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Resource

Interaction of Transcriptional Regulators with Specific Nucleosomes across the *Saccharomyces* Genome

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SUMMARY

A canonical nucleosome architecture around promoters establishes the context in which proteins regulate gene expression. Whether gene regulatory proteins that interact with nucleosomes are selective for individual nucleosome positions across the genome is not known. Here, we examine on a genomic scale several protein-nucleosome interactions, including those that (1) bind histones (Bdf1/SWR1 and Srm1), (2) bind specific DNA sequences (Rap1 and Reb1), and (3) potentially collide with nucleosomes during transcription (RNA polymerase II). We find that the Bdf1/SWR1 complex forms a dinucleosome complex that is selective for the +1 and +2 nucleosomes of active genes. Rap1 selectively binds to its cognate site on the rotationally exposed first and second helical turn of nucleosomal DNA. We find that a transcribing RNA polymerase creates a delocalized state of resident nucleosomes. These findings suggest that nucleosomes around promoter regions have position-specific functions and that some gene regulators have position-specific nucleosomal interactions.

INTRODUCTION

Genes and their promoters tend to have a canonical chromatin architecture, involving well-positioned nucleosomes at precise distances from the transcriptional start site (TSS). In the budding yeast *Saccharomyces*, the −1 and +1 nucleosomes are centered ~230 bp upstream and ~60 bp downstream from the TSS, respectively (Albert et al., 2007; Yuan et al., 2005). Between the two is an intervening ~140 bp nucleosome-free region (NFR) where the general transcription machinery assembles. A similar arrangement exists in multicellular eukaryotes (Barski et al., 2007; Mavrich et al., 2008b; Valouev et al., 2008). Little is known about how gene regulatory proteins and the transcription machinery function in the context of this organized state of chromatin. Indeed, although histone-binding domains have been identified (Bannister et al., 2001; Lachner et al., 2001) and factor-nucleosomal DNA interactions have been defined in vitro (Carey et al., 2006; Dang and Bartholomew, 2007; Gelbart et al., 2001; Hassan et al., 2001, 2002; Li et al., 1994; Prochasson et al., 2005; Rossetti et al., 2001; Saha et al., 2002; Sengupta et al., 2001), there is little direct evidence demonstrating the binding of regulatory factors to nucleosomes in vivo. Chromatin immunoprecipitation (ChIP) assays that measure in vivo occupancy do not distinguish between nucleosomal binding and direct binding to free DNA. Understanding whether and how transcription regulatory proteins interact with nucleosomes throughout a genome should provide key insights into how they function to regulate gene expression.

In principle, there are three nonmutually exclusive ways that a protein might engage a nucleosome: (1) through interactions with histones, (2) through interactions with nucleosomal DNA, and (3) through directed collisions having little or no intrinsic affinity. Proteins that interact with histones often have signature motifs such as bromodomains that recognize specific histone modifications (Ruthenburg et al., 2007). Proteins that interact with nucleosomal DNA might recognize the rotationally exposed DNA on the nucleosome surface or adjacent linker DNA entering and exiting the nucleosome (Polach and Widom, 1995; Rossetti et al., 2001). Proteins that potentially collide with nucleosomes in a directed manner include nucleic acid polymerases and helicases.

A number of questions arise regarding the interactions of the transcription machinery and its regulators with nucleosomes in their native context in vivo, which we examine here. (1) Given the fact that nucleosomes are evicted upon transcriptional activation and that promoters reside in nucleosome-free regions, do regulatory factors simply bind nucleosome-free DNA, or do some bind to nucleosomes, perhaps during the course of activation? (2) Do regulatory factors that bind nucleosomes discriminate among nucleosome positions? That is, do factors selectively interact with nucleosomes at the −1, +1, +2, etc. positions? (3) Do factors engage single nucleosomes or arrays of nucleosomes? If so, what might be the mechanistic significance? (4) In vivo, does a factor bind to rotationally exposed DNA on the nucleosome surface, or is the cognate site rotationally buried such that nucleosome disruption is required for binding? If binding is to rotationally exposed sites, do those sites reside near nucleosome borders, as model in vitro studies suggest (Polach and Widom, 1995)? (5) Are nucleosomes repositioned during transcription?

