Interactions between the Tetratricopeptide Repeat-containing Transcription Factor TFIIIC131 and Its Ligand, TFIIIB70

EVIDENCE FOR A CONFORMATIONAL CHANGE IN THE COMPLEX*

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In the transcription of tRNA and 5 S genes by RNA polymerase III, recruitment of the transcription factor (TF)IIIB is mediated by the promoter-bound assembly factor TFIIIC. A critical limiting step in this process is the interaction between the tetratricopeptide repeat (TPR)-containing subunit of TFIIIC (TFIIIC131) and the TFIIIB-related factor Brf1p/TFIIIB70. To facilitate biochemical studies of this interaction, we expressed a fragment of TFIIIC131, TFIIIC131-(1–580), that includes the minimal TFIIIB70 interaction domain defined by two-hybrid studies together with adjacent sequences, up to the end of TPR9, implicated in the assembly reaction. TFIIIC131-(1–580) interacts with TFIIIB70 in solution and inhibits the formation of TFIIIB70-TFIIIC-DNA complexes. In a coupled equilibrium binding assay, the formation of TFIIIC131-(1–580)-TFIIIB70 complexes was adequately described by a single-site binding model and yielded an apparent equilibrium dissociation constant of 334 ± 23 nM. CD spectroscopy and limited proteolysis experiments defined a well structured and largely protease-resistant core in TFIIIC131-(1–580) comprising part of the hydrophilic amino terminus, TPR1–5, the intervening non-TPR region, and TPR6–8. CD spectra showed that trifluoroethanol induced significant α-helical structure in TFIIIC131-(1–580). A more modest monovalent ion-dependent CD difference was observed in mixtures of TFIIIC131-(1–580) and TFIIIB70, suggesting that formation of the binary complex may proceed with the acquisition of α-helicity.

Transcription factor (TF) IIIC is a large multisubunit complex that is responsible for recruiting the RNA polymerase III (pol III) initiation factor, TFIIIB, to the DNA upstream of the transcription start site of 5 S and tRNA genes. Among these, an interaction between the 60-kDa subunit of TFIIIC (TFIIIC60) and TBP (22) together with other genetic and biochemical studies (discussed below), the TFIIIC-DNA complex is thought to interact initially with the TFIIIB-related subunit of TFIIIB (Brf1p/TFIIIB70) via its tetratricopeptide repeat (TPR)-containing subunit, TFIIIC131 (15, 16). Subsequently, protein-protein interactions between TFIIIB70 and TBP (16) facilitate the interaction of TBP with the DNA (17, 18). The presence of TBP helps to stabilize the complex via minor groove interactions similar to those seen in pol II transcription complexes (19). Complex stabilization may also be provided by the unmasking of a cryptic DNA-binding domain in the carboxyl terminus of TFIIIB70 (20). The TFIIIB complex is completed with the addition of TFIIIB90 (B*), which further buds the DNA between the site of TBP binding and the transcription start site (21) and alters the pattern of cross-linking of TFIIIC131, TFIIIB70, and TBP in the complex (6, 19). Recent studies have added to the complexity of these events by increasing the number of interacting components. Among these, an interaction between the 60-kDa subunit of yeast TFIIIC (TFIIIC60) and TBP (22) together with the high nuclear concentration of TBP and TFIIIB70 (23) have
suggested that TFIIIC131 and TFIIIC60 may function together in recruiting a preformed TFIIIB70-TBP complex. Alternatively, it is possible that the TFIIIC60-TBP interaction occurs transiently during the sequence of conformational changes that characterize TFIIIB recruitment (22). Similar alternative explanations apply to other reported interactions (e.g., those between the human homolog of yeast TFIIIC95 and the human homolog of TFIIIB70 and TBP, respectively) (10).

Biochemical and genetic data indicate that interactions between the TPR-containing subunit (TFIIIC131) and TFIIIB70 are biologically significant and thus play an important role in TFIIIB recruitment. In particular, mutations in and around TPR2 have been shown to increase transcription in vivo of a tRNA gene bearing a defective A block (24, 25). The same mutations have also been shown to increase transcription of wild-type and mutant templates in vitro by facilitating the recruitment of TFIIIB70 (25). Similar observations have been made in cells and in extracts of strains overexpressing TFIIIB70 (23, 26). These findings are complementary and reveal that the interaction between TFIIIC131 and TFIIIB70 represents a limiting step in the assembly of a pol III transcription complex. Solution studies with immobilized fusion proteins suggest that multiple regions of TFIIIC131 interact with TFIIIB70 (10, 16). One such region, defined by two-hybrid experiments as a minimal TFIIIB70 interaction domain, is contained within the hydrophobic amino-terminal region and TPR1 (r131-1TPR-1(–165)) (15). As noted above, mutations in adjacent TPRs also contribute to the TFIIIC131-TFIIIB70 interaction. However, the mechanism by which mutations in this region increase the recruitment of TFIIIB70 remains to be established. The favored model in the case of the PCF1-2 mutation (T167I in TPR2) involves a structural change within TFIIIC131 that affects the extent to which the binding reaction can proceed (25). Changes in the nature and/or accessibility of the TFIIIB70-binding site may also underlie the 4-fold decrease in the two-hybrid interaction that occurs when the minimal TFIIIB70 interaction domain, r131-1TPR-1(–165), is extended up to the end of TPR9 (15). The molecular and biochemical basis for these effects is not well understood at the present time and warrants further investigation.

