Reconstitution of the Yeast RNA Polymerase III Transcription System with All Recombinant Factors*

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Transcription factor TFIIIC is a multisubunit complex required for promoter recognition and transcriptional activation of class III genes. We describe here the reconstitution of complete recombinant yeast TFIIIC and the molecular characterization of its two DNA-binding domains, τA and τB, using the baculovirus expression system. The B block-binding module, τB, was reconstituted with τr138, τr91, and τr60 subunits. τr131, τr95, and τr55 formed also a stable complex, τrA, that displayed nonspecific DNA binding activity. Recombinant rTFIIIC was functionally equivalent to purified yeast TFIIIC, suggesting that the six recombinant subunits are necessary and sufficient to reconstitute a transcriptionally active TFIIIC complex. The formation and the properties of rTFIIIC–DNA complexes were affected by dephosphorylation treatments. The combination of complete recombinant rTFIIIC and rTFIIIB directed a low level of basal transcription, much weaker than with the crude B+ fraction, suggesting the existence of auxiliary factors that could modulate the yeast RNA polymerase III transcription system.

RNA polymerase III is responsible for the transcription of some 300 different genes in yeast, encoding mostly tRNAs (1–3). Transcription by RNA polymerase III requires two general auxiliary factors, TFIIIC and TFIIIB, and a 5 S RNA gene-specific factor, TFIIIA (reviewed in Ref. 4). The primary step in RNA gene activation is the binding of TFIIIC to the intragenic promoter elements, the A and the B blocks. DNA-bound TFIIIC directs the assembly of TFIIIB, upstream of the transcription start site, and TFIIIB in turn recruits RNA polymerase III for multiple transcription cycles. Transcription of eukaryotic class III genes is a variation of this scheme that involves a cascade of protein–DNA and protein–protein interactions (4–7). Yeast (Saccharomyces cerevisiae) TFIIIC is a multifunctional, multisubunit factor comprising six polypeptides organized in two large subassemblies, τA and τB. Identified by limited proteolysis and electron microscopy (8, 9), τB binds tightly to the B block that is located at a variable distance from the start site. Biochemical and genetic evidences indicated that τB likely comprises three subunits, τr138, τr91, and τr60 (10–13). Although τr138 and τr91 cooperate in B block binding (11), τr60 appears to link τA and τB domains and, quite unexpectedly, to participate in TBP recruitment (12, 14). The τA domain, visualized by electron microscopy, probably comprises τr95 and τr55, which are thought to participate in A block binding (15, 16), and τr131, which is mostly responsible for TFIIIB assembly (17, 18). τr131 is the only subunit of TFIIIC extending upstream of the start site (19). All six genes of yeast TFIIIC have been cloned and found to be essential for yeast cell viability, as was each of the 17 subunits of RNA polymerase III and the three components of TFIIIB (6). TFIIIB is a multiprotein transcription factor comprising three polypeptides that do not form a stable complex when not bound to DNA (20, 21). It can be chromatographically separated into two subfractions, B– containing the TATA-binding factor TBP and Brf1 and B+ containing Bdp1. TFIIIC-dependent TFIIIB assembly onto TATA-less genes involves a stepwise series of interactions and conformational changes starting with the recruitment of Brf1 by τr131, the entry of TBP mediated by Brf1 and probably τr60, followed by the binding of Bdp1, directed by τr131, that stabilizes and locks the TFIIIB–DNA complex (12, 20, 22). TFIIIB is able to assemble autonomously in vitro, via the interaction of TBP with the strong TATA box of the SNR6 gene (23, 24), but TFIIIC is required in vivo to transcribe the few TATA-containing class III genes (1, 25, 26). This paradox was resolved by the observation that TFIIIC relieves chromatin repression in vitro (27, 28), and in vivo (28). However, yeast TFIIIC did not show any detectable histone acetyltransferase activity (6), at variance with purified human TFIIIC (29, 30).

The basal transcription system described above directs accurate initiation and termination of transcription in vitro on a variety of class III genes (31, 32). It is still possible, however, that additional components may be needed to reach the high transcription rates observed in vivo. Indeed, efficient transcription of the SNR6 gene, which has a degenerate A block and a distant B block located downstream of the termination signal, was shown to require the Nhp6 proteins in vivo and in vitro (33, 34). There was also the intriguing observation that recombinant Bdp1 directed accurate transcription of the SLI44 RNA gene at a low level and needed to be supplemented with TFIIIE to fully restore the transcription level obtained with purified B+ fraction (31). TFIIIE factor activity has not yet been characterized, and its mode of action is still unclear (35).

