The symmetry of the yeast U6 RNA gene's TATA box and the orientation of the TATA-binding protein in yeast TFIIB

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The central RNA polymerase III (Pol III) transcription factor TFIIB is composed of the TATA-binding protein (TBP), Brf, a protein related to TFIIB, and the product of the newly cloned *TFC5* gene. TFIIB assembles autonomously on the upstream promoter of the yeast U6 snRNA (*SNR6*) gene in vitro, through the interaction of its TBP subunit with a consensus TATA box located at base pair -30. As both the DNA-binding domain of TBP and the U6 TATA box are nearly twofold symmetrical, we have examined how the binding polarity of TFIIB is determined. We find that TFIIB can bind to the U6 promoter in both directions, that TBP is unable to discern the natural polarity of the TATA element and that, as a consequence, the U6 TATA box is functionally symmetrical. A modest preference for TFIIB binding in the natural direction of the U6 promoter is instead dictated by flanking DNA. Because the assembly of TFIIB on the yeast U6 gene in vivo occurs via a TFIIC-dependent mechanism, we investigated the influence of TFIIC on the binding polarity of TFIIB. TFIIC places TFIIB on the promoter in one direction only; thus, it is TFIIC that primarily specifies the direction of transcription. Experiments using TFIIB reconstituted with the altered DNA specificity mutant TBPm3 demonstrate that in the TFIIB-U6 promoter complex, the carboxy-terminal repeat of TBP contacts the upstream half of the TATA box. This orientation of yeast TBP in Pol III promoter-bound TFIIB is the same as in Pol II promoter-bound TFIID and in TBP-DNA complexes that have been analyzed by X-ray crystallography.

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It is now well established that the TATA-binding protein (TBP) is an essential component of the transcription machineries of all three nuclear RNA polymerases (for review, see Hernandez 1993; White and Jackson 1992b). During transcription by RNA polymerase III (Pol III) TBP functions as an integral subunit of the central transcription factor TFIIB (Kassavetis et al. 1992b; Lobo et al. 1992; Taggart et al. 1992; White and Jackson 1992a), in association with two other polypeptides: Brf, a protein related to TFIIB (Buratowski and Zhou 1992; Colbert and Hahn 1992; López-de-Léon et al. 1992), and the product of the *TFC5* gene, which is known as B'' (Kassavetis et al. 1995). The assembly of TFIIB on the promoter is the key step in initiation and is understood most completely at TATA-less Pol III genes, such as those encoding tRNA. The promoter elements of tRNA genes, *box A* and *box B*, are contained in the transcribed sequence and serve as the binding site of the multisubunit assembly factor

TFIIC, which directs TFIIB upstream of the start site of transcription with little apparent regard to DNA sequence (Kassavetis et al. 1990).

Unlike tRNA genes, the yeast U6 small nuclear (sn)RNA gene *SNR6* contains both internal and external promoter elements: an intragenic *box A* element located at its customary position 20 bp downstream of the transcription start site, a *box B* element aberrantly positioned 120 bp downstream of the terminator, and a consensus TATA box at bp -30. *SNR6* transcription in vivo requires *box B* and is thus TFIIC-dependent, but the TATA element additionally functions to position TFIIB accurately on the upstream promoter (Eschenlauer et al. 1993; Gerlach et al. 1995; Burnol et al. 1993b). In contrast, *box A* and *box B*, and thus TFIIC, are dispensable for transcription in vitro, as TFIIB can direct its own assembly onto the U6 gene with the TATA box being essential for this process (Margottin et al. 1991; Burnol et al. 1993b; Joazeiro et al. 1994). One presumes, therefore, that the interaction of the TBP subunit of TFIIB with the TATA box is required for accurately initiated U6

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transcription in vivo and in vitro. Autonomous TATA-dependent binding of human TFIIB has also been described (Mitchell et al. 1992; Mitchell and Benfield 1993), and this process is somewhat reminiscent of the well-characterized assembly of TFIID onto TATA-containing Pol II genes where the TBP-TATA interaction nucleates formation of the preinitiation complex (Zawel and Reinberg 1992; Serizawa et al. 1994).

The structure of TBP and its interaction with DNA have been characterized in great detail. The conserved core domain consists of two repeats that differ in sequence but are topologically and structurally much alike (Nikolov et al. 1992; Chasman et al. 1993). The determination of the three-dimensional structures of full-length TBP from *Arabidopsis thaliana* and the carboxy-terminal core of yeast TBP interacting with different TATA elements has revealed that the antiparallel β -sheets on the concave underside of TBP form extensive hydrophobic contacts with the minor groove of the TATA box, severely deforming the DNA (J. Kim et al. 1993; Y. Kim et al. 1993). The structures allow one to rationalize the results of extensive biochemical studies (Lee et al. 1991; Starr and Hawley 1991; Horikoshi et al. 1992) and are, in particular, consistent with genetic studies, which imply that the carboxy-terminal (second) repeat is responsible for recognition of the 5'-half of the TATA box (Strubin and Struhl 1992; Arndt et al. 1994).

Despite the wealth of information concerning the interaction of TBP with DNA, some aspects of the mechanism of binding remain puzzling. Both the DNA-binding domain of TBP and the TATA box have nearly two-fold symmetry. Furthermore, recognition through the minor groove does not distinguish A:T from T:A base pairs. The mechanism by which TBP recognizes the specific direction of the TATA box is acknowledged as not understood (Kim and Burley 1994). That both cocrystal structures reveal no evidence for bidirectional binding of TBP has been used to argue that TBP is inherently able to determine the directionality of binding (Kim and Burley 1994). However, there is some evidence suggesting that the DNA-binding specificity of TBP can be modified according to the proteins with which it is associated (Lee et al. 1992; Heard et al. 1993).

In this study we have examined the ability of TBP as part of TFIIB to recognize the direction of the *SNR6* TATA box. We find TFIIB capable of binding to the *SNR6* promoter in both directions, and its constituent TBP unable to discern polarity at the TATA box. In contrast to TBP-mediated TFIIB assembly, TFIIC serves to recruit TFIIB to the promoter in one orientation only. Other experiments using TFIIB reconstituted with the mutant TBPm3 demonstrate that during U6 transcription complex assembly, the carboxy-terminal repeat of TBP contacts the upstream half of the TATA box. Thus, contrary to previous suggestions from work with higher eukaryotic systems (Mitchell et al. 1992; Mitchell and Benfield 1993; Wang and Stumph 1995), yeast TBP has the same orientation in DNA-bound TFIIB, in the TBP-DNA crystal structures, and in TFIID.

