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Molecular evidence for a positive role of Spt4 in transcription elongation

Ana G.Rondón, María García-Rubio, Sergio González-Barrera and Andrés Aguilera[1]

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Avd. Reina Mercedes 6, E-41012 Sevilla, Spain

[1]Corresponding author
e-mail: aguilo@us.es

We have previously shown that yeast mutants of the THO complex have a defect in gene expression, observed as an impairment of *lacZ* transcription. Here we analyze the ability of mutants of different transcription elongation factors to transcribe *lacZ*. We found that *spt4*Δ, like THO mutants, impaired transcription of *lacZ* and of long and GC-rich DNA sequences fused to the *GAL1* promoter. Using a newly developed *in vitro* transcription elongation assay, we show that Spt4 is required in elongation. There is a functional interaction between Spt4 and THO, detected by the lethality or strong gene expression defect and hyper-recombination phenotypes of double mutants in the W303 genetic background. Our results indicate that Spt4–Spt5 has a positive role in transcription elongation and suggest that Spt4–Spt5 and THO act at different steps during mRNA biogenesis.
Keywords: *lacZ*/*SPT4*/*SPT5*/THO complex/transcription elongation

Introduction

Transcription is a complex cellular process that involves three differentiated steps: initiation, elongation and termination. During elongation, RNA polymerase II (RNAPII) has to overcome situations derived from transient pausing caused by regulatory signals. This is achieved with the help of positive and negative transcriptional elongation factors. Positive factors include TFIIS (Wind and Reines, 2000), TFIIF (Bengal *et al*., 1991), human Elongin (Aso *et al*., 1995), ELL (Shilatifard *et al*., 1996), FACT (LeRoy *et al*., 1998; Orphanides *et al*., 1998; Wada *et al*., 2000), Elongator (Otero *et al*., 1999; Wittschieben *et al*., 1999), CSB/Rad26 (van Gool *et al*., 1997) and the 19S regulatory particle of the proteasome (Ferdous *et al*., 2001). Negative elongation factors include DSIF and NELF (Hartzog *et al*., 1998; Wada *et al*., 1998a; Yamaguchi *et al*., 1999a, 2002).

DSIF was isolated from HeLa cell nuclear extracts as a transcription elongation factor that causes pausing of RNAPII when cells are treated with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Wada *et al*., 1998a; Yamaguchi *et al*., 1999b). It is formed by two subunits, which are the human homologs of the *Saccharomyces cerevisiae* transcription factors Spt4 and Spt5 (Wada *et al*., 1998a; Yamaguchi *et al*., 1999b). Consistent with the role of Spt4–Spt5/DSIF in transcription elongation, *Drosophila* Spt5 has been shown to colocalize with RNAPII at highly transcribed sites of polytene chromosomes and at heat-shock genes upon induction (Andrulis *et al*., 2000; Kaplan *et al*., 2000). Also, *Drosophila* and yeast Spt5 proteins are bound to open reading frames (ORFs) when they are transcriptionally active, as determined by chromatin immunoprecipitation (ChIP) analysis (Andrulis *et al*., 2000; Pokholok *et al*., 2002). Furthermore, yeast Spt4 and Spt5 are tightly associated in a protein complex that interacts with RNAPII (Hartzog *et al*., 1998).

In vitro studies indicate that DSIF acts as a negative elongation factor, increasing the pausing of RNAPII (Yamaguchi *et al*., 1999b; Renner *et al*., 2001). The pausing is reversed by P-TEFb, which phosphorylates the RNAPII CTD and the DSIF hSpt5 subunit (Wada *et al*., 1998b; Kim and Sharp, 2001; Renner *et al*., 2001). Spt4–Spt5/DSIF complex has also been suggested to have a positive role in transcription elongation, although this has not been demonstrated yet. Thus, *spt4*Δ mutants are sensitive to 6-azauracil (6-AU; Hartzog *et al*., 1998; Costa and Arndt, 2000), a phenotypic landmark of transcription elongation defects caused by the *in vivo* depletion of GTP and UTP pools provoked by 6-AU (Shaw and Reines, 2000). In addition, it has been shown that *S.cerevisiae spt5-194* mutation causes a decrease in the levels of some RNAPII transcripts (Compagnone-Post and Osley, 1996) and that purified human DSIF stimulates transcription under limiting NTP concentrations (Wada *et al*., 1998a) or when added in excess to isolated early elongation complexes (Renner *et al*., 2001).

THO was identified in yeast as a four-protein complex that included Tho2, Hpr1, Mft1 and Thp2 (Chávez *et al*., 2000). Previous genetic and functional evidence indicated that mutations in the THO complex caused a strong transcription-dependent hyper-recombination phenotype and a defect in gene expression (Chávez and Aguilera, 1997; Piruat and Aguilera, 1998; Chávez *et al*., 2001). THO mutants, such as *hpr1*, are preferentially affected in their ability to express long and GC-rich DNA sequences driven from a strong promoter, as is the case of *lacZ* fused to the *GAL1* promoter (Chávez *et al*., 2001). It has recently been shown that THO is present, together with the components of the mRNA export machinery Sub2 and Yra1, in a larger complex conserved in yeast and humans, termed TREX. This complex is associated with ORFs in a transcription-dependent manner (Strässer *et al*., 2002). THO and mRNA-export mutants show, indeed, similar defects in transcription suggesting a connection between both transcription and mRNA metabolism (Jimeno *et al*., 2002). Our actual view is that THO has a major role in the

