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Nucleosomes and the accessibility problem

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Eukaryotic DNA is packaged in nucleosomes. How does this sequestration affect the ability of transcription regulators to access their sites? We cite evidence against the idea that nucleosome positioning is determined primarily by the intrinsic propensities of DNA sequences to form nucleosomes – such that, for example, regulatory sites would be 'nucleosome-free'. Instead, studies in yeast show that nucleosome positioning is primarily determined by specific DNA-binding proteins. Where nucleosomes would otherwise compete with regulatory protein binding (a modest but potentially biologically important effect), this obstacle can be relieved by at least two strategies for exposing regulatory sites. In contrast to their lack of effect on nucleosome positioning, DNA sequence differences do directly affect both the efficiencies with which nucleosomes form in regions flanking regulatory sites before induction, and the extent of their removal upon induction. These nucleosomes, evidently, inhibit basal transcription but are poised to be removed quickly upon command.

The DNA sequence *per se* does not suffice to position nucleosomes as found *in vivo*

Some 40 years ago an assay for nucleosomes was discovered that, with variations, is still used today [1]. Light digestion of chromatin (DNA and associated histones) with micrococcal nuclease (MNase), and extraction of the DNA, yields a series of DNA fragments of size modulo ~150 bp. This result, which holds for chromatin from many different eukaryotes, is caused by the relative inaccessibility of DNA segments wrapped around octamers of histones. To our knowledge, no protein or protein complex (other than one that includes a nucleosome) has been found to protect fragments larger than about 60 bp in this assay [2,3], and thus DNA registering as 'naked' in this assay may be occupied by proteins other than nucleosomes [4]. Students of gene regulation have long faced two related problems: how serious an obstacle does nucleosome formation present to the binding of regulatory proteins, and how might that obstacle – to the extent that it is one – be avoided or overcome?

An early analysis of a set of chicken DNA sequences protected from MNase digestion, taken with surmised DNA structural constraints, suggested that particular sequences would form nucleosomes more readily than others [5–13]. In particular, it was proposed that a sequence comprising sets of AA/TT/TA and GC base pairs, each pair separated by 5 bp, would have a high tendency to form nucleosomes [5,10,12]. And, the more such elements present in a given sequence the higher would be its 'nucleosome-forming potential' (NFP). These considerations led to the interesting idea that genomic DNA sequences might have evolved so as to spontaneously form nucleosomes in specified positions [12,14,15] and, as a corollary, that positioning might leave regulatory protein-binding sites 'naturally' nucleosome-free.

Indeed, nucleosomes are not positioned randomly on yeast DNA *in vivo* [16–19]. Were this non-random positioning determined by differing NFPs of different DNA sequences, then positions of nucleosomes formed *in vitro* with yeast DNA and purified histones should mimic those found *in vivo*. Such a correlation, based on genome-wide analyses, was reported [12,14], but was soon contradicted [20]. For example, it was noted [20] that there tends to be a nucleosome covering the +1 site at the 5′ end of genes *in vivo*, but no such preferential nucleosome positioning was detected *in vitro*. The correspondence between nucleosome positioning *in vitro* and *in vivo* can be improved, it is claimed, by addition of cellular extract plus ATP in nucleosome reconstitution experiments [21,22]. Thus there would appear to be only weak support for the idea that yeast DNA segments differ sufficiently in their NFPs to account for nucleosome positioning *in vivo* [13,23,24].

One general area of agreement between experiments performed *in vivo* and *in vitro* is that yeast promoter regions tend to be depleted of nucleosomes [14,20,25–27]. Promoters, defined as the 100–1000 bp regions lying immediately upstream of the transcription start-site of yeast genes, include sites of binding of regulatory proteins. Understanding nucleosome disposition in promoters requires distinguishing regulatory binding sites from the remaining regions of promoters. Two intensively studied cases in yeast, *CLN2* and *GAL1/10*, illustrate these (and other) points.