We have initiated a biochemical study to investigate the interaction between TFIIIC131 and TFIIIB70. In this work, we report the expression and characterization of a fragment of TFIIIC131, TFIIIC131-(1–580), that interacts with TFIIIB70 in solution and inhibits the formation of TFIIIB70-TFIIIC131-DNA complexes. We describe a coupled equilibrium binding assay that indirectly quantifies the interaction between TFIIIC131-(1–580) and TFIIIB70. Limited proteolysis of TFIIIC131-(1–580) together with circular dichroism experiments in the presence and absence of TFIIIB70 provide insights into the structure of the protein and suggest that a conformational change may occur upon formation of the TFIIIC131-(1–580)/TFIIIB70 complex.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—A wild-type clone of the gene encoding the 131-kDa subunit of yeast TFIIIC (TFIC4/PCF1) (24) was used as a template for polymerase chain reaction amplification by Pwo polymerase (a high fidelity polymerase). A truncated gene, encoding amino acids 1–580, was cloned as an NcoI-XhoI fragment into pET21-d. The resulting protein, TFIIIC131-(1–580), incorporated six histidine residues at the carboxyl terminus and a single amino acid substitution, N579L. Insert DNA was sequenced to confirm that no mutations had been introduced.

**Expression and Purification of TFIIIC131-(1–580)**—Recombinant TFIIIC131-(1–580) was prepared under native conditions and purified on Ni²⁺-nitrilotriacetic acid-agarose resin (Qiagen Inc.) following the manufacturer’s recommendations with the addition of 300 mM NaCl to the lysis buffer and 0.01% octyl β-D-glucoside (Pierce) to the elution buffer. Protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) were included in all purification steps. Further purification was achieved by heparin-agarose (type 1, Sigma) chromatography in 50 mM sodium phosphate, pH 6.0, 10% glycerol, 5 mM MgCl₂, 0.01% octyl β-D-glucoside, 300 mM NaCl, and 1 mM DTT. A second round of purification on a Ni²⁺-nitrilotriacetic acid-agarose resin from 300 mM to 1.0 M NaCl led to elution of the protein in a peak at 840 mM NaCl. Pooled fractions were loaded onto a column of hydroxyapatite (Bio-Gel HTP, Bio-Rad) equilibrated in the same buffer. A linear gradient from 50 to 250 mM sodium phosphate was applied, and the protein eluted in a peak at 140 mM sodium phosphate. Total protein recovery after these two columns was 45%. Octyl β-D-glucoside was increased to 0.1% in the pooled fractions, and the protein was concentrated to ~10 mg/ml. Purified TFIIIC131-(1–580) was stored at ~70 °C after dialysis into 20 mM Tris acetate buffer, pH 7.6, 25 mM sodium acetate, 5% glycerol, 1 mM DTT, and 0.1% octyl β-D-glucoside. Protein concentration was determined by direct absorbance at 280 nm using ε = 86,190. Silver-stained SDS-polyacrylamide gels of TFIIIC131-(1–580) were analyzed by laser scanning densitometry.

**Yeast Fractions and Recombinant Proteins**—Recombinant TFIIIB90 was purified under native conditions (to be described elsewhere) and was kindly provided by Tim Cloutier. Recombinant TFIIIB70 was purified by heparin-agarose (type 1, Sigma) chromatography in 50 mM sodium phosphate, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 4% urea. TFIIIB70 eluted at 1.4 M NaCl with a linear gradient from 50 mM to 2.0 M NaCl. TFIIIB70 was renatured, and its protein concentration was determined by direct absorbance as described previously (17). Purity was confirmed by Western analysis and silver staining of SDS-polyacrylamide gels.

