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When transcription goes on Holliday: double Holliday junctions block RNA polymerase II transcription *in vitro*

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Abstract

Non-canonical DNA structures can obstruct transcription. This transcription blockage could have various biological consequences, including genomic instability and gratuitous transcription-coupled repair. Among potential structures causing transcription blockage are Holliday junctions (HJ), which can be generated as intermediates in homologous recombination or during processing of stalled replication forks. Of particular interest is the double Holliday junction (DHJ), which contains two HJs. Topological considerations impose the constraint that the total number of helical turns in the DNA duplexes between the junctions cannot be altered as long as the flanking DNA duplexes are intact. Thus, the DHJ structure should strongly resist transient unwinding during transcription; consequently, it is predicted to cause significantly stronger blockage than single HJ structures. The patterns of transcription blockage obtained for RNA polymerase II transcription in HeLa cell nuclear extracts were in accordance with this prediction. However, we did not detect transcription blockage with purified T7 phage RNA polymerase; we discuss a possible explanation for this difference. In general, our findings implicate naturally occurring Holliday junctions in transcription arrest.

Graphical abstract

1. INTRODUCTION

Unusual nucleotide sequences and consequent non-canonical DNA structures often present obstacles for essential DNA transactions including transcription (reviewed in [1–3]).

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Conflict of interest

We declare no conflicts of interest.

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Transcription blockage by these structures not only affects gene expression but they may be perceived as damage and become substrates for unnecessary repair events involving elements of the transcription-coupled repair (TCR) machinery, which specifically recognizes stalled RNA polymerases (RNAP) as a signal to trigger local repair processes ([4, 5], reviewed in [6–8]). This “misdirected” repair (termed “gratuitous TCR” [6]) could lead to genomic instability, through mutations, nucleotide deletions and expansions.

Important examples of gratuitous TCR may include the polyglutamine-related neurodegenerative diseases [9]. A model has been proposed to explain how a long stretch of CTG/CAG triplet repeats might form slipped-strand structures during the passage of RNAP and then arrest the following RNAP. If the arrested RNAP initiates a repair event that includes a DNA break in the transcribed strand then either expansion or contraction could result, depending upon the locations of the slipped-strand structures. This example provides an underlying mechanism for the initiation of a gratuitous TCR event that could lead to a change in repeat copy number [10, 11]. In accordance with this model, significant transcription blockage by slipped-strand structures was observed in HeLa cell nuclear extracts [12].

The present study focuses on another family of unusual DNA structures that could interfere with transcription: single and double Holliday junctions (HJ and DHJ, respectively). These structures play important roles in numerous DNA transactions, e.g. as intermediates for homologous recombination, double-strand break repair, and the processing of stalled replication forks (reviewed in [13–15]). In addition, branched DNA structures similar to HJ could transiently appear during final stages of genomic replication when different replisomes converge [16].

Since the above-mentioned processes could occur simultaneously with transcription on the same DNA template (in particular, co-occurrence of DNA replication and transcription is well-documented (reviewed in [17, 18])), it is important to learn what happens when a transcribing RNAP encounters a Holliday junction. This question is particularly interesting in the case of the DHJ. Topological considerations impose the constraint that the total number of helical turns in the DNA duplexes between the junctions (further referred to as “internal duplexes”) cannot be altered as long as the flanking DNA duplexes are base-paired [19] (Fig. 1). (More rigorously, the topological parameter that cannot be altered in this structure is the “linking number” (reviewed in [20]), rather than “the number of helical turns.” However, if we do not consider the three-dimensional re-arrangement of the internal duplexes upon overwinding, we can visualize the linking number as the number of helical turns within the duplexes.)

