Effects of DNA strand breaks on transcription by RNA polymerase III: insights into the role of TFIIB and the polarity of promoter opening

George A. Kassavetis¹, Anne Grove^{1,2} and E. Peter Geiduschek

Division of Biological Sciences and Center for Molecular Genetics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0634, USA

²Present address: Louisiana State University, Division of Biochemistry and Molecular Biology, 534 Choppin Hall, Baton Rouge, LA 70803, USA

¹Corresponding authors
e-mail: gak@ucsd.edu or agrove@lsu.edu

Certain deletion mutants of the Brf1 and Bdp1 subunits of transcription factor (TF) IIB retain the ability to recruit RNA polymerase (pol) III to its promoters, but fail to support promoter opening: deletions within an internal Bdp1 segment interfere with initiation of DNA strand separation, and an N-terminal Brf1 deletion blocks propagation of promoter opening past the transcriptional start site. The ability of DNA strand breaks to restore pol III transcription activity to these defective TFIIB assemblies has been analyzed using U6 snRNA gene constructs. Breaks in a 21 bp segment spanning the transcriptional start rescue transcription in DNA strand-specific and subunit/mutation-specific patterns. A cluster of Bdp1 internal deletions also reverses the inactivation of transcription with wild-type TFIIB generated by certain transcribed (template) strand breaks near the transcriptional start site. A structure-based model and topological considerations interpret these observations, explain how Bdp1 and Brf1 help to enforce the general upstream→downstream polarity of promoter opening and specify requirements for polarity reversal.

Keywords: promoter opening/RNA polymerase III/*Saccharomyces cerevisiae*/TFIIB/transcriptional initiation

Introduction

The central transcription factor (TF) IIB recruits yeast RNA polymerase (pol) III to its promoters. DNA strand opening in the pol III promoter complex appears to nucleate at the upstream end of the nascent transcription bubble, near bp −9 (relative to the transcriptional start site as +1), and unidirectional DNA strand separation expands the bubble in the downstream direction beyond the transcriptional start site, to approximately bp +7. Bacterial RNA polymerases follow a similar reaction pathway (Kassavetis *et al.*, 2001).

The σ^{70} family subunits of the bacterial RNA polymerase holoenzymes play the key roles in locating and opening their promoters, recognizing the −10 promoter element as double-stranded DNA, initiating promoter

opening within the −10 site and binding sequence-specifically to the non-transcribed (non-template) strand of the −10 site as the transcription bubble forms (Marr and Roberts, 1997; Helmann and de Haseth, 1999; Guo *et al.*, 2000; Matlock and Heyduk, 2000). In a variation of this scheme, σ^{54} family subunits recognize their −12 promoter sites as duplex DNA, and generate an isomerization at the adjacent initiation site for promoter opening in an activator-requiring reaction (Cannon *et al.*, 2000).

TFIIB apparently plays a comparable role in initiating transcription by pol III, not only recruiting its polymerase to the promoter but also participating in promoter opening. Evidence for the post-recruitment function of *Saccharomyces cerevisiae* TFIIB was first provided by analysis of deletion mutants of its Bdp1 and Brf1 subunits (formerly called B'' and Brf, respectively). TFIIB assembled with these deletion proteins substantially retains the ability to recruit pol III and accurately position it over the promoter, but does not allow the promoter to open (Kassavetis *et al.*, 1998, 2001). Both regions of Bdp1 and Brf1 are essential for viability (Hahn and Roberts, 2000; Ishiguro *et al.*, 2002). Experiments analyzing the restoration of transcription by partial promoter opening (unpairing 5 or 3 bp segments) point to two stages of participation: deletions within an internal 65 amino acid segment of Bdp1 interfere with DNA strand separation at the initiating upstream end of the transcription bubble, while removing an N-terminal domain of Brf1 interferes with downstream propagation of the bubble beyond the transcriptional start site (Figure 1A; Kassavetis *et al.*, 2001).

