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A Tetratricopeptide Repeat Mutation in Yeast Transcription Factor $IIIC_{131}$ ($TFIIIC_{131}$) Facilitates Recruitment of TFIIB-Related Factor $TFIIIB_{70}$

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Transcription factor IIIC (TFIIIC) plays an important role in assembling the initiation factor TFIIIB on genes transcribed by RNA polymerase III (Pol III). In *Saccharomyces cerevisiae*, assembly of the TFIIIB complex by promoter-bound TFIIIC is thought to be initiated by its tetratricopeptide repeat (TPR)-containing subunit, $TFIIIC_{131}$, which interacts directly with the TFIIB-related factor, $TFIIIB_{70}$/Brf1. In this work, we have identified 10 dominant mutations in $TFIIIC_{131}$ that increase Pol III gene transcription. All of these mutations are found within a discrete 53-amino-acid region of the protein encompassing TPR2. Biochemical studies of one of the mutations (*PCF1-2*) show that the increase in transcription is due to an increase in the recruitment of $TFIIIB_{70}$ to TFIIIC-DNA. The *PCF1-2* mutation does not affect the affinity of TFIIIC for DNA, and the differential in both transcription and TFIIIB complex assembly is observed at saturating levels of $TFIIIB_{70}$. This indicates that mutant and wild-type TFIIIC-DNA complexes have the same affinity for $TFIIIB_{70}$ and suggests that the increased recruitment of this factor is achieved by a nonequilibrium binding mechanism. A novel mechanism of activation in which the TPR mutations facilitate a conformational change in TFIIIC that is required for $TFIIIB_{70}$ binding is proposed. The implications of this model for the regulation of processes involving TPR proteins are discussed.

The multisubunit transcription factor IIIC (TFIIIC) is an assembly factor that directs the binding of the general initiation factor, TFIIIB, upstream of the transcription start site of RNA polymerase III (Pol III) genes (21, 37). On tRNA gene templates, TFIIIC interacts with intragenic A and B block promoter elements. High-affinity binding of TFIIIC to DNA is mediated by the B block. This element is positioned at variable distances from the start site and, in at least one example, can function, albeit less efficiently, in an inverted orientation (5). These properties, together with the requirement for TFIIIC in transcription from chromatin templates (antirepression), provide a conceptual view of TFIIIC as an enhancer binding factor (5, 6). TFIIIC, through interactions with the A block, also plays a role in start site selection. Typically, the start site is located about 20 bp upstream of the A block. However, its position can be shifted within a 20- to 30-bp window by altering the placement of TFIIIB on the DNA (7, 10, 12, 15). Redirection of TFIIIB involves its TATA-binding protein (TBP) subunit, in addition to TFIIIC, and can be achieved by inserting, repositioning, or mutating an upstream TATA element. The conformational flexibility required for this variable placement of TFIIIB has been suggested to reside in the TFIIIB-assembling subunit of TFIIIC, $TFIIIC_{131}$ (15).

Specific transcription by Pol III can be achieved in the absence of TFIIIC on nucleosome-free templates which contain a suitable TATA box and an initiation site. Transcription of the *Saccharomyces cerevisiae* U6 gene, which contains the sequence TATAAATA 30 bp upstream of the start site, has been analyzed extensively in this regard and defines the concept of basal transcription in the Pol III system (5, 6, 10, 12, 14, 38). TATA box-directed transcription requires the same three components of TFIIIB (TBP, $TFIIIB_{70}$, and $TFIIIB_{90}$) that are necessary for TFIIIC-dependent reactions. Interestingly, on a nucleosome-free yeast U6 gene, addition of TFIIIC stimulates the formation of a TFIIIB complex (14). This is indicative of a role for TFIIIC in activating Pol III transcription that is independent of its antirepression activity.

In addition to its functions in recruiting and positioning TFIIIB, TFIIIC participates in a step which is limiting for transcription. This limiting step is defined by a dominant mutation, *PCF1-1*, which increases Pol III transcription in vivo and in vitro (39). The *PCF1-1* mutation causes a histidine-to-tyrosine change in the second of 11 tetratricopeptide repeats (TPRs) found in $TFIIIC_{131}$ (32) (Fig. 1a). However, the specific step facilitated by this mutation and its mechanism of action have not been determined. In this work, we report the identification of nine new dominant *PCF1* alleles. These mutations as well as the original *PCF1-1* allele map to a functionally unique 53-amino-acid region involved in transcriptional activation. Biochemical characterization of one of the new alleles, *PCF1-2*, shows that the mutation activates Pol III transcription by increasing the recruitment of the TFIIB-related subunit, $TFIIIB_{70}$, into the preinitiation complex. In addition, we demonstrate that the increased recruitment of $TFIIIB_{70}$ is achieved by a nonequilibrium binding mechanism.