**Transcription and Complex Assembly Assays**—Transcription with purified yeast TFIIIC, pol III, B′, and recombinant TFIIIB70 and TBP was performed under multiple-round conditions as described previously using a T7 RNA polymerase (Promega). Transcription complex assembly and electrophoresis on native polyacrylamide gels were also performed as described previously (25). Briefly, reactions in 20 μl contained a sup3-eST probe (10 fmol; quantified by fluorescence using PicoGreen reagent, Molecular Probes, Inc.), yeast TFIIIC (10 fmol), TFIIIB70 (3000 fmol), TBP (1000 fmol), and TFIIIB90 (400 fmol) as required in various experiments and were incubated at 20 °C for 60 min in a defined level of input TFIIIC. TFIIIB70 was limiting, and both TBP and TFIIIB90 were present in saturating amounts for TFIIIB-DNA complex assembly. In assays containing TFIIIC131-(1–580), the protein was added to preformed TFIIIC131-DNA complexes prior to the addition of the TFIIIC subunits.

**Quantitation and Data Analysis**—Digital images collected on phosphor storage screens were quantified using ImageQuant™ Version 5.0. Individual lines, one lane wide, were analyzed using Peak Finder to calculate peak areas corresponding to the TFIIIB-DNA complex. These values, when paired with the corresponding TFIIIC131-(1–580) concentration, yielded a transition curve describing the inhibition reaction. Importantly, the TFIIIC-DNA concentration in complex assembly assays never exceeded 0.25 mM. This is >500-fold lower than the apparent equilibrium dissociation constant determined for the TFIIIC131-(1–580)/TFIIIB70 complex. Under these conditions, the difference between total and free TFIIIC131-(1–580) in the reactions is negligible. The upper limit of complex assembly was determined by nonlinear least-squares analysis using the Hill equation and Microcal Origin™ Version 5.0 software. This limiting value was used to generate a scaled inhibition isotherm in which the relative level of TFIIIB-DNA complex formation is expressed as a function of TFIIIC131-(1–580) concentration. Multiple scaled data sets were then simultaneously refit to the Hill equation. Errors associated with the apparent equilibrium dissociation constant and the Hill coefficient were determined during curve fitting.

**Limited Proteolysis**—Proteases of varying specificities were screened to evaluate TFIIIC131-(1–580) structure. All enzymes were resuspended to 20 mg/ml with 25 mM Tris/HCl pH 7.5, 5 mM MgCl₂, 0.1 M NaCl, and 1 mM DTT. The following enzymes were informative: α-chymotrypsin (type VII Sigma), trypsin (Sigma), subtilisin (protease type VIII, Sigma), and endoprotease Glu-C (Sigma). Endoproteinase Arg-C (Sigma) did not digest TFIIIC131-(1–580) appreciably at high enzyme/substrate ratios, and protease X (Sigma)
Fig. 1. TFIIIC131-(1–580) inhibits transcription of a tRNA<sup>Leu</sup> gene. A shows a schematic representation of the structural domains in TFIIIC131. TFIIIC131-(1–580) encompasses the hydrophilic amino-terminal region, TPR1–5, an intervening non-TPR region, and TPR6–9. B shows a silver-stained SDS-polyacrylamide gel of the purified TFIIIC131-(1–580) protein. Protein size markers are annotated in kilodaltons. C shows the results of a multiple-round pol III transcription assay performed using a tRNA<sup>Leu</sup> gene with increasing concentrations of TFIIIC131-(1–580). The addition of TFIIIC131-(1–580) to these reactions preceded the addition of TFIIIB70 and the other components of TFIIIB, FL, full-length; bHLH, basic helix-loop-helix.

RAPIDLY DEGRADED TFIIIC131-(1–580). TFIIIC131-(1–580) at 0.05 mg/ml was digested in 20 mM Hepes-KOH, pH 7.8, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 5 mM CaCl<sub>2</sub> at 22 °C for various times and enzyme concentrations to establish conditions for rapid initial cleavage and generation of stable large fragments (27). The follow-
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Circular Dichroism—Circular dichroism (CD) spectra were measured on a Jasco J-720 spectropolarimeter at room temperature with the following parameter settings: step resolution, 0.5 nm; scan speed, 50 nm/min; response time, 1 s; bandwidth, 1 mm; and three to five accumulations/sample. Spectra were measured at a range of protein concentrations (TFIIIC131-(1–580), 275 nm to 4.66 μM; and TFIIIB70, 965 nm to 3.04 μM) in cells with path lengths from 0.1 to 2 mm. Samples were prepared in 10 mM sodium phosphate, pH 7.0 or 7.8, and 2–80 mM NaCl or KCl. After subtraction of the signal for the buffer alone, CD spectra were converted to molar ellipticity and fit using a constrained statistical regularization procedure (25) implemented by the program CONTIN Version 2 (29). Errors associated with secondary structural predictions represent S.D. from multiple experiments rather than the errors of estimation inherent in the predictions themselves.

