Published in final edited form as:

Nat Struct Mol Biol. 2006 July ; 13(7): 603–610.

A DNA-tethered cleavage probe reveals the path for promoter DNA in the yeast preinitiation complex

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Abstract

To directly map the position of promoter DNA within the RNA Polymerase II (Pol II) transcription Preinitiation Complex (PIC), FeBABE was tethered to specific sites within the *HIS4* promoter and used to map exposed surfaces of Pol II and the general transcription factors in proximity to DNA. Our results distinguish between previously proposed models for PIC structure and demonstrate that downstream promoter DNA is positioned over the central cleft of Pol II with DNA upstream of TATA extending toward the Pol II subunit Rpb3. Also mapped were segments of TFIIB, IIE, IIF, and IIH in proximity to promoter DNA. DNA downstream of the transcription bubble maps to a path between the two helicase subdomains of the TFIH subunit Rad25 (XPB). Together, our results show how the general factors and Pol II converge on promoter DNA within the PIC.

Keywords

RNA Pol II; preinitiation complex; hydroxyl radical; general transcription factor

Transcription of protein-coding genes by Pol II requires the recruitment of Pol II and the general transcription factors (GTFs) to promoters to form a PIC¹⁻³, analogous to the Closed Complex state of bacterial RNAP. Prior to transcription initiation, the PIC transitions into the Open Complex state, where ATP and the TFIH helicase subunit XPB promote melting of DNA surrounding the transcription start site and positioning of the template strand of the promoter within the active site cleft of Pol II^{1,4-7}. In metazoans, ~12 base pairs (bp) of DNA are melted in the Open Complex, and transcription typically initiates at a single start site ~30-bp downstream from the TATA element. However, transcription start site selection in *S. cerevisiae* is more complex since initiation occurs between 40–120-bp from TATA⁸. Despite these differences in start site position, the upstream edge of the transcription bubble maps to ~20-bp downstream of TATA both in metazoan systems and *S. cerevisiae*^{7,9-11}. These observations led to the suggestion that yeast Pol II “scans” downstream DNA for a suitable initiation site⁹.

Much insight into the mechanism of Pol II transcription has been gained from crystal structures of yeast Pol II¹²⁻¹⁶. Structural studies have shown that for elongating Pol II, the template DNA strand lies inside the deep central cleft located between Rpb1 and Rpb2, the two largest

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subunits of Pol II^{5,16}. However, the mechanism of how Pol II assembles with the GTFs and promoter DNA in the PIC remains unclear.

Protein-DNA cross-linking studies have provided important insights into PIC structure at the adenovirus major late promoter (AdMLP)¹⁷⁻²². These studies showed that the transcription machinery makes extensive interactions with promoter DNA in minimal PICs assembled from purified Pol II and GTFs. Cross-linking performed after treatment with the detergent Sarkosyl^{19,20} revealed that Rpb1 and Rpb2, the two largest subunits of Pol II, interact with promoter DNA over a 60-bp region. TFIIB and the small subunit of TFIIF (RAP30) both cross-link to DNA upstream and downstream of TATA whereas the large subunit of TFIIF (RAP74) cross-links to DNA downstream of TATA^{19,20}. These studies agree with results obtained using yeast Pol II and a subset of the GTFs at the AdMLP²². Both subunits of human TFIIE cross-link to the promoter region overlapping the transcription bubble region, and the human TFIIF subunit XPB cross-links to DNA downstream of the transcription bubble region¹⁹. However, more extensive cross-links with human TFIIE, TFIIF and TFIIF were detected in the absence of Sarkosyl^{17,18,21}. These observations led to different models for the structure of the PIC, and distinct mechanisms were proposed for promoter melting by the TFIIF helicase^{17,19}.

More recently, separate studies examining TFIIB interaction with Pol II have provided two additional models of PIC structure²³⁻²⁵. X-ray structure analysis of a complex of Pol II and TFIIB²³ showed that the TFIIB B-finger domain projects into the active site cleft and suggested a location for the TFIIB core domain outside of the cleft region near the Pol II dock domain. This led to a model for PIC structure where promoter DNA runs along the outer edge of the Pol II clamp domain below the active site cleft. In a separate study, photo-cross-linking and hydroxyl radical probes were placed on TFIIB and assembled into PICs to map the interaction surfaces between TFIIB and Pol II^{24,25}. These studies showed that in the PIC, the TFIIB core domain is in a location considerably different from that predicted from the Pol II-TFIIB structure. These results led to a model for PIC structure in which promoter DNA is positioned directly over the central cleft of Pol II rather than the outer edge of the clamp.

