A minimal RNA polymerase III transcription system

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Transcription factor (TF) IIIB recruits RNA polymerase (pol) III for specific initiation of transcription. All three subunits of TFIIB, TBP, Brf (the TFIIB-related subunit) and B'', are required for transcription of supercoiled and linear duplex DNA, but we show here that B'' is non-essential on a promoter that has been partly pre-opened by unpairing a short segment of the transcription bubble. These findings expose a striking similarity between transcriptional initiation by pol II, pol III and bacterial RNA polymerases: a preformed single-stranded DNA bubble upstream of the transcriptional start removes the dependence of pol II on TFIIE, TFIIH and ATP hydrolysis, and the dependence of pol III on B''; the favored placement of the transcription bubble for B''-independent transcription by pol III overlaps a DNA segment that interacts sequence specifically as single-stranded DNA with the σ^{70} initiation subunit of *Escherichia coli* RNA polymerase holoenzyme.

Keywords: B''/Brf/RNA polymerase III/*Saccharomyces cerevisiae*/transcription initiation

Introduction

The core transcription apparatus of yeast RNA polymerase (pol) III comprises the 18-subunit polymerase (Chédin *et al.*, 1999), together with its transcription initiation factors TFIIA, B and C. TFIIB, which is responsible for directing pol III to its promoters, is composed of the TATA-binding protein (TBP), Brf, the TFIIB-related and archaeal TFB-related subunit, and the pol III-specific B'' (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; López-De-León *et al.*, 1992). (Brf is also referred to as TFIIB70 and B'' is also referred to as TFIIB90.) TFIIC is a large general assembly factor that places TFIIB on the DNA template, and the multi-zinc finger TFIIA is a 5S RNA gene-specific assembly factor for TFIIC. TFIIB also binds autonomously to promoters with strong TATA boxes through a direct interaction of its TBP subunit. When pol III is brought to the transcriptional start site by TFIIB, the DNA duplex around the transcriptional start site is spontaneously and thermoreversibly opened in linear as well as in supercoiled DNA (Kassavetis *et al.*, 1990, 1992a; White, 1998).

Recent functional dissections of the Brf and B'' subunits

of TFIIB have shown that certain internal deletions in B'' and N-terminal deletions in Brf generate TFIIB assemblies that retain competence for directing accurately-initiating transcription of supercoiled plasmid DNA by pol III, but are inactive in the context of linear DNA. The defective TFIIBs recruit pol III and position it appropriately over the transcriptional start site, but the promoter does not open. In consideration of these findings, we have proposed that the normal function of TFIIB combines pol III recruitment with an essential role in subsequent steps of transcriptional initiation (Kassavetis *et al.*, 1998b).

The subsequent analysis of these defective TFIIB assemblies has focused on how to restore promoter opening in linear DNA. It is known that pol III opens its *SUP4* tRNA gene promoter non-coordinately, in two segments, one region surrounding the transcriptional start and extending downstream, and another region that opens at a lower temperature centered ~7 bp further upstream (Kassavetis *et al.*, 1992a). Accordingly, the effect on transcription of unpairing these segments of the transcription bubble by forming loops has been examined. In the course of that analysis, we have found conditions under which Brf and TBP alone secure accurately-initiating transcription by pol III: the requirement for participation of B'' is circumvented if the pol III promoter is partially pre-opened by unpairing DNA upstream of the transcriptional start site. The exploration of this B''-independent pol III transcription, and a discussion of its implications for the existence of a common reaction pathway to initiation of transcription by eukaryotic and bacterial RNA polymerases, are presented.

Results

B''-independent transcription

The pol III transcription bubble opens non-coordinately: its upstream segment (bp -9 to -5) opens at lower temperature, and its downstream segment (bp -3 to +11) responds selectively to the presence of the initiating nucleotide in a temperature downshift (Kassavetis *et al.*, 1992a). In order to explore the defects of certain Brf and B'' deletion proteins that assemble pol III over the start site of transcription on linear DNA templates but are unable to form a transcription bubble, we constructed a series of DNA templates, based on the TFIIC-independent *SNR6* (U6) gene, containing short single-stranded bubbles spanning the region unwound by pol III in the *SUP4* gene open complex (Figure 1). One of these DNA templates, construct -9/-5, yielded the surprising finding that full-length Brf and TBP alone assemble pol III for transcription at a significant level (Figure 2): pol III alone only weakly utilizes the -9/-5 bubble construct, initiating transcription near bp +1, and the level of this background transcription is unchanged by TBP, B'' or Brf alone, or by B'' with TBP

