Formation of the RNA polymerase II (Pol II) open complex (OC) requires DNA unwinding mediated by the transcription factor TFIIH helicase-related subunit XBP/Ssl2. Because XBP/Ssl2 binds DNA downstream from the location of DNA unwinding, it cannot function using a conventional helicase mechanism. Here we show that yeast TFIIH contains an Ssl2-dependent double-stranded DNA translocase activity. Ssl2 tracks along one DNA strand in the 5' → 3' direction, implying that it uses the nonextendable promoter strand to reel downstream DNA into the Pol II cleft, creating torsional strain and leading to DNA unwinding. Analysis of the Ssl2 and DNA-dependent ATPase activity of TFIIH suggests that Ssl2 has a processivity of approximately one DNA turn, consistent with the length of DNA unwound during transcription initiation. Our results can explain why maintaining the OC requires continuous ATP hydrolysis and the function of TFIIH in promoter escape. Our results also suggest that XPB/Ssl2 uses this translocase mechanism during DNA repair rather than physically wedging open damaged DNA.

Double-stranded DNA translocase activity of transcription factor TFIIH and the mechanism of RNA polymerase II open complex formation

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How ATP hydrolysis is coupled to promoter DNA unwinding and open complex formation at RNA polymerase II (Pol II) promoters is a longstanding question. Of the multisubunit RNA polymerases, only Pol II requires ATP for DNA unwinding. Here we show that the general transcription factor TFIIH subunit Ssl2 is a double-stranded DNA translocase. These and other data suggest that Ssl2 promotes DNA opening by tracking downstream from the site of DNA unwinding in the OC (20–24). Therefore, XBP/Ssl2 cannot function as a conventional helicase to promote OC formation (20). Three models have been proposed to explain the role of XBP/Ssl2 in transcription. First, it was postulated that XBP acts as a molecular wrench, binding to its site on downstream DNA and using its ATPase to rotate upstream DNA within the Pol II cleft (20). Because upstream DNA is constrained by TBP, TFIIH, and other factors, DNA rotation could lead to DNA opening. Second, it was proposed that the XBP ATPase activity promoted DNA opening via a conformational change in PIC components, leading to a structural rearrangement of both protein and DNA, analogous to the ATP-dependent mechanism of OC formation in the bacterial σ34 system (25). Third, comparing structure models of the PIC and OC suggested that ∼15 bp of downstream promoter DNA inserts into the Pol II cleft upon OC formation (22). Based on this and other data, it was proposed that XBP/Ssl2 functions as a double-stranded DNA (dsDNA) translocase (22, 23, 26). According to this model, XBP/Ssl2 attempts to track away from the PIC, but, because it is bound to the PIC via TFIIH, it instead feeds and rotates dsDNA into the Pol II cleft, leading to DNA opening. However, a key feature of this latter model, whether XBP/Ssl2 has dsDNA translocase function, had not been tested.

Here we show that yeast TFIIH has Ssl2-dependent dsDNA translocase activity and that it primarily tracks along one DNA strand in the 5' → 3' direction. The kinetic properties of the enzyme suggest a rate-limiting step between DNA binding and translocation and a processivity on the order of a turn of DNA.

Significance

Author contributions: J.F., E.T., E.G., and S.H. designed research; J.F. and E.T. performed research; J.F. and E.T. performed research; J.F. and E.T.; E.G., and S.H. analyzed data; and J.F., E.T., E.G., and S.H. wrote the paper. The authors declare no conflict of interest.

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consistent with the amount of DNA thought to be unwound in the OC. Our findings have important implications for the mechanism of OC formation, the relative instability of the OC, and the role of XPD/Ssl2 in NER.

