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## Uncoupling promoter opening from start site scanning

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### SUMMARY

Whereas RNA polymerase II (pol II) transcription start sites (TSSs) occur about 30–35 bp downstream of the TATA box in metazoans, TSSs are located 40–120 bp downstream in *S. cerevisiae*. Promoter melting begins about 12 bp downstream in all eukaryotes, so pol II is presumed to “scan” further downstream before starting transcription in yeast. Here we report that removal of the kinase complex TFIK from TFIH shifts the TSS in a yeast system upstream to the location observed in metazoans. Conversely, moving the normal TSS to an upstream location enables a high level of TFIK-independent transcription in the yeast system. We distinguish two stages of the transcription initiation process: bubble formation by TFIH, which fills the pol II active center with single stranded DNA; and subsequent scanning downstream, also driven by TFIH, which requires displacement of the initial bubble. Omission of TFIK uncouples the two stages of the process.

### Graphical abstract

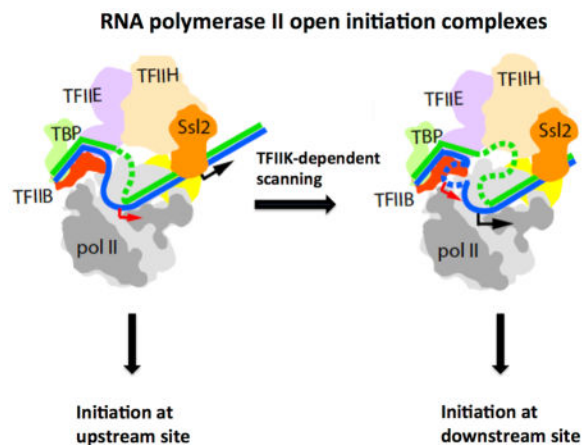
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#### Author Contributions

K.M. and R.D.K. designed the experiments. K.M., P.-J.M., R.E.D. purified proteins. K.M., R.E.D., H.J., and C.D.K. performed the experiments. K.M. and R.D.K. wrote the paper.

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## INTRODUCTION

RNA polymerase II (pol II) assembles with five general transcription factors (GTFs) in a pre-initiation complex (PIC) on promoter DNA (Conaway and Conaway, 1993; Kornberg, 2007). The largest GTF, TFIIH, comprises the helicase Ssl2, a six-subunit core complex, and a three-subunit kinase termed TFIID (Gibbons et al., 2012; Schaeffer et al., 1993; Schultz et al., 2000; Svejstrup et al., 1995). Ssl2 unwinds the promoter duplex to form a “transcription bubble” (Holstege et al., 1997; Kim et al., 2000; Pal et al., 2005; Fishburn et al., 2015), starting about 12 bp downstream from the TATA box, and in metazoans, pol II initiates transcription about 30 bp downstream; in *S. cerevisiae*, pol II scans further downstream and initiates 40–120 bp from the TATA box. Start site recognition is due in part to properties of pol II (Li et al., 1994; Yang and Ponticelli, 2012; Zhang et al., 2014), but the basis for scanning is unknown. The initiation of transcription is accompanied by phosphorylation of the C-terminal domain (CTD) of pol II (Feaver et al., 1994; Laybourn and Dahmus, 1990; Roy et al., 1994), due to the action of TFIID. Kinase activity is, however, dispensable for basal transcription *in vitro* (Li and Kornberg, 1994; Serizawa et al., 1993). Here we report on a requirement for TFIID for initiation at downstream sites, and thus a likely role of TFIID in promoter scanning.

