Published in final edited form as:

Biochem Biophys Res Commun. 2007 December 21; 364(3): 583–588.

A nucleosome positioned by $\alpha 2$ /Mcm1 prevents Hap1 activator binding *in vivo*

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Abstract

Nucleosome positioning has been proposed as a mechanism of transcriptional repression. Here, we examined whether nucleosome positioning affects activator binding in living yeast cells. We introduced the cognate Hap1 binding site (UAS1) at a location 24-43 bp, 29-48 bp or 61-80 bp interior to the edge of a nucleosome positioned by $\alpha 2$ /Mcm1 in yeast minichromosomes. Hap1 binding to the UAS1 was severely inhibited, not only at the pseudo-dyad but also in the peripheral region of the positioned nucleosome in α cells, while it was detectable in **a** cells, in which the nucleosomes were not positioned. Hap1 binding was restored in α cells with *tup1* or *isw2* mutations, which caused the loss of nucleosome positioning. These results support the mechanism in which $\alpha 2$ /Mcm1-dependent nucleosome positioning has a regulatory function to limit the access of transcription factors.

Keywords

nucleosome positioning; chromatin; activator binding; *in vivo* footprinting; yeast

In a general view of the process of transcriptional activation, activators have to gain access to an array of nucleosomes, and chromatin remodeling complexes and/or histone modification enzymes change the structural features of chromatin to recruit transcriptional machinery to the promoter regions [reviewed in 1,2]. Regarding activator binding, several regulatory factors such as steroid hormone receptor, Gal4-derivatives, USF and Sp1, bind to reconstituted nucleosomes *in vitro* under some circumstances, although their affinities for nucleosomal DNA decrease by one to three orders of magnitude, relative to those for naked DNA [see reviews, 2-4]. Positioned nucleosomes assembled with *Drosophila* embryo extracts preclude NF1 binding to its cognate site [5]. In contrast, NF- κ B binds to its recognition sites within a positioned nucleosome with an affinity close to that for free DNA *in vitro* [6]. Yeast Gal4 is one of the best characterized activators binding to nucleosomes *in vivo*. Gal4 can bind to its cognate site within a positioned nucleosome to perturb the nucleosome in yeast cells [7-11]. Also, the Pho4 activator can reportedly bind its nucleosome-occupied site before nucleosome disassembly *in vivo* [12]. However, only a few activators have been examined for their access

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to DNA within the nucleosome *in vivo*. Although nucleosome positioning has been observed in a number of promoters and proposed as a mechanism of repression, it has seldom been possible to alter nucleosome positioning and determine the effect on activator binding.

Here, we have utilized the yeast minichromosome system, which consists of an array of nucleosomes positioned by the yeast $\alpha 2$ /Mcm1 repressor complex, to examine activator binding to DNA within a nucleosome. We have shown that the binding of the activator Hap1 is severely limited at any location tested within the nucleosome *in vivo*.

Materials and methods

Plasmids and strains

The plasmid TALS-pBR322 Δ RI [13] was digested with EcoRI to remove 356 bp of the *STE6* promoter fragment, and was ligated, forming TRP1ARS1-pBR322 Δ RI. A pair of oligonucleotides with the UAS1 sequence in the *CYC1* promoter, 5'-AAT TCG AGC TCT GGC CGG GGT TTA CGG ACG ATG AGC TCG-3' and 5'-AAT TCG AGC TCA TCG TCC GTA AAC CCC GGC CAG AGC TCG-3', were synthesized chemically, annealed and ligated into the EcoRI site in TRP1ARS1-pBR322 Δ RI, forming TRP1ARS1-UAS1-pBR322 Δ RI. Then, the UAS1 fragment was excised by digestion of TRP1ARS1-UAS1-pBR322 Δ RI with SacI, and it was sub-cloned into the SacI site of TALS-pBR322 Δ RI, forming TALS-UAS1a-pBR322 Δ RI. To construct the TALS-UAS1b-pBR322 Δ RI and TALS-UAS1c-pBR322 Δ RI plasmids, portions of the TALS sequence were substituted with the UAS1 sequence (5'-TGG CCG GGG TTT ACG GAC GAT-3') by two-step PCR. All of the constructed plasmids were verified by DNA sequencing. The pBR322 Δ RI portion was eliminated by a digestion with HindIII, and the portions of the yeast plasmids were self-ligated, and transformed into yeast [13]. The yeast strains used were AMP107 [*MATa ura3 trp1 leu2 lys2 ho::LYS2*] and AMP108 [isogenic to AMP107 except for *MATa*] [14]. The *isw2* mutant strains, MHS303 [*MATa ura3 trp1 leu2 his3 lys2 ho::LYS2 isw2::HIS3*] and MHS314 [isogenic to MHS303 except for *MATa*] and the *tup1* mutant strain, YMH202 [*MATa ura3-52 leu2-3, 112 trp1 Δ his3 Δ ade2-101 lys2-801 tup1::URA3*], were described previously [15,16].