DNA is sharply bent in transcription initiation complexes. In the pol III promoter complex, significant DNA distortion is generated by TFIIB through its TATA box-binding protein (TBP) and Bdp1 subunits (Léveillard *et al.*, 1991; Braun *et al.*, 1992; J.L. Kim *et al.*, 1993; Y. Kim *et al.*, 1993; Juo *et al.*, 1996; Grove *et al.*, 1999). The multi-subunit RNA polymerases introduce additional large-scale DNA bending (Robert *et al.*, 1998; Coulombe and Burton, 1999). In probing the architecture of pol III promoter complexes, we have examined the effects of breaks into either DNA strand on initiation of transcription. Single-strand breaks destabilize duplex DNA and modestly increase DNA flexibility (Koudelka *et al.*, 1988; Erie *et al.*, 1989; Pieters *et al.*, 1989; Mills *et al.*, 1994). Single-strand breaks also facilitate σ^{70} -dependent promoter opening by *Escherichia coli* RNA polymerase (Li and McClure, 1998). One way to probe the connection between the architecture of pol III promoter complexes and initiation of transcription would therefore be to perturb constraints on DNA flexibility by introducing breaks into either strand of the DNA segment lying between the TATA box and the transcriptional start site. The experiments that are reported here focus on the ability of such DNA breaks to restore transcriptional activity to TFIIB

Fig. 1. Two defects of promoter opening generated by mutations in the Brf1 and Bdp1 subunits of TFIIIB and a test for restoration of transcriptional activity. (A) An N-terminal deletion in Brf1 and a group of small internal deletions in Bdp1 (Δ) block separate steps of promoter opening (middle line; Kassavetis *et al.*, 2001). Identified features of Brf1 include its N-proximal zinc-binding domain, direct repeats in its TFIIIB-related N-proximal half, and three conserved segments in its pol III-specific C-proximal half. A core segment of Bdp1 was defined by a deletion analysis (Kumar *et al.*, 1997). (B) A series of linear DNA constructs with single breaks at locations marked by vertical arrows. The sequence of the U6 gene (*SNR6*) is shown from the TATA box (bp -30 to -23) to 10 bp downstream of the normal transcriptional start (defined as +1). Transcription is to the right. DNA with a break in the top, non-transcribed (non-template) strand, 5' to bp -10 is designated as N-10t, for example. DNA with a break in the bottom strand, which is the transcribed (template) strand, 3' to bp -8 is designated as N-8b, for example.

assembled with the already referred to Bdp1 and Brf1 deletion proteins. Rescue of transcriptional activity is conferred by breaks in the DNA segment that forms the transcription bubble and by transcribed strand breaks in the adjacent upstream A₇ block of the U6 (*SNR6*) promoter. The rescue patterns (i.e. the dependence on location of DNA breaks) for the Bdp1 and Brf1 deletion mutants differ. These effects are explained in terms of previously proposed ideas about the role of transcribed strand continuity in constraining the initiation site of pol III transcription (Grove *et al.*, 2002), and in terms of facilitated initiation of DNA strand separation at a break.

An unanticipated property of the Bdp1 deletion proteins is their ability to reverse the inactivation of transcription with wild-type TFIIIB that is generated by transcribed strand breaks in the vicinity of the transcriptional start site (specifically at bp -4, +1 and +3). We propose that this striking effect is due to elimination of two constraints that impose the normal (upstream→downstream) polarity of

Fig. 2. Transcription of DNA with breaks: (A) transcribed strand breaks and (B) non-transcribed strand breaks. Transcription complexes were formed with wild-type TFIIIB (wt), TFIIIB assembled with Bdp1 Δ 388-409 or TFIIIB assembled with Brf1 Δ 68. Locations of DNA breaks are indicated above each lane. d, duplex DNA. The U6 transcript and recovery marker (r.m.) are shown, as indicated at the left side. Lanes 1, 10 and 17 in (A), and lanes 1 and 13 in (B) show transcription of intact duplex DNA with wild-type TFIIIB. Lanes 2, 11, 18 and 26 in (A) and lanes 2 and 14 in (B) show that TFIIIB assembled with mutant Brf1 or Bdp1 is defective in transcription of intact duplex DNA. In (A), lanes 1-9 are from one gel, as are lanes 17-25; lanes 10-16 and 26-31 are also from one gel. In (B), lanes 1 and 2-12 are from separate gels of one experiment analyzed in the same exposure of the phosphoimage plate; lanes 13 and 14-25 are similarly from one experiment, one exposure and two gels run in parallel.

promoter opening: (i) the transcribed strand break diminishes the energy barrier to DNA unwinding between fixed downstream and upstream points of protein attachment and (ii) these internal Bdp1 deletions prevent initiation of transcription bubble opening with normal upstream→downstream polarity.