U6RboxB

-30 -20 -10 -1+1 10 20

ACTATAAATAAATGTTTTTTCGCAACTATGTGTTTCGCGAAGTAACCCCTTCG
TGATATTTATTTACAAAAAAGCGTTGATACACAAGCGCTTCATTGGGAAGC

-14/-10 bubble

ACTATAAATAAATGTTTTTTCGCAACTATGTGTTTCGCGAAGTAACCCCTTCG
TGATATTTATTTACAAAAAAGCGTTGATACACAAGCGCTTCATTGGGAAGC

-9/-5 bubble

ACTATAAATAAATGTTTTTTCGCAACTATGTGTTTCGCGAAGTAACCCCTTCG
TGATATTTATTTACAAAAAAGCGTTGATACACAAGCGCTTCATTGGGAAGC

-9/-7 bubble

ACTATAAATAAATGTTTTTTCGCAACTATGTGTTTCGCGAAGTAACCCCTTCG
TGATATTTATTTACAAAAAAGCGTTGATACACAAGCGCTTCATTGGGAAGC

-7/-5 bubble

ACTATAAATAAATGTTTTTTCGCAACTATGTGTTTCGCGAAGTAACCCCTTCG
TGATATTTATTTACAAAAAAGCGTTGATACACAAGCGCTTCATTGGGAAGC

-4/+1 bubble

ACTATAAATAAATGTTTTTTCGCAACTATGTGTTTCGCGAAGTAACCCCTTCG
TGATATTTATTTACAAAAAAGCGTTGATACACAAGCGCTTCATTGGGAAGC

+2/+6 bubble

ACTATAAATAAATGTTTTTTCGCAACTATGTGTTTCGCGAAGTAACCCCTTCG
TGATATTTATTTACAAAAAAGCGTTGATACACAAGCGCTTCATTGGGAAGC

+7/+11 bubble

ACTATAAATAAATGTTTTTTCGCAACTATGTGTTTCGCGAAGTAACCCCTTCG
TGATATTTATTTACAAAAAAGCGTTGATACACAAGCGCTTCATTGGGAAGC

Fig. 1. Transcription templates with single-stranded DNA bubbles. The DNA, whose sequence is based on the *SNR6* gene in pU6RboxB (Whitehall *et al.*, 1995), extends from bp -60 to +138 (relative to the transcriptional start as +1). The template (bottom) strand is identical in all constructs.

(Figure 2, lane a and data not shown). Addition of full-length Brf (wt) and TBP together (referred to as B'; Kassavetis *et al.*, 1991) increases transcription 5- to 6-fold (Figure 2, lane b), but is ineffectual for promoting transcription of the fully duplex DNA (Figure 2, lane h), as expected. B'-directed transcription is at ~5% of the level generated by the complete TFIIB-DNA complex (compare Figure 2, lanes b and c). Removing Brf segments that are not present in the *Candida albicans* and *Kluyveromyces lactis* homologues (Khoo *et al.*, 1994; based on two alternative alignments) generates two variant proteins that are somewhat more active for transcription of bubble-containing DNA and elevate B'-independent transcription to >10-fold over background (Figure 2, compare lanes d and f with a). We have therefore used these variant proteins for experiments that are shown below (but principal observations have been confirmed with wild-type Brf).

The ability of the B'-DNA complex to direct transcription is highly sensitive to bubble placement (relative to the *SNR6* TATA box) (Figure 3A; BrfΔ383-424 was used for this experiment). Bubble-containing templates function less well for TFIIB-directed transcription than does fully duplex DNA, and bubble templates -4/+1, +2/+6 and +7/+11 are consistently less active than -14/-10 and -9/-5 (Figure 3A, left panel, top, and data not shown). All templates specify TFIIB-dependent initiation at bp

Fig. 2. A TBP-Brf-DNA complex that assembles pol III for specific transcription. TFIIB-DNA and B' (i.e. TBP-Brf)-DNA complexes were formed on fully duplex and -9/-5 bubble DNA, as indicated at the top of the figure, for 60 min (TBP was present in all reaction mixtures). Pol III was added for a further 10 min and multiple rounds of transcription were then allowed for 30 min. The yield of U6 RNA is specified below each lane relative to transcription elicited by the TFIIB(BrfΔ383-424)-duplex DNA complex, set at 100. The U6 transcript, ³²P-labeled -9/-5 bubble construct ('DNA'; used as a tracer during template preparation) and a labeled DNA recovery marker (r.m.) are identified on the left.

+1 (Figure 3A, left panel, bottom), with bubble template +2/+6 and +7/+11 also specifying initiation at bp +5 and +7, respectively (Figure 3A, compare the left- and right-hand panels); initiation at bp +10 with construct +7/+11 is not factor-dependent. B'-directed transcription proves to be more selective (Figure 3A, middle panel): constructs -9/-5 and -4/+1 generate initiation at bp +1 that is 7 and 10%, respectively, of transcription provided by complete TFIIB (left panel), and 30- and 5-fold, respectively, above the no-Brf background (Figure 3A, right panel; note that the background initiation at bp +1 is elevated with bubble template -4/+1). Fully duplex DNA, and constructs -14/-10, +2/+6 and +7/+11 fail to support B' complex-dependent transcription at significant levels above the no-Brf background (at each potential site of initiation; compare the middle and right-hand panels). Thus, among the 5 bp bubble templates that were tested, construct -9/-5 is optimal for B'-dependent transcription. Reducing the size of the bubble to 3 bp (constructs -9/-7 and -7/-5) allows retention of some capacity for B'-dependent transcription, but at less than one-eighth the efficiency of the 5 bp bubble template (Figure 3B).

