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Yeast H2A.Z, FACT complex and RSC regulate transcription of tRNA gene through differential dynamics of flanking nucleosomes

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ABSTRACT

FACT complex is involved in elongation and ensures fidelity in the initiation step of transcription by RNA polymerase (pol) II. Histone variant H2A.Z is found in nucleosomes at the 5′-end of many genes. We report here H2A.Z-chaperone activity of the yeast FACT complex on the short, nucleosome-free, non-coding, pol III-transcribed yeast tRNA genes. On a prototype gene, yeast *SUP4*, chromatin remodeler RSC and FACT regulate its transcription through novel mechanisms, wherein the two gene-flanking nucleosomes containing H2A.Z, play different roles. Nhp6, which ensures transcription fidelity and helps load yFACT onto the gene flanking nucleosomes, has inhibitory role. RSC maintains a nucleosome abutting the gene terminator downstream, which results in reduced transcription rate in active state while H2A.Z probably helps RSC in keeping the gene nucleosome-free and serves as stress-sensor. All these factors maintain an epigenetic state which allows the gene to return quickly from repressed to active state and tones down the expression from the active *SUP4* gene, required probably to maintain the balance in cellular tRNA pool.

INTRODUCTION

Eukaryotic genome is organized into transcriptionally inactive, condensed heterochromatin and transcriptionally active euchromatin in different epigenetic states. Chromatin formation, which results in repressive effect on elongation, can suppress non-specific initiations from the transcribed regions as well. A complex, facilitates chromatin transcription (FACT) with two subunits Spt16 and SSRP1 in humans, can counter this nucleosomal obstacle during elongation and even ensure the fidelity of transcription initiation by RNA polymerase (pol) II (1–3). FACT travels with pol II and removes/reinstates the H2A–H2B dimer from the nucleosome after passage of the pol II during elongation (4,5). Yeast FACT consists of three proteins: Spt16, Pob3 and a small, HMG box-containing, non-histone protein Nhp6 (6). Cells with deletion of both the copies of Nhp6 are viable (7), while Spt16/Pob3 are essential for cell viability (8), suggesting their functions are independent of each other.

Nucleosomal arrangement on pol II-transcribed genes follows a general pattern, wherein a nucleosome-free region (NFR) is flanked by two positioned nucleosomes (9). Several studies suggest that tDNAs are generally devoid of nucleosomes (9–11) and hence, refractory to chromatin mediated repression. Out of approximately 280 targets of pol III in the *Saccharomyces cerevisiae* genome (12,13), 275 genes code for different tRNAs. The most abundant ATP-dependent chromatin remodeler of yeast, RSC which interacts with all the three RNA polymerases, is found on most of the pol III-transcribed genes in yeast (14). Genome-wide localization studies of the chromatin modifying complexes also suggest that chromatin may be having an important regulatory role in expression of these genes (14–16). Recent studies have revealed that histone H2A variant H2A.Z (coded by Htz1 in *S. cerevisiae*) localizes to hundreds of repressed or basal, preferably TATA-less promoters of pol II (9). It acts as boundary mark for pol II-transcribed genes and is found around −200 and +200 bp from the transcription start site (TSS) of the tRNA genes (9). However, the role of H2A.Z in pol III transcription is largely an unexplored area. Earlier studies from our lab had shown that *in vivo* chromatin structure of a yeast pol III-transcribed gene, *SNR6* is remodelled by RSC in

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response to nutritional stress but H2A.Z, present in the upstream, regulatory nucleosome is not required for its activation *in vivo* (17).

Much of the *in vitro* transcription by pol III has been studied using yeast *SUP4*, one of the $tRNA^{Tyr}$ gene family members. Like a typical pol III-transcribed gene, *SUP4* has two internal promoter elements 55 bp apart, boxes A and B, but no upstream regulatory elements. The transcription complex formation is initiated with binding of the transcription factor (TF) IIIC to the boxes A and B, followed by the recruitment of the initiation factor IIIB, 30 bp upstream of TSS at +1 (18). Short length of genes facilitates terminator-dependent recycling of pol III which involves direct transfer of pol III to the TSS after termination in each round, giving several fold increase in transcription *in vitro* (19). Transcription by pol III is tightly regulated in response to any stress by a central regulator Maf1 (20,21). Nutrient deprivation like nitrogen and carbon starvation leads to immediate downregulation of transcription by pol III (22) and pol III is lost from its target genes under the conditions of repression (23).

In this study, we have shown the role of H2A.Z, RSC and FACT in modulating the transcription of the yeast $tRNA^{Tyr}$ gene. Working as H2A.Z chaperone, Spt16 with the help of SWR1 complex deposits as well as removes H2A.Z in the gene flanking nucleosomes. RSC promotes loading of FACT on the gene, maintains the transcribed region of *SUP4* nucleosome-free and keeps a nucleosome close to the gene terminator region *in vivo*, which probably prevents the facilitated recycling of pol III, resulting in lower transcription from *SUP4* in active state. These studies show a novel mechanism of transcriptional regulation of *SUP4* by all these activities which together keep the *SUP4* expression at a low level.

MATERIALS AND METHODS

Plasmid DNAs, primers, yeast strains and growth

Plasmid pLNwt containing the 256 bp DNA from the yeast *SUP4* gene locus was gift. Sequences of the primers and list of the yeast strains used in this study are given under the Supplementary Tables S1 and S2, respectively. Yeast cultures were grown in YEP (yeast extract and peptone) media containing 2% glucose. Cells were nutritionally deprived by shifting to 0.15× YEP without any carbon source (23) and allowed to grow for further 1.5 h, unless otherwise stated. Yeast cultures of temperature sensitive mutants were rapidly shifted to 37°C by adding pre-warmed media to the cultures grown at 30°C.

In vitro chromatin assembly and transcription

Chromatin was assembled on pLNwt using S-190 extract of Drosophila embryos and *in vitro* transcription using pure TFIIIB, TFIIIC and pol III was performed as previously described (24). 6× His-tagged Nhp6A was purified from the overexpression clone (gift from Ian Willis). Spt16–Pob3 dimer was purified from strain carrying TAP-tagged Spt16 using standard tandem affinity purification protocol (25).

Antibodies

Antibodies against H3 (Ab46765), H2A.Z (Ab4626) and Myc (9E10) were purchased from Abcam. Anti-HA, anti-myc (05-904, 05-724MG; Millipore), IgG sepharose (GE Healthcare), FLAG M2 agarose (Sigma) were purchased while anti-Spt16 and anti-Pob3 (Tim Formosa) were gifts.

Chromatin structure, chromatin immunoprecipitation and RNA *in vivo*

Chromatin structure analysis by the IEL method, chromatin immunoprecipitation (ChIP) and real time PCR, RNA extraction and quantification were performed as described (17), and repeated at least three times for each experiment. Fold enrichments in ChIP and real time PCR assays were calculated as occupancy normalized against *TELVIR* region and average from three independent experiments with error bars are shown. For high-resolution footprinting, digestion of chromatin with MNase *in vivo* and sample preparations were as described for the IEL method. Samples were used for primer extension and products resolved on 8 M urea–6% polyacrylamide gel as described earlier (24). Further details for each method and primers can be found in Supplementary Data. Secondary digestion of DNA for IEL analysis was with a HindIII site present 860 bp downstream of the *SUP4* gene and probe was 150 bp DNA abutting HindIII site.

Co-immunoprecipitations and pull-downs

Co-immunoprecipitation (IP) experiments (26) using yeast whole cell extract and *in vitro* pull downs to follow physical interactions of tagged H2A.Z and Spt16 proteins were performed as described under Supplementary Data.