From the above consideration of a DHJ, it follows that local unwinding within one or both of the internal duplexes created by transcribing RNAP would lead to compensatory energetically unfavorable overwinding within the internal duplexes (Fig. 2B, C). This creates an apparent force acting against RNAP entering the DHJ region, thus interfering with transcription. Note that in the case of a natural “mobile” (i.e. capable of branch-migration) HJ, an RNAP could “push” the DHJ in front of it instead of entering the DHJ region (Fig. 2D). In this case, the two HJs within the DHJ would branch-migrate simultaneously, keeping

the winding of the internal duplex regions constant, thus avoiding topological strain within the DHJ. However, such “pushing” is also likely to create an impediment for transcription because each step in branch-migration is associated with overcoming some energetic barrier [21]. Additionally, for sufficiently long DNAs, the viscous resistance to DNA rotation upon directed HJ movement might also be substantial. Of course, topological stress within the DNA *in vivo* would eventually be resolved by topoisomerases; but, before this happens, the transcription impediment by topological constraints could trigger important biological effects.

In this work, we have studied transcription encounters with various HJ-containing structures, including the DHJ, using a simplified model system with stable immobilized HJs. Since no publications have yet dealt with the encounter of transcription and HJs, an analysis of this interaction will augment our understanding of RNAP behavior at this potential, naturally-occurring obstacle.

2. MATERIALS AND METHODS

2.1. DNA substrates

DNA substrates were obtained by ligating a promoter-containing fragment to a structure of interest (e.g. DHJ) assembled from synthetic single-stranded DNA oligonucleotides (Figure 3). Two different promoter fragments were used: one with the T7 RNAP promoter and the other with the RNAPII cytomegalovirus (CMV) promoter. The T7 RNAP fragment was prepared via BamHI and BsmFI (New England Biolabs [NEB]) restriction of the pBluescript SK(+) plasmid as described in [12]. The RNAPII promoter fragment was prepared via EcoRI and BseRI (NEB) restriction of the pCMV β plasmid (Clontech) as described in [22]. The restricted promoter-containing fragments were then purified by agarose gel electrophoresis as described previously [12].

Gel-purified single-stranded DNA oligonucleotides were purchased from Integrated DNA Technologies. The oligonucleotide sequences within the assembled structures are shown in Fig. S1.

The DHJ was assembled in two steps (Fig. 3). In the first step, two sets of single-stranded oligonucleotides were hybridized (each set in separate test tubes) to form the internal duplexes with non-complementary “tails.” The mixture containing 4 μ M of the each oligonucleotide, 10 mM Tris-HCl and 10 mM MgCl₂ was incubated for 10 min at 65°C and then 3 h at 37°C. In the second step, the products of the first step were mixed together to allow their “tails” to hybridize to form the flanking duplexes. This step was performed for 3 h at 37°C. Other structures were obtained in a similar manner.

To characterize the assembled DNA structures, they were radioactively end-labeled in a total volume of 15 μ L containing: 2 μ M of DNA substrates, 10 μ Ci of [γ ³²P]-ATP, and 8 units of T4 polynucleotide kinase (Invitrogen) in the forward reaction buffer (Invitrogen). The reaction was carried out for 20 min at 37°C and the products were purified using the QIA quick Nucleotide Removal Kit (Qiagen). Purified, radioactively labeled products were incubated in 1 $\times$ NEBuffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM DDT)

for 2 hr at 37°C to ensure re-hybridization of the short flanking duplexes, in case of partial denaturing due to low ionic conditions or the presence of organic solvents during purification steps. The samples were analyzed by gel-electrophoresis in a 5% non-denaturing polyacrylamide gel running at low voltage (80 V) overnight, to avoid structure disruption from overheating. The gel was dried and then visualized using a Bio-Rad personal molecular imager system (Fig. S2).

The complete set of HJ forming oligonucleotides (e.g. R1, R2, D1, D2 for DHJ in the lane 1, Fig. S2) generates a predominant product, which is not present among the hybridization products obtained with incomplete sets of oligonucleotides (lanes 4–18); this indicates that all four oligonucleotides are present in the structure. However, some minor fractions, which correspond to incomplete sets of oligonucleotides are also present. For example, lane 1 contains additional minor bands consistent with the family of structures in lanes 4–7, and 11–12. To ensure that the transcription blockage pattern is due to the DHJ rather than to these minor products, all possible structures containing the incomplete set of oligonucleotides capable of ligation to the promoter fragment (e.g. containing R1 and R2) were also tested in the transcription experiments, in parallel with those for the DHJ (Fig. 4, 5; see the Results section for details).

