Marking the start site of RNA polymerase III transcription: the role of constraint, compaction and continuity of the transcribed DNA strand

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The effects of breaks in the individual strands of an RNA polymerase III promoter on initiation of transcription have been examined. Single breaks have been introduced at 2 bp intervals in a 24 bp segment that spans the transcriptional start site of the U6 snRNA gene promoter. Their effects on transcription are asymmetrically distributed: transcribed (template) strand breaks downstream of bp –14 (relative to the normal start as +1) systematically shift the start site, evidently by disrupting the normal mechanism that measures distance from DNA-bound TBP. Breaks placed close to the normal start site very strongly inhibit transcription. Breaks in the non-transcribed strand generate only minor effects on transcription. A structure-based model interprets these observations and explains how the transcribed strand is used to locate the transcriptional start site.

Keywords: RNA polymerase III/*S.cerevisiae*/TFIIIB/transcriptional initiation/U6 promoter

Introduction

Yeast RNA polymerase (pol) III is brought to its promoters primarily by interactions with its central transcription initiation factor, TFIIIB. TFIIIB is composed of three subunits: TBP, Brf1 and Bdp1. (Brf1 was called Brf, and Bdp1 was called B'' in previous work from this laboratory. See Materials and methods for a note on the new nomenclature.) Although TBP secures attachment of TFIIIB to strong TATA boxes, most budding yeast (*Saccharomyces cerevisiae*) pol III-transcribed genes do not have strong TATA boxes; recruitment of TFIIIB to their promoters is secured by TFIIIC, which binds to the gene-internal boxA and boxB promoter elements. On 5S rRNA genes, TFIIIC attaches to a transcription unit-internal protein platform created by TFIIIA. The connection between TFIIIB and TFIIIC is cemented primarily by their respective Brf1 and Tfc4 subunits (reviewed by White, 1998). Brf1 derives its acronym (for TFIIIB-Related Factor) from the homology of its N-proximal half with the pol II transcription factor TFIIIB; the C-proximal half

contains determinants primarily responsible for holding TFIIIB together through a strong interaction with TBP and a somewhat weaker interaction with Bdp1, the third subunit of TFIIIB (Kassavetis *et al.*, 1998a).

TBP kinks sharply and bends DNA, and Bdp1 introduces an additional DNA bend downstream of the TATA box (Grove *et al.*, 1999). It is the complexly bent TFIIIB–DNA complex that brings budding yeast pol III directly to the promoter (Kassavetis *et al.*, 1990). The principal interactions securing pol III recruitment involve Brf1 and the C34 subunit of pol III (Brun *et al.*, 1997; Andrau *et al.*, 1999). (C34 is part of a three-protein pol III subassembly that is specifically required for transcription initiation.) Additional pol III–TFIIIB interactions involve Brf1 and the C17 subunit of pol III (Chédin *et al.*, 1998; Flores *et al.*, 1999; Ferri *et al.*, 2000; reviewed by White, 1998; Geiduschek and Kassavetis, 2001). TFIIIB also plays an essential post-recruitment role in initiation of transcription by pol III by guiding promoter opening. This role has been exposed by finding that removal of certain segments of Brf1 and Bdp1 generates TFIIIB assemblies that retain the ability to recruit pol III to the promoter but fail to open the transcription bubble (Kassavetis *et al.*, 1998b).

It has been appreciated for some years that bacterial and eukaryotic RNA polymerases have common evolutionary roots (Schnabel *et al.*, 1983; Langer *et al.*, 1995). Recent determinations of the structures of a bacterial RNA polymerase from *Thermus aquaticus* and of yeast pol II, together with corresponding models of transcription elongation complexes, brilliantly bring this commonality into precise focus (Zhang *et al.*, 1999; Korzheva *et al.*, 2000; Cramer *et al.*, 2001; Gnatt *et al.*, 2001). Promoter complexes of *Escherichia coli* RNA polymerase holo-enzyme bend their DNA ~300° by wrapping it around the polymerase. It has been proposed that the strain introduced into DNA in forming the promoter complex is transduced into DNA strand separation (discussed in Buckle and Buc, 1994; Buckle *et al.*, 1999) initiating approximately one helical turn upstream of the start site of transcription (Helmann and de Haseth, 1999; Cannon *et al.*, 2000; Fenton *et al.*, 2000; Guo *et al.*, 2000; Matlock and Heyduk, 2000; Panaghie *et al.*, 2000). It is conceivable that DNA bending by TFIIIB might also be functionally related to its participation in promoter opening.

Our interest in exploring the connection between the architecture of the pol III–TFIIIB–promoter complex and initiation of transcription prompted us to examine whether introducing flexibility into DNA by placing breaks in one or the other strand would affect initiation of transcription. Our exploration focused originally on rescuing the transcriptional inactivity of certain Bdp1 and Brf1 deletion mutants (Kassavetis *et al.*, 1998b). The results of those experiments will be reported elsewhere. Here we present

Fig. 1. Effects of breaks in the transcribed and non-transcribed strands on transcription of the U6 gene. **(A)** Sites of DNA interruption. The sequence of the U6 (*SNR6*) gene extending from the TATA box (underlined) to 10 bp downstream of the normal transcriptional start (designated +1) is shown; transcription is to the right. DNA constructs with a simple break at the locations indicated are used for transcription and designated as follows (for example): the construct with a break in the non-transcribed (top) strand upstream of bp -10 is designated N-10t; the construct with a break in the transcribed (bottom) strand upstream of bp +5 is N+5b. **(B)** and **(C)** Transcription of DNA with breaks in the transcribed and non-transcribed strands, respectively. The locations of the breaks are specified above each lane. Lanes 1: controls, showing transcription of intact duplex DNA (d). In **(B)**, lanes 1–8 and 9–13 are from separate experiments.

and interpret striking effects of nicked DNA templates on initiation of transcription with wild-type TFIIB and pol III.