## RESULTS

### Omission of TFIID shifts transcription initiation upstream

TFIIH bearing a TAP tag on the Tfb4 subunit was purified from a yeast strain lacking the *TFB6* gene as described (Murakami et al., 2012). TFIIH bound to IgG-Sepharose through the TAP tag was stripped of TFIID by washing with high salt (300 mM potassium acetate plus 400 mM ammonium sulfate); following elution and gel filtration, essentially pure TFIIH devoid of TFIID (TFIIH- TFIID) was obtained (Fig. 1A). TFIIH- TFIID in combination with pol II and other general transcription factors proved capable of assembly in a complete pre-initiation complex on a *HIS4* promoter fragment as described (Murakami et al., 2013b), and also of transcription initiation (Fig. 1B). In the presence of TFIIH-

TFIID, however, run-off transcripts were longer than expected (indicated by red arrow in Fig. 1B). Transcription required TFIIH- TFIID (Fig. 1B), TBP (Fig. 1D) and TFIIB (Fig.

S1B), confirming promoter-dependence of the reaction. When TFIK was added back, the expected distal TSS utilization was restored (indicated by black arrows in Fig. 1B) with a concurrent decrease in proximal site use, and a 4-fold increase in total run-off transcription; the same result was obtained when TFIK bearing a mutation in the Kin28 subunit (TFIK-L83G) that confers sensitivity to the kinase inhibitor NA-PP1 (Liu et al., 2004) was added to the reaction in the presence of the inhibitor (Figs. 1C, S1A). Evidently TSS determination is dependent on a feature of TFIK other than the kinase activity. Similar results were obtained with two additional promoters tested (Fig. 1D). Primer extension analysis showed that in the presence of TFIK, transcription was initiated 63–103 bp downstream of the TATA box, whereas in the absence of TFIK, transcription was initiated 31–34 bp downstream of the TATA box (summarized in Fig. S2), a location characteristic of transcription initiation in metazoans rather than *S. cerevisiae* (Ponjavic et al., 2006). TFIIS had a slight effect on TSS utilization, and TFIIA and Mediator had no effect (Fig. S1D–E). All the other general transcription factors were required.

### Transcription from an upstream start site does not require TFIK

If omission of TFIK shifts transcription initiation to sites upstream, then it may be dispensable for transcription of a promoter whose TSS is normally located upstream. We created such a promoter by deletion of downstream DNA. We brought the TSS of the *SNR20* promoter from the wild type location 91 bp downstream of the TATA box (Fig. 2A, Fig. S2, fragment 91W) to locations 29, 31, and 33 bp downstream (Fig. 2A, Fig. S2, fragments 29D, 31D, 33D); a C+4G mutation was introduced in the TSS to yield only one rather than multiple closely spaced initiation sites. Transcription initiation on fragment 91W was diminished and shifted upstream by the omission of TFIK (Fig. 2B, lanes 4 and 8), whereas initiation on fragments 31D and 33D was at the same high level (0.25 transcripts per pol II) in the presence or absence of TFIK (Fig. 2B, lanes 2, 3, 6, 7). It is noteworthy that in the presence of TFIK, initiation on fragments 31D and 33D was mostly 31 or 33 bp downstream of the TATA box, the distance characteristic of metazoan transcription, and to a lesser extent about 60 bp downstream (Fig. 2B, lanes 6, 7, 10, 11), whereas, in the absence of TFIK, initiation was exclusively 31 or 33 bp downstream (Fig. 2B, lanes 2, 3), confirming a role of TFIK in initiation at further downstream sites. Initiation on fragment 29D was markedly diminished, further resembling metazoans, in which a 30–31 bp spacing between the TATA box and TSS is most frequent and a spacing shorter than 29 bp is rarely observed (Ponjavic et al., 2006).

The upstream TSS usage in yeast was not previously observed in the nuclear extract system (Lue et al., 1989) or *in vivo* (Faitar et al., 2001). We determined if the TFIK effect on TSS utilization might be revealed *in vivo* by nuclear depletion of Kin28, using the “anchor-away” method (Haruki et al., 2008; Jeronimo and Robert, 2014; Wong et al., 2014). We failed to observe shifting in TSS positions at *ADH1* or *SNR20* (Fig. S3). These results suggest that TFIK-subunit activity (Ccl1 or Tfb3) may still be intact when Kin28 is depleted, or that additional factors redundantly enforce downstream TSS usage.