Auxiliary proteins facilitate activator binding

CLN2

The 150 bp regulatory region of *CLN2* (which encodes a cyclin) bears three sites for the activator SBF [28–30], as well as binding sites for at least three auxiliary

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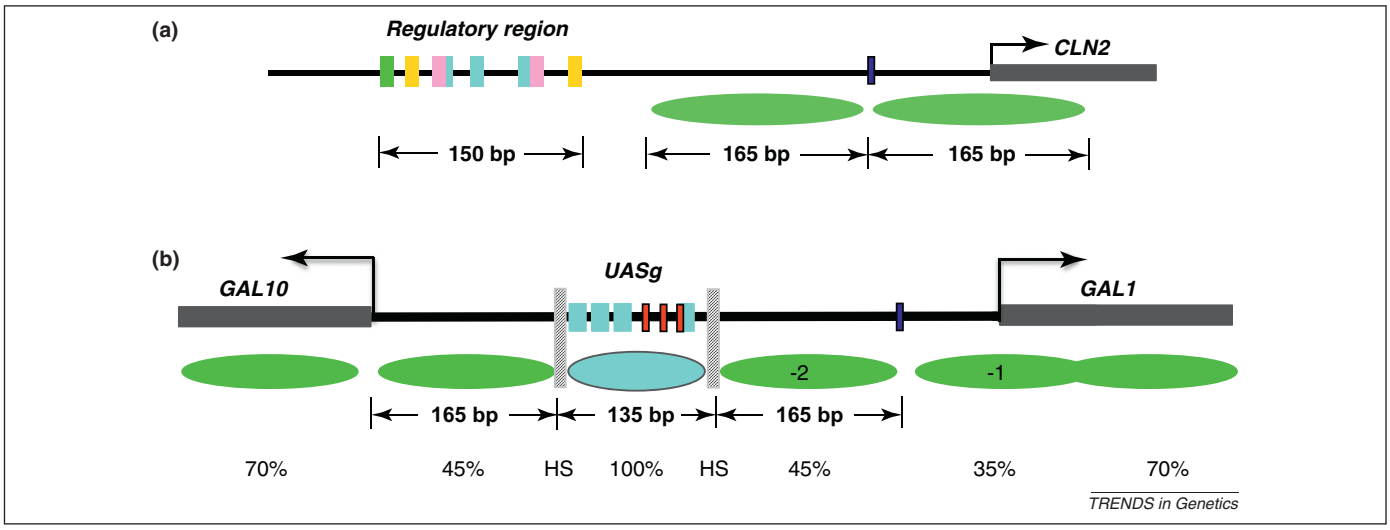

Figure 1. The regulatory regions and their flanking DNA at *CLN2* and *GAL1/10*. **(a)** *CLN2*. The 150 bp regulatory locus bears three binding sites for the activator SBF (cyan), as well as binding sites for the auxiliary proteins Reb1 (green), Mcm1 (yellow) and Rsc3 (pink). The constitutive presence of these auxiliary proteins at the locus has been confirmed by ChIP analysis, although not their precise locations. The flanking green ovals are nucleosomes that protect the canonical 165 bp (measured center to center), and occupy their sites inefficiently (Box 1). Both promoter nucleosomes are removed upon induction, presumably by Swi/Snf recruited by SBF. The start site of transcription of the *CLN2* gene is indicated by the arrow. **(b)** *GAL1/10*. The divergently transcribed *GAL1/10* genes are activated by Gal4, an activator that binds to four sites (in cyan) in the UASg (upstream activating sequence galactose) a regulatory element lying approximately midway between the two transcription start sites. Three putative RSC sites (in red) are also indicated, and the deletion that removes these sites, mentioned in the text, encompasses approximately the right half of the UASg. The RSC/nucleosome complex at the UASg is indicated by a cyan oval. Three phased promoter nucleosomes of ordinary size (165 bp measured center to center) are indicated by the green ovals. Whereas the UASg is occupied in 100% of the cells, phased sites are occupied in only some 35–45% of wild-type cells. HS (hypersensitive) sites are ~10 bp regions that are essentially naked throughout the population.

DNA-binding proteins – Mcm1, Reb1 and Rsc3 [31] (Figure 1a). The standard MNase protection assay indicates that this region is nucleosome-free throughout the cell cycle. SBF itself enters the nucleus, binds to its DNA sites, and activates expression of *CLN2* only at a specific stage of the cell cycle [28–30,32]. Mutating the binding sites for the auxiliary proteins allows nucleosomes to encroach over the region and, in this scenario, SBF can still find its sites and activate transcription, but only sporadically [31]. The picture that emerges is that the auxiliary proteins compete with, and displace, nucleosomes that otherwise would form over the region, and thereby maintain the interspersed SBF binding sites naked and readily accessible to SBF. This 150 bp region has a high NFP as assayed *in vitro* [14,31], but nevertheless the auxiliary proteins maintain it (including the SBF binding sites) in a nucleosome-free state [31]. Flanking this control region are well-positioned nucleosomes (Figure 1a), a matter we return to in the discussion of the *GAL* regulatory region.