Results

Breaks in the transcribed strand

Transcription has been examined on linear DNA extending from 60 bp upstream to 138 bp downstream of the transcriptional start of the *S.cerevisiae* U6-derived gene in the previously described construct U6_RboxB (Figure 1A). Although TFIIB binds to the TATA box of the U6 gene with either polarity, generating divergent transcription (Whitehall *et al.*, 1995), the upstream end of the DNA template is too close to the TATA box to allow proper pol III docking in a left-facing promoter complex. Consequently, only the rightward transcript of this DNA is produced.

Breaks have been introduced into either strand of an ~25 bp stretch of this DNA at 2 bp intervals. At its upstream end, this segment includes the stretch of DNA

Fig. 2. Primer extension analysis to determine the locations of transcriptional start sites. Selected examples (intact duplex DNA, constructs N-14b, N-12b, N-10b and N-8b) are shown in lanes 1–5, respectively, flanked by C and T sequence ladders.

that is bent when the Bdp1 subunit enters the TFIIB–DNA complex. Increasing DNA flexibility in this segment facilitates binding of TFIIB to a weak TATA box (Grove *et al.*, 1999). Breaks placed further downstream change the connectivity of the transcription bubble (Kassavetis *et al.*, 1992) and of the initially transcribed DNA. Attention has been devoted to assembling and purifying these templates in a way that assures the removal of partial assemblies with single-stranded ends (described in Materials and methods). DNA constructs are identified by the location of the strand break. For example, the DNA template with a 3' OH on nucleotide (nt) –7 and 5' OH on nt –6 of the transcribed (bottom) strand is designated as N-6b; the template with a break at the same position of the non-transcribed (top) strand is designated N-6t. Figure 1B compares the production of transcripts on intact duplex DNA and DNA interrupted in the transcribed strand (the bottom strand in Figure 1A) at the locations indicated. The two features of these experiments that are immediately apparent are: (i) the yield of transcripts, in other words the transcription activity of these DNA templates, varies greatly, with breaks in the transcribed strand placed near the normal start site strongly inhibiting productive transcription; (ii) breaks in the transcribed strand systematically affect the lengths of transcripts. To confirm that this is due to changes of initiation site, RNA 5' ends were identified and quantified by primer extension (Figure 2). The averaged results of this analysis are presented in Figure 3A. Transcribed strand breaks downstream of bp –16 elicit initiation at new sites located downstream of the break, at the expense of initiation at +1. All of the alternative initiation sites conform to the pyrimidine–purine –1/+1 consensus of natural pol III transcription initiation sites (Raymond *et al.*, 1985; Fruscoloni *et al.*, 1995; Zecherle *et al.*, 1996), i.e. with a pyrimidine in the non-transcribed strand immediately upstream of the initiating purine nucleotide. Every pyrimidine–purine step between positions –10 and +7 is represented in the seven detected start sites. The residual transcription of DNA with nicks in the vicinity of the normal start site (templates N-2b, N+1b and N+3b) initiates primarily at +1. Utilization of these new initiation sites, correlating with locations of breaks, is essentially completely TFIIB dependent: omission of Brf1 and Bdp1 reduces transcription by >99%. Introducing breaks into the template strand does not raise non-specific TFIIB-independent transcription substantially above the level of that of duplex DNA (bottom panel of Figure 3A).

Partially pre-opening the promoter relieves the absolute dependence of accurately initiating transcription on Bdp1

Fig. 3. The distributions of transcriptional start sites and total transcriptional activities of DNA with breaks in the transcribed (bottom) strand (**A**) and non-transcribed (top) strand (**B**). Total transcription is normalized relative to intact duplex DNA; error bars present the standard error of the mean for five experiments (two experiments for constructs N-4b to N+5b). The distribution of start sites was quantified by primer extension (two or three determinations with no significant differences). Start sites are color coded as shown in (**A**). Each panel also shows that the background of TFIIB-independent transcription (i.e. without Brf1 and Bdp1) is low (note the difference in scale), and not significantly affected by the introduction of breaks into DNA.

(Kassavetis *et al.*, 1999). This minimal pol III transcription system, consisting of pol III, TBP, Brf1 and a U6 promoter partly open between bp -9 and -5, operates at a low level (Figure 4). Introducing breaks into the transcribed strand, particularly within the span of the fully open transcription bubble (i.e. N-8b, N-6b, N-4b and N+5b DNA), also allows some Bdp1-independent transcription, at a slightly lower level, but clearly above any corresponding background of Brf1-independent transcription. Thus, these control experiments establish that the response of initiation of transcription to the placement of breaks in the transcribed DNA strand is specific, in the sense that it depends on TFIIB and is not generated by transcription factor-independent binding of pol III to the break.

Fig. 4. Breaks in the transcribed strand induce some Bdp1-independent transcription. Total transcription for all start sites represented in Figure 3A is shown as a fraction of transcription of intact duplex DNA with complete TFIIB. Open bars, transcription without Bdp1; closed bars, background of transcription without Brf1 or Bdp1.

It is known that introducing sites of potential deformability into an ~15 bp segment directly downstream of a weak TATA box increases TFIIB binding (Grove *et al.*, 1999). One might therefore suppose that introducing these breaks into the transcribed (bottom) DNA strand between bp -14 and -4 could also shift TFIIB placement, and that initiation upstream of the normal start site of transcription might be a direct consequence of upstream shifts of TFIIB. This eventuality was examined by MPE-Fe(II) and DNase I footprinting. For technical reasons that are specific to these nicked DNA probes (explained in Materials and methods and Figure 5 legend), this analysis accurately specifies the location on DNA of the upstream edge of the TFIIB complex, but does not specify its downstream edge. Nevertheless, the analysis showed that placements of TFIIB on probes N-14b (transcriptional initiation primarily at bp -10 and -8), N-10b and N-6b (transcriptional initiation primarily at bp -4 and -2), N-2b (transcription greatly diminished) and intact duplex DNA are indistinguishable. The DNase I footprints of TFIIB placement on intact duplex DNA and N-14b DNA are compared in Figure 5, as an example (other footprints are not shown). Evidently, TFIIB-dependent initiation of transcription at novel sites is not caused by relocating TFIIB. We suppose, instead, that the new initiation sites result directly from the removal of a constraint on the topography of the initiating transcription complex that is normally imposed by the continuity of the transcribed DNA strand.