## TFIIK affects downstream DNA conformation of PIC as determined by exonuclease III digestion

Exonuclease III mapping of the PIC revealed barriers to digestion at about position -9 relative to the TSS in the presence of TFIIK (Fig. 3, black arrow) and at about position -14 in the absence of TFIIK (Fig. 3, red arrow). No such barriers were observed in complexes formed without TFIIE and TFIIF, showing the barriers are characteristic of a complete PIC. The barriers are located on the opposite (downstream) side of the transcription bubble from sites of interaction with TFIIB (Pardee et al., 1998), TFIIF (Chen et al., 2010; Ghazy et al., 2004) and pol II (Kaplan et al., 2012; Majovski et al., 2005), shown by genetic analysis to be involved in TSS utilization. It is noteworthy that omission of TFIIK had a much greater effect on TSS utilization than a TFIIB mutation (E62K) known to confer a downstream TSS shift (Pinto et al., 1994) (Fig. S1B–C).

## DISCUSSION

The difference between initiation of pol II transcription about 30 bp downstream of the TATA box in metazoans and initiation further downstream in *S. cerevisiae* is commonly attributed to scanning downstream by pol II before initiation in *S. cerevisiae* (Giardina and Lis, 1993). Our findings point to a role of TFIIK in scanning: initiation is shifted upstream in the absence of TFIIK; and movement of a downstream TSS to an upstream location obviates the requirement for TFIIK. Exonuclease protection provides a physical basis for involvement of TFIIK in scanning.

Previous studies are consistent with the participation of TFIIK in TSS determination. A minimal PIC containing TBP, TFIIB, and pol II is sufficient to initiate transcription from artificial bubble templates (Holstege et al., 1995; Parvin and Sharp, 1993), but fails to support proper TSS utilization (Fishburn and Hahn, 2012). TFIIF is required not only for promoter opening but also for subsequent events, including initiation and the transition to RNA chain elongation (Bradsher et al., 2000; Dvir et al., 1997; Spangler et al., 2001). All polypeptides of the closed PIC are retained following promoter opening (Murakami et al., 2013a). The helicase subunit of TFIIF (Ssl2, yeast homolog of XPB, Fig. 4) is an integral component of the PIC and is almost certainly the molecular motor for scanning (Fazal, F. M., Meng, C. A., Block, S. M., personal communication). TFIIK must play an auxiliary role.

From the combination of our functional studies with previous structural information, we arrive at a picture of the initiation process (Fig. 4). Initial melting through the action of TFIIF draws some 24 bp of DNA into the polymerase cleft, filling the single stranded DNA-binding region of the active center. If a TSS is recognized in the single stranded bubble, transcription begins, an RNA-DNA hybrid helix develops, TFIIB is ejected as proposed (Bushnell et al., 2004; Liu et al., 2010), and transcription elongation ensues. Alternatively, if no TSS resides within the initial single stranded bubble, then further action of TFIIF drives scanning downstream. Additional single stranded DNA entering the pol II cleft must displace that which was bound before, through sliding and the formation of a larger bubble. Evidently TFIIK is necessary for this process, interacting with the DNA either directly (as suggested by exonuclease III mapping) or indirectly (for example, by bridging between TFIIF and pol II). In the absence of TFIIK, the DNA may disengage from the entrance of

the pol II channel and its unwinding by the TFIIF helicase may not be coupled to the delivery of a TSS to the active center of pol II (Fig. S4).

## EXPERIMENTAL PROCEDURES

### Protein purification

TFIIA, TFIIB, TBP, and TFIIS were available in recombinant form, and TFIIF, TFIIFH-TFIIK, and pol II were isolated from yeast as previously published (Murakami et al., 2013a; Murakami et al., 2012).