Results

Transcription has been examined on linear DNA extending from bp -60 to bp +138 of the U6 (*SNR6*) gene-derived transcription unit U6_{boxB}, and incorporating breaks in the non-transcribed (top) and transcribed (bottom) strands at locations that are indicated in Figure 1B. Active transcription by pol III of this U6 gene in linear duplex DNA is directed by TFIIIB (Figure 2A and B, lanes 1), but TFIIIB assembled with certain Bdp1 (Figure 2A and B,

lanes 2) or Brf1 deletion mutants (Figure 2A, lane 18 and B, lane 14) is almost completely inactive for transcription of intact linear duplex DNA. Partial promoter opening restores activity to these defective TFIIBs; the defect generated by deleting amino acids 388–409 of Bdp1 is suppressed by artificially opening upstream segments of the transcription bubble, while the defect generated by deleting the N-proximal 68 amino acids of Brf1 is suppressed by opening the downstream end of the transcriptional bubble (Figure 1A; Kassavetis *et al.*, 2001). In searching for connections between DNA deformation, architecture of promoter complexes and transcription, we examined whether introducing flexibility into DNA by placing breaks in either strand would also relieve the transcriptional defects of these Bdp1 or Brf1 mutants. We recently showed that interrupting the continuity of the transcribed DNA strand strongly affects the transcriptional activity of pol III and wild-type TFIIB, both qualitatively and quantitatively (Grove *et al.*, 2002): (i) the distribution of transcriptional start sites varies with the position of transcribed strand breaks; and (ii) overall transcript yields also vary, with particularly pronounced inhibition of transcription generated by transcribed strand breaks in the vicinity of +1. Suppression of the transcriptional defects of Bdp1 and Brf1 deletion mutants has to be interpreted in that context.

Breaks in the transcribed strand

An experiment examining transcriptional activity of TFIIB assembled with Bdp1 Δ 388–409 (lanes 3–9 and 12–16) and with Brf1 Δ 68 (lanes 19–25 and 27–31) is shown in Figure 2A. The salient features are that appropriately placed transcribed (template) strand breaks can substantially restore activity. Several specific properties of the restored transcription are evident: the lengths of these transcripts suggest shifts of the transcriptional start site. This was verified and quantified by primer extension analysis of RNA 5' ends (Figure 3). Restoration of activity can be assessed quantitatively by comparison with previously determined transcriptional efficiencies of these DNA templates with wild-type TFIIB (gray symbols). The distribution of start sites substantially follows the previously analyzed pattern of transcription with wild-type TFIIB for breaks upstream of +1: transcription shifts to upstream-lying alternative sites conforming to the pyrimidine/purine –1/+1 (non-transcribed strand) consensus of natural pol III transcription start sites, and start sites with mutant and wild-type TFIIB on a template with the same break point are comparable (Figure 3; Grove *et al.*, 2002). The restored transcription is essentially completely TFIIB dependent: omission of Brf1 and Bdp1 reduces transcription by >99% of the reference level in Figure 2A, lane 1. Introducing breaks into the transcribed strand segment that is covered by the fully open transcription initiation bubble does allow a low level of Bdp1-independent but Brf1- and TBP-requiring transcription (Kassavetis *et al.*, 1999), corresponding to 2–5% of transcription with intact duplex DNA (Grove *et al.*, 2002), but this background is greatly exceeded by the transcription levels that are generated by complete TFIIB assembled with Bdp1 Δ 388–409 (Figure 3).