To distinguish between all these models proposed for PIC structure, we used a strategy to directly establish the path of promoter DNA in the *S. cerevisiae* PIC. The Fe-EDTA probe FeBABE was tethered to specific locations on promoter DNA and hydroxyl radical cleavage was used to map proximity and position of both Pol II and the GTFs relative to the promoter. In contrast to previous DNA-protein cross-linking studies, this approach maps the position of amino acids on proteins that are exposed and in proximity to specific positions of promoter DNA. We find that DNA downstream of TATA is positioned over the central cleft of Pol II and that DNA upstream of TATA bends around the back face of Pol II toward the Pol II subunit Rpb3. This approach also located the surfaces of the general factors TFIIB, TFIIE, TFIIF and TFIIF near to DNA allowing us to distinguish between previously proposed models for PIC structure and helicase function. Finally, our data shows that despite differences in transcription start site between yeast and human Pol II, the yeast PIC assembles at a position equivalent to human Pol II with respect to the TATA element.

Results

FeBABE-tethered *HIS4* templates support *in vitro* transcription

To directly probe the path of promoter DNA in the yeast PIC, the hydroxyl radical generating reagent FeBABE²⁶ was tethered to specific positions on the yeast *HIS4* promoter. FeBABE is ~12 Å in length and has previously been linked to nucleic acids via a phosphorothioate on the nucleic acid backbone²⁷⁻²⁹.

We conjugated FeBABE to the *HIS4* promoter between nucleotides –87 and +1, where +1 indicates the most upstream transcription start site. From alignment of TATA elements, this corresponds to promoter positions –55 to +33 of the AdMLP (Fig. 1a), and is equivalent to the region of the AdMLP used in previous cross-linking studies, allowing a direct comparison between the two systems. FeBABE was conjugated to both the non-template and template DNA strands in clusters of 5 with an estimated conjugation efficiency of 70–80% (Fig. 1a, Supplementary Fig. 1a, and data not shown). In preliminary studies, we found that insertion of a single FeBABE at *HIS4* gave very weak protein cleavage, leading us to construct promoters with multiple FeBABE positions to circumvent this problem. 17 different probes were constructed with FeBABE conjugated to the non-template strand spanning a total of 10 bases (i.e., 1 FeBABE every 2 bases) (Fig. 1a). The 17 probes were numbered from –5 to –1 upstream of TATA and from 1 to 11 downstream of TATA. All 17 probes are essentially identical except for the position of the 5 FeBABEs; each probe is 590-bp, contains the *HIS4* promoter (encompassing regions upstream and downstream of the TATA element including the proposed transcription bubble region and the transcription start site), a cluster of 5 FeBABEs, a single upstream Gal4 binding site and is biotinylated at the 5' end to allow coupling to Dynabeads for *in vitro* transcription and for PIC formation/hydroxyl radical cleavage experiments.

To evaluate the ability of Pol II to transcribe RNA from phosphorothioate-modified and FeBABE-conjugated DNA, *in vitro* transcription assays were performed using immobilized promoter templates (Fig. 1b). Phosphorothioate-modification of promoters showed equivalent activity when compared to a control promoter containing no phosphorothioate residues. However, a ~50% decrease in transcription was observed when FeBABE was conjugated to probe 11, which was not surprising since this covers the transcription start site. Otherwise, conjugating FeBABE to the promoter had little or no effect on the ability of Pol II to transcribe *HIS4*.

Mapping the domains of Pol II close to promoter DNA in the PIC

Hydroxyl radical cleavage was first used to locate the surfaces of Rpb1 and Rpb2 in close proximity to *HIS4* promoter DNA within the PIC. Nuclear extracts containing a triple Flag-tag on either the C terminus of Rpb1 or Rpb2 were used to form PICs on immobilized FeBABE-conjugated *HIS4* templates. These PICs contain not only Pol II and GTFs but also a single dimer of the Gal4-VP16 activator and co-activators such as SAGA, Mediator and NuA4^{30, 31}. After PIC assembly, templates were washed to purify the complexes. Cleavage of peptide bonds near the iron center of the tethered FeBABE was activated by the addition of sodium ascorbate and hydrogen peroxide to generate hydroxyl radicals. Because the hydroxyl radicals diffuse to a limited extent under the conditions used, FeBABE probes for proteins located within ~15–20 Å of the Fe-EDTA center^{24-26,32}. Anti-Flag Western blots were used to visualize the Rpb1 and Rpb2 cleavage products (Figs. 2a, 2b and 3). Compared to a control experiment using *HIS4* promoter DNA with no phosphorothioate residues, specific cleavage fragments were observed using FeBABE-conjugated probes. In Figures 2a and b, additional experiments were carried out with probe 6 in which either FeBABE or hydrogen peroxide was individually omitted.