To address these questions, we developed a genome-wide factor-nucleosome interaction assay to examine proteins that

potentially make contact with nucleosomes in vivo. We examined proteins that we surmised to belong to the three broad interaction types described above, as well as a control protein that is not expected to interact with nucleosomes at all. Our goal was to identify unique, as well as general, principles regarding the genomic location and regulation of individual factor-nucleosome interactions. Our results suggest that regulatory proteins operate at cognate nucleosome positions at the 5′ end of genes.

RESULTS

Identification of Factor-Nucleosome Interactions In Vivo

We employed an in vivo factor-nucleosome interaction assay, which is derived from the standard ChIP assay involving protein-DNA crosslinking. In this assay, the chromatin was solubilized into nucleosome core particles using high levels of MNase (Yuan et al., 2005), rather than fragmented via sonication. We also employed multiple purification steps associated with the use of TAP-tagged proteins. The resulting immunoprecipitated factor-bound mononucleosomal DNA was detected by LM-PCR as a nucleosomal-sized band (Figure 1A), ultimately mapped across the genome using massively parallel DNA sequencing (AB SOLiD), and verified with high-density tiling microarrays (Affymetrix, 5 bp probe spacing). These genome-wide methods are expected to define a subset of all nucleosome positions in the genome that are in very close proximity (a few angstroms) to the tested factor.

We detected nucleosomal crosslinks for representatives in each type of interaction (Figure 1B and quantified in Table 1, data column 1): (1) Htz1, Srm1, Vps72, and Bdf1; (2) Rap1 and Reb1; and (3) Rpo21 (RNA polymerase [Pol] II). No crosslinks were detected using an untagged (BY4741) control. No crosslinks were detected with the general transcription factor Sua7 (TFIIB), indicating that not all nuclear proteins are in close crosslinkable proximity to nucleosomes. TFIIB binds in the middle of the NFR (~100 bp from −1 and ~40 bp from +1) and thus is not expected to interact with nucleosomes (Venters and Pugh, 2009). These findings substantially increase the number of proteins demonstrated to crosslink with nucleosomes in vivo, rather than with DNA only, which the standard ChIP assay does not distinguish.

A number of addressable caveats are associated with the factor-nucleosome LM-PCR assay. First, it does not distinguish between a protein that is bound directly to a nucleosome versus a protein that is bound to the adjacent linker/NFR regions but is close enough to be crosslinked. Below, we provide a means to distinguish these possibilities for sequence-specific DNA-binding factors. Second, without demonstration that binding is actually measurable in a standard ChIP assay, a negative result is not interpretable. Moreover, any crosslinking that is detected represents a net effect of intrinsic crosslinking (i.e., ChIP efficiency) and actual nucleosomal binding.

To distinguish between ChIP efficiency and actual nucleosome binding, we measured intrinsic crosslinking by standard genome-wide ChIP-chip experiments in which the chromatin is fragmented by sonication rather than by MNase overdigestion. In this assay, all binding events (nucleosomal and nonnucleosomal) are measured. To assess intrinsic ChIP efficiency, we calculated the ratio of hybridization values at the top 1% of bound sites (after probe normalization) to the bottom 10%, which we take to represent background levels of binding. ChIP efficiency is reported in data column 2 in Table 1. Factors like Rap1, Reb1, and Sua7/TFIIB have very high intrinsic ChIP efficiencies (40- to 70-fold more than that of the control BY4741).