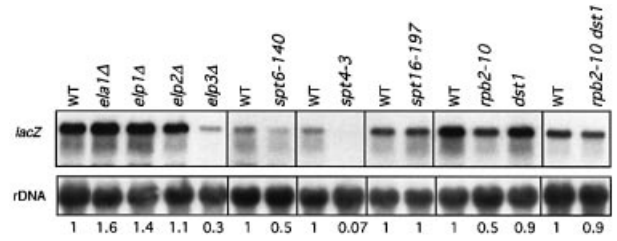

Fig. 1. Transcription analysis of a *leu2Δ3′::lacZ* fusion in different transcription elongation mutants and their respective isogenic wild-type strains. Northern analyses were performed from overnight cultures of yeast cells transformed with plasmid pSCh204. Strains used were W303-1A (WT), ela1Δ (*ela1*Δ), JSY102 (*elp1*Δ), elp2Δ (*elp2*Δ), JSY130 (*elp3*Δ), MH236H-1B (WT), MH236H-1D (*spt4-3*), MH137H-1B (WT), MH137H-4A (*spt6-140*), MF348-5B (WT), MF348-1A (*spt16-197*), ADW11-7C (WT), ADW11-11A (*rpb2-10*), ADW11-4B (*dst1*), ALY95-4A (WT), ALY95-1B (*rpb2-10 dst1*Δ). The DNA probes used were the 3 kb *Bam*HI 5′-end fragment of *lacZ* and a 589 bp 28S rDNA internal fragment obtained by PCR. The relative amount of *lacZ* mRNA values normalized with the corresponding WT value for each mutant analyzed is shown at the bottom.

metabolism of nascent mRNA during transcription, suggesting that the status of the nascent RNA becomes important for proper transcription (Jimeno *et al*., 2002).

In this study, we developed a new *in vitro* assay for the analysis of transcription elongation in whole-cell extracts (WCEs). With this assay, we showed that Spt4 is strongly required for transcription elongation. In addition, we found that *spt4*Δ mutants are strongly impaired in transcription of *lacZ* and other long and GC-rich DNA sequences driven from a strong promoter, phenotypes observed previously for the mutants of the THO complex. In contrast to THO mutations, *spt4* does not confer a transcription-dependent hyper-recombination phenotype. In the W303 genetic background, double mutant strains carrying *spt4*Δ and a THO mutation are either inviable or show stronger transcription and recombination phenotypes than the single mutants. We conclude that Spt4 has a positive role in transcription elongation and that this role is different from that of THO during mRNA biogenesis.

Results

Analysis of gene expression in different transcription elongation mutants: impairment of lacZ expression in spt4 and spt5

Mutants of the THO complex are defective in *lacZ* gene expression (Chávez *et al*., 2000). In order to establish whether the incapacity to express *lacZ* could be used as a phenotype linked to transcription elongation defects, we determined the ability of a number of known transcription elongation mutants to transcribe *lacZ*. We performed northern analysis of mutants and their respective wild-type isogenic strains transformed with centromeric plasmid pSCh204 (Chávez and Aguilera, 1997), in which the complete *lacZ* ORF is located between two truncated copies of *LEU2* transcribed from the *LEU2* promoter, yielding a 4.5 kb transcript. Figure 1 shows that the *spt4-3* mutant had a dramatic effect on the level of *lacZ* mRNA; reduced to 7% of the level of a wild-type strain. Among the other mutants tested (*ela1*Δ, *elp1*Δ, *elp2*Δ, *elp3*Δ, *spt6-140*, *spt16-197*, *rpb2-10*, *dst1* and *rpb2-10 dst1*), *lacZ* mRNA levels were slightly reduced in *elp3*Δ (30% of the wild-type levels), *spt6-140* and *rpb2-10* mutants (50% the wild-type levels) and was not affected in the rest of the mutants.

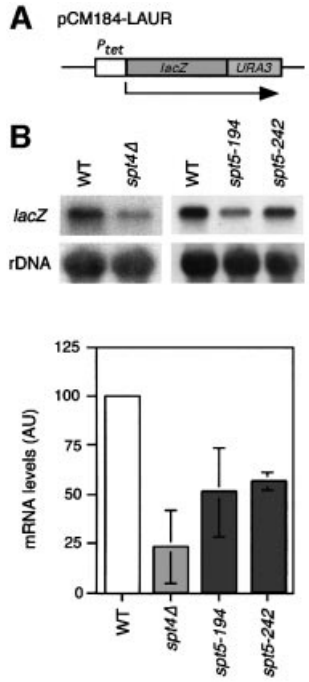

Fig. 2. Transcription analysis of *lacZ* in wild-type, *spt4*Δ and *spt5* mutant cells. (**A**) Scheme of the *lacZ-URA3* translational fusion under the control of the *tet* promoter. (**B**) Northern analyses of *lacZ-URA3* expression in wild-type (SPT4TW-1A or OY 97), *spt4*Δ (SPT4TW-1B), *spt5-194* (GHY 94) and *spt5-242* (GHY 828) strains transformed with plasmid pCM184-LAUR. DNA probes used were the 3 kb *Bam*HI 5′-end fragment of *lacZ* fragment and a 589 bp 28S rDNA internal fragment obtained by PCR. For more details, see Figure 1. Shown values are the mean of two independent experiments.

Spt4 forms a complex with Spt5 that is homologous to the human DSIF complex. Whereas Spt5 is essential for cell viability, Spt4 is not. For this reason, we decided to extend our functional analysis of *lacZ* transcription to null *spt4*Δ mutants. In this case, we analyzed gene expression in the P_{tet}*::lacZ-URA3* translational fusion (Jimeno *et al*., 2002). We have shown previously that mutants with an impaired ability to express *lacZ*, such as the mutants of the THO complex, are incapable of expressing this fusion. They yield a Ura^- phenotype and white colonies in X-gal-containing medium in contrast to the Ura^+ phenotype and blue-color forming colonies of wild-type cells (Jimeno *et al*., 2002). As expected, *spt4*Δ cells carrying P_{tet}*::lacZ-URA3* are Ura^- and they do not form blue-color colonies when adding X-gal to the medium (data not shown). They showed *lacZ* mRNA levels that were 20% of the wild type, confirming that *spt4*Δ cells are impaired in expression of *lacZ* driven from a P_{tet} promoter (Figure 2B). Next, we determined whether the leaky mutations *spt5-194* and *spt5-242* also conferred a defect in *lacZ* transcription. As can be seen in Figure 2B, both alleles reduced the efficiency of transcription of P_{tet}*::lacZ-URA3* to levels in the range of those of *spt4*Δ. As *spt4* and *spt5* have similar transcription phenotypes, we decided to continue our analysis with the *spt4*Δ null mutant only.

