The general transcription factor IIb (TFIIB) is required for accurate and efficient transcription of protein-coding genes by RNA polymerase II (RNAPII). To define functional domains in the highly conserved N-terminal region of TFIIB, we have analyzed 14 site-directed substitution mutants of yeast TFIIB for their ability to support cell viability, transcription in vitro, accurate start site selection in vitro and in vivo, and to form stable complexes with purified RNAPII in vitro. Mutations impairing the formation of stable TFIIB-RNAPII complexes mapped to the zinc ribbon fold, whereas mutations conferring downstream shifts in transcription start site selection were identified at multiple positions within a highly conserved homology block adjacent and C-terminal to the zinc ribbon. These results demonstrate that the N-terminal region of yeast TFIIB contains two separable and adjacent functional domains involved in stable RNAPII binding and transcription start site selection, suggesting that downstream shifts in transcription start site selection do not result from impairment of stable TFIIB-RNAPII binding. We discuss models for yeast start site selection in which TFIIB may affect the ability of preinitiation complexes to interact with downstream DNA or to affect start site recognition by a scanning polymerase.

Eukaryotic RNA polymerase II (RNAPII) requires the action of at least six accessory proteins to accurately initiate transcription. These accessory proteins, termed the general transcription factors (GTFs), have been the focus of much investigation and include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. The GTFs and RNAPII assemble in an ordered stepwise fashion on a class II promoter in vitro to form a functional preinitiation complex (PIC) (reviewed in Ref. 1). Assembly is initiated by the binding of TFIID to the TATA element via the TATA-binding protein (TBP) subunit, in some cases assisted by TFIIA. This complex is recognized by TFIIB, which binds and recruits RNAPII and TFIIF. PIC formation is completed by the association of TFIIE and then TFIIH, and the resulting complex can hydrolyze ATP and initiate mRNA synthesis. In contrast to this ordered-assembly model for PIC formation, it has been proposed that a preassembled holoenzyme, consisting of RNAPII, most of the GTFs, and additional factors, is recruited in one step to promoter-bound TFIID in vivo (reviewed in Ref. 1).

The general transcription factor TFIIB has an essential role in RNAPII transcription, and together with RNAPII and TBP, defines the minimal set of factors necessary for promoter-dependent transcription of a supercoiled DNA template in vitro (1). In both the ordered-assembly and holoenzyme-recruitment models of PIC formation, TFIIB recognizes promoter-bound TFIIID and facilitates association of the remaining GTFs and RNAPII. Consistent with this role, TFIIB interacts with DNA adjacent to the TATA box (2) and binds to TBP (3–5), the TBP-associated factor TAF40 (6), RNAPII (4, 7, 8), and both subunits of TFIIF (4, 9). TFIIB may also play a role in the regulation of transcription by gene-specific regulatory proteins, as many of these regulatory factors bind TFIIID directly (10–17). In addition, TFIIB is involved in the selection of transcription start sites, as mutations in the Saccharomyces cerevisiae SUA7 gene, encoding TFIIB, can alter transcription start site selection in vivo and in vitro (7, 18).

The conservation of TFIIB structure among eukaryotic organisms underscores its central role in transcription. The protein is comprised of a highly conserved N-terminal region and a C-terminal core domain (Fig. 1). Although no structural information has been obtained for full-length TFIIB, NMR structures have been obtained for the core domain of human TFIIB and for a portion of the N-terminal region of the archaeal TFIIB from Pyrococcus furiosus (19, 20). In addition, the crystal structure has been reported for the core domain, as well as for a DNA-TBP/TFIIB ternary complex (21). These studies have revealed several structural motifs, including a zinc ribbon fold in the N-terminal region, a pair of a-helical direct repeats in the core domain, and an amphipathic helix between the repeats.

The high degree of amino acid conservation of the N-terminal region of TFIIB suggests that important functions reside in this portion of the protein. Reflecting this, random PCR mutagenesis of the yeast TFIIB gene identified a number of cold- and temperature-sensitive mutants that mapped to this region (22). To determine the functions of the N-terminal region and to define functional domains, we have undertaken a site-directed mutagenesis study of yeast TFIIB. In previous work, we identified two substitution mutants, L50D and R64E, that exhibited impaired RNAPII binding and a downstream shift in transcription start site selection (7). To extend our analysis and to define the functional domains involved in stable RNAPII binding and transcription start site selection, we have in this work analyzed 14 additional site-directed substitution mutants for their ability to support cell viability, transcription.
**Functional Domains in the N-terminal Region of Yeast TFIIB**

