

RNA polymerase II senses obstruction in the DNA minor groove via a conserved sensor motif

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RNA polymerase II (pol II) encounters numerous barriers during transcription elongation, including DNA strand breaks, DNA lesions, and nucleosomes. Pyrrole-imidazole (Py-Im) polyamides bind to the minor groove of DNA with programmable sequence specificity and high affinity. Previous studies suggest that Py-Im polyamides can prevent transcription factor binding, as well as interfere with pol II transcription elongation. However, the mechanism of pol II inhibition by Py-Im polyamides is unclear. Here we investigate the mechanism of how these minor-groove binders affect pol II transcription elongation. In the presence of site-specifically bound Py-Im polyamides, we find that the pol II elongation complex becomes arrested immediately upstream of the targeted DNA sequence, and is not rescued by transcription factor IIS, which is in contrast to pol II blockage by a nucleosome barrier. Further analysis reveals that two conserved pol II residues in the Switch 1 region contribute to pol II stalling. Our study suggests this motif in pol II can sense the structural changes of the DNA minor groove and can be considered a “minor groove sensor.” Prolonged interference of transcription elongation by sequence-specific minor groove binders may present opportunities to target transcription addiction for cancer therapy.

Py-Im polyamide | transcription inhibition | minor groove | DNA

In eukaryotes, precursor mRNA synthesis is catalyzed by the RNA polymerase II holoenzyme (pol II), which frequently pauses during transcription elongation (1–3). In addition to regulatory factors that control pol II processivity, various obstacles encountered by pol II can also lead to stalling, and even backtracking, on the DNA template (4). Factors that affect pol II transcription elongation dynamics include intrinsic DNA sequences or structures (5, 6), endogenous epigenetic DNA modifications (7), embedded ribonucleotides (8), DNA lesions (9, 10), small-molecule DNA-binders (11, 12), DNA-binding proteins including nucleosomes (13), and even pol II itself (14, 15). Transient transcriptional pausing or short-lived transcriptional blockage can be rescued by the recruitment of transcription factor IIS (TFIIS) (16, 17), a transcription factor that facilitates the cleavage of backtracked transcript. In contrast, prolonged transcriptional arrest by some bulky DNA lesions triggers either ubiquitination and degradation of pol II, or transcription-coupled nucleotide excision repair, a special DNA repair pathway that preferentially repairs DNA lesions in the transcribed strand (17, 18). Structural, genetic, and biochemical studies have greatly advanced our understanding of how pol II copes with different kinds of covalent DNA lesions caused by oxidation (19, 20), alkylation (21–24), and photocyclo-addition (10). Less well understood are the interactions of the pol II machinery when confronted with a steric blockage by small molecules bound noncovalently in the minor groove of DNA (25–27).

Pyrrole-imidazole (Py-Im) polyamides are a class of small molecules that can be programmed to selectively target specific DNA sequences in the minor groove with high binding affinity (28, 29). This sequence-specificity arises from unique pairs of aromatic amino acids, which distinguish the edges of the four Watson–Crick

base pairs (30, 31). Eight-ring hairpin oligomers linked by a central aliphatic γ -aminobutyric acid unit have binding affinity comparable to TFs (32). Early studies suggested that cell-permeable Py-Im polyamides modulate gene expression by targeting TF binding (25, 33, 34). Importantly, inhibition of pol II occupancy at gene start sites leading to Rpb1 degradation has been observed (35). The question arises as to how polyamide occupancy in the DNA minor groove prevents pol II elongation. Do Py-Im polyamides pose a direct impediment to pol II elongation and for how long? In this study, we investigate the mechanism of how pol II transcription elongation is affected by Py-Im polyamide binding at a discrete site downstream from the pol II active site. From molecular modeling, a conserved pol II motif appears to function as a minor groove sensor and could be responsible for pol II elongation inhibition. This finding was verified by site-specific mutations of this region in pol II. These results suggest direct interaction between residues of the conserved motif and the bound Py-Im polyamides. In short, we have identified a sensor function of a conserved motif in pol II.

