

# A polar barrier to transcription can be circumvented by remodeler-induced nucleosome translocation

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## ABSTRACT

Many eukaryotic genes are regulated at the level of transcript elongation. Nucleosomes are likely targets for this regulation. Previously, we have shown that nucleosomes formed on very strong positioning sequences (601 and 603), present a high, orientation-dependent barrier to transcription by RNA polymerase II *in vitro*. The existence of this polar barrier correlates with the interaction of a 16-bp polar barrier signal (PBS) with the promoter-distal histone H3–H4 dimer. Here, we show that the polar barrier is relieved by ISW2, an ATP-dependent chromatin remodeler, which translocates the nucleosome over a short distance, such that the PBS no longer interacts with the distal H3–H4 dimer, although it remains within the nucleosome. *In vivo*, insertion of the 603 positioning sequence into the yeast *CUP1* gene results in a modest reduction in transcription, but this reduction is orientation-independent, indicating that the polar barrier can be circumvented. However, the 603-nucleosome is present at the expected position in only a small fraction of cells. Thus, the polar barrier is probably non-functional *in vivo* because the nucleosome is not positioned appropriately, presumably due to nucleosome sliding activities. We suggest that interactions between PBSs and chromatin remodelers might have significant regulatory potential.

## INTRODUCTION

Eukaryotic genomes are organized into chromatin in order to pack DNA efficiently into the nucleus and to regulate gene expression. The structural repeat unit of chromatin is the nucleosome, a complex containing two molecules of each core histone (H2A, H2B, H3 and H4) and 147 bp of DNA (1). The question of how transcription occurs in the presence of nucleosomes has been addressed extensively (2). *In vivo*, histones undergo displacement and exchange during intense transcription (3–6), but at the moderate levels of transcription typical of most genes, nucleosomes survive (5,7,8) and influence the rate of transcript elongation. Indeed, RNA polymerase II (Pol II) tends to stall at promoter-proximal sites (9–11), where it is often associated with the first nucleosome on the gene (12).

*In vitro*, the nucleosome is a potent inhibitor of transcription by Pol II (13,14). This can be ascribed to several factors, including: (i) intrinsic pausing signals specified by the underlying DNA sequence, which are observed even in the absence of nucleosomes, but tend to be greatly amplified when assembled into nucleosomes (15,16); (ii) nucleosome structure, which directs pausing at two specific sites within the nucleosome, at +15 and +45 with respect to the proximal nucleosome border (14); (iii) nucleosome stability, defined by the affinity of the DNA sequence for the histone octamer (14).

Nucleosomes assembled on the synthetic high-affinity 601, 603 and 605 positioning sequences (17), present a polar barrier to transcription *in vitro* (14). That is, Pol II transcribes through the nucleosome with much more

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difficulty when approaching from one side than when approaching from the other side, referred to as the 'non-permissive' and 'permissive' nucleosome orientations, respectively. Our recent studies suggest that the intra-nucleosomal location of a 16-bp sequence common to the 601, 603 and 605 positioning sequences ['R(2-3)'] might act as a polar barrier signal (PBS) (18). Mutations in the PBS decrease the strength of the polar barrier and reduce the affinity of the DNA for histones, without affecting nucleosome positioning. It was proposed that the location of the PBS in the distal half of the non-permissive nucleosome is critical for barrier function.

Here, we show that ISW2-mediated translocation of a non-permissive nucleosome over a short distance, such that the PBS no longer interacts with the distal half of the nucleosome, is sufficient to nullify the polar barrier. *In vivo*, we find that insertion of 603 into the yeast *CUP1* gene does not result in a strong polar barrier to transcription by Pol II, probably because the precise nucleosome position necessary for polar barrier function is not maintained.

## MATERIALS AND METHODS

See Supplementary Data for more details.

### Nucleosome assembly and remodeling *in vitro*

In the nomenclature used previously (18), the permissive orientations were 601R, 603 and 605; the non-permissive orientations were 601, 603R and 605R, where R indicates the reverse-complement of the published sequence (17). The 601, 603 and 605 templates were constructed using pGEM-3Z/601, pGEM-3Z/603 and pGEM-3Z/605 (17) and PCR primers listed in Supplementary Table S1. One primer was 5'-end-labeled. The resulting DNA fragments were digested with *TspRI* and reconstituted into nucleosomes by octamer transfer from long chicken erythrocyte chromatin (19). Remodeling was performed with purified ISW2 as described (20). Briefly, nucleosomal templates (5 ng/ $\mu$ l) were incubated with 35 ng ISW2 per 370 ng total DNA at 30°C for 30 min in 25 mM HEPES-KOH (pH 7.6), 5 mM MgCl<sub>2</sub>, 40 mM KCl, 0.1 mg/ml BSA, 6 mM Tris-HCl (pH 8.0), 5% glycerol, 30 mM NaCl, 0.3 mM ATP. Nucleosomal templates were analyzed by non-denaturing 4.5% PAGE (19).

### Assembly of elongation complexes and transcription

Pol II elongation complexes were assembled using purified His-tagged yeast Pol II, DNA templates and 9 nt RNA, and immobilized on Ni-NTA resin (21). Nucleosomal templates, before or after remodeling, were annealed to immobilized EC9 complexes through their *TspRI* sticky ends at 16°C for 30 min in TB300 (20 mM Tris-HCl (pH 7.9), 5 mM MgCl<sub>2</sub>, 300 mM KCl, 2 mM 2-mercaptoethanol). ISW2 dissociates from DNA in 300 mM KCl, preventing further remodeling. Annealed complexes were washed twice with TB300 and once with TB40 (as TB300, but with 40 mM KCl) and ligated in TB40 containing 0.5 mM ATP, 1% PEG8000, 0.2 mg/ml BSA and 400 U T4 DNA ligase (NEB) at 16°C for 30 min.

Ligated EC9 complexes were washed once with TB40 and twice with TB300. Limited transcription was initiated in TB300 with 20  $\mu$ M ATP, 20  $\mu$ M CTP and 20  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]-GTP (3000 Ci/mmol) for 15 min at room temperature. GTP was added to 10  $\mu$ M for 5 min to form EC45. Immobilized labeled EC45 complexes were washed twice with TB300, twice with TB40 and eluted with 0.1 M imidazole. Transcription was continued in TB with 0.4 mM NTPs at different KCl concentrations. Transcripts were resolved by denaturing 8% PAGE. Phosphorimages were quantified using ImageQuant (GE). The procedure for quantitation of the amounts of the run-off transcripts is designed to take into account possible variations in the efficiency of ligation and/or in the amount of material loaded on the denaturing gel. First, total amounts of transcripts that are longer than 45 nt (and therefore can be produced only on ligated templates) in each lane were quantified. Second, the fraction of the run-off transcripts to total amount of >45-nt transcripts was quantified. Finally, the yields of the run-off transcript were normalized to the amount of run-off transcripts produced in 1 M KCl (was set at 100%).