MATERIALS AND METHODS

***PCF1* alleles.** *PCF1-1*, *PCF1-2*, and *PCF1-23* were cloned from genomic libraries of mutant strains which showed increased expression of the Pol III reporter gene *sup9-e A19-supS1* (32, 39). Unique dominant mutations were confirmed by sequencing the entire gene. The other mutations were obtained by PCR mutagenesis (26) of an 1,100-bp *Eag*I/*Aat*II fragment of the *PCF1*/*TFC4* gene (29, 32). Based on the error rate of the Stoffel fragment under the conditions employed (10^{-4} to 10^{-5} errors/bp), the size of the amplified fragment (1,100 bp), and 10 cycles of PCR amplification, we estimate that approximately 1 molecule in every 5 to 50 molecules would contain a mutation. The amplified DNA was used to replace the wild-type sequence of a *PCF1* clone in pRS313, and

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the resulting *Escherichia coli* plasmid library comprising 105,000 clones was transformed into a wild-type yeast strain, IW1B6U (IW1B6 containing a chromosomal copy of *sup9-e A19-supS1* [39]). A total of about 50,000 yeast transformants were replica plated to Trp^- Met^- medium to identify *supS1*-expressing colonies. A total of 92 Trp^+ Met^+ colonies were obtained. Forty-one of these were sequenced completely, yielding seven new mutations. Each mutant gene contained only a single point mutation. A uniform distribution of mutations throughout the amplicon was confirmed by sequencing of independently selected conditional *PCF1* alleles. Suppressor activity was quantified by measuring β-galactosidase activity (1) in strains transformed with the plasmid pUKC352, which contains an amber-suppressible *PGK1-lacZ* reporter gene (11). Transformants were grown in synthetic complete medium lacking uracil and leucine to select for both the suppressor and the *lacZ* reporter plasmid. Assays were conducted on six cultures for each strain.

Extracts and transcription factors. Whole-cell extracts were prepared from isogenic wild-type and *PCF1-2* strains (IW1B6 and an outcross of mutant 12-11) and were fractionated on BioRex70 (32, 39). TFIIIB, TFIIIC, and Pol III were resolved by chromatography on DEAE–Sephadex A-25 (17). TFIIIC fractions were free of detectable levels of TFIIIB components as assayed by transcription and gel shift analysis. B″ was isolated from chromatin by urea extraction and BioRex70 chromatography (20). Pure recombinant TBP (rTBP) was provided by M. Brenowitz. $rTFIIIB_{70}$ was purified to homogeneity by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (27). The same method was used to purify $rTFIIIB_{90}$. ^{35}S-$TFIIIB_{70}$ was synthesized in a rabbit reticulocyte lysate and quantified by Western blot analysis with $rTFIIIB_{70}$ as a standard (34). ^{35}S-TBP was prepared similarly.

Transcription and complex assembly assays. Transcription with the BioRex fraction BRα (41 μg of protein) was performed on a *SUP4* template (0.5 μg) under multiple-round (39), modified single-round (34), and nascent (17-mer) RNA synthesis (17) conditions. Reconstituted transcription was performed under multiple-round conditions on a $tRNA^{Leu}$ template (YEp13; 0.5 μg) with wild-type or mutant TFIIIC (0 to 5 μg), Pol III (0.6 μg), rTBP (200 fmol), $rTFIIIB_{70}$ (2,000 fmol), and B″ (4.3 μg). DNA beads were prepared by coupling a *Dra*I/*Bam*HI-cleaved PCR fragment of the *sup3-e G10* gene (−132 to +200) to CNBr-activated Sepharose CL4B (Sigma [16]). Beads with or without coupled DNA were incubated with each BRα fraction under transcription conditions (39) without nucleoside triphosphates in 50-μl reaction mixtures for 60 min at 4°C. Complexes were stripped with 0.5 M NaCl and washed extensively in 20 mM HEPES-KOH (pH 7.8)–100 mM NaCl–0.1 mM EDTA–1 mM dithiothreitol–20% glycerol, and the bound proteins were eluted into Laemmli buffer (25 μl) by heating at 85°C for 10 min. SDS-polyacrylamide gel electrophoresis, Western blotting for $TFIIIB_{70}$, and laser densitometry were performed as described elsewhere (34). TFIIIB complex assembly in the BRα fraction was performed under transcription conditions (see above) with the following exceptions: reactions were in 20 μl and contained a 360-bp *sup3-eST* fragment (32) (300 fmol, 30 Ci/mmol; labelled by PCR), duplex poly(dI-dC) (25 μg/ml), and BRα fraction (15 μg) and were incubated for 60 min at 20°C. Heparin was added to 300 μg/ml for an additional 10 min (no serum) or for 1 h at 4°C (with serum) before analysis on a 5% polyacrylamide high-ionic-strength (50 mM Tris, 380 mM glycine [pH 8.3], 2 mM EDTA) native gel. After the gel was dried, the TFIIIB complex was quantified from a phosphorimage with the ImageQuant peak finder to analyze lines one lane wide. Complex assembly with recombinant or in vitro-synthesized TFIIIB subunits was performed similarly with the *sup3-eST* fragment (10 fmol), TFIIIC (10 fmol), and TBP (500 fmol), $TFIIIB_{70}$ (2,000 fmol), and $rTFIIIB_{90}$ (100 fmol) as specified. After complex assembly, sonicated salmon sperm DNA (1 μg) was added for 10 min before electrophoresis on 4% low-ionic-strength (0.5× Tris-borate-EDTA) native gels containing 5% glycerol and 1.5 mM magnesium acetate. TFIIIC-DNA complexes were assembled and separated similarly for determination of apparent K_d (2). Complexes were quantified as described above. Complexes for gel filtration were assembled for 30 min at 20°C in 50-μl reaction mixtures containing a *sup9-e* tRNA gene on a 9.5-kb plasmid (84 fmol), TFIIIC (60 fmol), and ^{35}S-$TFIIIB_{70}$ (2,000 fmol). Reaction mixtures were loaded onto 1-ml columns of Sepharose CL2B in 20 mM HEPES-KOH (pH 7.8)–80 mM NaCl–10 mM $MgCl_2$–0.1 mM EDTA–1 mM dithiothreitol–0.01% Brij 58–5% glycerol at room temperature (19). One-drop fractions were collected, subjected to SDS-polyacrylamide gel electrophoresis and analyzed with a phosphorimager.