Analysis of chromatin structure

Isolation and digestion of nuclei with micrococcal nuclease (MNase) were described previously [13,17]. MNase cleavage sites were analyzed by primer extension mapping, using the IR-Dye800-labeled MS-2 primer (1735-1701 nt in TALS) [17] with the LI-COR 4200L DNA sequencer.

In vivo UV-photofootprinting

In vivo UV-photofootprinting was performed as described [18,19]. The sites of the UV-photoproducts were analyzed by primer extension mapping, using the IR-Dye800-labeled MS-2 primer (1735-1701 nt in TALS) [17] with the LI-COR 4200L DNA sequencer.

Results

Experimental design

The yeast $\alpha 2$ /Mcm1p positions nucleosomes adjacent to the $\alpha 2$ operator in α cells [17,20,21]. Fig. 1 shows the chromatin structures of TRP1ARS1 and TALS minichromosomes, as determined previously [20,22]. The $\alpha 2$ operator is located in the region of 1535-1565 nucleotides (nt) in TALS. Nucleosome IV is positioned both translationally and rotationally adjacent to the $\alpha 2$ operator in the region of 1376-1522 nt in TALS in α cells, but not in α cells, as shown previously [17,23]. These minichromosomes have been utilized to examine the binding of Gal4 [7-11] and Bicoid [10], as well as to determine the effect of DNA sequences

on nucleosome formation [13] *in vivo*. We chose to examine the binding of the Hap1 activator [24], which is a member of a family of fungal transcription factors with the Zn₂Cys₆ binuclear cluster domain [25]. The UAS1 site was introduced at locations 24-43 bp, 29-48 bp and 61-80 bp interior to the edge (1522 nt) of the positioned nucleosome IV in TALS-UAS1a, TALS-UAS1b and TALS-UAS1c, respectively (Fig. 1). We also introduced the UAS1 site into the nuclease hypersensitive region B, a nucleosome-free region, in TRP1ARS1 (denoted as TRP1ARS1-UAS1) (Fig. 1).

Chromatin structures of TALS and TRP1ARS1 minichromosomes containing the UAS1 site

We analyzed the chromatin structures of TALS and TRP1ARS1 derivatives containing the UAS1 site, by limited digestion of nuclei with MNase and subsequent high-resolution primer extension mapping (Fig. 2). For the TALS derivatives, the nucleosome IV region was protected from MNase digestion in *a* cells, as compared with naked DNA and chromatin in *α* cells (lanes 1-6 for TALS-UAS1a, lanes 7-12 for TALS-UAS1b, and lanes 13-18 for TALS-UAS1c). Thus, in *a* cells, the UAS1 site is located around the dyad of the positioned nucleosome IV in TALS-UAS1a, whereas it is located in the peripheral region in the nucleosome in TALS-UAS1b and TALS-UAS1c. In *α* cells, nucleosomes were not positioned in these TALS minichromosomes. We also analyzed the chromatin structure of TRP1ARS1-UAS1 (lanes 25-30). The HSR B region, where the UAS1 was inserted, was hypersensitive to MNase digestion in chromatin, as compared to naked DNA samples. Thus, the UAS1 site is located in the nucleosome-free region in TRP1ARS1-UAS1 minichromosomes.

