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Conserved functional domains of the RNA polymerase III general transcription factor BRF

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In *Saccharomyces cerevisiae*, two components of the RNA polymerase III (Pol III) general transcription factor TFIIIB are the TATA-binding protein (TBP) and the B-related factor (BRF), so called because its amino-terminal half is homologous to the Pol II transcription factor IIB (TFIIB). We have cloned BRF genes from the yeasts *Kluyveromyces lactis* and *Candida albicans*. Despite the large evolutionary distance between these species and *S. cerevisiae*, the BRF proteins are conserved highly. Although the homology is most pronounced in the amino-terminal half, conserved regions also exist in the carboxy-terminal half that is unique to BRF. By assaying for interactions between BRF and other Pol III transcription factors, we show that it is able to bind to the 135-kD subunit of TFIIIC and also to TBP. Surprisingly, in addition to binding the TFIIB-homologous amino-terminal portion of BRF, TBP also interacts strongly with the carboxy-terminal half. Deleting two conserved regions in the BRF carboxy-terminal region abrogates this interaction. Furthermore, TBP mutations that selectively inhibit Pol III transcription in vivo impair interactions between TBP and the BRF carboxy-terminal domain. Finally, we demonstrate that BRF but not TFIIB binds the Pol III subunit C34 and we define a region of C34 necessary for this interaction. These observations provide insights into the roles performed by BRF in Pol III transcription complex assembly.

[*Key Words*: Transcription; general transcription factor; RNA polymerase III; TFIIIB; BRF; TBP]

In eukaryotic cell nuclei there are three DNA-dependent RNA polymerases. Each relies on a set of basal transcription factors to direct it to transcriptional promoters and to transduce signals from regulatory proteins. Although the basal factors employed by a particular polymerase are distinct from those employed by the others, one factor, the TATA-binding protein (TBP), is a component of all three systems (for reviews, see White and Jackson 1992b; Hernandez 1993; Rigby 1993; Struhl 1994). TBP is able to participate in these disparate roles by associating with three distinct sets of TBP-associated factors (TAFs) to form the complexes SL1, transcription factor IID (TFIID), and TFIIIB, which are dedicated to Pol I, Pol II, and Pol III, respectively (Dynlacht et al. 1991; Buratowski and Zhou 1992; Colbert and Hahn 1992; Comai et al. 1992; Kassavetis et al. 1992; Lobo et al. 1992; Pugh and Tjian 1992; Simmen et al. 1992; Taggart et al. 1992; White and Jackson 1992a; Chiang et al. 1993; Eberhard et al. 1993; Huet and Sentenac 1993). The most important Pol III basal transcription factor is TFIIIB, as this is the only factor utilized by all Pol III templates. TFIIIB comprises TBP and polypeptides of ~70 and 90 kD (Kassavetis et al. 1992). Although TFIIIB contains TBP as one of its sub-units, it does not recognize Pol III promoters primarily by sequence-specific DNA recognition. Instead, it is recruited to class III genes by protein–protein interactions with other Pol III basal factors, such as TFIIIC, which are bound to specific promoter sequences (Geiduschek and Tocchini-Valentini 1988; Kassavetis et al. 1992; Willis 1993; White 1994). In the process of associating with class III templates, however, TFIIIB interacts intimately with DNA upstream from the transcription initiation site (Kassavetis et al. 1989, 1991; Bartholomew et al. 1991) and, in yeast at least, is sufficient to direct several rounds of transcription initiation, even after TFIIIC has been removed by heparin treatment (Kassavetis et al. 1990). It has thus been concluded that TFIIIB mediates the specific recruitment of Pol III to class III templates.

Recently, genetic screens in *Saccharomyces cerevisiae* have identified a gene that, when overexpressed, serves as an allele-specific suppressor of point mutations in TBP (Buratowski and Zhou 1992; Colbert and Hahn 1992). This same gene was also isolated in a separate screen selecting for suppressors of a mutation in a tRNA gene promoter (Lopez-de-Leon et al. 1992). Biochemical and genetic analyses have established that the protein product of this gene, variously named BRF, TDS4, or PCF4 and henceforth referred to as BRF, is the 70-kD component of TFIIIB and is essential for yeast viability

(Buratowski and Zhou 1992; Colbert and Hahn 1992; Kassavetis et al. 1992; Lopez-de-Leon et al. 1992). Interestingly, sequence analysis reveals that the amino-terminal half of BRF is related to the Pol II basal factor TFIIB. TFIIB interacts with both TBP and Pol II and, by serving as a molecular bridge between these proteins, recruits Pol II to the promoter (Tschochner et al. 1992; Wampler and Kadonaga 1992; Barberis et al. 1993; Ha et al. 1993; Hisatake et al. 1993; Malik et al. 1993; Yamashita et al. 1993). TFIIB has also been implicated in selecting the Pol II transcription start site and in responding to regulatory factors (Lin et al. 1991; Lin and Green 1991; Pinto et al. 1992; Roberts et al. 1993). The homology between BRF and TFIIB implies that these factors play parallel roles in the Pol III and Pol II transcriptional machineries. More specifically, it suggests that BRF interacts directly with TBP and Pol III and that BRF may be involved in transducing regulatory signals to the Pol III transcriptional apparatus.

A powerful approach in defining the functional domains of a protein is to determine the regions most conserved in sequence throughout evolution. For example, the comparison of TFIIB polypeptides from diverse eukaryotes has identified conserved regions that are essential for TFIIB activity (Wampler and Kadonaga 1992; Buratowski and Zhou 1993; Na and Hampsey 1993). Because BRF has so far only been cloned from S. cerevisiae, its conserved domains have not been defined. Here, we describe the isolation and analysis of BRF genes from two yeast species that are evolutionarily highly diverged from S. cerevisiae. By comparing the sequences of these three homologs, we identify regions that are strongly conserved and likely to be crucial for BRF function. Furthermore, through in vitro protein–protein binding assays, we investigate interactions between BRF and other Pol III transcription factors and define the regions of the proteins necessary for these interactions. These approaches lead us to a better understanding of the roles played by BRF in Pol III transcription complex assembly and suggest a mechanistic basis for polymerase specificity.

Results

Cloning the BRF gene of Kluyveromyces lactis

Although basal transcription factors are generally conserved well throughout evolution in terms of overall structure and mechanism of action, the level of amino acid sequence homology varies considerably from one factor to another. For example, whereas the carboxy-terminal 180 amino acid residues of TBP are >80% identical between S. cerevisiae and human, TFIIB is only 35% identical between these species (Pinto et al. 1992; Hernandez 1993). To determine how well BRF is conserved and, thus, define functionally important regions, we attempted to clone its homologs from other species.

Initially, we focused on the yeast Kluyveromyces lactis, which is estimated to have diverged from S. cerevisiae ~10^8 years ago (Barns et al. 1991; Van de Peer et al.

1992). The strategy was to employ low-stringency nucleic acid hybridization, using the S. cerevisiae BRF gene as the probe. Southern blotting experiments revealed a single hybridizing locus in the K. lactis genome (data not shown). Using conditions optimized for these Southern blots, a K. lactis genomic DNA library screen yielded several hybridizing clones. Sequencing one of these revealed a single long open reading frame capable of encoding a protein of 556 amino acid residues with predicted mass of 62,243 daltons. Sequence analysis shows that this protein has strong homology to S. cerevisiae BRF (Fig. 1A). In particular, the region encompassing the amino-terminal 270 amino acid residues is ~75% identical. Although still extensive, the homology in the carboxy-terminal half of these molecules is less pronounced (overall 51% identity) and contains several short gaps. The strong homology between S. cerevisiae and K. lactis BRF proteins is reflected in very similar hydropathy profiles (Fig. 1B) and secondary structure predictions (data not shown).