### Preparation of core particles

Yeast strains were constructed as described in Supplementary Data. Cells were grown in synthetic complete medium at 30°C to A<sub>600</sub> ~0.5. Copper induction was for 20 min in 50  $\mu$ M CuSO<sub>4</sub>. Nuclei were prepared from ~250 A<sub>600</sub> units of cells as described (22), resuspended in 2.4 ml 10 mM HEPES-K pH 7.5, 35 mM NaCl, 0.5 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, divided into six aliquots of 400  $\mu$ l and digested with micrococcal nuclease (Worthington) at 100, 150, 225, 340, 500 or 750 U/ml for 2 min at room temperature. The reaction was stopped with 10 mM EDTA and 1% SDS. DNA was purified and dissolved in 50  $\mu$ l 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100  $\mu$ g/ml RNase A. The extent of digestion was determined by analysis in agarose gels stained with ethidium bromide. Core particle DNA from samples in which almost all of the chromatin had been digested to core particles was gel-purified and repaired using the PreCR DNA repair kit (NEB). The quality of the core particle DNA was verified in 8% native and 10% denaturing polyacrylamide gels. Core particle DNA was fully trimmed to 147–150 bp.

## RESULTS

### ISW2-induced nucleosome translocation relieves the polar barrier

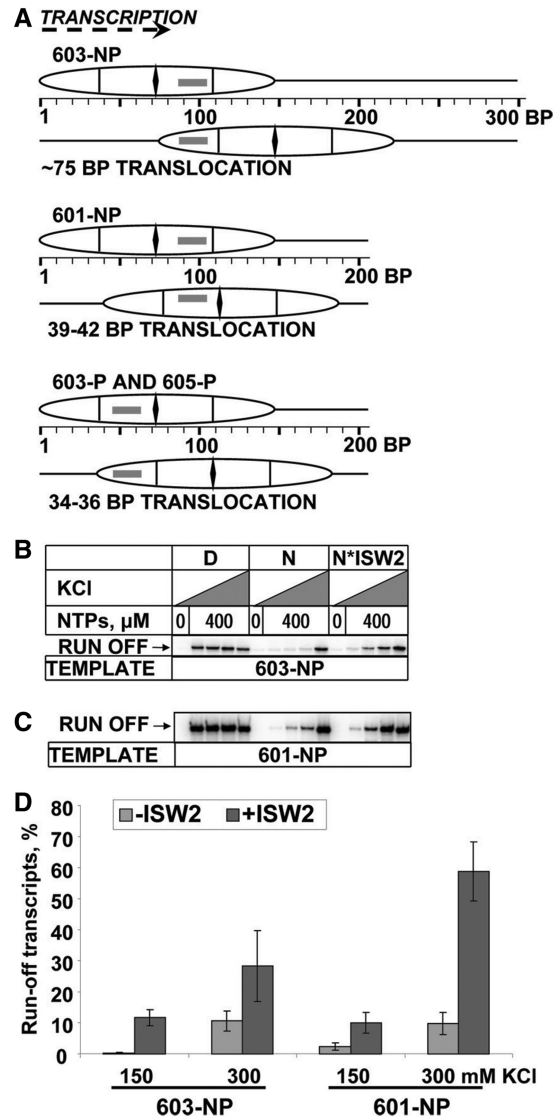
To evaluate the effect of re-location of the PBS on transcription by Pol II, an ATP-dependent chromatin remodeler was employed to translocate nucleosomes from the original 601, 603 or 605 positioning sequences to alternative positions. ISW2 was selected because it minimally perturbs nucleosome structure and moves nucleosomes over predictable distances, depending on the DNA length and the original nucleosome position (23,24). Templates were designed for translocation over short distances, placing the PBS in different locations with respect

to the nucleosome, such that it interacts with either the promoter-proximal H2A–H2B dimer, the promoter-proximal H3–H4 dimer, or the promoter-distal H3–H4 dimer (Figure 1A). Nucleosome positions before and after ISW2 remodeling were verified by analysis in a native gel (Figure 2A), restriction enzyme sensitivity (Figure 2B) and DNase I footprinting (Figure 2C).

The translocated nucleosomes were transcribed using pre-assembled yeast Pol II elongation complexes (EC). Briefly, EC9 complexes (where the number indicates the length of the transcript) were pre-assembled using synthetic oligonucleotides. The properties of these elongation complexes are very similar to those of promoter-initiated elongation complexes (14,25). The EC9 complexes were then annealed to the nucleosomal templates before or after ISW2 treatment, ISW2 was removed by washing, and the template was ligated. The nascent RNA was pulse-labeled with ATP, CTP and radioactive GTP to produce EC45 complexes, with Pol II stalled upstream of the promoter-proximal nucleosome boundary, awaiting the addition of UTP. The nucleosomal templates were transcribed in the presence of all four NTPs and at different concentrations of KCl to determine the strength and intranucleosomal location of the barrier to Pol II (Supplementary Figure S1).

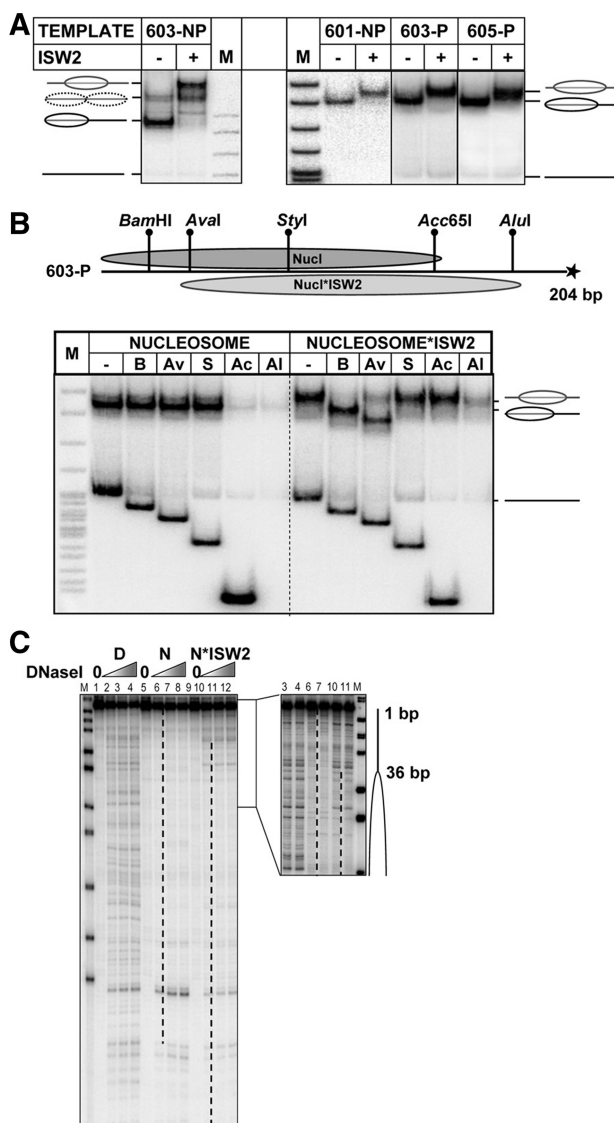
The effect of ISW2-induced nucleosome translocation on the polar barrier in non-permissive 601 and 603 nucleosomes (601-NP and 603-NP, Figure 1A) was analyzed. In these nucleosomes, the PBS is located at +87 to +102 with respect to the promoter-proximal nucleosome boundary, where it interacts with the promoter-distal H3–H4 dimer (18). ISW2 induced the translocation of the 603-NP nucleosome to a new position  $\sim 75$  bp downstream of the original position, such that the PBS now interacts with the promoter-proximal H2A–H2B dimer (Figure 1A). Transcription of the 603-NP template before remodeling was characterized by strong pausing at +45 (Supplementary Figure S2):  $>99\%$  of transcribing Pol II failed to yield run-off transcript in 150 mM KCl (Figure 1B and D). The 603-NP nucleosome barrier was relieved only in 1 M KCl, when nucleosome structure is disrupted. However, after nucleosome translocation by ISW2, the strength of the barrier was dramatically reduced, by  $\sim 30$ -fold in 150 mM KCl (Figures 1B and D, and 3D). In 150 mM KCl, only  $\sim 0.4\%$  of Pol II transcribed through the 603-NP nucleosome, whereas  $\sim 12\%$  was able to transcribe through the nucleosome after translocation. In 300 mM KCl, the fraction of Pol II able to transcribe through the 603-NP nucleosome increased from  $\sim 10$  to  $\sim 30\%$  (a  $\sim 3$ -fold decrease in barrier strength) (Figure 1D).