Ultimately, future advances in the definition and analyses of the RNA polymerase III transcription system will require its reconstitution with recombinant proteins. As a step toward this goal, we describe here the reconstitution of functional TFIIIC by expression of its subunits in insect cells. The τA and the τB domains of the factor could be produced independently and analyzed. The transcription system reconstituted with recombinant TFIIIC, recombinant TFIIIB, and highly purified RNA polymerase III directed a level of specific transcription similar to the one obtained with affinity-purified endogenous TFIIIC.

MATERIALS AND METHODS

Production and Expression of Recombinant Baculoviruses—The open reading frame of FLAG–τr138, HA–τr95, τr91, τr60, τr55, or Brf1–His were inserted in PVL1392 vector (Pharmingen) and then recombinated with baculovirus DNA (Bacvector 3000 DNA; Novagen) in Spodoptera frugiperda (SF9) cells. The recombinant viruses were plaque-purified, and viral stocks were prepared by three-step growth amplifications. The

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open reading frames of r138, His-r131, and r60-His were subcloned in a pFastbac1 vector (Invitrogen). The resulting plasmids were used for bacmid production according to the manufacturer’s protocol (Invitrogen). High Five cells (typically 2 × 10^7 cells) were infected with one baculovirus or co-infected with combinations of recombinant baculoviruses (from two to six) as indicated. Multiplicities of infection were adjusted so as to balance the amount of recombinant proteins simultaneously expressed from each virus. The cells were collected 72 h post-infection. The protein extracts were prepared as described (36).

Purification of the Recombinant Proteins—Preparation of r7A. GST-r131^4 (36), HA-r75 and r55 were co-expressed in High Five cells. The three polypeptides were co-purified successively by anti-HA-tag affinity column (Sigma) followed by GSH affinity column (Amersham Biociences) chromatography to yield the r7A subcomplex (according to the manufacturer’s instructions).

Preparation of r7B—High Five cell extract co-expressing FLAG-r138, r91, and r60-His was subjected to chromatography on heparin D (Biospepra) equilibrated in 50 mM Tris, pH 7.5, 100 mM NaCl, 20% glycerol, 5 mM β-mercaptoethanol, and protease inhibitor mixture (Complete™, Roche Applied Science). The proteins were eluted with a 30-column volume linear gradient of NaCl from 0.1 to 1 M. The fractions were then tested in gel shift assay using tDNA^Lew gene as a probe (see below). rTFIIICa and rTFIIICb were purified from High Five cells (2 × 10^9 cells) co-expressing r138, His-r131, HA-r75, r91, r60, and r55, using the Sprint Biocad system (Applied Biosystem) at 10 ml/min. The extracts, prepared in buffer B0 (50 mM Tris, pH 8, 20% glycerol, 5 mM β-mercaptoethanol, and protease inhibitor mixture) containing 40 mM ammonium sulfate (AS), were first adjusted to 250 mM AS and then loaded onto a 35-ml heparin HyperD column previously equilibrated with the same buffer. The resin was washed with 10 column volumes of buffer B0 containing 360 mM AS. A 15-column volume linear gradient of AS from 360 to 750 mM was then applied. Fractions were collected and assayed for TFIIIC-DNA binding activity (see below). rTFIIICa (fractions 30–40) or rTFIIICb (fractions 60–80) were pooled separately, adjusted to 550 mM AS, and subjected to fast liquid chromatography in a 0.8-ml Poros MC 20 (Applied Biosystem) column charged with Cobalt. rTFIIICa or rTFIIICb were eluted with buffer B0 containing 40 mM AS and 300 mM imidazole (pH adjusted to 8).

His-TBP (36), and His-Brf1 were expressed in High Five cells and purified successively by metal chelate chromatography on Poros MC 20 loaded with nickel and heparin chromatography (Poros 20 HE; Applied Biosystem). rBdp1 was prepared as described (37).