RESULTS

We studied the role of chromatin in regulating *SUP4* transcription, *in vitro* as well as *in vivo*. Recruitment of various activities to *SUP4 in vivo* under two different nutritional states in yeast was ascertained by ChIP and real time PCR assays.

Chromatin influences the transcription of *SUP4* gene

Naked DNA transcription of *SUP4* is known to be TFIIIC-dependent (Figure 1A, lanes 1 and 2). Transcription from *SUP4* using pure TFIIIC, pol III and recombinant TFIIIB gives two additional transcripts, corresponding to downstream initiations from the positions +4 and +8 with respect to the TSS at +1. Similar to *SNR6* (24), addition of TFIIIC to the chromatin assembled on the plasmid pLNwt yields 5-fold more transcript (Figure 1A, lane 2 versus 4). Chromatin formation suppresses the non-specific transcription from the plasmid backbone (data not shown) which may contribute to the observed higher than naked DNA (lane 2) level of transcription from chromatin (lane 4). Unlike *SNR6*, TFIIIC addition does not result in a nucleosome positioning on

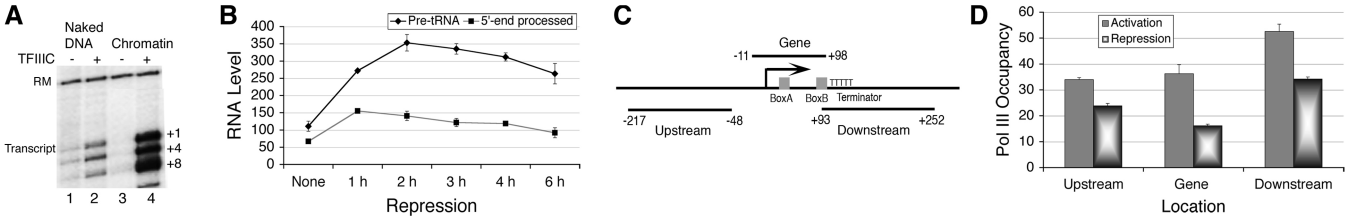

Figure 1. Transcription of *SUP4* gene and chromatin. (**A**) Transcription from chromatin is higher than naked DNA *in vitro*. TFIIIC-dependent transcription of naked DNA (cf. lanes 1 and 2) and chromatin (cf. lanes 3 and 4) produces two additional transcripts due to downstream initiations from the positions +4 and +8 with respect to TSS at +1. RM represents the position of recovery marker in the gel. Transcript from +1 site is marked. (**B**) *SUP4* RNA synthesis under nutritional stress. The total *SUP4* transcript levels at different time points of repression are normalized against the U4 transcript used as an internal control. Average and scatter of RNA levels estimated from three independent experiments are plotted. (**C**) Schematic diagram showing the amplicon sizes and primer pairs used in real time PCR and chromatin immunoprecipitation studies. Positions of the gene sequence elements with respect to the three amplicons representing the upstream, gene and downstream regions of *SUP4* gene are shown. Bent arrow represents TSS. For all ChIP data, averages from three independent experiments with error bars, normalized against *TELVIR* region, are calculated. (**D**) Relative occupancy of FLAG-tagged C160 subunit of pol III on *SUP4* in both active and repressed conditions. Nutrient deprivation with 0.15× YEP medium lacking glucose was used to repress the pol III transcription for 80 min.

the transcribed region of the gene (Supplementary Figure S1) suggesting absence of nucleosome on the gene may be due to the ATP-dependent chromatin remodelling activities present in the S-190 extract, which probably mobilize the nucleosome away from the gene region, making it transcriptionally active in the presence of TFIIIC. These *in vitro* observations suggest that chromatin may influence *SUP4* expression *in vivo* as well.

We measured *SUP4* RNA levels under active and repressive conditions of pol III transcription (Figure 1B). Surprisingly, *SUP4* pre-tRNA levels increase for the first 2 h while levels of 5′-end processed transcript do not change after 1 h of repression. Levels do not change much with prolonged starvation due to high stability of tRNAs. This suggests pre-tRNA levels increase after 1h of repression because processing stops but the increase during the first hour may be due to increased transcription. Starvation induced repression is accompanied by loss of pol III from the target genes (23). ChIP of Flag-RPC160 subunit using amplicons shown in the Figure 1C showed that after 80 min of nutritional stress, pol III levels on the gene drop by only 50% on the transcribed region (Figure 1D). This suggests that transcription from the gene may be continuing during initial repression and a change in the transcript level may be related to even chromatin structure around the gene.

A nucleosome-free gene region is flanked by two nucleosomes

Using micrococcal nuclease (MNase) digestion instead of sonication, we probed the *SUP4* gene region (Figure 1C) for the presence of core histones by the ChIP and real time PCR method. As compared to nucleosome-dense *TELVIR* region, the gene region is found histone-depleted where levels appear close to background level (Figure 2A and B). Comparatively lower enrichment of H2B and H3 in downstream region as compared to upstream, suggests that the nucleosomes may be present on both sides of the *SUP4* gene *in vivo* (−1 and +1, Figure 2C), but the +1 nucleosome is not well positioned. We confirmed the histone-depleted nature of the gene region *in vivo* by high-resolution MNase footprinting. As compared to naked DNA, chromatin shows MNase hypersensitivity at the position from +93 to +96 in the gene region (Figure 2D), while the region downstream of the terminator is protected (lanes 3, 4 versus 1, 2), suggesting presence of nucleosomes. The MNase digestion profile of rest of the gene sequence is same for both chromatin and naked DNA, suggesting the observed hypersensitivity probably represents the 5′-boundary of the +1 nucleosome, downstream of the gene.

Nucleosome positions near the gene were further confirmed by analysing the chromatin structure of *SUP4* tDNA locus *in vivo* using the indirect-end-labelling (IEL) approach (Figure 2E). As compared to MNase digestion pattern of naked DNA, the chromatin structure *in vivo* shows presence of an array of positioned nucleosomes upstream of the gene sequence (cf. lanes 1, 2 and 3, 4). The transcribed region of the gene is not cut well by MNase, but the broad band around +98 bp, downstream of the box B at +80 bp, shows a MNase hypersensitivity of the gene, not seen on naked DNA. Thus, considering the results from the Figure 2A and B, and the low-resolution nature of the IEL mappings, the region from −47 to +98 (±20) bp encompassing the *SU4* transcribed region is devoid of a nucleosome. Protection of naked DNA sites at −151 and −63 bp positions shows the presence of the −1 nucleosome positioned between −242 and −47 bp giving a median at −140 bp as opposed to −130 bp mapped by the ChIP-seq method (http://h2az.atlas.bx.psu.edu) for the same nucleosome. The protection of naked DNA sites, including the +192 bp position but absence of any strong boundaries between +100 and +350 bp positions (cf. lanes 3, 4 and 1, 2) suggests that as compared to the −1 nucleosome, the boundaries of +1 nucleosome are comparatively diffused. The span of this nucleosome matches well with the position of a fuzzy nucleosome between +90 and +300 bp (http://h2az.atlas.bx.psu.edu). Fuzzy nature of the +1 nucleosome may also be the reason that H2B and H3 levels in the downstream region are lower as compared to the upstream region in active state of the gene (Figure 2A and B). Increase in H2B levels only in +1 nucleosome under repression (Figure 2A)