Cloning the BRF gene from Candida albicans

Next, we attempted to isolate a BRF homolog gene from the more highly diverged yeast Candida albicans. Because preliminary low-stringency Southern blotting experiments were ambiguous, we turned to a polymerase chain reaction (PCR) strategy that has been used successfully to isolate TBP clones from evolutionarily diverse species (Kao et al. 1990; Rowlands et al. 1994). From the sequence comparison of S. cerevisiae and K. lactis BRFs (Fig. 1), we identified several regions of extended amino acid identity. As described in the legend to Figure 2A, degenerate oligonucleotide primers corresponding to several of these motifs were generated and used in low-stringency PCR reactions with the genomic DNA of C. albicans. Through this route, we isolated a gene encoding the entire putative C. albicans BRF homolog. Sequencing revealed a single long open reading frame encoding a protein of 553 amino acid residues with a predicted mass of 61,834 daltons. A three-way comparison of the S. cerevisiae, K. lactis, and C. albicans BRF homologs is shown in Figure 2A. Conservation is most pronounced in the amino-terminal TFIIB homologous region, especially in the area comprising the direct repeats. However, three blocks of strong homology are also identifiable in the BRF-specific carboxyl terminus (Fig. 2A,B; also see Discussion). Particularly in the amino-terminal half of these molecules, sequence homologies are reflected in very similar hydropathy profiles and secondary structure predictions (data not shown).

C. albicans BRF complements an S. cerevisiae BRF mutant

The fact that the C. albicans protein sequence described above has extensive homology throughout its length with S. cerevisiae BRF suggests strongly that it corresponds to a functional BRF homolog. To test this, we determined whether the growth defect of an S. cerevisiae

A

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KLBRF  MASTLQVSSRKCKNCGSTDFVRDISNTTNELICKVCGLVTEENSIVSELAFGEASNGAAV
SCBRF  MPV-------CKNCHGTEFERDLSNANNDLVCKACGVVSEDNPIVSEVTFGETSAGAAV
       *.       ****  .*.* **.** . *.*.** **.*.*.*.**** .***.* ****

KLBRF  IQGAFVSANQAHPTFMSHSGQNALMSRETTLNNARRKLKAVSYALNIPEYVTDAAFQWYR
SCBRF  VQGSFIGAGQSHAAF---GGSSALESREATLNNARRKLRAVSYALHIPEYITDAAFQWYK
       .**.*..* *.*.*       .* .** ***.********.****** .****.********

KLBRF  LALSNNFVQGRKSQNVIAACLYIACRKERTHHMLIDFSSRLQVSVYSIGATFLKLAKKLQ
SCBRF  LALANNFVQGRRSQNVIASCLYVACRKEKTHHMLIDFSSRLQVSVYSIGATFLKMVKKLH
       ***.********* ***** .*** .***.***********************  .***.

KLBRF  IVKLPLADPSLFIQHFAEKLELGDKKIKVIRDAVKLAQTMSRDWMYEGRRPAGIAGACLL
SCBRF  ITELPLADPSLFIQHFAEKLDLADKKIKVVKDAVKLAQRMSKDWMFEGRRPAGIAGACIL
       *  ***************** .****** .* .*******  **.*  **** ***********.*

KLBRF  LACRMNNLRRTHSEIVAISHVAEETLQQRLNEFKNTTSAKLSVKEFRDDETEVNEGERSA
SCBRF  LACRMNNLRRTHTEIVAVSHVAEETLQQRLNEFKNTKAAKLSVQKFRENDVE------DG
       ************.****.******************  .*** ***.* **...  *

KLBRF  ESKPPSFDKNRLKEKKIKDSLDTKEMLETSEEAVSRNPILTQVLGAQELSSKEVLYYLKK
SCBRF  EARPPSFVKNRKKERKIKDSLDKEEMFQTSEEALNKNPILTQVLGEQELSSKEVLFYLKQ
       * .**** *** ** *******  **  .**** . .******** ********* .***..***

KLBRF  LSERRKAEFSHIKATHGIDGEDLHKTEKD---KKRSLDE--------------------
SCBRF  FSERRARVVERIKATNGIDGENIYHEGSENETRKRKLSEVSIQNEHVEGEDKETEGTEEK
       .****     .****.*****..       . .** * *

KLBRF  ----------------------IDGYSLEKDPYRPRNLHLLPTTASLLSKVSDHPENLD
SCBRF  VKKVKTKTSEEKKENESGHFQDAIDGYSLETDPYCPRNLHLLPTTDTYLSKVSDDPDNLE
                             ******* *** ********** . ***** .**..

KLBRF  DVDDAELDSHLLDEEASKLKERIWIDINGDYLIEQESKRLKQEADLASGNTSLRKKRSKR
SCBRF  DVDDEELNAHLLNEEASKLKERIWIGLNADFLLEQESKRLKQEADIATGNTSVKKKRTRR
       ****.**..***.************ .*.*.*.************.*.****.***..*

KLBRF  TNRRNQSSASIVKVQVD---GL--------PLDVSVDDAD-AVDVVAAGGVKNLLQKTTFS
SCBRF  RNNTRSDEPTKTVDAAAAIGLMSDLQDKSGLHAALKAAEESGDFTTADSVKNMLQKASFS
       *  .*   . *.    **      *. .. *. * .* *..***.***..**

KLBRF  KKINYDAINGLFGQK
SCBRF  KKINYDAIDGLF--R
       ********.***  .
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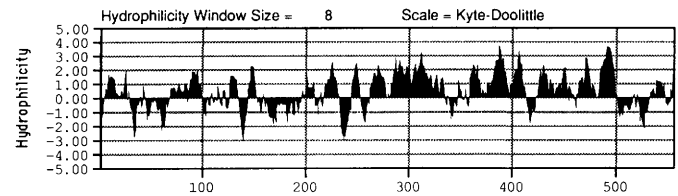
B

S. cerevisiae BRF

K. lactis BRF

Figure 1. (A) Cloning and sequence analysis of the K. lactis BRF gene. A K. lactis genomic DNA plasmid library was probed with a radiolabeled DNA fragment encompassing the amino-terminal region of S. cerevisiae BRF that is related to TFIIB. Low-stringency hybridization and washing protocols were employed (final wash of 2× SST at 50°C). A hybridizing clone was thus identified and sequenced. The single long open reading frame in the K. lactis DNA insert is presented in a comparison with the S. cerevisiae BRF protein sequence. Identities and similarities are indicated by asterisks and dots, respectively. The putative zinc finger and direct repeats in the TFIIB-homologous domain are indicated by the bracket and arrows, respectively. (B) Hydropathy plots of S. cerevisiae and K. lactis BRF homologs were derived by the method of Kyte and Doolittle (1982).

strain containing a temperature-sensitive mutation in BRF could be complemented by the introduction of a plasmid expressing the C. albicans BRF homolog. As a control we employed a vector expressing S. cerevisiae BRF. When incubated at 38°C, the untransformed temperature-sensitive mutant strain grew extremely slowly (doubling time in mid-log phase was 26.5 hr). However, when plasmids expressing S. cerevisiae BRF or C. albicans BRF were introduced, the growth rate of the strain was increased substantially (doubling times of 5.3 hr and 6.5 hr, respectively). These data are consistent with the strong conservation of BRF throughout evolution and indicate that the C. albicans protein is a bona fide BRF homolog.

