Alternative outcomes in assembly of promoter complexes: the roles of TBP and a flexible linker in placing TFIIIB on tRNA genes

Cláudio A.P. Joazeiro,1 George A. Kassavetis, and E. Peter Geiduschek

Department of Biology and Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92093-0634 USA

Saccharomyces cerevisiae transcription factor (TF) III, a TATA-binding protein (TBP)-containing multisubunit factor, recruits RNA polymerase (Pol) III for multiple rounds of transcription. TFIIIC is an assembly factor for TFIIIB on TATA-less tRNA gene promoters. To investigate the role of TBP–DNA interactions in tRNA gene transcription, we generated sequence substitutions in the SUP4 tRNA gene TFIIIB binding site. Purified transcription proteins were used to analyze the selection of transcription initiation sites and the physical structures of the protein complexes formed on these mutant genes. We show that the association of TFIIIB with tRNA genes proceeds through an initial step of binding-site selection that is codirected by its TBP subunit and by TFIIIC. TFIIIB is assembled in a predominantly metric manner with regard to box A, the start site–proximal binding site of TFIIIC, but TFIIIC opens a window within which wild-type TBP can select the TFIIIB-binding site. Despite its clear preference for AT-rich sequences, TBP can mediate TFIIIB assembly at diverse DNA sequences, including stretches containing only G and C. However, a mutant TBP, m3, which recognizes TATAAA and TGTAAA and is active for Pol III transcription, utilizes other sequences only poorly. We also show that alternative alignments between DNA-bound TFIIIB and TFIIIC are possible, implying a remarkably flexible linkage, and suggest that Tfc4, the TFIIIB-assembling subunit of TFIIIC, could be responsible for such elasticity. The relevance of these findings to alternative initiation of Pol II- and other Pol III-transcribed genes is discussed.

[Key Words: TFC4; RNA polymerase III; promoter complexes; TBP; TFIIIB; TATA-less genes]

Received December 12, 1995; Accepted February 6, 1996.
That the role of TFIIIC in vivo is not only to prevent repression of transcription by chromatin is evident from the observation that selection of start sites for U6 gene transcription in vivo is influenced both by box A and by the TATA box (Chalker and Sandmeyer 1993; Eschenbauer et al. 1993; Gerlach et al. 1995).

The binding of TFIIIB to tRNA and 5S rRNA genes is less well understood. The initial step in the formation of rDNA gene transcription complexes is recognition by TFIIIC of box A [consensus TRGYBARY(y)GGY, where R stands for purine, Y for pyrimidine, and lowercase for weaker conservation] and box B [consensus GGTT-CRANTCC], both located intragenically. box B is the high-affinity binding site, whereas the start site–proximal box A anchors the TFIIIC subunits (the τ₄ domain) that are more directly involved in the assembly of TFIIIB upstream of the transcription start site (for review, see White 1994; Geiduschek et al. 1995). No obvious sequence conservation has been observed upstream of tRNA genes, where TFIIIB binds. Sequence substitutions in this region are well tolerated, in contrast to the extremely deleterious effect of certain box B point mutations on the binding of TFIIIC and, indirectly, of TFIIIB (Kurjan and Hall 1980; Baker et al. 1986; Léveillard et al. 1993; Kaiser and Brow 1995).

Despite their different assembly pathways in vitro, the complexes of TFIIIB with the TATA-containing U6 gene and TATA-less RNA genes exhibit indistinguishable properties (of high stability, resistance to stripping at high ionic strength and by polyamions, and structural characteristics as determined by DNase I footprinting) and suffice for directing Pol III to multiple rounds of transcription (Joazeiro et al. 1994). This equivalence raises questions about the internal structures of TFIIIB bound to TATA-less and TATA-containing genes. Some biochemical and genetic data have been interpreted to suggest that TBP holds the components of TFIIIB together in TATA-less tRNA gene transcription but does not bind directly or tightly to DNA (for review, see Struhl 1994). TBP bends DNA sharply (J. Kim et al. 1993; Y. Kim et al. 1993), one might expect, accordingly, that TFIIIB complexes with TBP bound to a TATA box on the one hand, and with TBP loosely contacting DNA on the other, would have grossly different properties. However, TFIIIB does sharply bend the DNA of TATA-less genes (Léveillard et al. 1991; Braun et al. 1992b) although there is no direct evidence implicating TBP in this phenomenon.

In the work that is presented here, we analyze the TFIIIC-mediated assembly of TFIIIB onto the SUP4 tRNA⁶⁰ gene, and describe the transcription properties and physical structures of initiation complexes assembled on tRNA genes with sequence substitutions in the upstream-binding region. We derive three main conclusions from this work: [1] The binding of TFIIIB to TATA-less genes is codirected by TFIIIC and, unexpectedly, TBP, creating a potential for clashes of protein–protein and protein–DNA interaction that results in heterogeneous placement of transcription initiation complexes, generating multiple transcription start sites. Similar results were obtained for the U6 gene in vivo (Eschenlauer et al. 1993; Chalker and Sandmeyer 1993; Gerlach et al. 1995). Thus, the classification of Pol III genes according to the presence or absence of a TATA box does not reflect a mechanistic dichotomy in the assembly of TFIIIB.

[2] “Nonspecific” TFIIIB complexes with GC-rich DNA and “specific” complexes with AT-rich DNA have similar properties and neither type of complex is electrostatically stabilized. [3] A flexible linker connects TFIIIB and TFIIIC in the initiation complex. We suggest that the flexible linkage is located in Tfc4, the TFIIIB-assembling subunit of TFIIIC.

**Results**

**SUP4 can operate as a completely TATA-less gene**

The 5′-flanking regions of the yeast tRNA genes are generally AT-rich but do not suffice for TBP-mediated TFIIIB assembly in the absence of TFIIIC (Kassavetis et al. 1989, 1990). To determine whether the SUP4 tRNA gene would function as a completely TATA-less gene, we generated a promoter mutant (omniGC) in which most of the DNA normally protected by TFIIIB from DNase I cleavage was turned very GC-rich (Fig. 1A), and examined the effects of this substitution on TFIIIB binding and transcription.