RESULTS

Identification of dominant activating mutations in $TFIIIC_{131}$. To better define the limiting function of $TFIIIC_{131}$ in transcription, additional *PCF1* alleles were identified from our mutant strain collection while others were generated by PCR mutagenesis. The latter was conducted on an 1,100-bp fragment containing the first nine TPRs of the protein (Fig. 1a). A total of 10 mutations were recovered by genetic selection in yeast based on their ability to increase the expression of a reporter tRNA gene (*sup9-e A19-supS1*) in a dominant or codominant manner (Fig. 1b and c). Nine of the mutations had

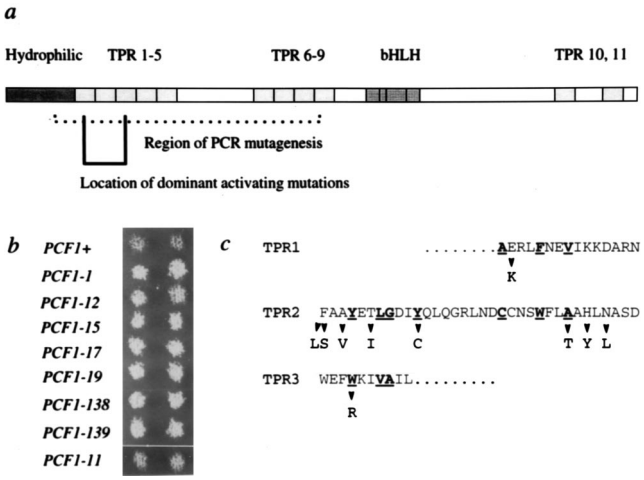

FIG. 1. Identification of dominant activating mutations in $TFIIIC_{131}$. (a) Distribution of TPRs in $TFIIIC_{131}$ (1,025 amino acids) and other structural domains (29, 32), the region of PCR mutagenesis, and the location of the dominant activating mutations. bHLH, basic helix-loop-helix. (b) Comparison of the phenotypes of wild-type and dominant *PCF1* alleles, in duplicate, in a wild-type merodiploid strain. (c) Partial amino acid sequence for TPRs 1 to 3 and the location of the dominant mutations. The corresponding *PCF1* allele numbers, listed from the amino-terminal end, are *PCF1-17*, *PCF1-12*, *PCF1-139*, *PCF1-19*, *PCF1-2*, *PCF1-11*, *PCF1-23*, *PCF1-1*, *PCF1-138*, and *PCF1-15*. Residues which define the TPR motif are in boldface and underlined.

strong suppressor phenotypes comparable to that of the *PCF1-1* allele described previously (32, 39), while one mutation (*PCF1-11*) exhibited only modest suppressor activity (Fig. 1b and data not shown). To relate the increased growth on selective medium (Fig. 1b) to a quantitative measure of the increase in nonsense suppressor activity, an amber termination codon readthrough assay was used (11). In this assay, readthrough of an in-frame amber termination codon in a *PGK1-lacZ* gene fusion allows suppression to be quantified by measuring β-galactosidase activity. The differential in *sup9-e A19-supS1* suppressor activity between a wild-type and a *PCF1-1* strain was found to be 2.9- ± 0.4-fold.

Seven of the dominant mutations were obtained by PCR mutagenesis of a DNA fragment encoding more than one-third of the 1,025 amino acids that comprise $TFIIIC_{131}$. Considering the relatively large size of this fragment, it is striking that these mutations as well as the three ethyl methanesulfonate (EMS)-generated mutations mapped to nine sites within a 53-amino-acid region (Fig. 1c and Materials and Methods). This region encompasses TPR2 and extends into the carboxy-terminal half of TPR1 and the amino-terminal half of TPR3. The dominant suppressor active phenotype of these mutations represents a unique property that is distinct from other mutations in this region. For example, in a screen for conditional *PCF1* alleles we have identified five mutations in TPRs 1 to 3 which are not suppressor active at the permissive temperature. Interestingly, 3 of the 10 suppressor mutations result in nonconservative changes in residues that define the predicted knob and hole structure of the repeat (25). The remaining mutations occur at positions that are not conserved among the TPR family of proteins. These data suggest that the increase in transcription conferred by the mutations may result from either local disruption of the hypothetical TPR structure or the formation of additional interactions involving nonconserved TPR residues in this region.