DNA Binding and in Vitro Transcription Assays—TFIIIC-DNA interactions were monitored by gel shift assays as described previously (38) using a ^32P-labeled DNA fragment carrying the tRNA^Lew or the SLIP4 tRNA^Tyr gene as a probe. The amounts of proteins used in the various gel shift assays were as follows: affinity-purified rTFIIIC (10 ng), heparin-purified rTFIIIC (50 ng), heparin-purified r7B (30 ng), and Mono Q endogenous TFIIIC (100 ng). The final KCl concentration was adjusted to 180 mM instead of 120 mM (used with TFIIIC) when r7B was assayed. The limited proteolysis assays were performed as described (12), using 50 ng of heparin-purified r7B or 150 ng of Mono Q-purified endogenous TFIIIC (38). The apparent dissociation constant (K_diss) of rTFIIICa-rTFIIICb, or rTFIIIC-tDNA^Lew complexes was determined as described previously (38).

Standard in vitro transcriptions were performed as previously described (12, 39), using the following amounts of proteins: 20 ng of affinity-purified rTFIIIC (or 100 ng of Mono Q-purified endogenous yTFIIIC), 0.5 µg of partially purified B^* fraction (22, 37), or 10 ng of pure rBdp1 when indicated, 20 ng of rTBP, 10 ng of rBrf1, 100 ng of highly purified RNA Pol III and 100 ng of the indicated DNA plasmid templates. As estimated by SDS-PAGE analysis followed by Coomassie Blue staining, 0.5 µg of the crude B^* fraction used provide no more than 20 ng of Bdp1 polypeptide. When 5 S RNA gene was transcribed, 40 ng of purified rTFIIIA (40) was added to the transcription mixture. The transcriptions reactions were allowed to proceed for 45 min at 25 °C, and the transcripts were analyzed by electrophoresis on 6% polyacrylamide, 8 M urea gel.

To analyze the initiation of transcription, a 17-mer assay was performed as described previously (41). Stable ternary complexes were formed by incubating the transcriptions proteins (same amount as for the standard in vitro transcription presented in this study) for 20 min at 25 °C. Purified RNA Pol III, ATP, CTP, and α-^32P-labeled UTP were then added, and the transcription was allowed to proceed for 20 min at 25 °C. The reaction products were separated by electrophoresis on 15% polyacrylamide, 8 M urea gel.

Footprint—Binding reactions were calibrated using heparin-purified r7B or Mono Q-purified endogenous TFIIIC (38) to obtain a complete retardation of the probe. The TFIIIC-DNA complexes obtained were then subjected to DNase protection as described (42), and the DNA fragments were resolved on an 8% polyacrylamide sequencing gel.

Phosphatase Treatments—Potato acid phosphatase (PAP; Fluka) in ammonium sulfate suspension was centrifuged for 30 min at 15,000 rpm at 4 °C and dissolved in PPA buffer (10 mM Pipes, pH 6, 100 mM NaCl, and 3 mM MgCl2) at a final concentration of 0.5 unit/ml. 50 µg of heparin-purified rTFIIIC, 15 ng of affinity-purified rTFIIIC, or 150 ng of Mono Q-purified endogenous yTFIIIC were incubated with increasing amounts of PAP (from 0.025 to 0.75 unit) as indicated, or bovine serum albumin in PPA buffer for 30 min at 30 °C. Phosphatase inhibitor mixture II (Sigma) was then added at a 1:20 dilution to the reaction mixtures before DNA binding or in vitro transcription assays.

Southwestern blot—The Southwestern blot was performed as previously described (39). Briefly 5–10 µg of each purified protein was subjected to 8% SDS-PAGE and blotted to nitrocellulose. The filters were first washed with the a buffer containing 20 mM Heps, pH 7.5, 0.1 mM EDTA, 5 mM MgCl2, 100 mM KCl and then incubated for 30 min with 20 mM phosphate-buffered saline, pH 7.2, containing 2.5% (v/v) Nonidet P-40, 1% (w/v) gelatin, 40 mM NaCl, 0.5 mM EDTA, and 10% (v/v)
Characterization of the Two Structural Domains of TFIIIC—To attempt the in vivo assembly of yeast TFIIIC, we constructed various recombinant baculoviruses for directing the production of each individual TFIIIC subunit in insect cells. The six subunits were overexpressed quite efficiently, without much noticeable proteolysis (data not shown). Some subunits were epitope-tagged to allow affinity purification of protein complexes. In co-expression experiments to produce partial or complete TFIIIC, insect cells were co-infected with appropriate amounts of viruses so as to co-express a similar level of each subunit. Next, we looked for protein assemblies that could form stable complexes with tDNA. High Five cells were co-infected with various combinations of recombinant baculoviruses to produce the six polypeptides (τ138, GST-τ131, HA-τ95, τ91, τ60, and τ55) or all possible combinations of five subunits. All of the polypeptides were present in similar amounts in the different extracts, as shown by immunoblotting (Fig. 1).