Transcription complexes were assembled with highly purified TFIIIC and TFIIIB (in the experiments presented in this work, TFIIIB and its subunit B′′ were limiting). Transcription was analyzed by primer extension. As shown in Figure 1B, the omniGC mutant (lane 5) is less well transcribed than is the wild-type gene (lane 1). This result was obtained with all materials tested: the Brα crude extract [Braun et al. 1989] containing TFIIIC, TFIIIB and Pol III, TFIIIB at each of four intermediate steps of purification, and TFIIIB reconstituted from all three recombinant subunits. However, transcription levels of the omniGC gene varied from approximately one-half (BRα) to less than one-tenth (Cibacron blue TFIIIB) of wild type (data not shown). We attribute at least a part of this variation to a combination of two effects: varying but relatively slow rates of assembly of stable TFIIIB–DNA complexes (possibly accentuated for the omniGC gene), combined with a time-dependent inactivation of unbound TFIIIB (S.K. Whitehall, pers. comm.). Stabilization by accessory factors yet to be identified could yield these kinds of differential effects, but we did not explore this issue further.

As determined previously (Léveillard et al. 1993), transcription initiation of the wild-type gene takes place mostly at bp + 1, but with lower efficiency also at bp + 4 and +8 [Fig. 1B, lane 1]. Most omniGC gene transcription initiates correctly, at bp + 1 [lane 5], but there is also initiation at a very low rate at bp +15, +18, +21, and +35. [This transcription is dependent on TFIIIC; data not shown].

TFIIIB–DNA complexes are extraordinarily stable and also resist stripping by very high concentrations of electrolyte [Kassavetis et al. 1990]. We set out to determine
whether transcriptionally active TFIIIB complexes with the omniGC gene are as resistant to stripping by high concentrations of NaCl as are complexes with the wild-type gene (Fig. 1B). TFIIIC(C+B) complexes were assembled under standard salt concentrations (100 mM NaCl), treated with 0.5 M NaCl, and diluted to 150 mM NaCl, at which latter concentration TFIIIB assembly can no longer take place (Fig. 1B, lanes 4, 8). Under these conditions, RNA synthesis results from interactions between Pol III and TFIIIB-DNA complexes that formed prior to the high ionic strength challenge. Transcription initiating at all sites in both the wild-type and omniGC genes is not differentially affected by prior stripping of initiation complexes with 0.5 M NaCl for 5 min (Fig. 1B, cf. lanes 2 and 3, and 6 and 7). Similar results were obtained with a 10-min challenge with 1 M NaCl (data not shown).

Figure 1. (See following page for legend.)
The stepwise assembly of transcription factors on the wild-type and omniGC genes was analyzed by gel retardation (Fig. 1C). As expected, TFIIIC binds equally well to wild-type and omniGC DNA (lanes 2 and 3, respectively). A fraction of the complexes of TFIIIC with wild-type DNA is converted into two slower migrating species in the presence of TFIIIB (lane 4). The faster-migrating of these is presumed to contain TFIIIC + Brf + TBP. [Brf and TBP stably bind TFIIIC–DNA in the absence of B', and reconstitute all of the properties of the fraction referred to as B' (Kassavetis et al. 1992); B" is limiting in the Cibacron blue TFIIIB preparations analyzed, data not shown]). The slower-migrating of these is presumed to contain TFIIIC + TFIIIB. The omniGC gene instead generates a smear of faint, slowly migrating bands (lane 5). Heparin treatment strips TFIIIC but leaves TFIIIB bound to DNA (Kassavetis et al. 1990). TFIIIC(C + B')–DNA complexes are also dissociated by heparin (Kassavetis et al. 1991). Heparin-resistant TFIIIB–DNA complexes are formed with the wild-type gene (lane 6) and, at a lower efficiency, with the omniGC gene (lane 7). The inefficiency of the omniGC DNA in binding TFIIIB can therefore account for the lack of shift of the TFIIIC complex by the TFIIIB fraction and also correlates with this template's poorer transcription activity.

To determine the location of the TFIIIB binding site on the omniGC DNA, it was necessary to perform two-dimensional footprinting, because of this DNA's lower efficiency in binding TFIIIB. This technique consists of treating preformed protein–DNA complexes with DNase I and subsequently separating binding from free DNA by native polyacrylamide gel electrophoresis. Individual bands are identified, cut from the gel, and the DNA is eluted and analyzed by sequencing gels. Under these conditions, whereas heparin-resistant TFIIIB complexes with the wild-type gene reveal complete protection of bp -9 to -40 on the top strand (Fig. 1D, lane 2), the TFIIIB–omniGC DNA complexes generate a DNase I footprint suggestive of a more heterogeneous placement of TFIIIB onto upstream DNA sequence (lane 4, data not shown): [1] The central portion of the TFIIIB footprint is complete (between bp -22 and -31) but partial on either side; and [2] protection extends farther downstream than on the wild-type gene to bp + 8 and perhaps beyond, as enhanced cleavage between bp -6 and +2 upon binding of TFIIIB to the wild-type gene (lane 2) is greatly diminished on the omniGC DNA probe (lane 4).

We conclude that although TFIIIB normally binds to AT-rich regions in naturally occurring tRNA genes, it can also form complexes with GC-rich DNA, albeit at lower efficiency. Once those complexes are formed, their properties are indistinguishable from those of TFIIIB complexes with AT-rich DNA: [1] They are transcriptionally active and resistant to stopping by high concentrations of salt and polyamines; and [2] they have nearly the same electrophoretic mobility when placed comparably on DNA [Fig. 1C, lanes 6, 7], suggesting comparable DNA bending (cf. Léveillard et al. 1991; Braun et al. 1992b). We also conclude that TFIIIC has a preferred assembling geometry, as it is capable of placing TFIIIB predominantly at the same site relative to box A in the wild-type and omniGC genes, regardless of the presence or absence of an AT-rich DNA sequence at that site.

A 6-bp TATA box can codirect TFIIIB binding with TFIIIC

Chalker and Sandmeyer (1993) generated mutant SUP2 tRNA genes to study the physical relationship between Pol III transcription start sites and insertion sites of the yeast transposon Ty3. These templates had a consensus 6-bp TATA box at different positions upstream of the transcription start site and exhibited some alterations in start site selection in vivo [Chalker and Sandmeyer 1993]. Our own experiments in vitro with the same tem-