Suppression of the transcriptional defect generated by the amino acid 388–409 deletion in Bdp1 is effected by

Fig. 3. Relative transcriptional activities of TFIIB assembled with Brf1 Δ 68 (top panel) or Bdp1 Δ 388–409 (bottom panel) on linear DNA with breaks at the indicated positions of the transcribed strand. Activities are shown relative to transcription of intact duplex DNA using wild-type TFIIB. Each column is divided up by color (code shown at the right side of the top panel) to indicate the distribution of start sites associated with each DNA template; error bars indicate the average deviations of two experiments. The gray continuous curve shows the transcriptional activity of the same DNA templates with wild-type TFIIB (Grove *et al.*, 2002).

transcribed strand breaks at bp –10 (template construct N–10b) and further downstream; there is no suppression of the transcriptional defect by the break at bp –14 and almost none by the break at bp –12 (Figure 3, bottom panel). Suppression by breaks at bp –10 and –8 is complete, in the sense that transcription with wild-type TFIIB and TFIIB(Bdp1 Δ 388–409) is comparable. Transcription with mutant TFIIB exceeds the wild-type TFIIB-associated level for the breaks at bp –6 and –4. Transcribed strand breaks in the vicinity of the normal start site (bp –2, +1 and +3) almost entirely block transcription directed by wild-type TFIIB, and breaks at bp –4 and +5 severely inhibit transcription. It is surprising that the Bdp1 amino acid 388–409 deletion restores activity. The residual transcription of wild-type TFIIB on N+1b and N+3b DNA initiates primarily at +1 (Grove *et al.*, 2002). In contrast, transcription of template N+1b that is restored by this Bdp1 deletion initiates almost exclusively at bp +5 and the restored transcription of N+3b DNA initiates at bp +5 and +7.

The break at bp -2 is exceptional. It generates maximal inhibition of transcription with wild-type TFIIIB, and the amino acid 388–409 deletion in Bdp1 does not relieve this effect. Evidently two defects of transcription mechanism are generated by breaking the transcribed strand in the vicinity of bp $+1$ (Grove *et al.*, 2002). The Bdp1 amino acid 388–409 deletion only compensates for one of these defects, as discussed below.

Similar effects have been noted in experiments with Bdp1 Δ 355–372: (i) TFIIIB assembled with this Bdp1 mutant is also defective for transcription of intact linear duplex DNA; (ii) suppression of this defect is only generated by breaks in the transcribed strand downstream of bp -12 ; and (iii) the Bdp1 amino acid 355–372 deletion suppresses the defect of wild-type TFIIIB in transcribing DNA with non-transcribed strand breaks at bp -4 , $+1$, $+3$ and $+5$, but not at bp -2 (data not shown). Thus, a block to transcription with wild-type TFIIIB that is generated by interrupting the continuity of the transcribed DNA strand around the transcriptional start is suppressed by Bdp1 deletions in a segment lying N-proximal to its SANT domain. The same segment of Bdp1 is involved in initiation of promoter opening (Figure 1A).

The N-terminal 68 amino acids of Brf1 include a Zn-binding motif that is essential for function *in vivo* (Hahn and Roberts, 2000). Removing this Brf1 segment nearly destroys the ability of TFIIIB to direct pol III transcription of intact linear duplex DNA (Figure 2A, lane 18). TFIIIB assembled with Brf1 Δ 68 yields a distinctive pattern of transcriptional activity: (i) breaks at bp -16 , -14 and -12 restore activity to TFIIIB assembled with Brf1 Δ 68, but are essentially without effect on transcription with TFIIIB assembled with Bdp1 Δ 388–409; (ii) breaks at bp -10 to -4 and at bp $+5$ permit substantial transcriptional activity relative to wild-type TFIIIB, just as they do for TFIIIB assembled with Bdp1 Δ 388–409; and (iii) Brf1 Δ 68 does not suppress the inactivating effects of breaks at bp -4 , -2 , $+1$, $+3$ and $+5$ (Figure 3, top panel).