Pol III is able to initiate transcription in the absence of TFIIB on a DNA template containing a 4 nucleotide (nt) 3' overhanging end, with initiation most efficiently primed by the dinucleotide corresponding to the first 2 nt of the recessed 5' end. Dinucleotides further into the duplex region fail to prime initiation (Bardeleben *et al.*, 1994). It is conceivable that the increased initiation at bp +1 that is mediated by B' on the -4/+1 bubble construct (Figure 3A) results from its stabilization of pol III binding to the -4/+1 bubble, enhancing what would otherwise be a transcription factor-independent initiation process. On the

Fig. 3. Requirements for the location and size of the DNA bubble generating B''-independent transcription. **(A)** Effect of bubble placement on transcription and initiation site selection. TFIIB-DNA (left panel), B'-DNA (middle panel) and TBP-DNA complexes (right panel) were used for multiple rounds of transcription by pol III. The RNA products are analyzed directly by denaturing gel electrophoresis (upper segment of each panel) and primer extension (lower segment). Upper segment of each panel: 32 P-labeled RNA; the size of the more slowly migrating transcripts is consistent with read-through (rt) of the *SNR6* transcriptional terminator. A labeled DNA recovery marker is not shown. Lower segment of each panel: otherwise identical samples with unlabeled transcripts were processed for mapping of RNA 5' ends by primer extension with reverse transcriptase. The accompanying sequence ladder is not shown. Transcripts (excluding the read-through and +10 initiated products) and reverse transcripts were quantified and are specified below each lane: relative to transcription with TFIIB on duplex DNA, set to 100, for the upper segments, and relative to the reverse transcript for +1-initiating RNA formed with TFIIB on duplex DNA, set to 100, for the lower segments. Brf Δ 383-424 was used for this experiment. **(B)** Three-base-pair bubbles also elicit B''-independent transcription. B'-pol III-DNA and TBP-pol III-DNA complexes (identified above each lane by the presence or absence of full-length Brf) were used for multiple rounds of transcription and analyzed as specified in (A) by direct examination of transcripts (left panel) and primer extension (right panel). U6 RNA synthesis and +1 initiation are quantified below each lane relative to B'-dependent transcription of the -9/-5 bubble template, set to 100. The lane at the far right (which is blank) shows a control reaction mixture lacking pol III analyzed by primer extension.

other hand, the B'-dependent initiation at bp +1 with constructs -9/-5, -9/-7 and -7/-5 (Figure 3A and B) requires a promoter unwinding downstream of the pre-formed bubble that pol III appears to be incapable of executing on its own.

Constraints on Brf and limitations on initiation

The structure of the TFIIB-DNA complex is redundantly supported by protein-protein (and protein-DNA) interactions: B'' and TBP interact with both the N- and C-proximal halves of Brf, and TBP also interacts with B'' (Khoo *et al.*, 1994; Kassavetis *et al.*, 1997, 1998a; Colbert *et al.*, 1998; Shen *et al.*, 1998); removal of some of these

individual interactions does not destroy the transcriptional activity of TFIIB for supercoiled DNA (Kumar *et al.*, 1997; Kassavetis *et al.*, 1998a).

Omitting B'' removes a large part of this structural redundancy, as shown in Figure 4. In the context of a supercoiled *SNR6* gene template with full-length B'' and TBP, the N-terminal, TFIIB-related half of Brf (amino acids 1-282) assembles into an unstable TFIIB-DNA complex that is highly active for transcription (Figure 4A, lane f), while the C-terminal half of Brf (amino acids 284-596) assembles into a stable TFIIB-DNA complex that retains only very weak transcriptional activity (lane g; barely visible on the original; see Kassavetis *et al.*,

Fig. 4. B''-independent transcription with N- and C-terminal truncations of Brf. (A) The N- and C-terminal halves of Brf complement for TFIIB-dependent transcription of a linear duplex *SNR6* gene template. Supercoiled pU6LboxB and a 366 bp linear DNA fragment derived from pU6LboxB were used for transcription directed by TFIIB complexes containing the Brf variants indicated at the top. The U6LboxB construct generates two divergent transcripts (identified on the left) due to TBP binding to the *SNR6* TATA box in either orientation. (B) The N- and C-terminal halves of Brf do not complement for B''-independent transcription of -9/-5 DNA. Primer extension analysis as described for Figure 3 with the Brf components specified above each lane. Initiation is quantified relative to B''-independent transcription with BrfΔ383-424 set to 100 below each lane. (C) B''-independent transcription of -9/-5 DNA requires the N-terminal zinc-ribbon domain of Brf. B'-pol III-DNA complexes were formed with the Brf variant protein specified above each lane. U6 RNA synthesis is analyzed directly and quantified relative to transcription with full-length Brf and B'' (set to 100 and not shown) below each lane.

1998a). On linear duplex DNA, TFIIB complexes containing the individual Brf halves are transcriptionally inactive (lanes b and c), but these Brf fragments complement each other efficiently (lane d; Kassavetis *et al.*, 1998b). In addition, Brf(165-596), lacking its putative N-terminal zinc ribbon and first TFIIB-related repeat, is inactive for transcription of supercoiled DNA only when combined with certain B'' internal deletions (Kassavetis *et al.*, 1997). In contrast, complementation of Brf(1-282) and Brf(284-596) is extremely inefficient for B'-only transcription (Figure 4B). Removal of the N-terminal 68 amino acids containing the putative zinc ribbon of BrfΔ366-407 does not interfere with TFIIB-directed transcription of the supercoiled *SNR6* gene (data not shown), but destroys B'-directed transcription of the -9/-5 bubble template (Figure 4C).