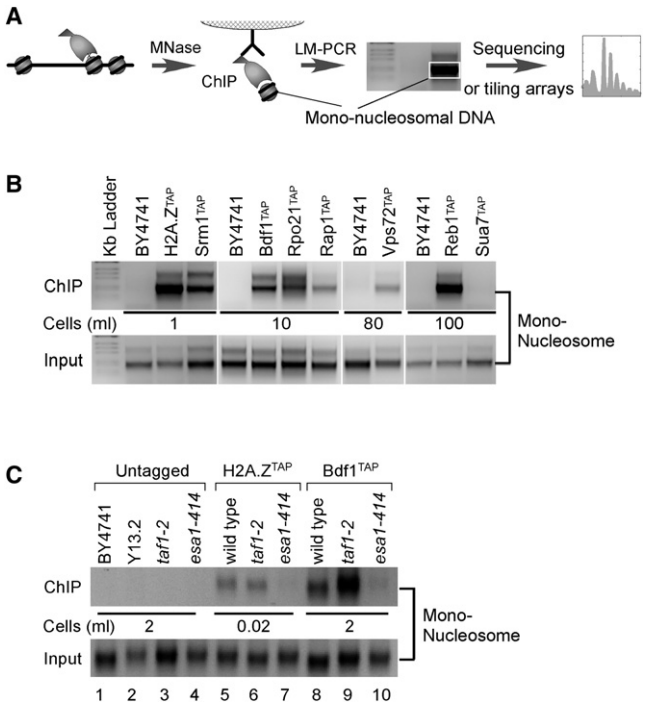

Figure 1. Identification of Factor-Nucleosome Interactions In Vivo

(A) Diagram outlining the factor-nucleosome interaction assay. Cells encoding a TAP-tagged protein are treated with formaldehyde. Chromatin is then isolated and solubilized to mononucleosomes with MNase and then subjected to TAP purification. Adaptor capture of mononucleosomal DNA produces an ~200 bp LM-PCR product, which can be subsequently mapped genome-wide.

(B) LM-PCR detection of the indicated factor-nucleosome interaction. BY4741 is a negative control that lacks the TAP tag; H2A.Z is a positive control. Input represents the equivalent of 10^{-5} ml of cell culture used in LM-PCR and is the material used for the immunoprecipitation. The volume of cell culture (at OD_{600} = 0.8) used in the TAP purification is indicated.

(C) Bdf1-nucleosome interactions are dependent on Nua4 (Esa1)-directed acetylation. LM-PCR experiments were performed on the indicated strains as described in (A) and (B).

We next calculated the nucleosome interaction ratio (data column 3 in Table 1), which equals the observed LM-PCR nucleosomal interaction signal normalized to ChIP efficiency (essentially, data column 1 divided by data column 2). As expected, the highest nucleosome interaction ratio was seen with Htz1/H2A.Z, which is a nucleosome subunit. The lowest ratio was Sua7/TFIIB, indicating that, despite its strong ChIP signal, it does not crosslink to nucleosomes. Thus, despite the nucleus being crowded with nucleosomes, not all competent gene regulatory factors will crosslink with nucleosomes. We conducted further analysis to assess the physiological and mechanistic significance of such interactions.

Table 1. Summary of Factor-Nucleosome Interactions

Protein	Complex	Complex Function	LM-PCR[a]	ChIP Efficiency[b]	Nucleosome Interaction Ratio[c]
Htz1	H2A.Z	Nucleosome subunit	934 ± 270	23	250
Srm1		Nucleotide exchange factor	151 ± 31	8	120
Rpo21	Pol II	Transcription	13.0 ± 3.2	11	7.0
Bdf1	SWR1 and TFIID	H2A.Z deposition	10.5 ± 2.5	19	3.3
Rap1		Sequence-specific DNA binding	5.2 ± 1.2	72	0.44
Vps72	SWR1	H2A.Z deposition	0.6 ± 0.4	11	0.35
Reb1		Sequence-specific DNA binding	2.2 ± 0.5	42	0.32
Sua7	TFIIB	General transcription initiation factor	0.1 ± 0.0	37	0.02
No tag	BY4741	Negative control	0.2 ± 0.1	1	

[a] Mononucleosomal LM-PCR signal normalized to input and volume of cells used. The standard error of the mean is shown for at least three replicates.
[b] ChIP efficiency ratio (CER) = T/B, in which T = hybridization probe value (after local background subtraction) at the top 1 percentile of occupancy (standard sonication-based ChIP) and B = hybridization probe value at the bottom 10 percentile of occupancy. The ratios are relative to that obtained for the BY4741-negative control. Similar results were obtained using the top 5 percentile versus bottom 20 percentile and top 10 percentile versus bottom 30 percentile.
[c] Nucleosome interaction ratio (NIR) = LM-PCR signal/CER. Values were adjusted by dividing through by the BY4741 value (0.16).