Figure 1 SUP4 can operate as a completely TATA-less gene. [A] Sequences, sites of transcription initiation, and TFIIIB footprints of wild-type, omniGC, and TA genes. The extent of the top-strand TFIIIB footprint is indicated by a solid bar above the corresponding DNA sequence, and locations relative to the major transcription start site of the wild-type gene (as bp +1) are marked at the top. Start sites are indicated by arrows, with thicker arrows indicating relatively greater utilization. [B] The omniGC gene is transcribed more weakly than the wild-type gene, but TFIIIB forms high salt-resistant transcription complexes with both genes. Wild-type (lanes 1-4) or omniGC (lanes 5-8) templates (200 fmoles) were incubated with TFIIIC (14 fmoles), TBP (50 fmoles), Brf (40 fmoles), and purified B' (35 fmoles) in 100 mM NaCl for 50 min. Equal volumes of solutions were then added to provide 100 mM (lanes 1, 5), 150 mM (lanes 2, 6) or 500 mM (lanes 3, 7) NaCl. After a 5-min incubation, each reaction mixture was diluted to 150 mM NaCl for transcription with Pol III (5 fmoles) and NTPs for 25 min. For lanes 4 and 8, TFIIIC and TFIIIB components were added to a mixture containing template DNA in 500 mM NaCl, and incubated for 5 min, and the mixture was diluted to 150 mM NaCl and provided with Pol III and NTPs for 25 min. Transcription products were analyzed by primer extension. Sites of transcription initiation are indicated at right. [Note that if pausing by reverse transcriptase contributes to the production of any of these bands, it is quantitatively more significant for the wild-type gene, which yields higher total levels of longer transcripts.) The global rate of transcription (all initiation sites) of the omniGC gene was 14% of the rate for the wild-type gene. [C] The omniGC gene binds TFIIIB less efficiently. TFIIIC (14 fmoles) and purified TFIIIB (0.5 fmoles) were added to be incubated with 2 fmoles wild-type or omniGC probes, as specified above each lane. Heparin was added to the reaction mixtures for lanes 6 and 7 prior to native gel electrophoresis. Free DNA [F], DNA complexes with TFIIIC (C), TFIIIC + Brf + TBP (C + B'), TFIIIC + TFIIIB (C + B), and TFIIIB (B) are indicated at right. [D] TFIIIB forms at the same site in wild-type and omniGC DNA: two-dimensional footprinting. Top strand-labeled wild-type or omniGC probes (4-5 fmoles) were incubated with or without TFIIIC (14-21 fmoles) and TFIIIB (1.5 fmoles), treated with heparin, and then digested partially with DNase I. Free DNA [F] and TFIIIB–DNA complexes [B] were isolated by native gel electrophoresis as noted (top), and partial DNase I digestion products analyzed on a sequencing gel are shown. The extent of protection by TFIIIB is indicated by the solid bar at left.
plates (plasmid-bearing strains were generously provided by S. Sandmeyer) yielded similar results [data not shown]. However, only a small fraction of the transcripts were found to initiate at sites expected from TFIIIB misplacement directed by the TATA box. We presume that this might be attributable to the retention of a competing AT-rich sequence at bp -30 in the Challer–Sandmeyer mutant templates. To determine whether the TFIIIC-mediated binding of TFIIIB to tRNA genes could be influenced by a TATA box (TATAAA), we generated similar mutant genes in the background of the poorly transcribed omniGC template, in an attempt to obtain less heterogeneous and more quantitatively altered material for direct biochemical analyses. A 6-bp TATA box was chosen to minimize TFIIIC-independent binding of TFIIIB [Joazeiro et al. 1994]. In these TA mutants (Fig. 1A), two other base pairs (−13 and −15) were substituted in the omniGC background to generate a sequence anticipated to be more favorable for promoter opening in the vicinity of bp −10, based on the consensus sequence around tRNA gene transcription start sites and on the lower melting temperature of A:T base pairs.

Transcription of these TA genes differed quantitatively and qualitatively (Fig. 2A): All mutants exhibit alterations in the utilization of transcription start sites relative to the wild-type gene that were mapped by primer extension of in vitro-synthesized RNA [presented below]; the mutant templates also differ in promoter activity. Construct TA-30 and the wild-type gene have similar promoter strengths, the TA-25 and TA-21 templates are somewhat less active, and the TA-40 template is very poorly active. It is critical to stress that the 6-bp AT-rich sequence created by these mutations do not suffice for TFIIIB binding as measured by transcription in the absence of TFIIIC (lanes 7–10).

To gain a better understanding of the mechanisms and structures responsible for these promoter mutant phenotypes we analyzed DNase I footprints and examined the distribution of initiation sites for transcription by primer extension analysis [Fig. 2B,C; the footprint of TFIIIB in the wild-type gene, and the analysis of its transcription initiation sites have been described previously [Kassavetis et al. 1989, 1992; Léveillard et al. 1993]].

The coordinates of the DNase I footprints of the TA-30 gene–TFIIIB complex and the wild-type gene–TFIIIB complex are very similar [cf. Figs. 1D, lane 2, and 2B, lane 4], with protection on the top strand extending from bp −8 to −40. Most of the TA-30 gene transcripts initiate at bp +1 and, to a lesser degree, at bp +4; initiation at bp +8 is much lower than for the wild-type gene [Fig. 2C, lanes 1,4]. The differences in transcription start site utilization between wild-type and TA-30 templates may be related to a higher degree of heterogeneity in TFIIIB placement [and, consequently, of Pol III placement] on the wild-type gene. Although the corresponding TFIIIB footprints are not obviously different, that is expected, as the utilization of the start site at bp +8 in the wild-type gene is relatively minor, and a small fraction of TFIIIB molecules displaced so as to generate those additional transcripts would be difficult to detect with the DNase I protection assay. On the other hand, the footprint of TFIIIB on the TA-21 gene shifts 7 bp downstream, extending from bp −3 to −34 [Fig. 2B, lane 6], and transcription initiates mostly at bp +8 [Fig. 2C, lane 6]. The TA-40 template forms a complex with TFIIIB that is shifted 10 bp upstream, protecting bp −20 to −51 from DNase I [Fig. 2B, lane 2] and transcription initiates inefficiently at several sites [bp +1, +4, +15, +18], but more efficiently at bp −11 [Fig. 2C lane 3; see also Fig. 3B]. Initiation at all of these sites is TFIIIC-dependent [data not shown]. Identical footprints were obtained with TFIIIB reconstituted entirely from recombinant components [data not shown; the results in Fig. 2B were obtained with recombinant TBP, recombinant Brf, and purified B’”.

Despite their different transcription activities, the wild-type and TA genes are similarly efficient in binding TFIIIC [Fig. 2D, lanes 2–5] and in forming heparin-resistant complexes with TFIIIB [Fig. 2D, lanes 6–9]. Most of the TFIIIB bound to the TA-40 gene probe is appropriately positioned to direct initiation of transcription −10 bp upstream of the natural SUP4 gene initiation site. We suggest that the apparent inconsistency between equally effective formation of TFIIIB complexes and inefficient initiation of transcription is mostly attributable to the lack of an efficient start site promoter element around bp −11 [cf. Fruscoloni et al. 1995].