ISW2 induced translocation of the 601-NP nucleosome over a short distance to a new position only 38–42 bp away from the original position (Figure 1A) (26). This resulted in a large increase in run-off transcript produced at 150 and 300 mM KCl (Figure 1C and D). The strength of the barrier was reduced  $\sim 5$ -fold in 150 mM KCl (Figure 1D): only  $\sim 2\%$  of Pol II transcribed through the 601-NP nucleosome, whereas  $\sim 10\%$  was able to transcribe through the nucleosome after translocation. In 300 mM KCl, the fraction of Pol II able to transcribe



**Figure 1.** ISW2-induced nucleosome translocation relieves the polar barrier. (A) Nucleosome positions and translocation distances on permissive (603-P and 605-P) and non-permissive (603-NP and 601-NP) templates before and after remodeling by ISW2. The PBS is indicated by a rectangle. (B) 603-NP or (C) 601-NP nucleosomes (N) or nucleosomes remodeled with ISW2 (N\*ISW2), or free DNA (D) were transcribed by Pol II in 40 mM KCl without NTPs, or in 40, 150, 300 or 1000 mM KCl with 0.4 mM NTPs. Pulse-labeled RNA was analyzed by denaturing PAGE. Only the run-off products are shown (see Supplementary Figure S2 for the entire gel). (D) Quantitation of run-off transcripts in (B) and (C). The amount of run-off transcript produced in 1 M KCl was set at 100%. The data represent means of three independent experiments, with standard errors.

through the 601-NP nucleosome increased from  $\sim 10$  to  $\sim 60\%$  (a  $\sim 6$ -fold decrease in barrier strength). Thus, the effect of translocation on the 603-NP nucleosome was greater than on the 601-NP nucleosome, probably because the 603-NP nucleosome is intrinsically more difficult to transcribe. In conclusion, ISW2-induced translocation of the non-permissive 601 and 603 nucleosomes over distances of 38–75 bp relieved the polar barrier to



**Figure 2.** Analysis of ISW2-induced nucleosome translocation. (A) Nucleosomes were reconstituted on 210- or 300-bp end-labeled DNA fragments containing the 601, 603 or 605 nucleosome positioning sequences (Figure 1A), incubated with ISW2 and analyzed by native PAGE. Nucleosome positions are indicated. M, end-labeled pBR322-*MspI* digest. (B) Mapping of 603-P nucleosome positions: restriction enzyme sensitivity assay. Top: Expected positions of nucleosomes on permissive 603-P template before (Nucl.) and after ISW2 remodeling (Nucl\*ISW2). Unique sites for restriction enzymes are indicated. Bottom: Analysis of end-labeled 603-P nucleosomes by native PAGE before and after remodeling and digestion with a restriction enzyme. Histone-free DNA was added to the nucleosomal templates as an internal control for restriction enzyme activity. (C) Mapping of 603-P nucleosome positions before and after remodeling by ISW2: DNase I footprinting. ISW2 induces translocation of the 603-P nucleosome by 36 bp. Footprinting of DNA (D) and the nucleosome (N) was conducted before and after remodeling. Analysis by denaturing PAGE. Nucleosome positions are indicated (dashed lines and ovals).

transcription, even though the translocated nucleosomes still contain the PBS.

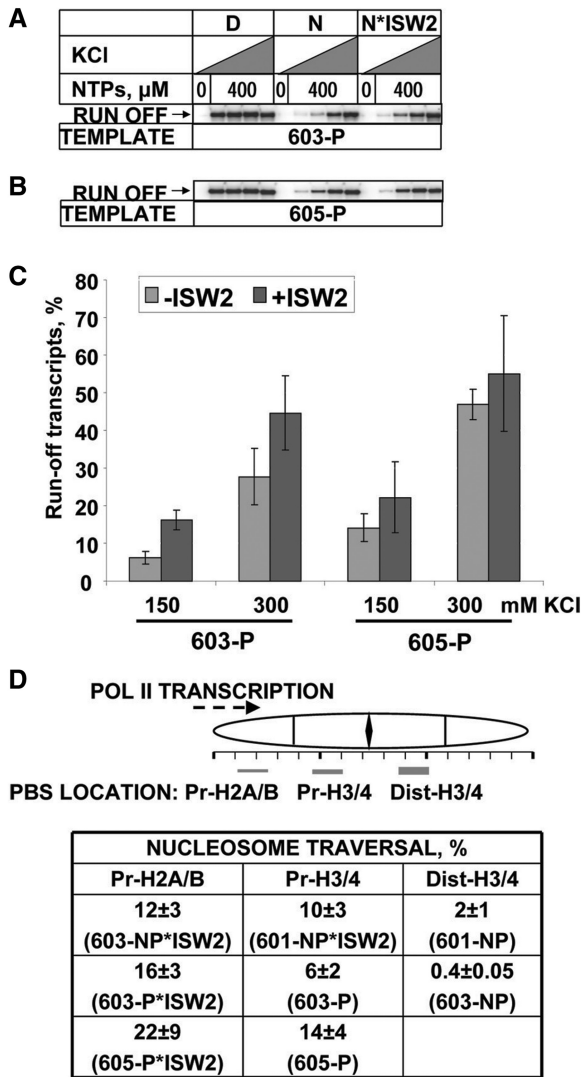
### ISW2-induced nucleosome translocation has little effect on the non-polar barrier