Bdf1 Interacts with NuA4-Acetylated Nucleosomes In Vivo

Bdf1 (type I interaction) is a component of SWR-C/SWR1 (Kobor et al., 2004; Krogan et al., 2003), which is responsible for incorporating H2A.Z into nucleosomes at promoters. Bdf1 binds to acetylated lysines on isolated histone H4 tails (Jacobson et al., 2000; Matangkasombut and Buratowski, 2003), and this acetylation is catalyzed by the Esa1 subunit of the NuA4 complex (Allard et al., 1999). As further validation of Bdf1-nucleosome interactions in vivo, we found that $Bdf1^{TAP}$-nucleosomal interactions were lost in a catalytically dead *esa1-414* mutant (Figure 1C, lane 8 versus 10). As expected, H2A.Z incorporation was also lost (Figure 1C, lane 7). Bdf1 also interacts with TFIID (Matangkasombut et al., 2000; Sanders et al., 2002), which is responsible for assembling the preinitiation complex. However, loss of the main TFIID subunit in a *taf1-2* strain failed to eliminate Bdf1-nucleosomal interactions (Figure 1C, lane 9). Together, the results indicate that Bdf1-nucleosomal interactions are mediated through NuA4-directed histone acetylation rather than TFIID. Thus, the factor-nucleosome interaction assay is further validated by the demonstration that the expected NuA4-dependent Bdf1-histone interactions that have been largely defined in vitro produce the expected dependencies in vivo.

Bdf1 Interacts with the +1 and +2 Nucleosomes

The genomic locations of Bdf1-crosslinked nucleosomes were determined by sequencing 1,202,352 of these nucleosomes (examples of mapped positions are shown in Figure 2A) and were verified by hybridization to high-density tiling arrays. Approximately 3% (1,853) of all 54,753 nucleosomes in the yeast genome were significantly crosslinked to Bdf1 ($p < 0.05$, Figure S1A and listed in Table S1 available online), many of which may represent low levels of binding. We selected the genes having the strongest 150 Bdf1-bound nucleosomes as a robust subset for further analysis (listed in Table S2; cutoffs of 50, 450, and 1,853 produced essentially the same results, as shown in Figure S1).

Surprisingly, at individual genes, Bdf1 bound predominantly to either the +1 or the +2 nucleosome (top versus bottom panels in Figures 2B, 2C, S1B, and S1C). This was not a consequence of misidentifying the +2 nucleosome because the hallmark of the +1 nucleosome, H2A.Z, was enriched at the +1 nucleosome in both cases (cyan-filled plot in Figure 2C). Moreover, the NFR that is adjacent to the +1 nucleosome is evident in both cases. We also found many cases in which Bdf1 bound to the −1 and −2 positions, but these turned out to also be the +1 and +2 nucleosomes of divergently transcribed genes (Figure S2B). Approximately 63% of the top 150 bound nucleosomes were found at the +1/+2 positions, compared to 15% expected by chance ($p = 10^{-59}$); 51% of all 1853 significantly bound nucleosomes were at this position ($p = 0$). Therefore, Bdf1 is selective for the +1 and +2 nucleosomes. Those not at +1/+2 positions may represent a combination of false positives, occupancy at nonprotein-encoding genes, and/or additional functionalities associated with Bdf1.

The selectivity of Bdf1 for the +1/+2 nucleosomes was not due to any intrinsically strong positioning of these nucleosomes, making them more detectable, because Bdf1-bound nucleosomes were about average for positioning strength when compared to all nucleosomes (Figure S1D). Furthermore, the distribution of Bdf1-bound nucleosomes from −1 kb to +1 kb of the TSS did not follow the canonical distribution of all nucleosomes at the same set of genes (Figure 2C), which would be expected if the interactions were simply selecting the best-phased nucleosomes.

Because Bdf1 is part of the SWR1 complex, we examined the genome-wide distribution of SWR1-nucleosomal interactions (via its Vps72 subunit). Genes having Bdf1- and Vps72-bound nucleosomes were statistically coincident ($P = 10^{-99}$) (Figure 2D). Moreover, when Bdf1 was enriched at the +1 nucleosome, SWR1 (Vps72) was as well; when it was enriched at +2, SWR1 (Vps72) was as well (Figure 2C, blue versus gold traces). This further supports the notion that the SWR1(Vps72)/Bdf1 complex together segregates between either the +1 or the +2 nucleosome, depending on the gene. Both clusters of genes tended to be transcriptionally active (red bar graph in Figure 2C), indicating that the +1/+2 Bdf1 interactions are