Results

Transcriptional symmetry at the U6 promoter

The interaction of TBP with the U6 TATA box is essential for TFIIB complex formation and thus for transcription in vitro (Burnol et al. 1993b; Joazeiro et al. 1994; Gerlach et al. 1995). Because both the TATA sequence and the DNA-binding domain of TBP exhibit nearly two-fold rotational symmetry, we examined whether TFIIB binds the U6 promoter in both directions. To address this question, we constructed bidirectional transcription units in which a pseudogene was engineered in the opposite orientation to the natural U6 gene. The pseudogene comprises a U6 start site sequence (−4 to +5) and a U6 terminator (+103 to +123) separated by a stretch of irrelevant yeast DNA, and contains no Pol III-specific internal promoter elements. In the interests of clarity and convenience we refer to the natural U6 gene as right-hand and the pseudogene as left-hand. The U6 promoter region (−48 to −5) was then cloned in both directions between the two opposing transcription units (see Fig. 1A). The resulting constructs, termed U6_R and U6_L, contain transcriptional start sites that are correctly spaced in both orientations relative to the TATA box. The natural direction of the promoter sequences in U6_R is toward the right-hand gene and, conversely, toward the left-hand transcription unit in U6_L.

The orientation of TFIIB-promoter complexes was monitored by analyzing multiple rounds of transcription. Two principal transcripts were observed: The larger RNA was the expected length of a right-hand (natural) U6 gene transcript, whereas the smaller had the size predicted for left-hand gene transcripts (Fig. 2A, lanes 1,2,6). Bidirectional transcription implies that TFIIB complexes assemble with left-handed and right-handed polarity on the upstream region of the U6 gene. Bidirectional transcription was also observed in experiments that employed TFIIB purified from yeast or reconstituted from entirely recombinant proteins (data not shown). Nonetheless, transcription favored the natural orientation of the U6_R promoter: right-hand transcription was, on average, 3.8-fold greater than left-hand transcription (Fig. 2B; Table 1). Conversely, when the entire promoter region was turned to face the left-hand gene (U6_L), left-handed transcription was favored, although the average preference was now only 2.4-fold (Fig. 2B; Table 1). This transcriptional skewing against the left-hand pseudogene is addressed in more detail below.

The role of the TATA box in determining the direction of transcription was assessed by mutating the U6_R TATA element. The U6_R background contains a single A to G change at −22 which effectively insulates the TATA box (bp −30 to −23) from adjacent AT-rich sequence, preventing the utilization of cryptic TATA sequences. However it is important to note that the other sequences flanking the TATA box are unchanged from the natural *SNR6* gene and remain in their original orientation with respect to the right-hand (natural) U6 gene. A single point mutation in the U6_R TATA element generates a perfectly symmetrical TATA box (5'-

Figure 1. (A) Bidirectional transcription constructs *U6_R* and *U6_L*. The right-hand (natural *U6*) gene is represented by the solid (black) bar and the left-hand (pseudo) gene is shaded gray. Both genes have *SNR6* start site sequence extending from 4 bp upstream to 5 bp downstream of the transcriptional start site (bp -4 to +5, represented by a short solid bar). The orientation of the promoter region (open area) is indicated by arrowheads and faces right in *U6_R*, and left in *U6_L*. (B) Mutations introduced into the *U6_R* TATA box. The position of the TATA sequence is indicated with respect to the start site of the right-hand gene, and the polarity of the TATA element is indicated by an arrow. Note that the sequences flanking the TATA box remain in their natural orientation.

TATATATA-3'; Fig. 1B), and in this context bidirectional transcription was again observed (Fig. 2A, lane 3). Moreover the bias in favor of right-hand transcription was still 2.9-fold (Fig. 2B; Table 1). Thus, even with the TATA box itself allowing no distinctions of polarity, transcription still had a preferred orientation. The introduction of A → T changes at TATA box positions 4 and 5 (bp -27 and -26) results in a *U6* gene in which the TATA sequence is effectively reversed (Fig. 1B). This construct (*U6* reverse), in which the TATA box faces the left-hand gene leaves the TATA box-flanking DNA sequence in its natural orientation. In this case, a substantial preference for right-hand transcription was maintained (on average 2.6-fold) despite the left-handed polarity of the TATA box (Fig. 2, A, lane 4, and B; Table 1). The A → T changes at positions 4 and 5 did not reduce the level of *U6* transcription (Fig. 2B), implying that these substitutions do not have an adverse effect on TFIIB binding. In contrast, the equivalent changes in a *his3* TATA box reduced TFIID binding, as assayed by transcription efficiency, to 41% of the control (Wobbe and Struhl 1990). This suggests that the DNA-binding specificity of TBP is modulated by the proteins with which it is associated. The creation of an extended 11-bp TATA box (*U6* extended), which comprises alternating TA sequence from bp -30 to -21, generated our most transcriptionally symmetric *U6* promoter with a 2:1 preference of right-hand over left-hand transcription, on average (Fig. 2, A, lane 5, and B; Table 1). The extended TATA box reduced the efficiency of right-hand transcription but was fully able to bind both TBP and TFIIB as judged by DNase I footprinting (data not shown). Moreover, we saw no evidence for suboptimal placement of TFIIB. It is possible that the extended TATA box has unfavorable consequences for other steps in transcription initiation.