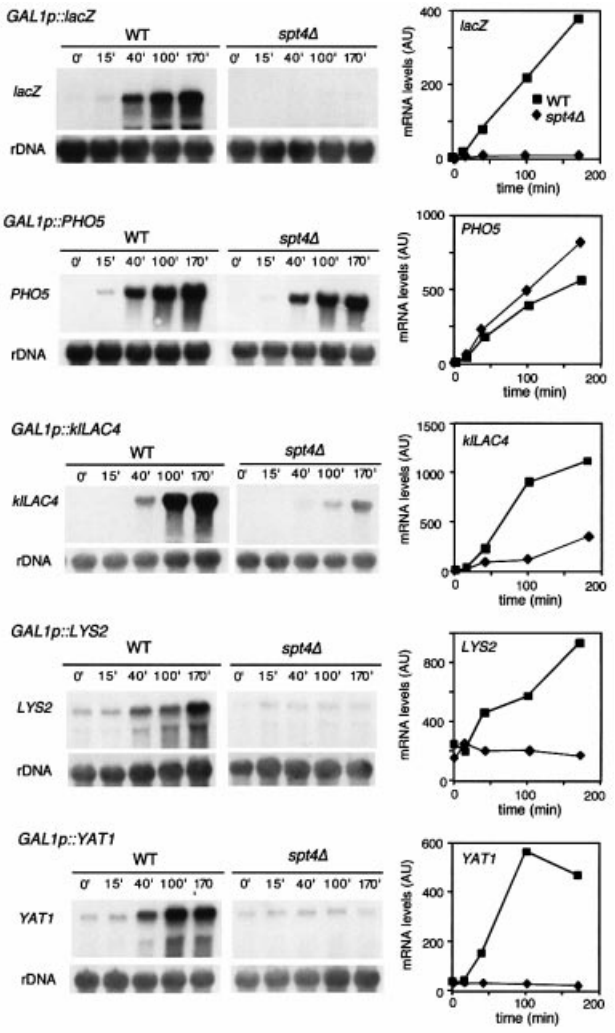

Fig. 3. Transcription analysis of *GAL1p::lacZ*, *GAL1p::PHO5*, *GAL1p::klLAC4*, *GAL1p::LYS2* and *GAL1p::YAT1* in wild-type and *spt4*Δ cells. Northern analyses of *lacZ*, *PHO5*, *klLAC4*, *LYS2* and *YAT1* mRNAs driven from the *GAL1* promoter in the strains BY4741 (WT) and YGR063c (*spt4*Δ) transformed with plasmids p416*GAL1lacZ*, pSCh202, pSCh255, pSCh227 and pSCh247 are shown. Mid-log phase cells grown in 2% glucose SC medium lacking uracil were washed and diluted into SC 3% glycerol-2% lactate medium lacking uracil to an OD_{600} of 0.8 and incubated for 14 h. Galactose was then added and samples were taken for northern analyses at different times, as specified. DNA probes used were the 3 kb *Bam*HI 5′-end fragment of *lacZ*, a 0.9 kb *Eco*RV *PHO5* internal fragment, 3 kb *Xba*I fragment containing *klLAC4* ORF, a 2 kb *Xho*I–*Xba*I fragment containing *YAT1*, *LYS2* internal fragment obtained by PCR and a 589 bp 28S rDNA internal fragment obtained by PCR. Plotted mRNA values are given in arbitrary units (AU) with respect to rRNA levels. For more details, see Figure 1. The *YAT1* and *LYS2* expression levels observed at 0 min is due to transcription of the endogenous genes copies. Other details are as in Figure 1.

spt4Δ cells have a strongly reduced capacity to express lacZ and long and GC-rich DNA sequences fused to the GAL1 promoter

Using a *GAL1p::lacZ* fusion, we showed, by kinetic analysis of transcription activation, that *spt4*Δ cells show low levels of accumulation of *lacZ* mRNA for 170 min after activation by galactose addition (Figure 3). The low mRNA levels are not due to a defect in activation of the *GAL1* promoter, but rather to an incapacity to properly transcribe *lacZ*. When an identical experiment was performed with a *GAL1p::PHO5* fusion, in which the bacterial *lacZ* sequence was replaced by the yeast *PHO5* sequence, accumulation of *PHO5* mRNA occurred with the same kinetics as the wild type, reaching similar wild-type levels of transcription after 170 min of galactose addition (Figure 3). This result demonstrates that it is the transcription through *lacZ*, and not the activation of the *GAL1* promoter, that is impaired in *spt4*Δ cells.

So far *spt4*Δ mutants have shown identical transcription defects to those described previously for mutants of the *THO* complex. In addition, the bacterial *lacZ* ORF is a 3 kb long sequence with a 56% GC content, whereas *PHO5* is a 1.5 kb long DNA sequence with a 40% GC content. We showed that *lacZ* sequences were not properly transcribed in an *hpr1*Δ mutant because these mutants were impaired in their ability to express long and GC-rich DNA sequences fused to the *GAL1* promoter (Chávez *et al.*, 2001). Consequently, in order to determine whether the inability of *spt4*Δ cells to express *lacZ* reflects their incapacity to properly express long and GC-rich DNA sequences, we extended the kinetic analysis of transcription activation of *spt4*Δ cells to the following three fusion constructs: *GAL1p::klLAC4*, *GAL1p::LYS2* and *GAL1p::YAT1*. The *klLAC4* sequence was a 3 kb long ORF from the yeast *Kluyveromyces lactis*, homologous to *lacZ* but with a typical yeast GC content (40%). *LYS2* was a 3.5 kb long *S.cerevisiae* DNA sequence with a standard yeast GC content (40%). *YAT1* was a 2 kb long *S.cerevisiae* ORF with an unusually high GC-content (58%). Kinetics of transcription activation of *GAL1p::klLAC4* showed that transcription was also impaired in this construct (Figure 3), presumably due to the fact that *klLAC4* was a long ORF (3 kb). However, in contrast to bacterial *lacZ* (56% GC content), significant levels of *klLAC4* transcript were accumulated after 170 min of galactose addition. Therefore, lowering the GC content of a 3 kb long ORF facilitated its transcription in *spt4*Δ cells. Figure 3 shows that *spt4*Δ cells accumulated almost undetectable levels of mRNA from either the *GAL1p::LYS2* or the *GAL1p::YAT1* constructs after 170 min of transcription activation by galactose addition. However, *spt4*Δ cells did accumulate *PHO5* mRNA (1.5 kb long with 40% GC content) like wild-type cells. Therefore, these results are consistent with the idea that Spt4 is required for proper transcription of long and GC-rich DNA sequences driven from the *GAL1* promoter.