The *PCF1-2* mutation increases TFIIIB recruitment. A biochemical basis for how the dominant mutations increase Pol III

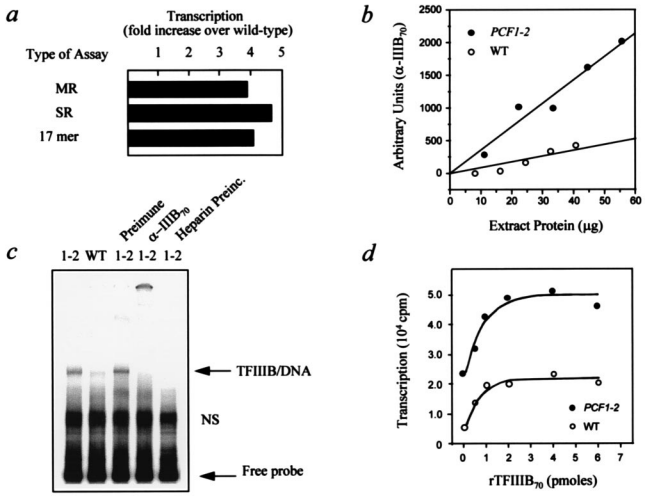

FIG. 2. Transcription and TFIIIB complex assembly in BioRex70-fractionated wild-type and *PCF1-2* extracts. (a) Bar graph showing the effect of the *PCF1-2* mutation on transcription of a *SUP4* template under conditions which permit multiple rounds of initiation (MR), limit initiation to approximately once per complex (SR), or measure nascent RNA synthesis (17-mer). Equal amounts of extract protein were used. (b) Assembly of $TFIIIB_{70}$ into salt-stable TFIIIB-DNA complexes in a solid-phase pull-down assay. The plotted data have been corrected for nonspecific binding of $TFIIIB_{70}$ to Sepharose CL4B in the absence of DNA. WT, wild type. (c) Formation of heparin-resistant TFIIIB-DNA complexes by native gel electrophoresis. The extract (wild-type [WT] or mutant [1-2]) used for complex assembly is indicated above each lane. The addition of preimmune or anti-$IIIB_{70}$ serum after complex formation or the addition of heparin before complex formation is also shown. NS, nonspecific. (d) Supplementation of wild-type (WT) and *PCF1-2* extracts with $rTFIIIB_{70}$. Multiple-round conditions and a $tRNA^{Leu}$ gene template were used.

transcription has emerged from studies of transcription and transcription complex assembly in crude extracts and with partially purified or recombinant factors. The following experiments were conducted with extracts from isogenic wild-type and *PCF1-2* strains. The *PCF1-2* mutation causes a threonine-to-isoleucine change at position 6 in TPR2 (Fig. 1c). In BioRex70-fractionated whole-cell extracts, the *PCF1-2* mutation caused a 5.0- $\pm$ 0.5-fold increase in transcription from a variety of Pol III templates (e.g., *sup9-e*, *SUP53*, and *SUP4*) under multiple-round conditions. The same templates showed a 4.7- $\pm$ 0.4-fold increase in transcription under single-round conditions. The results of representative assays conducted with the *SUP4* template are shown in Fig. 2a, which compares nascent transcript synthesis with multiple-round and single-round reactions. These results imply that the *PCF1-2* mutation increases the number of functional transcription complexes. Additionally, the nascent RNA synthesis assay showed that the mutant extract promotes correctly initiated Pol III transcription. Direct evidence that the *PCF1-2* mutation increases the number of TFIIIB-DNA complexes is shown by two independent methods in Fig. 2b and c. A TFIIIB-DNA complex pull-down assay was performed by assembling transcription complexes under equilibrium conditions on DNA beads. After extensive washing under conditions known to retain TFIIIB-DNA complexes (19), the bound proteins were eluted and analyzed by Western blotting with a $TFIIIB_{70}$-specific antibody. The linear regression lines in Fig. 2b reveal a fourfold differential in TFIIIB-DNA complex assembly commensurate with the transcriptional differential of the extracts. The same fourfold differential was observed in the ability of wild-type and *PCF1-2* extracts to assemble heparin-resistant TFIIIB-DNA complexes in a gel shift assay (Fig. 2c). The specificity of complex assembly in this assay is demonstrated by the ability of a $TFIIIB_{70}$-specific antibody, but not preimmune serum, to supershift the heparin-stripped complex and by the absence of the complex in a reaction to which heparin was added prior to the DNA.

We next determined whether the effect of the *PCF1-2* mutation on transcription (and presumably TFIIIB complex assembly) could be recapitulated in wild-type extracts by adding increasing amounts of the individual TFIIIB components. We anticipated that if the mutation affected the affinity of TFIIIC for any component of TFIIIB, the level of transcription at saturating concentrations of that factor would be the same in both wild-type and mutant extracts. In accordance with previous studies (34) which showed that neither TBP nor B″ is limiting for transcription in crude extracts, addition of rTBP (up to 10 pmol) or urea-extracted B″ (up to 6 μg of protein) did not increase transcription in either the wild-type or the *PCF1-2* mutant extract (data not shown). In contrast, the addition of $rTFIIIB_{70}$ increased transcription in both extracts (Fig. 2d). This result is consistent with $TFIIIB_{70}$ being the limiting factor in these extracts (34). Interestingly, at saturating levels of $TFIIIB_{70}$ (>2 pmol of exogenous factor) the *PCF1-2* extract was still 2.5-fold more active than wild type. Thus, the transcription differential between the wild-type and the *PCF1-2* extract persists at saturating levels of each TFIIIB component. Given the increase in TFIIIB complex assembly shown in Fig. 2b and c, this result indicates that *PCF1-2* increases transcription without affecting the affinity of TFIIIC for any TFIIIB subunit. Additional experiments supporting this conclusion are described below.