Despite the fact that the pseudogene has both *SNR6*

start site and terminator sequences, it yielded fewer transcripts in multiple-round transcription than did the natural *U6* gene. This results in a degree of difficulty in interpretation that we sought to avoid by restricting transcription to a single round and eliminating complications because of variations in chain elongation and reinitiation. In these experiments transcription complexes were allowed to form and transcription was initiated with a nucleotide mixture lacking ATP, so that RNA chain elongation halts before the first A residue, at +7 in both genes. The aligned complexes were subsequently chased with ATP for 15 sec to allow stable elongating complexes to form, whereupon heparin was added to prevent reinitiation. Single-round transcription retained the bias of multiround transcription, irrespective of the polarity of the TATA element (Fig. 2C,D; Table 1). The *U6_R* and *U6_L* constructs contain identical upstream promoter sequences, differing only in their respective orientations, so that quantitatively equal formation of preinitiation complexes can be expected. The molar yield of left-hand transcripts from the *U6_L* template and right-hand transcripts from *U6_R* were nonetheless unequal. Evidently, our estimations of the relative capacity for right- and left-hand transcription are biased, with right-handed transcription overvalued, and conversely, left-handed transcription undervalued because of sequence differences upstream of -48 or, more likely, downstream of +5. However, the level of this bias can be quantified (as described in Table 1), and appropriately corrected data are presented in the right-hand column of Table 1. The normalized ratio for right-hand to left-hand transcription level in the *U6_R* promoter was 3:1 (Table 1). In the context of the *U6* symmetrical or *U6* reverse TATA box, the right-hand gene was still favored approximately twofold, whereas the extended TATA box created a nearly symmetrical promoter, with right-hand transcription only preferred 1.4-fold. These results dem-

Figure 2. Transcription of the bidirectional U6 constructs. (A) Multiple-round transcription. Complexes were formed by incubating supercoiled plasmid DNA (70 fmoles) with TBP (50 fmoles), Brf (40 fmoles), B' (28 fmoles), and Pol III (5 fmoles) for 40 min. Transcription was initiated by adding ribonucleoside triphosphates and was allowed to proceed for 30 min. The templates employed were pU6_R (lane 1), pU6_L (lane 2), pU6-symmetrical (lane 3), pU6-reverse (lane 4), pU6-extended (lane 5) and p-539H6 (lane 6). The position of the right-hand (natural U6) transcript, the left-hand (pseudogene) transcript and the recovery marker (r.m.) are indicated. (B) Multiple-round transcription experiments were analyzed on a PhosphorImager, and molar yields of transcripts, adjusted for sample loss, were calculated. Transcription is expressed as a percentage, relative to transcription of p-539H6 (shown in A, lane 6, U6 w.t.). The average of three separate experiments is shown. The vertical lines above each bar indicate standard deviation of the mean. Bars representing right-hand transcription are solid and those representing left-hand

transcription are open. (C) Single-round transcription of bidirectional constructs. Transcription complexes were formed as described in A, but transcription was limited to a single cycle (as described in Materials and methods). (D) Single-round transcription data quantified as described in (B).

onstrate that the polarity of the natural U6 TATA sequence has relatively little influence on the direction of transcription and that such asymmetry that does exist is substantially due to sequences flanking the TATA sequence. However, we show below how to generate a context in which TBP-TATA interactions determine the directionality of TFIIB binding.

Structural analysis of bidirectional transcription complexes

We used DNase I footprinting to analyze the distribution of transcription complexes at the U6 promoter. The

TFIIB footprint on the U6 gene is almost symmetrical about the U6 TATA box (Joazeiro et al. 1994), which makes a direct determination of the proportion of TFIIB molecules bound in a particular orientation practically impossible. However, Pol III extends protection of the *SUP4* tRNA^{Tyr} gene downstream but not upstream of TFIIB (Kassavetis et al. 1990), creating asymmetry in the DNase I footprint that serves as a marker of TFIIB orientation. In these experiments preassembled transcription complexes (either TFIIB-DNA or TFIIB-Pol III-DNA) were resolved from each other and from the free DNA by nondenaturing electrophoresis after partial DNase I digestion. The separated complexes were ex-

Table 1. Asymmetry of transcription

Gene	Polarity ratio	Multiple-round transcription ^a	Single-round transcription	Single-round transcription, corrected
U6 _R	R/L	3.8 ± 0.4	4.0 ± 0.6	
U6 _L	L/R	2.4 ± 0.3	2.3 ± 0.1	3.0 ± 0.5 ^b
U6 symmetrical	R/L	2.9 ± 0.4	2.6 ± 0.2	2.0 ± 0.3
U6 reverse	R/L	2.6 ± 0.3	2.5 ± 0.3	1.9 ± 0.4
U6 extended	R/L	2.0 ± 0.05	1.9 ± 0.1	1.4 ± 0.2

^aEach value is a molar ratio of right-hand (R) to left-hand (L) transcripts (or vice versa, as specified in column 2). Errors are standard deviation of the mean.

^bThe geometric mean of 4.0 and 2.3. It is tacitly assumed, for the purpose of the calculation, that the general, and from the point of view of this work, irrelevant, bias toward rightward transcription can be assigned in equal measure to each polarity. Thus, R/L transcript ratios in column 4 are corrected by the corresponding factor $(2.3/4.0)^{0.5} \approx 0.75$ and are listed in column 5.

cised and their DNase I footprints were analyzed by denaturing polyacrylamide gel electrophoresis. An example of the analysis of transcription complexes formed on $U6_R$ is shown in Figure 3. The TFIIB–DNA complex was quantitatively shifted into a slower migrating species by the addition of Pol III, whereas no Pol III complex was observed in the absence of TFIIB (Fig. 3A), indicating that $U6$ promoter-bound TFIIB directs the efficient assembly of Pol III into the transcription complex. The DNase I footprint profiles of complexes formed on three separate templates ($U6_R$, $U6_L$, and $U6$ symmetrical) are shown in Figure 3B. TFIIB-protected $U6_R$ sequences from -40 to -10 (lane 3), in agreement with the previously reported dimensions of this footprint on a nearly identical *SNR6* gene (Joazeiro et al. 1994). The creation of a symmetrical TATA box did not detectably alter the TFIIB footprint (cf. lanes 3 and 11). The reversal of the orientation of the entire promoter region ($U6_L$) did generate some subtle differences in the TFIIB footprint (cf. lanes 3 and 7), but these are essentially only attributable to differences in the cleavage profile of the free DNA (cf. lanes 2 and 6). The addition of Pol III to the $U6_R$ –TFIIB complex extended the footprint most obviously, but incompletely, in the right-hand direction. The partial protection extended downstream over the natural $U6$ right-hand gene to $+19$. In contrast, the Pol III footprint on the *SUP4* tRNA^{Tyr} gene is essentially complete even in inactive closed promoter complexes at 0°C (Kassavetis et al. 1992a). As the complex was excised from a gel, the