Defective transcription elongation in spt4Δ WCEs

Our functional analysis of transcription in *spt4-3* and *spt4*Δ cells indicates that Spt4 may have a positive role in transcription elongation. In order to test this possibility directly, we assayed the ability of *spt4*Δ WCEs to promote transcription elongation *in vitro*. To accomplish this, we developed an *in vitro* transcription system based on plasmid pGCYC1-402 in which a hybrid *GAL4-CYC1* promoter containing a Gal4-binding site was fused to a 1.88 kb DNA fragment coding two G-less cassettes separated by 1.4 kb. The first G-less cassette was right downstream of the promoter and it was 84 nucleotides (nt) long. The second one was located at 1.48 kb from the promoter and was 376 nt long. In this assay, transcription activated by Gal4-VP16 led to an mRNA, which was then

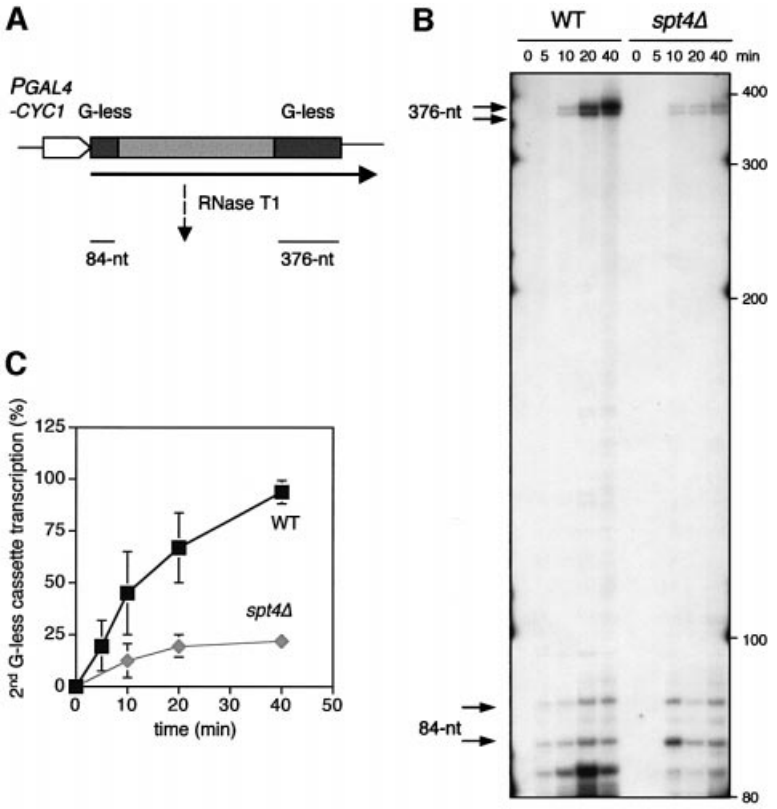

Fig. 4. *In vitro* transcription assays of wild-type and *spt4*Δ WCEs. (**A**) Scheme of double-G-less cassette system. RNase T1-treatment of mRNA driven from *GAL4-CYC1* promoter render two fragments corresponding to the G-less cassettes. (**B**) *In vitro* transcription assay using 400 ng of the double-G-less cassette-containing fragments as template, 100 μg of WCE from W303-1A (WT) and MGSC339 (*spt4*Δ) strains and 100 ng of Gal4-VP16. Reactions were stopped at 0, 5, 10, 20 and 40 min, treated with 160 U of RNaseT1 and run in a 6% PAGE. Two bands from each G-less cassette were obtained probably due to incomplete action of RNase T1. (**C**) Percentage of total transcripts that reach the 376 bp G-less cassette in both wild-type and *spt4*Δ cells with respect to the transcripts that cover the 84 bp cassette, taken as 100%. Each result is the mean value of two independent *in vitro* transcription assays. Radioactivity incorporated into the G-less cassettes was quantified in a Fuji FLA3000 and normalized with respect to the C content of each G-less cassette.

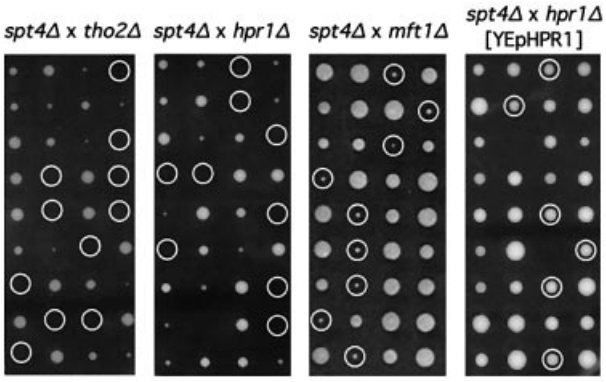

Fig. 5. Genetic analysis of the growth phenotype conferred by *spt4*Δ in combination with mutations of the THO complex. Tetrad analyses of the diploid strains obtained by crossing *spt4*Δ strain with either *tho2*Δ, *hpr1*Δ and *mft1*Δ null mutants are shown in the W303 genetic background. Whereas *tho2*Δ *spt4*Δ and *hpr1*Δ *spt4*Δ were synthetically lethal (double-mutant spores were able to germinate but died after several divisions), *mft1*Δ *spt4*Δ were able to grow. *hpr1*Δ *spt4*Δ double-mutant spores were rescued by the YEpHPR1 plasmid containing a wild-type *HPR1* copy. The THO null mutations, which contained the *KAN* cassette, were scored by G418 resistance and the *spt4*Δ*::URA3* by the Ura^+ phenotype. Double-mutant spores are indicated with circles.