The low transcriptional activity of start site-proximal nicks (Figures 1B and 3A) prompted an examination of whether lower transcriptional activity reflected decreased formation of TFIIB-pol III-DNA complexes. It does not. An electrophoretic mobility shift analysis (EMSA) of TFIIB-pol III-DNA complex formation is shown in Figure 6. TFIIB-pol III-DNA complex formation with N-6b DNA (lane 5; 48% of the transcriptional activity of intact duplex DNA), N-2b DNA (lane 6; 10% of the transcription activity of intact duplex DNA) and intact duplex DNA (lane 4) is comparable.

Fig. 5. Breaks in the transcribed DNA strand do not alter placement of TFIIIB. TFIIIB–DNA complexes (thick lines) were assembled onto intact duplex DNA (A) or N–14b DNA (B) followed by partial DNase I digestion, isolation of TFIIIB–DNA complexes by native gel electrophoresis and resolution of the partial cleavage ladders by denaturing gel electrophoresis, as described in Materials and methods. The resulting phosphor image density profiles are shown in each panel aligned to the DNase I cleavage profiles of naked DNA from mock binding reactions in which the free DNA was also purified by native gel electrophoresis (thin lines). Equivalent quantities of radioactivity were loaded on each lane of the denaturing gel. The black bars below these profiles specify the extent of the TFIIIB footprint. The downstream edge of the TFIIIB footprint on the N–14b probe is obscured by the loss, during native gel electrophoresis, of double-stranded DNA fragments formed by DNase I-generated nicks in the labeled non-transcribed strand within ~10 bp of the preformed break in the transcribed strand (indicated by hatching in the black box). Enhanced DNase I cleavage around the start site of transcription upon TFIIIB–DNA complex formation has been noted previously (Kassavetis *et al.*, 1989).

Breaks in the non-transcribed strand

Entirely different results emerge from the analysis of transcription templates with breaks in the non-transcribed (top) strand. Transcriptional initiation sites are essentially unaffected by the presence of breaks in the top strand (Figure 3B). The effectiveness of duplex DNA and of the top-strand-break constructs for transcription is also comparable (i.e. within a factor of three, Figures 1C and 3B), with a significant diminution of activity seen principally in conjunction with breaks placed downstream of the transcription start (N+3t and N+5t). The latter two templates also shift a small fraction of transcriptional initiation to bp +5 (Figure 3B).

This transcription is almost completely TFIIIB dependent; introducing breaks into the non-transcribed strand generates only a very low level of Brf1/Bdp1-independent transcription initiating in the vicinity of the nick (bottom panel of Figure 3B). The low level of transcription with

shifted initiation sites noted for transcription of N–10t, N–8t and N–6t DNA in Figure 3B is probably contributed by this non-specific background. Omission of Bdp1 only also yields the same low level of background transcription (data not shown). Thus, breaking the non-transcribed strand does not support specifically initiating but Bdp1-independent transcription.

In summary, transcription responds asymmetrically to the introduction of breaks into DNA strands. Novel properties of pol III transcription are introduced only by placing breaks in the transcribed strand. This asymmetry of effect for breaks systematically introduced over a span exceeding two DNA helical turns eliminates the possibility that these novel properties are due primarily to the introduction of flexibility into the DNA segment that links TFIIIB to pol III. The analysis raises two questions. What makes breaks in the vicinity of the normal start site deleterious for transcription? How does the introduction of

Fig. 6. Transcribed strand breaks do not interfere with TFIIB-dependent recruitment of pol III to the promoter. EMSA was used to compare the formation of TFIIB-pol III-DNA complexes by transcriptionally less active template N-6b and inactive N-2b with intact duplex DNA. The presence of pol III and TFIIB is indicated above each lane and complexes are identified at the side. Only the segment of the gel containing these complexes is shown; free DNA is not. Each DNA probe contained a common labeled non-transcribed strand at identical specific activity. All lanes are from the same gel.

nicks into the transcribed strand release the promoter complex from the normal constraints on the location of the transcriptional start site?

Promoter opening and abortive initiation

To address these questions, we probed promoter opening in constructs with breaks in the transcribed strand by KMnO_4 footprinting of the complementary (continuous) DNA strand (Figure 7). When the pol III-TFIIB complex opens the promoter in intact duplex DNA, the reactivity of T at bp -5, -3, -1, +2 and +3 increases, with the outlying T-12 and T+11 non-reactive. This DNA strand separation is completely TFIIB dependent (Figure 7A). Addition of GTP, CTP and UTP, which permits the production of 7mer RNA initiating at +1, further increases the reactivity of T in the non-transcribed strand and biases this increase in the downstream direction, relative to the no-NTP complex.

Construct N+1b retains <10% of the template activity of duplex DNA. TFIIB-pol III complexes form on this DNA, but the KMnO_4 footprint (Figure 7B) shows clearly that pol III opens the promoter aberrantly. The introduction of a break into the transcribed strand between bp +1 and -1 slightly increases the reactivity of DNA at T-1 in the absence of proteins; binding of TFIIB (alone) increases that reactivity, but pol III alone does not generate any significant change that might indicate factor-independent occupancy (data not shown). In the TFIIB-pol III complex with N+1b DNA, reactivity of T-5, T-3 and T-1 increases (T-1 to a lesser extent) while T+2 and +3 remain unreactive (Figure 7B). In aligned profiles (not shown), the sum of reactivity at T-5 and T-3 for complexes formed on intact duplex DNA (Figure 7A) and N+1b DNA (Figure 7B) is comparable, implying a comparable degree of opening of this upstream DNA segment.