To determine whether translocation has any effect on the non-polar barrier to transcription that is exhibited by all nucleosomes, permissive 603 and 605 nucleosomes were remodeled by ISW2 and transcribed (Figure 3). In the 603-P and 605-P nucleosomes, the PBS interacts with the promoter-proximal H3–H4 dimer. ISW2 induced nucleosome translocation to new positions 34 and 36 bp downstream of the original positions, respectively (Figure 1A). In both cases, the PBS was re-located such that it interacts with the promoter-proximal H2A–H2B dimer. In 150 mM KCl, there was a relatively modest increase in transcription of both the 603-P and 605-P nucleosomes (~2.5- and 1.5-fold, respectively) as a result of translocation (Figure 3). In 300 mM KCl, the increase in transcription due to translocation was slight for both nucleosomes (Figure 3C, Supplementary Figure S3). Thus, the strength of the barrier posed by the permissive nucleosomes decreased only slightly in response to translocation. The strength of the barrier was characteristic of nucleosomes formed on natural DNA, which allow progression of 10–40 and 45–90% of transcribing Pol II in 150 and 300 mM KCl, respectively (14). In conclusion, ISW2-induced nucleosome translocation over a short distance (34–75 bp) strongly relieved the polar barrier to transcription, by 5-fold (601) or 30-fold (603) (Figure 3D). The effect on the non-polar barrier was only 1.6–2.7-fold.

### The 603-positioning sequence does not form a polar barrier *in vivo*

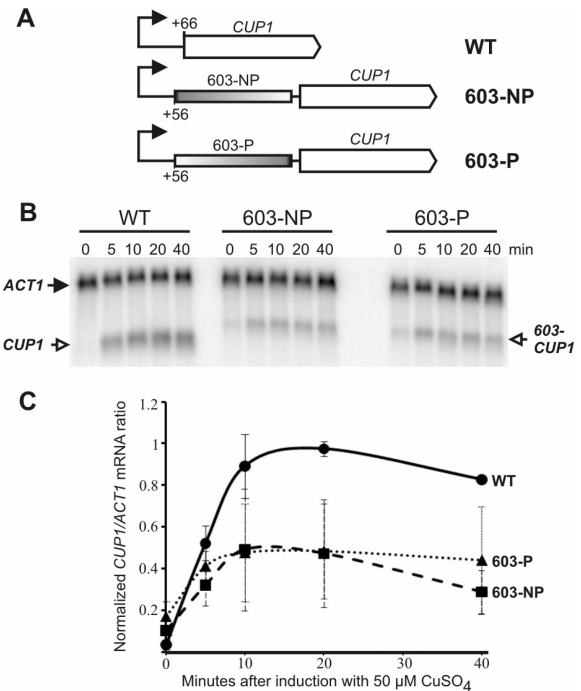
Is the PBS functional *in vivo*? The 603-sequence was inserted into the yeast *CUP1* gene at +56, between the major transcription start site at +1 and the start codon at +66, in the permissive or the non-permissive orientation (Figure 4A). *CUP1* encodes an inducible metallothionein which protects cells against the toxic effects of copper (27). Sequence-603 was chosen because it has a more polar barrier than 601 (Figures 1 and 3C). Haploid strains (WT, 603-NP and 603-P) were constructed in which a *CUP1* gene, with or without the 603-insert, was integrated into the chromosome, replacing the original *CUP1* locus (Figure 4A). These strains have only one copy of *CUP1* per cell. Growth assays showed that WT cells were resistant to 50  $\mu$ M copper, whereas both 603 strains grew moderately well only in 10  $\mu$ M  $\text{CuSO}_4$ . Thus, the 603-insert had an adverse effect on *CUP1* function.

The effect of 603 on copper-induction was determined (Figure 4B). *CUP1* was strongly induced in WT cells, increasing rapidly, peaking between 10 and 20 min, and then falling off somewhat (Figure 4C), as expected (28). The presence of the 603-insert was confirmed by the larger size of the 603-*CUP1* transcripts. Both 603-*CUP1* strains showed significantly elevated basal levels of *CUP1* expression and exhibited a robust induction, similar to that of wild-type cells, but reduced by ~2-fold, probably accounting for the increased copper-sensitivity of the 603-*CUP1*



**Figure 3.** The efficiency of transcription through permissive nucleosomes is minimally affected by ISW2. (A) 603-P or (B) 605-P nucleosomes (N) or nucleosomes remodeled with ISW2 (N\*ISW2), or free DNA (D) were transcribed by Pol II in 40 mM KCl without NTPs, or in 40, 150, 300 or 1000 mM KCl with 0.4 mM NTPs. Pulse-labeled RNA was analyzed by denaturing PAGE. Only the run-off products are shown (see Supplementary Figure S3 for the entire gel). Pulse-labeled RNA was analyzed by denaturing PAGE. (C) Quantitation of run-off transcripts in (A) and (B). (D) Intra-nucleosomal location of the PBS and the height of the nucleosomal barrier to transcription. Top: location of the PBS within different nucleosomes; the heights of the polar barrier to Pol II are indicated by the height of the rectangle which indicates the location of the PBS. Pr-H2A/B, Pr-H3/4 and Dist-H3/4: Pr = promoter-proximal; Dist = promoter-distal. Bottom: yields of run-off transcripts from the various nucleosomes in 150 mM KCl. The data represent the means of three independent experiments, with standard errors.

strains. Most importantly, 603-*CUP1* transcription was independent of the orientation of 603 (Figure 4C). Thus, 603 does not dictate a polar barrier to transcription *in vivo*. This could be due to nucleosome translocation by remodeling complexes, as shown above for ISW2 *in vitro*. Consequently, we asked whether the nucleosome is correctly positioned on 603 *in vivo*.

