly different way (on *box B*, rather than *box B+*, DNA-Sepharose, and in buffer containing Mg^{2+}). In addition to the TFIIIC-dependent features above, the footprint generated by this complex on the transcribed strand extended protection to *ca.* bp +37 and partial protection all the way to bp −20 (Fig. 3*C*, lane 3). The extension component of this footprint was distinguishable from the shared "core" TFIII(C+A) component (i.e., Fig. 3*A*) by preferential competition at higher concentrations of nonspecific DNA (data not shown). We think it likely that the TFIIIC purified on *box B* DNA-Sepharose contains additional protein with a relatively high nonspecific affinity for DNA but that interacts preferentially with the TFIII(A+C)–DNA complex. When these two TFIIIC preparations, which differ in their physical interactions with a 5S gene and TFIIIA, were tested on tRNA genes, their footprints were indistinguishable and the quantitative correlations of their transcriptional activities with their specific DNA binding were also similar (ref. 24 and data not shown). At this juncture, what is primarily interesting about the "extended" TFIII(A+C) complex shown in Fig. 3*C* is the demonstration of a specific modification of a highly specific DNA–protein interaction by protein that is apparently relatively nonspecific in its DNA binding.

The formation of TFIIIA and TFIII(A+C) complexes with the 5S rRNA gene could also be followed by nondenaturing gel electrophoresis (Fig. 4). A complex with a relatively modest retardation was formed between the 5S rRNA gene probe and TFIIIA (lanes 2 and 6). The specificity of this complex was confirmed by differential competition with unlabeled 5S rDNA (lane 4) and nonspecific pGEM-1 (vector) DNA (lane 3). The smeared distribution of radioactivity between the retarded and free probe bands suggests the possibility that TFIIIA–5S rDNA complexes may have dissociated in the gel.

The complex with TFIIIC (purified on *box B* DNA-Sepharose in the presence of Mg^{2+}) and TFIIIA was greatly retarded in the gel (lane 7) and sensitive to specific competition by unlabeled 5S rRNA gene plasmid (lane 9). (A complex with comparable mobility was formed with factor TFIIIC purified on *box B+* DNA–Sepharose; data not shown.) Competition by the SUP4 (G-62 → C) tRNA gene regenerated the TFIIIA complex, presumably by selectively competing for TFIIIC (lane 11), but the SUP4 (C-56 → G) tRNA gene was not an effective competitor (lane 10; cf., Fig. 3*B*). A plasmid containing only the 5′ half of a $tRNA^{Leu_3}$ gene (i.e., *box A* but not *box B*) in pBR322 also was an inefficient competitor, being no more effective than pBR322 alone (lanes 13 and 14). In contrast, the complementary 3′ half gene did compete for TFIIIC (lane 15). Thus, although the *S. cerevisiae* 5S gene has a *box A* consensus sequence (bp 49–60), the tRNA gene's *box A* is not determining for its ability to compete for TFIIIC. There was no significant complex formation with TFIIIC alone (lane 5).