For the isolation of TFIIK or TFIIK-L83G, yeast strain CB010 (Murakami et al., 2012) or SHY532 (Liu et al., 2004) harboring TAP-tagged Tfb3 was grown in 100L YPAD. The yeast culture was harvested at OD 7.0–8.0 and lysed by disruption with glass beads in buffer (400) (50 mM HEPES (pH7.6), 1 mM EDTA, 5% glycerol, 0.1% 3-(decyldimethylammonio) propanesulfonate (Sigma), with the mM concentration of potassium acetate in parentheses) supplemented with 1 mM benzamidine, 100  $\mu$ M leupeptin, 10  $\mu$ M pepstatin A, and 1 mM PMSF. The cell extract was stirred with 100 mM ammonium sulfate and 0.2 % polyethyleneimine (PEI) for 1 hour, centrifuged, loaded onto an IgG column, and washed with buffer (300). A mixture of holo-TFIIFH and TFIIK was eluted using buffer (300) after incubating with TEV protease for 15 hours at 4°C, and loaded onto UnoQ column (Bio-Rad). The flow-through fraction containing TFIIK was concentrated, and further purified by using Superdex 200 16/60 (GE healthcare) in a buffer containing 20 mM HEPES (pH7.6), 300 mM potassium acetate, 5 mM DTT, 10% glycerol, and 0.1% 3-(decyldimethylammonio) propanesulfonate.

For the isolation of TFIIE, *S. cerevisiae* harboring the TAP-tagged Tfa2 (Borggreffe et al., 2001) was grown in 100L YPAD. The yeast culture was harvested at OD 8.0–10.0 and lysed by disruption with glass beads in a lysis buffer (50 mM HEPES (pH7.6), 250 mM ammonium sulfate, 1 mM EDTA, 5% glycerol, 3 mM DTT, 1 mM benzamidine, 100  $\mu$ M leupeptin, 10  $\mu$ M pepstatin A, and 1 mM PMSF). The cell extract was stirred with 0.2 % PEI for 30 min, centrifuged, and was loaded onto an IgG column. The column was washed with buffer 300 (50 mM HEPES (pH7.6), 300 mM potassium acetate, 1 mM EDTA, 5% glycerol, 2.5 mM DTT, 1 mM benzamidine, 100  $\mu$ M leupeptin, 10  $\mu$ M pepstatin A) containing 400 mM ammonium sulfate, washed with buffer 300, and eluted after incubating with TEV protease for 15 hours at 4°C. The IgG elution was further purified by using Superose 6 XK 16/70 (GE healthcare) in a buffer containing 20 mM HEPES (pH7.6), 300 mM potassium acetate, 5 mM DTT, and 2 mM magnesium acetate.

For the purification of Mediator, yeast strain CB010 harboring TAP-tagged Med7 was grown in 16L YPAD. The yeast culture was harvested at OD 10–12 and lysed by disruption with zirconium beads in HGE(200) (50 mM HEPES (pH7.6), 5% glycerol, 1 mM EDTA with the mM concentration of ammonium sulfate in parentheses supplemented with 5 mM dithiothreitol, 1 mM benzamidine, 100  $\mu$ M leupeptin, 10  $\mu$ M pepstatin A, and 1 mM PMSF). The cell lysate was centrifuged in a JA-14 rotor at 8,000 RPM for 20 minutes. The soluble lysate was extracted with 0.25% PEI for 1 hour then centrifuged at 14,000 RPM for 45 minutes. The PEI soluble extract was further extracted with a 65% ammonium sulfate cut.

After the ammonium sulfate pellet was dissolved in 15% the original cell slurry volume of HGE(0), it was loaded onto an 8 ml IgG Fast Flow 4 column at ten column volumes per hour. After washing the IgG column with 20 column volumes of HGE(250), Mediator was eluted overnight (4°C) in 25 mM HEPES, 5% glycerol, 75 mM ammonium sulfate, and 2 mM dithiothreitol with 15 µg/ml of TEV protease. Eluted Mediator was applied to a 1ml HiTrap Q column (GE healthcare) and was eluted in a linear gradient from 75 mM to 1000 mM ammonium sulfate gradient in 25 mM HEPES, 5% glycerol, and 2 mM dithiothreitol. Peak fractions containing Mediator between 150 and 200 mM ammonium sulfate were pooled and concentrated to 3.1 mg/ml.