Transcription of the TA-25 gene was also examined. Transcription starts with comparable efficiency at bp +4 and +8 [Fig. 2C, lane 5; see also Fig. 2A], clearly unlike transcription of the TA-21 and TA-30 genes [Fig. 2C, lanes 4,6], whose AT-rich sequences are at the opposite side of the DNA helix. To determine whether the interaction between TFIIIC and box A imposes a constraint in the placement of TFIIIB at bp −25 we generated a combined box A–upstream binding region (UBR) mutant. Three nucleotide substitutions in box A that are known to diminish the ability the SUP4 gene to compete for limiting factors in a crude extract [Allison et al. 1983] were introduced in the TA-25 background, generating the T22T27Agl/TA-25 gene. The mutations in box A have very little effect on transcription [Fig. 2C, lane 7]: the ratio of transcripts initiating at bp +4 and +8 does not change, and the proportion of reverse transcription products that might correspond to initiation at bp +15, +18, +21, and +25 increases only slightly. [We have not verified whether these minor products are dependent on TFIIIC or whether they result from pausing in reverse transcription of tRNA templates with altered secondary structure due to mutations in box A].

The above results show that TFIIIB can select its binding site through interactions with short AT-rich sequences in a GC-rich background, provide strong evidence to suggest that the TFIIIB binding site determines the location of transcription initiation sites, and define a rule for this relationship: Transcription starts 28–30 bp downstream of the 5’ end of the AT-rich element selected for binding. We also conclude that multiple alignments are possible between the TFIIIB binding site and the box A promoter element that mediates TFIIIB assembly by anchoring the τA domain of TFIIIC. The assembly
Figure 2. A 6-bp TATA box specifies the TFIIIC-dependent placement of TFIIIB. (A) Transcription of wild-type and TA genes. Wild-type, omniGC, TA-40, TA-30, TA-25, or TA-21 DNA (100 fmoles of each) was incubated with TFIIIC (14 fmoles) and TFIIIB (0.5 fmoles) as specified above each lane before transcription. The recovery markers (r.m.) are labeled; RNA products are in the lower panel. (B) TFIIIB-binding sites on TA genes: two-dimensional footprinting. Top strand-labeled TA-40, TA-30, or TA-21 probes were incubated with or without TFIIIC and TFIIIB prior to heparin treatment and DNase I protection analysis as specified for Fig. 1D. The numbers at left indicate the positions of bands relative to the start site of transcription (bp +1). (C) Transcription initiation sites of wild-type and TA genes. The DNAs specified above each lane (100 fmoles) were incubated with TFIIIC (14 fmoles) and purified TFIIIB (1.0 fmoles), transcribed, and RNA products were analyzed by primer extension. Initiation sites are indicated at right. (D) Wild-type and TA DNAs bind similar amounts of TFIIIC and TFIIIB: Gel retardation analysis. TFIIIC (14 fmoles) and TFIIIB (0.5 fmoles) were allowed to bind to 5.5 fmoles of wild-type, TA-40, TA-30, or TA-21 probes for 40 min, as specified above each lane. Heparin was added to the reaction mixtures in lanes 6–9 for 2 min prior to loading onto the gel. Free DNA (F) and DNA complexes with TFIIIC (C) and TFIIIB (B) are indicated at left.

is flexible enough even to allow the placement of TFIIIB at opposite sides of the DNA helix. Mechanisms that might allow TFIIIC to mediate the assembly of TFIIIB at multiple positions covering a span of at least ~70Å along DNA are explored further in experiments that follow.

Compensatory mutations in the TBP subunit of TFIIIB suppress a promoter-down mutation

The results described to this point suggest that TFIIIB binding to DNA may take place through an initial step of selection of AT-rich sequences. To determine whether the TBP subunit of TFIIIB plays a role in this selection, we examined the consequences for TFIIIB binding of an A→G substitution at the second position of the TATAAA sequence in the TA-40 gene. This particular substitution was selected for two reasons: (1) It severely lowers TBP and TFIIID binding and Pol II promoter activity [Wobbe and Struhl 1990; Strubin and Struhl 1992]; and (2) the mutant TBP protein TBPm3 binds to both TATAAA and TGATAA [Strubin and Struhl 1992] and can be tested for its ability to suppress the A→G mutation on the TA-40 template when used to reconstitute TFIIIB.

As expected, the A→G mutation in TG-40 lowers initiation at bp -11 in reactions containing TFIIIB re-
TBP and TFIIIC codirect TFIIIB binding to tRNA genes

Figure 3. The TBP subunit of TFIIIB interacts with DNA on tRNA genes: TBPm3 suppresses a tRNA gene promoter-down mutation. TA-40 or TG-40 DNA (100 fmoles) was incubated with TFIIIC (14 fmoles), Brf (50 fmoles), purified B' (35 fmoles), and either TBPwt (200 fmoles; wt) or TBPm3 (200 fmoles; m3) as specified above each lane. Transcription products were analyzed by primer extension. Initiation sites are indicated at right.

These results show that a mutant TBP genetically selected for its ability to bind to TGTAAA confers, in TFIIIB, the equivalent ability to bind to this sequence, in a TFIIIC-dependent manner. The results also specify that the TBP subunit of TFIIIB interacts directly with DNA at some steps of TFIIIC-mediated assembly. We conclude that the binding of TFIIIB to tRNA genes is codirected by TFIIIC and TBP.

TFIIIB reconstituted with TBPm3 displays a narrowed binding site specificity

Three amino acid changes in TBPm3 allow this protein to bind to TGTAAA (Strubin and Struhl 1992). We expected that TFIIIBm3 might be able to bind to a larger variety of sequences than does wild-type TFIIIB and generate a broader utilization of start sites but found instead that the TA-40 and TG-40 genes only yield transcripts initiating at bp −11 in reactions with TFIIIBm3; in contrast, transcription with TFIIIBwt gives rise to several additional transcripts [Fig. 3].

To understand this effect better, the analysis was extended to other alleles [Table 1]. The effect of TFIIIBm3 on transcription of the TA-40 and TG-40 genes is not start site sequence specific: TFIIIBm3 can direct initiation efficiently from bp +1 [TA-40], +4 [TA-25], and +8 [GC-30]. Only initiation at bp +15 and +18 is inefficient with TFIIIBm3 on all analyzed templates. Also, the effect of TFIIIBm3 is not specific to minor transcription start sites: Transcription of the TA-25 template with TFIIIBwt initiates efficiently at both bp +4 and +8. TFIIIBm3 was found to increase initiations slightly at bp +4 and lower initiation at bp +8.