To test for the proper formation of the flanking duplexes, the flanking duplexes were designed with a unique restriction enzyme recognition site (Fig. S1, the relevant sites are shown in green). If a flanking duplex is properly formed, it should be cleaved by the respective enzyme, which would alter the electrophoretic mobility of the structure. The restriction reaction was performed for 2 h at 37°C in 1×NEBuffer 3 for SalI and BsiWI, BamHI NEBuffer for BamHI, and at room temperature in 1×NEBuffer 4 for SmaI; and terminated by addition of 20 mM EDTA. The products were analyzed on a 5% non-denaturing polyacrylamide gel as described above. The results of analysis for the DHJ are shown in Fig. S3. The efficient cleavage of the flanking duplexes by the respective enzymes indicates the proper formation of the flanks.

The assembled DNA structures were ligated to the respective promoter fragments via sticky ends formed by R1 and R2 oligonucleotides: BamHI sticky ends for the T7 RNAP promoter fragment or BseRI sticky ends for the RNAPII promoter fragment (Fig. S1). Ligation reactions were performed as in [22] except for heat inactivation of the ligase, to avoid thermal disruption of the structure. Since the oligonucleotides were not 5' phosphorylated, a nick was present in the non-template strand of the ligated product. Previously, it was shown that a nick in this position has no significant effect upon transcription [12].

2.2. RNAPII transcription assay in HeLa cell nuclear extracts

RNAPII transcription reactions were performed as previously described in [12]. To permit initiation complexes to form, the reaction was performed in a volume of 18 µL containing ~4 nM DNA substrate, 8 units of HeLaScribe nuclear extract (Promega), 3 µL of 1X HeLa nuclear extract transcription buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 20% glycerol), and 4.17 mM MgCl₂. The mixture was incubated at 28°C for 30 min. For elongation, 40 units of RNasin (Promega), 1 µL of NTPs (10 mM ATP, 10 mM GTP, 10 mM UTP and 0.4 mM CTP) and 40 µCi of [α -³²P]-CTP were added for a total

reaction volume of 25 μ L and incubated at 28°C for 30 min. To stop the reactions, EDTA was added to a final concentration of 6 mM. The RNA transcripts were purified using the Qiagen RNeasy MinElute cleanup kit. Samples were heated at 85°C for 2 min in 3 μ L of denaturing formamide loading dye and then loaded on a 5% denaturing polyacrylamide gel containing 8 M urea. Gel-electrophoresis was performed at 2000 V for 1.5 hr. The gel was dried and visualized by phosphorimaging.

2.3. T7 RNAP transcription assay

Each reaction was performed in a total volume of 12 μ L containing ~6.5 nM of DNA substrate, 2 μ L of 5X transcription buffer (Promega), 4 mM DTT, 16 units of RNasin (Promega), 1 μ L of NTPs (2 mM ATP, 2 mM GTP, 2 mM UTP, and 0.2 mM CTP), 10 μ Ci of [α -³²P]-CTP, and 18 units of T7 RNAP (Promega). The reactions were carried out at 37°C for 30 min. Termination of the reactions and purification of the products were performed as described previously [12]. Gel-electrophoresis was performed at 2000 V for 1.5 hr. Then the gel was dried and visualized by phosphorimaging.

2.4. Quantitation

Intensities of the transcript bands were measured by phosphorimaging and quantitated with Bio-Rad Image Lab software.

3. RESULTS

3.1. Experimental strategy

To test for transcription blockage by various HJs, we applied a strategy similar to one that we used previously [12, 22, 23]. Specifically, unusual DNA structures of interest are assembled from synthetic oligonucleotides and then ligated to double-stranded DNA fragments (obtained from an appropriate plasmid), which contain transcription promoter(s) for specific RNA polymerases (e.g. for RNA polymerase II, or T7 RNA polymerase, or both).