In gel retardation assays using crude cell extracts, two complexes of different migration rates were detected (data not shown). The larger one was only present when all six subunits were co-expressed, whereas the quickly migrating one was detected when τ138, τ91, and τ60 were co-expressed (data not shown). This complex was likely related to the protease-resistant complex previously characterized with TFIIIC preparations subjected to limited proteolysis (8). Indeed, τ138 was shown to be part of the protease-resistant τB domain (10), and biochemical studies have suggested that τ91 and τ60 also belonged to this DNA-binding subcomplex (11, 12, 16). We therefore attempted to reconstitute the minimal τB module using full-length polypeptides. Insect protein extracts containing recombinant FLAG-τ138, τ91, or τ60-His expressed either alone or in combination were partially purified by chromatography on a heparin column, and the fractions, eluted by a salt gradient, were analyzed by gel shift assays (Fig. 2A and data not shown). No protein-DNA complex was formed using the fractions from a control

To reconstitute τB, High Five cells were infected with recombinant baculoviruses encoding FLAG-τ138, τ91, and His-τ60. Protein extracts were chromatographed on a heparin column, and bound proteins were eluted using an NaCl gradient as described under “Materials and Methods.” Protein-DNA complexes were analyzed by electrophoresis and autoradiography. A gel shift assay. Heparin-purified fractions were incubated with a labeled DNA fragment harboring the tRNA<sup>3</sup> gene. The position of τB-DNA complexes is indicated on the left. B, polypeptide composition of τB. Preformed τB-DNA<sup>3</sup> complexes (lane 1) were incubated with 1 μg of control anti-T7 (lane 2), 0.3 or 1 μg of anti-Histidine (lanes 3 and 4, respectively), anti-τ91 (lanes 5 and 6), or anti-FLAG (lanes 7 and 8) antibodies. C, τB interacts with the B block of tDNA. τB (lane 2) and endogenous γTFIIIC (lane 3) were examined for their footprint on a DNA fragment encompassing the tRNA<sup>3</sup> gene (schematized on the left, with the location of the A and B blocks and of the initiation site) as described under “Materials and Methods.” Lane 1, control without protein. D, limited proteolysis of τB. Heparin-purified τB (lanes 2 and 3) or endogenous Mono Q-purified γTFIIIC (lanes 4 and 5) were complexed with the tRNA<sup>3</sup> gene and then subjected to limited proteolysis (lanes 3 and 5) or not (lanes 2 and 4) by incubation with 10 ng of α-chymotrypsin for 10 min at 25 °C. Lane 1, DNA probe alone.
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cell extract, indicating that no insect proteins were able to form a stable complex with the yeast tRNA probe under the rather stringent binding conditions used (125 mM KCl and 300 ng of competitor DNA; data not shown). Using extracts expressing one subunit, we only detected DNA binding activity with r91. The nonspecific DNA binding activity of r91 was previously reported (11). Although r91 was found to cooperate with r138 for DNA binding (11), these subunits did not appear to assemble strongly (data not shown). On the contrary, r91 and r60 can form a stable complex that could represent the scaffold of the r7 subcomplex (14). However, no specific DNA binding activity could be detected with this pair of subunits. In fact, the three subunits, r138, r91, and r60, were necessary to reconstitute a strongly DNA-binding complex (Fig. 2A). In the absence of any of these three subunits, no other DNA–protein complex similar in size could be detected (data not shown). To demonstrate the presence of these three polypeptides in the B block-binding complex, r7B-tDNA3Leu complexes were incubated for 1 h at 25 °C with increasing amounts of subunit-specific antibodies and analyzed by electrophoresis on a 5% polyacrylamide gel. As shown in Fig. 2B, anti-FLAG and anti-histidine monoclonal antibodies altered the migration of the r7B-tDNA complex (Fig. 2B, compare lane 1 with lanes 3, 4, 7, and 8). Anti-r91 polyclonal antibodies also interfered with complex formation (Fig. 2B, lanes 5 and 6), as observed when endogenous TFIIIC (yTFIIIC) is used in gel shift assays (11). On the other hand, r7B-tDNA complex was not affected by control anti-T7 antibodies (Fig. 2B, compare lanes 1 and 2). These results indicated that r138, r91, and r60 reconstituted r7B. r7B-DNA interaction was then analyzed by DNA footprinting and compared with the characteristic footprint observed with yTFIIIC over the tRNA3Leu gene (43) (Fig. 2C, compare lanes 3 and 1). As expected, r7B gave a partial footprint, spanning only the 3′ half of the gene, over the B block. The protection of the B block region was similar with r7B and yTFIIIC (Fig. 2C, compare lanes 2 and 3), which confirmed the binding specificity of the r7B complex. Limited α-chymotrypsin proteolysis of r7B generated a stable protein–tDNALeu complex of the same increased electrophoretic mobility as the protease-resistant B complex (8) generated from the endogenous yeast TFIIIC (Fig. 2D, compare lanes 3 and 5). Therefore, when bound to tDNA, the reconstituted r7B and the B domain of TFIIIC appeared to have the same accessibility to the protease, supporting the model of a transcription factor made of two structural modules. Together, these data demonstrate that, using full-length polypeptides, the minimum specific B block-binding domain is composed of r138, r91, and r60.