digested with RNase T1, an RNase that degrades all G-containing mRNA sequences, leaving the two G-less cassettes intact (Figure 4A and B). The efficiency of transcription elongation was determined as the percentage of all mRNAs reaching the 376 nt long G-less cassette among the total mRNA covering the 87 nt long G-less cassette (Figure 4C). As can be seen in Figure 4C, after Gal4-VP16 activation, *spt4*Δ cell extracts fully transcribed the 376 nt G-less cassette with a clearly reduced efficiency with respect to the wild type, as determined by kinetic analysis of transcription elongation. This is the first molecular demonstration that Spt4 has a positive role in transcription elongation.

Genetic interaction between Spt4 and the THO complex

Considering the similarity of *in vivo* transcription phenotypes conferred by *spt4*Δ and by the mutations of the THO complex, we decided to investigate whether there was a genetic relationship between these mutations by performing genetic analyses of double mutant combinations. With this purpose, we crossed a *spt4*Δ strain with single mutants of the *THO* complex (*tho2*Δ, *hpr1*Δ and *mft1*Δ) and performed tetrad analysis. Figure 5 shows that *tho2*Δ *spt4*Δ and *hpr1*Δ *spt4*Δ double mutants were inviable (*hpr1*Δ *spt4*Δ divided 5–10 times after germination). We show that the inviability of *spt4*Δ *hpr1*Δ mutants is, indeed, rescued by *HPR1* carried in a plasmid. The *mft1*Δ *spt4*Δ double mutant was viable but sick; it

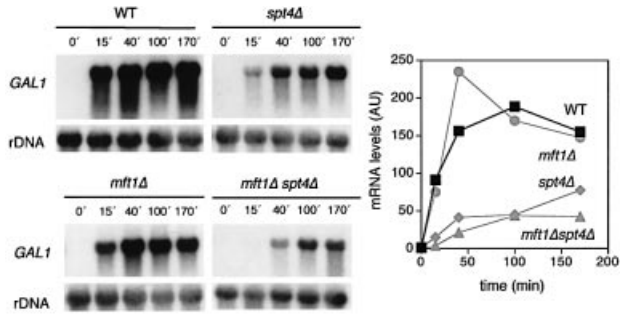

Fig. 6. Transcription analysis of *GAL1* in *spt4*Δ *mft1*Δ cells. Northern blot analyses of *GAL1* mRNA in the strains W303-1A (WT), MGSC339 (*spt4*Δ), WMS-1C (*mft1*Δ) and WMS-7C (*spt4*Δ *mft1*Δ). DNA probes used were the 750-bp *Ava*I–*Pvu*II fragment of *GAL1* and a 589 bp 28S rDNA internal fragment obtained by PCR. Other details are as in Figure 1.

grew poorly compared with single mutants (Figure 5). However, this result was dependent on genetic background. When we repeated the same experiments in the BY4741 instead of the W303 genetic background, we found that double mutants were viable. This result is consistent with our previous observation that Tho2 and Hpr1 are the most prominent components of the THO complex (Chávez *et al*., 2000).

As the most relevant features of the THO mutants were the transcription, hyper-recombination and plasmid-loss phenotypes, we took advantage of the viability of *mft1*Δ *spt4*Δ cells in the isogenic W303 genetic background to explore further the functional relationship between Spt4 and THO by analyzing these three phenotypes. We analyzed the effect on transcription by determining the kinetics of activation of the endogenous *GAL1* gene. As can be seen in Figure 6, both wild-type and *mft1*Δ cells had almost no effect on the kinetics of activation of *GAL1*. However, *spt4*Δ cells clearly showed slower kinetics of activation of *GAL1* mRNA accumulation, whereas the *mft1*Δ *spt4*Δ double mutant showed about half that of *spt4*Δ.

For the analysis of recombination, we first observed that *spt4*Δ had wild-type levels of recombination in the chromosomal direct-repeat construct *leu2-k::ADE2-URA3::leu2-k* (data not shown), a system in which *mft1*Δ and other mutations of the THO complex conferred an increase in deletions two orders of magnitude above wild-type levels (Piruat and Aguilera, 1998). We then analyzed recombination in the direct-repeat construct *leu2*Δ*3′::ADE2::leu2*Δ*5′* in centromeric plasmid pRS314-LA (Prado *et al*., 1997). As can be seen in Figure 7A, *mft1*Δ and *spt4*Δ single mutants showed 4- and 6-fold increases in recombination, respectively, whereas the *mft1*Δ *spt4*Δ double mutant showed a 25-fold increase.

Plasmid stability was analyzed for centromeric plasmid pRS313GZ containing the *GAL4-CYC1p::lacZ* fusion and the *HIS3* marker. As shown in Figure 7B, when transcription of *lacZ* was inactive, *mft1*Δ reduced the stability of the plasmid 3.7-fold below wild-type levels, *spt4*Δ 1.3-fold and the double mutant 13.5-fold. When transcription of *lacZ* was active, the reduction was 82-fold for *mft1*Δ cells, 0.6-fold in *spt4*Δ and 245-fold in the *mft1*Δ *spt4*Δ double mutant as compared with the wild type.