**Results**

**Immunoblotting**—To determine the steady-state protein levels of TFIIB mutants with altered growth properties, strains containing both wild-type or polyhistidine-tagged TFIIB constructs in plasmid p314/YIB1 (TRP1 selectable marker), selecting for Ura-, Trp+, colonies on CAA medium lacking uracil and tryptophan at 30 °C (7). Transformants were grown in liquid CAA medium lacking uracil and tryptophan at 30 °C, dilutions were spotted on SC medium containing 5-fluorouracil acid (5-FOA), and the plates were incubated at room temperature for 4 days. 5-FOA, toxic to cells containing a functional URA3 gene, selects for cells that have spontaneously lost the URA3-containing plasmid p316/YIB1 with wild-type TFIIB. Thus, the appearance of 5-FOA-resistant colonies reflects the ability of a TFIIB mutant to support cell viability in the absence of wild-type TFIIB. To further analyze the growth properties of mutants that supported viability, 5-FOA-resistant colonies were grown to mid-exponential phase in liquid YPD (1% yeast extract, 2% Bactopeptone, 2% dextrose) medium at room temperature, and dilutions in sterile samples were centrifuged at 15,000 × g for 3 min. Supernatants were transferred to fresh tubes containing 2.5 μl of anti-RNAPII monoclonal antibody (8WG16, Research Diagnostics) and incubated for 90 min at 4 °C with gentle rocking. Pansorbin (pre-blocked, 25 μl in P100) was then added, and the reactions were incubated for 60 min at 4 °C with gentle rocking. The samples were centrifuged at 15,000 × g for 2 min, and the pellets were washed three times with 250 μl of buffer P100 containing 0.1% Nonidet P-40 and once with 250 μl of P100. The pellets were resuspended in 40 μl of 1× SDS sample buffer, and 10 μl of each sample was resolved on a 10% SDS-polyacrylamide gel and transferred to Immobilon-P polyvinylidene difluoride membranes. Immunoblotting was performed as described above, using an affinity-purified anti-yeast TFIIB antibody (1:5,000), followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson Laboratories, 1:40,000).

**RESULTS**

**Identification of TFIIB N-terminal Substitution Mutants with Altered Properties in Vivo**—The N-terminal region of TFIIB contains several highly conserved sequence motifs (Fig. 1). To address the role of these motifs in the function of yeast TFIIB, we constructed and analyzed 14 site-directed substitution mutants. Substitutions were introduced at conserved residues that reside within the second rubredoxin knuckle of the zinc ribbon, the C-terminal β-strand of the zinc ribbon, and an adjacent homology block that is highly conserved among seven eukaryotic species. The mutants were initially tested for their ability to support yeast cell viability using a plasmid-shuffle complementation assay (see “Experimental Procedures”). Plasmid-shuffling of the mutants revealed that substitution mutants C45S/C46S and L52P did not support cell growth (Table I). The remaining 12 mutants, capable of supporting viability, were tested for their growth rates at several temperatures. Substitution mutants were indistinguishable from the wild-type, whereas five mutants (W63P, W63R, R64A, F66D, and H71E) exhibited varying degrees of cold sensitivity, with the W63P and H71E mutants also displaying sensitivity to elevated temperature. The mutants were also analyzed on a variety of other growth media, but the relative growth properties on these media were similar to those observed with YPD (data not...
The amino acid sequence of the N-terminal region of the archaeal TFIIB from P. furiosus is shown along with the position of the five β-strands (B1–B5) of the zinc ribbon. Presented below the P. furiosus sequence is a comparison of the amino acid sequences of TFIIB from seven eukaryotic organisms. Identical residues are in bold, and conserved residues are shaded. Asterisks below the S. cerevisiae sequence (amino acids 20–85) denote the positions of site-directed mutants analyzed previously (7). The position and nature of the site-directed substitutions analyzed in this work are denoted with arrows at the bottom of the figure. P. Fur, P. furiosus; K. Lac, Kluyveromyces lactis; S. Pom, Schizosaccharomyces pombe; X. Lac, Xenopus laevis; Dros, Drosophila melanogaster; Rat, Rattus norvegicus; Human, Homo sapiens; S. Cer, S. cerevisiae.

