A Gene-Specific Requirement for FACT during Transcription Is Related to the Chromatin Organization of the Transcribed Region

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The FACT complex stimulates transcription elongation on nucleosomal templates. In vitro experiments also involve FACT in the reassembly of nucleosomes traversed by RNA polymerase II. Since several features of chromatin organization vary throughout the genome, we wondered whether FACT is equally required for all genes. We show in this study that the in vivo depletion of Spt16, one of the subunits of Saccharomyces cerevisiae FACT, strongly affects transcription of three genes, GAL1, PHO5, and Kluyveromyces lactis LAC4, which exhibit positioned nucleosomes at their transcribed regions. In contrast, showing a random nucleosome structure, YAT1 and Escherichia coli lacZ are only mildly influenced by Spt16 depletion. We also show that the effect of Spt16 depletion on GAL1 expression is suppressed by a histone mutation and that the insertion of a GAL1 fragment, which allows the positioning of two nucleosomes, at the 5’ end of YAT1 makes the resulting transcription unit sensitive to Spt16 depletion. These results indicate that FACT requirement for transcription depends on the chromatin organization of the 5’ end of the transcribed region.

Organization of DNA into chromatin inhibits transcription in vitro at both initiation and elongation steps (24, 28). Conversely, transcription elongation in vivo is a very efficient process that is usually accompanied by the alteration of chromatin structure, indicating the high ability of RNA polymerases to overcome the nucleosomal barrier in the cell nucleus (59). Yeast genetics and in vitro experiments with animal cell extracts have defined a set of factors able to help RNA polymerase II (Pol II) to carry out transcription elongation in the chromatin context (22, 61). One of the main cellular functions allowing Pol II to transcribe chromatin is played by the FACT complex, which, so far, is the only known factor able to stimulate Pol II-dependent transcription elongation through chromatin in a highly purified system (42, 45).

The human FACT complex is composed of two proteins, p140 and SSRP1, closely homologous to the essential Saccharomyces cerevisiae proteins Spt16/Cdc68 (hereafter referred to as Spt16) and Pob3, respectively (43). Spt16 has been described elsewhere as a protein involved in transcription due to its Spt− phenotype (suppression of Ty insertions in yeast promoters) conferred by spt16 alleles (34). In addition, Spt16 and Pob3 have also been involved in the transcriptional regulation of cell cycle progression and in replication (50, 52, 64). Although a direct role of Spt16 in transcription initiation has been shown (6), there are several lines of evidence that support a role of Spt16 in transcription elongation, including sensitivity of certain spt16 alleles to 6-azauracil as well as physical and genetic interactions with known elongation factors (17). The in vivo association of FACT to elongating Pol II, both in Drosofila melanogaster and in yeast, also indicates a role in elongation (36, 51).

SPT16 belongs to the histone group of SPT genes. Other genes encoding transcription elongation factors also in this group are SPT4, SPT3, and SPT6 (68). In addition to the physical interactions with Spt4-Spt5 (32) and the Paf complex (30, 58), yeast FACT (yFACT) has been reported to interact with cell elements related to chromatin remodeling, such as Chd1 (56) and the NuA3 histone acetyltransferase complex (25). Yeast FACT and the HMG box protein Nhp6 combine to form the nucleosome-binding factor SPN (18), which is able to reorganize nucleosomes in vitro (48). Human Spt16 itself binds to nucleosomes and to H2A/H2B dimers, whereas SSRP1 interacts with H3/H4 tetramers (3). On the one hand, these interactions allow FACT to destabilize nucleosomes during transcription by promoting a loss of one H2A/H2B dimer, as shown by in vitro experiments (reviewed in reference 4). On the other, mutations in the yFACT subunits are synthetically lethal, with mutations affecting chromatin assembly (19), and spt16 mutations lead to the activation of cryptic transcription initiation sites within coding regions (26, 36). These lines of evidence suggest that FACT also plays a role in maintaining the integrity of chromatin structure during transcription by participating in the reassembly of those nucleosomes altered by Pol II transcription.

The two roles assigned to FACT during transcription elongation are related to chromatin structure. Since the features of chromatin organization (histone modifications, nucleosome spacing, and degree of nucleosome translational positioning) vary throughout the genome (63, 66, 69), we wondered whether the requirement of FACT during transcription elongation is the same for all genes. In order to answer this question, we decided to compare the influences of the in vivo depletion of Spt16 on the transcription of several genes, all of...
them sharing the same promoter to exclude differential influences at the initiation step. Our results showed that those genes, such as GAL1 and PHO5, exhibiting translationally positioned nucleosomes at the 5' end of the transcribed region, are clearly sensitive to the depletion of Spt16, whereas those genes showing a random nucleosome distribution are only mildly affected.

