Genome-wide location analysis reveals a role of TFIIS in RNA polymerase III transcription

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TFIIS is a transcription elongation factor that stimulates transcript cleavage activity of arrested RNA polymerase II (Pol II). Recent studies revealed that TFIIS has also a role in Pol II transcription initiation. To improve our understanding of TFIIS function *in vivo*, we performed genome-wide location analysis of this factor. Under normal growth conditions, TFIIS was detected on Pol II-transcribed genes, and TFIIS occupancy was well correlated with that of Pol II, indicating that TFIIS recruitment is not restricted to NTP-depleted cells. Unexpectedly, TFIIS was also detected on almost all Pol III-transcribed genes. TFIIS and Pol III occupancies correlated well genome-wide on this novel class of targets. *In vivo*, some *dst1* mutants were partly defective in tRNA synthesis and showed a reduced Pol III occupancy at the restrictive temperature. *In vitro* transcription assays suggested that TFIIS may affect Pol III start site selection. These data provide strong *in vivo* and *in vitro* evidence in favor of a role of TFIIS as a general Pol III transcription factor.

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Gene transcription is a complex and highly regulated process that, in eukaryotes, is carried out by three specialized RNA polymerases (Pol I, II, and III), dedicated to the transcription of different sets of genes. Transcription starts by the assembly of large preinitiation complexes (PIC) comprising TBP (the TATA-binding protein common to all three transcription systems) and general transcription factors specific to the RNA polymerase considered. Other transcription factors facilitate RNA elongation by Pol II (Shilatifard et al. 2003). One of these elongation factors, TFIIS, was initially discovered in human (Natori et al. 1973) and yeast cells (Sawadogo et al. 1980b), and is highly conserved in the eukaryotic and archaeal kingdoms (Hausner et al. 2000; Fish and Kane 2002). Structurally unrelated but functionally equivalent factors (GreA and GreB) also operate in bacteria (Borukhov et al. 1993; Opalka et al. 2003). TFIIS forms a binary complex with Pol II and stimulates an intrinsic transcript cleavage activity of RNA polymerase allowing elongating enzymes to resume RNA synthesis after accidental transcription arrest (Fish and Kane 2002; Kettenberger et al. 2003).

TFIIS is organized in three domains as determined by

limited proteolysis and nuclear magnetic resonance (NMR) (Awrey et al. 1997; Olmsted et al. 1998). The three-dimensional structure of the Pol II–TFIIS complex, without the TFIIS N-terminal domain, has been determined by crystallographic studies (Kettenberger et al. 2003). The N-terminal domain I of TFIIS protrudes from Pol II and is not required for elongation (Nakanishi et al. 1995; Awrey et al. 1998), but binds the yeast Mediator and SAGA coactivator (Wery et al. 2004). The TFIIS central domain II is inserted into the funnel-shaped pore of Pol II and brings the C-terminal conserved RSADE motif very close to the enzyme active site (Kettenberger et al. 2003). Remarkably, the C-terminal domain III of TFIIS shares the RSADE motif with the C-terminal parts of the Rpa12 (Pol I) and Rpc11 (Pol III) subunits, and is more distantly related to the corresponding Rpb9 subunit of Pol II, which has no RSADE motif. Rpc11 mediates the intrinsic transcription cleavage activity of Pol III (Chedin et al. 1998).

In the yeast *Saccharomyces cerevisiae*, TFIIS is encoded by *DST1*. The *dst1-Δ* mutant, or *dst1* mutants of the RSADE motif, grow like wild type, but are sensitive to the NTP-depleting effects of 6-azauracil (6AU) and mycophenolic acid (MPA), two drugs thought to compromise elongation efficiency (Exinger and Lacroute 1992; Ubukata et al. 2003). In the presence of 6-azauracil, deletion of *DST1* affects Pol II processivity without influencing its elongation rate (Mason and Struhl 2005). TFIIS was also

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found to stimulate transcription past an artificial arrest site *in vivo*, thus further supporting the notion that it overcomes elongation blocks (Kulish and Struhl 2001). Recent reports suggest that, in addition to its well-documented role as a Pol II elongation factor, TFIIS also contributes to transcription initiation (Malagon et al. 2004; Wery et al. 2004; Prather et al. 2005; Guglielmi et al. 2007; Kim et al. 2007). This initiation role does not depend on the C-terminal RSADE motif needed for transcript cleavage stimulatory activity (Guglielmi et al. 2007; Kim et al. 2007).

Although the role of TFIIS in stimulating Pol II transcript cleavage activity is well characterized *in vitro*, its *in vivo* function remains less obvious. In this study, we analyzed the genome-wide location of TFIIS using chromatin immunoprecipitation coupled with DNA microarray hybridization (ChIP–chip). TFIIS was detected on many Pol II-transcribed genes under normal growth conditions, and its occupancy closely correlated with that of Pol II. Unexpectedly, we identified a novel class of TFIIS targets corresponding to Pol III-transcribed genes. *In vitro* Pol III transcription assays suggested that TFIIS may contribute to correct start site selection. Taken together, our *in vivo* and *in vitro* data reveal a previously unsuspected role of TFIIS in Pol III transcription.

Results

TFIIS is located genome-wide on the Pol II- and Pol III-transcribed genes

Previous models proposed that TFIIS protein was recruited during transcription elongation in conditions where Pol II was stalled. This hypothesis was strengthened by ChIP assays, suggesting that TFIIS does not normally reside on DNA but is specifically recruited in the presence of NTP-depleting drugs that favor the transcriptional arrest of Pol II elongation complexes (Pokholok et al. 2002). However, recent ChIP analyses revealed the presence of TFIIS on promoters and coding regions of several selected genes in cells grown under normal conditions (Prather et al. 2005; Guglielmi et al. 2007). To better understand TFIIS function and resolve this discrepancy, we performed genome-wide location analysis of TFIIS under standard conditions, or in cells that have been NTP-depleted in the presence of MPA.

We examined the genome-wide distribution of TFIIS and Pol II by ChIP–chip experiments and compared their relative enrichments. The DNA arrays used contained >40,000 oligonucleotide probes covering 12 Mb of the yeast genome (see the Materials and Methods). This analysis was done using a strain (YGH2) carrying an active N-terminal HA-tagged version of TFIIS. We found 3652 oligonucleotides significantly bound (*P*-value <0.005) by TFIIS under standard conditions. Correlation between TFIIS and Pol II enrichment showed an unexpected pattern (Fig. 1A). The distribution was split into two highly different data sets. A first set of oligonucleotides, located within 1419 Pol II-transcribed genes, showed a good correlation between TFIIS and Pol II enrichment. As dis-

cussed below, a second group of oligonucleotides was enriched for TFIIS but not for Pol II, and corresponded to Pol III-transcribed genes.