Results

Py-Im Polyamides Are Roadblocks for Pol II Transcription Elongation.

To investigate the impact of a site-specific DNA-binding small molecule on pol II transcription elongation, we assembled a purified pol II elongation complex with a DNA scaffold (78 bp in

Significance

Transcription addiction is a hallmark of cancer and a potential therapeutic target. RNA polymerase II (pol II) is responsible for synthesizing precursor mRNA in all eukaryotic cells and can be blocked by obstacles, such as DNA lesions and nucleosomes on the DNA template. In this study, we demonstrate that sequence-specific minor groove binding pyrrole-imidazole polyamides can sterically block an elongating polymerase at the targeted binding site. We find this blockage is persistent and cannot be rescued by transcription factor IIS. We further show pyrrole-imidazole polyamides are detected in the minor groove via two conserved residues in the Switch 1 region of pol II. Collectively, these results provide mechanistic insights on how a noncovalent minor groove binder can obstruct pol II elongation.

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length) containing a full transcription bubble upstream and a specific 6-bp binding site downstream (59 bp from the scaffold end). The 6-bp site is bound specifically by Py-Im polyamides **1–4** that code for 5'-WGGWCW-3' according to the pairing rules (Fig. 1). To address the potential differences of hairpin Py-Im polyamide binding orientation relative to transcriptional directionality (upper black arrow in Fig. 1*B*), we also designed a second scaffold with the downstream polyamide binding site in an opposite orientation (upper black arrow in Fig. 1*C*). We termed the orientation in which the pol II encounters the γ -turn moiety of the hairpin Py-Im polyamide first (Fig. 1*B*) the “template strand (TS) binding orientation,” and the reverse orientation where pol II would encounter the “C-terminal linker” first the “nontemplate strand (NTS) binding orientation” (Fig. 1*C*).

Substitutions on the γ -turn or the C terminus of Py-Im polyamides influence DNA affinity as well as cellular uptake (36). To evaluate the effects of these substitutions on pol II transcription elongation, we compared Py-Im polyamides **1–4** (Fig. 1*A*). Hairpins **1**, **2**, and **3** have identical C terminus and vary on the γ -turn, where **1** has a chiral (R)- α -amino group, **2** has an acetylated (R)- α -amino group, and **3** has a (R)- β -amino substitution. Compound **4** is a cycle flanked by the same β -amino γ -turn as **3** and an unsubstituted γ -turn.

All four Py-Im polyamides (**1–4**) block RNA pol II transcription elongation at specific positions upstream of their binding

sites in both the TS and NTS orientations (Fig. 1*B* and *C*), whereas RNA pol II can transcribe through the DNA template to obtain full-length RNA transcripts in the absence of Py-Im polyamide treatment (see control lanes in Fig. 1*B* and *C*). Importantly, pol II cannot bypass the polyamide blockage even after prolonged incubation (2–20 h) (Fig. 1*B* and *C* and Fig. S1), implying that binding of Py-Im polyamides can induce prolonged arrest of pol II at the transcription elongation phase in a sequence-specific manner. To exclude the possibilities that pol II could fall off from DNA, or RNA could be released during the long time incubation in the presence of polyamides, we performed a native gel analysis to test stability of the polyamide-arrested pol II complex and we observed that the complex is very stable and no obvious RNA transcripts are released (Fig. S1*C*). To determine the stability of the polyamide-arrested pol II complex (without RNA transcript release) and whether transcription can recover upon the removal of polyamide, we designed competition experiments to remove the polyamide from the elongation complex by addition of competitor duplex DNA with the same binding site, and found that the elongation can be slowly recovered (18 h) and reach the full transcripts in the presence of high concentration of competitor duplex DNA (Fig. S1*D*). We found that the arrested region generally has two to four pausing bands that are located 2–5 bp upstream of the 5'-WGGWCW-3' binding site (see the dashed box on the right of the gel image in Fig. 1*B* and *C*, named