BRF interacts with the 135-kD subunit of TFIIIC

For most genes transcribed by Pol III, initial promoter recognition is mediated by the large multiprotein complex TFIIIC. Protein–protein interactions between TFIIIC and TFIIIB are then employed to recruit TFIIIB to the DNA. Previous studies have shown that BRF contacts TFIIIC (Kassavetis et al. 1991, 1992) and that BRF is positioned directly upstream from the second largest subunit of TFIIIC (τ135) in the Pol III transcription com-

plex (Bartholomew et al. 1991). As an approach to analyze potential interactions between BRF and τ135, we performed in vitro protein–protein binding "pulldown" assays. To do this, a fusion of glutathione S-transferase (GST) with full-length S. cerevisiae BRF was synthesized in Escherichia coli and purified by adsorption to glutathione–agarose beads. The immobilized proteins were then incubated with [35S]methionine-labeled derivatives of S. cerevisiae τ135 that had been generated by in vitro transcription and translation. After washing the beads extensively, the quantity of protein bound was assessed by SDS-PAGE and fluorography. As can be seen from Figure 3, the amino-terminal, middle, and carboxy-terminal thirds of τ135 (residues 1–363, 364–714, and 715–1025, respectively) all bind to the GST–BRF fusion. In contrast, little or no binding is observed to GST protein alone or to several control fusions (data not shown). These studies indicate that multiple regions of τ135 can mediate specific contacts with BRF and suggest that these interactions may play an important role in Pol III transcription complex assembly. Interestingly, τ135 contains 11 copies of the tetratricopeptide repeat (TPR) motif (Marck et al. 1993), and each of the three portions of this protein that we synthesized contains several of these motifs. As TPR motifs have been suggested to mediate protein–protein interactions (Goebl and Yanagida

Khoo et al.

Figure 2. (A) Sequence comparison of *C. albicans*, *K. lactis*, and *S. cerevisiae* BRFs (CABRF, KLBRF, and SCBRF, respectively). For cloning the *C. albicans* BRF gene, low-stringency PCR reactions were performed as described in Materials and methods, using *C. albicans* genomic DNA and primer pairs corresponding to conserved sequences in *S. cerevisiae* and *K. lactis* BRFs. In one case (primer pair 153/156; corresponding to amino acid sequences THHMLID and QQLTEEAV), a product was generated of the anticipated size. This, in turn, was radiolabeled and used to isolate a BRF homolog gene from a *C. albicans* genomic DNA library. The translation product of the *C. albicans* BRF homolog gene is presented in a comparison with the *S. cerevisiae* and *K. lactis* BRF protein sequences. Identities and similarities are indicated by asterisks and dots, respectively. The TFIIB-homologous domain is boxed; within this domain, the putative zinc finger region is indicated by a bracket, the imperfect direct repeats by arrows, and the region that coincides with a predicted basic amphipathic helix of TFIIB by a broken underline. The three regions of strong conservation in the carboxyl terminus (denoted I, II, and III) are underlined. (B) Schematic representation of the BRF sequence conservations shown in A. Percentages refer to identities between *K. lactis* or *C. albicans* proteins with *S. cerevisiae* BRF.
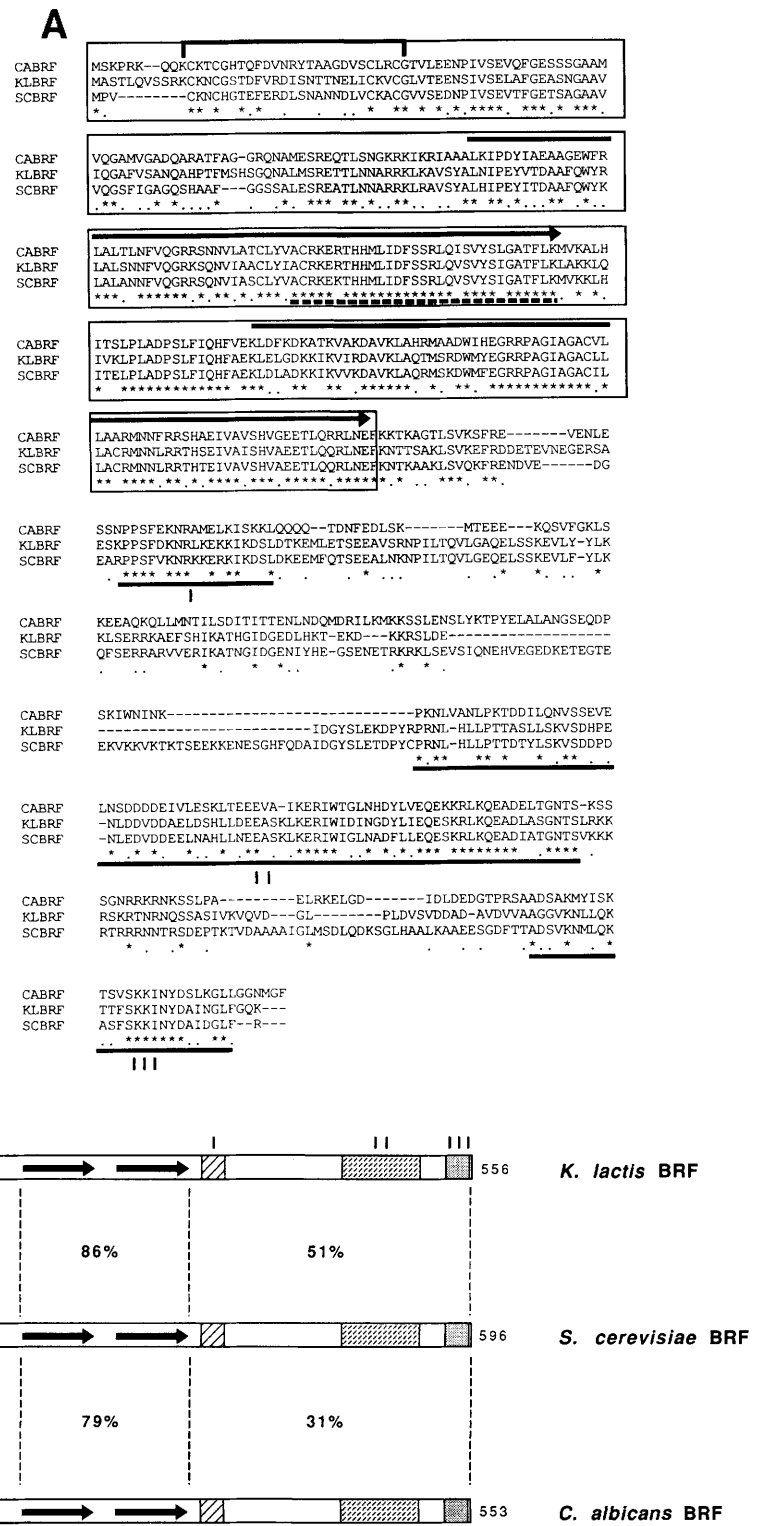
1991), it is possible that these regions direct interactions between τ135 and BRF.