incomplete footprint cannot be attributable to subsaturating levels of Pol III. Instead, we believe that it reflects the ability of TFIIB to bind bidirectionally to the $U6$ promoter, thereby generating the Pol III-dependent partial protection. Flipping the $U6$ promoter sequence to face the left-hand gene ($U6_L$) generated a reversal of the protection profiles. Pol III now extended the footprint into the left-hand gene, but again with only partial protection. Weak protection could also be discerned extending into the right-hand gene (lane 8). These results support our interpretation of the transcription analysis in that they provide structural evidence for the formation of transcription complexes at the $U6$ gene that point in both directions. The incomplete footprint over the right-hand gene can be used to estimate the proportion of TFIIB complexes facing in that direction. Thus, comparison of $U6_R$ and $U6_L$ footprints allows the proportion of complexes that bind in the natural direction of the promoters to be estimated.

An analysis of several two-dimensional footprinting experiments (three each for $U6_R$ and $U6_L$, two for $U6$ symmetrical) is presented in Table 2. The fraction of right-facing TFIIB with its bound Pol III positioned over the right-hand gene was estimated by comparing Pol III-dependent protection of bp $+10$ to $+19$ of the right-hand gene relative to free DNA. When promoter sequences were directed to the right (in $U6_R$), the indicator sequences were, on average, 69% protected by Pol III, compared with 29% when promoter sequences were directed

Figure 3. Two-dimensional DNase I footprinting of transcription complexes formed at the $U6$ gene. (A) Nondenaturing electrophoresis, after partial digestion with DNase I, of protein–DNA complexes formed on the $U6_R$ probe. Conditions of binding, DNase I treatment and electrophoretic separation are described in Materials and methods. The contents of reaction mixtures are indicated above each lane, and complexes are identified at the side. (Free) The no-factor control. (B) DNase I digestion patterns of isolated complexes formed on $U6_R$ (lanes 1–4), $U6_L$ (lanes 5–8), and $U6$ symmetrical (lanes 9–12) DNA. The identity of the complex is marked above the lane; (–) the free DNA of a no-factor control. (Lanes 1,5,9) Undigested probe DNA. The promoter region and left-hand and right-hand genes are indicated at right: (L) Position (in bp) relative to the left-hand gene start site ($+1L$); and (R) position relative to the right-hand gene start site ($+1R$).

Table 2. The orientation of TFIIB, determined by footprinting of TFIIB–Pol III–promoter complexes

Gene	Percent protection of the right-hand gene [bp (+10 to +19)]
U6 _R	68.6 ± 3.3
U6 _L	28.5 ± 3.1
U6 symmetrical	64.5 ± 2.1

to the left (in U6_L). This result implies that there is only a modest (2.4-fold) asymmetry of TFIIB binding to the *SNR6* gene. A similar analysis of footprints generated on the U6 symmetrical gene indicated that the indicator sequences were 65% protected, suggesting that the limited preference for rightward orientation still exists in the context of a symmetrical TATA element.

Breaking the transcriptional symmetry

The above experiments describe a high degree of transcriptional symmetry at the U6 promoter. We reasoned that this symmetry could be broken by introducing specific mutations asymmetrically into the TATA box. It has been noted that mutations in the 5' half of the TATA box generally have a much more severe influence on transcription, and by implication TBP binding, than do mutations in the 3' half (Wobbe and Struhl 1990). With this in mind we attempted to make changes in the U6 TATA box that would support the unidirectional association of TFIIB (Fig. 4A). An A → G substitution at bp –29 in the *SNR6* gene dramatically reduces in vitro transcription (Gerlach et al. 1995). In the context of the bidirectional transcription unit U6_R, the A29G change disrupts the TATA sequence for the right-hand gene but leaves a good left-facing TATA box (5'-TATTTACA-3') on the complementary strand, leading to the expectation that the A29G promoter would support expression of the left-handed gene only. Conversely, the equivalent substitution at position 7 in the other half of the TATA box (T24C; 5'-TATAACA-3') was anticipated to allow right-hand transcription while severely limiting left-hand transcription.

In multiple-round transcription, the A29G and T24C changes severely compromised gene expression in both directions (Fig. 4B, lanes 4,5), indicating that the DNA contacts provided by nucleotides 2 and 7 of the TATA box are equally important for U6 transcription complex formation, a further manifestation of functional symmetry. This contrasts with the situation implied for yeast TFIID, where an A → G change at position 2 in the *his3* TATA box reduces transcriptional efficiency 100-fold while the equivalent change at position 7 reduces transcription approximately threefold (Wobbe and Struhl 1990).

To suppress the TATA box mutations, we reconstituted TFIIB with TBPm3, which carries three amino acid changes in its carboxy-terminal repeat that alter its DNA-binding specificity and allow the recognition of A

Figure 4. (A) Asymmetry-generating mutations in the U6_R TATA box. The substitutions are highlighted; positions are marked relative to the start site of the natural right-hand gene. (B) Multiple-round transcription with the asymmetric TATA box mutations was performed as described for Fig. 1 (and Material and methods), except that wild-type TBP was replaced with TBPm3 [50 fmoles (lanes 6–10)], and the B'' concentration was doubled from 14 fmoles per reaction (lanes 1–5) to 28 fmoles (lanes 6–10). The positions of transcripts and the recovery marker (r.m.) are indicated, and templates are identified as in Fig. 1.

or G at TATA box position 2 (Strubin and Struhl 1992). TFIIB reconstituted with TBPm3 transcribed U6_R and U6_L bidirectionally (lanes 6,7). TFIIB containing TBPm3 also restored activity to the A29G promoter, but only for transcription of the right-hand gene (Fig. 4B, cf. lane 8 with lane 4), indicating TFIIB binding in one orientation only. Similarly, TBPm3-containing TFIIB directed only left-hand transcription from the T24C promoter. These experiments demonstrate that TFIIB containing TBPm3 is equally able to recognize A or G at position 2 in the TATA box.