Partially purified TFIIIC preparations from wild-type and *PCF1-2* extracts were used to analyze DNA binding, TFIIIB complex assembly, and transcription activity. Both TFIIIC preparations have the same affinity for binding to a *sup3-e* tRNA gene probe (Fig. 3a). The apparent dissociation constants calculated by Scatchard analysis were $(2.7 \pm 0.4) \times 10^{-10}$ and $(2.8 \pm 0.3) \times 10^{-10}$ M for wild-type and mutant TFIIIC, respectively. Additionally, both TFIIIC fractions have equivalent levels of $TFIIIC_{131}$ protein as determined by Western analysis (data not shown). However, the mutant TFIIIC fraction is approximately threefold more active per femtomole of DNA binding activity than wild type in a reconstituted transcription system comprising rTBP, $rTFIIIB_{70}$, and either $rTFIIIB_{90}$ or a urea-extracted chromatin fraction (B″) containing this factor (Fig. 3b and data not shown). As in Fig. 2d, the transcription differential seen here is independent of the concentration of the individual TFIIIB components, since a functional excess of each TFIIIB component has been determined empirically.

***PCF1-2* facilitates the recruitment of $TFIIIB_{70}$.** To investigate which step in TFIIIB complex assembly is affected by the *PCF1-2* mutation, wild-type or mutant TFIIIC-DNA complexes were analyzed for their ability to recruit pure recombinant or in vitro-synthesized TFIIIB components. In all of the gel shift experiments which follow, TFIIIC is the limiting factor and the results are representative of numerous experiments. After an incubation to establish equilibrium binding, the complexes were resolved on native gels. Under the conditions employed, the numbers of wild-type and mutant TFIIIC-DNA complexes formed are equal (Fig. 3c, lanes 2 and 3). In contrast, an approximately threefold difference in the number of TFIIIB-TFIIIC-DNA complexes was detected with either ^{32}P-DNA (Fig. 3c, lanes 11 and 12), ^{35}S-TBP (Fig. 3d, lanes 7 and 8), or ^{35}S-$TFIIIB_{70}$ (Fig. 3d, lanes 13 and 14) probes. Complexes assembled in the absence of $TFIIIB_{90}$, i.e., complexes containing TBP, $TFIIIB_{70}$, TFIIIC, and DNA (Fig. 3c, lanes 7

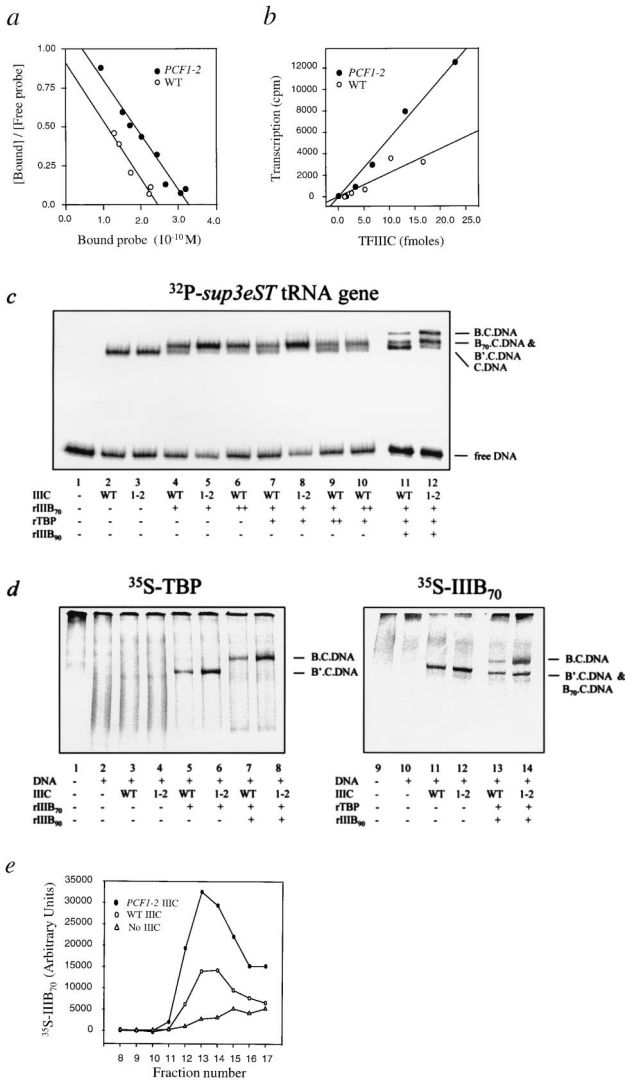

FIG. 3. Analysis of DNA binding, transcription, and TFIIIB complex assembly by wild-type (WT) and *PCF1-2* TFIIIC fractions. (a) Scatchard analysis of TFIIIC binding to a *sup3-eST* tRNA gene promoter. Apparent K_ds were determined from the negative reciprocals of the slopes of each line. (b) Transcription of a $tRNA^{Leu}$ gene was reconstituted under multiple-round conditions with wild-type or mutant TFIIIC, rTBP, $rTFIIIB_{70}$, a B″ fraction containing $TFIIIB_{90}$, and Pol III. The presence of a functional excess of each component (other than TFIIIC) was established by the concentration independence of transcription at or above the amounts used. TFIIIC femtomoles represent DNA binding activity. (c) Native gel electrophoresis of transcription complexes assembled on a ^{32}P-labelled *sup3-eST* tRNA gene. Reaction components included saturating amounts of each recombinant TFIIIB component as indicated under each lane. ++ indicates that twice the amount of a particular factor was used. B′.C.DNA complexes contain both $TFIIIB_{70}$ and TBP and comigrate with the B_{70}.C.DNA complexes. (d) Native gel electrophoresis of complexes containing ^{35}S-TBP (lanes 1 to 8) or ^{35}S-$TFIIIB_{70}$ (lanes 9 to 14). The reaction components indicated under each lane included the unlabelled *sup3-eST* gene and the same saturating concentration of each TFIIIB subunit as for panel c. The indicated positions of specific complexes were confirmed with the ^{32}P-labelled *sup3-eST* probe. (e) Isolation of ^{35}S-$TFIIIB_{70}$–TFIIIC–DNA complexes by size exclusion on Sepharose CL2B. Wild-type or mutant TFIIIC complexes formed on a plasmid containing the *sup9-e* tRNA gene in the presence of saturating amounts of ^{35}S-$TFIIIB_{70}$ were loaded onto 1-ml Sepharose CL2B columns. The graph shows a phosphorimage quantitation of $TFIIIB_{70}$ in the excluded fractions.