Two regions of BRF interact with TBP

The overexpression of BRF has been shown previously to suppress the growth defects of *S. cerevisiae* strains containing point mutations in TBP that impair transcription by Pol III (Buratowski and Zhou 1992; Colbert and Hahn 1992). Although this suppression could be mediated by mechanisms that do not involve direct interactions between BRF and TBP, the most likely interpretation is
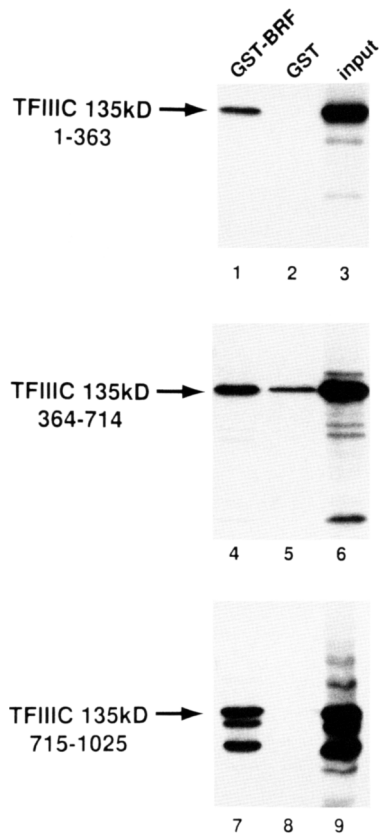
Figure 3. BRF binds to the 135-kD subunit of TFIIIC (τ135). Glutathione beads bearing GST/full-length BRF (lanes *1,4,7*) or GST alone (lanes *2,5,8*) were used in interaction assays as described in Materials and methods with the following ^{35}S-labeled in vitro-translated derivatives of τ135: amino acid residues 1–363 (lanes *1–3*), 364–714 (lanes *4–6*), or 715–1025 (lanes *7–9*). Bound proteins were detected by SDS-PAGE followed by autoradiography. To assess the efficiency of protein–protein interactions, the input amount of τ135 derivatives are included in lanes *3, 6*, and *9* and the positions of these proteins on the gel are indicated by arrows. The percentages of the input protein bound were 54%, 0%, 46%, 18%, 62%, and 0% for lanes *1, 2, 4, 5, 7*, and *8*, respectively.

that BRF binds to TBP and that BRF overexpression stabilizes TBP in its active conformation. To see whether BRF can bind TBP, we again performed in vitro protein–protein interaction assays employing GST–BRF fusions. These experiments revealed that *S. cerevisiae* TBP binds to the GST fusion bearing full-length BRF (Fig. 4A, lane 1), whereas no significant binding is observed to GST alone (lane 2) or to several control fusions (data not shown). These results provide strong support for the existence of interactions between BRF and TBP in the Pol III transcription complex.

To elucidate which regions of BRF mediate the interaction with TBP, we tested TBP binding to GST fusions bearing either the amino-terminal TFIIB homologous half of BRF or the carboxy-terminal half that has no counterpart in TFIIB (Fig. 4B). Perhaps surprisingly, in

addition to detecting interactions between *S. cerevisiae* TBP and the BRF amino-terminal region (lane 1), strong TBP binding was obtained with the carboxy-terminal BRF domain (lane 4). To further dissect the amino-terminal half of BRF, we synthesized both the putative zinc finger region and the direct repeat region separately as GST fusions and then tested them for TBP binding. This revealed that TBP binds the repeat domain, but only very weakly to the zinc finger region (Fig. 4B, lanes 3 and 2, respectively). Similar results were also obtained using TBP that had been expressed in bacteria, indicating that the interactions between BRF and TBP are likely to be direct rather than being mediated by other proteins in the in vitro transcription/translation system (data not shown). We therefore conclude that two distinct regions of BRF bind TBP: One maps to the amino-terminal direct repeat domain; the other to the carboxy-terminal half of the molecule. The specificity of these interactions is indicated by the fact that other derivatives of BRF, such as 1–89 and 263–431, do not bind TBP detectably despite the fact that these proteins are expressed at high levels in soluble form (Fig. 4C).

Recent studies have identified mutations in *S. cerevisiae* TBP that inhibit Pol III transcription in vivo but do not affect transcription by Pol II (Cormack and Struhl 1993; Kim and Roeder 1994). These mutations map to a solvent-exposed region of TBP that defines a groove across the top of the protein (Kim et al. 1993). It has therefore been suggested that this region specifies a surface of TBP that mediates important interactions with the Pol III transcriptional apparatus. Interestingly, overexpression of BRF in strains bearing temperature-sensitive Pol III-specific TBP mutations results in a partial reversal of the growth-defect phenotype (Cormack and Struhl 1993). We therefore tested whether these mutations affected interactions between TBP and BRF. To do this, three different Pol III-specific TBP mutants were synthesized and compared with wild-type *S. cerevisiae* TBP for binding to various BRF derivatives. Strikingly, compared with wild-type TBP (Fig. 5A), the TBP mutant F155S (Fig. 5B) is impaired drastically in its ability to interact with the carboxy-terminal portion of BRF (Fig. 5, A and B, cf. lanes 3). In contrast, it can still bind effectively to the amino-terminal region of BRF and full-length BRF protein (Fig. 5B, lanes 1,2) and actually displays slightly increased binding to these derivatives. Similar results are also obtained with the other two Pol III-specific TBP mutants tested, K138L (Fig. 5C) and R137W (data not shown). These data show that the three Pol III-specific TBP mutations tested all specifically impair the binding of TBP to the carboxy-terminal region of BRF and suggest that this protein interaction defect may be responsible for reduced Pol III transcription in strains bearing these mutations.