**Results**

TFIIH Has Ssl2-Dependent dsDNA Translocase Activity. We first measured DNA helicase activity of purified yeast TFIIH using a substrate consisting of a 100-base oligonucleotide with a labeled 25-base oligonucleotide annealed at either the 3′ or 5′ end (Fig. 1A). Consistent with the function of human TFIIH, we found that yeast TFIIH contains 5′ → 3′ helicase but not 3′ → 5′ helicase function (Fig. 1B). The 5′ → 3′ helicase activity requires both hydrolysable ATP and the Rad3 ATPase, because a TFIIH derivative containing a mutation in the Rad3 Walker B motif (Rad3 E236Q) that is predicted to be defective in ATP hydrolysis (27) is defective in helicase function. In contrast, both WT and Rad3 mutant TFIIH preparations are equally active in supporting transcription with purified factors and Pol II from the yeast HIS4 promoter (Fig. S1).

As an initial test for dsDNA translocase function, we assayed whether TFIIH can displace a 22-nucleotide DNA triplex-forming oligonucleotide (TFO) from a 142-bp dsDNA containing a complementary polypurine sequence at one end (Fig. 1A). The triplex is disrupted by heating, but is stable at 26 °C with or without the addition of ATP (Fig. 1C, lanes 1–3). Incubation of WT TFIIH, ATP, and the triplex substrate results in near complete dissociation of the TFO (Fig. 1C, lanes 4–6). The triplex displacement activity of TFIIH is unaffected by the Rad3 E236Q ATPase mutation but is abolished by the equivalent Walker B ATPase mutation in Ssl2 (E489Q; Fig. 1C, lanes 7–11). To assay whether TFIIH can simply bind and displace the triplex without translocation, we used a 22-nucleotide triplex substrate that did not contain additional dsDNA (Fig. 2A). The short triplex substrate is less stable than the longer triplex, likely due to the lack of stabilizing dsDNA. Nevertheless, as predicted for a dsDNA translocase, TFIIH cannot displace the TFO from this shorter substrate lacking dsDNA (Fig. 2B). The TFIIH triplex displacement activity was not dependent on a free DNA end, because TFIIH can also displace the TFO annealed to a 3.2-kb double-stranded plasmid, although with much slower kinetics (Fig. 2C). Both ATP and dATP function to promote OC formation (7) and, as expected, both nucleotides can promote triplex displacement (compare Fig. 1C and Fig. 2C, Right). Our combined results are consistent with the prediction that TFIIH contains an Ssl2-dependent dsDNA translocase activity.