RESULTS

An Amino-terminal Fragment of TFIIIC131 Inhibits pol III Transcription by Blocking the Recruitment of TFIIIB70 to TFIIIC131-DNA Complexes—As a starting point for more detailed biochemical studies on the TFIIIC131-TFIIIB70 interaction, we expressed and purified a truncated TFIIIC131 protein that includes the hydrophilic amino-terminal region, TPR1–5, an intervening non-TPR region, and TPR6–9 (Fig. 1A). This con-
struct, TFIIIC131-(1–580), includes the minimal TFIIIB70 inter-
action domain defined by two-hybrid studies (15) and adja-
cent sequences (centered around TPR2) that represent a hot

spot for activating mutations affecting TFIIIB70 recruitment (25). The TFIIIC131-(1–580) protein was expressed in Esche-
richia coli and purified under native conditions by chromatography on Ni<sup>2+</sup>-nitrilotriacetic acid resin, heparin-agarose, and hydroxylapatite. Analysis of the resulting preparation showed it to be >90% pure (Fig. 1B) and soluble at high concentrations (up to 20 mg/ml). Like the full-length TFIIIC131 protein in yeast, the apparent molecular mass of TFIIIC131-(1–580) on SDS-polyacrylamide gels (79 kDa) is considerably larger than its predicted molecular mass of 68.8 kDa (which includes the additional six histidine residues).

TFIIIC131-TFIIIB70 interaction studies predict that the TFIIIC131-(1–580) protein will bind to TFIIIB70 in solution and inhibit its incorporation into functional transcription complexes. To test for biochemical activity, the native TFIIIC131-
(1–580) protein was titrated into an RNA polymerase III tran-
scription assay under conditions in which TFIIIB70 was the limiting component. As shown in Fig. 1C, transcription of a tRNA<sup>Leu</sup> gene was inhibited in a TFIIIC131-(1–580) concentra-
tion-dependent manner. Notably, transcription start site selec-
tion was unaffected by the addition of TFIIIC131-(1–580), sug-
gest ing that the protein acts by perturbing complex assembly rather than the mechanism of assembly.

To define the step in transcription complex assembly affected by TFIIIC131-(1–580), varying amounts of the protein (up to the 300-pmol limit used to inhibit transcription) were tested for effects on the formation and stability of the TFIIIC131-DNA complex (Fig. 2A). Neither preincubation of TFIIIC131-(1–580) with DNA prior to the addition of TFIIIC nor its addition following the formation of TFIIIC-DNA complexes was found to affect this step (Fig. 2A and data not shown). Importantly, the addition of TFIIIC131-(1–580) did not supershift TFIIIC-DNA complexes as detected by native gel electrophoresis (Fig. 2A), and there was no change in the DNase I protection pattern of TFIIIC over the promoter elements (data not shown). These data exclude a possible stable association of TFIIIC131-(1–580) with TFIIIC-DNA complexes that could block the recruitment of TFIIIB components. In a separate experiment, we found that TFIIIC131-(1–580) did not affect the rate of dissociation of TFIIIC-DNA complexes (data not shown). TFIIIC131 can be photocross-linked to DNA both upstream and downstream of the transcription start site (19, 30). These data suggest that the factor may have intrinsic DNA binding activity. However, using native gel electrophoresis and a tRNA gene template, we found no evidence for either specific or nonspecific DNA binding activity by TFIIIC131-(1–580) up to a concentration of 15 μM (data not shown).

To assess the effect of TFIIIC131-(1–580) on transcription
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complex assembly involving TFIIIB70, the titration in Fig. 2A was repeated in the presence of TFIIIB70 (Fig. 2B). As expected, the protein inhibited the recruitment of TFIIIB70 to TFIIIC-DNA complexes. Importantly, the concentration dependence of this inhibition paralleled that seen in the transcription assay (Fig. 1C). These data indicate that the formation of a TFIIIC131-(1–580)/TFIIIB70 complex in solution prevents the assembly of TFIIIB70 into functional transcription complexes.

The ability of TFIIIC131-(1–580) to inhibit TFIIIB70 recruitment to TFIIIC-DNA complexes does not preclude additional interactions of the protein with other components of TFIIIB. Indeed, a weak interaction between the human homolog of TFIIIC131 and TBP has been reported (10). Moreover, a positive two-hybrid interaction has been described between a TFIIIC131 mutant deleted for TPR2 and TFIIIB90 (31). These findings raise the possibility that TFIIIC131-(1–580) could inhibit TFIIIB-DNA complex assembly by binding to all three components of TFIIIB. We therefore examined the inhibition of TFIIIB-DNA complex assembly by TFIIIC131-(1–580) and tested whether any rescue of the inhibition could be achieved by increasing the amount of TBP or TFIIIB90. Conditions for TFIIIB complex assembly were established such that TFIIIB70 was limiting and both TBP and TFIIIB90 were present in saturating amounts. Because of the high stability of the TFIIIB-DNA complex, the addition of TFIIIC131-(1–580) in these assays preceded the TFIIIB components. This ensures that TFIIIC131-(1–580) and TFIIIC-DNA complexes have an equal opportunity to bind TFIIIB70. At the upper limit of TFIIIC131-(1–580) used in Figs. 1C and 2, TFIIIB-DNA complex assembly (visualized after heparin stripping) was completely inhibited. The addition of a 2- or 4-fold higher amount of either TBP or TFIIIB90 could not detectably restore TFIIIB complex assembly in the presence of TFIIIC131-(1–580). We conclude that under the conditions employed, TFIIIC131-(1–580) does not inhibit the function of TBP or TFIIIB90 in complex assembly. Additional data consistent with this conclusion are described below.