The source of the differences in transcription initiation with TFIIIBwt and TFIIIBm3 is suggested by comparing DNA sequence −30 bp upstream of each transcription initiation site with the effect of substituting TFIIIBwt with TFIIIBm3 (Table 1). For most of the genes we have analyzed, the major transcription initiation site is located 28–30 bp downstream from the 5′ end of a

| Template | Start site | Percent TFIIIBwt | Sequences in upstream window |
|----------|------------|-----------------|-----------------------------|
| Wild type | +1         | 32              | TGTATATGTTG                 |
|          | +4         | 15              | ATATGTTGTTA                 |
|          | +8         | 9               | GTTATATGTTA                 |
| T22T27A31| +1         | 32              | TGTATATGTTG                 |
|          | +4         | 12              | ATATGTTGTTA                 |
|          | +8         | 9               | GTTATATGTTA                 |
|          | +15        | 5               | GTATCTTCTT                  |
|          | +18        | 7               | GTATCTTCTT                  |
| TA-40    | −11        | 46              | AATTATAAAC                  |
|          | +1         | 6               | TGGCGGCAGGC                 |
|          | +4         | 6               | CGGCAGGCAG                  |
|          | +15        | 4               | GTCCCTTACT                  |
|          | +18        | 1               | CTTACTTTT                   |
| TA-30    | +1         | 48              | TGTATAAAGC                  |
|          | +4         | 26              | ATAAGCCGC                   |
| TA-25    | +4         | 111             | CGGCTATAAA                  |
|          | +8         | 22              | TATAAAGTGTC                 |
| TA-21    | +8         | 59              | CGCCTATAAA                  |
| GC-30    | +8         | 75              | CGGCTATAGA                  |
|          | +15        | 2               | GGTATAGTACT                 |
|          | +18        | 3               | GTATCTCTT                   |
| omniGC   | +1         | 6               | TGGCGGCAGGC                 |
|          | +4         | 6               | CGGCAGGCAG                  |

*Table 1. Comparison of upstream DNA sequence with effects of TFIIIBm3 on transcription*

*Single determinations of transcription with TFIIIBm3, expressed as percentage of transcription with TFIIIBwt.

The window was selected to show sequences 23–32 bp upstream of each initiation site.

*The very low signal-to-noise ratio for this very minor transcript precludes quantification.
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TATA-like sequence, whereas the same spacing applied to the remaining initiation sites reveals a variety of upstream sequences (see, e.g., TA-40). Only the major initiation site is affected little by TFIIIBm3 or not at all. For the omniGC gene, the 5' end of the sequence GCGGCC is 30 bp upstream of the major initiation site (bp +1), and that site is poorly utilized in reactions with TFIIIBm3 (this observation cannot be accounted for by the low transcription levels of omniGC). These results show that the ability of TFIIIBm3 to direct initiation to a particular site correlates with the similarity of the sequence beginning 28-30 bp upstream of that site to a TATA box.

We conclude that the TBP mutant m3, despite having gained the ability to interact with G in addition to A at position 2 of the 6-bp TATAAA site, has a diminished capacity to bind to sequences that diverge from the 6-bp consensus TATA box outside of position 2, at least in the context of TFIIIC-mediated TFIIIB binding. We speculate that the triple amino acid substitution in a highly conserved DNA-binding domain of TBPm3 may compromise its ability to make a critical structural adaptation that is required for DNA binding and/or bending in the context of TFIIIB.

The results analyzed in Table 1 reinforce our conclusion that TBP is the subunit of TFIIIB that selects its binding site upstream of tRNA genes, when assembled by TFIIIC, through interactions with AT-rich sequences.

**TFIIIB naturally binds to either of two AT-rich sites in the SUP4 tRNA gene UBR**

In the TATA-less SUP4 tRNA<sup>Tyr</sup> gene short AT-rich sequences are present within the region protected by TFIIIB from DNase I cleavage, at bp −43, −30, and −22. These AT-rich clusters do not suffice for TFIIIB binding to SUP4 in the absence of TFIIIC and are footprinted only poorly by TBP at very high concentrations of this protein (T. Léveillard, unpubl.). We generated promoter mutants to determine whether these sequences had quantitative or qualitative effects on TFIIIB binding (Fig. 4A).

As described previously (Kassavetis et al. 1989), the DNase I footprint of TFIIIB on the wild-type SUP4 gene bottom strand extends from bp −8 to −43 (Fig. 4B, lanes 1,2). The complex of TFIIIB with a mutant gene bearing a GC-rich sequence between bp −31 and −39 has identical footprint boundaries (GC-40 mutant; Fig. 4B, lanes 7,8). Transcription levels of wild-type and GC-40 mutant DNAs with purified TFIIIB and TFIIIC are also comparable, with occasional variations (Fig. 4C), and the utili-
zation of start sites is identical for these templates (major start site at bp +1 but also at +4 and +8; Fig. 4C). Therefore, substitutions of sequences between bp −31 and −39 have little or no effect on the parameters that we analyze. On the other hand, a GC-rich sequence located between bp −13 and −21 (GC-20 mutant) has clear-cut effects on SUP4 gene transcription: Transcription levels are increased relative to the wild-type gene and, as for the TA-30 mutant, transcription initiates at bp +1 and +4, but not at bp +8 [Fig. 4C, lane 4]. However, the TFIIIB footprint coordinates are identical to the wild type (bp −8 to −43). The effects of the placement of the GC-rich sequence between bp −22 and −30 (GC-30 mutant) are similar to those generated by the TA-21 mutation: The TFIIIB-protected region is shifted ~10 bp downstream, relative to the wild-type gene [bp +5 to −35; Fig. 4B], and the major transcription initiation site shifts from bp +1 to bp +8, +15, and +18 [Fig. 4C, lane 3]. If these multiple initiation sites reflect heterogeneous TFIIIB placement, the natural AT-rich sequence at bp −21 of this mutant is probably a less efficient competitor for TFIIIB placement than is the TATAAA sequence in the TA-21 gene, which generates less diversity of transcription start sites (starts almost entirely confined to bp +8; Fig. 2C).

These results indicate that the TFIIIC-mediated placement of TFIIIB on the TATA-less wild-type SUP4 gene takes place through interactions of TBP with the AT-rich sequence at bp −30 and, in a small fraction of the templates, with the AT-rich sequence at bp −21.

**TFIIIC has an extendable arm**

box A is the promoter element that serves as an anchoring site for start site–proximal subunits of TFIIIC that are involved in TFIIIB recruitment to the upstream promoter region [Bartholomew et al. 1991; Kassavetis et al. 1991]. The results presented so far show that TFIIIC can be assembled by TFIIIB at multiple distances from box A. Assembly of TFIIIB at multiple sites could be mediated by interaction of TFIIIC with surrogate pseudo-boxes A, as when the spacing between box A and box B is suboptimal [Baker et al. 1987; Fabrizio et al. 1987; Eschenlauer et al. 1993; Joazeiro et al. 1994]. To further explore the mechanisms underlying the assembly of TFIIIB at multiple sites, the structures of TFIIIC(B+C) complexes with TA-21, TA-30, and TA-40 genes were analyzed by DNase I footprinting (Fig. 5).