These results specify that a transcribed strand break just upstream of the start site of transcription blocks the stable downstream propagation of promoter opening, monitored in the non-transcribed strand. One might wonder whether the downstream end of the transcription bubble nevertheless opens transiently and recloses readily. If this were the case, and if the template strand were transiently retained in

Fig. 7. Effects of breaks in the transcribed strand on promoter opening. Complexes with intact duplex DNA (A), constructs N+1b, N-6b and N-12b (B, C and D, respectively) were probed in the non-transcribed (top) strand with KMnO_4 . Densitometric traces of phosphor images of footprints are shown, and the locations of T residues are indicated. In each panel, complexes are identified as follows: black, TFIIB only; blue, TFIIB + pol III; green, GTP, UTP and CTP added to the complex with TFIIB and pol III; red (background), GTP, UTP and CTP added to the complex with pol III and TBP only.

the catalytic center, short abortive transcripts might be produced, even if full-length RNA is not. However, N+1b DNA is also defective in the production of abortive transcripts (data not shown). Addition of GTP, UTP and CTP barely changes the KMnO_4 footprint of the TFIIB-pol III complex with N+1b DNA (Figure 7B).

The N-6b template specifies initiation principally at bp -2 and to a much lesser extent at bp -4 and +1 (Figure 3A). The KMnO_4 footprint of N-6b DNA (Figure 3C) is free of any significant factor-independent pol III background. The footprint of the TFIIB-pol III complex with this DNA is also aberrant, with T-3 and T-5 reactive to KMnO_4 , T-1, 1 bp downstream of the principal site of initiation, barely reactive, and T+2 and T+3 unreactive. Evidently, stable

downstream propagation of promoter opening is impaired by the bottom strand break at bp -6. However, in contrast to the situation with N+1b DNA, transient, NTP-dependent opening of the promoter downstream of bp -2 does occur: full-length as well as abortive transcripts (with GTP and UTP only, or with all four NTPs) are generated in ~2-fold lower yield than with intact duplex DNA (Figure 3A and data not shown); and addition of GTP, UTP and CTP, which allows initiation at bp -2, and RNA chain elongation to bp +7, generates KMnO₄ reactivity for T-5 to T+3 (Figure 3C). [The reactivity of T+3, T+2 and T-1 relative to T-3 and T-5 is somewhat lower with the N-6b DNA-initiated complex (Figure 3C) than with the duplex DNA-initiated complex (Figure 3A). This may reflect the presence of a fraction of pol III that is poised for initiation at bp -4 but unable to do so in the absence of ATP.]

The N-12b template specifies initiation principally at bp -8, -4 and -2, with much smaller contributions from initiation at bp -10 and +1 (Figure 3A). The KMnO₄ footprint of the TFIIB-pol III complex with N-12b DNA qualitatively reflects this distribution of initiation sites (Figure 3D): increased reactivity is conferred principally at T-16 to T-12, T-5 and T-3, with some reactivity at T-17, and only barely increased reactivity at T-1, T+2 and T+3. The N-12b construct generates a minor background of TFIIB-independent pol III binding and strand opening throughout the T₇:A₇ DNA segment. The span of the KMnO₄ footprint from T-17 to T-1 suggests a mixture of complexes opening promoters at overlapping locations, pol III poised for initiation at bp -2 and -4, generating reactivity at T-1, T-3 and T-5 (as for N-6b in Figure 3C), and pol III poised for initiation at bp -8 and -10, generating reactivity at T-12 to T-17. The distribution of reactive Ts would also be compatible with a hyper-extended transcription bubble, reflecting a unique aberrant open promoter complex. Addition of GTP, CTP and UTP substantially changes this complex footprint, with reactivity greatly increasing at T+3, T+2, T-1, T-3 and T-5, increasing slightly at T-12, and diminishing slightly at T-15 and T-16. The appearance of reactivity at T+3, T+2 and T-1 likely reflects initiation and downstream expansion of the transcription bubble by pol III poised for initiation at bp -2, as with construct N-6b. The increase in reactivity at T-3 and T-5 may indicate that pol III poised for initiation with ATP at bp -4 is able to shift 2 bp downstream in the presence of GTP and absence of ATP. The lack of substantial change in reactivity of the T₇ stretch upon addition of GTP, CTP and UTP suggests that pol III poised for initiation with ATP at bp -8 cannot shift downstream for initiation at bp -2 with GTP.

KMnO₄ footprinting with construct N-16b was also examined. This template specifies initiation primarily at bp +1 (Figure 3A) and, accordingly, the KMnO₄ footprints of TFIIB-pol III complexes with N-16b DNA and intact duplex DNA are the same between T-5 and T+3 (data not shown). A significant background of TFIIB-independent DNA opening by pol III alone was observed between T-12 and T-20 of N-16b DNA. However, this opening does not give rise to significant TFIIB-independent transcription (Figure 3A), perhaps due to the absence of a suitable transcriptional start site within the T₇:A₇ segment.

Discussion

Pol III identifies the site at which it initiates transcription on the basis of distance from DNA-bound TBP (Joazeiro *et al.*, 1994). This spacing is not completely fixed (Fruscoloni *et al.*, 1995) because pol III hunts over a small DNA segment ($\pm 1-2$ bp) to locate the -1/+1 pyrimidine-purine signal (in the non-transcribed strand) that specifies RNA 5' ends. Breaking the continuity of the transcribed DNA strand disrupts this measuring mechanism, generating shifts of start site selection that greatly exceed the normally modest range of adjustment. Breaking the transcribed strand in the immediate vicinity of the transcriptional start generates a different aberration—a very strong inhibition of transcription—evidently by removing a constraint that normally holds DNA in contact with the pol III catalytic center (Figure 3A).