In this work, we have studied stable Holliday junctions that are incapable of branch-migration due to lack of homology between the branches. This is in contrast to mobile HJs formed between homologous DNA regions, which would eventually dissociate into two duplexes through branch-migration. Our strategy for preparing transcription substrates containing immobile DHJs is shown in Fig. 3. Other substrates were prepared similarly, using different sets of oligonucleotides (see Fig. S1 for details).

These constructs were subjected to *in vitro* transcription in the presence of radioactively labeled NTP with either purified T7 RNAP or HeLa nuclear extracts as the source of RNAPII and other necessary transcription factors, and the transcription products were separated on denaturing (sequencing) acrylamide gels. Unobstructed transcription proceeds to the very end of the substrate, producing the longest (“run-off”) product, while transcription blockages generate truncated transcripts, from which the position of the blockage site on the DNA template can be estimated.

3.2. Branched DNA structures cause RNAPII transcription blockage in HeLa extracts with superior blockage in the case of double Holliday junctions

Products of transcription for various DNA structures in HeLa extracts are shown in Fig. 4. The scheme at the left of the gel aligns the location of various products relative to the structure (using DHJ as an example), and the schemes at the right of the gel show all DNA substrates used in this experiment. As mentioned in the Introduction, HJ-containing substrates (2–4) could model replication and replication intermediates. The other substrates functioned primarily as controls to determine which blockage signals are specific for the HJ. However, D-loop-like structures similar to substrates 5 and 6 (Fig. 4, the right panel) also could appear at the initial stages of homologous recombination [14]. The transcription product from the promoter fragment alone (lane 1) was used as an additional reference for the positioning of the transcripts. Lane 2 corresponds to DHJ. Lanes 3 and 4 correspond to the structures containing only promoter-proximal HJ or only promoter-distal HJ, respectively; instead of a second HJ, they contain three-way Y-shaped junctions, which we will refer to as “Y-junction” or YJ. Bubble-containing substrates in lanes 5, 6, and 7 correspond to possible byproducts of DHJ annealing, in which one or two oligonucleotides, which form internal duplexes within the DHJ are missing. Lane 8 corresponds to the “perfect” Watson-Crick duplex without any unusual structures.

As expected, the perfect duplex (lane 8) predominantly yields complete (runoff) product. Some shorter transcripts (which we interpret as a result of transcription blockage, and further refer to as “blockage signals”) are barely distinguishable over background and might correspond to weak sequence-specific pausing sites.

For the bubble-containing substrates in lanes 5–7, blockage signals are widely distributed over the entire region of the bubble, in some cases extending into the double-stranded flanks. The run-off is the predominating product for substrates 6 and 7, while for substrate 5 the intensity of the runoff product is significantly reduced.

For the substrate containing the single promoter-proximal HJ (lane 3), the only blockage signals (shown by white block arrows) that are distinguishable over background are localized in the region 10–20 bp upstream of the HJ; and for the substrate with the single promoter-distal HJ (lane 4), additional blockage signals (gray block arrows) appear in the region 10–20 bp upstream of the HJ. (Note that the blockage signals are localized some distance upstream from the HJ, rather than exactly at the HJ. This is probably because the location of the blockage signal corresponds to the catalytic center of RNAP, which is localized some distance behind the front of the transcription complex (e.g. [24]) that encounters the HJ.)

Most importantly, in the case of the DHJ (lane 2), the blockages (shown by red block arrows) for both HJs are significantly stronger than those for either structure containing a single HJ (lanes 3,4) (see Fig. S4 and its legend for quantitation). As we will elaborate in the Discussion, this observation supports the “topological” mechanism for transcription blockage by DHJs.