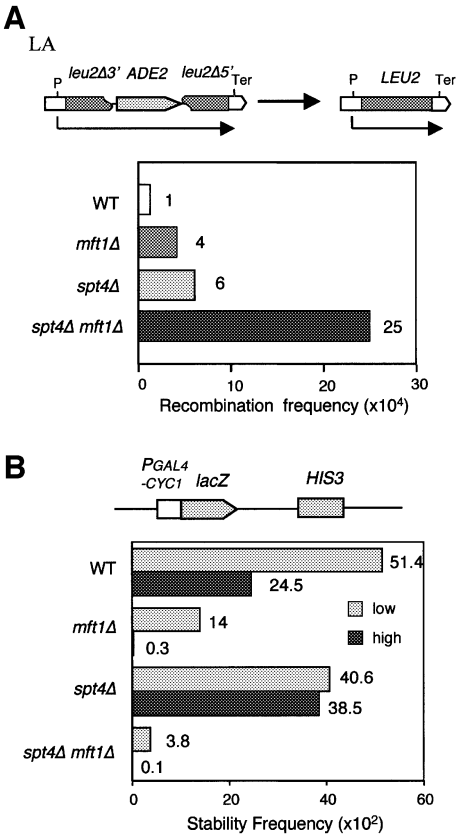

Fig. 7. Recombination and plasmid instability analyses in wild-type, *mft1*Δ, *spt4*Δ and *spt4*Δ *mft1*Δ cells. (**A**) Scheme of the deletion resulting from a recombination event between the direct repeats. Transcripts driven from the external *LEU2* promoter are indicated by arrows. Recombination frequencies were determined in the strains W303-1A, MGSC339 (*spt4*Δ), WMK-2A (*mft1*Δ) and WMS-7C (*spt4*Δ *mft1*Δ) transformed with pRS314-LA (LA). The median frequency of the six colonies is given. (**B**) Scheme of pRS313GZ used to assay plasmid instability. The frequencies of plasmid loss were determined in the same strains mentioned above under high or low transcription of the *GAL4-CYC1p*::*lacZ* construct. For this, six colonies were isolated in SC with either 2% glucose (low) or with 2% galactose (high). The frequency of colonies that have retained the plasmid were determined as the number of colonies growing in SC-his.

Discussion

We have shown that Spt4 has a positive role in transcription elongation. The ability of *spt4*Δ cell extracts to elongate transcription *in vitro* is clearly reduced with respect to the wild type. Furthermore, *spt4* and *spt5* cells are impaired in transcription of *lacZ* and other long and GC-rich DNA sequences driven from the *GAL1* promoter. There is a genetic interaction between Spt4 and THO, detected in the W303 genetic background by the lethality of *tho2*Δ *spt4*Δ and *hpr1*Δ *spt4*Δ double mutants, and by a stronger transcription and hyper-recombination phenotypes of the *mft1*Δ *spt4*Δ double mutant. These results are consistent with the idea that Spt4–Spt5 has a positive role in transcription elongation and that Spt4 and THO act at different levels during mRNA biogenesis.

Spt4 forms, together with Spt5, the yeast homolog of DSIF, a human factor whose negative role in transcription elongation has been well documented. *In vitro* it is required, together with NELF, for RNAPII pausing (Wada *et al*., 1998a; Yamaguchi *et al*., 1999a; Renner *et al*.,

Table I. Strains

Strain	Genotype	Source/Reference
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1*	R.Rothstein
SChY58a	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 hpr1Δ::kanMX4*	S.Chávez
RK2-6C	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 tho2Δ::kanMX4*	Piruat and Aguilera (1998)
WMK-2A	*MAT***a** *ade2-1 can1-100 his3-11 leu2-3, 112 trp1-1 ura3-1 mft1Δ::kanMX4*	Chávez *et al.* (2000)
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
YGR063c	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spt4Δ:: kanMX4*	Euroscarf
MGSC339	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 spt4Δ::URA3*	J.Brouwer
OY 97	*MAT***a** *his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52*	G.Hartzog
GHY 828	*MAT***a** *his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52 spt5-242*	G.Hartzog
GHY 94	*MAT***a** *his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52 spt5-194*	G.Hartzog
ela1Δ	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 ela1Δ::kanMX4*	W.Heyer
JSY102	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 elp1Δ::URA3*	J.Svejstrup
elp2Δ	*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 elp2Δ::HIS3*	J.Svejstrup
JSY130	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 elp3Δ::LEU2*	J.Svejstrup
SPT4TW-1A	*MAT***a** *ade2-1 his3 leu2 met15Δ0 trp1-1 ura3*	R.Luna
SPT4TW-1B	*MAT*α *his3 leu2 trp1-1 ura3 spt4Δ::URA3*	R.Luna
MH236H-1B	*MAT***a** *his3 leu2 lys2-128∂ trp1 ura3*	This study
MH236H-1D	*MAT***a** *his3 leu2 lys2-128∂ trp1 ura3 spt4-3*	This study
MH137H-1B	*MAT*α *his3 leu2 lys2-128∂ trp1 ura3*	This study
MH137H-4A	*MAT*α *ade2 can1-100 his3 leu2 lys2-128∂ trp1 ura3 spt6-140*	This study
MF348-5B	*MAT***a** *his4-912∂ leu2 lys2-128∂ trp1 ura3*	This study
MF348-1A	*MAT*α *his4-912∂ leu2 lys2-128∂ trp1 ura3 spt16-197*	This study
ALY95-4A	*MAT***a** *leu2Δ::hisG trp1Δ1 lys2-801$_{am}$*	This study
ALY95-1B	*MAT*α *ade2-101$_{oc}$ leu2Δ::hisG trp1Δ1 lys2-801$_{am}$ rpb2-10 dst1Δ::hisG*	This study
ADW11-7C	*MAT*α *ade2 his3Δ200 trp1 leu2Δ::hisG lys2-801*	This study
ADW11-4B	*MAT***a** *ade2 his3 trp1 leu2Δ::hisG dst1Δ:hisG*	This study
ADW11-11A	*MAT*α *ade2 his3 trp1 leu2Δ::hisG rpb2-10*	This study
WMS-1D	*MAT***a** *ade2-1 his3-11,15 leu2-3, 112 trp1-1 ura3-1*	This study
WMS-1A	*MAT*α *ade2-1 his3-11,15 leu2-3, 112 trp1-1 ura3-1 spt4Δ::URA3*	This study
WMS-1C	*MAT***a** *ade2-1 his3-11,15 leu2-3, 112 trp1-1 ura3-1 mft1Δ::kanMX4*	This study
WMS-7C	*MAT***a** *ade2-1 his3-11,15 leu2-3, 112 trp1-1 ura3-1 spt4Δ::URA3 mft1Δ::kanMX4*	This study