The relatively weak activity of B'-directed transcription has been further dissected in experiments that compare single and multiple rounds of transcription (Table I and Figure 5). B' recruits pol III to transcription complexes that execute a single round of transcription less effectively and more slowly than does TFIIB (Figure 5A). The rate difference is due to slower polymerase binding and/or

Table I. Single-round and multiple-round transcription with B' and TFIIB

	Single round ^a	Multiple round ^a	Rounds ^b
Brf wt	0.16 ± 0.04 (5)	0.29 ± 0.09 (5)	1.8 (5)
Brf(Δ366-407)	0.31 ± 0.07 (4)	0.79 ± 0.21 (4)	2.6 (4)
Brf(Δ383-424)	0.40 ± 0.02 (4)	1.10 ± 0.07 (3)	2.8 (3)
B' + Brf wt	1 (5)	5.25 ± 1.34 (5)	5.3 (5)
B' + Brf(Δ366-407)	1.29 ± 0.11 (4)	6.88 ± 0.73 (3)	5.3 (3)
B' + Brf(Δ383-424)	1.29 ± 0.17 (4)	7.06 ± 1.94 (4)	5.5 (4)

^aAverage of three to five experiments (as indicated in parentheses) normalized to single-round transcription with wild-type TFIIB; the standard error of the mean is also specified.

^bAfter 15 min of transcription.

Fig. 5. Comparison of TFIIB-directed and B'-directed assembly of pol III for multiple and single rounds of transcription. (A) Rate of formation of initiated transcription complexes. TFIIB and B' complexes with -9/-5 construct DNA (and using BrfΔ383-424) were formed for 60 min. A mixture of pol III, CTP, GTP and [α-³²P]UTP (i.e. lacking ATP) was added for the indicated interval before further addition of ATP and heparin to complete a single round of transcription, and to strip any pol III that had failed to initiate transcription. U6 RNA synthesis is quantified relative to a labeled DNA recovery standard. (B) The B'-DNA complex is relatively inefficient in promoting multiple rounds of transcription. TFIIB complexes (□) and B' complexes (●) with -9/-5 DNA (and BrfΔ383-424) were formed for 60 min. Pol III and a labeled nucleotide mixture lacking ATP were added for an additional 15 min to initiate transcription, but block elongation past bp +7. ATP was added to resume elongation and reinitiation for the time interval indicated. ATP and heparin were added to separate samples for 15 min to generate the normalizing single round of transcription.

Fig. 6. The transcription bubbles generated by pol III-bound B'-DNA and TFIIB-DNA complexes. Pol III was allowed to bind to -9/-5 bubble construct DNA (5' end-labeled in the non-transcribed strand) in the presence of TBP (left-hand panel), B' (middle panel) and TFIIB (right-hand panel). Buffer (■), GTP (▣), GTP + UTP (▨) or GTP + UTP + CTP (□) were added to 100 μ M final concentration for 10 min, followed by reaction with 25 mM KMnO₄ for 30 s. The reactivity of individual thymine residues between bp -14 and bp +11 (in the non-transcribed strand) was quantified and normalized for minor differences in the quantities of labeled DNA loaded in each gel lane. The reactivity values for a mock DNA-binding reaction (no pol III, no nucleotides) were then subtracted and the remainder is plotted in the upper portion of each panel for thymine residues that display significant hyper-reactivity to KMnO₄ oxidation. The lower portion of each panel shows the densitometric KMnO₄ footprint profile generated by pol III in the presence of GTP, UTP and CTP (GUC).

formation of the open complex, since addition of the first 7 nt to an open complex (preformed during 60 min) proceeds at almost the same rapid rate in B'- and TFIIB-directed transcription (data not shown). The number of rounds of transcription executed under the direction of B' is also lower than with TFIIB (Table I), and the relative disadvantage of B' becomes more pronounced as the time of RNA synthesis increases (Figure 5B). Since the rate of RNA chain elongation has been shown to be unaffected by the presence or absence of promoter-bound TFIIB (Bardeleben *et al.*, 1994; Matsuzaki *et al.*, 1994) and since termination, as measured by transcript release, is completed within 30 s in the absence of any transcription factor (Bardeleben *et al.*, 1994), the deficit of B' in directing multiple rounds of transcription must reside in a poorer ability to re-recruit pol III and reopen the promoter for reinitiation of transcription.

The B'-less pol III promoter complex

The physical basis of the limited ability of B' to direct transcription of the -9/-5 bubble construct is explored in footprinting experiments that are summarized in Figures 6 and 7. Open complex formation was probed with KMnO₄, which oxidizes thymine in single-stranded DNA,

but not in duplex DNA (Hayatsu and Ukita, 1967; Sasse-Dwight and Gralla, 1989). Although the non-transcribed strand of the -9/-5 construct contains two T residues within its 5 bp bubble (at bp -8 and -7; Figure 1), <10% of the 5' end-labeled DNA strand is oxidized by KMnO₄ at either position under the conditions used (data not shown), which makes it possible to assess pol III-induced oxidation of T on the 3' side of the -9/-5 bubble.