When assayed on the natural U6 promoter, TFIIB reconstituted with TBPm3 was less active than wild-type TFIIB. (Note that for Fig. 4B, the reactions with TBPm3 contained double the amount of the limiting B'' component of TFIIB). Presumably the three amino acid changes in TBPm3 result in some loss of DNA–protein contacts, which lower the intrinsic affinity for DNA and thus the transcriptional efficiency. Nevertheless, the demonstration of unidirectional transcription at the A29G and T24C variant promoters implies that, as part of yeast TFIIB, TBPm3 is only able to suppress A → G

changes at TATA box position 2 and has no ability to overcome an unfavorable base pair at TATA box position 7 (Fig. 5). This result also implies that TBP is so oriented in TFIIB at the U6 promoter that its carboxy-terminal repeat contacts the upstream half of the TATA box. Thus, the orientation of TBP as part of yeast TFIIB bound to the U6 gene is the same as in the DNA-TBP cocrystal structure (J. Kim et al. 1993; Y. Kim et al. 1993) and in TFIID (Strubin and Struhl 1992; Arndt et al. 1994). This is in contrast to some suggestions that TBP functions in the opposite orientation at Pol II and Pol III genes (Mitchell et al. 1992; Mitchell and Benfield 1993; Wang and Stumph 1995). Also, the observation of unidirectional transcription implies that Brf and B' interact with TBP in one orientation only.

The role of TFIIC in the determination of TFIIB-binding polarity

Autonomous TFIIB assembly at the U6 gene results in bidirectional complex formation. However, transcription complex assembly in vivo occurs via a TFIIC-de-

pendent mechanism (Brow and Guthrie 1990; Burnol et al. 1993b; Eschenlauer et al. 1993; Gerlach et al. 1995; Roberts et al. 1995). This prompted us to examine the influence of TFIIC on the binding polarity of TFIIB. The U6 gene has a properly positioned internal *box A*, but its extragenic *box B* imposes a *box B*-*box A* spacing that is completely suboptimal (Baker et al. 1987; Fabrizio et al. 1987). It has been suggested that the downstream U6 *box B* is brought into proximity of *box A* in vivo by some element of chromatin structure (Gerlach et al. 1995; Burnol et al. 1993a). The aberrant position of *box B* is presumably responsible for the inability to observe a consistent and significant positive influence of TFIIC on U6 transcription in a purified in vitro system (Joazeiro et al. 1994). The evidence suggests that TFIIC may actually misplace a proportion of TFIIB because of interaction with optimally positioned pseudo-*box A* sequences (Eschenlauer et al. 1993; Joazeiro et al. 1994).

To examine the role of TFIIC in determining transcriptional asymmetry with purified components we removed the downstream U6 *box B* and replaced bp +74 to +101 of the right-hand natural U6 gene with bp +74 to +101 of the *SUP4* tRNA^{Tyr} gene. This generated a right-hand gene that contains a *box B* optimally placed relative to the natural U6 *box A* (see Fig. 6A). The introduction of a strong *box B* element into the U6_R background (U6_R-*box B*) generates properly (i.e., naturally) oriented upstream and internal promoter elements for the right-hand gene. Conversely, in the U6_L background, the upstream promoter elements and TFIIC-recognition sequences face in opposite directions. In multiple-round assays the concurrent addition of TFIIC had only a small stimulatory effect on right-hand transcription when the *box B* element was positioned downstream of the right-hand terminator (U6_R and U6_L; Fig. 6B, lanes 1–4). However, in the context of an optimally spaced *box B* (right-hand gene), TFIIC generated a clear stimulation of rightward transcription, irrespective of the orientation of the upstream promoter elements (U6_R-*box B* and U6_L-*box B*; lanes 6,8). The greatest level of TFIIC-mediated transcription was observed when all parts of the upstream promoter element (Fig. 1) also face right (U6_R-*box B*, lane 6), thus suggesting that the upstream and internal promoter elements are able to cooperate in placement of TFIIB in vitro, as has been observed previously in vivo (Eschenlauer et al. 1993; Gerlach et al. 1995). When the polarity of the upstream promoter was reversed so that it faced away from the internal promoter elements (U6_L-*box B*), TFIIC was less able to enhance transcription of the right-hand gene (Fig. 6B, lane 8), suggesting that the potential for TFIIB-C cooperation may have been reduced. We also noted that in this case, some of the right-hand transcripts were slightly shorter; the possibility that this represented initiation at downstream sites was not examined.

With a strong internal *box B* in the right-hand gene, TFIIC addition reduced, but did not abolish, left-hand transcription. Thus, transcription complexes with a left-hand polarity were still able to assemble onto the promoter. In these experiments the DNA concentration was

Figure 5. Model for the interaction of the TBP subunit of TFIIB with the U6 promoter. TBP interacts with the natural TATA box in both directions, giving rise to bidirectional transcription. Mutations at TATA box position 2 or 7 (A29G and T24C promoters) prevent this interaction and abolish transcription in both directions. TFIIB containing TBPm3 binds the wild-type TATA box in both orientations but binds to A29G and T24C TATA boxes unidirectionally, supporting only right-hand transcription for the former and only left-hand transcription for the latter. Thus, TBPm3 suppresses only mutations in the upstream half (relative to the direction of transcription) of the TATA box. Because the suppressing mutations are in the carboxy-terminal pseudo-repeat of TBPm3, it is this repeat of TBP (open ovoid disc) that is shown contacting the 5'-half of the TATA box. The mutations in the carboxy-terminal repeat of TBPm3 are represented by an indentation, and the sites of the A29G and T24C TATA box mutations are indicated by solid triangles. Arrows indicate the direction of resulting transcription, and crosses indicate complexes that do not form.