GAL1/10

At the UASg (upstream activating sequence galactose) an auxiliary protein also facilitates binding of an activator (in this case Gal4), but the mechanism is different than that seen at *CLN2*. The UASg bears, in addition to its four Gal4 binding sites, binding sites for RSC [3], a member of the Swi/Snf chromatin remodeler family (Figure 1b). RSC binds to the UASg and traps it in an unusual, partially unwrapped nucleosome. The RSC/nucleosome complex protects from MNase digestion a fragment some 30 bp smaller than that protected by an ordinary nucleosome [2,3]. The small size of this protected fragment (i.e. 130 bp) explains, at least in part, why it has remained undetected until recently. The complex forms whether or not Gal4 is present, and is found on (i.e. protects) 100% of the UASg sites in the population. Short naked segments [hypersensitive (HS) sites], which mark the boundaries between the complex and the adjacent nucleosomes, flank the UASg (Figure 1b). Deletion of a portion of the UASg removes putative RSC binding sites, but leaves the three main Gal4 sites intact. This mutant UASg binds little if any RSC, is no longer flanked by HS sites, and the 130 bp salient protected fragment is no longer evident [3]. Gal4 binding to sites in the mutant UASg is delayed, and induction of *GAL1*, also delayed [3], is more sporadic (unpublished). The NFP of the UASg, like that of the *CLN2* regulatory region, is high [14,33]. For a discussion of this topic in other contexts see [34].

Thus, at both the *CLN2* regulatory region and the UASg, constitutively bound auxiliary proteins eliminate the competitive effect of nucleosomes on regulatory-protein binding. In neither case is the activator itself required for formation of the facilitating structure. In both cases, in contrast to the reasonable expectation that regulatory protein binding sites would have low NFPs, they in fact have high NFPs. This high NFP might be an unavoidable consequence of the rather high GC content of the activator binding sites at both *CLN2* and *GAL1/10*. Whereas at *CLN2* any competing nucleosome originally present would have to be competed away by binding of the auxiliary proteins, at the UASg a strong tendency to form a nucleosome might help formation of the RSC/partially unwrapped nucleosome complex. Some 2000 small MNase-protected fragments, possibly indicative of the presence of partially unwrapped nucleosomes, are found scattered throughout the genome, largely in promoter regions (unpublished). It remains to be seen how common will be the strategies for facilitating activator access as found at *CLN2* and *GAL1/10*.

Box 1. A nucleosome occupancy assay

There are several limitations inherent in the use of the standard micrococcal nuclease (MNase) protection assay to identify nucleosome disposition along a genome. The traditional assay is not highly quantitative. Thus, examining fragments protected at a single dose of MNase can reveal the presence of a nucleosome at a specific sequence, but only crudely can it reveal what fraction of the population (at an instant of crosslinking) bears a nucleosome at that position. Differences in inherent 'cuttability' of different DNA sequences can make the problem worse [23,24], especially when experiments are performed genome-wide. Moreover, because fragments are identified as nucleosomal only if they are ~150 bp in length, odd-sized fragments (particularly smaller ones) are undetected or ignored.

An assay that avoids these difficulties and that can directly distinguish occupancies and positioning uses, instead of one dose of MNase, some 16 levels of digestion spanning a 10 000-fold range [2]. The digestion curve of any specified ~60 bp segment of DNA is then displayed using PCR. The typical resulting curve is biphasic (Figure I): a fraction of the DNA is rapidly digested (i.e. is naked) and a fraction is highly protected (i.e. is occupied by a nucleosome). The inflection point for any given curve is taken as a measure of the fraction of the population that bears a nucleosome, at that position, at the instant of crosslinking. Because the crucial aspect of each curve is the inflection point, and not the absolute rate of digestion, effects of sequence on 'cuttability' are usually irrelevant. Analysis of a tiled array of PCR primers then can reveal the length of any protected fragment larger than ~60 bp (the length of the amplicon).