### MATERIALS AND METHODS

**Yeast strains, plasmids, and media.** The yeast strains used in this study are described in Table 1 and are isogenic to the S288C derivative BY4741 (7). All SYI strains were constructed by standard genetic methods of tetrad analysis or transformation. SYI-2C was constructed by splicing the 5'UTR of GAL1 to the BamHI site of pSCh247, previously constructed with the plasmid pCM189SPT16 (URA3 CEN TEToff::SPT16). As the addition of doxycycline did not completely switch off the expression of SYI-2C from this plasmid, we generated SYI-6 by crossing SYI-2C and Y17202 by and subsequent shuffling of pCM189SPT16 by pCM184SPT16 (TRPI CEN TEToff::SPT16). In SYI-6, SPT16 expression was completely switched off by the addition of doxycycline. SYI-25 was constructed by tagging genomic SPT16 at its 3' end with 18 Myc epitopes followed by 5' Myc epitopes lacta TRPI. It was made following a PCR-based strategy (29) using the plasmid GA2266 as the DNA template (kindly provided by Gustav Ammerer). SYI6 and SYI25 showed the same doubling time in the absence of doxycycline, and their time courses of growth inhibition in the presence of the drug were identical. The sequence of any primer used in this study is available upon request.

**Table 1. Yeast strains used in this work**

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<th>Strain</th>
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Ten micrograms of denatured DNA from each PHO5, GAL1, or YAT1 PCR fragment was immobilized on Hybond N+ filters with a pR600 slot blot (Boehrer). After nylon membranes were prehybridized for 1 h in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, and 0.5% SDS, hybridizations were performed with 2 ml of the same solution containing labeled RNA for 36 to 40 h in a roller oven at 65°C. After hybridization, filters were washed once in 2× SSC and 0.1% SDS for 20 min and twice in 0.2× SSC and 0.1% SDS for 30 min. We performed each experiment at least three times, swapping the filters in each replicate among the different type of samples. Filters were exposed for 4 days to an imaging plate (BAS-MP; FujiFilm) that was read at 100-μm resolution in a phosphorimager scanner (FLA-3000; FujiFilm). Values were corrected for radioactive hybridization with labeled genomic DNA and normalized with the signal given by a ribosomal DNA probe also immobilized on the filter.

**Chromatin immunoprecipitation assays.** For chromatin immunoprecipitation (ChIP) analysis of Pol II or Spt16-myc, the SJY6 or SJY29 yeast strain was treated with pSCh202, pSCh247, pSCh255, or p1416GAL1 LacI z-myc plasmid. Strains were grown to mid-log phase in synthetic complete medium lacking uracil (SC-Ura) with either 2% glucose or 2% galactose. For crosslinking, cells were treated with 1% formaldehyde for 15 min at room temperature. Chromatin immunoprecipitation assays were performed as described previously (2) with 8WG16 or e-Myc (9E10) antibody. We amplified a 300-bp-long region of each open reading frame in different positions (5', middle, and 3'). As a nontran-
scribed control, we amplified a region adjacent to FUS1. Primer mixes were empirically adjusted for balanced signals. PCR signals were quantified by a phosphorimager. Immunoprecipitation was defined as the ratio of each gene-specific product in relation to that of the nontranscribed region, always after normalization with the signal of its corresponding whole-cell extract. Several dilutions of the whole-cell extract were tested to make sure that the assays were in the linear range.

Nucleosome mapping. Yeast spheroplasts and micrococcal nuclease digestions were performed as described previously (14) with the modifications described in reference 10. Spheroplasts were prepared from mid-log-phase cultures grown in SC-Ura with 2% glucose. Cells were lysed and immediately digested with 7.5 to 125 µM of micrococcal nuclease. For naked DNA controls, genomic DNA was extracted as previously described and digested with 0.003 to 0.2 µM of micrococcal nuclease under the same conditions. Micrococcal nuclease-cleaved genomic DNA was digested with Sall [for YAT1, GAL1::pr::YAT1, and GAL1::pr::YAT1 (Ser)], BamHI (for GAL1::pr::LAC4), PvuII (for GAL1::pr::PHO5), or HindIII (for GAL1) and resolved in 1.5% agarose without ethidium bromide. For the analysis of GAL1::YAT1, the probe used was the 198-bp PCR fragment immediately upstream of the Sall site present in YAT1. For the analysis of GAL1::LAC4, the probe used was the 205-bp PCR fragment immediately upstream of the BamHI site present in LAC4. For the analysis of GAL1::PHO5, the probe used was the 195-bp PCR fragment immediately upstream of the Pvull site present in pRS416, close to the GAL1 promoter. For the analysis of GAL1, the probe used was the 200-bp PCR fragment immediately upstream of the HindIII site.