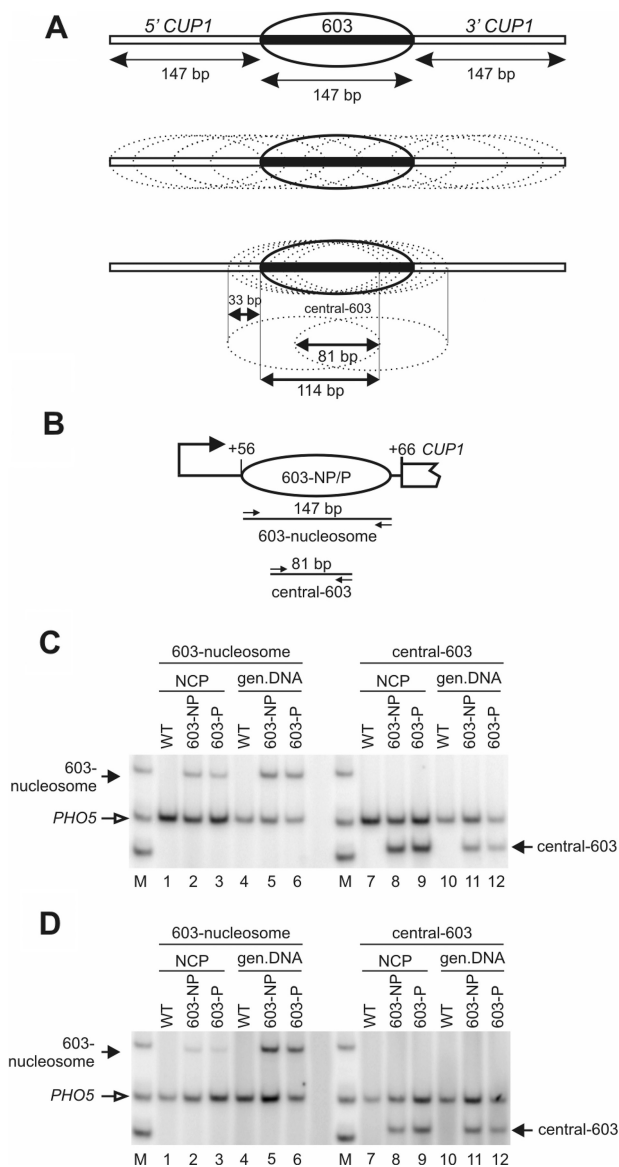


**Figure 4.** The 603-nucleosome positioning sequence does not form a polar barrier to transcription *in vivo*. (A) Yeast strains with 603 inserted at +56 in the 5'-untranslated region of *CUP1*. The major transcription start site (+1) is indicated by the arrow. The 603-NP and 603-P strains have 603 inserted in the non-permissive or permissive orientations, respectively. (B) Time courses of induction with 50  $\mu$ M  $\text{CuSO}_4$ . The phosphorimager scan of a northern blot probed for *CUP1* and *ACT1* transcripts is shown. (C) Time courses of induction: WT (circles); 603-P (triangles); 603-NP (squares). The data represent the means of two independent experiments, with standard deviations.

### The 603-nucleosome is positioned correctly in only a small fraction of cells

A quantitative PCR assay was used to measure the nucleosome occupancy of 603 *in vivo*. The assay depends on the fact that the 603-nucleosome position has been determined with high precision (29), corresponding exactly to the 147-bp inserted into *CUP1*. Thus, a pair of primers corresponding to the ends of the 603-insert yield the positioning sequence as a 147-bp PCR product. Using nucleosome core particle DNA trimmed to 145–150 bp by MNase as template (Supplementary Figure S4), this PCR product will be observed only if the nucleosome is located within a few base pairs of the expected position (Figure 5A). If trimming is incomplete, the occupancy of the 603-position would tend to be over-estimated.

The occupancy can be defined as the fraction of nucleosomes correctly positioned on 603 of all nucleosomes overlapping any part of 603 (Figure 5A). If the 603-nucleosome is present in all cells, the occupancy would be 1. Measuring the occupancy precisely is difficult, because all nucleosomes containing 1 bp or more of the 603-insert must be measured accurately and summed to provide the denominator. Instead, an approximate occupancy was determined by measuring all nucleosomes containing the central 81 bp of the 603-insert and using this as



**Figure 5.** The occupancy of the 603 nucleosome positioning sequence is low *in vivo*. (A) Nucleosome occupancy is defined as the fraction of nucleosomes precisely positioned on the 147-bp 603-insert (solid oval) of all nucleosomes containing any part of 603 (dotted ovals). Any nucleosome including part of 603 will physically prevent the formation of the correctly positioned 603-nucleosome. An approximate occupancy is measured by determining the fraction of nucleosomes correctly positioned on 603, of all nucleosomes containing the central 81 bp of 603 (lower panel). (B) The 603-*CUP1* promoter showing a nucleosome correctly positioned on 603. The locations of the 603-nucleosome and central 603 primer pairs are indicated (small arrows) with their respective amplicon sizes. (C) Measurement of 603 occupancy in non-induced cells. NCP: nucleosome core particles; gen. DNA: genomic DNA; M: marker (75, 100 and 150 bp). Lanes 1–6: multiplex PCR with the 603-nucleosome and *PHO5* primers (internal control). Lanes 7–12: PCR with the central-603 and *PHO5* primers. (D) Measurement of 603-nucleosome occupancy in cells induced with 50  $\mu$ M  $\text{CuSO}_4$  for 20 min. Phosphorimages are shown.

the denominator. This represents a significant over-estimate of 603 occupancy because many potential alternative positions are not included (Figure 5A). Primers corresponding to the central 101 bp of the

–1 nucleosome in the *PHO5* promoter (30) were used for normalization.

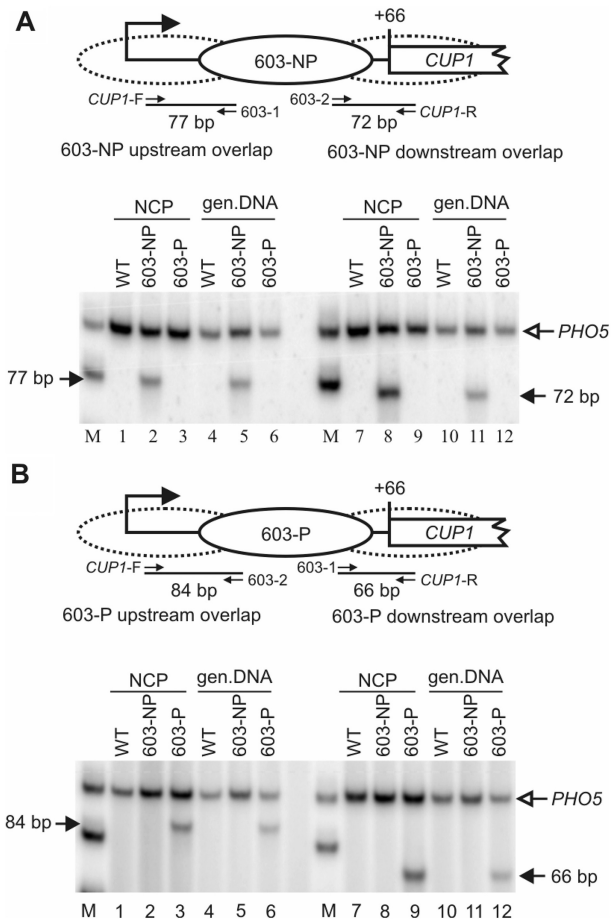
Multiplex PCR was performed using core particle DNA purified from the WT, 603-NP and 603-P strains, grown without copper induction (Figure 5B). The signals for core DNA were normalized using the signals from genomic DNA, since the amounts of 603 and *PHO5* must be equal in genomic DNA. Core particles from the 603-NP and 603-P strains gave rise to some 147 bp product (Figure 5C, lanes 2 and 3), proving that at least some 603-NP and 603-P cells contain a correctly positioned 603-nucleosome. However, for core particles from both strains, the intensity of the 603-nucleosome band was much lower than that of the *PHO5* internal control band (Figure 5C, lanes 2 and 3; ~5- and ~8-fold lower, respectively), whereas the ratio of the 603-nucleosome band to the *PHO5* internal control was close to 1 for genomic DNA (Figure 5C, lanes 5 and 6).