Quantitation of the TFIIIC131-(1–580)-TFIIIB70 Interaction in a Coupled Equilibrium Binding Assay—Rigorous quantitative assays are available to monitor TBP binding to DNA and the cooperative contributions of TFIIIB70 in the assembly of ternary TFIIIB70-TBP-DNA complexes (17, 18). However, analogous biochemical assays have not yet been developed to study interactions between TFIIIC (or its subunits) and the components of TFIIIB. The development of such assays is clearly needed to better understand the critical, limiting interaction between TFIIIC131 and TFIIIB70 and the effects of dominant TPR mutations in TFIIIC131. To this end, we tested whether the extraordinary stability of TFIIIB-DNA complexes would provide a reliable end point for quantifying, in an indirect manner, the interaction between TFIIIC131-(1–580) and TFIIIB70. A titration of TFIIIC131-(1–580) was performed (Fig. 3A) under the conditions described for Fig. 2C, and the heparin-stripped TFIIIB-DNA complexes were quantified using ImageQuant™ Peak Finder (see “Experimental Procedures”). The upper limit of complex assembly was determined by nonlinear least-squares fitting of the data to the Hill equation, and the resulting value was used to scale the data to compare different experiments. The results of four independent TFIIIC131-(1–580) titrations are shown in Fig. 3B. Global (simultaneous) analysis of these titrations yielded an isotherm describing the inhibition of TFIIIB-DNA complex assembly. Despite the molecular complexity of the reaction, the Hill coefficient (1.4 ± 0.2) approached the value of unity expected for single-site non-cooperative binding between two components. This result is consistent with the preceding data (Fig. 2) indicating that TFIIIC131-(1–580) inhibits TFIIIB-DNA complex assembly by binding to TFIIIB70. Moreover, since a single-site binding model adequately describes the data, the protein concentration at 50% inhibition (334 ± 23 nM) provides an apparent dissociation constant for the TFIIIC131-(1–580)/TFIIIB70 complex. It should be emphasized that the single-site binding model does not discriminate whether one or more regions of either protein contribute interactions in the complex. The data simply indicate that the sum of these interactions behaves according to single-site binding theory.

Protease Resistance Mapping of TFIIIC131-(1–580)—Schematically, TFIIIC131 (1–580) is composed of four distinct domains (Fig. 1A): the amino-terminal region, demonstrated to be important for a two-hybrid interaction with TFIIIB70 along
with TPR1; TPR1–5; an intervening non-TPR region; and TPR6–9. The two TPR domains are each predicted to have a-helical structure based on the reported crystal structure of TPRs in protein phosphatase 5 (32). The structure of the other regions within TFIIIC131-(1–580) is not known. One goal of this study was to ascertain whether the TPRs of TFIIIC131-(1–580) constitute independent structural domains. Limited proteolysis exploits that fact that site-specific proteases will cleave proteins in solvent-exposed unstructured regions rather than within a folded domain. Four enzymes of differing specificities were informative in probing the structure in regions rather than within a folded domain. The 34-kDa fragments were extremely stable to further digestion (data not shown). Subtilisin generated a similar pattern of bands, although the products in the 55-kDa region were heterogeneous (data not shown). Western blotting with an anti-peptide antibody raised against amino acids 109–124 of TFIIIC131 (immediately preceding TPR1) reacted with the 34-kDa fragments generated by both enzymes. These fragments have not been analyzed further.

MALDI-TOF mass spectrometry was used to define the limits of both the chymotrypsin- and trypsin-resistant regions of TFIIIC131-(1–580). The MALDI mass spectrum of the chymotrypsin-resistant fragment showed singly and doubly charged ions with a mass/charge ratio of 55,962.8. This allowed an unambiguous assignment of the protease-resistant region from amino acids 59 to 536 (mass of 55,979.7) (Fig. 4E). The MALDI spectrum of the trypsin digest revealed fragment heterogeneity, with multiple peaks ranging in mass from 51,805.7 to 53,935.4. Within this range, the masses for individual peaks could be attributed to tryptic cleavages within the R/K-rich region from amino acids 102 to 120 and at any one of four sites from amino acid 548 to the carboxyl terminus. A schematic representation of the trypsin- and chymotrypsin-resistant fragments is shown in Fig. 4E. It is apparent that the protease-sensitive regions of TFIIIC131-(1–580) reside in the amino terminus and in or downstream of TPR9. The chymotrypsin-resistant region includes more than half of the hydrophilic amino terminus, TPR1–5, the intervening non-TPR region, and TPR6–8.