RESULTS

Gene-specific effect of Spt16 depletion on mRNA levels. To investigate whether all genes are equally dependent on FACT for transcription elongation, we decided to compare several transcription units, which shared the same promotor but differed in the transcribed region, when depleting the in vivo levels of Spt16. Instead of using a degron approach, which would provoke a rapid destruction of the target protein after a heat shock, we decided to produce a slow reduction of Spt16 so as to lay out the conditions under which the protein could become limiting, but the cell physiology was not dramatically changed yet. In order to do so, we constructed the SJY6 strain, containing a Tet-controlled SPT16 gene. Doxycycline, the tetracycline analogue, did not affect the growth rate of a wild-type strain (not shown). In the absence of doxycycline, the SJY6 strain showed a wild-type growth (Fig. 1A). Although in the absence of doxycycline, the abundance of Spt16 was higher in SJY6 than in the wild type (Fig. 1B), the Tet::SPT16 construct did not produce an Spt− phenotype (not shown), suggesting that the excess of Spt16 was not sufficient to alter the correct function of Pol II machinery. Eight hours after 5 µg/ml doxycycline was added to SJY6 cells, the amount of Spt16 decreased below the wild-type levels (Fig. 1B). Two hours later, cells began to slow down their growth (Fig. 1A), but only after 12 h in the presence of doxycycline, cells began to accumulate in G1 (not shown) and cell viability started to diminish (Fig. 1A). Although in this kind of in vivo study it is formally impossible to rule out the involvement of additional elements between the input (FACT depletion) and the monitored output (changes in gene expression), we believe that the results obtained after the start of Spt16 limitation (8 h in doxycycline) and before the decrease in cell viability (12 h in doxycycline) are likely the direct consequences of FACT depletion on gene expression. According to this, for the following studies we compared samples taken 6, 8, 10, and 12 h after adding doxycycline.

The SJY6 strain was transformed with plasmids containing the following genes driven by the GAL1 promoter: PHO5 (pSCh202), YAT1 (pSCh247), Escherichia coli lacZ (p416GAL1-lacZ), and K. lactis LAC4 (pSCh255). We cultured these transformants in selective galactose medium both in the presence of 5 µg/ml doxycycline and in the absence of the drug. The mRNA levels of the mentioned genes and the endogenous GAL1 gene were then analyzed by Northern blotting. In the absence of doxycycline, the Northern conditions and time of exposure required to detect all hybridization signals were the same, suggesting that similar levels of mRNA accumulation were occurring in the cell for the five tested genes.

The depletion of Spt16 strongly affected the mRNA levels of the GAL1 gene. Ten hours after the addition of doxycycline, the mRNA levels of GAL1 were one-half of the untreated control, and 2 hours later, they hardly reached 25% (Fig. 1C). No expression of the endogenous PHO5 gene is detected in this high-phosphate medium due to the strong repressive nucleosomal organization of the PHO5 promoter (60). However, when PHO5 was transcribed from the GAL1 promoter, its mRNA levels were also severely affected by the depletion of Spt16. Just 8 h after the addition of doxycycline, a significant decrease in the PHO5 mRNA levels was detected compared to those of the control; 4 hours later, they were less than one-third of the control levels (Fig. 1C). In contrast, the levels of lacZ mRNA were only mildly affected by Spt16 depletion when lacZ mRNA was transcribed from the GAL1 promoter. No significant differences with the control were observed until 12 h after the addition of doxycycline, and at this time, the levels of lacZ mRNA still reached 60% of the control level (Fig. 1C). To exclude the possibility that this difference between GAL1 and PHO5 on the one hand, and lacZ on the other, was due to the larger length of lacZ mRNA (GAL1, 1.7 kb; PHO5, 1.5 kb; lacZ, 3.1 kb), we investigated the effect of Spt16 depletion on YAT1, a 2-kbp-long yeast gene that showed transcriptional behavior similar to that of lacZ in other mutants affected in gene expression (11). Since the mRNA levels of the endogenous YAT1 gene were undetectable under our culture conditions (not shown), the Northern results reflected the influence of Spt16 depletion on the GAL1::pr::YAT1 transcription unit. As shown in Fig. 1C, YAT1 mRNA was not affected by the depletion of Spt16 even 12 h after the addition of doxycycline. To further confirm that the length of the transcription unit was not related to the sensitivity to Spt6 depletion, we analyzed the mRNA levels of LAC4 (3.1 kb), a eukaryotic homologue of lacZ isolated from the yeast Kluyveromyces lactis. When LAC4 mRNA was transcribed from the GAL1 promoter, its accumulation was clearly affected by Spt16 depletion. Ten hours after the addition of doxycycline, the mRNA levels were already significantly far from the control, and 2 hours later, they reached only one-third of the control levels (Fig. 1C). Altogether, the results shown in Fig. 1C indicate a differential effect of Spt16 depletion on the mRNA levels of the tested transcription units. Since all shared the same promoter, we inferred that the detected differences should be related to the transcribed sequences.

It has been reported that spat16 mutations and in vivo depletion of Spt16 activate transcription initiation from cryptic promoters within coding regions (26, 36). As can be seen in Fig. 1D, after 12 h in the presence of doxycycline, the cryptic pro-
moter located in FLO8 also became slightly activated. However, no additional transcripts were detected in any of the cases where Spt16 depletion led to a marked decrease of the full-length transcript. As shown in Fig. 1E, neither GAL1 nor PHO5 or LAC4 produced secondary transcripts 12 h after the addition of doxycycline. We concluded that the detected declines in full-length transcripts were not mediated by the activation of cryptic initiation sites but rather were related to a postinitiation event.