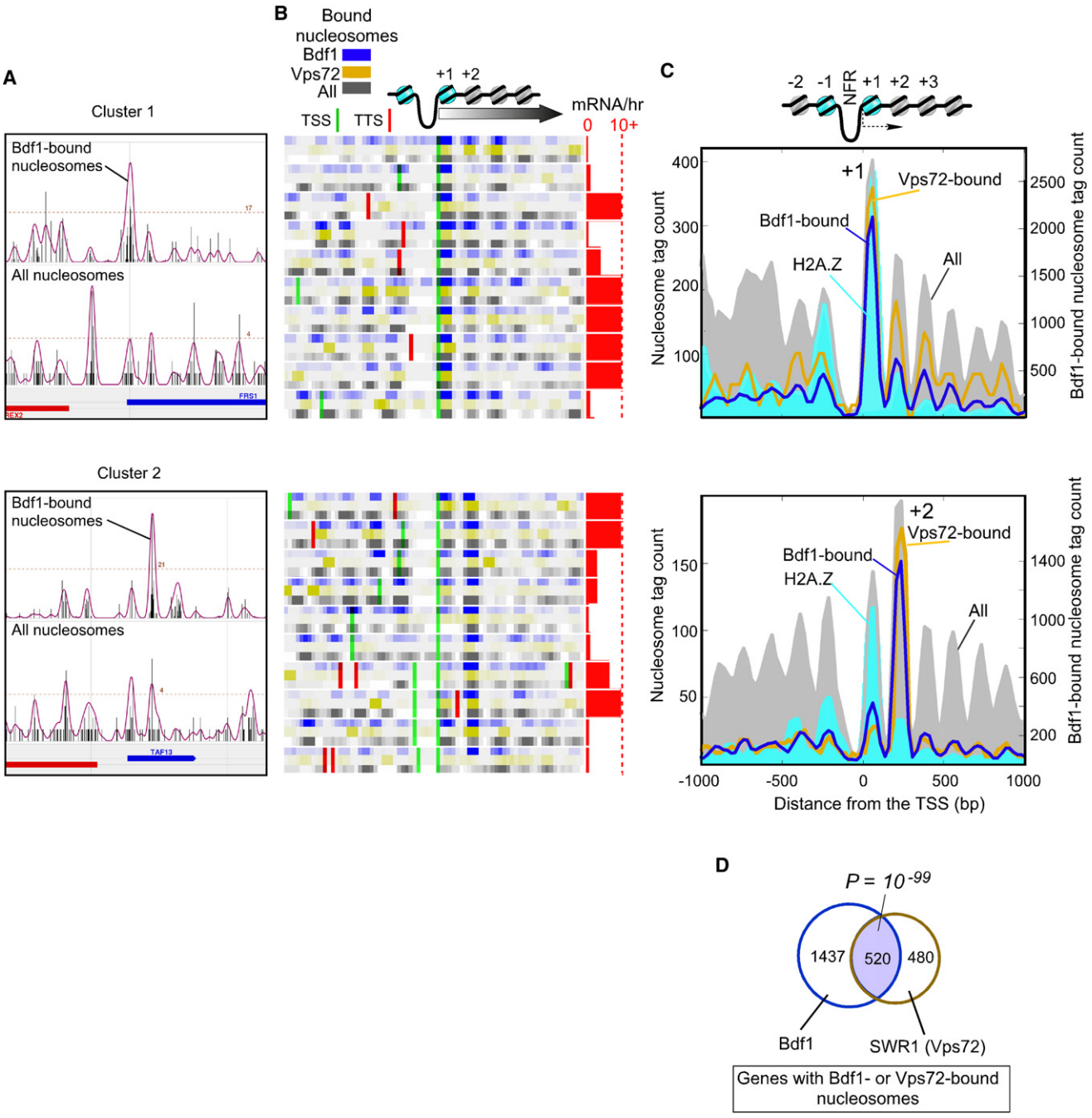

Figure 2. SWR1/Bdf1 Are Enriched at the +1 and +2 Nucleosomes

(A) Distribution of sequence tags for Bdf1-bound nucleosomes and all nucleosomes in a representative section of the genome. The top panel (cluster 1) displays the enrichment at the +1 nucleosome, and the bottom panel (cluster 2) displays the enrichment at the +2 nucleosome.