**TABLE I**

| Growth properties of yeast TFIIB N-terminal substitution mutants |
|---------------------------------------------------------------|
| Plus and minus designations correspond to relative growth rates compared to the wild-type (WT). Mutants indistinguishable from wild-type are: L52E, K55E, L56P, R60E, T65I, N68I, and P76A. |
|                  | 16°C | 30°C | 37°C  |
|------------------|------|------|-------|
| WT               | ++   | ++   | +++   |
| C45S/C48S        |      |      |       |
| L52P             |      |      |       |
| W63R             | +++  | +++  | +++   |
| W63P             | +/+  | +/+  | ++    |
| R64A             | ++   | ++   | +++   |
| F66D             | ++   | +++  | +/+   |
| H71E             | ++   | +++  | +     |

To confirm that the observed growth phenotypes were due to inherent functional impairments and not due to instability of the mutant proteins, we examined the steady-state levels of the mutant proteins by immunoblotting. Polyhistidine-tagged versions of the mutant proteins expressed from the wild-type (WT) were retested and confirmed to confer the same growth properties, we expressed and purified recombinant polyhistidine-tagged versions of the mutant TFIIB proteins and tested their abilities to support basal and activated transcription using a TFIIB-depleted whole-cell extract. The C45S/C48S double substitution mutant did not support any detectable level of transcription above background, whereas the L52P and W63P mutants were severely compromised for transcriptional activity (Fig. 3). The H71E mutant was indistinguishable from wild-type TFIIB, but significantly, the W63P, W63R, R64A, and F66D mutant proteins all conferred preferential usage of the more downstream transcription start site. The observed defects in transcriptional activity of the mutant proteins are unlikely to be due to gross misfolding, as all of the recombinant mutant proteins were competent to form stable TBP-TFIIB-DNA ternary complexes in a gel mobility-shift assay (data not shown). In addition, all of the mutant proteins that supported some basal transcription also supported a strong response to the transcriptional activator GAL4-VP16 (Fig. 3). These results demonstrate that the second rubredoxin knuckle and C-terminal β-strand of the zinc ribbon are critical motifs for basal transcription activity, whereas the adjacent homology block participates in transcription start site selection.

**Determination of Transcription Start Sites in Vivo**—The results of the in vitro transcription assays revealed that several of the mutants displayed an alteration in transcription start site selection on the CYC1 promoter. To confirm and extend these results, we examined in vivo start site utilization at the ADH1 and CYC1 promoters in the mutant strains. For comparison, we also analyzed RNA from strains containing the E62K and R64E substitution mutants, shown previously to alter start site utilization (7, 18). Consistent with the in vitro results, mutants W63P, R64A, and F66D displayed a downstream shift in start site selection from both the CYC1 and ADH1 promoters, whereas the shifts observed for mutant W63R were less pronounced (Fig. 4).

**Analysis of RNAPII-TFIIB Interaction**—In previous work,
we identified a substitution mutant in the C-terminal β-strand of the zinc ribbon (L50D) that was unable to bind purified yeast RNAPII in vitro (7). Although this result suggests that the zinc ribbon is involved in the interaction between TFIIB and RNAPII, additional mutants in the zinc ribbon would further establish its participation in RNAPII binding. Therefore, we determined the ability of the N-terminal mutants to form stable complexes with purified RNAPII using a co-immunoprecipitation assay. The C45S/C48S and L52P mutants did not form stable complexes with RNAPII, whereas the remaining mutants did complex with RNAPII, with the W63P protein exhibiting only slight impairment (Fig. 5). To confirm participation of the N-terminal region of yeast TFIIB in stable RNAPII binding, we tested the ability of recombinant core domain (residues 119–346) to form stable complexes with RNAPII. Full-length wild-type or L50D TFIIB protein was included in the binding reactions to act as internal positive and negative controls for stable RNAPII binding, respectively. As shown in Fig. 6, the core domain of TFIIB did not detectably bind RNAPII in these assays. Taken together, these results demonstrate that the second rubredoxin knuckle and the C-terminal β-strand of the zinc ribbon in the N-terminal region of TFIIB are involved in the direct interaction between TFIIB and RNAPII.

**DISCUSSION**

In this work, we have analyzed the in vivo and in vitro properties of 14 site-directed substitution mutants in the conserved N-terminal region of yeast TFIIB. Several structural motifs were targeted for mutagenesis, including the second rubredoxin knuckle and C-terminal β-strand of the zinc ribbon fold and an adjacent homology block that is conserved among seven eukaryotic species (Fig. 1). In vivo analyses revealed that two of the mutants, C45S/C48S and L52P, were incapable of supporting yeast cell growth, while an additional five mutants exhibited cold sensitivity and, in two cases, temperature sensitivity as well (Table I). In vitro transcriptional analysis demonstrated that both the C45S/C48S and L52P mutant proteins were severely impaired in their ability to support basal transcription (Fig. 3). These transcriptional defects correlated with an inability of these two mutant proteins to form stable complexes with purified RNAPII in vitro (Fig. 5). Similarly, the core domain, which lacks the N-terminal region and the zinc ribbon, also failed to bind RNAPII (Fig. 6). These results, combined with our previous determination that the L50D mutant is impaired for RNAPII binding, strongly suggest that the zinc ribbon fold, and in particular the C-terminal β-strand of the ribbon, are critical structural motifs for stable interaction of TFIIB with RNAPII.