**Differential effect of Spt16 depletion on transcription.** Assuming that the activity of GAL1 was not differentially influenced by the coding sequences inserted downstream, the differential effect of Spt16 depletion on the mRNA levels could be due either to a direct, but distinctive, influence of the Spt16 shortage on transcription elongation by Pol II or to an indirect effect of Spt16 scarcity on the stability of the different transcripts. To distinguish between these two possibilities, we determined Pol II processivity along the five tested genes 10 h after the addition of doxycycline. In order to do so, we measured the level of Pol II occupancy at the five transcription units. It has been reported for several genes and for the hybrid transcription unit GAL1-pr::YLR454 that the level of Pol II association is constant throughout coding regions (35, 36). However, after Spt16 depletion, Pol II occupancy in the middle and at the 3'end of GAL1 decreased to 0.6 and 0.7, respectively, in relation to the occupancy at the 5'end (Fig. 2A). Similar drops in Pol II occupancy were observed in the middle and at the 3'end of GAL1-pr::PHO5 and, to a lesser extent, at GAL1-pr::LAC4 (Fig. 2A). However, no effect of Spt16 depletion on Pol II occupancy was observed at GAL1-pr::lacZ and at GAL1-pr::YAT1 (Fig. 2A). The alteration in Pol II occupancy was limited to the three transcription units that also showed reduced levels of mRNA accumulation, and in all three cases, Pol II occupancy did not decrease further when being transcribed from the middle of the genes to the 3'ends. Taking into account that this technique detects only major defects in Pol II processivity (35, 38), we consider that these modest decreases in Pol II occupancy are significant enough and compatible with a processivity defect of Pol II at the 5'regions caused by Spt16 depletion. However, since Pol II molecules sitting at the promoter might also contribute to the 5'ChIP signals, a defect in the initiation-to-elongation transition cannot be completely excluded.

We also performed run-on assays with the endogenous GAL1 gene and GAL1-pr::PHO5, as representatives of the transcription units sensitive to Spt16 depletion, and GAL1-pr::YAT1, as the least sensitive to Spt16 shortage. We used 5'and 3'probes to measure the density of elongating polymerases at both ends of the transcribed regions. Eight hours after the addition of doxycycline, the three tested genes started to show a decline in their run-on signals that, only in the cases of the 3'ends of GAL1 and GAL1-pr::PHO5, was significantly different.
from that of the untreated control (Fig. 2B). Furthermore, the results showed that 12 h after the addition of doxycycline, the density of elongating Pol II at both ends of \( \text{GAL1} \) was around 10% of the control values (Fig. 2B). Very similar results were obtained when the run-on analysis was performed with the \( \text{GAL1pr}::\text{PHO5} \) transcription unit (Fig. 2B). In contrast, at the same time of depletion, \( \text{GAL1pr}::\text{YAT1} \) showed run-on signals five times higher at both ends of \( \text{YAT1} \) (Fig. 2B).
FIG. 3. GAL1pr::PHOS and GAL1pr::LAC4, but not GAL1pr::YAT1, exhibit translationally positioned nucleosomes on their transcribed regions. Chromatin and naked DNA samples of Y13232(pShc202) (A), BY4742(pShc247) (B), Y10425(pShc255) (C), and BY4742 (D) were treated with micrococcal nuclease (MN), digested with the indicated restriction enzymes, resolved in agarose gels, and hybridized with the noted DNA probes to map nucleosomes at GAL1pr::PHOS (A), GAL1pr::LAC4 (B), GAL1pr::YAT1 (C), and chromosomal YAT1 (D). Horizontal arrows indicate chromatin-dependent hypersensitive sites for micrococcal nuclease; white triangles indicate protections of micrococcal nuclease cuts present at the naked DNA lanes. Ovals show the positions of the mapped nucleosomes.

Considering the results of Pol II distribution by ChIP and run-on, we conclude that the different levels of mRNA accumulation measured after we added doxycycline were due to a differential effect of Spt16 depletion on transcription. We conclude that this effect is likely to take place at the elongation phase or at the initiation-to-elongation transition. The similar half-lives of GAL1 and GAL1pr::lacZ mRNAs during Spt16 depletion rule out changes in mRNA stability as the main explanation for the detected gene-specific effect of Spt16 shortage, although minor effects at this level cannot be completely excluded (not shown).

As the FACT complex travels with Pol II during elongation (36, 51), we considered whether the gene-specific differences detected were related to an unequal presence of FACT at the transcribed regions before Spt16 depletion. To answer this question, we carried out ChIP analysis of Spt16 under nondepleting conditions. To avoid influencing the ChIP analyses by possible Spt16 present at the GAL1 promoter, the PCR oligonucleotides selected corresponded to the central region of each gene and they were at least 700 bp away from the start codon. As shown in Fig. 2C, a transcription-dependent ChIP signal for Spt16 was detected in the five genes studied. These results indicated that Spt16 is present even in those genes, such as YAT1 and lacZ, whose transcription is least influenced by Spt16 depletion, might explain the degree of sensitivity to Spt16 shortage. Instead, we envisaged some intrinsic feature of the transcribed region to be the element that explains the differential response of the five tested genes to Spt16 depletion.