TFIIIC alone protects box B and, more weakly, box A to a similar degree for the TA-21, TA-30, and TA-40 genes (lanes 2, 5, and 8, respectively). The addition of TFIIIB to the interaction mixtures further stabilizes TFIIIC binding to box A and its vicinity on all three genes, and generates extended protection against DNase I cleavage at different sites upstream of the respective transcription start sites (lanes 3, 6, 9). The upstream borders of the TFIIIC(B+C) footprints do not change upon stripping with heparin (bp −34 for TA-21, bp −40 for TA-30, and bp −51 for TA-40; Fig. 2B) and are thus attributable to TFIIIC. The upstream border of the TFIIIC-alone DNase I footprint lies at about bp +11 [Kassavetis et al. 1990], whereas, as noted above, the downstream border of the TFIIIB-alone footprint (upon heparin treatment) lies at bp −3, −8, and −20 on TA-21, TA-30, and TA-40, respectively. In complexes containing both TFIIIC and TFIIIB, further protection that is continuous along DNA is generated between these borders. Site-specific DNA–protein photochemical cross-linking of TFIIIC–TFIIC–SUP4 DNA complexes previously implicated the 120-kD Tfc4 subunit of TFIIIC with this intervening protection [Bartholomew et al. 1991; Kassavetis et al. 1992]; the heparin-sensitivity of this protection also supports this contention. It is therefore notable that the span of interven-
ing protection, presumably attributable to Tfc4, increases as the location of TFIIIB shifts farther upstream: With TA-21 the protection extends from bp +10 to -2, with enhanced cleavage between bp +1 and +5; with TA-30, protection extends from bp +10 to -6, with bp +1 and -1 unprotected; with TA-40, protection extends from bp +10 to -19, with bp -12 less well protected. The presence of TFIIIC also diminishes enhanced DNase I cleavage attributable to the binding of TFIIIB (see Fig. 2B) on the TA-30 and TA-40 genes.

We conclude that a flexible component in the initiation complex allows multiple alignments between TFIIIC bound to box A and TFIIIB bound at the UBR.

Discussion

In this work we show that AT-rich DNA sequences within the TFIIIB-binding site of tRNA genes exert a quantitative and qualitative influence on TFIIIC-dependent TFIIIB binding. This observation suggests that the traditional classification of Pol III genes according to promoter architecture (see, e.g., White 1994) and to the presence of a TATA box does not reflect a mechanistic dichotomy in the assembly of TFIIIB.

Our experiments also unveil new aspects of the TFIIIC-mediated assembly of TFIIIB onto tRNA genes (Fig. 6): TFIIIC has a preferred geometry for assembling TFIIIB that places TBP at the DNA sequence beginning near bp -30 (top left). Nevertheless, TBP can take advantage of a flexible linkage between TFIIIB and TFIIIC, and, within the mechanical limits of that linkage, search for a preferred binding site (top right). Once bound, TFIIIB forms indistinguishable complexes with a variety of DNA sequences (bottom). The TFIIIB-binding site ultimately determines the site of transcription initiation. Below we discuss the evidence for this model.

TFIIIC assembles TFIIIB on nonconserved sequence upstream of tRNA genes (Kassavetis et al. 1989, 1990). Although there is a preferential placement of TFIIIB relative to box A (Fig. 1B,D), alternative alignments are allowed, as in the U6 gene in vivo: In the context of the wild-type and omniGC genes, AT-rich sequences placed at different sites push and pull TFIIIB binding efficiently over 20 bp (Figs. 2 and 4); in naturally occurring tRNA genes (Fig. 6). A model of structures and mechanisms in the assembly of TFIIIB onto tRNA genes. The box A-binding site of TFIIIC, start sites of transcription, and AT-rich and GC-rich blocks at the TFIIIB-binding site are shown. Only the subunits of TFIIIC that sit in the vicinity of box A and interact with TFIIIB are specified. The other TFIIIC subunits are referred to as "rest of TFIIIC." All three subunits of TFIIIB (TBP, Brf, and B") are represented. The diagrams at the top illustrate schematically the preferred conformation and one alternative folding state of Tfc4, the 120-kD TFIIIB-assembling subunit of TFIIIC. The hypothesized alternative conformational states of Tfc4 widen the window for the binding site search of TBP. Tfc4 preferentially places TBP by the GC-rich DNA sequence at bp -30, but the alternative Tfc4 conformation allows TBP to interact with the AT-rich DNA sequence at bp -40. The equilibrium between the allowed conformations of TFIIIC need not entirely determine the partition of differentially placed TFIIIB complexes: As the final TFIIIB complex is practically irreversibly bound to DNA, this partition could also be influenced by the rate of the irreversible step of TFIIIB binding at different DNA sequences and by the stability of intermediate forms of the TFIIIB-DNA complex (as indicated by assorted arrows and question marks). The placement of TFIIIB determines the site of transcription initiation [indicated by bent arrows at bottom]: RNA synthesis starts 28–30 bp downstream of the 5' end of the sequence selected by TBP. Whether TBP sits close to DNA in the final complex with TFIIIB remains unresolved.

Figure 6. A model of structures and mechanisms in the assembly of TFIIIB onto tRNA genes. The box A-binding site of TFIIIC, start sites of transcription, and AT-rich and GC-rich blocks at the TFIIIB-binding site are shown. Only the subunits of TFIIIC that sit in the vicinity of box A and interact with TFIIIB are specified. The other TFIIIC subunits are referred to as "rest of TFIIIC." All three subunits of TFIIIB (TBP, Brf, and B") are represented. The diagrams at the top illustrate schematically the preferred conformation and one alternative folding state of Tfc4, the 120-kD TFIIIB-assembling subunit of TFIIIC. The hypothesized alternative conformational states of Tfc4 widen the window for the binding site search of TBP. Tfc4 preferentially places TBP by the GC-rich DNA sequence at bp -30, but the alternative Tfc4 conformation allows TBP to interact with the AT-rich DNA sequence at bp -40. The equilibrium between the allowed conformations of TFIIIC need not entirely determine the partition of differentially placed TFIIIB complexes: As the final TFIIIB complex is practically irreversibly bound to DNA, this partition could also be influenced by the rate of the irreversible step of TFIIIB binding at different DNA sequences and by the stability of intermediate forms of the TFIIIB-DNA complex (as indicated by assorted arrows and question marks). The placement of TFIIIB determines the site of transcription initiation [indicated by bent arrows at bottom]: RNA synthesis starts 28–30 bp downstream of the 5' end of the sequence selected by TBP. Whether TBP sits close to DNA in the final complex with TFIIIB remains unresolved.
genes, the TFIIB-binding site is also not absolutely fixed relative to box A [Kassavetis et al. 1989].