For the experiment that is analyzed in Figure 6, pol III was incubated with TBP-DNA, B'-DNA and TFIIB-DNA complexes for 70 min prior to the addition of KMnO₄ (Figure 6, left, middle and right panels, respectively). GTP, GTP + UTP or GTP + UTP + CTP were added for the final 10 min, as indicated, in order to place GTP in the initiating nucleotide substrate site of polymerase, or to allow the synthesis of a 3 or 7 nt RNA chain, respectively. Addition of pol III to the TFIIB-DNA complex generates hyper-reactivity to permanganate at T-3, -1, +2 and +3 (Figure 6, right panel), but not at T-12 (or further upstream) or T+11 (Figure 6, right-hand panel, bottom). GTP has no effect on reactivity; addition of GTP + UTP, with or without CTP, increases reactivity at T-1 and T+2/+3 1.5- to 2-fold, but has no effect at T-3. Oxidation at T-7 and T-8 within the bubble also increases ~2-fold

Fig. 7. Identical (indistinguishable) pol III footprints in B' and TFIIB complexes with -9/-5 bubble DNA. DNA, 5' end-labeled in the non-transcribed strand, was incubated with TBP, Brf Δ 383-424 and B'', as required, for 60 min. Pol III with GTP and UTP (providing 200 and 100 μ M final concentration, respectively) or buffer was added for an additional 15 min, followed by addition of poly(dA-dT):poly(dA-dT) for 7 min to sequester non-specifically or weakly bound TFIIB components and pol III. Partial DNase I digestion, isolation of the appropriate complex by native gel electrophoresis and resolution of the partial cleavage ladders are specified in Materials and methods. Equivalent quantities of radioactivity were loaded in each lane of the sequencing gel. Phosphorimager density profiles are aligned and additionally normalized downstream of bp +49. The upper panel compares TFIIB-DNA and TFIIB-pol III-DNA complexes. The lower panel compares B'-DNA and B'-pol III-DNA complexes. The DNA segment protected by pol III is specified below each panel (the jagged ends indicating that protection upstream of bp -6 may be due to B' or TFIIB rather than pol III). The inset in the lower panel shows a segment of the footprint of a TBP-pol III-DNA complex that is discussed in the text.

upon addition of pol III to the TFIIB-DNA complex; no further effect is seen upon addition of GTP $\pm$ UTP $\pm$ CTP (Figure 6, right panel, bottom, and data not shown). Addition of pol III to the TBP-DNA complex does not generate increased permanganate reactivity and addition of nucleotides to these components is also without effect (Figure 6, left panel). Addition of pol III to the B'-DNA complex (Figure 6, middle panel) generates increased reactivity at T -3, -1, +2 and T +3, but at only 2-3% of the level generated by the pol III-TFIIB-DNA complex. GTP increases reactivity at T -3 ~3-fold with little or no increase at T -1, +2 or +3. As is the case for pol III-TFIIB-DNA complexes, addition of GTP + UTP with or without CTP increases reactivity at T -1 and T +2/+3, but to a greater extent (3- to 4-fold). In the presence of GTP, UTP and CTP, reactivity at T -3 to T +3 in pol III-B'-DNA complexes is 7-9% of reactivity with pol III-TFIIB-DNA complexes, considerably lower than predicted from the relative capacity for single-round

transcription (15-30%; Table I). This could be partly due to the 25-fold lower template concentration of the KMnO $_4$ footprint assay, but the larger effect of nucleotides on permanganate reactivity of the B'-pol III-DNA complexes (Figure 6, compare middle and right panels, top) also suggests that, even after 60 min of incubation with pol III, a substantial fraction of these promoter complexes (>50%) is not fully open.

The placement of pol III on B'-DNA and TFIIB-DNA complexes was compared by two-dimensional DNase I footprinting (Figure 7), in which individual DNase I-cleaved protein-DNA complexes are separated by native gel electrophoresis prior to analysis. Pol III-B' complexes of the -9/-5 bubble construct were not sufficiently stable to be isolated from native gels. Consistent with the notion that this instability might be contributed by incomplete promoter opening (Figure 6), we found that GTP + UTP improved the retention of B'-pol III-DNA complexes during gel electrophoresis ~2.5-fold relative to pol III-

DNA complexes. Because of its slightly lower mobility, conservative excision of bands from the gel minimized potential cross-contamination of the B'-pol III-DNA complex with pol III-DNA complexes (data not shown). The presence of GTP and UTP permits the formation of a trimer transcript that is synthesized reiteratively, along with dimers, as abortive initiation products (Bhargava and Kassavetis, 1999).

The accuracy of pol III recruitment by Brf and TBP to the -9/-5 bubble template was examined by two-dimensional DNase footprinting. TFIIB protects the -9/-5 construct DNA between ~bp -38 and -8, and enhances DNase I cleavage around the start site of transcription (Figure 7, top panel). Addition of pol III extends protection to bp +22, with enhanced DNase I cleavage at bp +28 and +30. The high degree of protection in this pol III footprint is unexpected, since TFIIB does not bind to the *SNR6* TATA box in a unique orientation, and places pol III either upstream or downstream of the TATA box according to its orientation (Whitehall *et al.*, 1995). It appears, therefore, that the -9/-5 bubble further specifies a unique (or predominant) orientation for the TFIIB-pol III-DNA complex.

DNase I footprinting detects B' protection of -9/-5 construct DNA between bp -32 and -18 (Figure 7, lower panel). Pol III binding to the B'-DNA and TFIIB-DNA complexes generates nearly identical footprint additions: for the B'-pol III-DNA complex, protection extends to bp +22 with enhanced cleavage at bp +28. Pol III also generates protection of this complex between bp -6 and -12. Since this DNA segment is protected by the TFIIB-DNA complex, this footprint difference cannot be interpreted as a difference in pol III placement. Indeed, on the basis of permanganate and DNase I footprinting, we conclude that the placements of pol III over the -9/-5 bubble promoter by B'-DNA and TFIIB-DNA complexes are indistinguishable.