## DNA templates

For a series of *SNR20* spacing promoters, *SNR20* (-122/+297) was subcloned into the pDrive Cloning Vector using forward (5'-CCC GGGCTGCAGGCCGTTTCCGATGGG-3') and reverse (5'-CCC GGGTAATGAGCCTCATTGAG-3') primers. A C+4G point mutation and a series of deletions were generated by the QuikChange Site Directed Mutagenesis. DNA templates were amplified by PCR, purified using Superose 6 10/300 column (GE healthcare) in a buffer containing 20 mM HEPES (pH7.6), 300 mM potassium acetate, 5 mM DTT, and 2 mM magnesium acetate, and concentrated up to 5–10 µM using Vivacon 500 3K MWCO (Vivaproducts), yielding ~0.3–0.5 nmol from 2 mL PCR reaction.

*HIS4* DNA fragment was prepared as previously published (Murakami et al., 2013a). *SNR19* DNA fragment was amplified from yeast genomic DNA using forward (5'-GATATCCTGCAGCTAAGGCGACGAGTTTTCC-3') and reverse (5'-GATATCGCTTAACGTCCTTCTACTATTGG-3') primers, and purified using Superose 6 10/300 (GE healthcare) in a buffer containing 20 mM HEPES (pH7.6), 300 mM potassium acetate, 5 mM DTT, and 2 mM magnesium acetate.

## Run-off transcription assay

All transcription assays were performed in an *in vitro* reconstituted system including TFIIA, TFIIB, TBP, TFIIE, TFIIF, TFIIH, and pol II unless otherwise indicated. 1.5 pmol of DNA fragment was combined with 3.7 pmol of TFIIA, 3.7 pmol of TFIIB, 1.5 pmol of TBP, 3.7 pmol of TFIIE, 1.5 pmol of pol II-TFIIF complex, and 1.5 pmol of TFIIH- Tfb6 with or without TFIIK in 5 µl of buffer 300 (50 mM HEPES (pH7.6), 300 mM potassium acetate, 5 mM DTT, and 5% glycerol), diluted with 5 µl of buffer 10 (20 mM HEPES (pH7.6), 10 mM potassium acetate, 5 mM magnesium sulfate, and 5 mM DTT), and incubated for more than 1hr on ice. The transcription was initiated by adding an equal volume of 2xNTP buffer containing 20 mM HEPES (pH7.6), 10 mM potassium acetate, 5 mM magnesium sulfate, 10 mM magnesium acetate, 1 unit of RNaseOut, 5 mM DTT, 1.6 mM ATP, 1.6 mM GTP, 1.6 mM CTP, 40 µM UTP, and 0.83 µM [ $\alpha$ -<sup>32</sup>P] UTP (2.5 µCi). The reaction was stopped in 15 min by adding 185 µl of stop buffer (300 mM sodium acetate (pH 5.5), 5 mM EDTA, 0.7% SDS, 0.1 mg/ml glycogen, 0.013 mg/ml of proteinase K (Sigma)). Transcripts were precipitated by adding 700 µL of ethanol, dried, and analysed by a denaturing 6% acrylamide gel.



### ***In vitro* kinase assay of PIC**

*HIS4* (−96/+112) DNA (1.5 pmol) was incubated with 3.7 pmol of TFIIB, 3.7 pmol of TFIIA, 1.8 pmol of TBP, 3.7 pmol of TFIIE, 1.0 pmol of RNA polymerase II-TFIIF complex, and 1.5 pmol of TFIIH- TFIIK with TFIIK or inhibitor-sensitive TFIIK mutant (TFIIK-L83G) (2.0 pmol). Phosphorylation was initiated by adding an equal volume of 2xATP buffer (20 mM HEPES (pH7.6), 10 mM potassium acetate, 5 mM magnesium sulfate, 10 mM magnesium acetate, 1 mM ATP, and 1.0 μCi/μl of [ $\gamma$ -<sup>32</sup>P]). The reaction was stopped in 6 min by adding 4x SDS-PAGE loading buffer and was analyzed by SDS-PAGE.