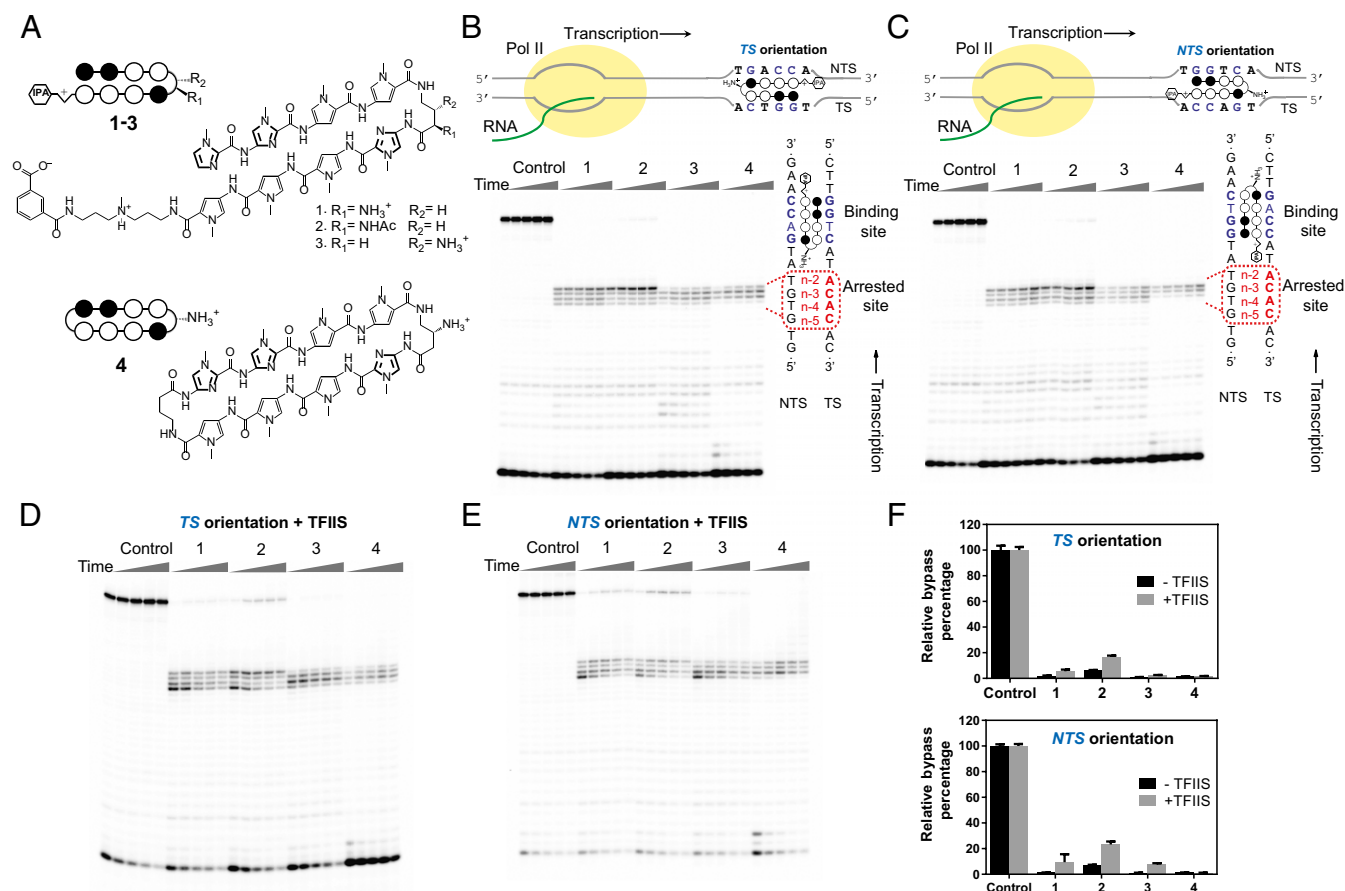


Fig. 1. Py-Im polyamides are strong roadblocks for RNA pol II transcription elongation. (A) Four Py-Im polyamides share the same 6-bp DNA target sequence (5'-WGGWCW-3'). The black solid circle refers to *N*-methylimidazole (Im), and the hollow circle refers to *N*-methylpyrrole (Py). (B) RNA pol II transcription blockage by Py-Im polyamides with a TS binding orientation and (C) with a NTS binding orientation. (D and E) PAGE gel analysis of TFIIS effect on Py-Im polyamide induced pol II arrest. The Py-Im polyamide binds DNA in a TS orientation (D) or NTS orientation (E). In these gel images, control lanes are RNA pol II transcription in the absence of Py-Im polyamides. Time points for transcription assays were 10 min, 20 min, 30 min, 1 h, and 2 h. The concentration of Py-Im polyamides was 0.5 μM . (F) Quantitative analysis of TFIIS effect on rescuing Py-Im polyamide-induced pol II arrest.

the n-2, n-3, n-4, and n-5 bands, from top to bottom). These results reveal that pol II is able to sense the barrier and becomes arrested several base pairs upstream of the bound Py-Im polyamide.

Quantitative analysis of the pol II pausing bands reveals specific patterns of polyamide-induced pol II arrest. The pattern of pausing bands depends on the chemical nature of the polyamides, the concentration of the compounds, the binding orientations, and the incubation time (Fig. 1 and Figs. S1–S5). In the TS binding orientation, where the γ -turn faces toward oncoming pol II, acetylation of the α -amine group in hairpin 2 causes more dominant pausing at the n-2 site than hairpin 1 (Fig. 1 and Fig. S1). Interestingly, moving the amine group into the β -position in hairpin 3 causes the major pausing sites to shift to n-3 and n-4 sites (Fig. 1 and Fig. S1). Finally, cycle 4 shares a similar pattern with hairpin 3 as might be expected, with a minor increase of the n-2 site with prolonged incubation (Fig. S1). In the NTS binding orientations where the C terminus faces toward the oncoming pol II, hairpins 1, 2, and 3 show similar stall patterns, which is consistent with their identical C-terminus moieties. In contrast, cycle 4 only has two major pausing sites (n-2 and n-3) and one minor pausing site (n-4), consistent with a shorter γ -turn facing pol II rather than a longer C-terminus linker. Despite the subtle pattern differences, both binding orientations had a common pausing region (n-2 to n-5) relative to the Py-Im polyamide binding site.