The sizes of the peptide fragments and their corresponding cleavage sites were calculated using *in vitro*-translated Flag-tagged Rpb1 and Rpb2 peptides as molecular standards²⁴, and the results are summarized in Supplementary Figures 2a-f and Supplementary Table 1. Based on the sizes of the peptide fragments, the Rpb1 cleavage sites are located in the Clamp core, Clamp head and Jaw domains¹⁴ (Figs. 2a and 2b). Rpb1 was cleaved by probes 1 to 8, covering an extensive region of promoter DNA downstream of TATA. Cleavage sites of Rpb2 were located in the protrusion, lobe and fork domains¹⁴ (Fig. 3). Rpb2 was cleaved by probes –3 and –2 and 3 to 6, spanning DNA both upstream and downstream of TATA.

Hydroxyl radical cleavage experiments of Rpb1 and Rpb2 were also carried out with FeBABE conjugated to the promoter template strand (Supplementary Figs. 1a and 1b). Results were nearly identical to those above except that cleavage was not detected in the protrusion domain of Rpb2 from probes 3 and 4 (Supplementary Fig. 1b). The Pol II cleavage sites are PIC-specific. When purified Pol II was subjected to hydroxyl radical cleavage generated by Fe/H₂O₂ in solution, different cleavage sites were observed (data not shown).

Site-specific hydroxyl radical cleavage was also used to probe the proximity of surfaces of the Pol II subunits Rpb3, Rpb4, Rpb5, Rpb7 and Rpb9 to promoter DNA. Of particular interest were Rpb5 and Rpb7 as these subunits had previously been shown to cross-link to the AdMLP^{19,22}. However, no cleavage was detected within any of these Pol II subunits suggesting that these subunits are not near DNA in the yeast PIC. An alternative explanation is that other proteins in the PIC block the access of the hydroxyl radical to these subunits.

Model for the path of promoter DNA in the PIC

The calculated FeBABE cleavage sites for Rpb1 and Rpb2 were mapped on the surface of the X-ray crystal structure of 12 subunit Pol II^{12,13} where strong/medium and weak cleavages are highlighted in blue and cyan, respectively (Fig. 4a). Four residues on either side of the calculated cleavage sites are highlighted to reflect the precision in measurement of cleavage fragments²⁴. Using the cleavage sites of Rpb1 and Rpb2 as a guide, we modeled the path of *HIS4* promoter DNA within the PIC. From the location of the cut sites on the Pol II surface, which are distributed on either side of the central cleft, the promoter DNA downstream of TATA most likely lies outside and runs along the middle of the central cleft as straight B-form DNA (Fig. 4b). Rpb2 was also cleaved by probes placed on DNA upstream of TATA. From the location of the cut sites on the Rpb2 protrusion domain, upstream promoter DNA most likely bends around the back face of Pol II near Rpb2 and extends toward Rpb3 (Fig. 4b). From this predicted path of the promoter DNA, all cleavage sites of Rpb1 and Rpb2 are within the ~30 Å range of FeBABE conjugated to the phosphorothioate group. The observed FeBABE cleavage pattern is not consistent with other proposed models for PIC structure where DNA is proposed to either wrap around Pol II for almost a complete turn^{18,21} or run along the outer edge of the Pol II clamp domain²³. In mammalian cells, melting of the DNA template occurs at -12-bp (-44, yeast numbering), located 19-bp downstream from TATA. In this model position -12 is located directly over the active site cleft (highlighted red/purple in Fig. 4).

The predicted position of promoter DNA is entirely consistent with the model for PIC structure proposed by Chen and Hahn²⁵, based on mapping the position of the TFIIB core domain proximal to the Pol II wall domain using TFIIB-linked FeBABE. The observed path of promoter DNA requires no deviation from B-form DNA other than the bend imposed by TBP. Also shown on these models are TBP (green ribbon) and TFIIB (yellow ribbon).

It is apparent from mapping the sites of cleavage to the Pol II surface that large regions of Rpb1 and Rpb2 are protected from cleavage. As demonstrated below, promoter probes giving rise to Rpb1 and Rpb2 cleavage are also in proximity to the other general transcription factors. A likely explanation for protection of Pol II from more extensive cleavage is that much of the Pol II surface in this region is not exposed and is protected by interaction with other general transcription factors.

Mapping the Rad25 helicase domains close to promoter DNA

DNA-protein cross-linking studies at the AdMLP have reported the positions of promoter DNA near the GTFs. However, these reported locations of TFIIE, TFIIF and TFIIH remain uncertain since two different cross-linking studies obtained distinct cross-linking patterns. For these reasons, we used FeBABE cleavage to locate the surfaces of TFIIE, TFIIF and TFIIH near

promoter DNA. Given that the interactions of TFIIB with promoter DNA, TBP and Pol II have been extensively studied, we first examined the cleavage of TFIIB as a control. FeBABE positioned on either side of TATA (probes –1 and 2 to 4) cleaves TFIIB as expected, in the B-finger, linker and core domains (Supplementary Figs. 3a, 3b, 4a, 4b and 4c; Supplementary Table 1). Cut sites within the core domain correspond to the specific contact points between promoter DNA and the core domain observed in the DNA-TBP-TFIIB crystal structure^{33, 34}.