As mentioned above, the structures containing a single HJ (and consequently, YJ on the opposite side of the bulge) (lanes 3, 4) have different blockage patterns. For the promoter-distal junction, a blockage signal (gray block arrow) was detected only for HJ (lane 4), but not for YJ (lane 3), while for the promoter-proximal junction the upstream blockage signals (white block arrows) were pronounced for both HJ and YJ. The reason for this difference in behavior of RNAPII at promoter-proximal and promoter-distal junctions remains to be elucidated. However, it does not affect our interpretation for blockage by DHJ (see Discussion) because strong blockage enhancement (red block arrows) is observed only in the presence of two HJs.

Widely distributed blockage signals for bubble-containing substrates in lanes 5, 6, 7 are probably due to some distortion of the normal elongation process across the structure. The origin of differences between the blockage patterns for these structures (e.g. stronger total blockage for the structure 5 versus structures 6 and 7) remains to be elucidated. However, in the context of this work, we primarily consider these structures as products of incomplete DHJ assembly, and for our purposes, it is important that none of these structures produces blockage patterns resembling the pattern for DHJ (lane 2).

For DHJ (lane 2), the presence of 2–3 distinctive bands upstream of the promoter-proximal HJ could be due to local sequence effects: our previous observations [25, 26] suggest that while the intensity of blockage signals and the region of their location is determined by the causative structure, the precise positions of the observed blockage signals could be defined by “hidden” sequence-specific pausing sites within DNA, which are exacerbated by the causative structure, but are too weak to be observed in the absence of this structure.

3.3. Branched DNA structures do not produce detectable blockage with purified T7 RNAP

The products of T7 RNAP transcription for the unperturbed duplex and various branched substrates (including DHJ) are shown in Fig. 5. In all cases, complete run-off product predominates and there is no detectable difference between the unperturbed duplex and the branched substrates.

4. DISCUSSION

Previous studies have shown that unusual DNA structures can arrest transcription by mechanisms that vary, depending upon the structure (reviewed in [1]). In this work, we have studied how the multi-subunit mammalian RNA polymerase II (RNAPII) and the simple single-subunit T7 RNA polymerase behave when they encounter DNA structures containing Holliday junctions (HJ). This study focuses on double Holliday junctions (DHJ) whose topological properties predict their increased ability to block transcription in comparison with single HJ (Figs. 1,2).

In accordance with this prediction, we observed strong transcription blockage by DHJ for human RNAPII in HeLa cell nuclear extracts (Fig. 4), while removal of either HJ from the DHJ significantly decreased the blockage signal. However, we did not detect any pronounced blockages for T7 phage RNAP (Fig. 5).

In order to account for the different results for the respective RNAPs, we will first consider two possible pathways of RNAP propagation into the DHJ region: with and without switching of the non-template DNA strand, which normally maintains contact with the RNAP during transcription. The initial stages for both pathways are schematically shown in Fig. 2B and C, respectively. In both cases, RNAP invasion into the DHJ region would induce energetically unfavorable overwinding in the internal duplexes, which would push the RNAP backwards, thus creating resistance for its forward propagation. However, an important difference between these two pathways (with and without switching of the non-template strand) is revealed upon further RNAP propagation into the DHJ region.

In the case of the non-template DNA strand switching (Fig. 6A), after RNAP completely invades the bottom internal duplex, the duplex could reform behind the RNAP (Fig. 6A, bottom). In this case, the total overwinding of internal duplexes would not increase further after RNAP invasion into the bottom internal duplex has been completed. Consequently, after overcoming the energetic barrier for entering the DHJ region, the resisting force either disappears or at least does not increase. (Note that in Figs. 2, 6 we did not show the spreading of the overwinding to the top internal duplex, which is topologically possible, but which might be sterically constrained (see the legend to Fig. 2). Taking this into account the spreading does not affect our argument).