Effect of the UAS1 locations in a positioned nucleosome on Hap1 binding *in vivo*

Previously, we showed that Hap1 binding may be detected sensitively, as the enhancement of UV-photoproducts in the center of UAS1 in the *CYC1* promoter by *in vivo* UV-photofootprinting [18,19]. Utilizing this advantage, we examined Hap1 binding to the UAS1 in TALS and TRP1ARS1 derivatives (Fig. 3). First, we analyzed Hap1 binding to the nucleosome-free region of the TRP1ARS1-UAS1 minichromosome in both *a* and *α* cells (lanes 1-6). In both cell types, Hap1 binding was detected as a hypersensitive site in UAS1 in chromatin samples, but not in naked DNA, consistent with previous studies of the *CYC1* promoter [18,19]. Then, we analyzed the TALS-UAS1a minichromosome (lanes 7-12), in which UAS1 was incorporated into the dyad axis of the positioned nucleosome IV in *a* cells. The hypersensitive site was detectable in *a* cells, in which nucleosomes were not positioned, but it was strongly diminished in *α* cells, indicating that Hap1 binding is severely limited at the dyad in the positioned nucleosome IV. Similar results for Hap1 binding were obtained for TALS-UAS1b (lanes 19-24) and -UAS1c (lanes 28-33), in which the UAS1 site was located in the peripheral region of the positioned nucleosome IV; that is, the hypersensitive site is present in *a* cells, but absent in *α* cells. It should be noted that the location of UAS1 in TALS-UASc is shifted to 5 bp exterior to the nucleosome edge, as compared to that in TALS-UAS1b. Thus, the rotational setting of the UAS1 in the nucleosome did not affect Hap1 binding. These results indicate that Hap1 cannot access its binding site in any of the tested locations within the nucleosome.

Hap1p binding is restored by an *isw2* or *tup1* mutation in MAT α cells

The Tup1-Ssn6 corepressor is required for repression and nucleosome positioning in *a*-cell-specific genes [26,27], and a *tup1* mutation causes the loss of nucleosome positioning in TALS minichromosomes [28]. The Isw2 chromatin remodeling complex positions nucleosomes in Tup1-regulated genes, such as *a*-cell-specific genes [15,28-31]. Here, we analyzed the chromatin structure of TALS-UAS1a in isogenic *isw2* mutant strains (Fig. 2). The MNase cleavage profile in the MAT α *isw2* strain (lanes 22-24) was identical to that in the MAT α wild

type (lanes 1-3) and *MATa isw2* strains (lanes 19-21), indicating the loss of nucleosome positioning in the TALS minichromosomes in the *isw2* mutant.

We next examined Hap1 binding in *MATa isw2* or *tup1* mutant strains. The results in Fig. 3 demonstrate that the hypersensitive site in UAS1 in TALS-UAS1a, -UAS1b and UAS1c was detectable in the *MATa isw2* and *MATa tup1* strains, as well as in *MATa* WT, indicating that Hap1 binding is restored by the *isw2* or *tup1* mutations in α cells. Thus, the inhibition of Hap1 binding in TALS minichromosomes in α cells is not due to the presence of $\alpha 2$, and the nucleosome positioning is responsible for inhibition of Hap1 binding.

Discussion

We have shown that a nucleosome positioned by the $\alpha 2$ /Mcm1 repressor complex prevents the binding of the Hap1 activator in living yeast cells. Previous studies revealed that the $\alpha 2$ /Mcm1 repressor, in conjunction with the Ssn6-Tup1 corepressor and the Isw2 complexes establishes an array of positioned nucleosomes to form a repressive chromatin structure [15,17,20,21, 28-30,32]. Our results imply that the positioned nucleosomes in the repressive chromatin function to limit the accessibility of at least a certain class of activators *in vivo*, therefore supporting the idea that nucleosome positioning plays a vital role in repression by $\alpha 2$ /Mcm1.

Unexpectedly, the Hap1 binding is diminished even at a site in the peripheral segment of nucleosomal DNA (24-43 bp and 29-48 bp from the edge) to nearly the same extent as that near the pseudo-dyad (61-80 bp from the edge). Thus, the inhibition of Hap1 binding does not depend on either the location or the rotational setting of the UAS1 in the nucleosome. In general, the accessibility at the pseudo-dyad is more limited than that in the peripheral region. For example, the *in vivo* accessibility of both the Dam and SssI methyltransferases is restricted beyond 20 to 29 bp into a positioned nucleosome [23,33]. The affinity of glucocorticoid receptor for the glucocorticoid response element (GRE) decreases, as the GRE is moved from the edge into the dyad *in vitro* [34]. Restriction enzyme digestion kinetic assays revealed that the equilibrium constants describing the dynamic site exposure decrease progressively from either end of the nucleosomal DNA toward the middle [35].