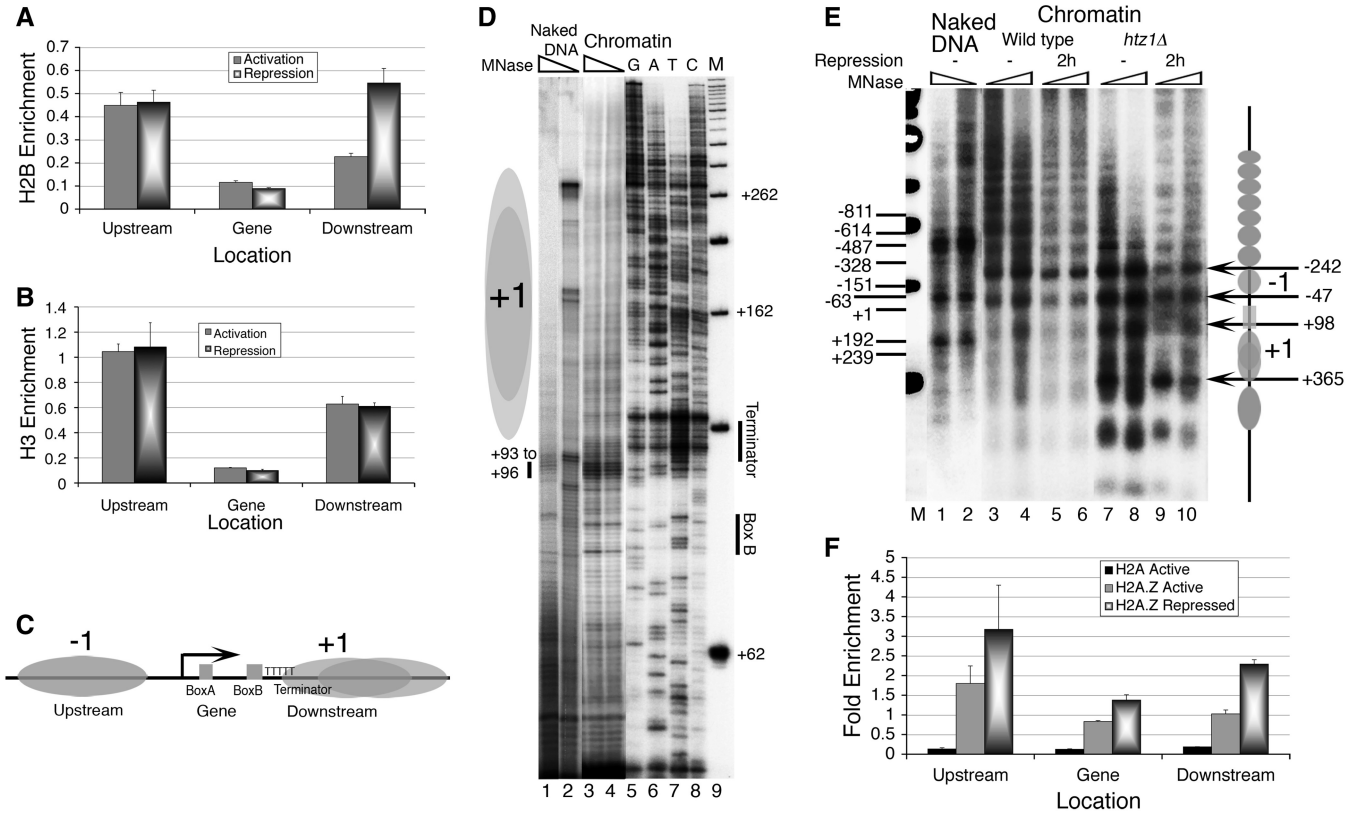

Figure 2. Effect of Repression and H2A.Z deposition on the *SUP4* gene locus *in vivo*. Chromatin organization at the *SUP4* locus is probed by ChIP/Real Time PCR and footprinting methods. (**A**) Relative occupancy of FLAG-tagged H2B. (**B**) Relative occupancy of Myc-tagged H3. (**C**) Nucleosomes flank the histone-free *SUP4* gene: Cartoon showing positions of the nucleosomes −1 and +1, relative to the *cis* elements of the gene, as marked in the panel 1C. (**D**) High-resolution MNase footprinting *in vivo*. MNase digested chromatin and naked DNA samples were used for extension with a primer which hybridizes to the bottom strand, 13 bp downstream of the TSS. Lane M shows a 50 bp ladder used as molecular size marker while GATC represent the sequencing reaction over genomic DNA. Two levels of MNase digestions are shown for *SUP4* as naked DNA (lanes 1 and 2) and chromatin (lanes 3 and 4). Positions of the box B and terminator are marked while grey ovals represent nucleosomes. Position of MNase hypersensitivity immediate upstream of the terminator is marked with a short vertical bar in the left-hand side. (**E**) Low-resolution chromatin structure analysis by the IEL method. Grey ovals denote the nucleosomal size protections and arrows mark the MNase cut sites in the chromatin. Gene region is marked with a rectangle. All numbers represent bp with respect to TSS at +1. Numbers on the left-hand side mark the MNase cuts seen on the naked DNA in lanes 1 and 2. Numbers on the right-hand side mark the MNase cuts seen on chromatin and −1, +1 mark the gene flanking nucleosomes. MNase cleavage pattern of wild type (lanes 3–6) and Htz1Δ cells (lanes 7–10) without (lanes 3, 4 and 7, 8) or with nutritional stress (lanes 5, 6 and 9, 10) for 2 h is shown. (**F**) H2A.Z deposition in the nucleosomes around the *SUP4* gene *in vivo*. Relative occupancies of FLAG-tagged H2A and H2A.Z on *SUP4* against *TELVIR* region are shown. H2A.Z levels increase with repression.

suggests a differential histone dynamics in both the nucleosomes.

Higher association of H2A.Z with *SUP4* gene under repressed state

Nucleosome mobility and occupancy on the promoter regions are often associated with the state of activity of a gene (11). As above data show absence of nucleosomes on the *SUP4* in its active state, we followed the nucleosome dynamics on the *SUP4* gene locus under repression conditions using IEL method. No change in the arrangement of nucleosomes upstream or downstream of the gene after repression (Figure 2D, lanes 5 and 6) is seen. But, the MNase sensitivity of the gene at +98 bp is reduced, suggesting a change in +1 nucleosome region, probably resulting in further terminator exposure and higher transcription in the repressed state (Figure 1B).

Nucleosomes flanking the tRNA genes (9) are reported to have H2A.Z. We looked for the presence of H2A and its only variant in the budding yeast, H2A.Z, in both the nucleosomes. As opposed to negligible H2A levels, H2A.Z shows significant enrichment in both, especially the −1 nucleosome (Figure 2F, Supplementary Figure S2). This profile of H2A.Z is similar to the general pattern of H2A.Z-containing nucleosomes flanking an NFR around the TSS, reported for active pol II-transcribed genes (10). However, IEL analysis shows that similar to *SNR6* (17); the chromatin structure remains unperturbed in the absence of H2A.Z, even under repression (Figure 2D, lanes 7–10), supporting a previous report showing no role of H2A.Z in general nucleosome positioning or maintenance of nucleosomal organization (27). H2A.Z associates with both active and inactive genes (28). On *SUP4*, H3 levels do not change (Figure 2B) but H2A.Z (Figure 2F) levels increase under repression. As compared to

wild-type cells, higher *SUP4* RNA levels found in *htz1Δ* cells (Figure 3A), suggest its repressive role in *SUP4* expression. Decrease in occupancy upon activation of *SUP4* is consistent with the reports showing that H2A.Z is lost during the active transcription of a gene (29,30).