Breaks in the non-transcribed strand

An experiment examining the effects of breaks in the non-transcribed strand on activity of TFIIIB assembled with Bdp1 Δ 388–409 and Brf1 Δ 68 is presented in Figure 2B; the quantitative analysis of transcriptional activities and start-site distributions is shown in Figure 4. Placing breaks at specific positions in the top strand weakly but unequivocally suppresses transcriptional defects generated by these two deletions. Most of the restored transcription retains the $+1$ start site. Although some initiation further upstream is generated by breaks at bp -8 , -6 and to a lesser extent bp -10 and -4 , most if not all of these events can be ascribed to non-specific initiation by pol III (Figure 4, open circles; see Grove *et al.*, 2002). The transcriptional defect generated by the Brf1 Δ 68 deletion is suppressed by non-transcribed strand breaks around bp $+1$ (Figure 2B, lanes 21–23 and Figure 4, top panel). Suppression of the defect generated by deleting amino acids 388–409 of Bdp1 is distributed more broadly among breaks located downstream of bp -10 (Figure 2B, lanes 5–11 and Figure 4, bottom panel). An experimental series with Bdp1 Δ 355–372 showed similar effects (data not shown). Globally, the asymmetry between the two DNA strands that was previously noted for the effects of strand breaks

Fig. 4. Relative transcriptional activities of TFIIIB assembled with Brf1 Δ 68 or Bdp1 Δ 388–409 on linear DNA with breaks at the indicated positions of the non-transcribed strand. Presentation as in Figure 3. The open red circles indicate background transcription with Brf1 and Bdp1 omitted.

on transcription with wild-type TFIIIB (Grove *et al.*, 2002) is, at least to some extent, reflected in the ability to compensate for the transcriptional defects of these Brf1 and Bdp1 mutants.

Discussion

We have examined the ability of single-strand DNA breaks to restore promoter opening to TFIIIB assembled with two classes of mutant subunits: (i) Bdp1 deletion proteins that interfere with the initial opening of the transcription bubble, a defect that can be repaired by pre-opening the upstream end of the bubble from bp -9 to -5 ; and (ii) Brf1 Δ 68, which prevents the downstream propagation of the transcription bubble, a defect that is explicitly repaired by pre-opening the downstream end of the transcription bubble from $+2$ to $+6$. Pre-opening the central segment of the transcription bubble (bp -4 to $+1$) restores some activity to both classes of mutants (Figure 1A) (Kassavetis *et al.*, 2001). Single-strand breaks destabilize duplex DNA and stimulate the nucleation of open promoter complex formation by *E. coli* RNA polymerase σ^{70} holoenzyme. Accordingly, it was anticipated that breaks in either DNA strand would rescue promoter opening defects of Bdp1 and Brf1 deletion

proteins if their locations correlated with the extent of preformed bubbles that rescue transcription. During the course of this analysis it was found that breaks in the transcribed and non-transcribed strands exert non-equivalent effects on transcription with wild-type TFIIB: breaks in the transcribed strand between bp -14 and -4 disrupt the normal mechanism that measures the distance from DNA-bound TFIIB to the start site and systematically shift the start site of transcription upstream; breaks in the transcribed strand between bp -2 and +5 severely inhibit transcription. These observations and other data led to a model based on the structure of the pol II elongation complex (Gnatt *et al.*, 2001) that rationalizes the dominant role of the transcribed strand in the pol III open complex (Grove *et al.*, 2002).

The model contains three basic elements (Figure 5A). (i) The unwound transcribed strand of the open complex engages the polymerase catalytic site as a U-shaped channel spanning nucleotides (nt) -9 to +4 that is constrained by non-specific protein-DNA interactions with the C128 and C160 subunits of pol III. This compresses an additional 4-5 nt into protein contact relative to linear duplex DNA. The complementary non-transcribed strand is relatively unconstrained in the complex. (ii) The stable association of pol III at its upstream end with the tightly bound TFIIB-DNA complex prevents both forward and backward translocation of polymerase and its associated transcription bubble in the absence of RNA synthesis. (iii) The pol III open complex is in temperature-dependent equilibrium with closed and partially closed states. Partial closure of the transcription bubble extrudes downstream duplex DNA only, due to the stable TFIIB-pol III interface at the upstream end. The catalytic center lies near the bottom of the U-shaped single-strand DNA binding channel of polymerase; partial closure of just a few base pairs pulls the transcribed strand away from the catalytic site, preventing initiation (Figure 5A).