If the binding of TBP to the carboxy-terminal half of BRF is of physiological significance, one might expect that the regions of BRF mediating this interaction would be conserved throughout evolution. Potential candidates for such regions are the three blocks of homology identified in Figure 2. We found that deletion of the carboxy-
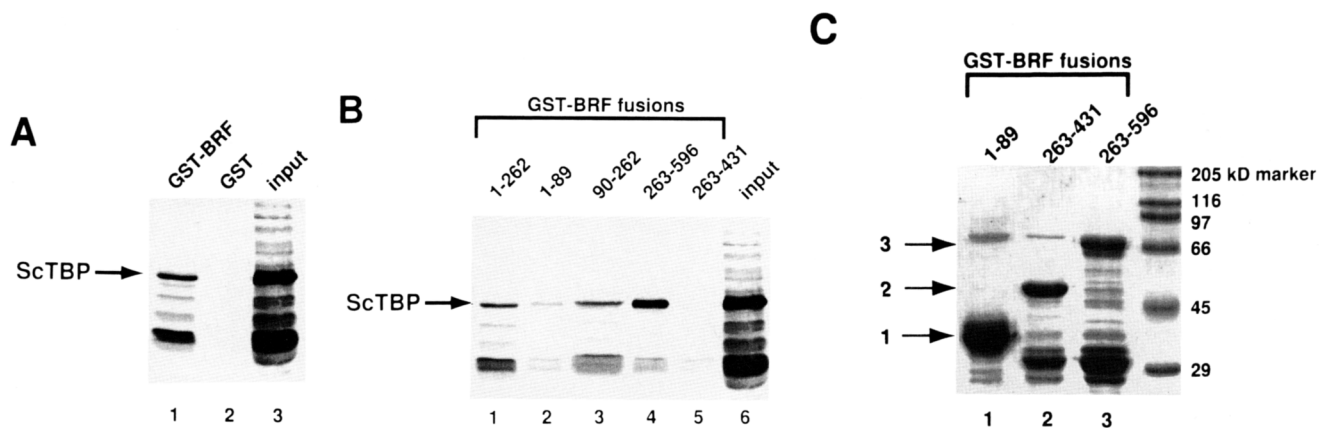
Figure 4. Interactions between BRF and TBP. (*A*) BRF binds TBP. Glutathione beads bearing GST/full-length BRF (lane *1*) or GST alone (lane *2*) were tested for binding of ^{35}S-labeled in vitro-translated wild-type *S. cerevisiae* TBP as described in Materials and methods. The amount of input protein used in these experiments is presented in lane *3* to allow an assessment of binding efficiency. Percentage of input protein bound in lanes *1* and *2* was 18% and 0%, respectively. (*B*) TBP binds to two regions of BRF. The binding of wild-type *S. cerevisiae* TBP was assessed using GST fusions to BRF residues 1–262 (zinc finger together with direct repeat region; lane *1*), BRF residues 1–89 (zinc finger region alone; lane *2*), BRF residues 90–262 (direct repeat region alone; lane *3*), BRF residues 263–596 (carboxy-terminal non-TFIIB-homologous domain; lane *4*), or BRF residues 263–431 (carboxy-terminal domain lacking homology blocks II and III defined in Fig. 2, lane *5*). Percentage of input protein bound in lanes *1–5* was 8%, 2%, 6%, 41%, and 0%, respectively. Although the very weak interaction between TBP and the BRF zinc finger region in lane *2* is likely to be artifactual, we cannot exclude the possibility that it is of physiological significance. (*C*) BRF derivatives that do not bind TBP are expressed at high levels in soluble form and are thus unlikely to be folded aberrantly. Portions of GST–agarose beads bearing purified derivatives 1–89 (lane *1*), 263–431 (lane *2*), or 263–596 (lane *3*) were electrophoresed on a 10% SDS–polyacrylamide gel and detected by Coomassie staining. The positions of the derivatives are indicated by arrows.

terminal 165 amino acid residues of BRF, including homology blocks II and III, abolishes binding of the carboxy-terminal half of BRF to TBP (Fig. 4B, lane 5). Consistent with this result, previous studies have indicated that deletions of the carboxy-terminal region abrogate BRF function in vivo (Buratowski and Zhou 1992; Colbert and Hahn 1992). We speculate that the inability to correctly transcribe class III genes in yeast strains bearing these carboxy-terminal BRF deletions may be a consequence of a TBP–BRF interaction defect.

Interactions between BRF and a subunit of Pol III

BRF has also been hypothesized to act in the recruitment of the Pol III enzyme to class III genes. The 34-kD subunit (C34), a subunit that is unique to Pol III, is known from photoaffinity DNA cross-linking studies to lie directly downstream from BRF in the assembled Pol III transcription complex (Bartholomew et al. 1993). To see whether BRF can interact specifically with C34, we again employed the GST–BRF pulldown assay. As shown in Figure 6A, C34 binds to fusions bearing both full-length BRF and the amino-terminal half of this protein (lanes 1 and 2, respectively). In contrast, essentially no binding is observed to either GST alone (lane 6) or to a fusion containing the carboxy-terminal half of BRF (lane 5). When we divided the amino-terminal half of BRF into its zinc finger and direct repeat regions, we found that the latter, but not the former, can bind C34 effectively (Fig 6A, lanes 3 and 4, respectively). Furthermore, when each of the two BRF repeat elements was tested separately, C34 bound well to the first but not the second (data not shown). We therefore conclude that the direct repeat region of BRF interacts specifically with C34 and that this interaction appears to be mediated primarily by the first repeat. To address which regions of C34 are involved in specifying the BRF interaction, we generated a version of C34 in which the carboxy-terminal 120 amino acid residues had been deleted and tested it for BRF binding (Fig. 6B). It was found to be unable to interact with BRF, indicating that the carboxyl terminus of C34 is required for BRF binding.

It is well established that TFIIB binds Pol II (Ha et al. 1993). Because the amino-terminal half of BRF that binds the C34 subunit of Pol III is homologous in sequence to TFIIB, we wondered whether TFIIB might also be able to bind this protein. To test this, we synthesized *S. cerevisiae* TFIIB as a GST fusion and tested for its ability to interact with C34. This test revealed that C34 can interact efficiently with BRF but not TFIIB (Fig. 6C, lanes 1 and 2, respectively). These findings suggest that although TFIIB and BRF are related in sequence, only BRF can associate with the Pol III-specific subunit C34. This specificity of interaction may, at least in part, be responsible for the ability of class III transcription complexes to selectively recruit the appropriate RNA polymerase.

Discussion

In this paper we have shown by protein–protein interaction assays that BRF binds specifically to several components of the Pol III transcriptional apparatus. Consis-
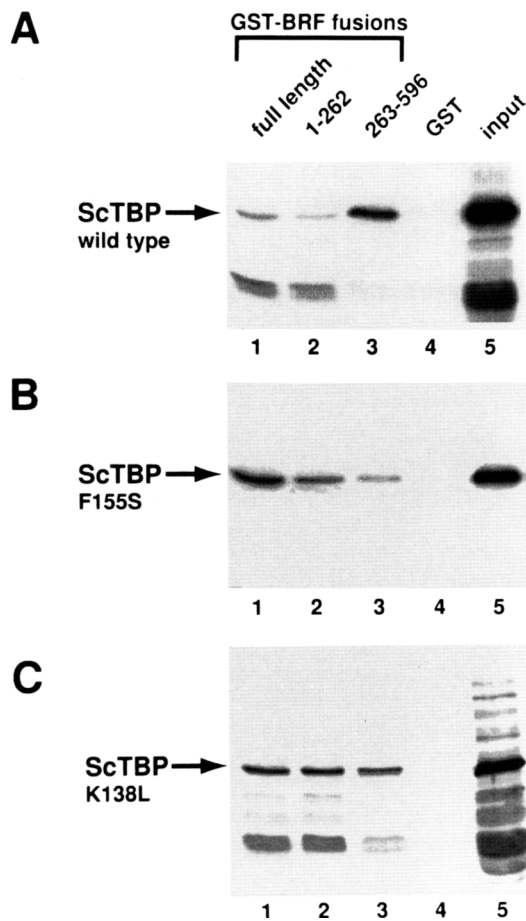
is located near both τ135 and C34 in assembled Pol III transcription complexes (Bartholomew et al. 1991, 1993). It is therefore likely that the interactions we observe in vitro are relevant functionally. Together with previous