### **Exonuclease footprinting**

Exonuclease footprinting was performed as described before (Murakami et al., 2013a). Briefly, <sup>32</sup>P-labeled *HIS4* (−96/+112) DNA (1.5 pmol) was incubated with 3.7 pmol of TFIIB, 3.7 pmol of TFIIA, 2.0 pmol of TBP, 3.7 pmol of TFIIE, 2.0 pmol of RNA polymerase II-TFIIF complex, and 2.0 pmol of TFIIH- TFIIK with or without 3.0 pmol of TFIIK in 5 μl of buffer 300 (50 mM HEPES (pH7.6), 300 mM potassium acetate, 5 mM DTT, and 5% glycerol), then combined with 5 μl of buffer 30 (50 mM HEPES (pH7.6), 5 mM magnesium sulfate, 30 mM potassium acetate, and 5 mM DTT), and incubated for more than 1 hr at 4°C. The reconstituted PIC was combined with an equal volume of 2xNTP buffer (1.6 mM NTP(s) or 1.6 mM dATP, 50 mM HEPES (pH7.6), 5 mM magnesium sulfate, 30 mM potassium acetate, 5 mM DTT, 10 mM magnesium acetate, and 5 units of RNaseOUT) and incubated for 4 min at 30°C. Exonuclease III digestion was performed with 5–10 units (NEB) for 9 min at 30°C, and was stopped by adding 185 μl of stop buffer (300 mM sodium acetate (pH 5.5), 5 mM EDTA, 0.7% SDS, 0.1 mg/ml glycogen, 0.013 mg/ml of proteinase K (Sigma), 0.5 mg/ml Salmon Sperm DNA (Invitrogen)). DNAs were precipitated by adding 700 μL of ethanol, dried, and analysed by a denaturing 6% acrylamide gel.

### ***In vivo* analysis of Kin28 function in TSS selection**

*kin28-FRB* or control “anchor-away” yeast strains (Jeronimo and Robert, 2014) were treated with 1 μg/ml Rapamycin (Sigma) for up to 1 hour followed by total RNA isolation and primer extension analysis as previously described (Kaplan et al., 2012). Primer extension products were resolved on denaturing 8% polyacrylamide (19:1 bisacrylamide:acrylamide) gels in 1× TBE. Primer sequences available upon request.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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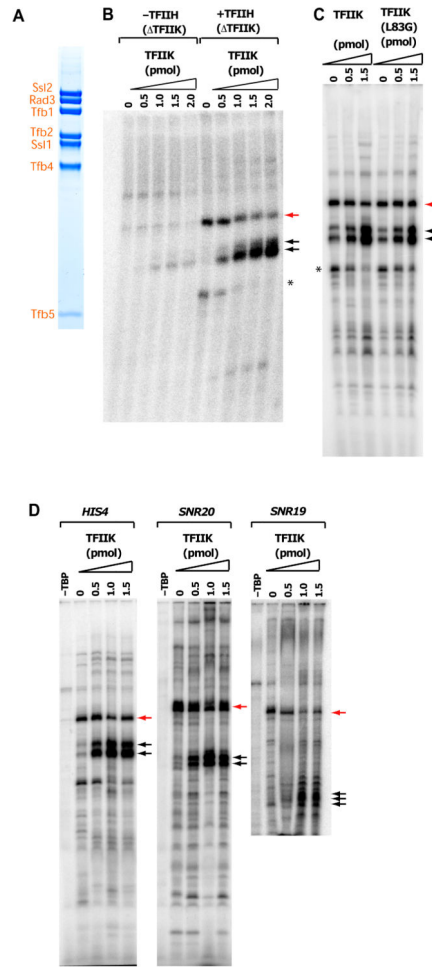