The other DNA-binding domain of TFIIIC, rA, has never been isolated. We tried to reconstitute a stable r7A complex using individual recombinant polypeptides. Interestingly, co-expressed GST–r131, HA–r95, and r55 co-purified during two successive affinity chromatographic steps (immunopurification on anti-HA-protein A-Sepharose followed by a glutathione affinity chromatography on fast protein liquid chromatography), as revealed by Coomassie Blue staining (Fig. 3A). Furthermore, the three proteins co-purified during gel filtration (data not shown). The stable complex formed by r131, r95, and r55 likely corresponded to the postulated rA module (8). We analyzed the DNA binding properties of this complex in gel shift assays using two different probes (LeuA and LeuB) harboring the A or B block of the tDNA3Leu gene, respectively. As shown in Fig. 3B, r7A retarded the migration of the two DNA fragments with the same efficiency. Similar results were obtained using a nonspecific 200-bp DNA fragment as a probe (data not shown). The three subunits r131, r95, and r55 were necessary to form a complex showing this nonspecific DNA binding activity. Indeed, none of the subunits, expressed alone or in pairs, was able to bind DNA detectably in gel shift assays under the conditions used (data not shown). To determine which subunit of rA was responsible for this DNA binding activity, partially purified recombinant subunits were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and then incubated with labeled poly(dA-dT) as described under “Materials and Methods.” Bound DNA was revealed by autoradiography (Fig. 3C, lower panel). A complex migrating slightly faster than yTFIIIC–tDNA complex of the same increased electrophoretic mobility as the protease-resistant B complex (8) generated from the endogenous yeast TFIIIC (Fig. 2D, compare lanes 3 and 5). Therefore, when bound to tDNA, the reconstituted r7B and the B domain of TFIIIC appeared to have the same accessibility to the protease, supporting the model of a transcription factor made of two structural modules. Together, these data demonstrate that, using full-length polypeptides, the minimum specific B block-binding domain is composed of r138, r91, and r60.