Transcribed strand breaks between bp -10 and -4 remove a constraint that is imposed by continuity of that strand and shift initiation to bp -2 and -4 because the compaction of an additional 4-5 nt of DNA into the bubble is no longer required to align the transcribed strand with the catalytic site. Transcribed strand breaks between bp -2 and +3 greatly inhibit transcription because extrusion of 2-4 bp of downstream duplex destroys alignment of the transcribed strand with the catalytic site. The discontinuity of the transcribed strand prevents the upstream transcribed strand-polymerase interactions from drawing sequence downstream of the break into the template strand DNA channel.

Single-strand DNA breaks rescue the promoter opening defect of Brf1 Δ 68

The ability of single-strand DNA breaks to rescue the downstream bubble propagation defect of Brf1 Δ 68 reflects both the helix-destabilizing effect of nicks and the relief of constraints imposed by the continuity of the transcribed strand. The partial restoration of Brf1 Δ 68 activity by non-transcribed strand breaks between bp -2 and +3 (Figure 4) correlates with the more complete rescue observed with pre-formed bubbles -4/+1 and +2/+6 (Kassavetis *et al.*, 2001). This rescue, therefore, may

Fig. 5. The continuity of DNA strands imposes constraints on promoter opening and initiation of transcription. (A) The TFIIB-pol III (B-DNA-pol) interface and continuity of the transcribed strand strongly constrain initiation to bp +1 and make transcription contingent on full opening of the transcription bubble. Breaks in the transcribed strand relax this constraint and allow transcription to initiate further upstream (Grove *et al.*, 2002). (B) Continuity of DNA strands and upstream tethering of pol III to DNA through TFIIB impose the upstream→downstream polarity of promoter opening. Opening of the transcription bubble with opposite polarity is blocked by the accumulation of topological strain between two sites of DNA attachment. (C) A strand break relieves the accumulation of topological strain associated with downstream→upstream progression of promoter opening.

solely result from the helix-destabilizing effect of these breaks. Breaks in the transcribed strand between bp -2 and +3 do not rescue transcription (Figure 3), but wild-type TFIIB is also inactive with these templates. We attribute these failures to the same cause: failure to align the transcribed strand with the catalytic site. Transcribed strand breaks between bp -4 and -10 also partially rescue Brf1 Δ 68, shifting the start site of transcription to bp -4 and -2, as observed with complexes containing wild-type TFIIB. The ability of these breaks, but not the pre-formed corresponding -9/-5 bubble, to restore transcription activity to Brf1 Δ 68 implies relief of a constraint imposed by the continuity of the transcribed strand. Breaks in either strand between bp -10 and +4 should enhance the formation of the upstream bubble with Brf1 Δ 68, but only breaks in the transcribed strand facilitate downstream propagation of the bubble: promoter opening in intact duplex DNA with Brf1 Δ 68 propagates as far downstream as bp -3 (Kassavetis *et al.*, 2001) and stops there.

Transcribed strand breaks in the upstream segment of the transcription bubble would allow single-strand DNA–pol III contacts upstream of the break to be established despite incomplete expansion of the bubble, and would also allow both sliding and maintenance of single-strand DNA–pol III contacts downstream of the break, thus facilitating an otherwise blocked expansion of the bubble downstream (Figure 5A).