Shift in the position of +1 nucleosome modulates *SUP4* transcription

In vitro results from the Figure 1A suggest involvement of ATP-dependent chromatin remodelers in *SUP4* transcriptional activation. Therefore, we monitored the *SUP4* tRNA levels in cells mutated for a subunit of the major chromatin remodelling complexes of yeast. Deletion of Isw2 or Chd1 does not have any effect on *SUP4* levels (Figure 3A), and both of them did not show enrichment near the gene (data not shown). In comparison, RSC localizes to the region (Figure 3B) and an increase in RNA level is found (Figure 3A) in cells with an Rsc4 mutation known to cause a loss of interaction with pol III (31). Similar to repressed state (Figure 1B), higher RNA levels in both H2A.Z and RSC mutants could be due to higher transcription as well as defective processing. Decrease in occupancy of RSC (Figure 3B) along with increase of H2B (Figure 2A) under repression, only in the downstream region, suggests that the repressive effect of RSC on *SUP4* expression may also be due to a remodelling of +1 nucleosome by RSC.

We used IEL method, which can map the nucleosomes with fair accuracy (32), to see the remodelling, suggested by above data. Digestion pattern of chromatin in *rsc4-Δ4* mutant does not show any apparent changes in the chromatin structure (Figure 3C) except a reduced MNase sensitivity (cf. lanes 1–4) near the box B (asterisk), which could be either due to loss of nucleosome and its boundary or further encroachment by a nucleosome from the downstream region. Comparison of the profiles of similarly digested DNA in lanes 2 and 4 (Figure 3D)

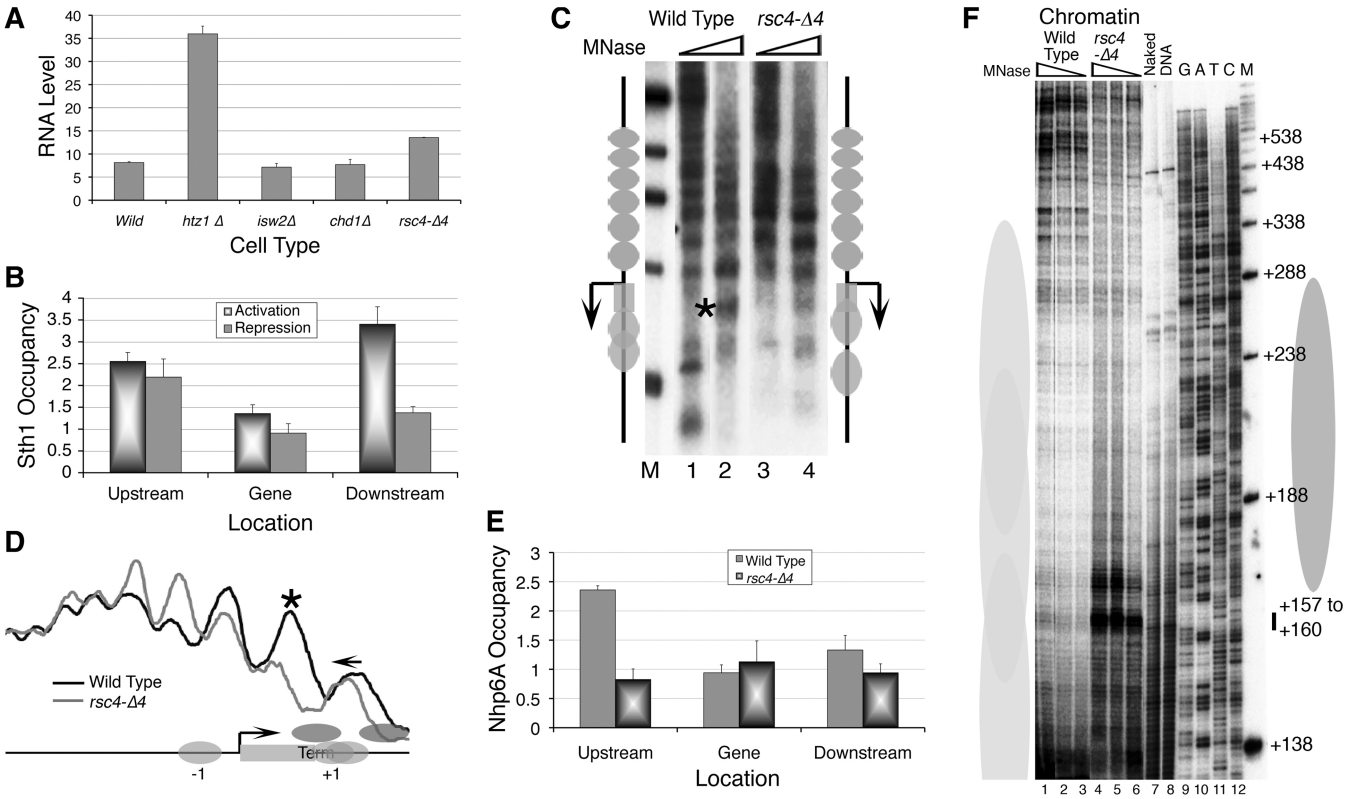

Figure 3. RSC has a repressive role in *SUP4* expression. (**A**) RNA was extracted from different mutants under active transcription conditions and *SUP4* levels estimated as given under the 'Material and Methods' section. U4 was used as the internal control to normalize the *SUP4* transcript levels. Average and standard deviations calculated from three independent isolations are plotted. (**B**) Relative enrichment of myc-tagged Sth1 subunit of RSC complex on *SUP4* in both active and repressed conditions. (**C**) IEL analysis of the chromatin structure in RSC mutant and wild-type yeast cells. Ovals represent the nucleosomes; arrow and rectangle mark the gene position. Bent arrow marks the TSS, asterisk marks the MNase hypersensitive site on the gene region. Digestions with two MNase levels are shown for each strain. (**D**) Comparison of the digestion profiles of the similarly digested DNA in lanes 2 and 4 from the panel C. Profiles were generated using the PhosphorImager and the Image Gauge (Fuji) software. Asterisk marks the MNase hypersensitive site on the gene region while short arrow marks the direction of nucleosome shift. Box marks the gene region, bent arrow the TSS and Term the gene terminator. Light and dark ovals represent the positioned nucleosomes in wild-type and mutant cells, respectively. (**E**) Relative enrichment of myc-tagged Nhp6 in *rsc4-Δ4* mutant in active condition. (**F**) High-resolution MNase footprinting. Samples were prepared and primer extension reactions carried out as described under the legends for the Figure 2D. The primer hybridizes to the bottom strand, 92 bp downstream of the TSS. Nucleosomes in *rsc4-Δ4* mutant are restricted to downstream of the terminator. Three levels of MNase digestions are shown for wild type (lanes 1–3) and mutant (lanes 4–6) cells. Dark grey oval represents the downstream nucleosome in mutant while light grey ovals represent fuzzy nucleosomes in wild-type condition. Numbers in the right hand side denote bp with respect to TSS while short vertical bar marks the position of MNase hypersensitivity in lanes 4–6.

shows a change further downstream, showing two positioned nucleosomes with new boundaries (right-side cartoon, Figure 3C; and darker ovals, Figure 3D), in place of a fuzzy +1 nucleosome. Physical and functional interaction of a subunit of yeast FACT, Nhp6 with RSC helps its loading onto the nucleosome *in vitro* (33). Figure 3E shows that the Nhp6 shows comparatively higher enrichment in the upstream region of *SUP4* from where it is lost in *rsc4-Δ4* mutant, suggesting RSC may help recruit Nhp6 on *SUP4*. Chromatin shows MNase hypersensitivity in the presence of Nhp6 *in vitro* (Supplementary Figure S3A) as Nhp6 binding changes the conformation of DNA (34). Thus, loss of MNase hypersensitivity of the gene region in the *rsc4-Δ4* mutant (Figure 3D) may be due to loss of Nhp6 as well as the shift of boundary of the +1 nucleosome.