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on a metal chelate affinity column by pooling separately fractions 30–40 and fractions 60–80 that do not form the upper complex. As revealed by silver staining, the recombinant complexes, rTFIIICa and rTFIIICb, eluted from the affinity column, were highly purified (Fig. 4B). Both complexes contained the six subunits of TFIIIC (rTFIIIC, rTFIIICa, and rTFIIICb) with varying concentrations of tRNA genes with varying intron length, indicating that the difference in complex mobility was not gene-specific (data not shown). To compare the relative or TFIIICb or endogenous TFIIIC had a similar sensitivity to salt concentrations and temperature (data not shown). To compare the relative dissociation constant (K_{app}) of TFIIIC-DNA complexes by titrating a fixed amount of TFIIIC (yTFIIIC, rTFIIICa, or rTFIIICb) with varying concentrations of tRNA_{3}^{55} gene under optimal binding conditions (38). TFIIIC-DNA complexes were resolved by gel electrophoresis and quantified by a PhosphorImager. The linearity of the Scatchard representation (Fig. 5A) suggested the presence of a single, major binding component in the protein-DNA complexes. From three independent tRNA_{3}^{55} titration experiments, the K_{app} estimated for rTFIIICa, rTFIIICb, and endogenous yTFIIIC was not significantly different (1.4 \times 10^{-11}, 2 \times 10^{-11}, and 2.6 \times 10^{-11} M, respectively). The DNA binding specificity of the endogenous or recombinant factor was further compared in DNA footprinting assays using the same amount of factor titrated by gel shift assays. As shown in Fig. 5A, the footprints of yTFIIIC and rTFIIICa on the tRNA_{3}^{55} gene were indistinguishable (Fig. 5A, lanes 1 and 2). In contrast, rTFIIICb yielded a comparatively weaker footprint over the A block region (Fig. 5B, lane 3). Enzymes and recombinant TFIIIC were then compared for their ability to direct specific transcription of various tRNA and 5 S RNA genes in vitro. Wondering whether the recombinant rTFIIIC had the same flexibility as the endogenous factor, we used tRNA genes with various spaced A and B blocks. Similar amounts of yTFIIIC (y), rTFIIICa (Ca), and rTFIIICb (Cb), based on gel shift data, were used in reconstituted transcription assays in the presence of the B’ fraction (containing Bdp1), recombinant TBP, rBrf1, highly purified RNA polymerase III, and rTFIIA when indicated. As expected, the in vitro transcription system was TFIIIC-dependent because no transcription could be detected in the absence of the factor (Fig. 5C). Both recombinant rTFIIIC, Ca or Cb, directed the transcription of the tRNA genes assayed. No difference in the length of the transcripts could be detected when compared with the transcripts obtained with endogenous yTFIIIC (Fig. 5C compare lanes y, Ca, and Cb), suggesting that the initiation and termination processes occurred correctly. In the presence of rTFIIICa, both forms of rTFIIIC could direct transcription of the 5 S RNA gene (Fig. 5C). Taking advantage of the SLI14 tRNA_{138}^{55} gene sequence, which allows the synthesis of a 17-mer transcript in the absence of GTP (Fig. 5D), we analyzed the specificity of the initiation reaction. Both recombinant and endogenous TFIIIC were able to synthesize the expected 17-mer RNA, indicating that recombinant rTFIIIC allowed initiation of transcription at the correct site (Fig. 5D, compare lanes 2–4).

Effect of Phosphatase Treatments on TFIIIC Activities—Three subunits of the yeast TFIIIC, r131, r95, and r138 have been found to be phosphorylated in vivo (6, 44), whereas r91 and r55 have been found to be phosphorylated in vitro (45). The function of these modifications has not been established yet. In view of the presence of two chromatographic forms of rTFIIIC, we explored the effect of the dephosphorylation of rTFIIIC on DNA binding using gel shift assays. As shown in Fig. 6A, PAP treatment of partially purified rTFIIICa resulted in the formation of DNA complexes of slightly increased electrophoretic mobility, comparable with that of rTFIIICb. In contrast, when phosphatase inhibitors were added together with PAP during the preincubation period with phosphatase, rTFIIICa-DNA complex migration remained unaffected (Fig. 6A, compare lanes 1 and 7). Preincubation of rTFIIICb with PAP under the same conditions did not result in a change in the mobility of the DNA complexes.