To further characterize the strength of Py-Im polyamides as inhibitors of pol II elongation, we performed concentration-dependent pol II inhibition analysis for the four polyamides 1–4 in both binding orientations (Figs. S2–S5). The full-length pol II transcripts are diminished and the arrested bands accumulate as polyamide concentration increases (Figs. S2–S5). To quantitatively evaluate the concentration-dependent inhibition data, we systematically determined the inhibition parameter K_i (the concentration of ligand that can inhibit 50% of pol II elongation) (see *SI Materials and Methods* for details) for each Py-Im polyamide and binding orientation (Table 1 and Figs. S2–S5). For each polyamide, the NTS binding orientation generally leads to a stronger inhibition effect than the TS binding orientation, suggesting an asymmetric inhibition of pol II transcription elongation. The K_i concentrations did not correlate with the binding affinity of these polyamides observed from duplex melting experiments (Table S1). A comparison of these four ligands reveals that 2 and 4 have the strongest inhibition. Comparison between hairpins 1, 2, and 3 clearly shows the influence of substitutions on the γ -turn, which faces the approaching enzyme in the TS binding mode. Acetylation of the γ -turn α -amine group in 2, which decreases DNA binding affinity, increases the inhibition ability by ~threefold, whereas moving the α -amine group to the β -position (3) decreases the inhibition ability by ~threefold. Interestingly, cyclization of 4 greatly increases the inhibition ability by ~16-fold over 3. Although modifications in the γ -turn and the C terminus can modulate the inhibition abilities of these molecules, all four molecules cause strong inhibition of pol II elongation with K_i values at nanomolar concentrations.

TFIIS Is Insufficient to Fully Rescue Py-Im Polyamide-Arrested Pol II.

Transcription elongation factor TFIIS can rescue arrested pol II and enhance pol II bypass of a variety of translocation barriers, such as nucleosomes, some DNA lesions, small-molecule DNA binders, and intrinsic pausing DNA sequences (12, 13, 16, 37).