Four of the five mutants that displayed conditional growth properties contain a substitution in the homology block adjacent to the zinc ribbon (Fig. 1). The one conditional mutant with a substitution outside of this homology block, H71E, exhibited a temperature-sensitive phenotype but was comparable to the wild-type with respect to transcription activity in vitro, RNAPII binding, and transcription start site selection. The precise biochemical defect of the H71E mutant protein remains to be determined. The four conditional mutants with a substitution in the homology block (W63R, W63P, R64A, and F66D) exhibited cold-sensitive phenotypes and a downstream shift in transcription start site selection both in vivo and in vitro (Table I, Figs. 3 and 4). These results demonstrate that multiple residues within the highly conserved homology block adjacent to the zinc ribbon participate in selection of transcription start...
sites. In previous work, Hampsey and co-workers reported that E62K or R78C mutations conferred downstream shifts in start site selection (18). Residue Glu-62 is also contained within this homology block, whereas Arg-78 is C-terminal to this block, but also is an invariant residue among seven species (Fig. 1). In ongoing studies, we have utilized a random PCR-generated mutant TFIIB library and a genetic selection scheme to directly

select for TFIIB mutants conferring downstream shifts in start site selection. To date, all of the mutants identified in this selection contain substitutions of residues in the homology block adjacent to the zinc ribbon or of residues Arg-78 or Val-79.2

Our results demonstrate that two separable and adjacent functional domains in the N-terminal region of yeast TFIIB govern stable RNAPII binding and transcription start site selection (Fig. 7). Mutations that impair stable RNAPII binding, as determined by a co-immunoprecipitation assay, extend from the second rubredoxin knuckle of the zinc ribbon through the C-terminal β-strand of the ribbon to residue Glu-62. We suggest that mutation of the first rubredoxin knuckle of the zinc ribbon would also impair RNAPII binding, because it is highly likely that the integrity of the ribbon fold is required for proper RNAPII interaction. Mutations altering start site selection encompass residues Glu-62 to Phe-66, and also include the more C-terminal pair of conserved residues Arg-78 and Val-79. Mutants W63R, W63P, R64A, and F66D, all conferring downstream shifts in start site selection, were proficient for stable RNAPII binding. Moreover, the L52P mutant was defective for stable RNAPII binding, but the low level of transcription supported by this mutant protein initiated at normal start sites in vitro (Fig. 3). Thus, our results indicate that downstream shifts in start site selection are not caused by impairment in the stable association of TFIIB and RNAPII. It was reported previously that the E62K and R78C mutants, both conferring downstream shifts in start site selection, were impaired for RNAPII binding in a surface plasmon resonance assay (8). We suggest that residue Glu-62 is involved in both start site selection and RNAPII binding, i.e. the adjacent domains for stable RNAPII binding and start site selection overlap at residue Glu-62.

We have determined that substitutions of residue Arg-78 other than R78C, such as R78L, confer downstream shifts in start site selection without impairing stable RNAPII binding by our assay (7).2 The failure of the R78C mutant to bind RNAPII perhaps results from the introduction of a cysteine residue adjacent to the zinc ribbon. The presence of this additional cysteine could interfere with normal coordination of zinc by the adjacent four cysteines in the zinc ribbon, thereby perturbing the ribbon structure and impairing RNAPII binding.

The mechanism by which transcription start sites are selected by RNAPII remains to be determined. In higher eukaryotes, transcription initiation usually occurs at a discrete start site located 25–30 base pairs downstream of the TATA element. In contrast, transcription initiation in S. cerevisiae frequently occurs at multiple start sites within a window of 30–120 base pairs downstream of the TATA element (26, 27). The window for transcription initiation in S. cerevisiae could result from preinitiation complexes that loop out a limited amount of intervening downstream DNA to interact with potential initiation sites. If so, TFIIB mutants could confer down-