Figure 6. TFIIC determines the binding polarity of TFIIB. (A) The relative positions of the TFIIC recognition elements *box A* and *box B*. The wild-type *SNR6* gene (p-539H6) and the bidirectional constructs (U6_R and U6_L) have an external *box B* element aberrantly positioned downstream of the transcriptional terminator, at +235. A right-hand gene-internal *box B* at +80, optimally spaced relative to the natural *box A* (at +20), was introduced into the bidirectional constructs by removing the natural U6 *box B* and replacing right-hand gene sequences (bp +74 to +101) with the equivalent sequences from the *SUP4* tRNA^{Tyr} gene. The upstream promoter region, right-hand (natural U6) gene, and left-hand gene are shown as in Fig. 1. The substituted *SUP4* tRNA^{Tyr} gene sequence is indicated by cross-hatching. (B) Multiple-round transcription. Transcription complexes were formed as described in Materials and methods either in the presence (lanes 2,4,6,8,10) or absence (lanes 1,3,5,7,9) of TFIIC (14 fmoles). Templates are indicated below the lanes. All templates have a *box B* element; only the presence of a right-hand gene-internal *box B* element is indicated by the suffix *box B* (lanes 5–8). (C) Multiple-round transcription of pU6_L-*box B*. To saturate DNA with TFIIC, the concentration of the pU6_L-*box B* template was reduced from 70 to 7 fmoles. Transcription complexes were formed in the presence of increasing amounts of TFIIC: (Lane 2), 7 fmoles; (lane 3) 14 fmoles; (lane 4) 28 fmoles. (Lane 1) No TFIIC. To reduce autonomous TFIIB binding and increase the dependence on TFIIC for complex formation, the preincubation time was reduced from 40 to 20 min and the time allotted to multiple rounds of transcription was shortened to 15 min. (D) Influence of TFIIC on multiple round transcription of A29G (lanes 1,2) and T24C (lanes 3,4) promoter variant constructs containing internal *box B* elements. Details as in B.

high (70 fmoles), such that TFIIC would not saturate the template and TBP was also in excess relative to the other TFIIB components. To perform transcription under TFIIC-saturating conditions the template concentration was reduced from 70 to 7 fmoles. Also, the time allowed for complex formation was shortened from 40 to 20 min as we have observed that TFIIC assembles TFIIB more rapidly than does TBP. Under these conditions, addition of increasing amounts of TFIIC to the U6_L-*box B* template almost abolished left-handed transcription while once again greatly increasing the level of right-handed gene expression (Fig. 6C). Nonetheless, residual levels of left-handed transcription were still detectable. It is probable that some limited level of complex assembly was still able to occur via a TFIIC-independent

mechanism such that a small proportion of TFIIB was placed in a leftward direction. However, an alternative and more provocative explanation is that TFIIC was able to assemble TFIIB in both orientations. To distinguish between these possibilities, we introduced an internal *box B* into the A29G and T24C backgrounds. In this case, autonomous assembly of TFIIB should be severely limited and thus transcription should be completely TFIIC dependent. The results shown in Figure 6D confirm this prediction, as efficient transcription required TFIIC. Only right-hand transcription was rescued by TFIIC (lanes 1–4), implying that TFIIC only places TFIIB in one direction. It is interesting to note that the T24C-*box B* gene was transcribed somewhat more efficiently than the A29G-*box B* gene, suggesting

that recognition of the TATA box may still be significant in this case, and that the contacts provided by TFIIC are better able to compensate for deficiencies in the 3' half of the TATA box than in the 5' half. Order-of-addition experiments indicated that promoter-bound TFIIB is refractory to redirection by TFIIC (data not shown). TFIIC does not provide a mechanism for recycling TFIIB, at least not on the relatively short time scale of these experiments.

Discussion

These experiments demonstrate that TFIIB binds the *SNR6* gene's upstream promoter with both polarities because its TBP subunit fails to recognize the direction of the TATA box. Consistent with this finding is the ability of reverse and symmetrical TATA boxes to function efficiently for U6 gene transcription in vitro (Fig. 2). In contrast, crystallographic studies of the TBP-TATA box complex reveal no evidence for bidirectional binding, and this has been used to argue that TBP is inherently able to recognize the polarity of the TATA box and thus to set the direction of Pol II transcription (Y. Kim et al. 1993; J. Kim et al. 1993; Kim and Burley 1994). Assuming this to be the case, what generates the difference between TBP binding alone and as part of TFIIB? The U6 TATA box deviates from perfect symmetry only at position 5; in this respect it is not surprising that its polarity is not recognized by TBP. However, the *CYC1* TATA box, which was cocrystallized with yeast TBP, also deviates from perfect symmetry at only one position (position 7). Although the distinction between those TATA boxes that support polar binding and those that do not may be extremely subtle, it is more probable that polarity is at least partly imposed by the flanking sequence and the location of ends of the short oligonucleotides that are used to produce well-diffracting crystals. Most importantly, our experiments measure the interaction of TBP with DNA in the presence of the other TFIIB subunits (Brf and B''). It is highly likely that these other proteins modulate the TBP-DNA interaction.

The modest preference for transcription in the natural direction of the *SNR6* promoter clearly results from a preferential orientation of initiation complexes (Tables 1 and 2). A bias for TFIIB binding that is maintained with a perfectly symmetrical TATA box must be determined by the structure or interactions of flanking DNA. TBP and TFIIB bend DNA, and so binding to DNA that is already bent to conform to its binding site, or is more readily bendable in a particular direction, would be energetically favored. For example, the affinity of TBP for DNA that is bent toward the major groove is 300-fold higher than for the equivalent DNA bent toward the minor groove (Parvin et al. 1995). If left- and rightward bound TFIIB bend DNA differently, that could select for one direction of binding over the other, and the Brf and B'' subunits of TFIIB could play a role in that selection.

The TBP-TATA box interaction that generates the autonomous assembly of TFIIB onto the U6 promoter and the action of TBP at TATA elements of Pol II promoters

appear to be nonidentical. A single A → G change at position 2 severely reduces yeast TFIID and TFIIB binding, as assayed by transcription in vitro (Wobbe and Struhl 1990; Gerlach et al. 1995). However, that U6 transcription should be so drastically reduced by a T → C mutation at position 7 would not be predicted on the basis of in vitro transcription experiments with TFIID (Wobbe and Struhl 1990) or from studies demonstrating that TBP binds specifically to a 6-bp TATA box (Strubin and Struhl 1992). TATA box position 7 must therefore be considered to have a special part in TFIIB binding. This requirement for strong contacts at both ends of the TATA box is perhaps one source of binding symmetry. That TBP recognizes the U6 TATA box in a TFIIB-specific way suggests an accessory role for at least one of the other TFIIB subunits in TATA box recognition. Another example of an associated protein that alters the DNA-binding properties of TBP is provided by TFIIA, which can direct the binding of mutant TBPs that alone do not recognize DNA (Lee et al. 1992). Also, the ability of TBPm3 to suppress different TATA box mutations in plant protoplasts was RNA polymerase-specific (Heard et al. 1993).