In contrast, if the RNAP does not switch to the non-template strand upon entering the HJ region (Fig. 6B), its propagation continues to tighten and wind the internal duplexes, creating increasing topological stress. In addition, for the immobile HJ, the strands (of the same color) within the internal duplexes are not complementary to each other and therefore can not hybridize with each other upon unwinding of the internal duplexes, rendering the growing flanking regions single stranded. The non-compensated unwinding of the internal duplexes together with the growing topological stress would make RNAP propagation in the mode shown in Fig. 6B extremely energetically unfavorable; eventually, it would either have to stop or switch to the non-template strand and continue transcription in the mode shown in Fig. 6A. However, for the “bulky” RNAPII transcription complex to switch and continue transcription in this mode might be challenging because that would require confinement of the RNAPII between the two internal duplexes (Fig. 6A) and steric clashes between the RNAPII and the top internal duplex during transcription. We speculate that these steric impediments contribute to RNAPII blockage by DHJ. In addition, the steric impediments for rotation of RNAPII while transcribing the bottom duplex could generate transient overwinding stress in the structure containing only the promoter-distal HJ (structure 4 in Fig. 4), which could contribute to the blockage at this junction. In contrast, these steric constraints are likely to be a less significant issue for the much smaller T7 RNAP; thus, it could easily translocate through the DHJ. This could be a plausible explanation for the differences in behavior of these RNAPs upon encountering a DHJ.

Since the RNAPII transcription was performed in HeLa nuclear extracts, we cannot exclude the possibility that the binding of proteins to HJs contributes to RNAPII blockage. However, although HJ-specific protein binding could be responsible for the blockage at a single HJ, it does not explain the significant increase in transcription blockage for HJs within the DHJ structure where the HJs are separated by sufficiently long (60 bp) double-stranded DNA

regions. Thus, even if we assume HJ-specific protein binding, the topological explanation for increased RNAPII blockage by DHJ remains valid. This argument is also valid whether the RNAPII is able to pass through the DHJ by itself or whether it requires the help of other factors (e.g. helicases), which might be present in the extracts. Further confirmation of the model could include experiments with purified RNAPII and determining whether type IB topoisomerases could facilitate transcription through DHJ by relaxing overwinding within the internal duplexes.

In this work we studied stable immobilized HJs, which in contrast to naturally occurring, mobile HJs are not capable of branch migration. In the case of mobile HJs, there is a third possibility for the encounter between RNAP and DHJ in which two mobile HJs within the DHJ could migrate simultaneously with the same rate as the RNAP, thus preventing accumulation of overwinding in the internal duplexes (Fig. 2D). However, since HJ migration is associated with axial rotation of all four DNA duplexes involved in this process, for very long flanking DNA duplexes (which would be the case *in vivo*), this process would be associated with strong viscous resistance. Consequently, significant overwinding of the internal duplexes (and associated resistance toward RNAP progression) would build up before the HJs began to move.

Our observation of RNAPII transcription blockage by HJ-containing structures, regardless of its underlying mechanism, may have significant biological importance: the processes that can lead to formation of these structures, (e.g. homologous recombination, double-strand break repair and processing of stalled replication forks) might occur within transcribed DNA regions, within which collision between these structures and the transcription complex could occur. Our observations with the model system suggest that this collision leads to transcription blockage that, in addition to affecting gene expression, could have a number of other biologically important outcomes, including genomic instability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Holliday junctions cause RNAPII transcription arrest in HeLa cell nuclear extracts
- Double Holliday junctions have stronger RNAPII blockage than single junctions
- Holliday junctions do not produce detectable blockage for purified T7 RNAP
- We discuss propose topological constraints as the mechanism for transcription blockage
- Holliday junctions are implicated as novel structures to block transcription

Fig. 1. Topology of double Holliday junctions (DHJ)

A: Standard representation of DHJ. DNA strands are shown in red and blue. Duplexes localized between the Holliday junctions are referred to as “internal duplexes” while the other duplex regions are referred to as “flanking duplexes”. **B:** DNA helical turns are shown within the internal DNA duplexes. **C:** From a topological point of view, the flanking duplexes, as long as they remain base-paired, could be simply replaced by connectors (shown by straight, short lines) between the respective DNA strands. Therefore, the internal duplex regions form two intertwined circles (red and blue), for which the total number of turns that one strand makes around the other cannot be changed as long as the circles are not disrupted; consequently, underwinding in one region generates compensatory overwinding in the rest of the structure.