Correlation between nucleosomal organization and sensitivity to Spt16 depletion. We have shown elsewhere that, when introduced in the yeast genome, lacZ does not exhibit transcriptionally positioned nucleosomes, even when it is fused to the GAL1 promoter (11). In contrast, the GAL1 gene shows an ordered array of nucleosomes on the transcribed region (11). Taking into account these previous results and the solid in vitro data assigning a chromatin-remodeling role to FACT in transcription elongation (42, 48), we decided to investigate the chromatin organization of the other three transcription units so far studied in this work.

Indirect end-labeling experiments of chromatin preparations, partially digested with micrococcal nuclease, showed clear patterns compatible with positioned nucleosomes at the transcribed regions of GAL1pr::PHOS and GAL1pr::LAC4 but not at the transcribed region of GAL1pr::YAT1 (Fig. 3A to C). In the GAL1pr::PHOS transcription unit, the comparison of the chromatin lanes to the ones of naked DNA gave a combination of hypersensitive sites and protections that predicted an array of at least six nucleosomes covering the GAL1 promoter and the first half of the PHOS gene (Fig. 3A). Additional hypersensitive sites are visible in the second half of the PHOS gene, suggesting that the nucleosomal array might extend further. A similar picture was obtained from the GAL1pr::LAC4 transcription unit (Fig. 3B). The intensity of the bands corresponding to the predicted linker regions was not always the same, but the differences between the lanes corresponding to chromatin and naked DNA are clear enough to conclude that chromatin is not positioned at random at the transcribed re-
GAL1 in both the chromatin and the naked DNA samples. We then "positioned" those genes whose micrococcal nuclease pattern is the same in all cases, hereafter we call "positioned" all genes showing a nonrandom pattern of chromatin and "nonpositioned" those genes whose micrococcal nuclease cuts already shown on the transcribed region of GAL1 were detected on the transcribed region of the chromosomal YAT1 gene. These results rule out a possible episcopal artifact and confirm that the presence of positioned nucleosomes in a promoter is not enough to transmit this positioning to any adjacent chromatin region, just as we have previously found with GAL1::lacZ (11). The DNA sequence of the transcribed region seems therefore to be essential for nucleosome positioning downstream of the GAL1 promoter.

Considering the previously published data and the results shown in Fig. 3, we can establish a good correlation between the sensitivity of a transcription unit to Spt16 shortage and the occurrence of translationally positioned nucleosomes on its transcribed region. This is the case for the endogenous GAL1 gene and the GAL1::PHO5 and GAL1::LAC4 transcription units. Vice versa, GAL1::lacZ and GAL1::YAT1, the two least Spt16-dependent transcription units, exhibit randomly positioned nucleosomes at their transcribed regions. Although the degree of nucleosome positioning is not exactly the same in all cases, hereafter we call "positioned" all genes showing a nonrandom pattern of chromatin and "nonpositioned" those genes whose micrococcal nuclease pattern is the same in both the chromatin and the naked DNA samples.

Since all transcription units so far analyzed were controlled by the GAL1 promoter, we wondered whether the correlations between nucleosome positioning and Spt16 dependency could be extended to other genes. We first measured the effect of Spt16 depletion on the mRNA levels of the chromosomal YAT1 gene, transcribed in ethanol-containing medium. As shown in Fig. 4A, Spt16 depletion did not cause a negative effect on YAT1 mRNA levels but rather an increase that might be caused by the up-regulation of the YAT1 promoter under these conditions (ethanol, absence of glucose, and limitation of Spt16).

Our nucleosome-mapping results could not be compared to the data obtained with tiled arrays for a substantial part of the Saccharomyces cerevisiae genome, as that study did not include the coding regions of YAT1, PHO5, or GAL1 (69). We decided to measure the mRNA levels of two genes, highly expressed in YPD, whose chromatin organizations have been described in that study: SRO9 and CIT2 (69). The first one displays a translationally positioned nucleosome at the 5’ end of the transcribed region, whereas the second one lacks translationally positioned nucleosomes at its 5’ end, displaying, however, several of them on the second half of its coding region. As shown in Fig. 4B, the mRNA levels of SRO9 were very sensitive to Spt16 depletion, while the amounts of CIT2 mRNA were not negatively affected by the Spt16 shortage but, like those of YAT1, were rather upregulated.

We also examined the effect of Spt16 depletion on the mRNA levels of the metallothionein-coding CUP1 gene, whose chromatin is not organized into a unique array of positioned nucleosomes when transcribed but into clusters of overlapping nucleosome positions (55). We measured the effect of Spt16 depletion on the accumulation of CUP1 mRNA in cells growing in copper-containing medium. No significant effect was observed (Fig. 4C).