Gal4 binding to DNA has been examined extensively in the positioned nucleosome in the TALS minichromosome system [8,10,11], which we have used here. In contrast to Hap1, Gal4 binds to its cognate site not only in the 41 bp from the left and right edges of the nucleosome, but also at the dyad, although the accessibility is greater at translational positions farther from the dyad [11]. Nucleosomes are remodeled by the binding of Gal4 or Bicoid in minichromosomes, when their recognition sites are incorporated in a positioned nucleosome [10]. Both Gal4 and Hap1 have the Zn₂Cys₆ DNA binding motif and bind as dimers, but Gal4 and Hap1 recognize two CGG triplets symmetrically and asymmetrically, respectively. In addition, the space between the two triplets, which is important for their affinity [36], is different between Gal4 and Hap1. Thus, the configuration of the CGG triplets in the nucleosome may affect their recognition ability. The differential features in their binding to the nucleosome may be due to differences in their DNA recognition modes. Alternatively, the binding ability to the nucleosome may depend on the action of coactivators associated with activators. Gal4 binding is assisted by SWI/SNF [37] and SAGA [38,39], and these complexes may disrupt nucleosomes to gain stable access to the cognate site. Regarding this possibility, since Gal4-dependent nucleosome disruption *in vivo* does not require SWI/SNF and Gcn5 [9,40], other factors acting with Gal4 could be involved in this process.

Chromatin immunoprecipitation studies have shown that general transcription factors bind promoters repressed by *Drosophila* Polycomb group proteins [41], and also that *SIR*-generated heterochromatin is permissive to the constitutive binding of an activator, HSF, and two

components of the preinitiation complex, TBP and Pol II, in yeast cells [42]. However, the relationships between the transcription factor binding sites and the nucleosome positions were not specified in these experiments. Previously, we showed that the activators Hap1 and Abf1 bind to the *CYC1* and *HOP1* promoters, respectively, even when the promoters are repressed by the yeast Ume6 complex [14]. In contrast, Hap1 was prevented from binding to the *CYC1* promoter, which is repressed by Rme1 (regulator of meiosis) [18]. Taken together, whatever the repression mechanisms are, our results emphasize that activators are not always able to bind to their recognition sites within the nucleosome *in vivo*, and thus nucleosome positioning plays an important role in repression.

Acknowledgements

We thank K. Hattori and S. Kokubo for technical assistance in the construction of plasmids in their undergraduate studies. This work was supported by Grants-in-Aid for Scientific Research (C) 17570147 and 19570169 from JSPS, and a Special Cooperative Research Grant (2007) from Meisei University to MS, and by NIH Grants R01 GM39531 and AI070272 to APM.

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Fig. 1.

Experimental design in this study. Chromatin structures of the TALS minichromosome in *MAT α* cells and the TRP1ARS1 minichromosome. The UAS1 site was introduced into the TALS and TRP1ARS1 minichromosomes, and its location is indicated by the shaded boxes in TALS-UAS1a, -UAS1b and UAS1c and TRP1ARS1-UAS1. The $\alpha 2$ operator and the nucleosomes are shown by the hatched boxes and the gray ellipses, respectively. The asterisk indicates the dyad of the positioned nucleosome IV.

Fig. 2.

High resolution mapping of MNase cleavage sites in TALS and TRP1ARS1 derivatives containing the UAS1 site. **a**WT and **α**WT are wild-type strains. The locations of the $\alpha 2$ operator ($\alpha 2$ op, the hatched box), UAS1 (the shaded box) and the nucleosome IV (the gray ellipse) are shown on the left side of the gel. In each set of data, lanes labeled C indicate MNase digestion of isolated nuclei (chromatin) at two nuclease levels, and lanes labeled D indicate MNase digestion of the naked DNA as a control. The degree and integrity of MNase digestion in each sample were judged from nucleosome ladders of genomic DNA analyzed by agarose gel electrophoresis.

Fig. 3.

In vivo UV-photo footprints of Hap1 in TALS and TRP1ARS1 derivatives containing the UAS1 site. Lanes marked C are samples from intact cells irradiated with UV light (500 and 750 mJ/cm²); lanes marked D are samples from purified DNA irradiated with UV light (240 mJ/cm²). Arrows indicate sites of enhancements of UV photoproducts in irradiated cells, as compared with irradiated purified DNA (lane D).