(B) Same as (A) for a collection of loci in which the tag counts have been binned (25 bp bins, smoothed via a three-bin moving average) and converted to color intensities. Each gene is represented by three tracks (Bdf1-bound nucleosomes in blue, Vps72-bound nucleosomes in gold, and all nucleosomes in gray). Genes are aligned by their transcriptional start site (TSS) (David et al., 2006). TSS (green lines) and transcript termination sites (TTS, red lines) in the region are shown. Transcription frequency (mRNA/hr) (Holstege et al., 1998) is shown as horizontal red bars, with 10 mRNA/hr or more indicated by the dashed line.

(C) A composite distribution of tags around the TSS for those genes having the top 150 Bdf1-bound nucleosomes (blue trace). Also shown for the same set of genes are tag distributions for nucleosomes bound by Vps72 (gold trace), H2A.Z (cyan fill), and H3/H4 ("All" in gray fill).

(D) Venn diagram of the overlap of genes having significant ($p < 0.05$) Bdf1- or Vps72-interacting nucleosomes.

associated with transcription. However, neither group was differentially enriched with any Gene Ontology function, which is consistent with such interactions being associated with the transcription process rather than any gene-specific control mechanism. We also examined more than 2000 genomic data sets in the public domain for differential properties between the two clusters. We found that cluster 1 tended to have higher levels of intergenic H4 acetylation (largely probing the status of the −1 and +1 nucleosomes) compared to cluster 2 (data not shown), which is consistent with cluster 2 being relatively depleted of crosslinkable +1 nucleosomes and cluster 1 having relatively high levels of acetylated +1 nucleosomes for Bdf1 binding.

Bdf1 Forms a Dinucleosome Complex Specifically with the +1 and +2 Nucleosomes

We next sought to understand the relationship between Bdf1 binding to the +1 versus +2 nucleosome by biochemically isolating native Bdf1-nucleosomal complexes (i.e., no formaldehyde and use of a less-chaotropic buffer). Surprisingly, these complexes were resistant to MNase (unlike other immunoprecipitated nucleosomal complexes), yielding predominantly dinucleosomes rather than mononucleosomes (Figure 3A). This observation suggests that a native Bdf1-containing complex simultaneously binds to two nucleosomes and protects the intervening linker DNA from MNase digestion.

To verify that the dinucleosomal complex represents interactions at the +1 and +2 positions, as opposed to minor or nonspecific complexes at other locations, the dinucleosomal DNA was mapped at high resolution to the yeast genome. The dinucleosomal DNA mapped to a region spanning the +1 and +2 nucleosomes (Figure 3B, note that occupancy between −1 and −2 is due to +1/+2 occupancy of divergent genes), which demonstrates that the Bdf1-bound dinucleosomal complex is indeed specific to the +1/+2 nucleosomes. Taken together, our findings suggest that the SWR1/Bdf1 complex binds to a NuA4-acetylated dinucleosomal complex that resides at the +1 and +2 positions of active genes (Figure 3C). The SWR1 complex then inserts H2A.Z preferentially at the −1 and +1 nucleosomes.

The strong bias of Bdf1 binding toward the +1 versus the +2 nucleosome position (or vice versa) at individual genes in the ChIP assay might be a consequence of greater intrinsic nucleosome occupancy levels at the biased position, as shown in Figure 2C (gray-filled plot). To identify a possible source of this bias, we hypothesized that, as Pol II transcription moves through this region, the +1 acetylated histones are ejected but perhaps retained locally by the SWR1/Bdf1 complex bound at +2 (Figure 3D). These histones are returned to +1, and a reciprocal process happens at +2 as Pol II moves through the +2 region.

Because Bdf1-bound nucleosomes might present a stronger barrier to Pol II movement, such a model predicts that Pol II occupancy (measured by standard sonication-based ChIP) would be enriched just before the nucleosome that SWR1/Bdf1 is bound to. In addition, the same SWR1/Bdf1-bound nucleosome might be preferentially crosslinked to Pol II due to their close proximity. Indeed, we find evidence to support these predictions at both the +1 (Figure 3E) and +2 (Figure 3F) nucleosome positions, where a local enrichment of Pol II (red trace) is found at a fixed distance just upstream of a SWR1/Bdf1-bound (blue-filled plot) and Pol II-crosslinked nucleosome (dark red trace). Additional Pol II is found in the body of the genes, as expected of their transcriptionally active state. Interestingly, in examining more than 2000 public genomic data sets for distinguishing features between cluster 1 and 2, one of the strongest distinctions was the enrichment of the Bye1 negative regulator of transcription at some cluster 1 genes (data not shown), which might indicate that the hold-up of Pol II before the +1 nucleosome might be regulated, at least in part, through Pol II at these genes.