TFIIB bound at different sites stabilizes TFIIC binding to adjacent DNA, without apparently changing the placement of TFIIC at box A [Fig. 5]. If both TFIIB and the box A-binding domain of TFIIC (τA) are fixed in place through interactions with DNA, what allows the spacing between the two binding sites to vary, and what generates the stretching of the footprint as the TFIIB–box A separation increases? The Tfc4 subunit of TFIIC (120 kD) is efficiently cross-linked to photoactive nucleotides located from within the TFIIB-binding site to downstream of box A [Bartholomew et al. 1991], is the only subunit of TFIIC to undergo specific conformational changes during assembly of TFIIB onto DNA [Kassavetis et al. 1991, 1992], and is the TFIIB-interacting subunit of TFIIC [Bartholomew et al. 1991; Kassavetis et al. 1992, Khoo et al. 1994, Chaussivert et al. 1995]. We interpret the generation of variable lengths of footprint between box A and the TFIIB-binding site in the TA templates [Fig. 5] as a result of protein being stretched out along DNA and imagine that the stretching may occur within Tfc4 [Fig. 6]. A striking feature of the derived amino acid sequence of Tfc4 is the presence of 11 tetratricopeptide repeats (TPRs; Marck et al. 1993; Rameau et al. 1994). TPRs are postulated to mediate protein–protein interactions [for review, see Lamb et al. 1995]. We envision that some of the TPR repeats of Tfc4 serve as a folding arm or provide multiple alternative binding sites for TFIIB and/or TFIIC [Khoo et al. 1994, Chaussivert et al. 1995]. Further structure analysis, especially of protein–DNA alignments in this segment of the transcription factor complex, will clearly be of some interest.

DNA sequence recognition during TFIIC-mediated assembly of TFIIB is performed by its TBP subunit: the TBPm3 mutant, which recognizes both TATAAA and TGTAAA, suppressed the transcription defect of the TG-40 gene [Fig. 3], the utilization of initiation sites is also affected by TBPm3 [Fig. 3, Table 1]. A comparison of the crystal structures of TBPm3 and the wild-type protein reveals no surface changes likely to alter interactions with other proteins [S. Burley, pers. comm.]. We surmise, accordingly, that the start-site selection effect of the m3 mutations is attributable to a narrowed DNA-binding specificity of TBPm3 that is manifested possibly through altered kinetics of binding to different sequences.

The conclusion that TBP interacts with DNA during assembly of TFIIB on tRNA genes is consistent with our previous observation that TFIIB complexes with TATA-containing U6 and TATA-less tRNA genes are indistinguishable [Joazeiro et al. 1994]. The following reasons might have accounted for the lack of success of previous work (including work from our laboratory) in deriving this conclusion. [1] The lack of conservation of sequence at the TFIIB-binding site of Pol III genes and tolerance for substitutions of sequence upstream of tRNA genes [Kurjan and Hall 1980; Kassavetis et al. 1989, Léveillard et al. 1993; G.A. Kassavetis, unpubl.] could be attributable to either the intrinsic flexibility of the TFIIC:TFIIB interaction, allowing TFIIB to probe for better binding sites in the vicinity of a substituted sequence, or to the high local concentration of TFIIB brought close to DNA by TFIIC. High local concentration, coupled to the very slow dissociation rate of TFIIB from DNA [Kassavetis et al. 1989] should bypass the requirement for a high-affinity binding site. As predicted by this model, certain mutations in the DNA-binding domain of TBP have a weaker effect on TATA-less Pol III transcription than on TATA box-dependent Pol II transcription, because the latter depends on an initial step of TATA recognition by TBP rather than an assembly factor-requiring step [Schultz et al. 1992, Poon et al. 1993]. [2] A low requirement for specific DNA sequence by TBP in the context of TFIIB may be the result of additional contacts established by Brf and B”. The influence of B” is profound: It is not until this protein is bound that TFIIB becomes heparin- and salt-resistant [Kassavetis et al. 1991]. [3] The lack of success in photo-cross-linking TBP within the TFIIB complex of either the SUP4 TRNA gene, SS rRNA gene, or U6 snRNA gene [Bartholomew et al. 1991, 1994, Braun et al. 1992a; Kassavetis et al. 1992, C. Bardeleben, pers. comm.] must have been attributable to either the spatial arrangement between this protein and the cross-linking group or to competition with other TFIIB components for cross-linking. (A slightly different reagent, also protruding from the major groove, but placed elsewhere, cross-links TBP efficiently, B. Bartholomew, pers. comm.). [4] The interaction of TBP with upstream DNA is an essential but transitory step during TFIIB complex formation, such that DNA contacts initially made by TBP are later replaced by Brf and B”. The concomitant loss in the photo-cross-linking of TBP upon incorporation of B” into the TBP–Brf–TFIIC–DNA complex [Kassavetis et al. 1992] and transformation of the TFIIB complex to its heparin-resistant state is consistent with interpretations 3 and 4.

If TBP sits close to DNA in the completely assembled complex of TFIIB with tRNA genes, how does it interact stably with GC-rich DNA on the omnitGC gene and with AT-rich DNA so as to generate apparently identical complexes? TBP establishes base pair contacts only in the minor groove [Starr and Hawley 1991; Lee et al. 1991], which allows less specific sequence discrimination. Specific and nonspecific DNA form complexes with TBP that have similar rates of dissociation, are more thermostable than the unbound protein, and are transcriptionally competent [Coleman and Pugh 1995]. Another example of a protein docking on its specific DNA site and a heterologous site in nearly identical ways is provided by work on the glucocorticoid receptor dimer bound to one specific half-site and one nonspecific half-site: there is a striking resemblance between the structures of the two half-complexes [Luisi et al. 1991].

Multiple initiation sites in eukaryotic transcription

We show that start site selection on tRNA genes is influenced by TBP, TFIIC, and Pol III [TFIIB is involved in accurate Pol II start site selection in yeast (Pinto et al.
Joazeiro et al.