Table 1. Concentration of Py-Im polyamides for 50% inhibition of RNA pol II bypass (K_i , nM)

Orientation	1	2	3	4
TS	50 ± 10	~17 ± 2	160 ± 30	~10 ± 2
NTS	45 ± 12	<5	63 ± 17	~8 ± 2

To investigate whether TFIIS can stimulate pol II bypass of polyamide-induced pol II arrest, we performed transcription elongation experiments in the absence or presence of TFIIS. As shown in Fig. 1 *D* and *E*, the majority of pol II remains arrested by Py-Im polyamides even in the presence of TFIIS, regardless of the binding orientation (TS or NTS), with only a small portion of transcriptional bypass observed for hairpins 1 and 2. These results indicate that TFIIS fails to effectively rescue polyamide-induced pol II arrest. The presence of TFIIS altered the patterns of the pausing bands, as the major arrested bands are shifted toward the upstream positions (Fig. 1 *D* and *E*, n-4 or n-5 positions). This finding suggests that pol II may adopt pretranslocation or backtracked states during Py-Im polyamide-induced arrest. TFIIS facilitates cleavage of short RNA oligos from the 3'-end of RNA transcripts, leading to the shortened transcripts and shifted pausing patterns.

Quantitative comparison of bypass percentage in the absence (Fig. 1 *B* and *C*) and presence of TFIIS (Fig. 1 *D* and *E*) reveals distinct responses to TFIIS among the four polyamides (Fig. 1*F*). For example, the lowest-affinity hairpin 2 is more sensitive to TFIIS treatment with ~12% transcript bypass of the blockage and ~88% arrest in the TS binding orientation. In contrast, the higher-affinity cycle 4 is resistant to TFIIS treatment. We observed no transcriptional bypass in the presence of TFIIS even after 2-h incubation. Similar TFIIS sensitivity profiles were also observed for NTS binding orientation, with cycle 4 most resistant to TFIIS treatment (Fig. 1*F*). These results demonstrate that TFIIS is insufficient to rescue Py-Im polyamide-induced pol II transcriptional arrest, indicating that Py-Im polyamide-induced transcriptional blockage is strong and stable. This finding is in contrast with other translocation barriers, such as nucleosomes or other small-molecule binders (Distamycin and DAPI) (12, 13).

Sequence-Specific RNA Pol II Transcriptional Blockage by Py-Im Polyamides.

Our results support the DNA target specificity of Py-Im polyamides (32), as evidenced by these polyamides only causing pol II stalling specifically 2–5 bp upstream of the match binding site but not in other sites in the template DNA. To further directly verify that pol II transcription stalling by polyamides is indeed a result of its discrete binding to the 5'-WGGWCW-3' sequence, we compared the transcriptional inhibitory effect of hairpin 1 upon its binding to DNA with a perfectly matched (PM) sequence (5'-TGGTCA-3') or with a single mismatched (MM) target sequence (5'-TGTTC^uCA-3') (single mutation underlined) (Fig. 2*A* and Fig. S6). As shown in Fig. 2, a single mutation in the binding sequence (MM) abolished Py-Im polyamide-induced pol II transcriptional arrest in the TS binding mode (both in the presence and absence of TFIIS). A similar effect is observed for the scaffold containing a single mutation in the NTS binding orientation (Fig. S6). Furthermore, DNA duplex melting experiments confirm that a single mutation significantly diminishes ligand binding affinity (Table S1), consistent with transcriptional inhibition results. These results demonstrate that polyamide-induced pol II transcriptional arrest is dependent upon the matched DNA sequence in both directions.

Structural Insight into Py-Im Polyamide-Induced Pol II Arrest Revealed by Molecular Modeling.

To gain further mechanistic understanding of how pol II stalls upstream of the polyamide binding sites (2–5 bp), we modeled a series of pol II-arrested complexes in the presence of Py-Im polyamide binding to mimic pol II progression into the pausing region in a stepwise manner. The models are built based on the superposition of crystal structures of the downstream DNA duplex of pol II elongation complex (PDB ID code 3M3Y) (23) with the structure of the polyamide bound DNA duplex (PDB ID code 3I5L) (31). Here we modeled each of the four major pausing states observed in our biochemical studies. Details for model set-up can be found in *SI Materials and Methods*.