2001). DSIF has been found tightly bound to hypo-phosphorylated RNAPII in HeLa nuclear extracts (Wada *et al*., 1998a; Yamaguchi *et al*., 1999b) as well as to hyper-phosphorylated RNAPII (Lindstrom and Hartzog, 2001). Consistent with a negative role at the early steps of transcription elongation, DSIF has been shown to inhibit only hypo-phosphorylated RNAPII (Yamaguchi *et al*., 1999a). It has been suggested that DSIF dissociates from RNAPII upon CTD phosphorylation by P-TEFb (Wada *et al*., 1998b). However, there are data suggesting that Spt4–Spt5/DSIF may have a positive role in elongation also. These data include the 6-AU sensitivity of yeast *spt4*Δ mutants (Hartzog *et al*., 1998; Costa and Arndt, 2000) and the stimulation of *in vitro* transcription by DSIF or under limiting NTPs (Wada *et al*., 1998a) or when purified DSIF was added in excess to early elongation complexes (Renner *et al*., 2001). Here we demonstrate, with a newly developed *in vitro* transcription assay, that Spt4 plays a positive role in transcription elongation. The *spt4*Δ null mutation reduces the efficiency of elongation at least 4-fold below wild-type levels (Figure 4). This result is consistent with our observation that *spt4*Δ cells are impaired in their ability to transcribe *lacZ in vivo*, regardless of the promoter from which transcription is driven, or in their ability to transcribe long and GC-rich DNA sequences (Figures 2 and 3). The *spt5-194* and *spt5-242* alleles reduced the efficiency of transcription of P_{tet}*::lacZ-URA3* to levels similar to those of *spt4*Δ (Figure 2B). This positive role in transcription elongation is consistent with recent data showing that in an HIV-Tat based system, Spt5, in cooperation with Tat, prevents the premature dissociation of RNA from the transcription complex at terminator sequences and reduces the amount of RNAPII pausing at arrest sites (Bourgeois *et al*., 2002). This function resembles that of bacterial NusG, which contacts and enhances RNA polymerase stability on DNA templates (Li *et al*., 1992; Sullivan and Gottesman, 1992) and has a region homologous to Spt5 (Hartzog *et al*., 1998; Wada *et al*., 1998a; Ponting, 2002).

Mutants of the THO complex, such as *hpr1* and *tho2*, show a similar incapacity to express long and GC-rich DNA sequences fused to the *GAL1* promoter (Chávez *et al*., 2001). Indeed, it has recently been shown by ChIP analysis that Spt5 and Tho2/Hpr1 associate with transcriptionally active ORFs (Andrulis *et al*., 2000; Pokholok *et al*., 2002; Strässer *et al*., 2002). These results suggest that both Spt4–Spt5/DSIF and THO act during transcription. This is consistent with the isolation of Hpr1 and Spt4–Spt5 in combination with components of the Paf1–Cdc73 complex associated with RNAPII (Chang *et al*., 1999; Squazzo *et al*., 2002).

It is important to note that THO is associated with the mRNA export proteins Sub2 and Yra1 in a larger complex (Strässer *et al*., 2002), that THO associates with RNA and that THO mutants accumulates mRNA in the nucleus (Strässer *et al*., 2002; Jimeno *et al*., 2002). This, together with the synthetic lethality of double mutants of THO and Mex67, indicates that THO may function at the level of the nascent mRNA. However, in contrast to THO mutants, spt4 cells do not accumulate mRNA in the nucleus (data

not shown) and, in contrast to *tho2 mex67-5* mutants, *spt4 mex67-5* double mutants are viable (S.Jimeno and A.Aguilera, unpublished data). The synthetic lethality of *hpr1 spt4* and *tho2 spt4*, the high genetic instability phenotype of *mft1*Δ *spt4*Δ (Figure 7) and the fact that the observed hyper-recombination in *spt4*Δ cells is not linked to transcription (data not shown; Malagón and Aguilera, 1996) suggests that THO and Spt4 act at different levels.

A distinctive feature of *lacZ* with respect to yeast ORFs such as *GAL1*, through which transcription is either poorly or not affected in *hpr1*Δ, is a lack of nucleosome positioning (Chávez *et al*., 2001). It is possible that the Spt4–Spt5 function was more acute during transcription of DNA sequences with a poorly organized chromatin. However, it is important to note that the positive role of Spt4 in transcription elongation is observed *in vitro* with naked DNA (Figure 4). This indicates that the major function of Spt4 in elongation is independent of chromatin structure. Nevertheless, we cannot discard that, in addition, Spt4 might have *in vivo* a positive role in transcription elongation related to chromatin structure (see Winston and Carlson, 1992). In this sense, it is worth mentioning that *spt4*Δ strains transcribe a *HTA1-lacZ* fusion with an efficiency 5-fold below wild-type levels, a defect that is suppressed by overexpression of histones H2A and H2B (Compagnone-Post and Osley, 1996). This may be due to a defect in the regulation and feedback repression of histone genes in *spt4* mutants (Compagnone-Post and Osley, 1996), but it could also be due to the inefficient *lacZ* transcription of *spt4* mutants or to the effect that histone imbalance may cause on the nucleosome assembly. In any case, since imbalance of histone stoichiometry alters transcription of a number of yeast genes (Wyrick *et al*., 1999), we cannot disregard that phenotypes associated with histone imbalance are due to the secondary effect on transcription regulation of other genes.