Fig. 2. Schematics for a transcribing RNA polymerase (RNAP) encountering a double Holliday junction

Helical turns within DNA duplexes are shown only for the duplexes localized between Holliday junctions. **A:** RNAP (gray oval) approaching DHJ. **B, C:** RNAP entering DHJ region with switching (from blue to red) or without switching the non-template DNA strand, respectively. In the first case (**B**), RNAP causes local unwinding only in the bottom internal duplex, thus generating compensatory overwinding in the rest of this duplex. Spreading of this overwinding to the top internal duplex is topologically possible, but this spreading might be associated with steric impediments because the transferring of the helical turns between internal duplexes would require alternating threading of the flanking duplexes through the space between the internal duplexes. An example of such threading is shown by the curved dashed arrowed lines: the threading of the top right flanking duplex (red) through the space between the internal duplexes would create one additional turn within the top internal duplex, while the threading of the bottom right flanking duplex (blue) would remove one turn from the bottom internal duplex; and alternating these threading events would transfer the helical turns from the bottom duplex to the top. In the second case (**C**), RNAP locally unwinds and generates compensatory overwinding in both internal duplexes, in effect “compressing” the DHJ structure. In both cases, the overwinding (which is energetically unfavorable) creates an apparent force that acts against RNAP entering the DHJ region. **D:** Both HJs of the DHJ structure could branch-migrate in the direction of transcription with the same speed as RNAP translocation. This would keep the length of the internal duplexes constant, and consequently, their overwinding does not occur.

Fig. 3. Strategy to obtain double Holliday junction substrates

The DHJ is assembled from two-step hybridization of four oligonucleotides D1, R1, D2, R2. Complementary sequences within the oligonucleotides are marked with the same color. The assembled structures were then ligated to promoter-containing DNA fragments. Since the oligonucleotides were not 5' phosphorylated, a nick was present in the non-template strand of the ligated product. All other substrates were obtained with the same protocol but with different sets of oligonucleotides. The drawings separated by double-headed block arrows correspond to different pictorial representations of the same DHJ structure.

Fig. 4. Transcription products obtained in HeLa nuclear extracts for various branched DNA substrates

See the main text for description. The designation of oligonucleotides (D1, R1, D2, R2) is the same as in Fig. 3. In addition, oligonucleotide R2c is complementary to R2. Red block arrows correspond to DHJ blockage signals, white block arrows correspond to promoter-proximal HJ blockage and grey block arrows correspond to promoter-distal HJ blockage. "RO" stands for runoff and "RO_p" stands for the promoter fragment runoff. See Fig. S4 for profile analyses of lanes 2–4.

Fig. 5. DHJ does not produce detectable blockage for T7 RNAP

Structures tested in the experiment are shown at the left, and the results are shown at the right (the right-most panel shows lower exposure of the same gel). “RO” stands for runoff, “lig” is the ligation site for the promoter fragment, “pJ” and “dJ” stands for promoter-proximal and promoter-distal junctions, respectively. It is seen that there is no detectable difference between the branched structures (lanes 1–4) including DHJ (lane 1) and the perfect duplex (lane 5).

Fig. 6. Transcription in DHJ area with (A) and without (B) non-template DNA strand switching

A: When RNAP enters the DHJ area (top) with switching of the non-template strand from blue (before encounter with DHJ) to red, the RNAP induces overwinding in the bottom internal duplex, and this is energetically unfavorable and thus creates a force that opposes the entering of RNAP. However, after the entering and continuation of transcription, the overwinding remains constant, so the resisting force does not increase further. **B:** As long as RNAP does not switch the non-template strand, overwinding accumulates during RNAP translocation and the resisting force continues to increase.