**Ssl2 Tracks in the 5′ → 3′ Direction Along the DNA Duplex.** dsDNA translocases are thought to track along one strand of the DNA backbone (31). If true for Ssl2, discerning the polarity of translocation will reveal which promoter strand is used by Ssl2 during DNA unwinding. As an initial test of polarity, we assayed triplex displacement using substrates where the TFO was annealed to either the top or bottom strand of the 142-bp duplex DNA (Fig. 3A). As shown above, TFIIH can readily displace the TFO from the bottom strand triplex substrate (Fig. 3B, lanes 4–6). In contrast, the top strand triplex substrate is almost completely resistant to TFO displacement (Fig. 3B, lanes 1–3). One model consistent with this result is that Ssl2 tracks along one strand of duplex DNA in the 5′ → 3′ direction and that this tracking is blocked by the annealed TFO. As a further test of this model, we introduced biotin as a tracking barrier on the triplex substrates (Fig. 3A). These substrates contain a single-strand DNA nick with biotin attached to the 5′ end of one strand of duplex DNA. We found that the top-strand biotin was a much stronger block to TFO displacement, with only one third of the TFO displaced after 6 h (Fig. 3C and D). Although the bottom-strand biotin modestly inhibited TFO displacement, the TFO was >80% displaced after 6 h, similar to the nonbiotinylated template. As a further test of whether the integrity of the top strand is the most critical for TFO displacement, we generated triplex substrates with 5-bp single-stranded gaps on either the top or bottom duplex strand, directly adjacent to the triplex (Fig. S2). Consistent with the above results, we found that TFO displacement was most inhibited by the gap on the top strand. Under conditions of the assay, 80–90% of the TFO was displaced from the nongapped template, 45% displaced from the template with the gap on the bottom strand, and only 20–25% displaced from the template with the gap on the top strand. Our combined results strongly suggest that Ssl2 primarily tracks along one strand of duplex DNA in the 5′ → 3′ direction.
Blocking SsII2 Translocation Inhibits Transcription Initiation. The above results predict that blocking SsII2 translocation should inhibit transcription initiation. Mapping the location of SsII2/XPB-DNA binding in PICs has suggested SsII2 binds 30–36 (yeast) and 40–50 bp (human) downstream from TATA (20–23). To test the role of SsII2 translocase function in transcription initiation, we created three promoter derivatives with Cy3 dye inserted in the phosphodiester backbone of the nontemplate DNA strand 37, 41, or 46 bp downstream from the HIS4 TATA (Fig. 4A). Cy3 positioned in the DNA backbone is a strong inhibitor of SsII2 translocation in the TFO displacement assay (Fig. S3). The modified and unmodified DNA templates were tested for in vitro transcription activity using the reconstituted system (32), and transcription from these templates was completely TFIIH dependent (Fig. 4B, compare lanes 4–7 and 8–11). We found that Cy3 positioned on the nontemplate strand 41 and 46 bp from TATA inhibited transcription approximately fivefold (Fig. 4B, lanes 4, 6, and 7), consistent with the dsDNA translocase model. Cy3 positioned 37 bp downstream from TATA inhibited transcription approximately twofold (Fig. 4B, lanes 4–5). We speculate that transcription escaping this more upstream Cy3 insertion may be due to inherent flexibility in the SsII2–DNA interaction, allowing SsII2 to escape the Cy3 block by binding DNA just downstream of Cy3 37 in a position nearly equivalent to the observed XPB-human promoter interaction (23).

To confirm that the observed transcription inhibition by Cy3 could not be explained by an inhibition of transcription elongation, we created HIS4 promoter derivatives containing a 12 nucleotide single-strand bubble beginning 21 bp downstream from TATA (Fig. 4C). Pol II initiating from this bubble in the absence of any general factors transcribed past the Cy3 block with 47–77% efficiency compared with a bubble template lacking Cy3 (Fig. 4D, lanes 4–7). Taken together, our results show that the integrity of the nontemplate strand is important for transcription initiation and is consistent with the dsDNA translocase model for open complex formation.

SsII2 Translocates with Low Processivity. To further characterize the SsII2 motor within the TFIIH complex, we next investigated stimulation of the ATPase by nucleic acid. Measurements of how the steady-state rate of ATP hydrolysis depends on DNA template length and concentration can be used to assay for translocation and to evaluate different models of translocation (33–35). For Fig. 2. Template requirements for triplex disruption by TFIIH. (A) Triple helix substrates used to monitor TFIIH translocation. TFO22 was generated by annealing the 22-nt TFO to the 22-bp triplex target sequence. This smaller triplex was somewhat less stable than the 142-bp triplex DNA, likely due to no additional dsDNA. To make the 142-bp and circular triplex templates, the TFO was annealed to a PCR-generated 142-bp duplex or a 3.2-kb plasmid containing the triplex target sequence. (B) Triplex disruption assay using the TFO22 triple helix template and the TFIIH derivative Rad3 E236Q. (C) Time course of triplex disruption comparing the circular 3.2-kb plasmid triplex and the linear 142-bp triplex templates. Intact triplex was measured at each time point and quantified by comparison with a standard curve. The percent disruption of each triplex is indicated. ATP and dATP both function in the TFO displacement assay (compare with Fig. 1C) and in OC formation (7).