The analysis that follows proposes a model that reconciles these findings; the recently determined structures of yeast pol II and of a pol II transcription complex (Cramer *et al.*, 2000; Gnatt *et al.*, 2001) permit the specification of a relatively high level of detail. Previously established facts about pol III transcription are also intrinsic elements of the model.

Chemical cleavage footprinting and cross-linking of the TFIIB-U6 snRNA gene promoter complex defines a downstream edge of the TFIIB footprint at bp -14, with Brf1 in close proximity to the major groove at bp -13/-12 (Kassavetis *et al.*, 1998a, 2001). Addition of pol III extends the MPE-Fe(II) footprint downstream to bp +14 (Kassavetis *et al.*, 2001). MPE-Fe(II) and DNase I footprinting specifies that the upstream edge of TFIIB, and therefore its placement, is not changed by the presence of breaks in the transcribed strand. An unchanged placement of TFIIB implies an unchanged placement of pol III.

Permanganate footprinting of the pol III open promoter complex on the *SUP4* tRNA gene suggests a transcription bubble that spans bp -9 to +7 (Kassavetis *et al.*, 1992), within 1-2 bp of the implied extent of the transcription bubble in the structure of a pol II elongation complex (Gnatt *et al.*, 2001), and with the proposed path of duplex DNA through pol II (Cramer *et al.*, 2001). Thus, we may view the pol II elongation complex as a model for an open pol III-promoter complex primed for initiation.

The dramatic start site shifts that are generated by breaks in the transcribed strand between bp -16 and +5 are not continuous (Figure 3), but are determined also by a requirement for the -1/+1 pyrimidine-purine start site motif already referred to.

Open complex formation by pol III and by bacterial RNA polymerase appears to nucleate at the upstream end of the nascent transcription bubble and expand downstream. One might think that helix destabilization by a nick would facilitate the nucleation of promoter opening at alternative sites, because strand breaks should also increase DNA flexibility. The resulting additional degrees of freedom might generate alternative sites for placement of the transcribed strand in the catalytic site. If any of this were the case, breaks in the non-transcribed and transcribed strands should generate similar effects on start site selection, contrary to our observations (Figure 3B); templates with preformed 5 bp bubbles between bp -14 and

Fig. 8. How DNA breaks in the transcribed strand alter start site selection by pol III. (A) View of the transcribed strand taken from the structure of the pol II elongation complex (Gnatt *et al.*, 2001). The transcribed strand is in red (phosphate in black) from nt -9 to +4 [pyrimidine at +1 specifying the incoming ribonucleotide (yellow) across the catalytic site Mg^{2+} (magenta)]. Atoms of pol II subunits RpbA (green) and RpbB (blue) that come within 3.5 Å of any atom of the transcribed DNA strand are also shown. (B) Diagram of the TFIIB-pol III open complex incorporating features shown in (A). TFIIB (red octagon) anchors pol III (light grey circle) preventing backward and limiting forward translocation in the absence of RNA synthesis. Thick lines representing DNA within the pol III open complex are divided into colored segments to indicate complementarity and position relative to the normal start site of transcription (+1; yellow circle). The transcribed strand is constrained in the complex by non-specific interaction with C128 (light blue) and C160 (light green), compacting an additional 4–5 bp of DNA into the structure to align nt +1 with the catalytic center [initiating ribonucleotide and Mg^{2+} color coded as in (A)]. The non-transcribed strand is not significantly constrained in the complex. The open complex may be in equilibrium with closed or partially closed states. Partial closure of the open complex (C) extrudes downstream duplex DNA, since stable TFIIB-DNA and TFIIB-pol III interactions prevent extrusion upstream. Partial closure at the downstream or upstream edge of the bubble (the latter may also necessitate downstream edge closure) pulls the transcribed strand away from the catalytic site, preventing initiation. The transcribed strand is therefore the conduit through which TFIIB placement specifies the transcriptional start site. Breaks in the transcribed strand within the segment that is melted in the open complex (D) remove the constraint imposed by the continuity of the transcribed strand, allowing both extrusion of downstream DNA and alignment of the transcribed strand with the catalytic site.

+1, should also nucleate promoter opening at new sites, but in fact leave start site selection unchanged (Kassavetis *et al.*, 1999).

The model

The recently determined structure of a pol II elongation complex (Gnatt *et al.*, 2001) identifies transcribed strand residues +4 to -9 as part of the transcription bubble, with residues +1 to -9 base paired to the nascent RNA. The transcribed strand engages the catalytic site as a U-shaped structure, compressing an additional 4–5 nt into protein contact (relative to linear duplex DNA). In the pol II complex, the transcribed strand is held in place by non-specific backbone interactions with Rpb1 at its downstream end (green in Figure 8A) and with Rpb2 upstream

(blue in Figure 8A). These transcribed strand residues are also part of the pol III open complex transcription bubble, and may be anchored in an analogous configuration. (The Rpb2 contacts may not specify the state of an open promoter complex perfectly, as nt -9 to +1 of the transcribed strand are in an RNA-DNA hybrid duplex.) Transcribed strand interactions with pol II and pol III must be weak enough to allow translocation during initiation and transcript elongation (Gnatt *et al.*, 2001). Indeed, DNA downstream of bp +4 is only partially ordered in the structure of the elongation complex, consistent with mobility of the DNA. The non-transcribed strand of the transcription bubble is not resolved and may not be uniquely positioned within pol II (or within pol III). This is also reflected in the complex pattern of pol III-DNA cross-linking with zero-spacer photoreactive nucleotides: five pol III subunits can be cross-linked concurrently to DNA at bp -3/-2 or -10/-9 in the non-transcribed strand of the pol III transcription bubble (Bartholomew *et al.*, 1994). These considerations immediately suggest that the qualitative difference between transcription of templates with top or bottom strand breaks is based on the requirement for anchoring the template strand with respect to the active site, and on a paucity of specific restraints of the non-template strand.