The occupancy of 603 was measured by normalizing the 603-nucleosome band and the central-603 band to their respective *PHO5* internal controls and then to their respective genomic 603/*PHO5* ratios, and dividing the 603-nucleosome ratio by the central-603 ratio. The occupancy of 603 was  $20 \pm 7\%$  in 603-NP cells and  $14 \pm 11\%$  in 603-P cells (averages of three independent experiments). Copper-induction had little effect on occupancies (Figure 5D): these were  $12 \pm 4\%$  in 603-NP cells and  $12 \pm 9\%$  in 603-P cells (averages of two independent experiments). In both cases, the orientation of 603 had no significant effect on occupancy. In conclusion, the large majority of cells do not have a correctly positioned 603-nucleosome.

#### Evidence for nucleosomes positioned over the *CUP1*-603 junctions

Given that nucleosomes are not positioned on 603 as expected, it should be possible to detect overlapping nucleosomes containing part of the 603-insert and some flanking up or downstream *CUP1* DNA. To verify the presence of such nucleosomes, different primer pairs had to be used for the 603-NP and 603-P strains, because of the orientation of 603 (Figure 6A). PCR performed with the upstream junction primers gave the expected 77 bp product using core particle and genomic DNA from the 603-NP strain (Figure 6A, lanes 2 and 5). The downstream junction primers gave a similar result. The results confirm the presence of nucleosomes in positions overlapping the junctions between *CUP1* and 603 in 603-NP cells. The same was shown to be true in 603-P cells using different primer combinations (Figure 6B).

A quantitative measure of the junction nucleosomes was obtained by comparison with nucleosomes containing the central 603-sequence (Supplementary Table S2). In non-induced 603-NP cells, nucleosomes were more likely to include the central 603-sequence or the downstream 603-*CUP1* junction DNA than the upstream *CUP1*-603 junction. In 603-P cells, the central 603 sequence was more likely to be nucleosomal than either junction sequence. However, these differences were small. Induction with copper had only minor effects on



**Figure 6.** Evidence for nucleosomes positioned over the *CUP1*-603 junction sequences in non-induced cells. Detection of nucleosomes positioned over the *CUP1*-603 junctions in (A) 603-NP cells and (B) 603-P cells. The schematics show a nucleosome correctly positioned on 603. The dotted ovals represent nucleosomes in positions overlapping the junctions of 603 with *CUP1*. The locations of the upstream and downstream *CUP1* primers and of internal 603 primers 1 and 2 are indicated (small arrows). Phosphorimages of PCR assays are shown. NCP: nucleosome core particles; gen. DNA: genomic DNA; M: marker (75 and 100 bp). Lanes 1–6: multiplex PCR with the upstream junction primer pair and *PHO5* primers (internal control). Lanes 7–12: PCR with the downstream junction primer pair and *PHO5* primers. Similar results were obtained with copper-induced cells (Supplementary Figure S5). The data are quantified in Supplementary Table S2.

nucleosome distribution (Supplementary Figure S5 and Table S2). Thus, overall, 603 was not strongly preferred over neighboring *CUP1* sequences for nucleosome assembly *in vivo*. The fact that the fraction of junction nucleosomes is high is consistent with the low occupancy of 603, because the presence of a junction nucleosome precludes the presence of the 603-nucleosome, as they both cannot be present on the same DNA molecule and therefore in the same cell.

## DISCUSSION

In summary, the presence of a strong polar barrier to transcription correlates with the interaction of the PBS with the promoter-distal H3–H4 dimer (Figure 3D). The

polar barrier is therefore dependent on precise nucleosome positioning: even a small change in position (35–75 bp) results in re-location of the PBS and loss of the barrier. Thus, ISW2, an ATP-dependent chromatin remodeler, can strongly affect the transcription rate by moving the nucleosome such that the PBS is now in a different location, even within the same nucleosome (Figure 3D). The PBS within 603, one of the strongest nucleosome positioning sequences known, does not act as a polar barrier *in vivo*, probably because in most cells, the nucleosome is not positioned as required.

## Circumvention of the polar barrier by ISW2-induced nucleosome translocation *in vitro*

Our data are consistent with the proposal that the polar barrier is negated by re-location of the PBS within the nucleosome. When ISW2 moves the PBS away from the distal H3–H4 dimer, the sequence contained within the nucleosome is changed and the histone octamer is likely to be bound at a less energetically favorable location. Although the sequence context has changed, there is a good correlation between the location of the PBS sequence within the nucleosome and the height of the barrier to transcribing Pol II (Figure 3D). The relative de-stabilization of the nucleosome *per se* is unlikely to account for the loss of the polar barrier, because the polar barrier does not depend on the overall stability of the nucleosome, but rather on its orientation with respect to the approaching polymerase (14). In the 601-NP and 603-NP nucleosomes, the PBS is located such that it interacts with the promoter-distal half of the H3–H4 tetramer (Figure 3D), resulting in a very high barrier to transcription. Translocation by ISW2, resulting in re-location of the PBS such that it interacts with the proximal H3–H4 dimer or with the proximal H2A–H2B dimer, greatly reduces the strength of the polar barrier. In the 603-P and 605-P nucleosomes, the PBS interacts with the promoter-proximal H3–H4 dimer and the barrier is relatively weak, similar to that exhibited by nucleosomes formed with natural DNA. After translocation, the PBS interacts with the promoter-proximal H2A–H2B dimer, with little effect on the weak barrier. Thus, there is a strong correlation between the presence of a strong polar barrier and the interaction of the PBS with the promoter-distal H3–H4 dimer.

## The absence of a polar barrier *in vivo*

How is the polar barrier circumvented *in vivo*? A likely explanation is the low occupancy of 603; most cells do not contain a nucleosome correctly positioned on 603, which is necessary for the PBS to function. In the 10–20% of 603-NP cells which do contain the 603-nucleosome, the polar barrier might be present but undetected, given the error in the measurements. In these cells, the polar barrier might cause Pol II to stall inside the 603-nucleosome. An alternative view is that nucleosomes on *CUP1* and 603 might be in flux, due to various nucleosome sliding activities, spending only a short time in the 603-position (31,32). If this is the case, a transient polar barrier might be formed in all

603-NP cells, active only when the nucleosome is in the 603-position. We propose that the polar barrier is absent *in vivo* because nucleosome sliding activities drive translocation of the 603-nucleosome from its critical position, preventing interaction of the PBS with the promoter-distal H3–H4 dimer.

Direct support for translocation of the 603-nucleosome by remodeling complexes could be obtained by examining nucleosome sliding mutants. However, in addition to ISW2, there are several chromatin remodeling complexes capable of mobilizing nucleosomes in yeast, including ISW1, SWI/SNF and RSC. This level of redundancy makes it difficult to eliminate nucleosome-sliding activity in yeast cells.