Fig. 3. Polarity of SsII2 catalyzed TFIIH translocation. (A) Triple helix substrates designed to test the polarity of SsII2 translocation. Top and bottom strand triplexes are annealed as shown using PCR-generated duplex DNA and the TFO. PAGE-purified oligonucleotides were annealed with the TFO to generate the biotin-containing triplexes where a 5′-biotin labeled oligonucleotide is positioned immediately upstream of the triplex on the top or bottom duplex strand. (B) Triplex disruption assay comparing templates with the TFO annealed to either the top or bottom strand of the duplex. Either WT or Rad3 E236Q TFIIH was used as indicated. (C) Time course of triplex disruption from biotin templates by TFIIH (Rad3-E236Q). Reactions were incubated for the indicated times with 1 mM dATP at 26 °C and quantified for intact triplex remaining. (D) Quantitation of results in C.
Fig. 4. In vitro transcription from promoters with DNA backbone blocks on the non-template DNA strand. (A) HIS4 promoter derivatives with Cy3 DNA backbone insertions. DNAs were constructed from synthetic oligonucleotides and contained Cy3 positioned 37, 41, or 46 bp downstream from HIS4 TATA. Transcription was assayed by primer extension using the lacI oligonucleotide as shown. (B) In vitro transcription reactions using the reconstituted yeast Pol II system and the promoters in A. Lanes 1–4 contain the indicated amounts of a transcription reaction used to generate a standard for quantitation of transcription signals relative to the unmodified template. Lanes 5–7 are transcription reactions using the indicated Cy3 templates. Percent transcription relative to the unblocked template is indicated. No transcription is observed when TFIIH is omitted (lanes 8–11). (C) HIS4 promoter derivatives identical to those in A except that they contain the 12 nucleotide single-stranded DNA bubble as shown. This bubble allows transcription initiation by Pol II in the absence of other general factors (33). (D) In vitro transcription using purified yeast Pol II on the bubble templates and assayed by primer extension. Lanes 1–4 contain the indicated amounts of a transcription reaction using the non-Cy3 bubble template. Lanes 5–7 are transcription reactions using the Cy3-modified bubble templates. Lanes 8–9 are mock transcription reactions lacking Pol II and assayed by primer extension. The products marked by * are due to primer extension of the DNA template, which is blocked by Cy3. These blocked products are not visible in B, lanes 9–11, as they are significantly longer than the RNA products initiated at the HIS4 TSS.

these measurements, we used the TFIIH preparation containing Rad3 E236Q so that Ssl2 was the only functional ATPase. TFIIH and DNA were preincubated for 40 min, and then ATP was added, and phosphate release was quantitated at different times (1–20 min). The steady-state rate of ATP hydrolysis was determined by a linear fit of these data (Fig. 5A). ATP hydrolysis in the absence of nucleic acid was undetectable, and dsDNA consistently stimulated ATPase activity four- to fivefold higher than that of single-stranded DNA (Table 1), consistent with the enzyme being a dsDNA translocase.

The steady-state rate of ATP hydrolysis was measured as a function of DNA concentration and DNA template length over a range of DNA lengths from 30 to 80 bp and on a circular plasmid representing an infinitely long template (Fig. 5 A and B). As the dependence of rate on DNA concentration follows a Michaelis–Menten curve, we were able to determine both a Michaelis constant (K_M) and a maximal velocity (V_max) for each length of DNA (Fig. 5B). Although K_M does not show a length dependence (Fig. 5C), V_max is clearly sensitive to DNA length (Fig. 5D). The dependence of V_max on template length is a clear indication that Ssl2 is a dsDNA translocase as nontranslocating enzymes do not show this behavior (33–35) (Fig. S4D).

Models of translocation can be used to fit the dependence of V_max on DNA length to extract quantitative values for parameters such as occluded site size, kinetic step size, and motor processivity, i.e., the probability that the motor takes a step forward instead of dissociating (Materials and Methods) (33–35). In the simplest model for translocation, the motor binds DNA at any site and steps forward coupled to ATP hydrolysis. At each site, the motor may either hydrolyze ATP and take a step forward or dissociate (Fig. S4B). At the end of the template, the motor either dissociates or translocates off the end of the DNA. This model predicts that K_M will vary with the length of the DNA template, whereas V_max will not (Fig. S4B). This behavior is inconsistent with our observed results (Fig. 5C and D).