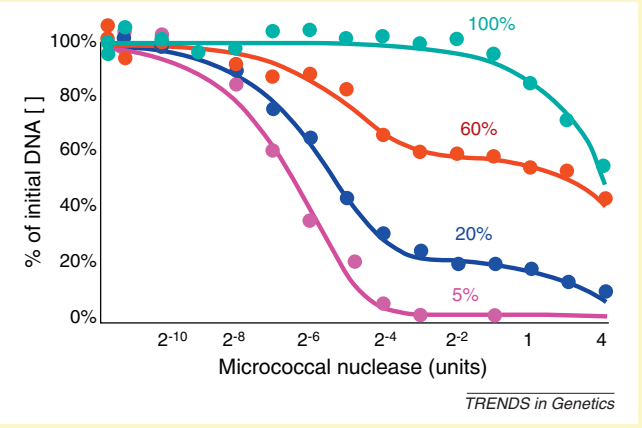

Figure I. Digestion curves for four different ~60 bp segments of yeast chromatin, generated as described in [2]. The middle two curves (red and blue) are obviously biphasic, as are most curves generated by digestion of various chromatin fragments taken from around the genome. The upper part of each of these two curves is generated by nucleosome-free DNA, and the lower part by nucleosome-occupied DNA. The inflection point provides the fractional occupancy of the fragment by a nucleosome (60% and 20%, respectively, in the cases shown). The top (cyan) and bottom (purple) curves are more nearly monophasic. They describe fragments, respectively, taken from the UASg, and another from a segment adjacent to the UASg that includes an HS site as shown in Figure 1b. The UASg is 100% occupied (by a RSC–nucleosome complex as revealed by later experiments), and the short (~10 bp) hypersensitive (HS) region is occupied in less than 5% of the population. To register as being 'protected' in this assay, no part of the fragment can be exposed.

Nucleosome positioning versus occupancies – effects on induction

For a specific DNA-binding protein such as *lac* repressor, high occupancy (tight binding) implies precise positioning. But for nucleosomes these two parameters (positioning and occupancies) can be determined independently using the occupancy assay described in Box 1. For example, as we have seen (Figure 1b), rather precisely positioned nucleosomes flank the UASg (on both sides) before induction. Despite being well-positioned, these promoter nucleosomes occupy their sites relatively infrequently. As indicated on the figure, these promoter sites are occupied about half as efficiently as is the UASg itself (by the RSC/nucleosome complex), and about 2/3 as efficiently as are more randomly positioned nucleosome sites in the ORFs [2]. A similar scenario describes the *CLN2* promoter nucleosomes (Figure 1a; unpublished). What determines the rather precise positioning but low occupancies of these promoter nucleosomes? And to what biological end? We first address positioning.

Positioning

At both the UASg and the *CLN2* regulatory region, positioning of flanking nucleosomes is determined by the auxiliary proteins bound to the respective regulatory regions. Thus (i) a UASg transplanted into the *GAL1* ORF causes flanking ORF nucleosomes to phase in a fashion similar to that seen with the UASg at its wild-type position, and (ii) deletion of RSC sites from the UASg blurs positioning of the flanking nucleosomes [3]. At *CLN2* an analogous effect is seen: mutation of the auxiliary protein binding sites causes not only encroachment of nucleosomes over the regulatory region, but also loss of phasing of the flanking nucleosomes [31].

The similar effects of the disparate structures at *CLN2* and *GAL1/10* suggest a common mechanism for determining nucleosome phasing in the two cases. We surmise that in each case phasing is a consequence of more-or-less random nucleosome formation, accompanied perhaps by nucleosome fluidity, around a zone of exclusion (the regulatory locus in each case). Thus, in each case the regulatory region comprises a barrier and phasing is a statistical consequence of more or less random nucleosome-formation in flanking regions [35]. What causes the phased promoter nucleosomes to occupy their sites so weakly? This cannot be caused by the barrier itself because when the UASg is transplanted to a position in the ORF (as mentioned above), the flanking nucleosomes, although phased, occupy their sites significantly more efficiently than do the nucleosomes flanking the UASg at its wild-type location in the *GAL1/10* promoter region [3].