The notion that Bdf1 might help retain nucleosomes at some promoters is in apparent conflict with the findings that nucleosomes are highly dynamic at promoter regions (Dion et al., 2007; Rufiange et al., 2007). We addressed this by comparing the dynamic state of Bdf1-bound nucleosomes to all other nucleosomes at the +1/+2 position. Strikingly, Bdf1-bound nucleosomes were as "cold" or even colder (i.e., slower exchange dynamics) than the coldest 5% of +1/+2 nucleosomes (Figure 3G). This finding lends further credence, from two independent data sets, to the idea that Bdf1 promotes retention of nucleosomes at promoters during the passage of Pol II.

Rap1 Selectively Binds to the −1 Nucleosome that Is Shared between Two Divergent Genes

As a representative of type 2 nucleosome-interacting proteins, the sequence-specific DNA-binding transcription factor Rap1 is both an activator and repressor of some of the most highly and lowly expressed genes in the cell (Kurtz and Shore, 1991; Shore, 1994). Rap1's positive role in transcription might be to direct nucleosome disruption and/or recruit TFIID to promoters (Garbett et al., 2007; Yu and Morse, 1999), whereas its negative role, paradoxically, may be to promote nucleosome formation (Gartenberg, 2000; Shore, 1994). These apparent opposing functions remain enigmatic but could be linked to the location of Rap1 and nucleosomes in promoter regions.

The genomic locations of Rap1-crosslinked nucleosomes were determined by sequencing 383,892 of these nucleosomes (Figure 4A) and were verified by hybridization to high-density tiling arrays. Approximately 0.4% (229) of all 54,753 nucleosomes in the yeast genome were significantly crosslinked to Rap1 ($p < 0.05$) (Figure S2A and listed in Table S1). Thirty percent of the previously determined Rap1-bound loci (Lieb et al., 2001) overlapped with these nucleosomes (the remainder being nucleosome-free sites). The genes associated with the top 150 Rap1-crosslinked nucleosomes were selected for further study (listed in Table S2).

Approximately 43% of the Rap1-bound nucleosomes were at the −1 position ($p < 10^{-58}$) (Figures 4B, 4C, S2B, and S2C). For the same reasons presented above for Bdf1, detection of the Rap1-crosslinked nucleosomes was not a consequence of biased selection of nucleosomes that are intrinsically the most detectable (Figures 4B, 4C, and S2B–S2D).

Rap1-nucleosome crosslinking was not a consequence of Rap1 binding to adjacent linker DNA and fortuitously crosslinking to a neighboring nucleosome because when crosslinking was omitted, Rap1-nucleosomal binding was still detected on fully digested nucleosome core particles (presumably