To resolve this further, we used the high-resolution MNase footprinting *in vivo* (Figure 3F). The presence of a strong cut around +160 bp position in the mutant as compared to the wild-type condition, suggests the presence of the common boundary of the two positioned nuclesomes at ~50 bp downstream of the terminator. Out of the two, positioning of the gene-proximal nucleosome places its terminator at the dyad axis, though its footprint is not easy to ascertain since the chromatin-specific cut in the gene region is broad and diffuse. Therefore, the remodelling activity of RSC in the wild type cells must be keeping the gene region nucleosome-free and +1 nucleosome fuzzy, making it encroach the second terminator of *SUP4*, T2 at +108 bp (35) in active state.

SUP4 gene *in vivo* is flanked by a Ty element in upstream and an ORF of unknown gene in the downstream region. The TSS of both the divergent genes are found two nucleosomes away from the *SUP4*. We did not find either transcript of the downstream gene or effect of repression or RSC mutation on the upstream transcript (data not shown), confirming the observed changes in the gene downstream region are related directly to *SUP4* expression.

Nhp6 regulates the transcription initiation from the *SUP4* chromatin

Nhp6 is reported to suppress the downstream initiations from naked *SUP4* DNA *in vitro* (36). In a titration experiment (Figure 4A), addition of purified, recombinant Nhp6A to chromatin also abolishes downstream initiations and the right *SUP4* transcript increases with increasing Nhp6 (lanes 1–3). However, in contrast to steady increase in naked DNA transcription with addition of upto ~300 ng Nhp6 (data not shown), transcription from chromatin drops after the saturation levels of Nhp6 at 60 ng (lanes 4–10, Figure 4A). Expecting a similar effect of Nhp6 *in vivo*, we used an intron-specific primer which also detects transcripts from two isogenes of *SUP4*, all differing from each other by 1 or 2 bp in TSS selection (36). As marked in the Figure 4B, −1 represents

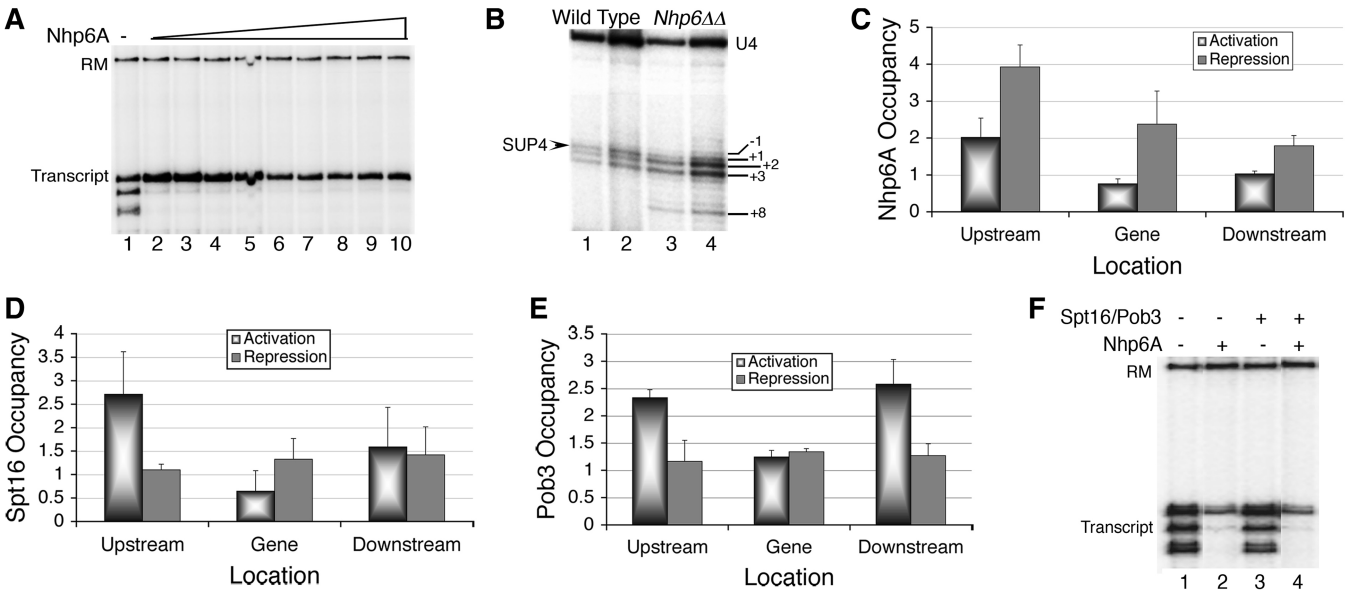

Figure 4. FACT has inhibitory effects on *SUP4* transcription. (**A**) Effect of Nhp6 on *SUP4* transcription *in vitro*. Transcription of S-190 assembled chromatin over *SUP4* was followed using pure yeast pol III transcription machinery in the absence (lane1) and presence (lanes 2–10) of increasing amounts (36, 60, 72, 84, 99, 108, 120, 132 and 144 ng) of pure Nhp6A. Transcript and RM are as given for the Figure 1A. (**B**) Nhp6 confers the fidelity of transcription *in vivo*. Total RNA was isolated and used to generate extension products using the intron-specific primer of *SUP4*. The three products obtained from the two yeast strains using 5 or 10 μg total RNA are resolved on a gel as described under methods. Arrow marks the major *SUP4* transcript initiating from the +1 bp position. Numbers on the right-hand side denote the positions of all the transcripts with respect to +1 site of *SUP4*, as explained in the text. (**C**) Relative enrichment of myc-tagged Nhp6A in both active and repressed states. (**D**) Relative enrichment of myc-tagged Spt16 in active and repressed states. (**E**) Relative enrichment of TAP-tagged Pob3 in both active and repressed states. (**F**) Effect of FACT on the chromatin transcription of *SUP4 in vitro*. Transcription of S-190 assembled chromatin over *SUP4* was followed using pure yeast pol III transcription machinery. Pure FACT components in excess were added as indicated.

barely visible *SUP2* transcript; *SUP3* and *SUP4* appear together at +1; while +2 and +3 represent downstream initiated transcripts from *SUP3* and *SUP2*, respectively. Deletion of Nhp6 is found to reduce transcription from the right positions in all three genes (−1 and +1 bands in lanes 3, 4 versus lanes 1, 2). In Nhp6*ΔΔ* cells, the downstream initiated transcripts from +2 and +3 (transcripts from *SUP3* and *SUP2* respectively), show an increase and a transcript initiated from +8 position, probably from *SUP4*, is also found, as seen *in vitro* (cf. Figures 1A and 4B).