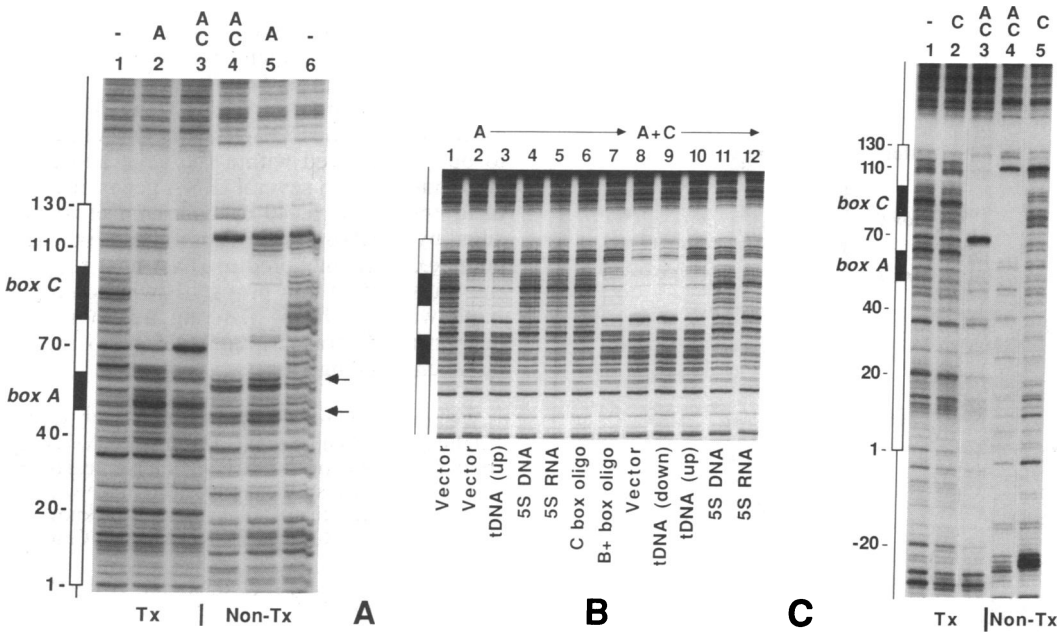

FIG. 3. TFIIIA–TFIIIC interaction on the 5S gene. (*A*) Affinity-purified TFIIIA (4 fmol of activity) was incubated with 2 fmol of probe (3′-end-labeled on the transcribed strand for lanes 1–3; 5′-end-labeled on the nontranscribed strand for lanes 4–6) for 40 min at 20°C prior to DNase I digestion for lanes 2 and 5. TFIIIC (5 fmol of transcription activity) was added to TFIIIA and probe for lanes 3 and 4. Lanes 1 and 6 were no-protein controls. Consensus *boxes A* (bp 49–60) and *C* (bp 80–97) are indicated. Transcription initiates at bp 1. Tx, transcribed. The arrows are referred to in the text. (*B*) DNase I protection in the presence of various competitors. Lanes: 1, no-protein control digestion of 2 fmol of probe; 2–7, samples contained 4 fmol of TFIIIA; 8–12, samples contained 4 fmol of TFIIIA and 8 fmol of TFIIIC. DNA (80 fmol; a 40-fold molar excess over probe DNA) of the following supercoiled plasmid DNAs were present during binding as follows. Lanes: 1, 2, and 8, pGEM-1 (vector); 3 and 10, pTZ1 (strongly TFIIIC-binding SUP4 mutant tRNA gene); 4 and 11, pBB111R (repeat 5S rRNA gene); 9, pLNG56 (weakly TFIIIC-binding mutant SUP4 tRNA gene); 6 and 7, competition with 200 fmol of DNA oligonucleotides representing *box C* (TFIIIA-binding; 4.5 ng) and *box B+* (TFIIIC-binding; 3.8 ng), respectively. Yeast 5S RNA (200 fmol) was added as competitor for lanes 5 and 12. (*C*) TFIIIC affinity purified on *box B* DNA-Sepharose in the presence of Mg^{2+} (7 fmol of DNA-binding activity assayed by gel retardation with a tRNA gene probe) was added to affinity-purified TFIIIA (4 fmol of activity) for DNase I protection experiments displayed in lanes 3 and 4. Samples for lanes 4 and 5 contained 3 fmol of 5′-end-labeled nontranscribed strand probe and those for lanes 1–3 contained 2 fmol of 3′-end-labeled transcribed strand probe. Lanes: 2 and 5, TFIIIC alone; 1, no factors were added. Each sample also contained 25 fmol of supercoiled plasmid pKD1 (as carrier DNA) and was incubated for 40 min at 20°C prior to DNase I digestion. (The transcribed strand probe contained a short fragment that interfered with accurate assessment of the upstream limit of DNase I protection.)

A further expansion of DNA contacts occurred upon addition of TFIIIB (Fig. 5, lane 3). On the transcribed strand, the DNase I footprint now extended to bp −45, with enhanced cleavage at bp 69, little or no protection at two locations (bp 46 and 36/37), and only partial protection just upstream of the transcriptional initiation site at bp −4 (arrow at left of figure). TFIIIB alone did not stabilize TFIIIA–DNA complexes (data not shown), TFIII(B+C) alone did not protect the DNA probe (lane 1).

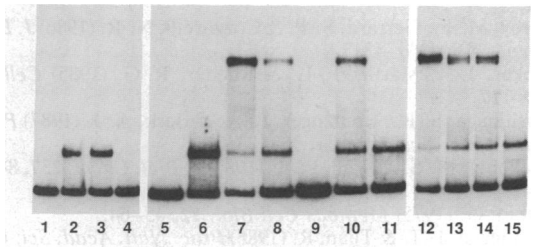

FIG. 4. Electrophoretic analysis of complexes formed with TFIIIA, TFIIIC, and 5S rDNA. TFs were incubated with a 5S rDNA probe and a 30-fold molar excess of various competitors. Lanes 1–4 contain TFIIIA complexes. TFIIIA was incubated with the probe alone (lane 2) or with various DNA competitors added (lane 3, nonspecific pGEM-1; or lane 4, 5S rDNA-containing pBB111F). No TFIIIA was added for lane 1. Lanes 5–11 contain TFIII(A+C) complexes. TFIIIA and -C were incubated with the probe alone (lane 7) or with the following plasmids also added (lane 8, pGEM-1; lane 9, pBB111F; lane 10, pLNG56; or lane 11, pTZ1). Samples in lanes 5 and 6 contained TFIIIC and -A, respectively, without competitor DNA. Lanes 12–15 show competition by tRNA half genes. TFIIIA and -C were incubated with the probe alone (lane 12) or with various competitors also added (lane 13, pBR322; lane 14, 5′ half $tDNA^{Leu_3}$-containing pJD5′-half; or lane 15, 3′ half $tDNA^{Leu_3}$-containing pJD3′-half).