FIG. 4. Effect of Spt16 depletion on the expression levels of several genes driven by their own promoters. (A) Northern analysis of the mRNA levels of YAT1 during Spt16 depletion. SJY6 cells were grown in SC with ethanol to induce YAT1 expression. (B) Northern analyses of the mRNA levels of SRO9 and CIT2 during Spt16 depletion. SJY6 cells were grown in SC. Nucleosome positioning at the two genes is depicted as described by Yuan et al. (69). (C) Northern analysis of the mRNA levels of CUP1 during Spt16 depletion. SJY6 cells were grown in SC plus copper. Overlapping phases of nucleosomes at the transcribed CUP1 gene are depicted as described by Shen et al. (55). The results of a typical experiment and the quantification of three independent experiments are shown in each case. Dox, doxycycline; prom, promoter; a.u., arbitrary units.
their chromatin organization. We first made use of the histone H4 allele hhf2-13, a dominant H4-R45H mutation that causes alterations of chromatin structure by disrupting essential DNA-histone interactions (40, 65) and favors nucleosome mobility in vitro (15). Overexpression of hhf2-13 suppressed the negative effect of Spt16 depletion on the GAL1 mRNA levels, indicating that the impairment of GAL1 expression caused by Spt16 depletion was mediated by chromatin structure (Fig. 5A). The absence of SPT16 mRNA in the doxycycline-treated hhf2-13 cells indicated that the suppression was not due to a deficient repression of the Tet promoter (Fig. 5A). The levels of GAL1pr::YAT1 mRNA remained unaffected. This rules out the suppression as a consequence of a general increase in either mRNA levels or GAL1 promoter activity caused by hhf2-13 (Fig. 5A).

We also engineered GAL1pr::YAT1 to introduce positioned nucleosomes in its transcribed region. We inserted a 430-bp fragment from the 5′/H11032 end of the GAL1 transcribed region into the GAL1pr::YAT1 transcription unit, immediately downstream of the promoter. The resulting GAL1pr::GAL1(5′/H11032)-YAT1 transcription unit became sensitive to Spt16 depletion, as shown in Fig. 5B. We checked that, as expected, the inserted GAL1 fragment is able to position two nucleosomes (Fig. 5B), confirming that even in a plasmid, both the promoter and the transcribed region of GAL1 keep their chromatin organization. Again in this transcription unit, a low number of positioned nucleosomes seems to be sufficient to make transcription Spt16 dependent, at least when they are located at the 5′ end of the transcribed region. We conclude that FACT seems to be required for transcription of the DNA sequences immediately downstream of the initiation site whenever they are organized into translationally positioned nucleosomes.

Since FACT seemed to be necessary for transcription when the 5′ end of the transcribed region was occupied by positioned nucleosomes, we decided to investigate possible chromatin reorganizations at that region of the GAL1 gene after transcription induction. As shown in Fig. 6A, the first three nucleosomes that were clearly positioned at the 5′ end of the transcribed region when wild-type cells were grown in glucose (+2, +3, and +4) became relocated when cells were grown in galactose.
In agreement with previous studies (14), nucleosome +2, occupying the region where the preinitiation complex (PIC) gets assembled, was substituted in galactose by a broad hypersensitive region. In addition, the space corresponding to nucleosomes +3 and +4 was occupied by a pattern of protections and hypersensitivities that are compatible with a single positioned nucleosome in the middle and two smaller structures at both sides (Fig. 6A). Further studies would be needed to clarify this aspect. In any case, this experiment shows that, although the 5' end of the GAL1 transcribed region suffers drastic chromatin reorganization after activation, the nucleosomal distribution of this region in galactose is not random but shows at least one positioned nucleosome. To test whether this nucleosomal pattern was still present during Spt16 depletion, we analyzed the nucleosomal organization of GAL1 in SJY6 cells growing in galactose plus doxycycline. Ten hours after doxycycline was added, the pattern of micrococcal nuclease cuts was very similar to that of the wild type in galactose (Fig. 6B). We conclude that GAL1, and likely other genes displaying positioned nucleosomes immediately downstream of the initiation site, keeps a positioned nucleosomal organization during transcription, which seems to require FACT to be transcribed.

**DISCUSSION**

Depletion of FACT affects transcription of genes differentially. Pol II cannot carry out productive transcription of DNA templates organized in chromatin, both at initiation and at elongation phases, in the absence of additional factors. In vitro transcription experiments have demonstrated the ability of FACT to stimulate transcription elongation of chromatin templates by Pol II (42) in cooperation with H2B monoubiquitination (45). In those in vitro experiments, the activity of FACT as a chromatin-dependent elongation factor was demonstrated by comparing its effects on naked DNA and chromatin. Since cell DNA is always organized in chromatin. However, the features of chromatin organization vary throughout the genome. In animal cells, some genomic regions show a higher tendency to establish translationally positioned nucleosomes than others (66). In Saccharomyces cerevisiae, 65% of the genome shows translationally positioned nucleosomes; promoter-proximal sequences within coding regions show a higher tendency toward nucleosome positioning, but coding regions almost entirely covered by nonpositioned nucleosomes are also found (69). Following an in vivo depletion strategy, we have tested the consequences of FACT scarcity on the expression of the studied genes, or at least a subset of them, are more reluctant to slide or to be transferred than those nucleosomes occupying nonpositioned genes. These stable nucleosomes would require the octamer disassembly-reassembly activity of FACT.