Circular Dichroism Analysis of TFIIIC131-(1–580)—CD spectroscopy can be used to estimate the overall content of secondary structure in a polypeptide and to follow changes in secondary structure resulting from ligand binding or altered solution conditions (33). We have used CD to analyze the secondary structural characteristics of TFIIIC131-(1–580). CD spectra were obtained at two pH values (7.0 and 7.8), at monovalent salt concentrations from 2 to 80 mM, and at protein concentrations from 275 nM to 4.66 μM. Individual spectra were fit using the program CONTIN and a set of reference spectra for 16 proteins of known structure (28, 29). In addition to robust curve fitting of the data, this analysis computes the fractions of the various structural classes (α-helix, β-sheet, β-turn, and random coil) in the protein. In analyzing the spectra of proteins with known structures, CONTIN is most accurate in estimating the amount of α-helix and β-sheet (28). For TFIIIC131-(1–580), the chosen solutions for seven independent spectra (see Fig. 5, solid line, for a representative spectrum) showed a constant amount (69 ± 4%) of these structures. With 38 ± 6% α-helix and 31 ± 4% β-sheet. Based on the known crystal structure of TPRs from protein phosphatase 5 (32), the amount of α-helix contributed solely by the TPRs in TFIIIC131-(1–580) is 39%. Thus, assuming that the TPRs in TFIIIC131-(1–580) adopt the same fold as in protein phosphatase 5, the CD data suggest that the non-TPR domains of the protein contain relatively little α-helix and are predominantly in a β-sheet structure. The non-TPR domains are also expected to contain a significant amount of random coil structure (16 ± 2%).

Trifluoroethanol (TFE) has been shown to induce α-helical structure in peptides and proteins with a propensity for α-helix formation (34). Given the prediction of random coil structure in TFIIIC131-(1–580), we examined the effect of TFE on the CD spectra of the protein. TFE concentrations up to 30% raised the α-helical content of the protein as indicated by the elevated maxima at 193 nm and the deeper double minima at 208 and 222 nm (Fig. 5). In contrast, only minor changes in the spectra were observed between 30 and 50% TFE, indicating that the maximum potential for α-helix formation had been reached.
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Fig. 4. Protease sensitivity of TFIIIC131(1–580). Shown is a time course of digestion of TFIIIC131(1–580) with three different proteases: chymotrypsin (A), trypsin (B), and endoproteinase Glu-C (D). Protein size markers are annotated in kilodaltons. The 55-kDa protease-resistant fragment is marked with arrows. In C are shown the results from far-Western analysis of a duplicate chymotrypsin digest (as in A) probed with 

35S-labeled TFIIIB70. In E is shown a schematic representation of chymotrypsin- and trypsin-resistant regions of TFIIIC131(1–580). Structural domains are the same as those shown in Fig. 1A. Vertical lines indicate potential protease cleavage sites. Horizontal lines define the limits of the protease-resistant regions determined by mass spectrometry. Dotted lines show cleavage heterogeneity generated by trypsin. The asterisk marks the sequence recognized by an anti-peptide antibody.

Fig. 5. Circular dichroism spectroscopy of TFIIIC131(1–580) in the presence and absence of trifluoroethanol. Samples containing 1.65 μM TFIIIC131(1–580) were prepared in 20 mM sodium phosphate, pH 7.0, and 25 mM NaCl. CD spectra were measured at room temperature in a 0.5-mm path length cell. After subtraction of the spectrum for buffer alone and conversion to molar ellipticity, the data were fitted using CONTIN. The plotted curves represent the best fits determined by CONTIN. The spectrum obtained in the absence of TFE is shown by a solid line. Spectra obtained in the presence of TFE are shown by broken lines. At 30–50% TFE, the spectra are essentially identical. deg, degrees.

Interestingly, the change in α-helicity predicted by CONTIN at 30–50% TFE was equal to the amount of random coil predicted in the absence of TFE. Presumably, the amount of unstructured protein in TFIIIC131(1–580) is minimal under these conditions.