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**Fig. 5.** Stable binding of TFIIB mutant proteins with purified RNAPII. Purified recombinant TFIIB proteins were tested for their ability to form stable TFIIB-RNAPII complexes by co-immunoprecipitation. Binding reactions contained 100 ng of the indicated recombinant TFIIB protein and 500 ng of purified yeast RNAPII where indicated. Proteins were precipitated with a monoclonal antibody specific for RNAPII (8WG16, see “Experimental Procedures”). The input TFIIB protein (25% of total) and the precipitates (25% of total) were analyzed by immunoblotting using an affinity-purified anti-yeast TFIIB polyclonal antibody. The RNAPII-independent species of greater molecular weight than TFIIB corresponds to the heavy chain of the 8WG16 mouse IgG, which weakly cross-reacts with the goat anti-rabbit IgG secondary antibody. Shown in the left and right vertical panels are the results from two separate experiments.

**Fig. 6.** The core domain of yeast TFIIB does not detectably bind RNAPII. Binding reactions contained 35 ng of recombinant core TFIIB (residues 119–346), 50 ng of wild-type or L50D proteins as positive or negative internal controls, respectively, and 500 ng of purified RNAPII where indicated. The input TFIIB proteins (50% of total) and the precipitates (50% of total) were analyzed by precipitation and immunoblotting as described in the legend to Fig. 5. The RNAPII-independent species of greater and lower molecular weight than TFIIB correspond to the heavy and light chains of the 8WG16 mouse IgG, respectively, which weakly cross-react with the goat anti-rabbit IgG secondary antibody. The minor species in lanes 1 and 3 that co-migrates with core domain is a degradation product of the full-length proteins.

**Fig. 7.** Summary of mutations that impair stable RNAPII binding or confer downstream shifts in start site selection. Yeast TFIIB residues 11–90 are marked with asterisks designating the positions of substitution mutants that impair stable RNAPII binding (asterisks above the amino acid sequence) or confer downstream shifts in transcription start site selection (asterisks below the amino acid sequence).

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2 S. L. Faitar and A. S. Ponticelli, unpublished data.
stream shifts in start site selection by altering preinitiation complex structure so as to impair the ability to loop out shorter segments of DNA, thereby impairing the ability of the complexes to utilize start sites closer to the TATA element. However, it has been reported that promoter melting for the S. cerevisiae GAL1 and GAL10 genes in vivo is comparable to other eukaryotes, occurring approximately 20 base pairs downstream of the TATA element (28). The extent of promoter melting appears to be independent of the distance between the TATA box and the transcription start sites, suggesting that the positioning and subsequent promoter melting of S. cerevisiae preinitiation complexes is similar to mammalian preinitiation complexes. Thus, transcription initiation in the downstream window from S. cerevisiae TATA elements may be the result of a "scanning" polymerase (28). If start site selection in S. cerevisiae involves a scanning polymerase, upstream or downstream shifts in start site utilization could result from RNAPII with enhanced or diminished initiation site recognition, respectively. Upstream shifts in start site selection have been observed both in vitro and in vivo with yeast strains containing a deletion of the small RPB9 subunit of RNAPII or mutation of the C-terminal zinc-binding motif in RPB9 (29–31). Mutation or deletion of the RPB9 subunit might alter the structure of polymerase on the DNA such that initiation sites are more efficiently recognized early in the scanning process. Conversely, downstream shifts, conferred by mutations in TFIIB or the RPB1 subunit of RNAPII (32), may be a consequence of a polymerase with diminished start site recognition. TFIIB might function in start site selection as a component of the scanning polymerase complex and assist in initiation site recognition and/or utilization (18). If so, mutations that impair the stable association of TFIIB and RNAPII might produce unstable scanning complexes, resulting in alterations in start site selection.

Although it remains possible that TFIIB is part of a scanning polymerase complex, our results demonstrate that most mutations that confer downstream shifts in start site selection do not affect stable association of TFIIB and RNAPII. Moreover, the L50D and L52P mutants, defective for stable RNAPII binding yet able to support low levels of transcription, support initiation at normal start sites. Therefore, if a scanning polymerase is involved in start site selection, our results are more consistent with TFIIB conferring properties for initiation site recognition upon RNAPII at the time of preinitiation complex assembly. In this model, the zinc ribbon of TFIIB has the major function in the stable interaction with RNAPII, whereas the adjacent homology block may interact with RNAPII in a more subtle manner to affect start site selection. Such an interaction between the homology block of TFIIB and RNAPII could affect the conformation of the polymerase on the DNA, thereby affecting subsequent start site recognition upon the onset of scanning. Continued biochemical analyses of the TFIIB mutants described here should provide additional insight into the mechanism of transcription start site selection and the role played by TFIIB.

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