The connection between positioning and nucleosome stability is also supported by the phenotype of some histone mutants which show defects in nucleosome positioning in vivo (65) and an increased nucleosome mobility in vitro (15), due to alterations of the histone-DNA interactions on the surface of the nucleosome (40). We have used one of these histone mutants (hhf2-13) to test our hypothesis, and we found that the impairment of GAL1 transcription after Spt16 depletion was clearly suppressed by hhf2-13, making GAL1 expression insensitive to Spt16. It is the central DNA wrap of the nucleosome which is affected by hhf2-13 (40). The same region of nucleosomal DNA is also perturbed by FACT action, according to the in vitro studies of FACT-nucleosome interaction (48). We find it full of sense, therefore, that hhf2-13 suppresses the absence of FACT at a gene displaying positioned nucleosomes. It is theoretically possible that this suppression is not caused by the histone mutation itself but by the increase in histone dosage produced by the introduction of extra copies of the H3 and H4 coding genes (12). We do not favor this interpretation, since it has been shown that histone imbalance does not affect GAL1 chromatin organization and does not derepress GAL1 transcription (41). But even if this were true, the results of this experiment would support that the gene-specific effect of FACT depletion is mediated by chromatin.

The analysis of the nucleosomal organization of GAL1 in glucose and in galactose indicates a deep reorganization of the 5' end of the coding region after activation, in agreement with
the inverse correlation between histone-DNA interactions (measured by ChIP) and transcriptional activity reported for GAL genes (54). It is worth mentioning that the transcription-dependent changes in chromatin structure that we have detected at the GAL1 coding region were not observed in a previous analysis of GAL1 chromatin (11). The main difference between the two analyses was the genetic background. In the previous study, we used W303-derived strains, whereas in the present study, all strains were isogenic to BY4741. Further studies would be needed to clarify this striking difference. In any case, in both BY4741 and W303 cells grown in galactose, the 5' end of the GAL1 coding region is not nucleosome free. At least one positioned nucleosome is present during transcription in that region, suggesting that the reported decrease in histone occupancy of GAL genes during transcription (54) does not involve a random nucleosomal distribution.

Finally, an important piece of evidence connecting FACT and positioned nucleosomes at the 5' end of the coding region comes from the insertion of two positioned nucleosomes between the promoter and the transcribed region of the Spt16-independent GAL1pr::YAT1 transcription unit. The resulting GAL1pr::GAL1(5')-YAT1 became sensitive to Spt16 depletion. Altogether, our results suggest that FACT is required for the transcription of those genes whose transcribed region is organized into positioned nucleosomes at the 5' end.

It has been shown that FACT plays a role in preventing the activation of cryptic initiation sites by contributing to the proper reposition of nucleosomes after the passage of elongating Pol II (26, 36). We have indeed shown here the slight activation of a cryptic initiation site present in FLO8 12 h after adding doxycycline. However, 8 or 10 h after doxycycline was added, times chosen for the functional analyses of this study, the cryptic initiation site present in FLO8 was not active yet, and the nucleosomal organization of GAL1 was similar to that of the wild type. We have shown that the negative effect of Spt16 shortage on the accumulation of GAL1, PHO5, and LAC4 mRNAs was not due to the activation of cryptic initiation sites within their coding regions. Moreover, the results of the run-on experiments show a lower density of elongating Pol II at PHO5 and GAL1 and do not support secondary transcripts emerging in these genes. Mason and Struhl (36) suggested that the overall negative effect of Spt16 depletion on transcription might be due to a competition between normal promoters and cryptic initiation sites for the transcriptional machinery. According to this hypothesis, the higher number of initiation sites originated in the cell by the depletion of Spt16 might affect the GAL1 promoter due to a subsequent scarcity of general transcription factors. Since the five genes driven by the GAL1 promoter do not behave the same, we can also exclude this explanation for the phenomenon described here, unless the sequences located downstream differentially affect the activity of the promoter (see below).

FACT has also been involved in PIC assembly by facilitating TATA-binding protein binding to the TATA box in the context of a nucleosome. According to this, FACT depletion might also affect transcription initiation in a promoter-specific manner. However, it is difficult to explain all of the results shown in this work in terms of transcription initiation. We have shown here that several transcription units driven by the same promoter (GAL1pr) exhibit different degrees of sensitivity to Spt16 depletion. The diverse nucleosomal distributions at the coding regions might differentially affect the chromatin organization of the GAL1 promoter. However, we did not find such differences at the nucleosomal mapping that we have carried out, although subtle differences cannot be completely ruled out. We would then expect a decrease in Pol II recruitment. In contrast, we have found accumulation of Pol II at the 5' end of the Spt16-dependent genes. We cannot exclude that a part of this accumulation corresponds to initiating Pol II due to the inherent inaccuracy of the ChIP technique, but in that case, the results would be compatible with a role of FACT in the transition from initiation to elongation and not in PIC assembly.