The results obtained by ChIP–chip experiments were confirmed by conventional ChIP on a set of selected Pol II- and Pol III-transcribed genes (Fig. 1B). All selected genes displayed a significant enrichment compared to background level measured on the coding region of the nontranscribed *GAL1* gene. Similar results were obtained in ChIP experiments using chromatin from a strain expressing native TFIIS and anti-TFIIS antibodies for immunoprecipitation (data not shown). We wondered whether TFIIS could also be detected on Pol I-transcribed 35S rDNA and Pol III-transcribed 5S rDNA templates. Since the array used had a poor coverage of that region, we performed conventional ChIP experiments on the rDNA locus. A slight enrichment on the 35S rDNA and no significant enrichment on the 5S rDNA were seen compared to the intergenic NTS1 and NTS2 regions (Fig. 1C). Since the binding of TFIIS to transcribed sequences would also be observed if TFIIS was an RNA-binding protein, we tested if the enrichment of TFIIS was dependent on the presence of the transcribed RNA by digestion with RNase A/T1 in ChIP experiments. Digestion did not affect TFIIS enrichment (data not shown). We also examined TFIIS occupancy of a gene transcribed by a heterologous T7 RNA polymerase (Chen et al. 1987). We did not observe any significant TFIIS enrichment (Supplemental Fig. S1), indicating that TFIIS does not localize to transcribed sequences thanks to an RNA-binding property.

Genome-wide TFIIS occupancy on Pol II-transcribed genes correlates with Pol II occupancy

Analysis of Pol II genome-wide occupancy revealed 15,911 oligonucleotides significantly bound (*P*-value <0.005). These probes corresponded to 3819 Pol II-transcribed genes. After removing all the oligonucleotides corresponding to Pol III-transcribed genes from the analysis, there was a fairly linear relationship between TFIIS and Pol II enrichment with a correlation coefficient of 0.642 (Fig. 1A). The example of Pol II and TFIIS enrichment profiles on the *PYK1* gene (Fig. 1D) confirms that TFIIS is present on coding regions even under standard growth conditions (i.e., in the absence of NTP-depleting drugs), and that TFIIS and Pol II occupancies are extremely well correlated. Other examples of enrichment profiles on *ADH1* and *PGK1* genes are shown in Supplemental Figure S2A,B. The presence of TFIIS is not limited to transcribed protein-coding regions, since we detected TFIIS on intergenic regions transcribed in short unstable RNAs. The examples of the cryptic transcript *NELO25C*, located on chromosome V between *RMD6* and *DLD3* genes (Wyers et al. 2005), and of *SRG1*, a transcriptional repressor of *SER3* (Martens et al. 2004), are shown in Supplemental Figure S2C,D. Pol II- and TFIIS-enriched genes were classified according to the biological process categories of gene ontology (GO). A standard hypergeometric test was used to determine the overrepresented

Figure 1. Genome-wide location analysis of TFIIS and Pol II. (A) Enrichment of Pol II versus enrichment of TFIIS. The genome-wide enrichment of Pol II and TFIIS in YPD medium was assessed from ChIP-chip experiments using YGH2 (3HA-TFIIS) strain. A linear regression for Pol II enrichment versus TFIIS enrichment on class II probes and its equation are indicated. Red dots correspond to class III probes. (B) Quantitative ChIP analysis of TFIIS enrichment on selected genes. Immunoprecipitated fragments from ChIP experiments were amplified with primers as indicated in the Supplemental Material. The *GAL1* ORF was used as a control. Error bars represent the standard deviation between at least three biological replicates. The background level was represented by a dotted line. (C) Quantitative ChIP analysis of TFIIS enrichment on rDNA. Immunoprecipitated fragments from ChIP experiments were amplified with primers as indicated in the Supplemental Material. The intergenic NTS1 and NTS2 regions were used as a control. Error bars represent the standard deviation between at least three biological replicates. The background level was represented by a dotted line. (D) Enrichment profile of Pol II and TFIIS on the *PYK1* gene. The enrichment of Pol II (blue) and TFIIS (red) on *PYK1* in YPD medium was assessed from ChIP-chip experiments using YGH2 (3HA-TFIIS) strain. The genomic positions of probe regions on chromosome 1 are indicated along the X-axis and represented by black points or circles. Watson strand-transcribed gene *PYK1* is colored in blue. The enrichment ratio is indicated along the Y-axis.

categories and associated *P*-values. Since the lists of Pol II and TFIIS overrepresented GO categories were largely overlapping (Supplemental Table S1), we concluded that TFIIS was not preferentially bound to a particular group of Pol II-transcribed genes. The smaller number of TFIIS-enriched genes compared to Pol II is best explained by the lower binding levels of TFIIS.

To explore the effect of NTP depletion on TFIIS genome-wide occupancy, yeast cells grown in minimal SD medium were exposed to 10 μ M MPA for 4 h (i.e., just when an effect on growth rate can be observed). We could detect a slight increase of TFIIS enrichment level compared to standard conditions (SD medium), but this effect was far less pronounced than in a previous study

using a different and nonfunctional tagged version of TFIIS (Supplemental Fig. S3A; Pokholok et al. 2002). The enrichment profiles of TFIIS on *PYK1* and *ILV5* genes illustrate that in the presence of mycophenolate, occupancy levels of TFIIS were only slightly increased all along the genes (Supplemental Fig. S3B,C). Note that TFIIS enrichment is much higher in yeast cells growing rapidly in rich medium as compared with minimal medium.