TFIIB containing TBPm3 yielded unidirectional transcription at the A29G and T24C promoters, right-hand for the former and left-hand for the latter. Here, TFIIB must have recognized the TATA box in a polar manner, as TBPm3 only suppressed mutations in the 5'-half (upstream half) of the TATA box. The mutations in TBPm3 (Ile-194 to Phe, Leu-205 to Val, Val-203 to Thr) are all contained in its carboxy-terminal repeat. In the structure of the yeast TBP-TATA box complex, Leu-205 contacts TATA box position 3, whereas the equivalent residue in the *A. thaliana* TBP-TATA box complex (Leu-163) contacts the adenine at position 2. Thus, in both crystal structures, Leu-205 (or its equivalent) is in close proximity to TATA box position 2. Moreover, modeling studies indicate that wild-type TBP does not bind to TGTA-containing TATA sequences because the exocyclic NH₂ of guanine generates a steric clash in the protein-DNA interface. Changing Leu-205 to Val is predicted to create a pocket that could accommodate the exocyclic NH₂ group (Kim and Burley 1994). Based on the pattern of promoter recognition by TFIIB containing TBPm3, and these structural correlates, we conclude that the suppression of the A29G and T24C mutations is direct, and that the carboxy-terminal repeat of TBP contacts the upstream half of the TATA box in the TFIIB-U6 gene complex. The Pol II promoter recognition patterns of two other individual yeast TBP mutants (Leu-205 to Phe and Leu-114 to Phe) indicate that as part of TFIID in vivo, TBP likewise binds the TATA box in a specific orientation, with the carboxy-terminal repeat of TBP contacting the upstream half of the TATA box (Arndt et al. 1994). We conclude that TBP has the same orientation, relative to the direction of transcription, when yeast TFIID and TFIIB bind to their respective promoters.

In contrast, it has been suggested that human and *Drosophila* TBP function in opposite orientations at Pol II and Pol III promoters (Mitchell et al. 1992; Mitchell

and Benfield 1993; Wang and Stumph 1995). The TATA box of the human U6 snRNA gene, which determines Pol III specificity (Lobo and Hernandez 1989; Lobo et al. 1991), resembles an inverted Pol II–TATA sequence. In a fractionated mammalian system, TATA-dependent Pol III transcription preferred a reverse TATA box (Mitchell et al. 1992; Mitchell and Benfield 1993), although Lobo et al. (1991) found no effect of TATA box inversion on transcription of the human U6 gene. Wang and Stumph (1995) have also demonstrated recently that Pol II and Pol III transcription with a *Drosophila* extract start exclusively from opposite sides of a common TATA box. Thus, it remains a possibility that the TBP of higher eukaryotes functions in opposite orientation in TFIID and TFIIB.

TFIIIC clearly directed unipolar assembly of TFIIB on the *SNR6* gene. Evidently the asymmetric interaction of TFIIIC with TFIIB [presumably through contacts between the TFIIIC 120-kD subunit and Brf (Khoo et al. 1994; Chaussivert et al. 1995)] ensures that TBP associates with the TATA box in one orientation only. At the same time, TFIIIC-dependent placement of TFIIB on *SNR6* in the absence of a TATA box results in the utilization of downstream start sites and an almost complete loss of correctly initiated transcription in vivo (Eschenlauer et al. 1993). Furthermore, TFIIIC can be made to place TFIIB at widely varying locations upstream of a tRNA gene box A, implying that the TFIIIC–TFIIB interaction is intrinsically flexible (C.A.P. Joazeiro, pers. comm.). Thus, whereas TFIIIC sets the direction of transcription, the TATA box helps to fix its precise location. The evidence of other experiments suggests that the interaction of DNA with TBP is also an integral part of TFIIB placement on TATA-less tRNA genes (C.A.P. Joazeiro, pers. comm.).

In conclusion, the ability of TFIIB to bind bidirectionally to the *SNR6* gene leads us to speculate that TFIID may be able to do the same at certain promoters. TFIIIC provides a mechanism by which TFIIB can be oriented correctly on Pol III genes and, by comparison, the interaction of TAF_{II}150 with the initiator element (Verrijzer et al. 1994, 1995) and other TAF_{II}–DNA interactions (Purnell et al. 1994) may serve not only to stabilize the TFIID–DNA complex but also to ensure its correct orientation.

Materials and methods

Oligonucleotides

The following oligonucleotides were used for plasmid construction: U6 term 1 (5′-AATTCGATAAAAAAAAAACGAAATAAAGGATCC-3′); U6 term 2 (5′-AATTGGATCCTTTATTTTCGTTTTTTTTTATCG-3′); TATA 1 (5′-CGAACACATTCACATATTTTCGGCTACTAT(A/T)(A/T)TA(G/T)ATGTTTTTTCGCAACTATGTGTTTCG-3′); TATA 2 (5′-CGAACACATAGTTGCGAAAAAACAT(A/C)TAT(A/T)(A/T)ATAGTAGCCGAAAATA-3′); TGTA 1 (5′-CGAACACATTCCACTATTTTCGGCTACT(A/G)TAAA(C/T)AGATGTTTTTTCGCAACTATGTGTTTCG-3′); TGTA 2 (5′-CGAACACATAGTTGCGAAAAAACATCT(A/G)TTTA(C/T)AGTAGCCG-

AAAATAGTGAATGTGTTTCG-3′); box B 1 (5′-AATCGGCGTTTCGACTCCCCCGGGGAGATATTTTCGTTTTTTT-TTTTATA-3′); box B 2 (5′-AGATTATAAAAAAAAAAACGAAATATCTCCCCGGGGGAGTCGAACGCCCGCAT-3′). Double-stranded oligonucleotides suitable for use in cloning experiments were made by hybridizing the following oligonucleotide pairs: U6 term 1 and 2; TATA 1 and 2; TGTA 1 and 2; box B 1 and 2. Mixtures of 300 pmoles of each oligonucleotide in 50 µl volume of 50 mM NaCl were heated to 65°C and cooled slowly to ambient temperature.