and 8, and d, lanes 5 and 6), also showed a threefold difference, suggesting that the mutation affected a step before the recruitment of $TFIIIB_{90}$ and at or before the recruitment of TBP. Indeed, wild-type and mutant TFIIIC-DNA complexes exhibited differential binding of $TFIIIB_{70}$, albeit at a reduced level (1.4- to 2.0-fold) compared to the higher-order complexes (Fig. 3c, lanes 4 and 5, and d, lanes 11 and 12). Importantly, the level of complex assembly observed on wild-type TFIIIC-DNA is not limited by the amount of $TFIIIB_{70}$ or TBP, since addition of twice the amount of these factors does not affect the differential (Fig. 3c: compare lanes 4 and 6 with lane 5 and compare lanes 7, 9, and 10 with lane 8). The persistence of the differential between wild-type and mutant TFIIICs in the assembly of $TFIIIB_{70}$-containing complexes at saturating concentrations of the TFIIIB subunits supports the conclusions reached from the transcription experiments (Fig. 2d and 3b) and indicates a nonequilibrium mechanism of action (see below).

The relatively low affinity of $TFIIIB_{70}$ for TFIIIC-DNA complexes (20, 27) and consequent likelihood of dissociation of the $TFIIIB_{70}$-TFIIIC-DNA complex during electrophoresis may explain why the differential in the assembly of these complexes is slightly less than that measured for higher-order complexes. We therefore examined the binding of $TFIIIB_{70}$ to TFIIIC-DNA by a comparatively rapid gel filtration assay which separates DNA-bound complexes from unbound factors (19). We found that the amount of ^{35}S-$TFIIIB_{70}$ excluded from Sepharose CL2B in a TFIIIC-dependent manner was threefold higher for the mutant TFIIIC fraction than for wild type (Fig. 3e). From these experiments, we conclude that *PCF1-2* increases transcription by facilitating the recruitment of $TFIIIB_{70}$ to the TFIIIC-DNA complex.

DISCUSSION

In this work, we have identified a functionally unique domain in the 131-kDa subunit of TFIIIC that is involved in activating Pol III gene transcription. In addition, we have determined the step in transcription complex assembly that is facilitated by one of the mutations in this domain (*PCF1-2*) and we have established that the mechanism of activation by this mutation follows a nonequilibrium binding model. Each of these points and their implications are discussed below. Our genetic selection for increased Pol III gene transcription has yielded 10 dominant mutations in the *PCF1* gene which encodes $TFIIIC_{131}$. Considering that $TFIIIC_{131}$ is a protein of 1,025 amino acids, the 10 mutations map to a relatively small region of about 50 amino acids encompassing the second TPR (Fig. 1). This localization of the mutations together with their common phenotype suggests that they affect the same biochemical function.

In addition to activating Pol III transcription in vivo, two of the mutations, *PCF1-1* and *PCF1-2*, have now also been shown to activate Pol III transcription in vitro (32) (Fig. 2). Biochemical studies reported here of the effect of the *PCF1-2* mutation demonstrate that mutant TFIIIC has a higher specific activity than wild-type TFIIIC in TFIIIB complex assembly and transcription assays but is equivalent to wild-type TFIIIC with regard to its DNA binding activity (Fig. 3). Moreover, the increase in TFIIIB complex assembly was shown to result from the facilitated recruitment of the TFIIB-related transcription factor, $TFIIIB_{70}$ (Fig. 3c and e). The ability of mutations in $TFIIIC_{131}$ to increase the recruitment of $TFIIIB_{70}$ is consistent with the known direct interaction between these polypeptides detected by a glutathione *S*-transferase pull-down assay in vitro (22) and by two-hybrid interactions in vivo (8).