Figure 5. Mutations of TBP that impair Pol III transcription specifically abrogate interactions with the BRF carboxy-terminal region. ^{35}S-Labeled in vitro-translated derivatives of *S. cerevisiae* TBP were assayed for binding to various GST–BRF fusions as described in Fig. 4. The assays presented here were all performed simultaneously. (*A*) Wild-type TBP was tested for interactions with GST fusions to full-length BRF (lane *1*), the amino-terminal half of BRF (residues 1–262; lane *2*), the carboxy-terminal half of BRF (residues 263–596; lane *3*), or GST alone (lane *4*). Wild-type TBP binds to the carboxy-terminal portion of BRF strongly (lane *3*) and also to the BRF amino-terminal region (lane *2*). Percentage of input protein bound in lanes *1–4* was 5%, 1%, 35%, and 0%, respectively. (*B*) The Pol III-specific TBP mutation F155S reduces binding to the carboxy-terminal half of BRF (lane *3*), whereas binding to full-length BRF (lane *1*) and the BRF amino-terminal region (lane *2*) is actually increased. Percentage of input protein bound in lanes *1–4* was 26%, 6%, 5%, and 0%, respectively. (*C*) The Pol III-specific TBP mutation K138L reduces binding to the carboxy-terminal half of BRF but does not affect interactions with the amino-terminal region of BRF significantly. Percentage of input protein bound in lanes *1–4* was 34%, 30%, 17%, and 1%, respectively.

tent with TBP and BRF being subunits of TFIIB, we find that these proteins interact with one another. We have also shown that BRF binds to the τ135 component of TFIIIC and to the C34 subunit of Pol III. Previous protein–DNA cross-linking studies have indicated that BRF
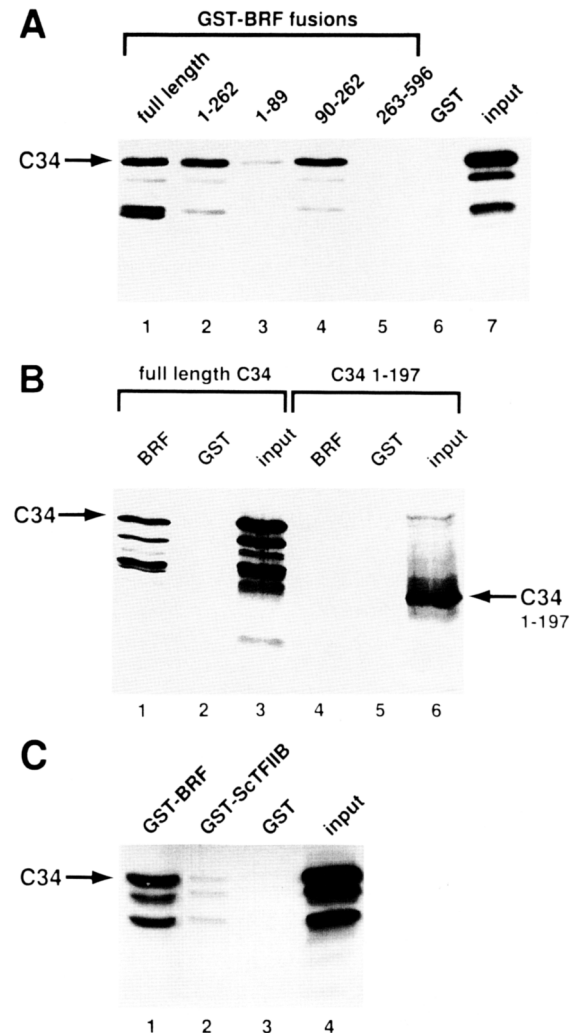
Figure 6. (*A*) BRF binds specifically to the C34 subunit of Pol III. ^{35}S-Labeled in vitro-translated C34 subunit of *S. cerevisiae* Pol III was tested for binding to GST fusions of the following: full-length BRF (lane *1*), BRF residues 1–262 (zinc finger together with direct repeat region; lane *2*), BRF residues 1–89 (zinc finger region alone; lane *3*), BRF residues 90–262 (direct repeat region alone; lane *4*), BRF residues 263–596 (carboxy-terminal non-TFIIB-homologous domain; lane *5*), or GST alone (lane *6*). Amount of input C34 is shown to allow assessment of binding efficiencies. Percentage of input protein bound in lanes *1–6* was 34%, 33%, 2%, 34%, 0%, and 0%, respectively. (*B*) ^{35}S-Labeled in vitro-translated wild-type C34 (lanes *1–3*) or C34 residues 1–197 (lanes *4–6*) were tested for binding to GST fused to full-length BRF (lanes *1,4*), or to GST alone (lanes *2,5*). Percentage of input protein bound was 41%, 0%, 0%, and 0% for lanes *1, 2, 4, 5*, respectively. (*C*) ^{35}S-Labeled C34 was tested for binding to GST fusions of the following: full-length BRF (lane *1*), *S. cerevisiae* TFIIB (lane *2*), or GST alone (lane *3*). Percentage of input protein bound in lanes *1–3* was 35%, 0%, and 2%, respectively.

observations, our data support a model in which BRF plays a central role in Pol III transcription. First, BRF forms part of TFIIIB by interacting with TBP and possibly the B'' component of this Pol III-specific TBP–TAF complex (Kassavetis et al. 1991, 1992). Second, by binding to the τ135 subunit of DNA-bound TFIIIC, BRF mediates the recruitment of TFIIIB to class III templates. Third, BRF confers Pol III specificity to the transcription complex by interacting specifically with the C34 subunit of Pol III.

Through cloning BRF homologs from *K. lactis* and *C. albicans* and comparing their sequences with *S. cerevisiae* BRF, we have defined evolutionarily conserved regions of BRF that may correspond to important functional domains. Although *K. lactis* and *C. albicans* are both yeasts, they are distant from *S. cerevisiae* from an evolutionary standpoint. For example, *K. lactis* is estimated by rRNA sequence comparisons to have diverged from *S. cerevisiae* $\sim 10^8$ years ago and the evolutionary distance between *S. cerevisiae* and *C. albicans* is considerably larger still (Barns et al. 1991; Van de Peer et al. 1992). Strikingly, the BRF proteins of *K. lactis* and *C. albicans* are 62% and 47% identical, respectively, to *S. cerevisiae* BRF. This conservation is much higher than that of gene-specific regulatory factors such as GAL4/LAC9 and heat shock factor (HSF), where homologies between the *K. lactis* and *S. cerevisiae* proteins are confined to relatively short regions (Salmeron and Johnston 1986; Wray et al. 1987; Jakobsen and Pelham 1991). The level of evolutionary conservation of BRF is similar to that of TFIIB, which is 67% identical between *K. lactis* and *S. cerevisiae* (Na and Hampsey 1993). (The amino-terminal portion of BRF that is related in sequence to TFIIB is actually more conserved from *S. cerevisiae* to *K. lactis* than are the TFIIB proteins of these two species.) The strong evolutionary conservation of BRF is consistent with this protein's role as the linchpin of the Pol III transcriptional apparatus.

From the three-way comparison of BRF proteins in Figure 2A, we can divide BRF tentatively into four highly conserved domains (Fig. 2B). The first lies at the extreme amino terminus and includes a cysteine-rich sequence postulated to form a zinc-binding "finger" structure (Buratowski and Zhou 1993; Ha et al. 1993). This region is well conserved in *K. lactis* and *C. albicans* BRFs, suggesting an important role. Deleting this region of *S. cerevisiae* BRF is lethal (Colbert and Hahn 1992). Because the analogous TFIIB domain is implicated in binding TFIIF (Barberis et al. 1993; Buratowski and Zhou 1993; Ha et al. 1993), this region of BRF might interact with a Pol III basal factor with homology to TFIIF.