Genome-wide TFIIS occupancy on Pol III-transcribed genes correlates with Pol III occupancy

We compared TFIIS and Pol III occupancies on Pol III-transcribed genes, since a global correlation would imply

that TFIIS might be a new Pol III general factor. A ChIP-chip experiment on Rpc160, the largest Pol III subunit, was performed. We found 964 enriched oligonucleotides located in or close to the class III genes that were described previously in genome-wide location analyses (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004). Four-hundred-thirty-two of the 3652 oligonucleotides significantly bound (P -value <0.005) by TFIIS under standard conditions were within or close (up to 500 bp upstream or downstream) to class III genes. They were significantly enriched by Pol III, and represented all Pol III-transcribed genes for which probes were located on the arrays; i.e., *tDNAs*, *SCR1*, *RPR1*, *SNR52*, and *ZOD1*. *SNR6* showed a low level of TFIIS enrichment on the B box located ~100 bp downstream from transcribed region (Supplemental Fig. S4A). No TFIIS enrichment was detected on the *ETC* loci that are occupied by TFIIB but not by TFIIC and Pol III (Moqtaderi and Struhl 2004). As noted above, the 5S rDNA also showed no detectable enrichment for TFIIS.

The overall correlation between Pol III and TFIIS occupancies (Fig. 2A) confirmed our previous observations on the genome-wide TFIIS distribution. Considering only the oligonucleotides corresponding to Pol III-transcribed genes, the analysis showed a linear relationship with a correlation coefficient of 0.718 (Fig. 2A). The enrichment profile of TFIIS on *SCR1*, a selected class III gene, is presented in Figure 2B. Other examples of TFIIS enrichment profiles on *RPR1* and *tDNA^{Gly}* are shown in Supplemental Figure S4B,C. In these examples, the Pol II-transcribed genes adjacent to class III genes were devoid of TFIIS, strongly suggesting that TFIIS could be recruited independently of Pol II transcription.

To establish that the presence of TFIIS on class III genes was specific to Pol III transcription and independent of Pol II transcription, we used specific Pol II and

Pol III mutations and examined how they affected the distribution of TFIIS. The *rpb1-1* mutant of Pol II rapidly stops transcription after a shift to 37°C (Nonet et al. 1987). When cells were incubated at 37°C for 30 min, Pol II occupancy of all Pol II-transcribed genes tested (*ADH1*, *RPL28*, *RPL17a*, and *ACT1*) was greatly reduced in the *rpb1-1* strain compared to the wild-type strain (Fig. 3A, left panel). TFIIS occupancy strongly correlated with that of Pol II, and was also greatly reduced after incubation at 37°C. Conversely, TFIIS and Pol III occupancies of all Pol III-transcribed genes tested (*tDNAMet*, *tDNAPhe*, *SCR1*, and *RPR1*) remained largely unchanged under the same conditions (Fig. 3A, right panels). These results demonstrated that the presence of TFIIS on Pol III-transcribed genes is independent of Pol II transcription.

The Pol III-specific mutant *rpc25-S100P* was similarly used to impair class III gene transcription. For this purpose, a more prolonged shift to the restrictive temperature (10 h at 37°C) was needed (Zaros and Thuriaux 2005). Under this condition, Pol III and TFIIS occupancies were reduced on class III genes, even though the decrease of TFIIS occupancy was somewhat less pronounced (Fig. 3B, right panel). As expected, no significant effect was observed on Pol II and TFIIS association to the Pol II-transcribed genes (Fig. 3B, left panel). Thus, the presence of TFIIS on class II or III genes is largely dependent on transcription by Pol II or Pol III, respectively.

Most class III genes are too small to allow a spatial resolution of the Pol III machinery location by ChIP, but *SCR1*, the longest Pol III-transcribed gene (522 bp), provided this possibility. We examined the spatial distribution of TFIIS on this gene compared with that of the Pol III basal machinery composed, in addition to Pol III, of TFIIB and TFIIC complexes (Geiduschek and Kassavetis 2001). Seven real-time PCR amplicons spanning the upstream, transcribed, and downstream regions of

Figure 2. Genome-wide location analysis of TFIIS and Pol III. (A) Enrichment of Pol III versus enrichment of TFIIS. The genome-wide enrichment of Pol III and TFIIS in YPD medium was assessed from ChIP-chip experiments with MW671 (3HA-RPC160) and YGH2 (3HA-TFIIS) strains, respectively. A linear regression and its equation are indicated. Red dots correspond to class III probes. (B) Enrichment profile of TFIIS on the *SCR1* gene. The genome-wide enrichment of TFIIS in YPD medium was assessed from ChIP-chip experiments. The genomic positions of probe regions on chromosome 5 are reported along the X-axis and represented by black points. Watson strand-transcribed genes are colored in blue and Crick strand-transcribed genes are colored in green. The enrichment ratio is reported along the Y-axis.

Figure 3. Effect of *rpb1-1* and *rpc25-S100P* mutations on TFIIS, Pol II, and Pol III occupancies on selected genes. Immunoprecipitations were performed using antibodies against 3HA (12CA5) for HA-TFIIS, 13Myc (9E10) for Rpc160-13Myc, and CTD (8WG16) for the Pol II Rpb1 subunit. Immunoprecipitated fragments from ChIP experiments were amplified with primers as indicated in the Supplemental Material. The *GAL1* ORF was used as a control. The values are the average of three independent experiments. Error bars indicate the standard deviation. (A) TFIIS, Pol II, and Pol III occupancies in the *rpb1-1* mutant. Standard ChIP assays were performed on chromatin prepared from the D788-4a strain transformed by *RPB1*-containing pYeB-B220 plasmid (wild type) or by empty vector Yep351 (*rpb1-1*). Cells were grown in selective SD medium complemented with amino acids at 30°C and then shifted for 30 min at 37°C. (B) TFIIS, Pol II, and Pol III occupancies in the *rpc25-S100P* mutant. Standard ChIP assays were performed on chromatin prepared from D792-3a strain transformed by *RPC25*-containing pRS315-RPC25 plasmid (wild type) or by empty vector pRS315 (*rpc25-S100P*). Cells were grown in selective SD medium complemented with amino acids at 30°C and then shifted for 10 h at 37°C.

SCR1 were designed (Fig. 4A). We analyzed the association profiles of the Rpc160 Pol III subunit, the Bdp1 TFIIB subunit, the Tfc1 TFIIC subunit, and TFIIS (Fig.