Plasmid constructions

The plasmid p-539H6 contains *Saccharomyces cerevisiae* sequence from −539 to +630 of *SNR6* (transcription start site = +1) (Brow and Guthrie 1990). The parent plasmid for the bidirectional transcription constructs, p-539H6-term, was made by cloning the double-stranded oligonucleotide term 1 + 2 into the *EcoRI* site of p-539H6. Bidirectional constructs pU6_R, pU6_L, pU6-symmetrical, pU6-reverse, and pU6-extended, were made by inserting the degenerate double-stranded oligonucleotide TATA 1 + 2 between the blunt-end *SnaBI* and *NruI* sites of p-539H6-term and selecting the appropriate clones by sequencing. The bidirectional constructs pA29G_R and pT24C_R were made as described for pU6_R except that the double-stranded oligonucleotide TGTA1 + 2 was used in place of TATA 1 + 2. An internal box B element was introduced into plasmids pU6_R, pU6_L, pA29G_R, and pT24C_R by cloning the box B 1 + 2 double-stranded oligonucleotide between *HindIII* and *EcoNI* restriction sites.

Transcription proteins

TFIIB (purified to the Cibacron blue Sepharose step), Pol III (Mono Q), wild-type TBP, and Brf were purified as described previously (Kassavetis et al. 1990; Colbert and Hahn 1992; Joazeiro et al. 1994). TBPm3 was purified as described for the wild-type protein. B'' was purified to the hydroxylapatite step as described (Kassavetis et al. 1995), BSA was added to 40 µg/ml followed by dialysis into buffer D + 100 [40 mM Na HEPES at pH 7.8, 7 mM MgCl₂, 20% (vol/vol) glycerol, 0.01% (vol/vol) Tween 20, 100 mM NaCl, 0.5 mM PMSF, 1 µg/ml of pepstatin, 1 µg/ml of leupeptin] and concentrated 5- to 10-fold (Centriprep 30; Amicon). This B'' fraction did not contain any detectable Brf or TBP activity as assayed by in vitro transcription. Quantities of TFIIB, Brf, and B'' are specified in terms of fmoles of DNA-binding activity, as described (Kassavetis et al. 1991). Quantities of Pol III are specified in terms of femtomoles of active molecules for specific transcription and represents a minimum estimate (Kassavetis et al. 1989). The concentration of wild-type TBP was determined from its extinction coefficient; TBPm3 concentration was estimated by the Bradford assay (Bradford 1976) using wild-type TBP as a standard. Recombinant B'' (Kassavetis et al. 1995) was generously provided by A. Kumar (University of California, San Diego).

Multiple-round transcription assays

Multiple-round transcription assays were performed essentially as described (Gerlach et al. 1995). Briefly, TBP (50 fmoles), B'' (14–28 fmoles) Brf (40 fmoles) and Pol III (5 fmoles) were preincubated with supercoiled DNA template (70 fmoles) for 40 min at room temperature (20–22°C) in transcription buffer containing 40 mM Tris-Cl at pH 8.0, 7 mM MgCl₂, 3 mM dithiothreitol, 25 mM potassium acetate, 35 mM NaCl, 100 µg of bovine serum albumin per ml and 0.5% (wt/vol) polyvinyl alcohol. The NaCl

concentration was raised to 70 mM and transcription was initiated by adding nucleotides to a final concentration of 200 μ M ATP, 100 μ M CTP, 100 μ M GTP and 25 μ M [α - 32 P]UTP at 10^4 cpm/pmol. Transcription was allowed to proceed for 30 min and was stopped by the addition of six volumes of stop solution [40 mM Tris-Cl at pH 7.5, 2 mM Na₂EDTA, 0.2% (wt/vol) SDS] containing a 32 P-labeled DNA fragment as a recovery marker. Samples were processed for electrophoresis, analyzed on 8% polyacrylamide denaturing gels, and molar yields of transcripts were quantified as described (Gerlach et al. 1995).

Single-round transcription assays

Transcription complexes were preassembled as described above. A nucleotide mixture lacking ATP (200 μ M GTP, 100 μ M CTP, 25 μ M [α - 32 P]UTP at 5×10^4 cpm/pmol) was added for 10 min, generating transcripts paused at +7 (on both the right-hand and left-hand genes). Stable elongation complexes were formed by adding ATP to 200 μ M for 15 sec before adding heparin to 30 μ g/ml, to prevent reinitiation. Transcription continued for 3 min and was terminated by adding six volumes of stop solution. Samples were processed and analyzed as specified above.

Two-dimensional DNase I footprinting

Probe DNA was prepared by end-labeling *Eco*NI-cleaved plasmid DNA with Klenow fragment DNA polymerase and [α - 32 P]dATP and removing unincorporated nucleotides. DNA was cleaved further with *Eco*RI, and the resulting 201-bp fragment was separated on nondenaturing acrylamide gels, passively eluted, and purified by passage over NACS 52 resin (GIBCO BRL). DNA-TFIIB-Pol III complexes were formed in two steps: TFIIB (0.75 fmole) purified to the (Cibacron blue-Sepharose step) supplemented with TBP (30 fmoles) and B'' (28 fmoles) was incubated with probe DNA (~6 fmoles) for 60 min at room temperature in buffer [40 mM Tris-Cl at pH 8.0, 7 mM MgCl₂, 3 mM dithiothreitol, 25 mM potassium acetate, 35 mM NaCl, 100 μ l of bovine serum albumin, 4% glycerol, 2.5 μ g/ml of poly(dG-dC):poly(dG-dC)]. The NaCl concentration was then raised to 90 mM and 1.25–2.5 fmoles of Pol III was added, along with 0.35 μ g of nonspecific linear plasmid DNA (*Eco*RI-cleaved pGEM1; Promega) to prevent Pol III binding to the ends of the labeled probe. Pol III complexes were allowed to form for 15 min before mild digestion with DNase I as described (Kassavetis et al. 1990). Transcription complexes were resolved by nondenaturing electrophoresis as described (Kassavetis et al. 1990) and excised from the gels. DNA was passively eluted, extracted with phenol-chloroform, precipitated with ethanol, and resolved on 8% acrylamide sequencing gels. Footprints were quantified on a PhosphorImager.

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