Cleavage experiments were then carried out for the Rad25 subunit of TFIIF. Yeast TFIIF is composed of 10 subunits including two helicases and a kinase^{1,3}, and helicase activity of the Rad25 subunit (XPB) is essential for open complex formation⁶. The human XPB helicase is the only TFIIF subunit observed to cross-link to promoter DNA and separate cross-linking studies place XPB at different locations on promoter DNA^{17,19}. Moreover, these studies could not unambiguously identify the helicase domain as the DNA proximal domain.

Rad25 in PICs was cleaved by probes 6 to 9, the region of DNA directly downstream of the predicted location of the metazoan transcription bubble^{7,10,11} and 12-bp downstream from the proposed upstream site of the yeast transcription bubble⁹ (Fig. 5a and Supplementary Fig. 5a). The sizes of the peptide fragments and their corresponding cleavage sites were calculated as described above, and the results are summarized in Supplementary Figures 3c and 3d and Supplementary Table 1. Yeast Rad25 (842 amino acids) is a member of the SF-2 family of DNA helicases and cleavage sites map to the DEVH box helicase and the C terminal helicase subdomains of Rad25 (Fig. 5b) showing that in the PIC, the helicase domain is exposed and in proximity to promoter DNA.

Although no structure of Rad25 has yet been obtained, the structure of several other SF-2 family members has been determined³⁵ including the recent structure of *Archaeoglobus fulgidus* XPB³⁶. DNA helicases contain two conserved subdomains, one encompassing the DEX(D/H) box subdomain and the other encompassing a C terminal helicase subdomain, with these two subdomains connected by a linker of variable length. ATP binds between these two subdomains and nucleic acid is observed to lie across the top groove between them. Conformational changes mediated by ATP hydrolysis are thought to induce strain into the bound nucleic acid to promote single strand DNA formation and/or remove secondary structure in single stranded nucleic acids. As described in Supplementary Methods, a structure model for the two Rad25 helicase subdomains (which lie between positions 349 and 714) was generated based on sequence alignment of eukaryotic XPB family members with Af XPB (Supplementary Fig. 6), the structure coordinates of Af XPB, and positioning of these models in the closed conformation by orientation of the subdomains relative to the HCV RNA helicase containing bound RNA^{35,36}.

The FeBABE cleavage sites within Rad25 were mapped on the surface of this structure model (Fig. 5c) where strong/medium and weak cleavages are highlighted in dark blue and cyan, respectively. From the location of the cleavage sites on either side of the central groove between the two helicase subdomains, the order of the probes generating cleavage, and the location of nucleic acid in other helicases of known structure, we predict that DNA downstream of the transcription bubble is likely to lie along the top of Rad25 between the two subdomains in the approximate direction shown by the arrow, where the arrowhead indicates more downstream promoter DNA. In this view shown, ATP would bind at the bottom of the structure model between the two subdomains.

Mapping the domains of the TFIIF subunits near promoter DNA

Yeast TFIIF likely binds to Pol II as a heteromer composed of three subunits, two of which, Tfg1 (RAP74) and Tfg2 (RAP30) are conserved among human, insects and yeast³. The third

yeast subunit Tfg3 is less tightly associated, has no mammalian counterpart, and is also a component of TFIID and the chromatin remodeling complexes RSC and INO80³⁷. The N termini of human Tfg1 and Tfg2 subunits form a dimerization domain, and the C termini of both subunits are winged helix domains³⁸. Cryo-EM has been used to determine the structure of yeast Pol II in complex with TFIIF³⁹, where Tfg2 has been observed to interact extensively with the surface of Pol II along the central cleft.

Both Tfg1 and Tfg2 were cleaved from an extensive region of promoter DNA both upstream and downstream of TATA (probes -4 to 6), in agreement with the cross-linking results of Coulombe and coworkers²¹ (Fig. 6; Supplementary Figs. 3e-h, 5b and c; Supplementary Table 1). The cleavage sites of Tfg1 map to the border of the Tfg2 dimerization domain/unstructured domain and to the middle of the unstructured domain as shown in Figure 6b. Cleavage of Tfg2 maps to the Tfg1 dimerization and the winged helix domains, as shown in Figure 6d.

Mapping the domains of the TFIIE subunits near promoter DNA

TFIIE contains two subunits, Tfa1 (TFIIE α) and Tfa2 (TFIIE β) and is thought to be a heterodimer. TFIIE is required for recruitment of TFIIF and for regulation of TFIIF kinase and helicase activities⁴⁰. Electron crystallography studies place TFIIE near the Pol II active center⁴¹, consistent with the observed cross-linking of IIE to DNA overlapping the transcription bubble region in the PIC¹⁹.