Base pairs –16 to –12 are located upstream of the normal open complex transcription bubble but lie in a stretch of seven consecutive T:A base pairs. Transcribed strand breaks between bp –16 and –12 also rescue the transcriptional defect of Brf1 Δ 68. We attribute this to a helix-destabilizing effect of breaks in this AT-rich region right next to the upstream edge of the transcription bubble, which would also promote downstream bubble expansion. We had noted that transcribed strand breaks between bp –16 and –12 shift start sites of transcription with wild-type TFIIIB to bp –10 and –8 (Grove *et al.*, 2002). Indeed, trial experiments with DNA in which the T₇:A₇ segment is broken up [non-transcribed changed from (–19)GTTTTT–TTTC(–12) to (–19)AGGTCGATC(–12); Gerlach *et al.*, 1995] suppressed this upstream shift of initiation (A.Grove, unpublished data). Initiation at bp –10 and –8 requires at least a partial disruption of the TFIIIB–DNA complex, presumably resulting from a cascading effect of pulling additional transcribed strand sequence into pol III (Grove *et al.*, 2002). The same events occur with TFIIIB containing Brf1 Δ 68. Further effects of DNA sequence on the ability of DNA breaks to rescue the transcriptional activity of defective TFIIIB assemblies remain to be explored.

Single-strand breaks rescue promoter opening-defective Bdp1 mutants: topological constraints impose an upstream-to-downstream progression on open complex formation

The partial rescue of the bubble-initiation defect of Bdp1 Δ 388–409 by non-transcribed strand breaks between bp –10 and –4 correlates with complete rescue by the pre-formed bp –9/–5 bubble and may solely result from helix destabilization. The complete rescue by transcribed strand breaks in the same region reflects the ability of these breaks to maintain stabilizing and productive single-strand DNA–pol III contacts without compacting 2–5 nt of additional single-strand DNA into polymerase. DNA breaks at bp –2 and +1 may fall into the same category as breaks between bp –10 and –4, since the pre-formed –4/+1 bubble also rescues the transcriptional defect of Bdp1 Δ 388–409.

Non-transcribed strand breaks at bp +3 and +5 partially restore activity to Bdp1 Δ 388–409 and transcribed strand breaks between +1 and +5 even generate levels of transcription that are well above those of complexes containing wild-type TFIIIB. However, a +2/+6 bubble fails to rescue the activity of promoter opening-defective Bdp1 deletion mutants. Evidently, this failure is imposed by the connectivity of both DNA strands. When promoter opening nucleates naturally at the upstream end of the transcription bubble, positive superhelical strain generated by bubble expansion downstream can dissipate (Figure 5B). The reverse polarity is disfavored by the topological strain that would accumulate between the

TFIIIB–pol interface and the DNA attachment site at the downstream end of the transcription bubble. TFIIIC binding to its low-affinity *boxA* binding site (located ~20 bp downstream of the start site of all *S.cerevisiae* pol III-transcribed genes) might also be thought capable of impeding the upstream-to-downstream propagation of open complex formation, but is prevented from doing so because entry of pol III into the TFIIIB–TFIIIC–DNA complex disrupts TFIIIC–*boxA* interaction (Kassavetis *et al.*, 1990).

We propose that promoter opening can be nucleated at the downstream end of the transcription bubble, but only when either DNA strand is discontinuous. This is most clearly demonstrated by the rescue of transcription with TFIIIB assembled with Bdp1 Δ 388–409 by the break at bp +3 on the non-transcribed strand. This break specifies initiation at bp +1, which, in the context of a continuous transcribed strand, should require nearly complete open complex formation. Breaks in the non-transcribed strand at the downstream end of the bubble and localized DNA melting should allow the complementary strand to swivel opposite the break (Mills *et al.*, 1994), which in turn should facilitate engagement of the transcribed strand with its protein channel. It should also be possible for some DNA to unravel from the upstream side of the break during the initial deposition of the transcribed strand into the protein channel without accumulation of (topological) strain. The reduction in strain may allow nearly complete opening of the transcription bubble from the downstream end (Figure 5C).

Other RNA polymerases

The problem of topological strain accumulation necessarily applies also to the polarity of promoter opening by the other eukaryotic nuclear RNA polymerases and by bacterial RNA polymerases. Pol II and pol I are also attached to DNA upstream of the transcriptional start site through interactions with core transcription factors, and bacterial polymerases are attached to DNA upstream of the transcription bubble by their σ subunits. The protein interactions that drive promoter opening generate a second point of DNA attachment. Separately from any other consideration, initiation of DNA opening at the downstream end and propagation in the upstream direction is energetically disfavored because of the attendant accumulation of superhelical strain within a short DNA segment that is constrained at each end by attachment to protein (Figure 5B). The bacterial RNA polymerase holoenzymes drive the energetically favored reaction pathway (Brodolin and Buckle, 2001) by placing the protein contact that initiates promoter opening near bp –11 (Marr and Roberts, 1997; Cannon *et al.*, 2000; Murakami *et al.*, 2002). A design that placed initiating contacts at the downstream end of the transcription bubble would be inferior on energetic grounds.