Nhp6 can recruit Spt16 and Pob3 to nucleosome to form the stable FACT complex (6). Accordingly, presence of Nhp6 specially in the upstream region of *SUP4* is matched with levels of other two FACT subunits Spt16 and Pob3 in active state (Figure 4C–E) and its increase in all three regions under repression (Figure 4C) is in agreement with its inhibitory role in *SUP4* transcription *in vitro*, at higher levels. However, unlike Nhp6, Spt16 and Pob3 show loss from the repressed gene (Figure 4D and E) and mutations in Spt16 and Pob3 did not affect the fidelity of pol III (not shown), suggesting the functions of Nhp6 and Spt16/Pob3 are independent of each other. In agreement to this, when purified Spt16 and Pob3 are added for *in vitro* transcription, downstream-initiated transcripts from chromatin are not abolished (lane 3 versus 1, Figure 4F). This result is in contrast to reported association of Spt16 with the increase in transcription fidelity for many of the pol II-transcribed genes (5,37). Being in excess, Nhp6, wherever added, shows increase in fidelity (lanes 2 and 4) but decrease in total transcript, whether added alone (lane 2) or with Spt16/Pob3 (lane 4). These results show that all the three components of yFACT occupy the *SUP4* gene simultaneouly *in vivo* but the ability to ameliorate the downstream initiated transcripts resides with Nhp6 alone.

Repressive role of FACT in tDNA transcription

Apart from being a part of the FACT complex, Spt16 has a specific role in chromatin organization on certain pol II-transcribed genes (38). The IEL analysis of Nhp6 as well as Spt16 mutants did not show any change in gross chromatin structure around *SUP4* (Supplementary Figure S3B), suggesting their association with *SUP4* locus may have effect at some other level.

Spt16 is known to differentially affect transcription of the pol II-transcribed genes (38). The *SUP4* RNA levels in cells defective in either Spt16 or Pob3 (Figure 5A) show an increase while pol II-transcribed genes *CMD1* and U4 do not show change in their transcript levels in Spt16 mutant cells (Supplementary Figure S4). In contrast to roles of FACT subunits in pol II transcription activation (5,6,39,40), the higher *SUP4* levels in Spt16 and Pob3 mutants suggest a repressive role for them in *SUP4* expression.

Spt16 is required for H2A.Z deposition on *SUP4* locus

Spt16 has been shown to work as chaperone for H2A (4) and H2A.X (41). It co-purifies with H2A.Z in the absence of Nap1 and H2A.Z-specific chaperone Chz1 and helps in SWR1-mediated exchange of H2A.Z–H2B dimer *in vitro* (42). We found a loss of H2A.Z levels on *SUP4* in the absence of Chz1 (data not shown) and Swr1 (Supplementary Figure S2) suggesting Swr1 and Chz1 are required for H2A.Z deposition at *SUP4* locus *in vivo*. A loss of Spt16/Pob3 (Figure 4D and E) but increase of H2A.Z (Figure 2F) under repression suggest Spt16 may be required for H2A.Z removal from nucleosomes. Similarly, a loss of H2A.Z in the absence of functional Spt16 (Figure 5B) indicates an involvement of Spt16 in the deposition of H2A.Z.

Spt16 is known to interact with histone H3. In the absence of Spt16, a rapid loss of H3 is seen on both active and inactive genes (43). This suggests that the observed H2A.Z dynamics on *SUP4* could be indirectly due to H3/nucleosome dynamics and may not have any direct correlation with Spt16. However, total H3 as well as H2A.Z levels in mutant and wild type cells did not show any significant difference (Supplementary Figure S5A), suggesting lack of Spt16 activity may be directly responsible for the lower H2A.Z levels in nucleosmes flanking the *SUP4* (Figure 5B). Mutant cells showed a differential behaviour of H3 in −1 and +1 nucleosomes (Supplementary Figure S5B), again suggesting both the nucleosomes follow different dynamics under repression, and may be targets of different chromatin modifiers. Preferential occupancy of FACT in the upstream region and NFR on the transcribed region of *SUP4* advocate for requirement of FACT activity only in the −1 nucleosome, which shows enrichment of H2A.Z.

While general pol III transcription is repressed within minutes of nutrient deprivation, the *SUP4* transcript (Figure 1B) and H2A.Z levels (Figure 5C) show steady increase till 2 h. The increase probably reflects the initial cellular response to the stress wherein H2A.Z plays a positive role as discussed later. The H2A.Z shows significantly higher level in the −1 nucleosome after 2 h repression. Levels drop after 2 h, in parallel to transcription levels but remain at higher than active state in the later stages of repression, suggesting its loss may be necessary for activation. Unlike H3, H2A.Z levels in both nucleosomes are comparatively lower in Spt16 mutant cells (Figure 5C) but time course of H2A.Z dynamics looks similar in both types of cells. While a slight increase in H3 could be the reason behind the small increase of H2A.Z in the −1 nucleosome in mutant cells (Supplementary Figure S5B) under repression, it could also be due to activity of the H2A.Z specific chaperone Chz1 (42). These differences in dynamics of the two suggest that H2A.Z does not follow H3 dynamics on *SUP4*. While Chz1 may be an alternate chaperone, Spt16 is required on *SUP4* for H2A.Z deposition during active state and removal in repressed state. Thus, working as H2A.Z chaperone along with Chz1, Spt16 may deposit as well as remove H2A.Z from the *SUP4* flanking nucleosomes. This is not surprising, as most of the histone chaperones show broad specificity and often work in redundant manner *in vivo* (44). Therefore, H2A.Z levels at any point of time may represent the outcome of the removal/deposition by Spt16 and Chz1.