Structural studies on the transactivation domains of several pol II transcription factors (e.g. VP16 and c-myc) have revealed a general lack of secondary structure in aqueous solution (35). The acquisition of structure upon interaction of these domains with components of the basal transcription machinery has demonstrated that the interactions proceed by an induced fit mechanism (35). Since the recruitment of TFIIIB70 by TFIIIC131 represents a functionally analogous interaction between pol III transcription components, we examined whether the binding of TFIIIB70 to TFIIIC131(1–580) could mimic the induction of secondary structure seen with TFE. Individual spectra were obtained for TFIIIC131(1–580) at 927 nm, for TFIIIB70 at 965 nm, and for a mixture of both proteins in buffer containing 80 mM NaCl. The interaction of TFIIIC131(1–580) and TFIIIB70 is strongly favored under these conditions since their concentrations are ~3.5 times the apparent dissociation constant determined under similar ionic conditions (see Fig. 3). CONTIN fits of the spectra for the two proteins alone were summed to produce the spectrum expected if no conformational change occurs upon binding (Fig. 6, solid line). A comparison of this spectrum with that observed for the mixture (Fig. 6, triangles) shows that there is a small but clear difference. As the presence of TFE, the elevated maxima at 193 nm and the deeper double minima at 208 and 222 nm reflect the formation of α-helical structure in the TFIIIC131-(1–580)-TFIIIB70 complex. This observation is supported by the fact that CD spectra acquired in 15 mM KCl under otherwise identical conditions did not exhibit a difference spectra; at this lower salt concentration, the sum of the individual spectra was superimposable with the mixture (data not shown). These data reveal a salt dependence for the interaction of TFIIIC131(1–580) with TFIIIB70. The existence of the CD difference spectra at the higher salt concentration has been independently confirmed in a mixture containing 4.66 μM TFIIIC131-(1–580) and 3.04 μM TFIIIB70 using a shorter path length cell (0.1 mm) to maintain an optimal signal (data not shown).

DISCUSSION

The interaction between TFIIIB70 and promoter-bound TFIIIC represents an important step in the recruitment of the initiation factor TFIIIB upstream of the transcription start site (6). Although multiple subunits of both TFIIIB and TFIIIC participate in this assembly reaction (10, 15, 16, 22), biochemical studies indicate that in yeast, the interaction between TFIIIB70 and TFIIIC-DNA is the major thermodynamically limited step (23, 25, 36). The binding of TFIIIB70 to the TFIIIC-DNA complex is mediated by the TPR-containing subunit, TFIIIC131 (15, 16), and potentially by TFIIIC95 (based on an interaction between the homologous proteins in humans) (10). However, mutations in TFIIIC131 increase the transcription of a mutant sup9-1 tRNA gene in vivo and facilitate the recruitment of TFIIIB70 to TFIIIC-DNA complexes in vitro (24,
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Fig. 6. A CD difference in the TFIIIC131-(1–580):TFIIB70 complex. Samples containing 927 nM TFIIIC131-(1–580), 965 nM TFIIB70, or a mixture of the proteins at these concentrations were prepared in 10 mM sodium phosphate, pH 7.8, and 80 mM NaCl. CD spectra were obtained at room temperature using a 0.5-mm path length cell. The measured molar ellipticities are represented by symbols, and the corresponding CONTIN fits are represented by broken lines. The calculated sum of the CONTIN fits for the individual TFIIB70 and TFIIIC131-(1–580) spectra is represented by the solid line.

Thus, the interaction between TFIIB70 and TFIIIC131 appears to be limiting for transcription complex assembly in vivo. To study this interaction in greater detail, we expressed a fragment of TFIIIC131, TFIIIC131-(1–580), that encompasses the minimum TFIIIB70 interaction domain (defined by two-hybrid studies) together with additional sequences implicated in the assembly reaction (16, 25). We showed that TFIIIC131-(1–580) inhibits pol III transcription in vitro (Fig. 1) by binding to TFIIB70 and by blocking preinitiation complex assembly (Figs. 2 and 3). In addition, we established a coupled equilibrium binding assay in which the formation of TFIIIC131-(1–580):TFIIB70 complexes in solution is linked to the inhibition of TFIIB8 complex assembly on a tRNA gene template. By quantifying TFIIB-DNA complexes resolved on native polyacrylamide gels as a function of TFIIIC131-(1–580) concentration, this assay has provided an indirect measure of the interaction between TFIIB70 and TFIIIC131-(1–580).

Simultaneous nonlinear least-squares fitting of multiple data sets to the Hill equation resulted in a Hill coefficient of $-1.4 \pm 0.2$, slightly higher than the theoretical value of unity expected for a noncooperative binary interaction. However, by applying the simplifying assumption that the data are adequately described by a single-site binding model, we determined an apparent dissociation constant of $334 \pm 23$ nM for the TFIIIC131-(1–580):TFIIB70 complex. This relatively low binding affinity is consistent with the limiting nature of the TFIIIC131-TFIIB70 interaction described above and, considering the size of the two proteins, suggests that the interface between them is quite small (37). The coupled binding assay used here is simple and, as indicated by the error, can be performed with high precision. In the absence of direct quantitative assays for this protein-protein interaction, the assay will be useful in analyzing the relative contributions of different regions/domains of TFIIIC131-(1–580) and in examining the effect of activating mutations in TPR2.