We believe that Spt4 acts upstream of the THO complex during transcription as a bona fide transcription elongation factor. The THO complex might act downstream of Spt4–Spt5 in association with the nascent mRNA and other proteins whose specific functions are related to mRNA metabolism (Strässer *et al*., 2002; Jimeno *et al*., 2002). It is likely that the effect of THO mutations in transcription is mediated by a possible role in the stabilization or processing of the nascent mRNA (Jimeno *et al*., 2002). We need to understand better the molecular basis of the requirement of Spt4–Spt5 for expression of DNA sequences such as *lacZ* in order to decipher why the effect of Spt4–Spt5 on *lacZ* expression differs from those of other putative elongation factors (Figure 1). However, our novel *in vitro* transcription analysis demonstrates for the first time that Spt4–Spt5 plays a positive role in transcription elongation, which might explain the *lacZ* expression defects of *spt4* and *spt5* cells.

Materials and methods

Yeast strains and plasmids

Yeast strains used are listed in Table I. Plasmids used for expression analyses were p416*GAL1lacZ* (Mumberg *et al*., 1994), pSCh202 and pSCh204 (Chávez and Aguilera, 1997), pSCh255, pSCh227 and pSCh247 (Chávez *et al*., 2001). pRS314-LA (Prado *et al*., 1997) and pRS313*GZ* (P.Huertas, unpublished data) were used to determine recombination frequencies and plasmid stability, respectively. The latter plasmid contains the *lacZ* fusion under *GAL4-CYC1* hybrid promoter cloned *in vivo* from pSEZT (J.Svejstrup, Cancer Research UK, South Mimms, UK) into pRS313 (Sikorski and Hieter, 1989) as described previously (Prado and Aguilera, 1994). YEpHPR1 used to rescue *hpr1*Δ *spt4*Δ derives from YEp13. Plasmid pRJRGAL4-VP16 (M.Ptashne, Sloan-Kettering Institute, New York, NY) was used to purify the His_6-tagged Gal4-VP16 recombinant protein from *E.coli*. For *in vitro* transcription, we constructed plasmid pGCYC1-402, which contains double-G-less cassettes of 84 and 376 bp separated by 1.4 kb fused to a *GAL4-CYC1* hybrid promoter. For the construction of this plasmid, the 300 bp *GAL4-CYC1* promoter was obtained by PCR from pGAL4CG (Lue *et al*., 1989) using primers CTAGAGGATCCGGGTGACAGCCCTCCG and CGATACATCGATAGGAGACTAGGGTGGTATAG and cloned into the *Sma*I site of pBSK+ to give pGCYC1. The new plasmid was then used to clone at the *Cla*I site generated at the 3′-end of the promoter, the double-G-less cassette system obtained by PCR from pSLG402 (Lee and Greenleaf, 1997) using primers CATGAAATCGATGGGTGTTCCTGAAGGGGGGC and GCAGGCCTAATCGATTCTAGAGGATCCCCG.

In vivo analysis of gene expression

Ten micrograms of total RNA was prepared from induced cultures and used for northern analysis following standard procedures (Chávez and Aguilera, 1997). DNA filters were hybridized first with ^{32}P-labelled DNA probes as specified. For determination of the total amount of RNA used, filters were stripped and re-hybridized with ^{32}P-labelled 28S rDNA obtained by PCR as described previously (Chávez and Aguilera, 1997).

Frequency of recombination and plasmid stability

For the determination of the recombination frequencies, six independent colonies were obtained from SC-trp and recombinants were scored as Leu^+ colonies on SC-trp-leu. Recombination frequencies were calculated as the median frequency of six independent cultures as described previously (Prado and Aguilera, 1995). Plasmid stability was determined as the median frequency of plasmid-containing cells (His^+ colonies) from a total of six independent colonies isolated from non-selective synthetic complete medium, as described previously (Piruat and Aguilera, 1998). Each recombination frequency value represents the mean of two independent experiments.

Preparation of yeast WCEs

Yeast cells were grown in rich medium YEPD at 30°C to an OD_{600} of 1. WCEs were prepared essentially as described in Schultz *et al*. (1991), with the exception that the extraction buffer and precipitation and dialysis were performed as described by Woontner *et al*. (1991). Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as standard. The final protein concentrations of the WCEs varied from 30 to 40 mg/ml. WCEs were distributed in aliquots and stored in liquid nitrogen. They were stable after repeated cycles of freezing and thawing.

In vitro transcription

Each reaction was performed in a final volume of 20 µl of buffer A 0.5 (20 mM HEPES pH 7.5, 20% glycerol, 1 mM EDTA, 1 mM DTT and 500 mM KAc) with 100 µg of WCE (3 µl) and 100 ng of Gal4-VP16 purified as described by Cho *et al*. (1997) and dialyzed in buffer A 0.05 (as A 0.5 but with 50 mM KAc). Final KAc concentration should be lower than 150 mM. The reaction was set up adding 20 µl of transcription buffer 2× [final concentration: 40 mM HEPES–KOH pH 7.5, 5 mM $MgCl_2$, 1 mM ATP, 1 mM GTP, 0.5 mM UTP, 0.03 mM CTP, 40 mM phosphocreatine, 32 µg of creatine kinase (CK), 5 mM dithiothreitol and 7.5 U of the RNase inhibitor RNAguard (Amersham)]. After 20 min of pre-incubation at room temperature 400 ng of pGCYC402 and 1 µl [α-^{32}P]CTP (3000 Ci/mmol) were added. The reaction was stopped at the indicated times by addition of 200 µl of stop buffer (10 mM Tris–HCl pH7.5, 0.3 M NaCl and 5 mM EDTA) and 160 U RNaseT1 for 15 min at room temperature. Samples were treated with proteinase K, phenol-extracted and run in a sequencing gel as described in Sayre *et al*. (1992). The amount of radiactivity incorporated was quantified with a Fuji FLA3000.

Miscellaneous

DNA isolation, ^{32}P-radiolabeling, genetic crosses and yeast transformations were performed according to standard procedures (Prado and Aguilera, 1995).

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