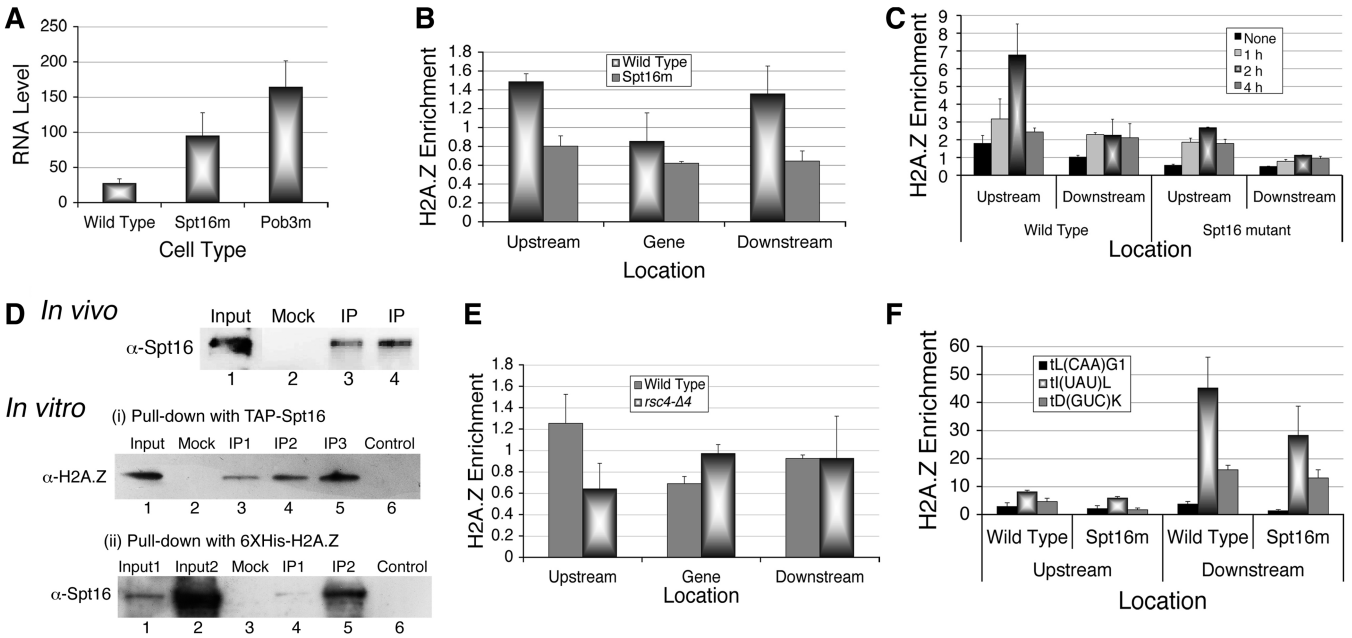

Figure 5. FACT is a H2A.Z chaperone. (**A**) *In vivo* analysis of *SUP4* expression in mutants of Spt16 and Pob3. Total RNA was isolated and intron-specific primer extension products of *SUP4* were visualized by phosphorImaging of the gel as described under methods. RNA levels were normalized against U4 and average from three independent experiments with scatter are shown. (**B**) Relative enrichment of HA-tagged H2A.Z in the absence of functional Spt16 in active condition. Comparative levels of H2A.Z are low in both the nucleosomes in Spt16 mutant cell. (**C**) HA-H2A.Z levels around the *SUP4* gene in wild type cells change with time in parrallel to transcriptional repression under nutrient starvation. (**D**) Co-immunoprecipitation of H2A.Z and Spt16 *in vivo* and *in vitro*. Mock was as described under Supplementary Data. Input lanes are loaded with one-third (*in vivo*) or one-fourth (*in vitro*) of the sample as compared to IP (immunoprecipitate) lanes. Spt16 was at increasing amounts in IP1, 2 and 3. Upper panel: *In vivo*, H2A.Z-HA is immunoprecipitated with anti-HA antibody and IP is probed with anti-Spt16 antibody. Lanes 3 and 4 are the duplicates of the immunoprecipitation (IP). Lower panel: (i) and (ii) show *in vitro* pull-downs, using purified Spt16/Pob3 or 6XHis-tagged H2A.Z; probed with anti-H2A.Z or anti-Spt16 antibodies, respectively. Control shows the immobilized bait protein alone on beads while mock lanes show incubation of the prey with bare beads. (**E**) Relative enrichment of HA-tagged H2A.Z in *rsc4-Δ4* mutant in active condition. (**F**) Relative enrichment of H2A.Z-HA on tDNA loci present on three different chromosomes.

Higher RNA and lower H2A.Z levels in Spt16 mutant and higher H2A.Z but lower Spt16 in repressed wild-type cells suggest a close relation between H2A.Z dynamics, FACT and transcription of SUP4. Unlike *SUP4*, H2A.Z on *CMD1* and U4, two Spt16-independent genes transcribed by pol II (6, Supplementary Figure S4) shows lower levels under active as well as repressed states in mutant cells (Supplementary Figure S5C). Therefore, Spt16 is required for the deposition of H2A.Z in active state although the transcription of these genes does not require Spt16. As Spt16 is a known chaperone for other H2A variants, loss of the variant H2A.Z from the genes in the mutant cells reflects a direct outcome of defect in H2A.Z deposition by Spt16 in active state.

FACT is a chaperone of H2A.Z for pol III-transcribed genes

A chaperone role would require direct interaction between H2A.Z and Spt16. Using yeast cells carrying HA-tagged H2A.Z, we found that Spt16 co-immunoprecipitates with H2A.Z *in vivo* (Figure 5D, upper panel), while an N-terminal mutant of Spt16 could not pull down H2A.Z (Supplementary Figure S6A), re-enforcing the possibility of Spt16 working as H2A.Z chaperone *in vivo*. For confirming direct interaction of the two *in vitro*, we purified TAP-tagged Spt16-Pob3 heterodimer from yeast cells and 6XHis-tagged recombinant yeast H2A.Z from *E. coli* cells (Supplementary Figure S6B). We used the tagged proteins immobilized on Calmodulin sepharose or Ni-NTA agarose beads, respectively, and added the other partner as pure protein (Figure 5D, lower panel). We found that both Spt16 and H2A.Z can efficiently pull-down each other, demonstrating a direct physical interaction between the two. As Nhp6/FACT are enriched specifically in the upstream region (Figure 4C and D), H2A.Z levels also show larger changes only in the −1 nucleosome (Figures 2F and 5D). All these results support a H2A.Z chaperone role for FACT on *SUP4* locus. In agreement with Nhp6 requirement for Spt16/Pob3 binding to nucleosome (45) and loss of Nhp6 in *rsc4-Δ4* mutant; H2A.Z is specifically lost from the −1 nucleosome in this mutant (Figure 5E), suggesting Nhp6/FACT may be a link between RSC and H2A.Z. Nucleosomes flanking NFRs containing tDNA sequences (9) are reported to have H2A.Z and recently human FACT was shown to associate with some pol III-transcribed genes (46). We monitored H2A.Z levels in the absence of functional Spt16 on some more tRNA genes (Figure 5F). Similar to *SUP4*, H2A.Z levels in the mutant cells show significant loss on all these genes, suggesting FACT may have a general role in H2A.Z dynamics on pol III-transcribed genes.

Taken together, results presented in this study show while Nhp6 is required for fidelity; loss of RSC activity, H2A.Z or the FACT components results in higher *SUP4* RNA levels in active state, suggesting they may be working in the same pathway, probably to reduce the *SUP4* expression. This may be a novel mode of gene regulation whereby all chromatin related activities target the gene to repress it rather than activate in active state. Importantly, regulation of H2A.Z levels in −1 nucleosome by FACT as H2A.Z chaperone and shift in position of +1 nucleosome by RSC in direct response to transcription repression show a differential role of both the gene flanking nucleosomes in the gene expression.

DISCUSSION

According to the general belief, the tRNA genes are largely devoid of well-defined, positioned nucleosomes obviating the need for epigenetic modes of regulation. In the genome-wide sequence-directed nucleosome position predictions (http://genie.weizmann.ac.il/software/nucleo_prediction.html), the *SUP4* gene sequence is predicted to exclude nucleosomes from the gene region. Actively transcribed *SUP4* gene on a multicopy plasmid, was shown to resist incorporation into a positioned nucleosome *in vivo* (47). Using salt-dilution chromatin assembly method, we found that the *SUP4* gene excludes nucleosome deposition over itself *in vitro* while a single nucleosome is positioned in the upstream region (not shown). Therefore, sequence-directed nucleosomal absence near the *SUP4* tDNA *in vivo* could potentially exclude role of all epigenetic mechanisms in regulating *SUP4* transcription. Results presented in this study have shown a central role for H2A.Z, RSC and FACT in tRNA transcription and suggest a complex interplay of a number of epigenetic processes in regulating *SUP4* gene.

H2A.Z as stress-receptor in *SUP4* expression

Presence of H2A.Z may have important biological implications (48). Yeast H2A.Z, present in many loci throughout the genome, is implicated in locus-specific activation and repression of genes (48–50). The initial increase of *SUP4* transcription under repression is similar to induction of many stress-response genes shortly after stress signal is received (51,52). During first 2 h, when pol III levels drop, increase in Swr1 levels and loss of Spt16 may increase the H2A.Z levels in −1 nucleosome. The drop in H2A.Z levels after prolonged repression probably poises the gene for the activation process through a quick H2A.Z-independent trigger. H2A.Z presence in the nucleosome reduces its mobilization by a chromatin remodeller (27). Thus, increase in H2A.Z under repressed condition may help keep the −1 nucleosome in its place while loss of H2A.Z and increase of RSC in active state would delocalize the downstream nucleosomes making the +1 nucleosome fuzzy. Thus, H2A.Z confers differential behaviour on these nucleosomes in response to repression while its dynamics on *SUP4* is not related to active transcription. These observations suggest H2A.Z serves as a starvation-respone factor for *SUP4*, similar to its recently suggested thermosensory role (53). Significantly, on two of pol II-transcribed genes as well, which show a delayed repression, late increase of H2A.Z levels is seen (Supplementary Figures S4 and S5C), suggesting H2A.Z may be a general stress-response factor.