Examination of the structural organization of TFIIIC131-(1–580) by partial proteolysis showed it to be remarkably resistant to cleavage by a variety of proteases. This was especially noteworthy for trypsin and chymotrypsin because of the large number of potential cleavage sites and because of the location of these sites; potential cleavage sites for one or the other or both enzymes are located in the loops linking each of the 18 TPR helices (Fig. 4E) (32). Loops and turns are typically found on the solvent-accessible surface in proteins (38) and are thus often susceptible to cleavage. The proteolysis data therefore imply that the tertiary structure of TFIIIC131-(1–580) is such that the accessibility of these sites in TPR1–8, but not those in TPR9, is restricted. Similarly, the intervening non-TPR region that separates repeats 5 and 6 must be well structured since it is not cleaved readily by trypsin, chymotrypsin, endoprotease Glu-C, or subtilisin (Fig. 4 and data not shown). This result together with secondary structural predictions based on CD (Fig. 5 and above) and the known structure of TPRs support the idea that the intervening non-TPR region is predominantly $\beta$-sheet. Accordingly, we suggest that the protease-resistant core of TFIIIC131-(1–580) is likely to result from the packing of the two TPR domains together with the intervening non-TPR domain as opposed to these three domains being structurally isolated from one another. In contrast, parts of the hydrophilic amino-terminal domain are clearly solvent-exposed since this domain is susceptible to cleavage by trypsin and chymotrypsin in two distinct regions.

Circular dichroism spectroscopy in the presence of TFE provided evidence that a conformational change involving the acquisition of $\alpha$-helical structure occurs in TFIIIC131-(1–580). Moreover, a small but reproducible monovalent ion-dependent increase in $\alpha$-helical structure was detected upon formation of TFIIIC131-(1–580):TFIIB70 complexes. With respect to the magnitude of this increase in $\alpha$-helical structure, we note that the TPR crystal structure predicts a minimum of 228 $\alpha$-helical residues in TFIIIC131-(1–580). The acquisition of a typical 10-amino acid helix would therefore contribute, at most, a 4% increase in molar ellipticity for this protein alone. In a mixture of TFIIIC131-(1–580) and TFIIB70, the percent change in molar ellipticity would be reduced by the $\alpha$-helical content of TFIIB70 (inferred from the structure of TFIIB) (39). Therefore, the observed modest CD difference spectra are consistent with expectations given the size and structure of TFIIIC131-(1–580) and TFIIB70.

The increase in $\alpha$-helical structure detected on complex formation could be attributed to either or both proteins. However, the following observations suggest that $\alpha$-helix formation in the complex occurs largely within TFIIIC131-(1–580). (i) Experiments with TFE demonstrated a significant potential for the acquisition of $\alpha$-helicity in TFIIIC131-(1–580) (Fig. 5). In contrast, only a small change in the CD spectra of TFIIB70 was observed in the presence of TFE (data not shown). (ii) The signal detected by CD (molar ellipticity) is proportional to the molar extinction coefficient of the protein, which is 2.7 times higher for TFIIIC131-(1–580) than for TFIIB70. Thus, in an approximately equimolar mixture of the two proteins, both of which contain significant amounts of $\alpha$-helix (see above and Refs. 32 and 39), the CD spectrum is predominantly that of TFIIIC131-(1–580) (Fig. 6).

An increase in $\alpha$-helicity in the TFIIIC131-(1–580):TFIIB70 complex may result from the stabilization of structure at the interface between the two proteins. This induced fit mechanism has been reported previously for other protein-protein interactions (35) and is a common feature of many protein-nucleic acid interactions (37, 39). An intriguing alternative possibility is that $\alpha$-helical structure may be acquired at a site distal to the binding interface (by the propagation of conformational changes, e.g., see Refs. 40 and 41) and that the resulting structure may be important for subsequent steps in transcription complex assembly or function. A structural change of this type is compatible with the concerted sequence of conformational changes documented by site-specific DNA-protein photocross-linking during TFIIC-dependent assembly of TFIIB (2). In particular, TFIIB70-dependent changes in the structure of
TFIIC appear to be important for allowing entry of TFIIIB90 into the preinitiation complex (42). TFIIIB70-dependent helix formation in TFIIC131 could potentially play a role at this step. Further examination of these possibilities will require delimiting the region of helix formation in TFIIC131-(1–580) and an analysis of mutants that fail to adopt this structure.

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Interactions between the Tetratricopeptide Repeat-containing Transcription Factor TFIIIC131 and Its Ligand, TFIIIB70: EVIDENCE FOR A CONFORMATIONAL CHANGE IN THE COMPLEX
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