The exposed amino acid surfaces of Tfa1 and Tfa2 (Fig. 7) near promoter DNA within the PIC were located and results are summarized in Supplementary Figs. 3i-l and Supplementary Table 1. Both Tfa1 and Tfa2 were cleaved from a relatively short region of the promoter (probes 2 to 4) just downstream of TATA and overlapping the transcription bubble region, in agreement with cross-linking data from Ebright and coworkers¹⁹ and with EM studies⁴¹. Other cross-linking studies indicating that TFIIE also binds upstream and downstream of the bubble region¹⁸ is not consistent with our findings. Cleavage of Tfa1 maps to the winged helix domain as shown in Figure 7b, while cleavage of Tfa2 maps to the C-terminal region, as shown in Figure 7d. The position of the cut sites in Tfa1 and Tfa2 are consistent with the findings of recent EM studies⁴² that locate the N-terminus of TFIIE α adjacent to the C-terminal third of TFIIE β in the human TFIIE dimer. Since only partial crystal structures are available for both TFIIE and TFIIF, these cleavage sites could not be modeled onto a structure.

Discussion

To distinguish between previously proposed models for PIC structure and to map the regions of the GTFs near promoter DNA in the PIC, we tethered FeBABE at specific locations of the *HIS4* promoter. FeBABE was positioned both upstream and downstream of the TATA box from positions -87 to +1 (-55 to +33, human numbering) and used along with yeast nuclear extracts to form PICs. Activation of FeBABE generated hydroxyl radicals, cleaving exposed regions of polypeptides in close proximity to these promoter positions. Our data provides a model for the path of promoter DNA in the PIC across the surface of Pol II and is clearly compatible with only one previously proposed model for PIC structure. From the locations of the cleavage sites on the Pol II surface, which are distributed among Rpb1 and Rpb2 on either side of the central cleft, the promoter DNA downstream of TATA most likely runs outside of and along the center of the Pol II cleft. DNA upstream of TATA bends around the back face of Pol II near Rpb2, extending towards Rpb3. Our model is entirely consistent with the position of TFIIB in PICs mapped using FeBABE tethered to cysteine residues in the TFIIB core, finger, and ribbon domains^{24,25} but is very different from other proposed models^{18,21,23}. The observed DNA bending around Pol II is in agreement with the DNA bend imposed by the binding of TBP to DNA visualized by X-ray structural analysis^{33,34}.

Positioning of downstream promoter DNA over the Pol II central cleft is in agreement with most of the DNA-protein cross-linking results in the human and yeast systems for Rpb1 and Rpb2. In these studies, extensive cross-linking between promoter DNA downstream of TATA and the Rpb1 and Rpb2 subunits was observed¹⁸⁻²². In contrast, our results also demonstrate that DNA upstream of TATA follows a path near Rpb2 that lies distant from Rpb1. This corresponds to the cross-linking between Rpb2 and upstream DNA observed in previous studies, but not with the weak cross-linking observed to Rpb1^{18,20-22} or to Rpb7²². Previous cross-linking studies utilized either partially assembled PICs or PICs formed from the minimal set of GTFs, in contrast to the studies reported here where the PICs contain Mediator, activator, and other coactivators. Since EM studies of Pol II-Mediator show that Mediator binds to the face of Pol II predicted to interact with upstream promoter DNA, it is possible that Mediator and/or another of these coactivators influences the DNA path in the PIC.

A summary of the hydroxyl radical cleavage results are shown in Figure 8 with specific probes cleaving the GTFs indicated in panel **a**, and results are mapped on the model of the PIC in panels **b-d**. From this summary, it is clear that promoter DNA downstream of TATA is very crowded, being positioned near Pol II, TFIIB, TFIIF, TFIIIE, and TFIIH. Of all the GTFs, TFIIF is in proximity to the most extensive region of promoter DNA, extending both upstream and downstream of TATA. The EM structure of the Pol II-TFIIF complex showed that Tfg2, the small TFIIF subunit, makes extensive contacts with a large surface of Pol II, consistent with our cleavage results. In contrast, the EM studies did not reveal appreciable density of Tfg1 in proximity to DNA. Our results demonstrate that in the PIC, Tfg1 is in proximity to nearly 60-bp of promoter DNA. Perhaps the position of Tfg1 is fixed only in higher order PIC complexes.