However, in contrast to the simple translocation model above, models that incorporate a slow step before unbinding and the active DNA-bound motor states result in a length-dependent V_max and a length-independent K_M (33–35). Two limiting cases of this behavior are (i) a slow step after binding and before translocation or (ii) a slow step before dissociation from the end of the DNA (Fig. S4C). These two cases predict different
Table 1. $V_{\text{max}}$ of the Ssl2 ATPase with single- or double-stranded DNA

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<thead>
<tr>
<th>DNA Length [bp (ds) or nt (ss)]</th>
<th>$V_{\text{max}}$ (ATP/Ssl2 s$^{-1}$)</th>
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<tr>
<td>ds 30</td>
<td>5.19</td>
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<tr>
<td>ds 40</td>
<td>6.82</td>
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<td>ds 60</td>
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<td>ss 60</td>
<td>2.46</td>
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<td>ss 80</td>
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Reactions with single-stranded DNA were carried out as described in Materials and Methods and contained 12.5 μM nt of each template. The average $V_{\text{max}}$ values from two independent experiments are given. For comparison, the predicted $V_{\text{max}}$ from an analogous ds DNA template is shown (data from Fig. 4D).

Pre-steady-state behavior in the ATPase rate (34). A slow step at the end of the DNA predicts a burst of rapid ATP hydrolysis on ATP addition compared with the steady-state rate. This burst phase arises because initial rounds of hydrolysis do not encounter the rate-limiting step that takes place at the DNA end. In contrast, a slow step only between DNA binding and translocation predicts a lag in ATP hydrolysis rate. This lag phase arises because the rate-limiting step occurs before translocation and before ATP hydrolysis occurs. Our data are consistent with the latter case, as there is a lag in ATP hydrolysis after adding ATP to TFIIH prebound to DNA (Fig. 5A). The observed lag suggests that Ssl2, in complex with TFIIH, first binds DNA in an inactive conformation and then, after ATP addition, isomerizes to the active state before translocation.

To extract quantitative measures of translocase parameters, we analyzed the DNA length dependence of the Ssl2 ATPase $V_{\text{max}}$ with an expression derived from the isomerization model (SI Materials and Methods) (34). Assuming a translocation step size of 1 bp and a contact size on the DNA of 16 bp (Table S1 and Materials and Methods), we determined a processivity of Ssl2 in the context of TFIIH of 0.90 with a fit error of 0.01 corresponding to an average of 10.0 ± 0.9 bp translocated before dissociation from the DNA. Because we have no independent measure of step size, we note that a larger step size would lead to a lower processivity. For example, analyzing the same data with a step size of 2 bp would correspond to 5.3 ± 0.3 bp translocated before dissociation from the DNA.

Discussion

Our results on the translocase activity of Ssl2 in the context of the TFIIH complex are completely consistent with the translocase model of OC formation and explain how TFIIH might generate torsional stress in promoter DNA, leading to unwinding. Our finding that Ssl2 tracks in the 5′ → 3′ direction implies that Ssl2 uses the non-template strand of promoter DNA as it attempts to track away from the PIC but instead reels downstream dsDNA toward the PIC, creating open DNA via torsional or mechanical stress that is then fed into the Pol II cleft (Fig. 6). Interestingly, this is the opposite direction to the characterized helicase activity of the Ssl2 subunit alone, suggesting that the enzyme does not use the same motor mechanism for helicase and dsDNA translocase activities. Our conclusion that the processivity of Ssl2 translocation is similar to the amount of DNA unwound in the OC can explain why the Pol II OC is unstable. We speculate that DNA opening requires continuous ATP hydrolysis because Ssl2 likely dissociates from the DNA after translocating a short distance (while remaining in complex with TFIIH and the PIC), resetting the initiation complex to its closed state. In contrast to the situation with other prokaryotic and eukaryotic RNA Pols, it seems likely that the contacts between Pol II and unwound DNA are not strong enough to stabilize the fully unwound state in the absence of continuous Ssl2 translocation activity.