1992), and we anticipate that Brf, its homolog in Pol III transcription, will be shown to have a comparable role, as it interacts with Pol III [Bartholomew et al. 1993; Werner et al. 1993; Khoo et al. 1994]. TBP and TFIIIC influence start site selection indirectly, by determining where TFIIIB will sit (see above). In the mutants that we analyzed, transcription initiation within a 30-bp window (Fig. 2C,D) is primarily not the result of "hunting" by Pol III, as the location of the major start site correlates with the major TFIIIB-binding site (Fig. 2A–C). Previous work had suggested this correlation (Léveillard et al. 1993). We extend that conclusion and reformulate it as a rule: Transcription initiates 28–30 bp downstream of the 5′ end of an AT-rich or TATA-like sequence. (Transcription of the yeast U6 gene also initiates 30 bp downstream of the 5′ end of the TATA box [Gerlach et al. 1995]). Pol III has a preference for certain DNA sequences around transcription start sites [Fruscoloni et al. 1995 and references therein] and can efficiently hunt at least 1 bp upstream and downstream for the best initiating nucleotide, but when forced to initiate 4 bp downstream of the normal site of the rRNA4-euk gene, it does so very inefficiently, or not at all [Fruscoloni et al. 1995]. In the context of the wild-type SUP4 gene, we suggest that start sites at bp +1 and +4 are not attributable to Pol III hunting but rather to distinctly placed TFIIIB complexes, assembled via TBP binding to partly overlapping sites, for two reasons: (1) The quantitative effects of TFIIIBm3 on initiation at bp +1 and +4 are different (Table 1); and (2) mutating the box A element alters the ratio between these two transcripts (data not shown).

Comparison of Pol II and Pol III promoters

TBP may interact with initiator (Inr)-containing and TATA-less Pol II promoters as it does with tRNA genes: (1) the TAF150 subunit of TFIID interacts with DNA around the transcription start site and farther downstream [Verrijzer et al. 1994], maybe in a functionally equivalent manner to the TFIIIC-box A interaction; (2) indirect evidence suggests that TBP interacts with TATA-less DNA in the −30 region of Inr-containing promoters [Wiley et al. 1992; Zenzie-Gregory et al. 1993]; and (3) mutations in the TBP DNA-binding domain that lower binding to the TATA box and lead to a decrease in transcription of TATA-containing Pol II genes have little effect on Pol III rRNA and Pol II Inr-containing gene transcription [Martinez et al. 1995]. These observations suggest how TFIID assembly onto TATA-less, Inr-containing promoters might proceed through an initial step of binding TAF150 to Inr, to create a high local concentration of TBP around DNA at bp −30 and promote the TBP-TATA-less-DNA interaction. If a relatively flexible linkage exists also in this system, TBP would be capable of similarly exercising binding site selection and play a role in determining the final geometry of the TFIID complex and fixing the sites of transcription initiation.

Materials and methods

DNA

The triple box A mutant SUP4 gene construct, pCJ Tz2Tz2A31 (numbers relative to the transcription start site as +1), combines three box A mutations described previously (U10, U15, and A19; Allison et al. 1983; Baker et al. 1986) and was made by site-directed mutagenesis. Plasmids pLNwt, containing the wild-type SUP4 tRNA gene, and pCJ Tz2Tz2A31 were used to construct the upstream mutant plasmids, taking advantage of a Bst1107I site centered 13 bp upstream of the transcription start site and a MunI site centered 21 bp farther upstream. Plasmid DNA were cut with MunI and Bst1107I and the large fragment was purified before ligating to the following annealed oligonucleotides (the complement of the bottom-strand oligonucleotide is underlined): wild-type gene, 5′-AATTITTTCAATTGTTATATGTGTTATAGTATA (note that a 31-bp oligonucleotide was used to replace a 23-bp fragment. The oligonucleotide design was such that the "wild-type" gene of this work has the sequence (−39) TTTCATT duplicated, and therefore gene-flanking sequences upstream of bp −41 are not the same as they occur in the original yeast SUP4 gene). omniGC, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-40, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-30, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-25, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-20, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-20, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-15, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-10, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-5, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-5, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-5, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-5, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-5, 5′-AATTGCTGCCGGGCCGCCTCGCCGG; TA-5, 5′-AATTGCTGCCGGGCCGCCTCGCCGG. Probes for gel retardation and footprinting assays were prepared as follows: Plasmid DNAs were cut with XbaI and treated with shrimp alkaline phosphatase prior to labeling with [γ-32P]ATP and T4 polynucleotide kinase. DNA was then purified from free label by gel filtration through 1-ml Sepharose CL2B columns. Subsequent digestion with EcoRI, PstI, and HindIII was used to generate the 302-bp end-labeled DNA probe, a 12-bp end-labeled fragment, and a 2.8-kb unlabeled fragment, which were not purified away.

Transcription proteins

Methods of preparation and activity assays for yeast TBP (recombinant), TFIIIB (purified to the Cibacron blue-Sepharose step), TFIIIC (purified by DNA affinity chromatography), Brf (recombinant, Mono S purified), Pol III (Mono Q purified), and B′ [yeast-derived, poly(dA-T)/poly(dA-T)] agarose-purified, recombinant Ni-NTA agarose purified] are described or referenced elsewhere [Kassavetis et al. 1990, 1992, 1995; Joazeiro et al. 1994]. Quantities of TFIIIC, TFIIIB, Brf, and B′ are specified in terms of femtomoles of active molecules for specific transcription and represent a minimum estimate [Kassavetis et al. 1989].

Plasmid pET-TPBm3, containing TBPm3 [Strubin and Struhl, 1992] on a pET11a T7 RNA Polymerase expression vector was constructed by replacing the 800-bp Bst36I–BamHI DNA fragment of pYTFIID [generously provided by M. Sayre (Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD) and R. Kornberg (Stanford University School of Medicine, Palo Alto, CA)] with the equivalent fragment possessing all three mutations of the TBPm3 protein from a yeast expression plasmid containing TBPm3 [a generous gift of K. Struhl [Harvard University School of Medicine, Boston, MA]]. TBPm3
was purified as for the wild-type protein, and its concentration estimated by the Bradford assay using wild-type TBP as a standard.

**Acknowledgements**

We are grateful to Simon Whitehall for advice, shared information, discussions, and comments on the manuscript, to David Brow for comments on the manuscript, to Suzanne Sandmeyer and Kevin Struhl for providing plasmids and to Ashok Kumar for recombinant Bt. C.A.F. gratefully acknowledges a fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil. Our research has been supported by a grant from the National Institute of General Medical Sciences.

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C A Joazeiro, G A Kassavetis and E P Geiduschek

*Genes Dev.* 1996, 10:
Access the most recent version at doi:10.1101/gad.10.6.725

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