The mapped position of TFIIF partially overlaps with that for both subunits of TFIIIE, which lies downstream of TATA and over the site for initiation of the transcription bubble. As TFIIF, TFIIIE, and TFIIB^{23,25} lie in this critical region, they are positioned to play important roles in the mechanism of Open Complex formation, where the double stranded DNA is disrupted and the template strand of DNA is inserted within the Pol II active site. Our results are consistent with the previous proposal that TFIIIE stabilizes single stranded DNA in the Open Complex⁴³ as well as with the findings that Tfg1 and the B-finger region of TFIIB lie in the active site of Pol II in the PIC^{23,25}. Notably, probes 4 and 5, which lie over the transcription bubble, cleave in the unstructured region of Tfg1, a region previously found to play an important role in transcription start site selection^{44,45}. Also, from the position of cleavage of Tfa1 at the N-terminus and of Tfa2 in the C-terminal third, we propose that the dimerization region of TFIIIE⁴² lies over the site for initiation of single stranded DNA formation. Moreover, probes 2 and 3, which cover this DNA region, cleave Tfa2, the small subunit of TFIIIE, in a segment adjacent to the Tfa2 Basic region. This Basic region encompasses the proposed site of single stranded DNA binding⁴³. From these positions, TFIIIE and/or TFIIF may play a role analogous to region 2.3 of bacterial sigma factor, where a conserved hydrophobic residue has been proposed to stack on the exposed downstream face of the base pair forming the upstream edge of the transcription bubble⁴⁶.

HIS4 promoter probes 6–9 all generate cleavage in Rad25, the helicase subunit of TFIIH that is critical for Open Complex formation. The position of Rad25 at this site is consistent with previous cross-linking data placing human XPB downstream of the transcription bubble region at the AdMLP¹⁹. Importantly, hydroxyl radical cleavage of Rad25 demonstrated that the two helicase subdomains were exposed and in proximity to this segment of the promoter. Mapping of these cleavage patterns to a structure model for the two helicase subdomains suggests that in the PIC, the promoter DNA lies in a groove between the subdomains in a manner similar to nucleic acid interacting with other diverse helicases³⁵. This suggests that in the PIC, the Rad25/XPB helicase is poised to induce torsional strain on promoter DNA. Consistent with the model proposed by Ebright and coworkers¹⁹ the helicase would act downstream from the segment

that initially becomes single stranded. An alternative model for XPB was recently suggested⁴⁷ in which the XPB ATPase, but not the DNA helicase activity, is required for Open Complex formation. In this model, the XPB ATPase serves to induce a conformational change in the PIC during Open Complex formation rather than the helicase acting directly to induce strain on DNA. Our findings suggest that the first model is more likely since Rad25 within the PIC seems in position to act on promoter DNA.

Finally, comparison of our results and the results of protein-DNA cross-linking in the human¹⁷⁻²¹ and yeast²² systems demonstrates that PIC formation occurs in a similar location with respect to the TATA element. This similar organization of the PIC is in contrast to the great disparity in transcriptional start sites between *S. cerevisiae* and human Pol II. A model for start site location in yeast, consistent with available data, is that upon ATP addition, Rad25 generates torsional strain in DNA upstream of its binding site. TFIIF and/or TFIIE would next insert residue(s) between double strand promoter DNA to initiate and stabilize single stranded bubble formation. This would be followed by insertion of the single stranded template strand into the Pol II cleft. Perhaps promoted by the helicase activity of Rad25, single stranded template DNA is fed through the Pol II cleft until a suitable transcription start site is encountered. This proposed movement would be somewhat analogous to the scrunching mechanism proposed for T7⁴⁸ and bacterial Pol at initiation. For bacterial Pol, single-molecule studies have shown that long range (>>8-bp) scrunching can occur, suggesting that the initiation complex can accommodate long-range bubble expansion (A. Revyakin, C.Y. Liu, T. Strick, and R. Ebright, Howard Hughes Medical Institute, Rutgers University, Piscataway, NJ, personal communication). In *S. cerevisiae*, this would involve long single-stranded DNA loops protruding from the central cleft while Pol II retained contacts with the GTFs at the promoter. Based on previous genetics and biochemical findings, TFIIB and TFIIF would play a critical role in the transcription start site selection^{44,49-52}. Future studies combining structural analysis, biochemistry and genetics should elucidate the mechanism for transcription start site selection and reveal the basis for the great differences in start site selection shown by yeast and mammalian systems.

Methods

Preparation of phosphorothioate-containing *HIS4* DNA templates

Phosphorothioate-containing templates were prepared by ligating the products of two PCR reactions, where one of the PCR products contains phosphorothioates incorporated at specific locations. This is described in detail in Supplementary Methods.

FeBABE conjugation to phosphorothioate-containing *HIS4* promoters

Conjugation reactions containing 6 mM FeBABE (iron (S)-1-(p-bromoacetamido-benyl) EDTA; Dojindo) and 860 ng phosphorothioate DNA were performed in 20 mM MOPS, pH 7.9. After 16 hours of incubation at 50 °C, the biotinylated phosphorothioate-containing *HIS4* promoters were immobilized to M280 streptavidin-coated dynabeads (Dyna) and excess FeBABE was removed by washing 3 times with 200 μ l of transcription buffer containing 0.05% (w/v) NP-40. FeBABE-tethered probes were directly used in the hydroxyl radical cleavage assay.