This mechanism predicts multiple cycles of DNA opening and closing during the initial stages of transcription. It was found that the Pol II OC is stabilized by a four-nucleotide RNA (8), so this may be the minimum length of transcript to prevent reversion to the PIC dsDNA state. However, translocase function may be necessary to assist in DNA opening until a 7- to 8-base RNA:DNA hybrid is formed. This model can explain the observation that TFIIH stimulates promoter escape from templates with a short stretch of premelted DNA from −9 to −1 with respect to the TSS (17).

Finally, our results have important implications for the action of XBP during NER. In the general genome repair pathway, TFIIH is recruited to DNA lesions bound by factor XPC, where it opens an asymmetric ~27-bp DNA bubble surrounding the lesion (18, 19). Both XBP and XPD are required for this DNA unwinding, although XBP does not seem to promote unwinding via a conventional helicase mechanism. Mutations that abolish the XBP ATPase abolish DNA unwinding activity during NER, but two XBP mutations with reduced 3′ → 5′ helicase function were reportedly active for NER (13). Based on these results, it was proposed that XBP functions indirectly in DNA opening by using its ATPase function to promote a conformational change in the XPC–DNA–TFIIH complex, physically wedging open 5 bp of the DNA duplex, and positioning the XPD helicase to open the DNA surrounding the DNA lesion (10, 18, 19). Given our results, it seems more likely that XBP opens 5 bp of DNA using a dsDNA translocase mechanism similar to that in OC formation. By generating torsional strain in rotationally fixed damaged DNA, the XBP subunit of TFIIH can lead to initial unwinding, generating a substrate for XPD to generate the fully opened 27-bp asymmetric bubble surrounding DNA lesions.

Materials and Methods

DNA Helicase Assay. TFIIH helicase activity was monitored using a fluorescent dye-labeled oligonucleotide (IR700-TTCACCAGTGAGACGGGCCACACGCG) annealed to PAGE-purified 100-bp oligonucleotides, with the resulting templates having 5′ or 3′ overhangs of 75 bases (Fig. 1). The assay was performed as previously described (36), with the following modifications: 10-μL reactions contained 10 mM Hepes (pH 7.6), 100 mM potassium glutamate, 10 mM magnesium acetate, 3.5% (vol/vol) glycerol, 1 mM DTT, 1 μg BSA, 60 fmol holo-TFIIH, and 30 fmol template DNA. ATP or ATPγS was added to 1 mM, and reactions were incubated 1–2 h at 26 °C. Controls received TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) in lieu of ATP or were heated to 95 °C for 30 s. Reactions were quenched by the addition of 10 μL 33% glycerol, 40 mM EDTA, and 0.5% SDS and analyzed by PAGE using 10% acrylamide gels in 0.5× TBE buffer (134 mM Tris, 44 mM boric acid, 2.5 mM EDTA, pH 8.8) plus 0.1% SDS. Following electrophoresis, gels were visualized using an Odyssey IR scanner (LI-COR).

![Fig. 6. dsDNA translocase model for open complex formation. The Ssl2 subunit of TFIIH tracks in the 5′ → 3′ direction on the non-template promoter DNA strand (red). Because TFIIH movement is constrained due to interaction with other PIC components, translocation results in insertion and rotation of promoter DNA into the Pol II cleft, leading to DNA unwinding (right arrows indicate rotation and direction of dsDNA movement). The short persistence length of Ssl2/TFIIH predicts that the OC state is unstable, in agreement with experimental observations (9, 10).](image-url)
In Vitro Transcription Assay. In vitro transcription using recombinant and purified factors was performed similarly to previously described assays (32). See SI Materials and Methods for additional information.