The upstream TATA box at bp –25 and downstream promoter element (DPE) at bp +25, which are binding sites of TFIID, pose an interesting problem in this context. A significant number of *Drosophila* promoters have both sites (Kutach and Kadonaga, 2000). Simultaneous tight binding upstream and downstream of the transcriptional start site should clamp it shut. An even greater number of *Drosophila* promoters have DPE sites, but no TATA

boxes. Tight binding by TFIID at the DPE should favor reversed polarity (downstream→upstream) of promoter opening. It remains to be seen whether pol II can facilitate the initiation of promoter opening at either end of the transcription bubble and whether ATP-driven, TFIID-dependent promoter opening plays a distinctive role at these promoters. It has been proposed that TFIID functions as a molecular crank, facilitating the nucleation and the downstream propagation of the bubble by incrementally rotating downstream DNA relative to fixed upstream protein–DNA interactions (Kim *et al.*, 2000).

Another possible pathway for reversing the polarity of promoter opening

The ability of a transcribed strand break at bp +1 and +3, but not the break at bp –2, to rescue the transcriptional defect associated with Bdp1Δ388–409 is striking. As noted above, transcribed strand breaks between bp –2 and +3 severely diminish transcription with wild-type TFIIB due to a failure of alignment of the transcribed strand with the pol III catalytic site. We speculate that this misalignment is a consequence of the preferential nucleation of DNA strand opening from the upstream end of the transcription bubble, even in the presence of DNA breaks at the downstream end. What distinguishes the Bdp1 amino acid 388–409 deletion is that it impairs nucleation of DNA strand opening from the upstream end. In the structure of the pol II elongation complex (Gnatt *et al.*, 2001), the downstream site of DNA strand separation lies at bp +5 (relative to the location of the catalytic site at +1), or possibly 1 or 2 bp further downstream. DNA breathing around breaks located at bp +1 to +5 would allow the transcribed strand 3' end downstream of the break to thread into the transcribed strand-binding channel, following the same entry path that the continuous transcribed strand uses during RNA chain elongation. Filling the channel sufficiently to allow initiation (to ~4 nt beyond the catalytic center) would not require DNA melting upstream of the break. Inactivity of these breaks with wild-type TFIIB and TFIIB containing Brf1NΔ68 may result from nucleation of DNA strand opening from the upstream end, which places the transcribed strand segment upstream of the break into the channel and blocks threading of the transcribed strand downstream of the break into the same site.

Materials and methods

The 198 bp (–60 to +138) pU6_RboxB-derived transcription template and its variants containing single-strand breaks in either strand have been described (Grove *et al.*, 2002). The purification and quantification of proteins have been described (Kassavetis *et al.*, 2001 and references therein). Quantities of pol III are specified as femtomoles of enzyme active for specific transcription (Kassavetis *et al.*, 1989); quantities of other proteins are specified as femtomoles of protein. Wild-type TBP and full-length Bdp1 were estimated to be nearly 100% active and full-length Brf1 20% active in the formation of heparin-resistant complexes. Brf1Δ366–409 (rtBrf1), the parental reference type for Brf1NΔ68, is 70% more active than Brf1 in specific transcription.

Transcription and transcriptional start-site mapping by primer extension with reverse transcriptase were performed as previously described (Grove *et al.*, 2002), with the following minor variations: assays comparing wild-type and mutant Bdp1 contained 120 fmol of Bdp1 and 360 fmol of Brf1; assays comparing Brf1NΔ68 and rtBrf1 contained 150 fmol of Bdp1 and 200 fmol of Brf1.

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