The footprint of the TFIII(A+C+B)–DNA complex was compared with that of a crude extract also containing pol III. Fraction BRα (rechromatographed), containing pol III, TIFIIB, and TFIIIC, gave no DNase I footprint on the 5S gene (Fig. 5, lane 7). When combined with TFIIIA, BRα generated a footprint with the same extension as the TFIII(A+C+B) complex (lane 6) but with little or no protection at bp 18–37 (bracket at right of figure) and full protection near the transcriptional start site (arrow), which could conceivably be due to bound RNA polymerase. Thus, TFIIIA alone, TFIII(A+C) (Figs. 3 and 4), TFIII(A+B+C), and a complete transcription system (Fig. 5) generated distinct DNA-binding states on the 5S rRNA gene.

DISCUSSION

In these experiments, a complex of transcription proteins has been shown to interact with the entire *S. cerevisiae* 5S rRNA transcription unit and ≈45 bp of 5′ flanking sequence. It is known that the assembly of these transcription complexes is sequential, in particular that *Xenopus* and yeast TFIIIA must bind first (3, 19, 28). We have found that the yeast TFIIIA protects a smaller segment of its homologous 5S rRNA gene from DNase I than does *Xenopus* TFIIIA (Fig. 1). Since proteolytically cleaved *Xenopus* TFIIIA can still bind to the 3′ end of its ICR (29), one might ascribe the limited binding

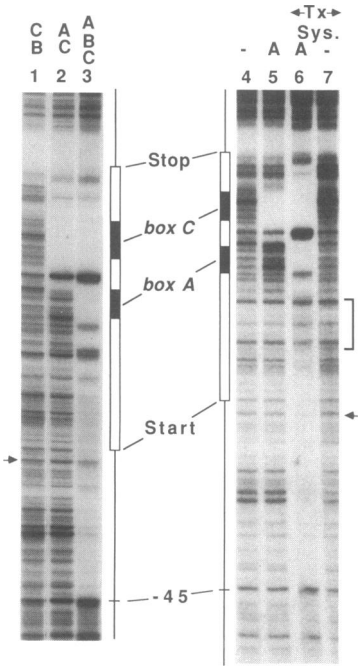

FIG. 5. Effect of TFIIIB on the 5S gene–TF complex and comparison with a complete transcription system. Protection of 2 fmol of transcribed-strand probe by 4 fmol of TFIIIA and 8 fmol of TFIIIC is displayed in lane 2 (identical with Fig. 3*A*, lane 2); 4 fmol of TFIIIB was added to TFIIIA and TFIIIC for lane 3. In lane 6, enough of the repurified BRα fraction to give 3 fmol of either tRNA or 5S transcription complexes was added to 4 fmol of TFIIIA. Lane 7 shows the same amount of BRα in the absence of TFIIIA. Lane 1 contains TFIIIC and TFIIIB in the absence of TFIIIA. Lane 4 contains the no-protein DNase I digestion control.

site of the yeast TFIIIA to proteolysis. However, the strict correspondence between its DNA-binding and transcription activities (Fig. 2), and the fact that most, if not all, of the TFIIIA–DNA complex is capable of successive interactions with TFIIIC and TFIIIB, yielding enlarged footprints (Figs. 3–5), argues against proteolysis. (Certain complex alternatives involving cleavage of TFIIIA that destroys affinity for part of the DNA binding site, although retaining TF activity cannot be formally excluded.)

We note parenthetically that numerous earlier attempts to footprint the yeast 5S gene in crude fractions proved unsuccessful, or only indicative (refs. 14 and 30 and unpublished data); we suspect that these failures may have been due to interference with TFIIIA-binding by nonspecific DNA-binding proteins.

When yeast TFIIIC binds to the TFIIIA–DNA complex, the state of the 5S rRNA gene changes. Two footprints have been seen with TFIIIC purified in somewhat different ways. One involves a limited extension of interaction along the gene in the 3′ and 5′ directions and the other extends partial DNA protection all the way to bp −20. The extension to bp −20 is probably due to an additional component that either binds preferentially to DNA adjacent to TFIII(C+A) or causes at least one of these transcription factors to change its mode of DNA binding.

Two segments of the 5S gene ICR are homologous with box A of tRNA genes (Fig. 1), yet competition by DNA bearing a $tRNA^{Leu_3}$ gene *box A* is ineffectual (Fig. 4*C*), and it is *box B* that instead determines ability to compete with a 5S gene–TFIIIA complex for TFIIIC (Figs. 3*B* and 4 *B* and *C*). If the *box A*-binding domain of TFIIIC is attached to DNA in the 5S gene transcription complex, that binding may be relatively weak and readily competed for by any DNA that binds tightly to TFIIIC through its *box B*.

The binding state of the 5S gene is further modified by extensively purified TFIIIB, with DNA interaction extending to bp −45, altered within the gene and greatly stabilized. Experiments with several tRNA genes also show extensions of footprints to *ca.* 40 bp upstream of transcriptional start sites by TFIIIB (24). Thus, these pol III transcription complexes are anchored to DNA next to, as well as within, the transcription unit proper. It will be interesting to determine whether the 5′ flanking attachment is involved in anchoring the transcription factors to the 5S gene during RNA chain elongation.

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