TFIH Purification. WT TFIH was purified from strain strain YH869 (RAD3-HA1-TAP tag, tfb63) as previously described (32) except that, following the ultracentrifugation step, potassium acetate was added to the extract to a final concentration of 0.6 M before binding to IgG-Sepharose (GE Healthcare). TFIH with ATPase-defective Rad3 was purified by the same method from strain YH887 (leu2 rad52 Ssl2::NdeI-FLAG, tfb63 H40N) carrying plasmid pJF82 (ars cen LEU2 RAD3 (E236Q)-(HA1)-TAP tag). Because the Ssl2 E489Q mutation is lethal, this TFIH derivative was purified from a WT strain containing the Tag-tailed Ssl2 mutation on a plasmid. Strain YH861 (leu2 Ssl2) carrying plasmid pJF62 (ars cen LEU2 sssl (E489Q)-(FLAG1)-TAP tag) was grown in glucose complete media lacking leucine, and TFIH was purified by the method described above.

Triplex Disruption Assay. Triplex DNA template formation and disruption reactions were performed as previously described (28), with the following modifications: templates were assembled from duplex DNA containing a triplex target sequence (AAAAGAAAAGAAAGAAAAGAAA) and a fluorescent or 32P-labeled TFO (TCTTTTCTCTTCTTTCTTT). The DNA and TFO were combined at 1 μM final concentration in 25 mM Mes (pH 5.5) and 10 mM MgCl2, heated to 57 °C for 15 °C and then cooled at 1 °C/min over 35 min to allow annealing. Triplex DNA was stored at -20 °C and diluted in 50 mM Tris HCl, pH 8.0, 10 mM MgCl2, and 1 mM DTT before the assay. Ten-microliter reactions contained 10 mM Heps (pH 7.6), 100 mM potassium glutamate, 10 mM magnesium acetate, 3.5% glycerol, 1 mM DTT, 1 μg BSA, 0.01% Nonidet P-40, 15 fmol holo-TFIH, and 30 fmol triplex DNA. ATP or dATP was added to 1 mM, and reactions were incubated at 26 °C for the indicated times before stopping with 2.5 μL 5x GSBM (15 mM glycine, 3% SDS, 250 mM Mops, pH 5.5, and 0.04% brophenol blue). The reactions were analyzed by PAGE using 6% acrylamide gels with buffer: 40 mM Tris-acetate pH 5.5, 5 mM sodium acetate, and 1 mM magnesium chloride. Gels were visualized using either an Odyssey IR scanner (LI-COR) or dried and visualized by Phosphorimager (Molecular Dynamics).

ATPase Assay. DNA-dependent ATPase activity of TFIH (Rad3-E236Q) was measured using a colorimetric assay kit (Innovaa, 601-0120). Forty-microliter reactions contained 10 mM Heps (pH 7.6), 100 mM potassium glutamate, 10 mM magnesium acetate, 3.5% glycerol, 1 mM DTT, 4 μg BSA, 40 fmol holo-TFIH, and 1.25 μM to 1.5 μM template DNA. After 40 min at room temperature, purified ATP was added to 0.5 mM, and reactions were incubated 1–20 min at 26 °C. Reactions were stopped by the addition of 10 μL gold mix and, after 4 min, 4 μL stabilizer Z. After 30 min at room temperature, absorbance was measured at 653 nm and plotted against DNA concentration. A standard curve was established for every experiment using the kit-included phosphate standard and used to determine TFIH catalyzed ATP hydrolysis. Templates from 30 to 80 bp were tested at nine concentrations in triplicate, spanning a 1,200-fold range of DNA concentration. See SI Materials and Methods for information on extracting transcription kinetic parameters from the ATPase data.

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