In vitro transcription with immobilized templates

Immobilized template assays for *in vitro* transcription were performed as described (www.fhcr.org/labs/hahn). 180 μ g nuclear extract and 48 ng activator Gal4-VP16 were used to assemble PICs on immobilized phosphorothioate-containing DNA templates or FeBABE-conjugated DNA templates (215 ng). After 40 min incubation, transcription was initiated by

addition of 100 mM of each rNTP. Products were mapped by primer extension as previously described³¹.

Hydroxyl Radical Cleavage Assay

Hydroxyl radical probing experiments were performed as previously described²⁴ except that 720 µg yeast nuclear extract, 192 ng Gal4-VP16 activator and 860 ng FeBABE-conjugated *HIS4* promoter immobilized template were used. After PIC formation on immobilized templates, PICs were washed 3 times with 400 µl of transcription buffer containing 0.05% (w/v) NP-40, then Fe-EDTA was activated to generate hydroxyl radicals as previously described²⁴. Cleavage fragments were visualized by anti-Flag Western blot using the LI-COR Bioscience Odyssey Infrared Imaging System.

Determination of the FeBABE Cleavage Sites

The molecular sizes of Rpb1-, Rpb2-, Rad25-, Tfg1-, Tfg2-, Tfa1-, Tfa2- and Sua7-Flag tagged fragments generated by FeBABE cleavage were determined as previously described²⁴ using a set of *in vitro* translated Flag-tagged constructs for each protein. Based on multiple experiments we estimate that the cleavage sites are mapped within 8–10 residues.

Yeast Strains, Nuclear Extracts and Antisera

A complete list is provided in Supplementary Methods.

Co-ordinates of the Rad25 model and of the Pol II-TFIIB-TBP-DNA model are available upon request.

Acknowledgements

We thank R. Ebright for the suggestion of the scrunching mechanism for yeast Pol II, H.T. Chen and L. Warfield for yeast strains and nuclear extracts, and L. Fan and J. Tainer for the coordinates of Af XPB before public release. We thank B. Schmidt and J. Roe for technical advice on efficient conjugation of FeBABE to DNA, and H.T. Chen, N. Mohibullah, B. Moorefield and I. Whitehouse for discussions and for comments on the manuscript. This work was supported by Grant 5R01GM053451 from the National Institutes of Health. G. Miller was supported in part as a postdoctoral associate of the Howard Hughes Medical Institute.

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Supplementary Material

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a **b**

Figure 1. *HIS4* promoter templates tethered to FeBABLE support transcription

(a) 17 DNA-FeBABLE probes were derived from the *HIS4* promoter with FeBABLE attached to the non-template strand. 11 FeBABLE-tethered probes were also derived from *HIS4* with FeBABLE attached to the template strand (Supplementary Fig. 1a). Positions of FeBABLE conjugation are indicated by the red dots. The major sites of transcription initiation at +1 and +12 are indicated by arrows, and the upstream and downstream bases are numbered with respect to +1. For comparison with the results of studies in the human system, the analogous numbering of the adenovirus major late promoter (AdMLP) is shown, based on alignment of TATA sequences and the site of transcription initiation in the human system. The TATA element is highlighted in blue and the position of the transcription bubble region in the human system is highlighted in red. (b) *In vitro* transcription activity using yeast nuclear extract from a subset of these promoters, in the absence (-) or presence (+) of tethered FeBABLE, is shown.

Figure 2. Directed hydroxyl radical probing of RNA Polymerase II subunit Rpb1

(a) Cleavage fragments of Rpb1, triple Flag tagged at the C terminus (Rpb1-Flag) were visualized by Western Blotting with either anti-Flag antibody or **(b)** anti-Rpb1_N200 antibody that recognizes the first 200 residues of Rpb1. All 17 probes and a control probe containing no phosphorothioates (Control) were conjugated to FeBABE and used in PIC formation/hydroxyl radical probing. Negative controls include control +FeBABE, probe 6 -FeBABE +H₂O₂, and probe 6 +FeBABE -H₂O₂. In **(a)** left hand blot, SDS-PAGE was carried out in MOPS NuPAGE buffer (Invitrogen) whereas for the 2 blots on the right hand side, SDS-PAGE was carried out in MES NuPAGE buffer (Invitrogen). In **(b)** cleavage products in the lower molecular weight range are shown with higher sensitivity due to their lower intensity (lower panel). The arrows point to full length Rpb1. Specific cleavage fragments are indicated by brackets and labeled to indicate the region of the protein cleaved. * Indicates non-specific polypeptide detected by antibodies. WT, wild type nuclear extract.

Figure 3. Directed hydroxyl radical probing of RNA Polymerase II subunit Rpb2

Cleavage fragments of Rpb2-Flag were visualized by Western Blotting with anti-Flag antibody as described in Figure 2. The arrow points to full length Rpb2. Specific cleavage fragments are indicated by brackets and labeled to indicate the region of the protein cleaved. P/L, Protrusion/Lobe domain. * Indicates non-specific polypeptides detected by antibodies. WT, wild type nuclear extract.