The second conserved region of BRF encompasses the two imperfect direct repeats of ~ 76 amino acid residues that are related to the repeat elements of TFIIB. In this region, 73% of the residues are identical in all three BRF proteins in Figure 2A, indicating that it is particularly crucial for BRF function. Our data suggest that the conservation of this domain reflects the fact that it mediates important interactions with several components of the Pol III transcription apparatus. One function of this re-

gion appears to be to interact with TBP, as we have shown that a GST fusion containing this domain binds TBP specifically in vitro. Consistent with this result, recent work has demonstrated that the analogous repeat region of TFIIB also binds TBP (Barberis et al. 1993; Ha et al. 1993; Hisatake et al. 1993; Yamashita et al. 1993).

We have also demonstrated that the BRF direct repeat region and the first BRF repeat in isolation can interact with the 34-kD subunit of Pol III. This is in line with previous studies that have suggested that BRF is involved in Pol III recruitment (Bartholomew et al. 1993) and with the fact that the homologous repeat region of TFIIB binds Pol II (Ha et al. 1993). It has been speculated previously that C34 might interact with class III transcription factors on the basis of the fact that antibodies against C34 inhibit specific transcription but not factor-independent nonspecific transcription (Huet et al. 1985). Furthermore, it has been shown that BRF interacts with C34 using a yeast two-hybrid assay (Werner et al. 1993), although this approach could not exclude the possibility of an indirect interaction. It should be noted, however, that interactions between BRF and Pol III may not be sufficient for efficient Pol III recruitment, because the 90-kD component of TFIIIB (B'') is necessary for transcription to proceed (Kassavetis et al. 1991, 1992). By synthesizing a deletion derivative of C34, we have shown that its carboxy-terminal 120 amino acid residues are required for effective BRF binding. This tallies with the observation that the carboxy-terminal region of C34 is essential for yeast viability (Stettler et al. 1992). Importantly, unlike several Pol III subunits, C34 is not present in either of the other two nuclear pols. The interaction between BRF and C34 may therefore play a crucial role in defining the pol specificity of Pol III transcription preinitiation complexes. In line with this prediction, we find that the Pol II general factor TFIIB is unable to bind C34 effectively. Perhaps TFIIB interacts with a Pol II-specific subunit that performs an analogous function to C34.

Other possible roles for the direct repeat domain of BRF include binding to DNA and interacting with Pol III regulatory factors. Previous work has identified a region at the end of the first TFIIB repeat that contains a cluster of basic residues and is predicted to form an amphipathic α-helix. This region has been implicated in mediating TFIIB interactions with TBP and upstream regulators such as GAL4–VP16, and point mutagenesis has suggested that the basic residues are crucial for TFIIB function (Lin et al. 1991; Ha et al. 1993; Roberts et al. 1993; Yamashita et al. 1993). Interestingly, the analogous region of BRF constitutes the most extensive region of homology between the three BRF proteins in Figure 2A (indicated by a broken underline), although the conservation with TFIIB here is marginal. It is tempting to speculate that this part of BRF might constitute an interaction surface for TBP, C34, and/or Pol III regulatory factors.

The remaining conserved regions of BRF lie within the carboxy-terminal portion of the protein that has no homology to TFIIB. The most obvious feature of this do-

main is its high charge density; for example, in *K. lactis* BRF, 20% of the residues in this region are acidic and 19% are basic. Overall, this part of BRF is less conserved than the amino-terminal region (the carboxyl termini of *K. lactis* and *C. albicans* proteins are 51% and 31% identical, respectively, to *S. cerevisiae* BRF), and several gaps must be introduced to optimize the alignments. Nevertheless, three units of strong homology can be discerned readily (indicated I, II, and III in Fig. 2, A and B). The longest of these is region II, which covers 77 amino acid residues and is 80% identical between *S. cerevisiae* and *K. lactis* and 43% identical between *S. cerevisiae* and *C. albicans*.

The surprising demonstration that the carboxy-terminal half of BRF associates strongly with TBP suggests that this interaction may be of importance to Pol III transcription. Significantly, we find that deletion of homology blocks II and III of the BRF carboxy-terminal region abrogates the binding of this domain to TBP. Interestingly, previous studies have indicated that deletion of the carboxy-terminal 35–100 residues of BRF that span homology blocks II and III disrupts BRF function in vivo (Buratowski and Zhou 1992; Colbert and Hahn 1992). It is tempting to speculate, therefore, that the Pol III transcription defect in yeast strains bearing these mutations is a result of the inability of the carboxy-terminal half of BRF to bind TBP.

Using a regional codon randomization strategy, Cormack and Struhl (1993) recently generated a large number of single amino acid residue mutations in TBP and tested them for their effects on TBP function in vivo. The majority either had no discernible phenotype or had generalized effects on transcription. However, of the 186 mutations examined, 65 were specifically defective in Pol III transcription and 85% of these mapped to an exposed surface of TBP forming a groove across the upper part of the protein. Kim and Roeder (1994) have also identified a Pol III-specific TBP mutation (K138L) that maps to this vicinity. In all cases tested, overexpression of BRF in strains bearing these Pol III-specific TBP mutations results in partial reversal of the growth defect (Cormack and Struhl 1993). This suggests that BRF interacts with the region of TBP defined by the Pol III-specific mutations.

Because the amino-terminal half of BRF is highly related to TFIIB, one would anticipate that these two regions would make similar contacts with TBP. If the TFIIB homologous region were the only region of BRF to interact with TBP, one would therefore expect that TBP mutations specifically affecting binding to TFIIB would arise at a similar frequency to those affecting interactions with BRF. This is not the case, however; the mutagenesis procedure of Cormack and Struhl (1993) obtained 65 TBP mutants that were Pol III specific but only 10 that were Pol II specific. Our data showing that the carboxyl terminus of BRF also contacts TBP and that Pol III-specific TBP mutants are defective in this interaction help resolve this enigma. Specifically, our data suggest that in addition to the amino-terminal half of BRF binding TBP in an analogous fashion to TFIIB, the carboxyl

terminus of BRF mediates interactions with the Pol III-specific surface of TBP.

BRF therefore appears to possess two distinct TBP-interacting domains. Although it is not yet clear why BRF has evolved in this way, it can be surmised that the two regions will perform different functions in Pol III transcription complex assembly. Interestingly, full-length BRF binds to TBP less effectively than the carboxy-terminal region alone and does so with a comparable efficacy to the amino-terminal BRF domain alone. This suggests that in the complete BRF molecule, the carboxy-terminal TBP-binding region is not normally accessible. It is tempting to speculate that initial binding of BRF to TBP is mediated by the TFIIB homologous domain of BRF. Subsequently, the carboxy-terminal region of BRF may become exposed by a conformational shift, perhaps induced by the binding of factors such as the 135-kD component of TFIIC, the other TFIIIB subunit B'', Pol III, or regulatory proteins. Previous studies have revealed conformational shifts in BRF during Pol III transcription complex assembly (Kassavetis et al. 1992). This type of mechanism may ensure an ordered assembly of TFIIIB on the promoter and might preclude the formation of non-productive interactions between BRF and other Pol III transcription factors in inappropriate non-promoter-bound contexts.