### The 603-insert does not position a nucleosome strongly *in vivo*

We have shown that most cells do not have the 603-nucleosome at the correct position. It is perhaps surprising that the occupancy of 603 is so low *in vivo*, given that it is one of the strongest positioning sequences known, and that DNA sequence is a major determinant of positioning in yeast (33–35). Perhaps 603 is not such a strong positioning sequence *in vivo*, as the related 601 sequence is a relatively poor competitor for histones under some conditions *in vitro* (36). The 5S RNA gene is a natural, strong positioning sequence and it also fails to position uniquely *in vivo* (37), although this is also true *in vitro* (38), unlike 601. More generally, these observations imply that the nucleosome positioning information [the ‘nucleosome code’ (39)] that is undoubtedly present in DNA is insufficient to predict the locations of all nucleosomes unambiguously *in vivo*. Other factors likely to be of critical importance include the binding of transcription factors, which may act as nucleosome phasing signals (31,32) and the formation of nucleosomal arrays *in vivo*, which imposes restrictions on the locations of neighboring nucleosomes once the first nucleosome has been assembled (e.g. during DNA replication) (28). The promoters of yeast genes tend to be relatively low-affinity sites for nucleosome formation *in vitro* and tend to exclude nucleosomes *in vivo* (31,32). However, like the *PHO5* promoter (26), the *CUP1* promoter is an exception: there is a nucleosome on the *CUP1* promoter in non-induced cells, which adopts one of several alternative positions *in vivo* (29) and *in vitro* (30). Insertion of 603 just downstream of the major transcription start site would necessarily interrupt these positioning signals, perhaps interfering with the positioning of the nucleosome on the 603-insert just downstream.

However, probably most important is the fact that ISW2 (Figure 1A) and RSC (40,41) can move the nucleosome quantitatively from 601 or 603 to a much less energetically favorable position *in vitro*. Thus, remodeling complexes can overcome the very high affinity of the histone octamer for 601 or 603, shifting the nucleosome to a higher free-energy state. This is also likely to be the case *in vivo*: nucleosome sliding activities use the free-energy gained from ATP hydrolysis to move nucleosomes to positions that are not necessarily those of the

lowest free energy. Thus, the insertion of 603 elsewhere in the yeast genome, where there is much less sliding activity, perhaps in a silenced region, might result in higher occupancies.

### Nucleosomal barriers and remodeling complexes

The polar barrier to yeast Pol II cannot be relieved by the elongation factor TFIIS or by the histone chaperone FACT, although IIS can facilitate transcription though the polar barrier by human Pol II (14). ISW2 is the first yeast factor to be identified that can relieve the polar barrier *in vitro*. Although the existence of polar nucleosomal barriers in natural DNA remains to be demonstrated, it seems likely that such sequences exist *in vivo*. Polar barriers might possess significant regulatory potential if combined with nucleosome sliding activities capable of controlling the occupancy of the critical nucleosome position relative to other positions.

More generally, our data suggest that nucleosome re-positioning could be involved in regulation of transcription. In particular, elongating Pol II is paused immediately downstream from the transcription start site (TSS) on thousands of genes in *Drosophila* and man (9,10). The first nucleosome downstream of the TSS (the +1 nucleosome) is positioned relatively well and is translocated during activation of transcription (12,42). Furthermore, elongating RNA polymerase II co-maps with the promoter-proximal boundary of the +1 nucleosome on many *Drosophila* genes (12). Finally, the +1 nucleosome can form a strong, activator-sensitive barrier to transcribing human Pol II *in vitro* (13). However, a causal relationship between +1 nucleosome translocation and transcription activation remains to be established.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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### REFERENCES

1. Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F. and Richmond, T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature*, **389**, 251–260.
2. Kulaeva, O.I., Gaykalova, D.A. and Studitsky, V.M. (2007) Transcription through chromatin by RNA polymerase II: Histone displacement and exchange. *Mutat. Res.*, **618**, 116–129.