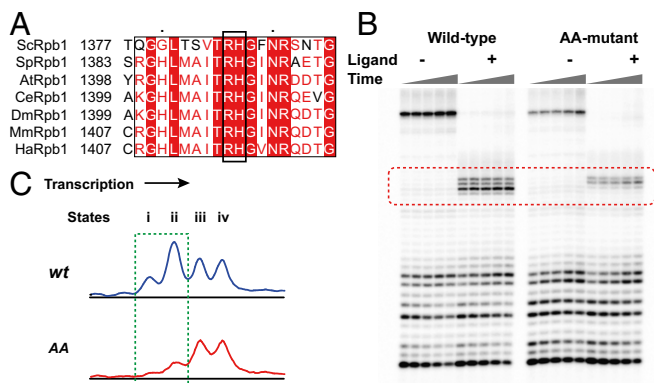


Fig. 4. Effects of Py-Im polyamide on the elongation of wild-type and AA-mutant are distinct. (A) Sequence alignment of Switch 1 region of Rpb1 of RNA pol II in different species. (B) Gel data of transcriptional elongation of the wild-type and AA-mutant. Time points were 10 min, 20 min, 30 min, 1 h, and 2 h. The different pausing patterns in the presence of 0.5 μ M Py-Im polyamide **1** are highlighted in the red dashed box. (C) Quantitative analysis of pausing patterns between the wild-type and AA-mutant. Different intensity peaks refer to different pausing states as described in Fig. 3. The major difference was highlighted in the green dashed box.

difference between the wild-type and AA-mutant confirmed the inspective role of Arg1386 and His1387 of the Rpb1 switch 1 motif in sensing the DNA minor groove before duplex unwinding. In addition, it is worth mentioning that compared with the wild-type pol II, the AA-mutant has a relatively strong sequence pausing effect during elongation (see the bottom part of gel in Fig. 4B), which may indicate possible roles of these two residues during normal transcriptional progression.

Discussion

Minor-Groove Sensor Motif Signals Transcription Pause and Blockage.

Although extensive mechanistic studies have been focused on how pol II recognizes and processes covalent DNA lesions (such as UV damage), or bypasses nucleosome barriers, less is known about the molecular mechanism of how pol II processes non-covalent DNA-binding small molecules. In this study, we investigated the effect of site-specific binding by Py-Im polyamides on pol II elongation in two binding orientations using biochemical assays, modeling, and mutation studies.

Our data from these studies revealed that an elongating pol II can sense polyamide binding, and pause as a stable complex immediately upstream of the binding site for over 20 h. This event is sequence-specific, where a single base pair mutation in the polyamide target sequence leads to the abolition of pol II arrest (Fig. 2). We further show the persistent pol II arrest induced by Py-Im polyamides cannot be rescued by TFIIS. This finding is remarkable given the fact that pol II itself has a strong capability to unwind the downstream DNA duplex to form the transcription bubble. This result is also in sharp contrast with the fact that TFIIS is able to rescue nucleosome-induced pol II arrest and a variety of pol II pausing/arrest events (13, 16, 40), considering that the binding affinity of histone–DNA interaction is very strong, within the similar nanomolar range as polyamides (41, 42). This observation may be explained by the kinetics of Py-Im polyamide DNA binding; the rate of dissociation is slow, comparable to DNA binding proteins, but the rate of association is much faster (43). Hence, the resistance to the effect of TFIIS may be attributed to the long-lifetime bound state of Py-Im polyamides during transcriptional elongation. These results support the idea that polyamides can block pol II elongation in a sequence-specific manner.

Furthermore, we find that substitutions of the functional moieties of Py-Im polyamides cause varied inhibitory potencies

toward pol II elongation with K_i values in the 1- to 100-nM range (Table 1). Intriguingly, the magnitude of inhibitory effects does not correlate directly with the polyamides' DNA binding affinities. This difference may reflect the difference between the aqueous solution where DNA binding affinity is measured and the amino acid-rich environment of the pol II downstream main channel where transcription inhibition is measured (Fig. S7).