4B). The distribution of the Pol III transcription machinery on *SCR1* was as described previously (Roberts et al. 2006). TFIIB binding was maximal on the TATA box,

Figure 4. Pol III, TFIIS, TFIIB, and TFIIC location analysis on the *SCR1* gene. (A) Schematic organization of the *SCR1* gene. The location of the PCR fragments amplified in ChIP analyses are indicated by gray and black lines. Black boxes represent TATA, A and B boxes, and Terminator (T). (B) Occupancy profile of Pol III, TFIIS, TFIIB, and TFIIC on the *SCR1* gene. Standard ChIP assays were performed on chromatin prepared from YGH11 strain using antibodies against 3HA-TFIIS (12CA5) and Rpc160-13Myc (9E10), from MW4035 strain using antibodies against Bdp1-3HA (12CA5), from yOH1 strain using antibodies against Tfc1-13Myc (9E10), and from YGH15 strain using antibodies against Rpc11-3HA (12CA5). Cells were grown in YPD medium at 30°C. The *GAL1* ORF was used as a control [c].

while TFIIC was cross-linked over the entire *SCR1* locus. TFIIS distribution on the *SCR1* gene resembled more closely that of Pol III with a maximum at the level of the A and B boxes at the beginning of the gene. However, the TFIIS occupancy profile differed from those of TFIIB and TFIIC. We also examined the association of Rpc11, a Pol III subunit homologous to TFIIS, and showed that the distribution of Rpc11 on the *SCR1* gene was similar to that of Rpc160 subunit (Fig. 4B). Thus, the TFIIS distribution on the *SCR1* gene follows closely that of Pol III, suggesting an active role of TFIIS in class III-gene transcription.

Since TFIIS interacts directly with Pol II, we tested the possible association of this factor with Pol III machinery by coimmunoprecipitation (co-IP) approach. No interaction of TFIIS with Pol III, TFIIC, and TFIIB was detected in crude extracts from noncross-linked cells (Supplemental Fig. S5A). However, when co-IP assays were performed with extracts from cross-linked cells (ChIP extracts), TFIIS

coimmunoprecipitated with Pol III and TFIIC, but not with TFIIB (Supplemental Fig. S5B), in agreement with our ChIP data. We detected TFIIS when Rpc160-Myc or Rpc11-HA were used to immunoprecipitate Pol III. These co-IP results showed a concomitant presence of TFIIS with Pol III and TFIIC on class III genes.

Effect of *dst1* mutations on tRNA synthesis and Pol III occupancy in vivo

Since tRNA suppressor genes are transcribed by Pol III, we tested the influence of deleting *DST1* on suppression efficiency to analyze the role of TFIIS in Pol III transcription in vivo. We therefore compared the growth of a yeast strain containing the chromosomally integrated *SUP11* tRNA^{Tyr}_{UAA} allele suppressing the *ade2-1* ochre mutation with that of the isogenic *dst1-Δ* strain. In the presence of adenine in the growth medium, the *SUP11*

dst1-Δ ade2-1 strain grew as well as the *SUP11 DST1 ade2-1* strain (Fig. 5A, +Ade), but a significant growth difference was observed between the two strains in the medium lacking adenine (–Ade), indicating a decrease in the level of *ade2-1* mutation suppression. This observation suggests that TFIIS could influence Pol III transcription *in vivo*.

In the course of this study, we observed that *dst1-Δ* impaired yeast growth at 16°C (Fig. 5B), a phenotype that has not been described previously. This phenotype was not complemented by a point mutation in the RSADE motif of TFIIS domain III, responsible for the cleavage activity (*dst1-E291A*) (Fig. 5C). This mutant, the most sensitive to cold temperature, actually had a growth defect even at 30°C. In contrast, the *dst1-R200A* mutant in Pol II-binding domain II grew like a wild type. We con-

cluded that the domain III of TFIIS is important for yeast growth at low temperature.

We analyzed the effect of *dst1* deletion on the levels of short-lived tRNA₃^{Leu} precursor at low temperature. Wild-type strain and a *dst1-Δ* mutant were grown at 30°C or shifted for 8 h at 16°C. Total RNAs were extracted and used for Northern blot analysis (Fig. 5D). The amount of mature tRNA₃^{Leu} was used as a loading control. The deletion of *dst1* significantly decreased the tRNA₃^{Leu} precursor levels in yeast cells grown at 16°C (2.1-fold).

To investigate the effect of *dst1* mutations on class III gene expression under low-temperature conditions (16°C) in more details, we performed a genome-wide analysis of Pol III transcriptome (Ciesla et al. 2007). Log₂ ratios of class III gene transcription levels in the mutant versus

Figure 5. In vivo effect of TFIIS on Pol III transcription. (A) Growth of the YGH10 (*SUP11 dst1-Δ ade2-1 ochre*) strain relative to the MB159-2c (*SUP11 DST1 ade2-1 ochre*) strain. Cells were grown at 30°C for 3 d on minimal medium supplemented (+Ade) or not (–Ade) with adenine. (B) Growth of the *dst1*-null mutant at 30°C or 16°C. Suspensions of YPH500 (wild-type) or ESH1 (*dst1-Δ*) strains were serially diluted, spotted on YPD-rich medium, and grown for 3 d at 30°C or at for 5 d 16°C. (C) Growth of *dst1* mutants at 30°C or 16°C. *dst1-Δ* (YGH12) strain was transformed by pRS425-pPGK vector, pRS425-DST1, pRS425-*dst1R200A*, or pRS425-*dst1E291A* plasmids bearing different *DST1* alleles. Cells were grown in liquid SC-leucine medium at 30°C or shifted at time 0 at 16°C. (D) Northern blot analysis of tRNA₃^{Leu} abundance in wild-type and *dst1-Δ* strains. RNAs were extracted from wild-type (ESH1/pRS425-DST1) and *dst1-Δ* (ESH1/pRS425-pPGK) cells grown in SC-leucine medium at 30°C or shifted at 16°C for 8 h. tRNA precursors abundance was quantified, and fold decrease of transcription in *dst1-Δ* strain was compared with that in wild type grown at the same temperature (arbitrary set to 1). Average values of three independent experiments and standard deviations (SD) are indicated. (E) Analysis of the Pol III transcriptome at 16°C. RNAs were extracted from the *dst1-Δ* (ESH1) strain transformed by pRS425-pPGK vector, pRS425-DST1, pRS425-*dst1R200A*, or pRS425-*dst1E291A* plasmids grown at 30°C or shifted for 8 h to 16°C, and analyzed by hybridization to Pol III-specific microarrays. Expression log₂ ratios of class III genes in the mutant strains compared with wild type are presented according to the red-green color scale.

wild-type strains were hierarchically clustered (Fig. 5E). At 30°C, the Pol III transcript profiles of the *dst1-Δ* and the wild-type strains were similar. In contrast, a significant decrease of several class III transcripts was observed in *dst1-Δ* mutant at 16°C. We found that transcription levels of class III genes were strongly diminished at 16°C in the *dst1-E291A* strain, whereas the *dst1-R200A* mutant had a Pol III transcript profile similar to that of the wild type. The *dst1-E291A* mutant had more pronounced effects on Pol III transcription than the *dst1-Δ* strain, consistent with its slower growth at all temperatures. Curiously, class III genes showing the most reduced transcript levels were different in *dst1-Δ* and *dst1-E291A* mutants.