3. Kristjuhan, A. and Svejstrup, J.Q. (2004) Evidence for distinct mechanisms facilitating transcript elongation through chromatin *in vivo*. *EMBO J.*, **23**, 4243–4252.
4. Schwabish, M.A. and Struhl, K. (2004) Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. *Mol. Cell. Biol.*, **24**, 10111–10117.
5. Dion, M.F., Kaplan, T., Kim, M., Buratowski, S., Friedman, N. and Rando, O.J. (2007) Dynamics of replication-independent histone turnover in budding yeast. *Science*, **315**, 1405–1408.
6. Rufiange, A., Jacques, P.E., Bhat, W., Robert, F. and Nourani, A. (2007) Genome-wide replication-independent histone H3 exchange occurs predominantly at promoters and implicates H3 K56 acetylation and Asf1. *Mol. Cell*, **27**, 393–405.
7. Thiriet, C. and Hayes, J.J. (2005) Replication-independent core histone dynamics at transcriptionally active loci *in vivo*. *Genes Dev.*, **19**, 677–682.
8. Rufiange, A., Jacques, P.E., Bhat, W., Robert, F. and Nourani, A. (2007) Genome-wide replication-independent histone H3 exchange occurs predominantly at promoters and implicates H3 K56 acetylation and Asf1. *Mol. Cell*, **27**, 393–405.
9. Zeitlinger, J., Stark, A., Kellis, M., Hong, J.W., Nechaev, S., Adelman, K., Levine, M. and Young, R.A. (2007) RNA polymerase stalling at developmental control genes in the *Drosophila melanogaster* embryo. *Nat. Genet.*, **39**, 1512–1516.
10. Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R. and Young, R.A. (2007) A chromatin landmark and transcription initiation at most promoters in human cells. *Cell*, **130**, 77–88.
11. Muse, G.W., Gilchrist, D.A., Nechaev, S., Shah, R., Parker, J.S., Grissom, S.F., Zeitlinger, J. and Adelman, K. (2007) RNA polymerase is poised for activation across the genome. *Nat. Genet.*, **39**, 1507–1511.
12. Mavrich, T.N., Jiang, C., Ioshikhes, I.P., Li, X., Venters, B.J., Zanton, S.J., Tomsho, L.P., Qi, J., Glaser, R.L., Schuster, S.C. *et al.* (2008) Nucleosome organization in the *Drosophila* genome. *Nature*, **453**, 358–362.
13. Brown, S.A., Imbalzano, A.N. and Kingston, R.E. (1996) Activator-dependent regulation of transcriptional pausing on nucleosomal templates. *Genes Dev.*, **10**, 1479–1490.
14. Bondarenko, V.A., Steele, L.M., Ujvari, A., Gaykalova, D.A., Kulaeva, O.I., Polikanov, Y.S., Luse, D.S. and Studitsky, V.M. (2006) Nucleosomes can form a polar barrier to transcript elongation by RNA polymerase II. *Mol. Cell*, **24**, 469–479.
15. Izban, M.G. and Luse, D.S. (1991) Transcription on nucleosomal templates by RNA polymerase II *in vitro*: inhibition of elongation with enhancement of sequence-specific pausing. *Genes Dev.*, **5**, 683–696.
16. Kireeva, M.L., Hancock, B., Cremona, G.H., Walter, W., Studitsky, V.M. and Kashlev, M. (2005) Nature of the nucleosomal barrier to RNA polymerase II. *Mol. Cell*, **18**, 97–108.
17. Lowary, P.T. and Widom, J. (1998) New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning. *J. Mol. Biol.*, **276**, 19–42.
18. Kulaeva, O.I., Gaykalova, D.A., Pestov, N.A., Golovastov, V.V., Vassilyev, D.G., Artsimovitch, I. and Studitsky, V.M. (2009) Mechanism of chromatin remodeling and recovery during passage of RNA polymerase II. *Nat. Struct. Mol. Biol.*, **16**, 1272–1278.
19. Gaykalova, D.A., Kulaeva, O.I., Bondarenko, V.A. and Studitsky, V.M. (2009) Preparation and analysis of uniquely positioned mononucleosomes. *Methods Mol. Biol.*, **523**, 109–123.
20. Kassabov, S.R., Henry, N.M., Zofall, M., Tsukiyama, T. and Bartholomew, B. (2002) High-resolution mapping of changes in histone-DNA contacts of nucleosomes remodeled by ISW2. *Mol. Cell. Biol.*, **22**, 7524–7534.
21. Kireeva, M.L., Walter, W., Tchernajenko, V., Bondarenko, V., Kashlev, M. and Studitsky, V.M. (2002) Nucleosome remodeling induced by RNA polymerase II: loss of the H2A/H2B dimer during transcription. *Mol. Cell*, **9**, 541–552.
22. Kim, Y. and Clark, D.J. (2002) SWI/SNF-dependent long-range remodeling of yeast HIS3 chromatin. *Proc. Natl Acad. Sci. USA*, **99**, 15381–15386.
23. Gangaraju, V.K., Prasad, P., Srour, A., Kagalwala, M.N. and Bartholomew, B. (2009) Conformational changes associated with template commitment in ATP-dependent chromatin remodeling by ISW2. *Mol. Cell*, **35**, 58–69.
24. Zofall, M., Persinger, J., Kassabov, S.R. and Bartholomew, B. (2006) Chromatin remodeling by ISW2 and SWI/SNF requires DNA translocation inside the nucleosome. *Nat. Struct. Mol. Biol.*, **13**, 339–346.
25. Walter, W., Kireeva, M.L., Studitsky, V.M. and Kashlev, M. (2003) Bacterial polymerase and yeast polymerase II use similar mechanisms for transcription through nucleosomes. *J. Biol. Chem.*, **278**, 36148–36156.
26. Kagalwala, M.N., Glaus, B.J., Dang, W., Zofall, M. and Bartholomew, B. (2004) Topography of the ISW2-nucleosome complex: insights into nucleosome spacing and chromatin remodeling. *EMBO J.*, **23**, 2092–2104.
27. Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welch, J. and Fogel, S. (1984) Primary structure and transcription of an amplified genetic locus: the CUP1 locus of yeast. *Proc. Natl Acad. Sci. USA*, **81**, 337–341.
28. Shen, C.H., Leblanc, B.P., Neal, C., Akhavan, R. and Clark, D.J. (2002) Targeted histone acetylation at the yeast CUP1 promoter requires the transcriptional activator, the TATA boxes, and the putative histone acetylase encoded by SPT10. *Mol. Cell. Biol.*, **22**, 6406–6416.
29. Morozov, A.V., Fortney, K., Gaykalova, D.A., Studitsky, V.M., Widom, J. and Siggia, E.D. (2009) Using DNA mechanics to predict *in vitro* nucleosome positions and formation energies. *Nucleic Acids Res.*, **37**, 4707–4722.
30. Svaren, J. and Horz, W. (1997) Transcription factors vs nucleosomes: regulation of the PHO5 promoter in yeast. *Trends Biochem. Sci.*, **22**, 93–97.
31. Kim, Y., McLaughlin, N., Lindstrom, K., Tsukiyama, T. and Clark, D.J. (2006) Activation of *Saccharomyces cerevisiae* HIS3 results in Gcn4p-dependent, SWI/SNF-dependent mobilization of nucleosomes over the entire gene. *Mol. Cell. Biol.*, **26**, 8607–8622.
32. Clark, D.J. (2010) Nucleosome positioning, nucleosome spacing and the nucleosome code. *J. Biomol. Struct. Dyn.*, **27**, 781–793.
33. Shen, C.H. and Clark, D.J. (2001) DNA sequence plays a major role in determining nucleosome positions in yeast CUP1 chromatin. *J. Biol. Chem.*, **276**, 35209–35216.
34. Kaplan, N., Moore, I.K., Fondufe-Mittendorf, Y., Gossett, A.J., Tillo, D., Field, Y., LeProust, E.M., Hughes, T.R., Lieb, J.D., Widom, J. *et al.* (2009) The DNA-encoded nucleosome organization of a eukaryotic genome. *Nature*, **458**, 362–366.
35. Zhang, Y., Moqtaderi, Z., Rattner, B.P., Euskirchen, G., Snyder, M., Kadonaga, J.T., Liu, X.S. and Struhl, K. (2009) Intrinsic histone-DNA interactions are not the major determinant of nucleosome positions *in vivo*. *Nat. Struct. Mol. Biol.*, **16**, 847–852.
36. Wu, C. and Travers, A. (2005) Relative affinities of DNA sequences for the histone octamer depend strongly upon both the temperature and octamer concentration. *Biochemistry*, **44**, 14329–14334.
37. Chipev, C.C. and Wolffe, A.P. (1992) Chromosomal organization of *Xenopus laevis* oocyte and somatic 5S rRNA genes *in vivo*. *Mol. Cell. Biol.*, **12**, 45–55.
38. O'Donohue, M.F., Duband-Goulet, I., Hamiche, A. and Prunell, A. (1994) Octamer displacement and redistribution in transcription of single nucleosomes. *Nucleic Acids Res.*, **22**, 937–945.
39. Segal, E., Fondufe-Mittendorf, Y., Chen, L., Thastrom, A., Field, Y., Moore, I.K., Wang, J.P. and Widom, J. (2006) A genomic code for nucleosome positioning. *Nature*, **442**, 772–778.
40. Carey, M., Li, B. and Workman, J.L. (2006) RSC exploits histone acetylation to abrogate the nucleosomal block to RNA polymerase II elongation. *Mol. Cell*, **24**, 481–487.
41. Shukla, M.S., Syed, S.H., Montel, F., Faivre-Moskalenko, C., Bednar, J., Travers, A., Angelov, D. and Dimitrov, S. (2010) Remosomes: RSC generated non-mobilized particles with approximately 180 bp DNA loosely associated with the histone octamer. *Proc. Natl Acad. Sci. USA*, **107**, 1936–1941.
42. Koerber, R.T., Rhee, H.S., Jiang, C. and Pugh, B.F. (2009) Interaction of transcriptional regulators with specific nucleosomes across the *Saccharomyces* genome. *Mol. Cell*, **35**, 889–902.