Role of FACT components on *SUP4* gene

Our results suggest that FACT may be responsible for the reported presence of H2A.Z near tDNAs in NFR-flanking nucleosomes (9), a role not attributed to the FACT till now. Different core histone dimers can be exchanged by more than one histone chaperone in the chromatin assembly/disassembly reactions *in vivo* on different genes, suggesting a broad range of carriers for them (44). Events like histone exchange or nucleosome loss on the transcribed or promoter regions of the genes may be either the cause or the consequence of transcription. Nevertheless, these events are neither spontaneous nor autonomous and require the assistance of a chromatin remodeler and chaperone. Moreover, transcription-associated loss/exchange of histones due to passage of the elongating RNA polymerase II takes place with the help of FACT only in the transcribed region of a gene (2–5). The N-terminal domain of Spt16 is known to interact with H3 and in its absence a rapid loss of H3 is seen on both the active and inactive genes (43). Thus, on *SUP4*, the observed effect of Spt16 on the dynamics of H2A.Z in the flanking nucleosomes, which are regulatory and not structural in nature, need not be transcription process-associated, but very well be due to the Spt16 chaperone activity. Spt16 was shown to co-purify with H2A.Z in the absence of Nap1 and H2A.Z-specific chaperone Chz1 and help in SWR1-mediated exchange of H2A.Z–H2B dimer *in vitro* (42). We have shown that FACT interacts with H2A.Z both *in vitro* and *in vivo*. Thus, FACT may be a chaperone for the dimer of H2B with H2A or its major variants on different genes which may or may not require FACT for their expression.

Nhp6 stimulates the transcription of pol III-transcribed U6snRNA, while the deletion of both the copies of Nhp6 abolishes the TFIIIB footprint in the TATA box region (54). Although de novo synthesis of bulk of tRNA is not affected in Nhp6*ΔΔ* strain (55), we have found multiple effects of Nhp6 on *SUP4*. It physically interacts with RSC and blocks remodelling action of RSC *in vitro* (33). As it is required to load Spt16/Pob3 (45), mutation in RSC or loss of Nhp6, results in probably the same effect; loss of Spt16/Pob3 recruitment, and hence, H2A.Z. Therefore, increase in *SUP4* RNA levels in RSC, Spt16 and Pob3 mutants could be due to the loss of H2A.Z in these strains, a condition similar to *htz1Δ* cells.

RSC plays multiple roles in *SUP4* gene expression

Loss of RSC is reported to shrink NFR (56) on pol II-transcribed genes and increase the nucleosome density on pol III-transcribed genes (57). We have shown that RSC is required to keep the nucleosome immediate downstream of the *SUP4* terminator, fuzzy *in vivo* (Figure 6A). It changes the local density of nucleosmes near the *SUP4* gene (Figure 6B and C), maintains nucleosome positions

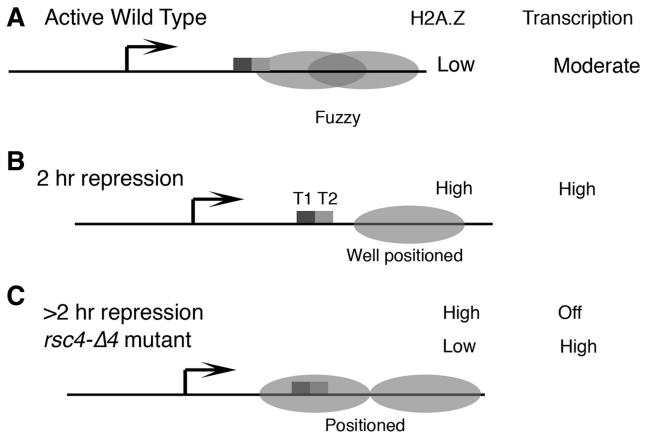

Figure 6. Relationship of transcription from *SUP4* with H2A.Z level and nucleosome positions. Ovals represent nucleosomes, boxes and T1, T2 denote two terminators, bent arrow marks the TSS. Only downstream nucleosomes are shown with H2A.Z levels and transcriptional state of the gene shown on the right-hand side. (**A**) In wild-type cells, active state, nucleosmes are fuzzy and block T2. (**B**) In wild-type cells, following 2 h repression, +1 nucleosome is positioned at a short distance from the terminators. (**C**) In wild-type cells, after repression longer than 2 h, two nucleosomes may be positioned similar to that seen in *rsc4-Δ4* cells. Both terminators are near the dyad axis of the gene-proximal nucleosome.

to suppress the transcription in active state, helps loading Nhp6/FACT for transcriptional fidelity and differential marking with H2A.Z according to the expression state. While leaving the +1, RSC persists on the −1 nucleosome under repression, keeping the gene poised for activation under repressed state.

Loss of Sth1, the catalytic subunit of RSC, from the downstream region of *SUP4* within first hour during repression, may result in a nucleosome positioning similar to that seen in the *rsc4-Δ4* mutant (which shows loss of remodelling activity on *SNR6*, 17). On some of pol III-transcribed genes, loss of Sth1 activity was earlier found to result in gradual increase of histone density but as RSC was not lost under repression, histone density on these genes did not show any change (57). However, despite an increase in histone density near the gene terminator at +100 bp (Figure 2A) we could not see a similar nucleosomal protection in the gene proximal region after 2 h of repression. As Sth1 is lost from the downstream of *SUP4*, a gradual increase in histone density in the gene region may take place with further repression. Accordingly, a nucleosome was seen to encroach the gene after 4 hr repression (not shown), suggesting an organization similar to *rsc4-Δ4* cells may be generated after prolonged satrvation.

Regulation of *SUP4* from downstream

Absence of the transcription terminator of pol III-transcribed genes results in loss of the facilitated recycling of pol III and transcript yield (19). As the cartoon in the Figure 6 depicts, due to its fuzzy nature, the +1 nucleosome encroaches the gene terminator T2 at +108 bp (Figure 6A). This may allow transcription termination only at T1, ensuring utilization of only one gene terminator *in vivo*; but interfere with terminator-directed recycling of pol III (19), resulting in reduced transcription rate in active state. Under initial hours of repression, +1 nucleosome positioning away from the gene would make both the terminators equally exposed (Figure 6B) and the resultant increase in the transcription rate would give higher RNA levels from *SUP4* as observed under repression for some time (Figure 1B). Interestingly, presence of the gene terminators at the dyad axis of a positioned nucleosme in the *rsc4-Δ4* mutant, similar to that seen on the *SNR6* gene in active state (17), may allow the terminator-dependent recycling of pol III, giving higher transcription rate. In comparison, prolonged stress may completely abolish the transcription despite the same positioning in the wild type cells. This may be related to different signals from H2A.Z dynamics in both conditions (Figures 5C and F, and 6C).

Thus, a fine tuning of the +1 nucleosome position by RSC along with H2A.Z as the stress-sensor, probably regulates the gene output under different states from pol III-transcribed genes. These observations suggest *SUP4* may be an example where diverse chromatin related activities come together and co-ordinately keep the gene expression low in active state generating a milieu in which gene remains in a poised state for release from the repressed state.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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