To examine the effect of the *dst1-Δ* mutation on genome-wide Pol III occupancy at low temperature (16°C), wild-type and *dst1-Δ* strains containing a C-terminal 13Myc tag on the Rpl60 Pol III subunit were grown in YPD-rich medium at 30°C and then shifted to 16°C for 8 h. At low temperature, all class III genes were bound by Pol III. We compared Pol III genome-wide occupancy in a *dst1-Δ* and a wild-type strain grown at 16°C (Fig. 6A). A general reduction of Pol III occupancy in the *dst1-Δ* mutant was observed, suggesting that TFIIS could stabilize Pol III on class III genes. Regression analysis indicated a 1.5-fold decrease of Pol III binding in the mutant (the slope of the correlation line, shown in red, equal to 0.645) with a high correlation coefficient ($R^2 = 0.965$). The high correlation coefficient indicated that the association of Pol III to almost all class III genes was significantly reduced. A similar analysis was performed to examine the effect of *dst1* deletion on Pol II genome-wide occupancy at low temperature (Fig. 6B). The binding of Pol II was found to be reduced 1.6-fold in the mutant (the slope of the correlation line equal to 0.621) with a reduced correlation between wild-type and *dst1-Δ* strains ($R^2 = 0.647$). In the case of Pol II, the *dst1* deletion could have indirect effects on gene expression regulation that may explain the lower correlation coefficient between the wild-type and the mutant strains. Examples of enrichment profiles on specific genes are shown in Supplemental Figure S6. The overall reduction of Pol II and Pol III occupancies that was observed at low temperature when *dst1* was deleted suggested that TFIIS could stabilize Pol II and Pol III on their target genes.

We extended our genome-wide Pol III and Pol II location analysis to the *dst1-E291A* and *dst1-R200A* point mutants grown at 16°C. The binding of Pol II was found to be reduced 1.4-fold in the *dst1-E291A* mutant and 1.3-fold in the *dst1-R200A* mutant (the slope of the correlation lines equal to 0.723 and 0.788, respectively) (Fig. 6D,F). As expected, both *dst1* mutations affected Pol II occupancy. In contrast, the binding of Pol III was significantly diminished (3.1-fold) in the *dst1-E291A* mutant, but not at all in the *dst1-R200A* mutant (the slope of the correlation lines equal to 0.325 and 1.088, respectively) (Fig. 6C,E). Thus, the *dst1-E291A* mutant affected both Pol II and Pol III binding, whereas the *dst1-R200A* mutant had a small but significant effect only on Pol II association.

We further analyzed the occupancy of the Bdp1 TFIIB subunit and the Tfc1 TFIIC subunit in *dst1-Δ* and wild-type strains on several class III genes at 16°C (Fig. 6G,H). The association of TFIIC was unchanged, but the binding of TFIIB was reduced in the *dst1-Δ* mutant compared with the wild type.

TFIIS affects Pol III transcription in vitro

To examine the role of TFIIS in Pol III transcription in vitro, we expressed wild-type TFIIS and the TFIIS-E291A mutant form that is unable to stimulate Pol II elongation (Ubukata et al. 2003) as 6xHis fusion proteins in *Escherichia coli*, and purified the corresponding polypeptides to near homogeneity. We first checked that the wild-type TFIIS was active in stimulating nonspecific Pol II transcription on calf thymus DNA (Sawadogo et al. 1980b), while the TFIIS-E291A protein was not (data not shown). The effect of the wild-type and the E291A mutant TFIIS on Pol III transcription in vitro were examined in a Pol III transcription system reconstituted with all recombinant TFIIC and TFIIB and highly purified Pol III (Ducrot et al. 2006). Multiple-round transcription assays were performed with the *SUP4* tRNA gene as a template. As observed previously, the *SUP4* transcripts generated with TFIIB recombinant components migrated as two or three diffuse bands on polyacrylamide gels (Fig. 7A, lane 1), which was not the case in the presence of the crude fraction B' (Andrau and Werner 2001). This fraction contains additional factors, like Nhp6 (Braglia et al. 2007), that can restore transcriptional initiation specificity (Kassavetis and Steiner 2006). Western blotting analysis also revealed the presence of TFIIS in the B' fraction (data not shown). Remarkably, adding the purified wild-type TFIIS to the reconstituted transcription system resulted in the formation of the correct length transcript (Fig. 7A, lanes 2–6), in contrast to the purified TFIIS-E291A mutant protein at the same concentration (Fig. 7A, lanes 7–11). The same results were obtained after mixing wild-type and mutant TFIIS preparations, indicating that the mutant TFIIS preparation did not contain interfering components (Fig. 7A, lane 12). Quantification of the transcription signals revealed that the total amount of transcripts did not significantly change in the presence of wild-type or E291A mutant TFIIS. High concentrations of TFIIS (40 ng/μL) started to inhibit the transcription (Fig. 7A, lanes 6–11). In the subsequent assay, we used 5 ng/μL TFIIS.