Reconstitution of Yeast TFIIIC—In gel shift assays, rTFIIICa and rTFIIICb formed two distinct complexes (Fig. 4A). The same results were obtained with different tRNA genes with varying intron length, indicating that the difference in complex mobility was not gene-specific (data not shown). DNA complex formation with recombinant TFIIICa or TFIIICb or endogenous TFIIIC had a similar sensitivity to salt concentrations and temperature (data not shown). To compare the relative affinity to tDNA of both forms of rTFIIIC, we determined the apparent dissociation constant (K_{app}) of TFIIIC-DNA complexes by titrating a fixed amount of TFIIIC (yTFIIIC, rTFIIICa, or rTFIIICb) with varying concentrations of tRNA_{3}^{55} gene under optimal binding conditions (38). TFIIIC-DNA complexes were resolved by gel electrophoresis and quantified by a PhosphorImager. The linearity of the Scatchard representation (Fig. 5A) suggested the presence of a single, major binding component in the protein-DNA complexes. From three independent tRNA_{3}^{55} titration experiments, the K_{app} estimated for rTFIIICa, rTFIIICb, and endogenous yTFIIIC was not significantly different (1.4 \times 10^{-11}, 2 \times 10^{-11}, and 2.6 \times 10^{-11} M, respectively). The DNA binding specificity of the endogenous or recombinant factor was further compared in DNA footprinting assays using the same amount of factor titrated by gel shift assays. As shown in Fig. 5A, the footprints of yTFIIIC and rTFIIICa on the tRNA_{3}^{55} gene were indistinguishable (Fig. 5A, lanes 1 and 2). In contrast, rTFIIICb yielded a comparatively weaker footprint over the A block region (Fig. 5B, lane 3). Enzymes and recombinant TFIIIC were then compared for their ability to direct specific transcription of various tRNA and 5 S RNA genes in vitro. Wondering whether the recombinant rTFIIIC had the same flexibility as the endogenous factor, we used tRNA genes with various spaced A and B blocks. Similar amounts of yTFIIIC (y), rTFIIICa (Ca), and rTFIIICb (Cb), based on gel shift data, were used in reconstituted transcription assays in the presence of the B’ fraction (containing Bdp1), recombinant TBP, rBrf1, highly purified RNA polymerase III, and rTFIIA when indicated. As expected, the in vitro transcription system was TFIIIC-dependent because no transcription could be detected in the absence of the factor (Fig. 5C). Both recombinant rTFIIIC, Ca or Cb, directed the transcription of the tRNA genes assayed. No difference in the length of the transcripts could be detected when compared with the transcripts obtained with endogenous yTFIIIC (Fig. 5C compare lanes y, Ca, and Cb), suggesting that the initiation and termination processes occurred correctly. In the presence of rTFIIICa, both forms of rTFIIIC could direct transcription of the 5 S RNA gene (Fig. 5C). Taking advantage of the SLI14 tRNA_{138}^{55} gene sequence, which allows the synthesis of a 17-mer transcript in the absence of GTP (Fig. 5D), we analyzed the specificity of the initiation reaction. Both recombinant and endogenous TFIIIC were able to synthesize the expected 17-mer RNA, indicating that recombinant rTFIIIC allowed initiation of transcription at the correct site (Fig. 5D, compare lanes 2–4).

Effect of Phosphatase Treatments on TFIIIC Activities—Three subunits of the yeast TFIIIC, r131, r95, and r138 have been found to be phosphorylated in vivo (6, 44), whereas r91 and r55 have been found to be phosphorylated in vitro (45). The function of these modifications has not been established yet. In view of the presence of two chromatographic forms of rTFIIIC, we explored the effect of the dephosphorylation of rTFIIIC on DNA binding using gel shift assays. As shown in Fig. 6A, PAP treatment of partially purified rTFIIICa resulted in the formation of DNA complexes of slightly increased electrophoretic mobility, comparable with that of rTFIIICb. In contrast, when phosphatase inhibitors were added together with PAP during the preincubation period with phosphatase, rTFIIICa-DNA complex migration remained unaffected (Fig. 6A, compare lanes 1 and 7). Preincubation of rTFIIICb with PAP under the same conditions did not result in a change in the mobility of the DNA complexes.
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Reconstitution of a Basal RNA Polymerase III Transcription System—Next we attempted to reconstitute a defined RNA polymerase III transcription system using recombinant factors, rTBP, rBrf1, rBdp1, rTFIIIC, and highly purified RNA polymerase III. Recombinant TFIIIC and the recombinant components of yeast TFIIIB were necessary and sufficient for accurate transcription of the SUP4 tRNA gene (Fig. 7). Purified rTFIIIA was the only additional factor necessary to transcribe the 5 S RNA gene in our reconstituted system (Fig. 7). However, in each case, the level of transcription was low when rBdp1 was used instead of the B’ fraction (Fig. 7, compare lanes 1 and 2). We estimated that the transcription level was stimulated 6-fold when the B’ fraction replaced rBdp1 (Fig. 7). Similar results were previously obtained with yTFIIIC, suggesting that the B’ fraction might contain some transcriptionally active components in addition to Bdp1 (31, 46). The fact that the 5 S RNA transcripts obtained with B’ were processed, whereas only precursor of 5 S RNA was obtained with rBdp1, indicated that additional activities were present in the B’ fraction (Fig. 7, lanes 9 and 10).

DISCUSSION

In this study, we showed that active yeast TFIIIC can be reconstituted from its six known recombinant subunits. The two DNA-binding domains, rA and rB, were characterized and could be assembled independently. Our results define a minimal transcription system, comprising all the general class III transcription factors, which could be stimulated by some unidentified components present in the B’ fraction, underscoring the need for additional auxiliary factors.