The low level of complex formation of pol III with the TBP-DNA complex hampers an examination of pol III alone bound to the -9/-5 bubble. (Even the footprints of gel-isolated TBP-pol III-DNA complexes are less distinct and complete, possibly due to contamination with polymerase bound at DNA ends.) We have generally observed weak protection at bp -8 and between bp +1 and +17 (Figure 7, lower panel, inset), indicating that the -9/-5 bubble does not by itself 'correctly' place pol III over the *SNR6* promoter.

Discussion

These experiments firmly establish a role for TFIIB in the initiation of transcription by pol III that extends beyond polymerase recruitment. We show that the absolute requirement for the B'' subunit of TFIIB in pol III transcription can be circumvented by opening a small part of the transcription bubble (Figure 2). This B''-less transcription requires Brf, specifically directs pol III to the normal start site of the *SNR6* gene (Figure 7) and demands specific placement of a DNA loop (Figure 3A). Thus, the TATA box-bound B' (i.e. TBP + Brf) complex imposes a rigid geometry on transcriptional initiation. Some residual B'-dependent transcription is even retained when the DNA loop is reduced in size from 5 to 3 bp

(Figure 3B). Five-base-pair bubbles elicit a weak background of Brf-independent transcription, primarily initiating within the bubble, that varies considerably between different bubble constructs (Figure 3A, right-hand panel). This is probably due to sequence-dependent differences in internal structure within different 5 bp loops (Figure 1) and to sequence preference for initiation of transcription (Fruscoloni *et al.*, 1995; Zecherle *et al.*, 1996).

What do B'' and Brf normally contribute to the formation of the open promoter complex?

Physical recruitment of pol III to the preformed partial transcription bubble by B' is accurate (Figure 7), but inefficient relative to intact TFIIB and the polymerase is weakly held (as judged by gel retardation). Brf interacts with the C34 subunit of pol III (Brun *et al.*, 1997) through epitopes located in two conserved segments of its pol III-specific C-proximal half (Andrau *et al.*, 1999). Pol III also interacts with the N-proximal, TFIIB-homologous half of Brf (Khoo *et al.*, 1994; Kassavetis *et al.*, 1998a). Some of these interactions are likely to be direct, since they are detected by two-hybrid screens and by affinity probing (Werner *et al.*, 1993; Khoo *et al.*, 1994; Wang and Roeder, 1997; Andrau *et al.*, 1999). However, these direct Brf-C34 interactions do not suffice for pol III recruitment to linear DNA in the absence of B''.

Thus, B'' must supply one or more auxiliary interactions with pol III and/or change the conformation of Brf in a way that exposes additional pol III interaction sites. Indeed, a specific pol III-B'' interaction has been detected by affinity chromatography (Kumar *et al.*, 1997). On the other hand, if B'' merely provided a single additional pol interaction site, one would expect that B'' deletions eliminating pol III recruitment to linear duplex DNA should already have been identified (Kumar *et al.*, 1997; Kassavetis *et al.*, 1998b). B'' clearly reconfigures Brf around the TBP-DNA complex, as evidenced by photochemical cross-linking and footprinting analysis (Kumar *et al.*, 1997; Colbert *et al.*, 1998; Kassavetis *et al.*, 1998a; see also Figure 7). It is more likely, therefore, that the B''-generated reconfiguration of Brf is required for effective pol III interaction (and it is conceivable that Brf reciprocally reconfigures B'').

In the absence of B'', the DNA loop probably serves as an auxiliary pol III-binding site (cf. Guo and Gralla, 1998), but it is not a required site for pol III binding in the context of recruitment by TFIIB (since pol III binds stably to the TFIIB-DNA complex at 0°C, at which temperature the transcription bubble is closed). The absence of B'' also changes the pol III-promoter interaction in other ways. For example, pol III attachment to the B'-bubble promoter complex is greatly stabilized by a short nascent transcript or by binding the ribonucleoside triphosphates (GTP and UTP) that form the initial phosphodiester bond. In addition, the reactivity of T -3 to KMnO₄ increases ~3-fold upon insertion of GTP into the catalytic site of pol III (Figure 6), indicating that, even with bp -9 to -5 pre-melted, expansion of the bubble remains defective in the absence of B''. Binding of the *Escherichia coli* σ^{70} holoenzyme to the *rrnB* (ribosomal RNA) promoter is similarly dependent on ATP concentration (Gaal *et al.*, 1997), but other *E.coli* promoters do not show that effect. Thus, one can interpret the role of B'' (at least

partly) as changing the effective strength of the *SNR6* promoter.

B''-independent transcription is also highly demanding of the integrity of Brf (Figure 4). Most striking is the finding that splitting Brf at amino acid 283, which leaves the physical properties and transcriptional activity of TFIIB essentially unchanged (Figure 4A, and Kassavetis *et al.*, 1998a), reduces B''-independent transcription to near-background levels (Figure 4B). Evidently B'' serves as a scaffold for holding Brf fragments in a TFIIB–DNA complex.