TFIIIC is typically referred to as an assembly factor based on its ability to recruit the initiation factor TFIIIB upstream of the start site of Pol III genes (19). This function of TFIIIC is achieved through its sequence-specific recognition of A and B

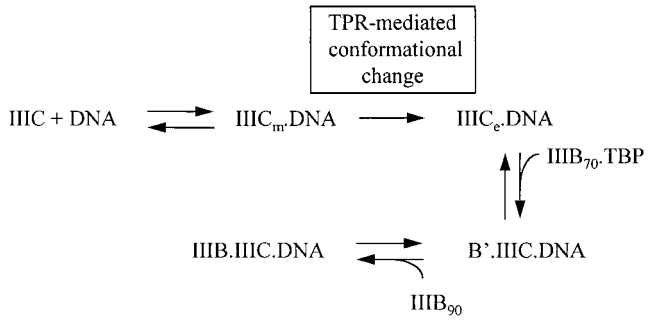

FIG. 4. A model for transcriptional activation by dominant TPR mutations in TFIIIC$_{131}$. The assembly of TFIIIC and the components of TFIIIB onto a Pol III template such as a tRNA gene is shown as sequential binding equilibria (19) except for the hypothesized TPR-mediated conformational change. IIICm.DNA and IIICe.DNA represent TFIIIC-DNA complexes in which the binding site for TFIIIB$_{70}$ is masked and exposed, respectively. The binding of TFIIIB$_{70}$ and TBP may occur as a complex as indicated or may proceed sequentially as demonstrated in vitro (20).

block promoter elements in tRNA-type genes and through protein-protein interactions between one of its subunits, TFIIIC$_{131}$, and the 70-kDa subunit of TFIIIB (8, 22). After recruiting TFIIIB to DNA, TFIIIC is dispensable for initiation and thus is not considered a general initiation factor (19). Indeed, on TATA-containing Pol III genes such as the yeast U6 gene, TFIIIC is not required for basal transcription in vitro since TFIIIB can be assembled via its TBP subunit (5, 6, 12, 14, 38). Importantly, the addition of TFIIIC significantly stimulates TFIIIB complex assembly and transcription on this template (14, 24). These features of TFIIIC are clearly analogous to those of classical activator proteins that function in transcription by RNA Pol II (31, 35). Our finding that mutations in a TFIIIC subunit stimulate the recruitment of one of the Pol III basal transcription factors further strengthens this analogy.

One common mechanism of activator function involves effects on the equilibrium of formation of the basal transcription complex (31). Interestingly, our results show that the increased recruitment of TFIIIB$_{70}$ by mutant TFIIIC-DNA complexes is not achieved in this manner but instead proceeds by a nonequilibrium mechanism: (i) the transcription differential between wild-type and mutant TFIIIC persists in cell extracts that have been supplemented with saturating levels of rTFIIIB$_{70}$ (Fig. 2d); (ii) the transcription differential is maintained in a reconstituted system containing a functional excess of TFIIIB$_{70}$, TBP, and TFIIIB$_{90}$ (Fig. 3b); and (iii) saturating levels of TFIIIB$_{70}$ (or other TFIIIB components) do not drive complex assembly with wild-type TFIIIC to the extent observed with the mutant TFIIIC fraction (Fig. 3c: compare lanes 4 to 6 and 7 to 10). This indicates that all of the available TFIIIB$_{70}$ binding sites on TFIIIC are occupied under these conditions. How then is the differential recruitment of TFIIIB$_{70}$ achieved? The data are consistent with a mechanism of activation by TFIIIC$_{131}$ in which the TPR mutations facilitate a conformational change that exposes the TFIIIB$_{70}$ binding site (Fig. 4). In this way, the number of mutant TFIIIC molecules capable of binding TFIIIB$_{70}$ is increased relative to the wild-type factor. Further support for this mechanism comes from photocross-linking studies which suggest that at least two conformational changes occur in TFIIIC$_{131}$ during transcription complex assembly (upon binding TBP and TFIIIB$_{90}$, respectively [20]). The conformational flexibility of TFIIIC$_{131}$ has also been suggested to contribute to the variable placement of TFIIIB on templates with altered 5′ flanking sequences (15). Additionally, the existence of intramolecular interactions in TFIIIC$_{131}$ that shield the TFIIIB$_{70}$ interaction domain(s) has been suggested by a two-hybrid study of their interactions (8). Finally, the characteristics of 3 of the 10 TPR mutations described here (*PCF1-11*, *PCF1-15*, and *PCF1-23*) favor a conformational model since they are predicted to disrupt the local TPR structure (Fig. 1c). Accordingly, the ability of the dominant mutations to increase Pol III transcription in vivo and in vitro indicates that the proposed TPR-mediated conformational change is normally limiting for the assembly of TFIIIB$_{70}$ into the preinitiation complex. The idea that a binding site for a general initiation factor might be masked or exposed by a conformational change in an activator represents a potentially novel mechanism for regulating the function of this class of proteins.

The biochemical relationship between the proposed TFIIIC conformers (Fig. 4) is currently unknown. However, studies conducted in several laboratories including our own have shown that TFIIIB-TFIIIC-DNA complex assembly at salt concentrations of 80 to 100 mM is complete (i.e., has apparently reached equilibrium) within 30 to 40 min at 20°C (9, 18). Since saturating concentrations of TFIIIB$_{70}$ cannot drive the assembly reaction on wild-type TFIIIC-DNA complexes (Fig. 3c), we suggest that the rate of formation of the TFIIIC$_e$-DNA conformer must be very slow relative to the rate of TFIIIB-TFIIIC-DNA complex assembly.