To demonstrate that TFIIS could contribute to correct start site selection, RNAs generated in a standard multiple-round Pol III transcription assay were purified and analyzed by primer extension. Figure 7B showed that, in the absence of TFIIS, transcription initiation occurred at base pairs +1, +4, and +8 relative to the start site used in vivo. The addition of B' fraction or of wild-type TFIIS restored a correct start site selection, while the TFIIS-E291A mutant had no effect. Similar results were obtained on transcripts produced in a single-round assay (Supplemental Fig. S7A). The transcription start sites were analyzed in vivo on *SUP4* gene in a *nhp6a-Δ nhp6b-Δ* con-

Figure 6. Pol II and Pol III genome-wide enrichment in TFII mutants at 16°C. The genome-wide enrichment of Pol III (A,C,E) or Pol II (B,D,F) in YPD medium (A,B) or SC-leucine medium (C–F) at 16°C was assessed from ChIP–chip experiments. A linear regression (red line) and its equation are indicated. The dotted line corresponds to $y = x$. Enrichment of Pol III or Pol II in *dst1-Δ* (YGH12) was compared with the wild-type strain (YGH11) (A,B). Enrichment of Pol III or Pol II in *dst1-E291A* (YGH12/pRS425-*dst1E291A*) (C,D) and *dst1-R200A* (YGH12/pRS425-*dst1R200A*) (E,F) strains was compared with the wild-type strain (YGH12/pRS425-DST1). (G,H) Quantitative ChIP analysis of TFIIC and TFIIB enrichment on selected class III genes. *dst1-Δ* and wild-type strains were grown in YPD medium at 30°C and shifted for 8 h to 16°C. Immunoprecipitations were performed using antibodies against 13Myc (9E10) for Tfc1-13Myc and 3HA (12CA5) for Bdp1-3HA. Immunoprecipitated fragments from ChIP experiments were amplified with primers as indicated in the Supplemental Material. The *GAL1* ORF was used as a control. Error bars represent the standard deviation between at least three biological replicates.

text and in the presence or absence of *DST1* gene. The *nhp6* mutant background was used because these proteins were previously implicated in start site selection and

could mask the effect of *dst1-Δ*. We could not identify any effect of *dst1* deletion on start site selection in this background (Supplemental Fig. S7B).

Figure 7. TFIIS stimulates faithful Pol III transcription in vitro. (A) Effect of TFIIS on Pol III transcription. Standard in vitro Pol III transcription on the *SUP4* template has been performed as described in the Materials and Methods, in the absence (lane 1) or presence of increasing quantities (0.1, 0.2, 0.4, 0.8, and 1.6 μ g) of wild-type TFIIS (lanes 2–6) or mutant TFIIS–E291A (lanes 7–11) or with a mix of wild-type and E291A protein (M, lane 12). Transcription start sites are indicated. (B) Primer extension analysis of start sites used by Pol III on the *SUP4* gene in vitro. Transcription reactions were performed in the absence of TFIIS, in the presence of wild-type TFIIS, mutant TFIIS–E291A, or with a B' fraction. Transcription and primer extensions were performed as described in Materials and Methods using a probe hybrid-

izing within the *SUP4* intron. Positions of the major transcription start site are indicated. (C) Time-course analysis of RNA cleavage by Pol III. Pol III (lanes 1–14) or Pol III Δ (lanes 15–21) (Chedin et al. 1998) ternary complexes formed in the presence of 3XTPs were isolated on Sepharose CL-2B as described in the Supplemental Material and then incubated for 10 min with a 50 M excess of purified TFIIS (lanes 8–21) or without TFIIS (lanes 1–7). Ternary complexes were then incubated at 16°C in transcription buffer containing 5 mM MgCl₂ in the absence of nucleotides for various periods of time. The transcript sizes are indicated. Transcription with Pol III Δ results in the formation of an 18-mer RNA instead of a 17-mer for the wild-type enzyme (Chedin et al. 1998).

To examine whether TFIIS could affect the elongation step of Pol III transcription, the elongation kinetics of Pol III were analyzed on a *SUP4* template that can produce a stalled ternary complex after the synthesis of a 17-mer transcript in the absence of GTP. The ternary complex was allowed to resume transcription by adding the four NTPs. No significant changes in elongation kinetics were observed in the presence or absence of purified wild-type TFIIS (Supplemental Fig. S8). TFIIS being a cleavage stimulatory factor in Pol II transcription, we wanted to know whether TFIIS could influence the intrinsic cleavage activity of Pol III. Labeled ternary complexes halted by omission of GTP in the transcription reaction were purified and incubated for various periods of time in the presence of MgCl₂ to activate the Pol III cleavage activity (Chedin et al. 1998). TFIIS did not stimulate the cleavage activity of wild-type Pol III (Fig. 7C, lanes 1–14, look at the disappearance rate of the 17-mer transcript). Pol III Δ , a RNA polymerase mutant that lacks the Rpc11, Rpc37, and Rpc53 subunits, is not competent for RNA cleavage activity (Chedin et al. 1998; Landrieux et al. 2006). Pol III Δ was purified from *rpc37HA-Ct* mutant as described previously (Landrieux et al. 2006). We observed that the addition of TFIIS did not restore an efficient cleavage by Pol III Δ (Fig. 7C, lanes 15–21). Taken at face value, in vitro transcription assays suggest a role for TFIIS in start site selection during Pol III transcription.

Discussion

In this study, we performed a genome-wide location analysis of the TFIIS transcription factor. TFIIS was detected across the whole genome of exponentially growing cells, indicating that the binding of this transcription factor to chromatin is not restricted to NTP-depleted cells. TFIIS and Pol II genome-wide occupancies correlated very well, suggesting that TFIIS is not recruited only when Pol II is stalled. A second and more surprising outcome of our study was that TFIIS could be detected

on almost all Pol III-transcribed genes. This result raised the intriguing possibility that TFIIS might operate as a general Pol III-associated factor. We provided substantial in vivo and in vitro data demonstrating that TFIIS is important for Pol III transcription.

Concerning the TFIIS function in Pol II transcription, we observed that TFIIS was associated with a large number of Pol II-transcribed genes in cells growing exponentially in rich medium, in line with previous ChIP analyses of TFIIS on a few class II genes (Prather et al. 2005; Guglielmi et al. 2007). The distribution of TFIIS over distinct Pol II-transcribed regions, including intergenic regions transcribed in short unstable RNAs (Martens et al. 2004; Wyers et al. 2005; Davis and Ares 2006), precisely correlated with Pol II itself, which is consistent with the role of TFIIS as an elongation factor but is not contradictory with an additional role during initiation (Prather et al. 2005; Guglielmi et al. 2007; Kim et al. 2007). In the presence of MPA, the enrichment level of TFIIS genome-wide was only slightly increased compared with that in normal growth condition. Essentially, two models for TFIIS recruitment to Pol II-transcribed genes may be envisioned. TFIIS could be only recruited to arrested Pol II complexes. Alternatively, it might associate and dissociate from the elongating Pol II, independently of transcription arrests, shifting to the cleavage-prone conformation of Pol II if need arises. The latter model would better account for the fact that GTP depletion by MPA, which is likely to promote arrest, does not strongly increase TFIIS occupancy.