A model reflecting these considerations is presented in Figure 8. The pol III open complex is in temperature-dependent equilibrium with closed and partially closed states (Figure 8B and C). Even fully closed promoter complexes are stable, presumably reflecting primarily TFIIB-pol III interactions (Kassavetis *et al.*, 1992). Full opening of a partially open promoter is accompanied by insertion of additional DNA from the downstream end of the initiation complex (Figure 8C), since upstream TFIIB-pol III interactions restrain forward and backward translocation of polymerase as well as insertion of additional DNA from the upstream side. Partially closed states do not properly align the transcribed strand with the catalytic site, preventing initiation of transcription. This is the principal mechanism that fixes the relationship between the placement of TFIIB and the transcriptional start site. Because the non-transcribed strand is not held tightly by pol III, breaks in it do not alter the effect of partial promoter closing on alignment of the transcribed strand with the catalytic center. Transcribed strand breaks within the bubble or proximal to the bubble relax constraints on the placement of this strand (Figure 8D) because extrusion of downstream DNA no longer prevents engagement of the transcribed strand with the catalytic site. Breaks upstream of +1 allow an upstream shift of the start site without altering the connectivity of pol III with TFIIB; breaks downstream of +1 allow a downstream shift of the start site if they permit further DNA compaction within polymerase (Cheetham and Steitz, 1999).

This model rationalizes the principal effects of DNA strand breaks on transcription by pol III. Transcribed strand breaks between bp -4 and -10 shift the start site to bp -2 and/or -4 (Figure 3A), accompanied by DNA strand closure at bp -1, +2 and +3 (Figure 7C) because they relieve a strain in the transcribed strand that is generated by placing the +1 site at the catalytic center (Figure 8A and B). Breaking the transcribed strand between bp -4 and -10

allows non-specific DNA–polymerase contacts upstream of the break to be maintained, while re-associating 2–4 bp of downstream DNA (normally compacted as single strands) and realigning non-specific transcribed strand–pol III interactions (Figure 8D).

Specific features

More detailed consideration is required to rationalize the following observations: (i) the preponderance of initiation at bp –2 relative to bp –4 and +1 with templates N–4b and N–6b, and persistence of initiation at bp –2 with templates N–8b, N–10b and N–12b (Figure 3A); (ii) inactivity of templates N–2b, N+1b and N+3b, which specify their residual initiation primarily at bp +1; (iii) partial restoration of transcription with template N+5b, specifying initiation at bp +7; (iv) low KMnO₄ reactivity of template N–6b at T–1 on the non-transcribed strand, a site that should be part of the transcription bubble for transcription initiating at bp –2.

Breaks in the transcribed strand shift initiation to bp –2 and –4 because alignment of the template strand with the catalytic center no longer requires the pulling in and compressing of an additional segment of the transcribed strand in order to satisfy the requirement for linked contacts at the catalytic center and, at the upstream end, with TFIIB. In other words, normal initiation of transcription at the +1 site with duplex DNA is the direct result of a constraint imposed by the continuity of the transcribed strand, and becomes energetically unfavorable when that constraint is removed. Persistence of initiation at bp –2 with transcribed strand breaks between bp –12 and –8 indicates that compacting only 2–3 nt of the transcribed strand (instead of 4–5 nt) is not significantly disfavored. Consequently, the existence of additional stabilizing non-specific pol III–transcribed strand interactions upstream of the catalytic site (Figure 8A) provided by aligning nt –2 with the catalytic center explains preferred initiation at bp –2 for templates N–6b and N–4b. For templates N–12b, N–10b and N–8b, retention of initiation at bp –2 stems from these and additional non-specific protein–DNA interactions.

The inactivity of templates N–2b, N+1b and N+3b is explained in a related way. These strand breaks render the 4–5 nt compaction of the transcribed strand unfavorable, and at least 2 bp of downstream DNA that would be strand dissociated in the open promoter complex of continuous DNA remain paired. For N–2b, this should allow the positioning of nt –2 close to the catalytic site, but the vicinal strand break eliminates interactions necessary to maintain the required orientation of nt –2 relative to the catalytic center (Gnatt *et al.*, 2001). The break at +1 disrupts template strand alignment with the catalytic site. The break at +3 may do the same, or lead to abortive initiation. That pol III still initiates predominately at bp +1 with these templates (albeit at a low level and presumably with the initially transcribed RNA continuous across the break in the case of template N+3b) is puzzling. Evidently, the energetically disfavored alignment of bp +1 with the catalytic site provides the residual path to productive transcription in this unfavorable situation.

The re-emergence of transcriptional activity with template N+5b, with initiation shifted to bp +7, might imply additional compaction of the transcribed DNA strand with

pol III, but we favor an alternative interpretation: initiation of transcription at bp +1 with elongation to bp +5, at which step TFIIB–pol III contacts may be partially broken, allowing translocation and realignment of the bubble with *de novo* initiation at bp +7. [Abortive initiation leading to productive initiation has also been proposed as a possible mechanism for moving yeast pol II to bona fide transcription initiation sites located up to 100 bp downstream of the TATA box (Guzman and Lis, 1999).] Analysis of abortive initiation and KMnO₄ footprinting should provide additional insights into initiation at bp +7 with the N+5b template.

In view of the structure of the pol II transcription bubble and the extent of the normal pol III transcription bubble, it is unlikely that T–1 on the non-transcribed strand is base paired when construct N–6b specifies initiation at bp –2. The low permanganate reactivity of T–1 accordingly requires another explanation. We propose that the decompression allowed by the break at position –6b generates an extrusion of 2 bp of downstream duplex DNA, and more effectively buries the separated non-transcribed strand in the cleft between the two largest pol III subunits, limiting accessibility of permanganate ion to T–1 for steric reasons or due to (fortuitous) protein interaction.