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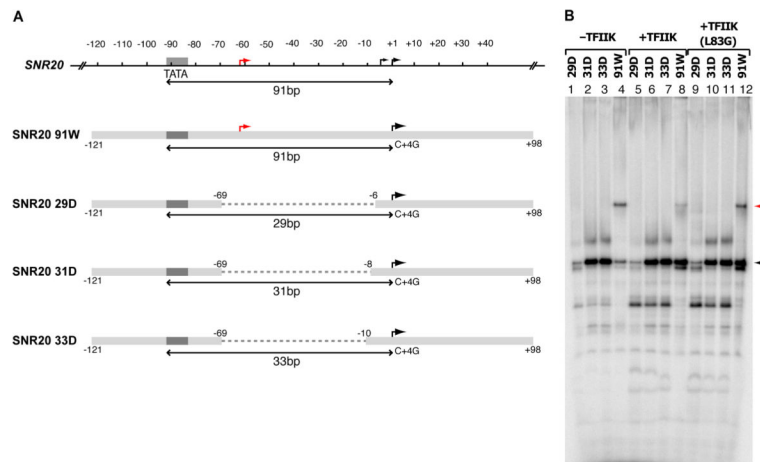
### Highlights

- Omission of TFIIK shifts the TSS in a yeast system to a metazoan location
- Moving the TSS to a metazoan location enables transcription in the yeast system
- Omission of TFIIK uncouples promoter opening from start site scanning



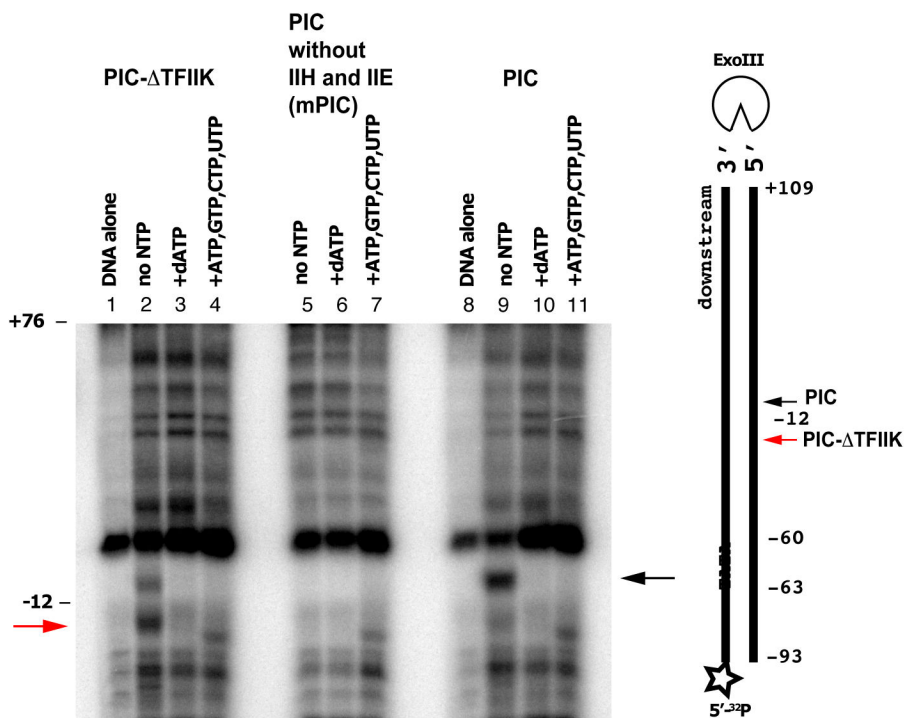
### Figure 1. Role of TFIK in transcription initiation

(A) SDS polyacrylamide gel electrophoresis of purified TFIH- TFIK. (B) Run-off transcription with increasing amounts of TFIK. *HIS4* promoter DNA (-96/+112) (Murakami et al., 2013a) was combined with TFIIA, TFIIB, TBP, TFIIE, TFIH- TFIK, pol II-TFIIF complex, and the amounts of TFIK indicated. Transcripts initiated from upstream and downstream TSSs are indicated by red and black arrows. The asterisk indicates a non-specific product that was not observed by primer extension analysis. (C) Run-off transcription with increasing amounts of TFIK (left panel) or inhibitor-sensitive TFIK mutant (TFIK-L83G) (Liu et al., 2004) (right panel). The reactions were performed as in (B) except with the addition of 50  $\mu$ M NA-PP1. (D) Same as (B) with different promoters. See also Fig. S1.