Although we cannot completely rule out an initiation component, the simplest interpretation of our results suggests that an involvement of FACT in transcription elongation may be immediately after initiation has occurred. According to this perspective, FACT would be required for facilitating transcription through those nucleosomes less prompt to slide or to be transferred. In the absence of FACT, Pol II would pause in front of such nucleosomes and would eventually become arrested. The comparison of the patterns of Pol II distribution after Spt16 depletion, obtained by ChIP and by run-on, detects a difference: in GAL1 and GAL1pr::PHO5, the amounts of immunoprecipitated Pol II located at 5' are higher than in the rest of the gene; in contrast, the densities of active Pol II are roughly similar at the 5' and 3' ends of both genes. The simplest explanation for this phenomenon would be that the excess of Pol II present at 5' became arrested after suffering backtracking and therefore was undetectable by a run-on assay. This hypothesis is also in agreement with published results showing how nucleosomes induce Pol II arrest in vitro by stabilizing its backtracked conformation (27).

In Drosophila polytene chromosomes, Saunders et al. (51) have shown that FACT is not recruited to RNA polymerase III-dependent genes, which are known to undergo nucleosome transfer rather than disassembly during in vitro transcription elongation. It may be possible that FACT-dependent nucleosome disassembly/reassembly would be required only by Pol II to transcribe positioned nucleosomes, whereas those nucleosomes not exhibiting a fixed translational positioning might be more likely to transfer or slide during transcription elongation. We do not have data to distinguish which of the two proposed functions of FACT, disassembly or reassembly, is critical for transcription of GAL1 and the other Spt16-dependent genes studied here. However, if we consider positioning as an indication of nucleosome stability, as discussed above, it seems more likely that transcription elongation of highly organized chromatin requires the nucleosome disassembly activity of FACT. If this interpretation is true, an explanation must be provided for the predominant requirement for FACT at the 5' end of the coding regions. Either nucleosomes positioned at these regions are particularly stable, or the capability of Pol II machinery to interact with nucleosomes changes along the transcribed region by including perhaps other histone chaperones such as Asf1, also acting during transcription elongation (53). Alternatively, the accumulation of positive DNA supercoiling ahead of Pol II might facilitate nucleosome reorganization once genes have been transcribed to some extent, as it has been shown elsewhere (31), reducing the FACT requirement at these regions.
Does every gene require its own menu of factors after transcription initiation? Biochemical and genetic analyses during the last 15 years have described a numerous set of factors playing auxiliary roles in Pol II-dependent mRNA biogenesis after transcription initiation, favoring mainly processivity (35). However, little is known about the relative importance of each of these factors in terms of the number of genes that requires their function. Since they measure the combination of initiation, elongation, and mRNA stability, global transcriptome analyses have not been very useful in this respect. In this work, by comparing five genes under the control of the same promoter, we have shown that FACT is not equally required for all genes during transcription. It was recently reported that, although recruited to the transcribed region of the human p21 gene in a carboxyl-terminal domain (CTD) kinase-dependent manner when it becomes activated by p53, FACT is dispensable for p21 expression (21). In fact, p21 transcription does not require CTD phosphorylation at Ser2, indicating that the requirement of P-TEFb for transcription elongation is also gene specific (21). It is worth mentioning that CUP1, one of the genes whose expression is not affected by Spt16 depletion, can be transcribed by a mutant version of Pol II that lacks the CTD (37). Altogether, these elements suggest a relationship between the requirements for CTD phosphorylation and FACT. In this respect, it would be interesting to analyze the nucleosomal organization of p21 and other possible P-TEFb- and FACT-independent mammalian genes.

By using the same five transcription units driven by the GAL1 promoter that have been analyzed in this work, we have shown elsewhere that the THO complex, involved in the connection between transcription elongation and mRNA transport, is also not uniformly needed for all of them (11). It is interesting that those transcription units whose elongation is highly dependent on FACT are not strongly affected by theo mutations; this is the case for GAL1, GAL1p::PHO5, and GAL1p::LAC4. However, GAL1p::lacZ and GAL1p::YAT1, dramatically affected by theo mutants, are only mildly affected by Spt16 depletion. According to the chromatin analysis presented here, the THO complex seems to be specially needed at genes with random chromatin organization. Since THO plays a role in preventing the formation of R loops by nascent mRNA (23), a contribution of positioned nucleosomes in preventing R loops can be suggested.

Another gene-specific factor involved in postinitiation events is TFIIIS, an elongation factor dispensable for the expression of most genes, which plays a capital role in transcriptional activation of Drosophila hsp70. It does so by releasing promoter-proximal paused Pol II from arrest (1). Pol II pausing in hsp70 at the transcription elongation step seems to be influenced by the nucleosomal organization of the promoter-proximal region (8, 57). Considering FACT, P-TEFb, THO, and TFIIIS, the emerging picture is that the intrinsic properties of the transcribed region of a given gene determine the set of factors required for its proper mRNA biogenesis.

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