Comparisons with prokaryotic RNA polymerases

Transcribed strand breaks generate distinctive effects on transcription by the single-subunit T7 RNA polymerase (Weston *et al.*, 1997; Jiang *et al.*, 2001), and it is instructive to examine how these differences are rationalized in terms of the structure of this enzyme (Cheetham and Steitz, 1999; Cheetham *et al.*, 1999). In particular, a break 5 bp upstream of the normal transcriptional start does not shift the T7 polymerase start site (c.f. Figure 3), but instead eliminates a slippage synthesis of G oligomers that accompanies transcription of duplex DNA. Oligo(G) synthesis has been interpreted as due to a barrier to template strand translocation during the addition of the first few nucleotides by T7 RNA polymerase. Since the T7 promoter-recognition and double-stranded DNA-binding site of T7 RNA polymerase extends to within 5 bp of the transcriptional start (Li *et al.*, 1996; Weston *et al.*, 1997), it is easy to see why the transcriptional start would not shift upstream. On the other hand, early steps of template strand translocation take place without releasing the double-stranded DNA recognition site and instead are proposed to require a compression into a hydrophobic pocket as already referred to (Cheetham and Steitz, 1999). The transcribed strand break circumvents this requirement (Jiang *et al.*, 2001).

The effect of missing nucleosides (gaps) on the ability of *E. coli* RNA polymerase to initiate transcription and form stable elongation complexes has also been examined recently (Levin *et al.*, 2000). Missing nucleosides in the transcribed strand between bp –13 and +11 and in the non-transcribed strand between bp –13 and –4 substantially inhibit formation of elongation complexes. Although this may appear to differ substantially from what is seen with pol III, it is quite possible that much of the difference is due to conditions that limit initiation to +1 with a dinucleotide primer: if downstream DNA extrusion did occur as a consequence of introducing gaps into the transcribed strand, the resulting complexes would require

different dinucleotide primers and would therefore appear to be inactive as assayed. Additional differences probably stem from the interaction of σ^{70} with the non-transcribed strand in the upstream part of the transcription bubble (Marr and Roberts, 1997), which helps to maintain the open complex. If the bacterial RNA polymerase holo-enzyme restrains the non-transcribed strand through its σ subunits, gaps in the non-transcribed strand may also generate DNA extrusion.

Comparison with pol II

Bacterial and archaeal RNA polymerases, and eukaryotic nuclear pol I and pol III, spontaneously open their promoters. In view of this apparent unity of mechanism, and of the extraordinary conservation of structure between eukaryotic and bacterial RNA polymerases, it is striking that pol II instead requires the action of a DNA helicase, the XPB subunit of TFIIF (Bradsher *et al.*, 2000), for promoter opening in linear duplex DNA. Two models for the mechanism of TFIIF-dependent promoter opening have been proposed. The first (Kim *et al.*, 2000) invokes a cranking mechanism in which TFIIF injects negative DNA twist downstream of the start site of transcription through an ATP hydrolysis-dependent process. With the upstream end of the pol II–DNA complex rotationally fixed through its interaction with TFIIB and TBP, the twist deficit translocates to, and initiates the opening of, the transcription bubble. The second model (Douziech *et al.*, 2000) invokes a wrapping and peeling mechanism in which TFIIF tightens the wrapping of DNA around the promoter complex, somehow induces localized DNA unwinding near the start site of transcription and utilizes the XPB helicase to complete ATP hydrolysis-dependent promoter opening.

It has been known for some time that negatively supercoiled DNA circumvents the absolute requirement of TFIIF for promoter opening, allowing pol II, TBP, TFIIB and the RAP30 subunit of TFIIF to initiate transcription accurately at some promoters (Parvin and Sharp, 1993; Tyree *et al.*, 1993). The cranking model implies similar pathways to open initiation complex formation in negatively supercoiled and relaxed or linear DNA. In contrast, a direct involvement of the XPB subunit of TFIIF in separating the DNA strands at the transcriptional start site implies the operation of two different mechanisms in open complex formation by remarkably similar RNA polymerases. Analysis of factor-dependent promoter opening with interrupted-strand DNA templates similar to the analysis presented here should help to discriminate between models of action of TFIIF in pol II promoter opening. In particular, a cranking mechanism would be expected to make promoter opening strongly dependent on the continuity of both strands in the DNA segment between upstream-bound TBP and downstream-bound TFIIF.

A special case

The ability of templates N–12b and N–14b to specify initiation at bp –10 and –8 is perplexing in view of the extension of the transcription bubble 9–10 bp upstream of the transcriptional start, and the juxtaposition of TFIIB and pol III near bp –14 referred to above. Placement of the catalytic center at bp –8 (the major site of initiation for

N–12b and N–14b DNA) should extend the transcription bubble upstream to bp –17, and upstream DNA would be predicted to emerge from pol III near bp –23 (at the downstream edge of the TATA box, a site that is in close contact with TBP). On the contrary, template N–14b does not shift the upstream edge of the TFIIB footprint in the absence of pol III (Figure 5). Evidently, initiation at bp –8 requires disruption of TFIIB–DNA interactions downstream of the TATA box.