Materials and methods

Cloning, analysis, and expression of BRF proteins

Standard molecular cloning techniques were as described by Ausubel et al. (1991). Dideoxy chain-termination sequencing was performed using the Sequenase version 2.0 system (U.S. Biochemical) according to the manufacturer's recommendations. Sequence analysis was by the MacVector program package (International Biotechnologies, Inc., New Haven, CT) and by the program CLUSTAL V for multiple alignments (Higgins et al. 1992). Low-stringency PCR reactions were performed as described previously (Rowlands et al. 1994). PCR products were cloned into pBluescript KS(+).

Cloning and expression of GST–BRF and GST–TFIIB fusions

Full-length *S. cerevisiae* BRF was cloned by amplifying the gene from genomic DNA using the following oligonucleotides (all oligonucleotides read 5' → 3'): GTCCATCGATATGCCAGT-GTGTAAGAACTGT and GTCCATCGATCCTAAACAAAC-CGTCAATGGC. The 1.8-kb fragment thus generated was cut with *Cla*I and ligated into the *Cla*I site of pBluescript KS(+) (Stratagene) as well as into the *Cla*I site of the vector pGEX-20 (R. Treisman, unpubl.) prepared from the *E. coli* dam⁻ dcm⁻ strain GM272. To generate the 1–262 and 263–596 constructs, a full-length 1.8-kb fragment was also generated from genomic DNA using oligonucleotides as above, except that the 5' oligonucleotide contained an *Eco*RI site (vide infra). This fragment was cut with *Eco*RI and *Cla*I to generate a 0.8-kb fragment (cut at the natural *Eco*RI site within the BRF gene) encoding residues 1–262 and a 1.0-kb fragment encoding the rest of BRF. The 0.8-kb fragment was ligated into *Eco*RI-cut pGEX-2TK (Pharmacia); the 1.0-kb fragment was ligated into *Eco*RI–*Cla*I-cut pGEX-20. The other constructs were generated by PCR cloning from the pBS KS(+) BRF construct into the *Eco*RI site of pGEX-2TK,

using the appropriate oligonucleotides: BRF 1–89, TGAC-GAATTCGAAT GCCAGTGTGTAAGAACTGT and TGAC-GAATTCGGCGCGTAATTTCCTTCTTGC; and BRF 90–262, TGACGAATTCGCGTTTCTTACGCATTACAT and TGAC-GAATTCGTTCAACCGTTGCTG. BRF 263–431 was constructed by cutting pGEX-20 BRF 263–596 with XbaI and religating the 5.5-kb fragment thus generated. The GST–ScTFIIB construct was made in a similar fashion by PCR from genomic DNA with the following oligonucleotides: TAGAGAATTC-GAATGATGACTAGGGAGAGCATA and TATCGAATTCT-TATTTCTTTTCAACGCCCGGTA and cloned into the EcoRI site of pGEX-2TK.

Cloning and expression of other factors

Each S. cerevisiae transcription factor (τ135, RNA Pol III C34 subunit, or TBP) was cloned using the following oligonucleotides to amplify the gene by PCR from genomic DNA; TFIIIC 135-kD 1–363, TGTAAGATCTATGGCAGCAGGAAAATTG-AAAAAG and AGAACTGCAGTCAGAGAATGTTCAAAGA-AGACCAGTC; TFIIIC 135-kD 364–714, TTTGAGATCTATG-GCGGAACTTTTCTTAAAACTCGCA and CTCTCTGCA-GTCATAGCGGCCCTTCAAACACACTATC; TFIIIC 135-kD 715–1025, AGGGAGATCTATGGAGGAAAGAGTAACCT-TAACT and AAACCTGCAGTCAAATACTTAAATACTTC-TCCAT; Pol III subunit C34, TCGACAGCTGATGAGTG-GAATGATAGAAAATGGG and AAAACTGCAGTCAAAT-TGTCCATTCATCGAAATA; and TBP, TTTTCAGCTGA-TGGCCGATGAGGAACGTTTAAAG and TCCTCTGCAGT-CACATTTTTCTAAATTCACTTAG.

The resulting fragments were then ligated into appropriately digested ping 14 in vitro transcription–translation vector (Bannister et al. 1991), and the resulting construct was translated and radiolabeled with [35S]methionine using the TNT rabbit reticulocyte lysate coupled transcription–translation system (Promega) according to the manufacturer's instructions. Radiolabeled human TBP was expressed from the construct pARhTFIID (GPP-63), a generous gift of M.G. Peterson (Peterson et al. 1990). The missense mutants of ScTBP were constructed using oligonucleotide-directed site mutagenesis of a uracil-containing single-stranded DNA (ssDNA) template produced from the ping 14–ScTBP construct, essentially as in Ausubel et al. (1991). The carboxy-terminal deletion of C34 (1–197) was synthesized by linearizing the ping 14–C34 template with BstBI. This linearized template was then transcribed and translated to generate labeled protein in a conventional two-step in vitro translation reaction (Ausubel et al. 1991).

Protein–protein interaction studies

Generation and purification of fusion proteins consisting of BRF domains linked to GST was accomplished by the method of Smith and Johnson (Smith and Johnson 1988; Lin and Green 1991; Bannister et al. 1993). Fusion proteins were purified by adsorption onto glutathione–agarose beads (Smith and Johnson 1988; Bannister et al. 1993) and analyzed by SDS-PAGE and Coomassie staining. Equal amounts of each protein were used in the protein–protein interaction studies, except for unfused GST, which was used, at least, at a concentration fivefold higher. Identical negative results were also obtained when lower amounts of unfused GST were employed. Interaction assays were conducted as follows: 10-μl samples of glutathione–Sepharose beads bearing either GST alone or the GST–BRF fusion were preincubated at room temperature for 15 min on a rotating wheel with 30 μg of BSA in buffer Z' 200 (25 mM HEPES–KOH at pH 7.5, 200 mM KCl, 12.5 mM MgCl$_2$, 20%

glycerol, 0.1% NP-40, 20 μM ZnSO$_4$, 2 mM DTT). A sample of radiolabeled in vitro-translated protein was then added, and incubation continued for another 60 min. After extensive washing with buffer NETN/200 (20 mM Tris-HCl at pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP-40), the agarose beads were mixed with SDS sample buffer and boiled; then samples were analyzed by SDS-PAGE followed by fluorography. All assays were repeated multiple times with at least two different preparations of fusion protein. Quantitations were carried out from autoradiographs using a Quantimet (Cambridge Instruments).

Yeast transformation and complementation

A S. cerevisiae strain (rad3-52; leu2-3,112; his3Δ200; tds4Δ::LEU2) containing a plasmid bearing the HIS3 gene and BRF temperature-sensitive allele tds4-144 (restrictive temperature 38°C) was provided by S. Buratowski (Whitehead Institute, Cambridge, MA). Transformation with plasmids containing the URA3 gene and either CaBRF or the wild-type S. cerevisiae BRF/TDS4 was performed using the lithium acetate procedure of Schiestl and Gietz (1989). To measure growth rates of resulting strains, cells were grown at the permissive temperature to saturation and then diluted to the same OD$_{650}$ values in 20 ml of YNB/2% glucose supplemented with 20 μg/ml of uracil prewarmed to the appropriate temperature. Aliquots of the cultures were taken for measurement of culture density by OD$_{650}$ at intervals.

Acknowledgments

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