Comparison with pol II

RNA polymerases II and III bind to transcription factor–DNA complexes that are anchored by TBP bound to an AT-rich sequence. TFIIB and the TFIIB-related N-terminal half of Brf bind to this TBP–DNA complex in a similar orientation (Kassavetis *et al.*, 1998a). The demonstration that the Brf–TBP–DNA complex assembles pol III on bubble-containing templates for specific initiation expands the range of similarities between pol II and pol III transcription complex assembly and initiation. Transcription of linear duplex DNA by pol II requires TBP, TFIIB, TFIIF, TFIIE and TFIIH. Stable association of pol II requires only TBP, TFIIB and TFIIF, but promoter opening requires ATP hydrolysis catalyzed by the XPB helicase of TFIIH, which is brought into the initiation complex by TFIIE (Jiang and Gralla, 1995; Holstege *et al.*, 1996; Tirode *et al.*, 1999; reviewed by Orphanides *et al.*, 1996). An intrinsic role of TFIIE in open complex formation beyond its involvement in TFIIH recruitment has also been discerned (Holstege *et al.*, 1995).

A preformed single-stranded DNA bubble upstream of the transcriptional start entirely removes the dependence of specifically initiated transcription on TFIIE, TFIIH and ATP hydrolysis (Pan and Greenblatt, 1994; Holstege *et al.*, 1995, 1996). Even the requirement for TFIIF is no longer absolute in this case: the TBP–TFIIB–bubble DNA complex alone inefficiently assembles pol II for specific initiation, and TFIIF stimulates this basal transcription >100-fold (Pan and Greenblatt, 1994; Holstege *et al.*, 1996). Similarly, B'' stimulates transcription by the Brf–TBP–bubble DNA complex 7- to 20-fold (Figures 3 and 5, and Table I). In the context of bubble-containing templates, B'' and TFIIF also share the common function of generating a more stable complex by providing additional interactions with polymerase and TFIIB or Brf, respectively. Both TFIIF-independent and B''-independent transcription require placement of the preformed bubble within the 10 bp segment upstream of the transcriptional start (Figure 3; Pan and Greenblatt, 1994; Holstege *et al.*, 1996).

It is likely that the N-terminal Zn-ribbon domains of TFIIB and Brf, which probably project toward the respective start sites of transcription, play key roles in these minimal pol II and pol III transcription systems. Deletion of the Brf Zn-ribbon abolishes B''-independent transcription (Figure 4C), but does not impair Brf–TBP–DNA complex formation (Kassavetis *et al.*, 1997, 1998a; Colbert *et al.*, 1998), suggesting a role in interaction with pol III. [The Brf–C34 subunit of pol III interaction appears not to involve the Zn-ribbon domain (Khoo *et al.*, 1994; Andrau *et al.*, 1999).] Removing its Zn-ribbon domain destroys the stable association of yeast TFIIB with purified pol II

(Pardee *et al.*, 1998). Point mutations in this part of TFIIB also alter transcriptional start site selection or greatly destabilize pol II–TFIIB association (Pardee *et al.*, 1998). The point mutations in TFIIB that impair pol II binding change amino acids that are conserved or identical in fungal Brf, suggesting that the N-terminal Zn-ribbon domains of TFIIB and Brf interact with a common or conserved surface feature of pol II and pol III.

Comparison with bacterial RNA polymerase holoenzyme

Pol III non-coordinately opens a large transcription bubble at a tRNA gene promoter in two segments (Kassavetis *et al.*, 1992b). One imagines that separate sets of protein–DNA interactions generate these two parts of the transcription bubble. The existence of two sets of promoter-opening interactions is also suggested by analyses of promoter opening by *E.coli* and *Bacillus subtilis* holoenzymes (Chen and Hermann, 1997; Zaychikov *et al.*, 1997). Certain mutants in the *E.coli* RNA polymerase β subunit readily open an upstream segment of the transcription bubble, even at low temperature, but are defective in DNA strand separation at the transcriptional start (Severinov and Darst, 1997). An extensive analysis of DNA binding by the *E.coli* σ^{70} holoenzyme also supports a model invoking two separate interactions with single-stranded DNA: action at the –10 promoter element nucleates promoter opening (Marr and Roberts, 1997; Hermann and de Haseth, 1999), and the core enzyme stabilizes strand separation around the transcriptional start site (Guo and Gralla, 1998).

The conserved region 2.3 of *E.coli* σ^{70} and *B.subtilis* σ^A contributes a single-stranded DNA binding activity that probably participates in the nucleation of DNA melting at bp –10. Certain mutations in this aromatic amino acid-rich segment generate impaired promoter opening that can be suppressed by DNA supercoiling and by short preformed DNA bubbles (Aiyar *et al.*, 1994; Juang and Hermann, 1994). It is tempting to think of B'' playing a similar role in open complex formation: B'' also contains an aromatic residue-rich segment (between amino acids 402 and 420); small internal deletions within this region generate pol III–TFIIB–DNA complexes that support promoter opening on supercoiled but not on linear DNA (Kassavetis *et al.*, 1998b), and we have shown here that the absolute requirement for B'' is circumvented by performing the upstream segment of the pol III transcription bubble. The weak cross-linking of B'' that is observed at bp –13/–12 and bp –5 on the *SNR6* gene localizes to its amino acid 371–594 C-terminal segment; B'' internally deleted between amino acids 409 and 421 is also somewhat defective in cross-linking to bp –5 (S.M.A.Shah, A.Kumar, E.P.Geiduschek and G.A.Kassavetis, unpublished observations). However, probing protein–DNA interactions in pol III–TFIIB–DNA complexes by photochemical cross-linking has so far failed to provide direct evidence for B'' contacts with the non-transcribed strand in this region of the transcription bubble (Bartholomew *et al.*, 1993, 1994). Clearly, further analysis of protein interactions with the transcription bubble should prove highly informative on this issue.