The problem is to explain how the simple introduction of a break into the transcribed strand at bp –14 generates such a dramatic effect when, in contrast, a break introduced 2 bp further upstream on the same strand has little effect, and breaks in the non-transcribed in this vicinity have no effect on start site selection. We suspect that initiation at bp –8 (and at bp –10) results from a combination of special circumstances that are specific to the sequence of the *SNR6* promoter. First, breaks at bp –14 and –12 lie within a stretch of seven T:A base pairs proximal to the upstream edge of the normal open transcription bubble (Figure 1A). Weak A:T base pairing, and breaking the continuity of the transcribed strand in proximity to the bubble favor melting of this DNA. (A break located 2 bp further upstream, at bp –16, may be distant enough to diminish bubble expansion greatly.) Secondly, the transcribed strand break and upstream expansion of the bubble relieves compression, and allows the catalytic center to shift upstream relative to the transcribed strand (initially by 2–4 nt). Finally, entry of just 2 bp of upstream DNA sequence into the bubble may sufficiently disrupt TFIIB–DNA interactions downstream of the TATA box to destabilize the TFIIB–DNA complex (Colbert *et al.*, 1998) and set off a cascade of further upstream encroachment by pol III.

Materials and methods

DNA templates and probes

The 198 bp (–60 to +138) pU6_RboxB-derived transcription template and its –9/–5 heteroduplex bubble variant have been described (Kassavetis *et al.*, 1999). Promoter specificity has been verified previously (Whitehall *et al.*, 1995). Interrupted-strand versions of this transcription template were generated by annealing their three purified single-stranded DNA components. Continuous strands and strands extending from the break to bp +138 were produced by PCR amplification with pairs of purified primers, one of which contained a ribonucleotide at its 3'-end. After alkaline hydrolysis at the ribonucleotide, the shortened, amplified DNA strands were isolated on denaturing gels. DNA strands extending from bp –60 to the position of the break were purchased and purified on denaturing gels. DNA strands (all with 5' and 3' OH ends) were annealed at stoichiometric (1:1:1) equivalence (quantified by UV absorbance at neutral pH). Purification of nicked DNA by simple native gel electrophoresis was not always adequate for removing trace amounts of partially annealed and/or unannealed products, which proved to be inhibitory for transcription. Consequently, all nicked DNAs were purified by electrophoresis through native gels with a 1 cm upper zone containing two co-polymerized 5'-acrydite-modified oligonucleotides, each at 10 μ M final concentration. For DNA constructs with an interrupted top strand, the acrydite-modified oligonucleotide sequence corresponded to either nt –59 to –31 of the top strand or nt +135 to +106 of the bottom strand. The complementary acrydite-modified oligonucleotides were used for DNA with an interrupted bottom strand. The contiguous top or bottom strands in the annealing reactions contained ³²P label at their 5' ends (25 c.p.m./fmol) for quantifying recovery. Fully duplex and bottom strand nicked DNA probes for EMSA and footprinting were made by the same procedure with the contiguous top strand 5'-³²P labeled with T4 polynucleotide kinase.

Proteins and nomenclature

The purification and quantification of proteins has been described (Kassavetis *et al.*, 2001). Quantities of pol III are specified as fmol of enzyme active for specific transcription (Kassavetis *et al.*, 1989); quantities of the other proteins are specified as fmol protein. TBP and Bdp1 were estimated to be nearly 100% active and Brf1 20% active in the formation of heparin-resistant TFIIB–DNA complexes. Brf1Δ366–409 is 70% more active than Brf1 in specific transcription.

A common nomenclature newly agreed upon for components of TFIIB and TFIIB-like factors specifies Brf1 as the new designation of the protein previously called Brf, TFIIB70, Pcf4, Tds4 and human TFIIB90; the new name Bdp1 (for B double prime) is specified for the protein previously called B'', Tfc5, TFIIB90, Sfc5 and human TFIIB150. Wild-type genes encoding Brf1 and Bdp1 are designated by the corresponding italicized capital letters (*BRF1*; *BDP1*).

Assays

TFIIB–DNA complexes for transcription, EMSA and footprinting with KMnO₄, MPE-Fe(II) and DNase I were formed for 40 min at 20°C (25°C for KMnO₄ footprinting) in 18 μl of reaction buffer containing 40 mM Tris–HCl pH 8, 7 mM MgCl₂, 3 mM dithiothreitol, 100 μg/ml bovine serum albumin, 5 μg/ml poly(dG–dC)–poly(dG–dC), 6–8% (v/v) glycerol and 80–90 mM NaCl. Transcription reactions contained 30 fmol DNA template, 100 fmol TBP, 120 fmol Bdp1, and 360 fmol Brf1 or 200 fmol Brf1Δ366–409. B'–DNA complexes were assembled for transcription as above, but with 50 mM NaCl and 200 fmol TBP. TBP–DNA complexes were assembled for transcription control assays to match the conditions for the corresponding TFIIB or B' complexes. Reaction mixtures for EMSA and footprinting contained 8–12 fmol DNA probe, 150 fmol TBP, 150 fmol Bdp1 and 540 fmol Brf1. Two microliters of pol III (5 fmol for transcription; 15 fmol for EMSA and footprinting) was added for an additional 20 min. Multiple-round transcription during 30 min was initiated by adding 5 μl of reaction buffer containing 1 mM GTP, ATP and CTP, and 125 μM [α -³²P]UTP (10 c.p.m./fmol). Reactions were stopped, samples were processed for denaturing gel electrophoresis and quantified by phosphor image plate analysis (Kassavetis *et al.*, 1989). Primer extension analysis of unlabeled transcripts followed Kassavetis *et al.* (1999). Analysis of abortive initiation products produced under multiple-round transcription conditions (with 25 μM UTP or CTP as the labeled nucleotide) followed Bhargava and Kassavetis (1999). EMSA, KMnO₄ footprinting and two-dimensional MPE-Fe(II) and DNase I footprinting followed Kassavetis *et al.* (1999, 2001). Two-dimensional footprinting is only compatible with defining the upstream edge of the TFIIB footprint on DNA probes with bottom strand breaks due to loss, during native gel electrophoresis, of DNA fragments that were formed by nicking the non-transcribed strand within 10 bp of the preformed transcribed strand break. Comparison of cleavage patterns of bare DNA (control) and TFIIB DNA complexes is further complicated by partial retention of short TFIIB-bound DNA fragments.

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