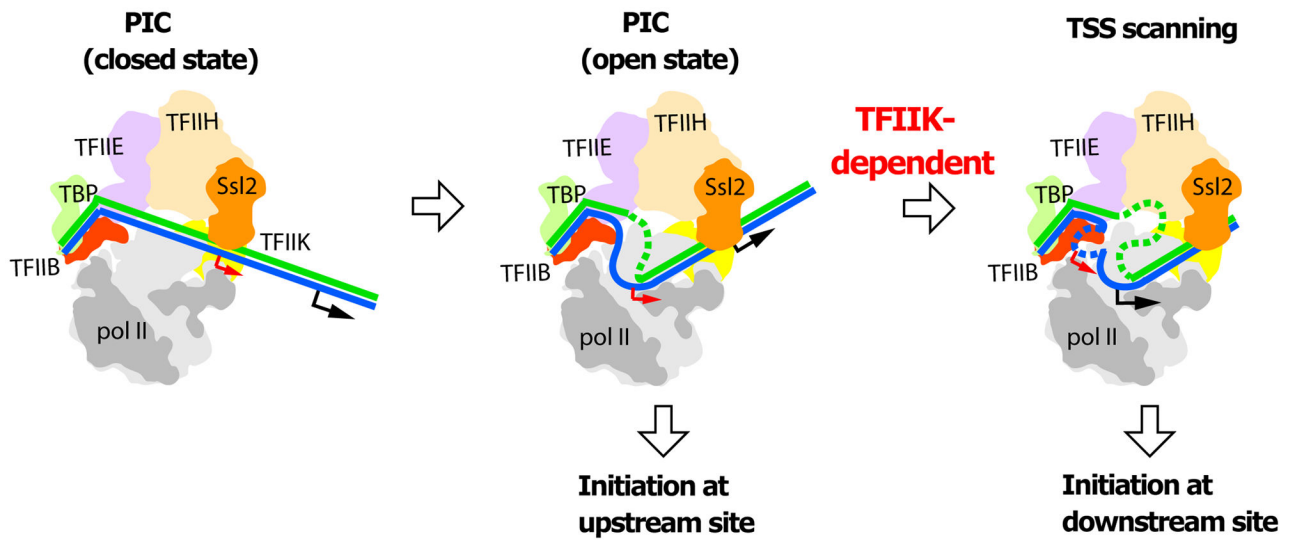
**Figure 2. Run-off transcription with modified promoters**

(A) Schematic illustration of modified promoters (also see Fig. S2). The wild type *SNR20* promoter, the *SNR20* promoter with a C+4G mutation introduced in the TSS (*SNR20 91W*), and the *SNR20* promoter with deletions (indicated by dashed lines) bringing the +1 TSS to positions 29, 31, and 33 bp from the TATA box (*SNR20 29D*, *SNR20 31D*, *SNR20 33D*). (B) Run-off transcription with the modified *SNR20* promoters. For reactions with the inhibitor-sensitive mutant TFIID-L83G, 1.1 mM NA-PP1 was added.



**Figure 3. Exonuclease III Footprints of the PIC and PIC- TFIK**  
<sup>32</sup>P-labeled *HIS4* (-96/+112) DNA was incubated with TFIIA, TFIIB, TBP, TFIIE, TFIIF, TFIK, and pol II-TFIIF complex with or without nucleotides as indicated over the lanes, followed by treatment with exonuclease III and gel electrophoresis. Positions of protected fragments are indicated by black (PIC) and red (PIC- TFIK) arrows.





**Figure 4. Schematic representation of promoter opening and TSS scanning**

Schematic representation of the PIC (left panel) and proposed changes upon promoter opening (middle panel) and TSS scanning (right panel). DNA is blue and green. TFIIB is yellow. Upstream and downstream TSSs are indicated by red and black arrows. See also Fig. S4.