How does the TFIIIB$_{70}$ binding site on TFIIIC$_{131}$ become exposed as a result of the proposed conformational change? Although additional studies will be required to answer this question, some suggestions can be advanced based on current information. The TFIIIB$_{70}$ binding site on TFIIIC$_{131}$ appears to be multipartite since different regions of the protein, including an amino-terminal fragment containing TPR2, can interact with TFIIIB$_{70}$ in solution (22). However, residues in TPR2 probably do not contribute a large number of direct contacts (if any) with TFIIIB$_{70}$, since TPR2 can be deleted without affecting significantly a two-hybrid interaction between these proteins (8). In contrast, the loss of this two-hybrid interaction upon deletion of TPR1 or the acidic amino terminus of TFIIIC$_{131}$ suggests that these two regions comprise the primary binding site(s) for TFIIIB$_{70}$ (8). Since 3 of our 10 mutations result in nonconservative changes in residues that define the predicted knob and hole structure of the TPR, they could potentially disrupt intramolecular TPR-TPR interactions between TPR2 and the adjacent repeats (Fig. 1c). It seems likely that unfolding of these repeats would expose residues in TPR1 and promote the formation of the TFIIIB$_{70}$ binding site.

Additional studies with the yeast two-hybrid system have shown that deletion of TPR2 increases an interaction between TFIIIC$_{131}$ and the 90-kDa subunit of TFIIIB (33). This suggested a negative role for TPR2 in the recruitment of TFIIIB$_{90}$ and provided a possible explanation for the effect of the dominant *PCF1* mutations; increased transcription might result from the mutations overcoming the negative influence of TPR2 on the binding of TFIIIB$_{90}$. We have examined the association of TFIIIB$_{90}$ with wild-type and mutant TFIIIC complexes in the presence of saturating TFIIIB$_{70}$ and TBP by native gel electrophoresis. In these experiments, the formation of heparin-resistant TFIIIB-DNA complexes was quantified as a function of TFIIIB$_{90}$ concentration by a nonlinear least-squares analysis (4). The resulting binding isotherms showed that complexes containing mutant TFIIIC do not have a higher affinity for TFIIIB$_{90}$ than wild-type complexes (30). The results of these experiments will be published elsewhere. Consequently, the increase in transcription resulting from the *PCF1-2* mutation can be accounted for solely by the increase in

$TFIIIB_{70}$ recruitment (e.g., an approximately threefold differential is seen in Fig. 2d and 3b to 3). Interestingly, the magnitude of this biochemical effect appears to be sufficient to account for the increased growth exhibited by the strong *PCF1* alleles, since the *PCF1-1* mutation was shown to increase *sup9-e A19-supS1* suppressor activity in vivo about threefold.

The increase in transcription resulting from the *PCF1-1* and *PCF1-2* mutations is readily apparent in crude extracts (including the BRα fraction) and in reconstituted systems comprising purified TFIIIC and entirely recombinant TFIIIB factors or urea-extracted B″ (reference 32 and this work). However, attempts to reconstitute the transcription differential with solely fractionated components from yeast have been unsuccessful (reference 32 and data not shown). Reactions containing either wild-type TFIIIC or mutant TFIIIC and a functional excess of partially purified yeast TFIIIB show no transcription differential. In fact, for wild-type TFIIIC, the yeast TFIIIB fraction supports approximately threefold more transcription than does recombinant TFIIIB. This results in a level of transcription similar to that of reactions containing mutant TFIIIC (Fig. 3b and data not shown). These findings suggest that partially purified TFIIIB fractions contain an activity which mimics the stimulatory effect of the dominant mutations. This activity is evidently not present at significant levels in crude extracts, but it appears to have been concentrated in yeast TFIIIB fractions. We have also reported previously that the *PCF1-1* mutation increases the amount of $TFIIIB_{70}$ in partially purified TFIIIB fractions and increases the activity of the B″ fraction (32). Subsequent Western analysis has shown that while wild-type and mutant extracts contain equal amounts of $TFIIIB_{70}$, the recovery of the protein in *PCF1-1*- or *PCF1-2*-derived TFIIIB fractions is four- to sevenfold higher than that in the wild-type TFIIIB fraction (30). Western blot analysis also detected $TFIIIB_{70}$ in our native MonoS-purified B″ fractions. Thus, the increase in the activity of the *PCF1-1*-derived B″ fraction (32) may be attributed to the enhanced recovery of $TFIIIB_{70}$ rather than an effect on $TFIIIB_{90}$. The mechanism by which mutant $TFIIIC_{131}$ enhances the recovery of $TFIIIB_{70}$, but not $TFIIIB_{90}$ or TBP, in the TFIIIB fraction is under investigation.

The ability of dominant mutations in a specific TPR-containing region of $TFIIIC_{131}$ to facilitate the extent to which transcription complex assembly can proceed has important implications for the functionally diverse TPR family of proteins. In recent years, the function of TPRs as protein-protein interaction domains has been established (25) and the motif has become associated with an increasing number of regulated cellular processes. For example, recruitment of the SSN6-TUP1 corepressor to promoters regulated by glucose or oxygen requires specific TPRs in SSN6 (36). TPR structure is also critical for the activity of the 20S cyclosome-anaphase promoting complex (25). This complex contains at least four TPR proteins, including CDC16, CDC23, and CDC27, and is responsible for the cell-cycle-regulated ubiquitination of a variety of proteins whose destruction is critical for cell cycle progression (23). TPR proteins have also been implicated in the regulation of protein synthesis, protein folding, and signal transduction (3, 13, 28). Our model (Fig. 4) suggests that regulation of some of these processes may be achieved by affecting TPR structure and the ensuing TPR-mediated interactions with target proteins. For example, the activities of cell-cycle-regulated protein kinases could affect changes in the conformation of particular TPR domains in the anaphase promoting complex such that specific substrate binding sites become exposed.

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