Figure 4. Model for the path of promoter DNA in the Preinitiation Complex

(a) Left hand panel: Hydroxyl radical cleavage sites of Rpb1 and Rpb2 were mapped to the surface of the complete Pol II crystal structure^{12,13} from data in Figures 2 and 3. Right hand panel: Same as in left hand panel but rotated clockwise by 40° as indicated. (b) A model for the predicted path of *HIS4* promoter DNA within the Preinitiation Complex (two views, rotated by 40°). TBP and TFIIBc were fitted into the complex based on the crystal structure of human TFIIBc-TBP-TATA box complex³³, hydroxyl radical mapping of TFIIB to RNA Pol II²⁵, and based on data from this study (Supplementary Fig. 4). TFIIB and TBP are shown as yellow (magenta sphere, zinc) and green backbone models, respectively. The DNA non-template strand is colored pink, and the template strand is colored light blue. DNA base pair -12

(numbering based on the AdMLP), the site of DNA strand melting, is colored red for the template strand and purple for the non-template strand, respectively.

Figure 5. Hydroxyl radical probing of TFIIH subunit Rad25

(a) FeBABE-DNA cleavage assay in PICs using nuclear extract containing Rad25-Flag. Rad25 cleavage fragments were visualized by Western blotting with anti-Flag antibody. The arrow points to full length Rad25 and specific cleavage fragments are indicated by the brackets and labeled as to which region in the protein is cleaved. * Indicates non-specific polypeptides detected by antibodies. WT, wild type nuclear extract. (b) Schematic indicating Rad25 helicase domains based on sequence alignment (Supplementary Fig. 6) and summary of cleavage sites. DRD, damage recognition domain (c) Hydroxyl radical cleavage sites were mapped to a structure model of the Rad25 helicase domain. A nine-residue segment centered on the calculated site is colored dark blue (strong/medium cleavage) and cyan (weak cleavage). The

red arrow indicates the suggested path of promoter DNA, where DNA close to the transcription bubble is nearer the start of the arrow and downstream DNA (closer to the yeast transcription start site) is nearer the arrowhead.

Figure 6. Directed hydroxyl radical probing of TFIIIF subunits Tfg1 and Tfg2

(a) Cleavage fragments of Tfg1-Flag were visualized by Western blotting with anti-Flag antibody. Arrow points to full length Tfg1 and specific cleavage fragments are indicated by the brackets and labeled as to indicate the region of the protein that is cleaved. C, control probe. * Indicates non-specific bands detected by antibodies. (b) Schematic indicating Tfg1 domains³⁹, arrows indicate cleavage sites. (c) Cleavage fragments of Tfg2-Flag were visualized by western blotting with anti-Flag antibodies, as in (a). (d) Schematic indicating Tfg2 domains³⁹ and arrows indicate the cleavage sites.

Figure 7. Directed hydroxyl radical probing of TFIIIE subunits Tfa1 and Tfa2

(a) Cleavage fragments of Tfa1-Flag were visualized by Western blotting with anti-Flag antibody. Arrow points to full length Tfa1 and specific cleavage fragments are indicated by the brackets and labeled as to indicate the region of the protein that is cleaved. C, control probe. * Indicates non-specific bands detected by antibodies. (b) Schematic indicating Tfa1 domains⁵³ and arrows indicate cleavage sites. (c) Cleavage fragments of Tfa2-Flag were visualized by Western blotting with anti-Flag antibodies, as in (a). (d) Schematic indicating Tfa2 domains and arrows indicate the cleavage sites.

Figure 8. Summary of hydroxyl radical cleavage of Pol II and GTFs within the Preinitiation Complex

(a) Summary of the results of hydroxyl radical cleavage where FeBABLE was tethered to the non-template strand of the *HIS4* promoter. Results of hydroxyl radical cleavage from FeBABLE attached to the template strand are nearly identical. Pol II subunits Rpb1 and Rpb2 are highlighted in blue, TFIIF subunits Tfg1 and Tfg2 are highlighted in orange, TFIIE subunits Tfa1 and Tfa2 are highlighted in purple, TFIIH subunit Rad25 is highlighted in red and TFIIIB is highlighted in yellow. (b-d) DNA probes resulting in cleavage of TFIIF, TFIIE, and Rad25 are color-coded. (b) Left panel: Model of PIC structure (Fig. 4a) where DNA in proximity to Tfg1 is colored orange. Right panel: same as left panel but rotated by 40° as indicated (c) Same

as **(b)** except DNA in proximity to Tfg2 is colored orange. **(d)** Same as **(b)** except DNA in proximity to Rad25 and TFIIIE is colored red and purple respectively. DNA base pair -12 (numbering based on the AdMLP), the site of DNA strand melting, is colored red for the template strand and black for the non-template strand.