Additionally, our results revealed that pol II senses polyamide binding in the minor groove via its conserved Switch 1 region and becomes stalled 2–5 bp upstream. The early polyamide-induced blockage of pol II elongation (n-4, n-5) is because of a steric clash with minor groove residues Arg1386 and His1387 during transcriptional progression toward the binding site (before formation of the transcriptional bubble), whereas the latter arrest (n-2, n-3) is attributed by direct prevention of DNA unwinding (Figs. 3 and 4 and Fig. S7).

Biological Implications and Consequences. Unlike DNA damaging agents, such as cisplatin, that covalently modify DNA in a global manner, Py-Im polyamides bind to the DNA minor groove noncovalently in a sequence-specific manner. Previous studies have indicated that polyamides can inhibit pol II elongation and trigger the degradation of Rpb1 *in vivo* (35). Our studies provide a mechanistic explanation of these observations. We found that polyamide-induced pol II arrest is stable for hours and cannot be rescued by TFIIS. Such prolonged stalled pol II is likely a target for ubiquitination and degradation in a similar manner to pol II stalled by cisplatin-induced DNA damage, and subsequent apoptosis (44, 45). The molecular processing by pol II transcription machinery may provide important insights into the understanding of how cells respond to this kind of noncovalent DNA minor groove binder *in vivo*. Given the potent sequence-specific inhibition by polyamide interference, and the demonstrated antitumor activity in xenografts with low host toxicity, it would be very attractive to consider whether there is a role in cancer therapy for these molecules. Transcription inhibition has been explored as a therapeutic strategy in cancer treatment (46–49). Inhibition of a ubiquitous and essential pathway in cancer could overcome, in part, the drug resistance conferred through the genetic heterogeneity of the disease. Indeed, a recent study has shown that many cancers have a concomitant hemizygous loss of *POLR2A* (*RPB1*) and p53, rendering them more vulnerable to transcription inhibition (49). Development of small molecules that selectively obstruct the pol II transcription machinery in a sequence-specific manner would present a therapeutic strategy to target transcription addiction in cancer cells.

Finally, this study reveals a sensor function of the Switch 1 region to detect downstream DNA minor groove variations during pol II elongation. A highly conserved motif in pol II (Arg1386 and His1387 of Rpb1) has an inspective role within the DNA minor groove and can sense DNA minor groove binders. During transcription elongation, this motif likely interrogates the downstream duplex DNA before the formation of the transcription bubble. This motif and our previously identified major groove “epi-loop” (50) both exhibit DNA-sensing roles. Working together, these motifs can detect abnormalities in the downstream DNA template from both the major and minor grooves, which may play important roles in terms of controlling transcriptional fidelity and DNA damage recognition. Future studies may aim to reveal the function roles of this motif in the normal pol II transcription process, which may possibly unveil new insights into RNA pol II transcription regulation.

Materials and Methods

RNA Pol II Purification and *In Vitro* Transcription Assay. Wild-type RNA pol II was purified from *Saccharomyces cerevisiae* as previously described, unless otherwise stated (51, 52). For the transcription inhibition assay, the RNA/DNA scaffold is preformed and incubated with various Py-Im polyamides for 3 h before the addition of pol II.

DNA Template Design. DNA sequences of template strand used for TS and NTS binding orientation were 5'-GACTCTTCTGACTTGGTCATACACACTTCTCTCTCGTTGTTCTCTCGATTGTTAAGTGATGTGCTGTTGTAAT-3' and 5'-GACTCTTCTGACTTGGTCATACACACTTCTCTCTCGTTGTTAAGTGATGTGCTGTTGTAAT-3' (1–4 binding site is underlined).

Py-Im Polyamide Synthesis. Compounds 1–4 were synthesized on solid support as previously described (53–55). All compounds were purified by RP-HPLC and correct masses were verified by MALDI-TOF.

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