The presence of TFIIS at nearly all Pol III-transcribed genes strongly suggests that it is a Pol III transcription factor. This hypothesis is supported by several lines of evidence. (1) TFIIS occupancy correlated well with that of Pol III genome-wide. (2) The occupancy profile of TFIIS closely followed that of Pol III on the *SCR1* gene, the longest class III gene. (3) TFIIS coimmunoprecipitated with Pol III after formaldehyde cross-linking. (4) A temperature-sensitive Pol II mutation (*tpb1-1*) strongly

reduced TFIIS enrichment at Pol II-transcribed genes but had no effect on its association with Pol III-transcribed genes. Conversely, a temperature-sensitive Pol III mutation (*rpc25-S100P*) diminished TFIIS binding at Pol III-transcribed genes with no effect on its association with Pol II-transcribed genes. Thus, the presence of TFIIS on Pol III-transcribed genes depends on Pol III activity and is independent of Pol II. (5) Under low-temperature conditions, the *dst1-Δ* mutation affected growth, and diminished Pol III and TFIIB association with class III genes. (6) The *dst1* deletion impaired the translational suppression of *ade2-1* (a nonsense UAA mutant) by the *SUP4^{ochre}* suppressor, and reduced the Pol III transcript levels under low-temperature conditions. (7) TFIIS improved Pol III transcription start site selection in vitro. Altogether, these data indicate that TFIIS is a bona fide component of the Pol III transcription machinery. It has been suggested previously that TFIIS could also play a role in Pol I transcription (Sawadogo et al. 1980a; Sawadogo et al. 1981; Schnapp et al. 1996), but a recent report demonstrated that the intrinsic cleavage activity of Pol I requires the Rpl2 subunit, sharing sequence homology with TFIIS (Kuhn et al. 2007). Our ChIP results showed that TFIIS was enriched on the Pol I-transcribed rDNA templates. TFIIS could thus be implicated in Pol I transcription. Interestingly, the Pol III-transcribed 5S rRNA genes, arranged in tandem with the 35S rRNA genes, were not enriched by TFIIS. The reason for the absence of TFIIS on 5S rRNA genes is unknown but could stem from the intragenic binding of TFIIA factor that is required for 5S transcription.

As a Pol II elongation factor, TFIIS strictly depends on the RSADE domain, since inactivating this domain (or deleting the entire TFIIS) makes cells sensitive to NTP-depleting drugs (mycophenolate, 6-azauracil), which is generally seen as a consequence of a defective Pol II-associated cleavage (Exinger and Lacroute 1992; Ubukata et al. 2003). As a factor involved in Pol II initiation, TFIIS is needed for the full recruitment of Pol II to several promoters, especially in the absence of the Med31 subunit of Mediator. This, however, does not depend on the RSADE motif but is impaired in *dst1-R200A*, a mutation of the TFIIS Pol II-interacting domain (Guglielmi et al. 2007). Turning now to the Pol III-associated role(s) of TFIIS, we found that the RSADE motif is clearly important in this context, since the corresponding mutant form of TFIIS alters start site selection by Pol III in vitro, and since Pol III occupancy is strongly diminished in *dst1-E291A* cells grown at 16°C, with a strongly perturbed Pol III transcriptome. The reason why class III genes, the transcription of which is most diminished, differ in *dst1-Δ* and *dst1-E291A* is presently unknown but might be the consequence of the strong perturbation of Pol III transcription in the latter background. An indirect effect of altered Pol I or Pol II transcription is unlikely because *dst1* mutations do not affect 35S rRNA precursor transcription at 16°C (data not shown) and because Pol II occupancy defect is not increased in *dst1-E291A* compared with *dst1-Δ*. In contrast, *dst1-R200A* had a limited but significant effect on Pol II occupancy, with no effect

at all on Pol III, suggesting that this mutation might specifically affect a Pol II-associated function (Guglielmi et al. 2007).

In the course of this study, we examined the possible implication of TFIIS in the different steps of in vitro Pol III transcription. Omitting TFIIS did not detectably influence Pol III elongation or cleavage activity in vitro but altered start site selection, suggesting that TFIIS might primarily act at the level of Pol III recruitment and/or transcription initiation. This is further supported by our ChIP assays showing that *dst1-Δ* strongly reduces the presence of Bdp1, the TFIIB-related subunit of the TFIIB initiation factor of Pol III, with no effect on the TFIIC initiation factor. In Pol III transcription, start site selection and initiation require the precise targeting of the enzyme by its initiation factors (TFIIC and TFIIB) and the opening of the transcription bubble around the start site (Kassavetis and Geiduschek 2006). TFIIS might participate in the initial steps of the Pol III transcription cycle in different ways. TFIIS could bend DNA and facilitate appropriate DNA binding by the transcription machinery as do Nhp6A and Nhp6B (Kassavetis and Steiner 2006). Alternatively, TFIIS could facilitate productive initiation by influencing the interaction of Pol III with basal factors for more accurate enzyme positioning. We favor the second hypothesis since direct binding of TFIIS to DNA has never been shown and since TFIIS and Pol III coimmunoprecipitated in cross-linked extracts.

One could be initially surprised to find TFIIS associated with class III genes, since Pol III has an intrinsic transcript cleavage activity that depends on Rpl1 subunit (Chedin et al. 1998; Alic et al. 2007). Rpl1 has a C-terminal Zn loop that bears an RSADE motif, critical for transcript cleavage, that closely resembles the C-terminal domain of TFIIS (Chedin et al. 1998). Our results suggest that both Rpl1 subunit and TFIIS are required for efficient Pol III transcription, but that their roles are not identical. Recombinant TFIIS (Fig. 7), in contrast to recombinant Rpl1 alone (Chedin et al. 1998), could not restore cleavage activity of Pol IIIΔ variant lacking Rpl1, suggesting that TFIIS does not participate in this reaction. Further, we found that Rpl1 and TFIIS bound throughout *SCR1* gene as Rpl160 (used as a proxy for Pol III) did. Moreover, TFIIS and Rpl1 coimmunoprecipitated in cross-linked extracts, as did Rpl160, indicating that their binding to class III genes is not mutually exclusive.