Occupancies

Occupancies of the phased nucleosome sites in the *GAL1* promoter are determined by the NFPs of the underlying sequences [36]. This was revealed by substituting, at site –1 (Figure 1b), for the wild-type sequence, six sequences with increasing numbers of AA/TT/TA and GC elements, and measuring nucleosome occupancies. Before induction, occupancy increased monotonically with the number of AA/TT/TA and GC elements. The occupancies spanned a range of some 35% for the wild-type case to 100% for a 'superbinder' sequence comprising 26 AA/TT/TA or GC elements. That these differences are biologically important is shown by the following experiments.

Upon induction of the *GAL* genes (effected by adding galactose) promoter nucleosomes are removed by Swi/Snf,

a step essential for rapid induction [2]. Swi/Snf is recruited to the *GAL* genes by Gal4 [2,37], as it is recruited by the activator Pho4 to the *PHO5* gene where it removes promoter nucleosomes [38,39]. For wild-type *GAL1*, the promoter nucleosomes are removed essentially completely. But, as the occupancy before induction is increased (by substituting DNAs with higher NFPs), the fractional occupancy after induction also increases. Experiments with substitutions at site –2 (the site adjacent to the UASg) show that, as would be expected, increased nucleosome occupancy is accompanied by decreased induction. Further experiments show that recruited Swi/Snf works more efficiently on nucleosomes at –2 than that at –1, but that, nevertheless, the same rule applies to both sites: higher occupancy before induction – as dictated by the NFP of the underlying sequence – is associated with higher occupancy after induction [36]. We do not know whether this effect is caused by less removal as the NFP increases, or whether it reflects nucleosome reformation – which would be more rapid the higher the NFP – following removal. For *CLN2*, we assume that low occupancies by phased promoter nucleosomes are explained by similar considerations.

General propositions

We suggest the following general formulations to explain nucleosome positioning and occupancies, and their effects on gene regulation.

For most genomic DNA, sequences do not differ sufficiently in their NFPs to determine nucleosome positioning *in vivo*. Instead, nucleosomes are directed to, or held at, specific positions by specific DNA-binding proteins. For example, RSC traps a partially unwrapped nucleosome at the UASg, and the RSC/nucleosome complex causes flanking nucleosomes to adopt more or less specific positions (i.e. are 'phased') as a result of the barrier erected by the complex [3]. We imagine that a protein(s) holds nucleosomes at other positions where they are precisely positioned – at the +1 site of many genes, for example – and that phasing of downstream nucleosomes, diminishing with distance, results from a barrier effect [35]. Evidently a similar barrier (or zone of exclusion) is erected by the auxiliary proteins bound to the *CLN2* regulatory region [31].

DNA sites need not be inherently (i.e. in the absence of auxiliary factors) naked to allow the access of regulatory proteins. In fact, both the *CLN2* regulatory region and the UASg have high NFP as assayed *in vitro* (as mentioned above). Instead, auxiliary proteins can clear out nucleosomes (as in the *CLN2* case), or trap a nucleosome in an unusual form (as in the *GAL1/10* case), such that crucial binding sites are readily accessible [3,31]. In the absence of these auxiliary factors nucleosomes inhibit the binding of regulatory proteins where they compete for the same regions on DNA. However, where analyzed, such inhibition applies more to the rate of binding than to the final level [3]. Thus, at both *CLN2* and *GAL1/10*, the activators (SBF and Gal4, respectively) operate in the absence of auxiliary factors (an effect of directed mutations), but do so more sporadically and slowly that they do in the presence of those factors.

The *GAL1/10* and *CLN2* promoters each comprise two parts: one binds regulatory proteins whereas the other forms 'promoter nucleosomes'. The latter, positioned by barriers manifested by specific DNA-binding proteins, occupy sites of low NFP – such that they can be quickly and completely removed upon induction. These promoters are relatively depleted of nucleosomes before induction because of the low NFP of the sequences flanking the regulatory regions [6,36] and, following induction, these nucleosomes are removed by recruited chromatin remodelers [2,36,38].