With the reconstitution of active TFIIIC from recombinant subunits, the characterization of the polypeptide composition of the yeast factor is now completed. The six subunits r138, r131, r95, r70, r55, and r55 are necessary and sufficient to form active rTFIIIC, which was functionally indistinguishable from endogenous TFIIIC in terms of DNA binding
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specificity and affinity as well as transcription factor activity. Yeast (S. cerevisiae) TFIIIC likely corresponds to human TFIIIC2, although the later comprises only five subunits and apparently lacks the ortholog of TFIIS (47–49). Interestingly, the most conserved subunits (r131 and r55) are involved in A block binding and TFIIIB recruitment. The highly divergent subunits are located at the downstream end of the genes (11, 15).

We present evidence that modification of endogenous or recombinant TFIIIC by phosphorylation is important for its DNA binding activity. TFIIIC is phosphorylated in yeast on several subunits (6, 44). Whether the same sites are phosphorylated in recombinant TFIIIC remains to be determined. Nevertheless, our observations suggest a structural and potentially regulatory role for TFIIIC modification by phosphorylation.

The present work establishes the organization of the six subunits of TFIIIC in two large globular domains, 7A and 7B (8, 9), that can be assembled independently. r7B was reconstituted with r131, r95, and r55. r7B displayed the expected DNA binding specificity for the B block region and had similar affinity for the tRNA3Gal gene as TFIIIC (data not shown), confirming that rB is most likely responsible for the overall stability of the TFIIIC-DNA complex (50). Interestingly r7B appears to have protease sensitive extension(s) that can be removed without affecting DNA binding and that might possibly participate in rB-7A interaction.

PRCURE 6. Effect of phosphatase treatment on TFIIIC activities. A, dephosphorylation of TFIIIC and DNA binding activity. Heparin-purified rTFIIICa (lanes 1–7) or Mono Q-purified endogenous yTFIIIC (lanes 8–12) were preincubated during 30 min at 30 °C with varying amounts of PAP before DNA complex formation on a labeled tDNA3Gal probe. Protein-DNA complexes were analyzed by electrophoresis and autoradiography. Lanes 1 and 8, no PAP; lanes 2–5, 0.05, 0.15, 0.25, or 0.5 unit of PAP, respectively; lane 3, rTFIIICa, no PAP; lane 7, 0.5 unit of PAP in the presence of Phosphatase inhibitor mixture; lanes 9–12, 0.25, 0.5, or 0.75 unit of PAP, respectively. Lanes 12, 0.75 unit of PAP in the presence of phosphatase inhibitor mixture. The migration of native yTFIIIC, rTFIIICa, rTFIIICb, and rB-TDNA complexes are indicated. B, dephosphorylation of TFIIIC and transcriptional activity. Highly purified rTFIIICa was preincubated with 0, 0.025, 0.075, 0.22, or 0.41 unit of PAP (lanes 1–5, respectively) or with 0.41 unit of PAP in the presence of phosphatase inhibitor mixture (lane 6). Phosphatase inhibitor mixture was then added, and TFIIIC activity was tested on a labeled tDNA3Gal probe. DNA complex formation and transcriptional activity were then plotted as percentages of control activity in the absence of PAP.

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stutized system (53). Transcription of the yeast U6 RNA gene, that has a strong TATA box allowing proper TFIIIB assembly in vitro through TBP binding, was simply reconstituted with the recombinant components of yeast TFIIIB, TBP, Brf1, and Bdp1 and purified RNA Pol III (31, 32, 54). Remarkably, in our hands, this basal system was suboptimal and was strongly stimulated by TFIIIE, a factor that remains to be characterized (31, 35). Suboptimal transcription with entirely recombinant TFIIIB was ascribed to a defective reinitiation rate (37). Transcription reinitiation strongly influences the level of transcription, which is well described for the Pol III system (55). These observations suggested that some important component or catalytic activity was missing. Phosphorylation of TBP (56) and of the Pol III complex (53) by CK2 is required by its rylation of TBP (56) and of the Pol III complex (53) by CK2 is required for transcription. However, the fact that the yeast CK2 can be replaced by its Drosophila ortholog in yeast cells (57) suggested that recombinant TBP was correctly modified in insect cells. A number of accessory factors have been proposed to stimulate Pol III transcription, without apparent gene specificity, including TFIIIE, the La protein, NF1, PC4, and the Topo I (reviewed in Ref. 51). The high mobility group Nhp6 protein specifically increases the transcription efficiency of SNR6 gene in vivo and in vitro by facilitating transcription complex assembly (33, 34). The present development of a reconstituted Pol III transcription system with all of the recombinant factors opens new avenues for the structural and functional analysis of TFIIIE and for the exploration of accessory effectors of the Pol III transcription system.

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