In conclusion, there is now mounting evidence that TFIIS controls several levels of DNA transcription. At a subset of Pol II-transcribed gene promoters, it could be recruited and act together with Mediator to recruit Pol II (Prather et al. 2005; Guglielmi et al. 2007; Kim et al. 2007), independently of its transcript cleavage stimulatory activity. TFIIS could also dynamically associate and dissociate from Pol II and stimulate the enzyme intrinsic RNA cleavage activity when needed. In addition, the present study shows that TFIIS is a Pol III transcription factor that stimulates Pol III transcription and may contribute to precise start site selection.

Materials and methods

Protein purification

DB3.1 *E. coli* cells containing either pDEST17-*DST1* or pDEST17-*dst1E291A* were grown at 30°C to 0.6 OD₆₀₀. Expression of the 6xHis-TFIIS fusion protein was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested after 3 h of induction and resuspended in 20 mM HEPES buffer (pH 7.5) containing 10 μM ZnCl₂, 300 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, and a set of protease inhibitors (phenyl-methyl-sulfonyl fluoride, Complete [Roche]). After lysis by sonication at 4°C, the lysate was clarified by centrifugation in a Beckman JA20 rotor for 25 min at 12,000 rpm. The 6xHis-TFIIS proteins were purified using an ÄKTA purifier (Amersham Biosciences) on a Hi Trap Chelating HP 5-mL column with a gradient of imidazole from 10 mM to 1 M. SDS-PAGE analysis, followed by Coomassie brilliant blue staining, showed that the fusion proteins were purified to near homogeneity.

ChIP and genome-wide ChIP-chip

Cross-linked chromatin was prepared essentially as described previously [Kuras and Struhl 1999; Kuras et al. 2003]. Cells were grown exponentially to 0.6 OD₆₀₀ and cross-linked with 1% formaldehyde for 10 min. The 3HA- and 13Myc-tagged proteins were immunoprecipitated with 12CA5 and 9E10 antibodies, respectively; Pol II was immunoprecipitated with 8WG16 anti-CTD antibody (Covance), and bound to IgG magnetic beads (Dynabead). Immune complexes were washed as described previously [Kuras and Struhl 1999]. Cross-link reversal and DNA purification were performed as described [Kuras et al. 2003], except that the final elution was in 50 μL. Immunoprecipitated DNA was analyzed by quantitative real-time PCR on an ABI Prism 7000 or 7300 machine (Applied Biosystems). The PCR reactions were carried out in 25 μL containing 0.4 μM each primer, and 12.5 μL of mastermix SYBR green PCR reaction (Applied Biosystems). Relative quantification using a standard curve method was performed and the occupancy level for a specific fragment was defined as the ratio of immunoprecipitated DNA over total DNA. *GAL1* ORF region was used as a non-transcribed control.

Ligation-mediated PCR was done as described previously [Ren et al. 2000], except that amino-allyl conjugated dUTP (150 μM final) was used and only 30 cycles of PCR were performed. The PCR products were purified using a Microcon YM-30 filter. Amino-allyl modified DNA was recovered with 20 μL of H₂O and the DNA was lyophilized. DNA was labeled as previously described [Harismendy et al. 2003], except that unincorporated dyes were removed using a QIAquick PCR Purification Kit (Qiagen). Labeled DNA was recovered with 100 μL of buffer EB, and ethanol precipitated. Hybridization and washing conditions were as described previously [Lee et al. 2006]. Microarrays were obtained from Agilent Technologies and feature 41,418 (G4486A) or 41,776 (G4493A) 60-mer oligonucleotide probe spots, with an average density of one probe each 266 bp of the yeast genome. Images of Cy5 and Cy3 fluorescence intensities were generated using the Genepix 4000B scanner (Molecular Devices) and extracted using GenePix Pro 6 software (Molecular Devices). At least two biological replicates were performed for each experiment.

Data analysis

The computational data analysis was performed in R using the *limma* package [Smyth et al. 2005] from the bioconductor project (<http://www.bioconductor.org>). After subtraction of the local

background, the data from both channels were median normalized, and log ratios of signal intensities were generated for each feature. The log ratios were processed by fitting a linear model for each feature in order to calculate the average log ratio between replicates. *P*-values were then calculated by performing an empirical Bayes moderated *t*-statistic test, and adjusted for multiple testing by Benjamini and Hochberg false discovery rate (FDR) method. The complete raw data set is available at Array Express (<http://www.ebi.ac.uk/arrayexpress>) under accession number E-MTAB-10. Visualization of ChIP-enriched genomic regions was performed using an adaptation of the Ringo package [Toedling et al. 2007]. GO analysis was performed using GOstats package.

Pol III transcriptome analysis

Microarray hybridization was performed as described previously [Ciesla et al. 2007]. Briefly, 20 μg of total RNA was reverse transcribed with specific primers designed to hybridize to the 3' end of all mature tRNAs. cDNA was labeled with Cy3 and Cy5 dyes (Amersham) and hybridized on a Pol III-specific microarray (O. Harismendy, pers. comm.) harboring all the different tRNA genes, as well as all the other genes transcribed by Pol III.

In vitro transcription and primer extension assays

Standard Pol III in vitro transcription reactions on the *SUP4* template were performed as described previously [Ducrot et al. 2006; Alic et al. 2007] in 40 μL of transcription buffer. pRS316-*SUP4* plasmid (150 ng) was incubated for 20 min at 25°C in the presence of 100 ng of rTFIIIC, 20 ng of rTBP, 10 ng of rBdp1, 10 ng of rBrf1, 100 ng of highly purified Pol III, and 0.5 μg of purified B' fraction or different concentrations of TFIIS protein (wild-type or E291A mutant). Transcription was started by the addition of 600 μM A/C/GTP, 300 μM UTP, 25 μM [α-³²P]UTP (400 Ci/mmol), and allowed to proceed for 15 min.

Primer extension assays were performed as described previously [Andrau and Werner 2001] on the *SUP4* template with 200 μM unlabeled ATP, CTP, UTP, and GTP. Single-round assays were performed as described, except that 125 μM unlabeled ATP, CTP, UTP, GTP, and 0.3 mg/mL heparin were used [Kasavetis and Steiner 2006].

The reaction products were analyzed by electrophoresis on denaturing polyacrylamide gels (7% for in vitro transcription and primer extension assay). Gels were autoradiographed using MR film with an intensifying screen (Kodak). Quantifications were performed with Image Quant software (Molecular Dynamics).

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