Concluding remarks and future perspectives

Transcription activation in eukaryotes, and often in bacteria as well, is effected by recruitment: a transcription activator binds to DNA and, in a binding reaction involving a separate surface on the activator, binds to (and thereby recruits) a component(s) of the transcription machinery to a promoter [40]. The recruiting reaction imparts specificity (as determined by the where the activator binds). However, the recruiting interaction is weak and, in the absence of any inhibitor, spontaneous binding of the transcription machinery (i.e. in the absence of an activator) would lead to significant basal levels of transcription. All recruiting reactions – which govern the behavior of many enzymes that work on other macromolecules – are subject to this potential problem [40]. As emphasized elsewhere [41], evolution has added inhibitors – some specific, some general – to discourage basal reactions while poising systems such that these inhibitors are overcome upon command. Nucleosomes, we believe, provide this inhibitory function for transcription. Thus, nucleosomes have been reported to inhibit spontaneous, spurious transcription originating at internal positions in ORFs [42]. We imagine that, in yeast, nucleosomes form at promoters efficiently enough to also inhibit such basal reactions – a conjecture not yet demonstrated – but weakly enough that they can be quickly removed upon command [36]. This scenario would explain why in bacteria (which do not have nucleosomes), unlike in eukaryotes, activators that work by recruitment are apparently always associated with specific repressors – those repressors have as one of their jobs the elimination of basal transcription in the absence of the activator. In eukaryotes, general inhibition by promoter nucleosomes, by hypothesis, suffices to reduce basal transcription such that, in general, no specific repressor is required in the absence of an activator [43]. Deletion of Gal4, for example, results in a very low level of basal transcription in cells growing in medium containing the sugar raffinose despite the absence of any known specific repressor. The *GAL* genes are subject to repression, but this is not related to a basal level problem. Instead, a 'glucose-repression' mechanism ensures that glucose, the favored carbon source, will be preferentially utilized when cells encounter a mix of galactose and glucose.

Perhaps, as others have suggested, proteins (either auxiliary proteins as at *CLN2,* or activators themselves), that bind to closely spaced DNA sites, function cooperatively to remove competing nucleosomes [44]. Cooperativity would make the reverse reaction – in which nucleosomes encroach and displace regulatory proteins – infrequent. It remains to be seen how such binding reactions are affected by their affinities for DNA, the clustering

of their sites, their concentrations, and so on. In any event there is a striking superficial similarity between the *CLN2* regulatory region and the *Drosophila eve* enhancer [45] – in both cases we have a closely packed array of specific DNA-binding proteins. At the *Drosophila* enhancer, as for *CLN2*, perhaps these proteins ensure access of crucial regulators to their sites. A prediction of this idea is that strict alignment of the auxiliary DNA-binding proteins, as found naturally, is not required. Rather, spacing, such that each protein could contribute to competition with a nucleosome, would be key. Evidence consistent with this idea has been presented [46–48].

This line of thought would emphasize the close relationship between 'accessibility' and 'specificity'. Consider a solution of naked bacterial DNA and lambda repressor. At the proper concentrations, repressor will more likely be found bound to its proper operator site than to accidental sites that might be found around the genome. This specificity is accounted for, of course, by cooperativity, repressor dimers contacting each other and binding together to adjacent sites [49,50]. Where a nucleosome obstacle is present, the same rule applies – the higher the cooperativity the more specific the binding. In this scenario, the various DNA-binding proteins need not interact to compete cooperatively with a nucleosome. Facilitating structures – such as those found at the UASg and at *CLN2* – are specificity factors that encourage binding to their associated sites [3,31].

It remains to be seen how many activators, in yeast and in higher eukaryotes, must compete with nucleosomes for binding, and how many are aided by auxiliary factors such as those we have described for *GAL1/10* and for *CLN2*. RSC in higher organisms lacks the DNA-binding determinants of yeast RSC [51–53], and therefore where Gal4 is used to drive gene expression from genes fused to the UASg [54,55], the activator is apparently competing, unaided, with nucleosomes for access to DNA. Preliminary experiments suggest that gene activation under these conditions is sporadic (unpublished).

As a simple extension of these findings, silencing (such as that found at a silent mating type region in yeast) could be achieved by proteins that effectively increase the occupancy of promoter nucleosomes without affecting activator binding sites. We imagine that even a factor of 10–50 would suffice to prevent nucleosome removal and activation of transcription. Such a mechanism need not impede activator binding [56–58]. Assays that distinguish occupancy from positioning (Box 1) should support or refute this idea. And, using the occupancy assay, it should be straightforward to determine whether histone modifications (levels of acetylation, for example) affect occupancy.

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