Materials and methods

The transcription templates for this work are based on the *SNR6* gene-containing plasmid pU6RboxB (Whitehall *et al.*, 1995) with a single

G→A substitution at bp -22 (relative to the transcriptional start at +1) reversing an original A→G substitution. Fully duplex DNA was generated by PCR amplification with DNA primers specifying 5' ends at bp -60 and +138; sequence substitutions corresponding to the top strands shown in Figure 1 were oligonucleotide mediated (Kunkel *et al.*, 1987). DNA bubble constructs were made by annealing complementary DNA strands. These were generated by PCR amplification with pairs of primers, one of which contained a single ribonucleotide. Alkaline hydrolysis of these PCR products yielded DNA strands that were readily separated on denaturing gel (on account of their differing lengths). The shorter DNA strands were isolated, combined to form the bubble templates specified in Figure 1, repurified on native gels and quantified by UV absorbance. The identity of final products was confirmed by dideoxy sequencing. (Complete sequences and detailed methods are available on request.) Plasmid pU6LboxB and its 366 bp derived fragment used in Figure 4A have been described (Whitehall *et al.*, 1995; Kassavetis *et al.*, 1998b).

Purification and assays have been described previously for pol III (Mono Q-purified; Kassavetis *et al.*, 1990), recombinant TBP (Joazeiro *et al.*, 1994), recombinant B' (native purification; Kassavetis *et al.*, 1997), full-length Brf (N- and C-terminally His₆-tagged), Brf(1-282) and Brf(284-596) (Kassavetis *et al.*, 1998a). BrfΔ366-407, BrfΔ383-424 and NΔ68BrfΔ366-407 were derived from pET21d-based expression clones of Brf(2-596) and Brf(69-596) containing a His₇ tag followed by a TEV protease cleavage site at their N termini (generously provided by J.Ernst and P.Sigler, Yale University). Deletions were generated by PCR amplification and reinsertion into the same vector for expression. A unique *Cla*I or *Afl*III site, introduced during amplification, facilitated recombining of N- and C-terminal parts of the Brf open reading frame to generate internal deletions: BrfΔ366-407 contains an additional leucine at the deletion point and BrfΔ383-424 contains an additional isoleucine at the deletion point. Primer and Brf expression vector sequences are available on request.

Protein-DNA complexes for transcription and footprinting were formed at 20°C in 20 µl of reaction buffer [40 mM Tris-HCl pH 8.0, 7 mM MgCl₂, 3 mM dithiothreitol, 7-8% (v/v) glycerol, 100 µg/ml bovine serum albumin and NaCl as specified below], containing 100 ng poly(dG-dC):poly(dG-dC), 50 fmol template DNA (for transcription) or 2-6 fmol 5' end-labeled template DNA (for footprinting), 100-400 fmol TBP, 200-600 fmol Brf, 150 fmol B', 10 fmol pol III and 40 mM (Table I, and Figures 2 and 6), 60 mM (Figure 4A), 70 mM (Figures 3, 4B, 4C and 5) or 90 mM NaCl (Figure 7). B'-directed transcription was found to be optimal at 40 mM NaCl with full-length Brf, and at 40-70 mM NaCl with BrfΔ366-407 or BrfΔ383-424. For multiple rounds of transcription, 20 µl samples of pre-assembled promoter complexes were supplemented with 5 µl of nucleotides in reaction buffer providing 200 µM ATP, 100 µM GTP and CTP, and 25 µM [α-³²P]UTP for 30 min. For single- and multiple-round transcription assays presented in Table I and Figure 5, B'-DNA and TFIIB-DNA complexes were formed for 60 min in 18 µl of reaction buffer; 2 µl of pol III and 2.5 µl of a nucleotide solution providing GTP, CTP and UTP (as specified above) were added together for 10 min (Table I), 15 min (Figure 5B) or the indicated time (Figure 5A), followed by 2.5 µl of a solution providing 100 µM ATP without (multiple round) or with (single round) 200 µg/ml heparin for an additional 15 min. Transcription was terminated and samples were processed for gel electrophoresis as described (Kassavetis *et al.*, 1989). RNA intended for primer extension analysis was prepared as indicated above, but with unlabeled nucleotides. Following extraction with phenol and precipitation with ethanol, samples were resuspended in 15 µl of 10 mM Tris-HCl pH 8.8, 250 mM NaCl, 1 mM dithiothreitol containing 2 U placental ribonuclease inhibitor and 20 fmol of a 5'-³²P-labeled transcribed strand primer (bp +67 to +46). Samples were annealed for 1 h at 53°C and diluted 3-fold; the subsequent reaction and processing conditions followed Joazeiro *et al.* (1996). KMnO₄ footprinting and two-dimensional DNase I protection analysis were performed as described (Kassavetis *et al.*, 1992b; Kumar *et al.*, 1997), and noted in the figure legends.

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