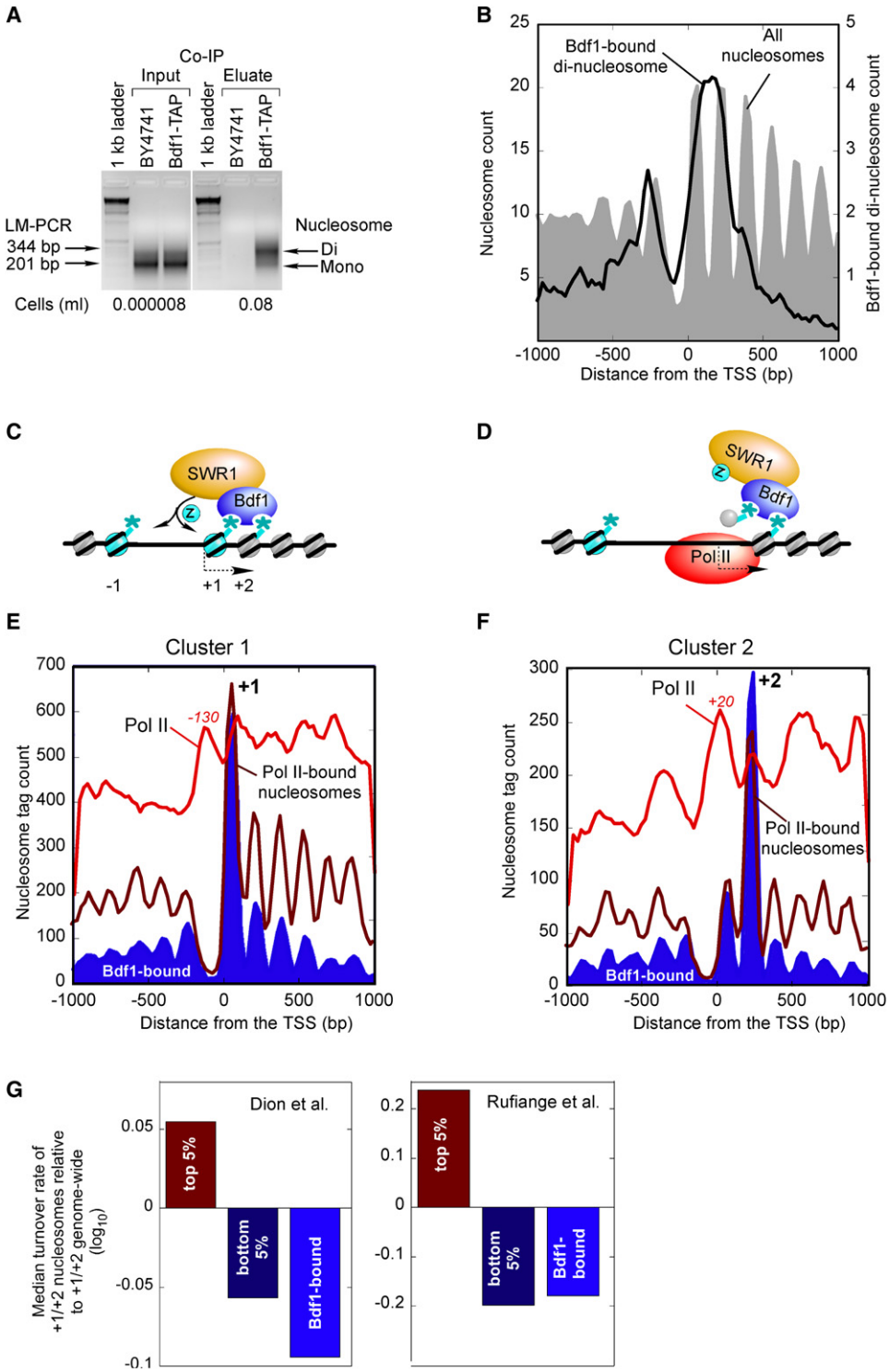

Figure 3. Bdf1 Forms a Dinucleosome Complex with the +1 and +2 Nucleosomes

(A) LM-PCR of Bdf1-bound dinucleosomes. The assay was performed as in Figure 1A except that formaldehyde crosslinking was omitted and less-chaotropic extraction buffers were used (termed "CoIP").

(B) Bdf1 dinucleosome material from (A) was analyzed by Affymetrix high-density tiling arrays. The distribution of the highest 4722 ($p < 0.05$) peaks, representing the dinucleosome midpoint, around the nearest TSS is shown.

(C) Model of how binding of Bdf1 to acetylated +1/+2 nucleosomes might promote H2A.Z incorporation via the SWR1 complex. The asterisks represent histone acetylation marks. "Z" denotes H2A.Z.

eliminating linker sites) (Figure S2E). Moreover, the MNase-resistant DNA present in Rap1-bound nucleosomes was not longer than that found in other nucleosomes (see Figure 5D), indicating that Rap1 was not protecting an additional flanking sequence as a potential consequence of adjacent binding. More importantly, 80% of Rap1-bound nucleosomal DNA possessed a Rap1-binding site within its borders, and very few had sites in adjacent linker regions (Figure 4D, black-filled plot). Rap1-bound sites (Buck and Lieb, 2006) that were not detected as Rap1-bound nucleosomes in our study were found adjacent to nearby nucleosomes (red trace). This further confirms that Rap1 in linker/NFR regions does not fortuitously crosslink to adjacent nucleosomes. Interestingly, telomeric Rap1 sites tended to be internal to nucleosomes (green trace), suggesting that nucleosomal Rap1 interactions may be different in telomeric regions compared to promoter regions.

Strikingly, 23% of Rap1-bound −1 nucleosomes were shared between two divergently transcribed genes (i.e., the same nucleosome serving the −1 role for both genes), compared to < 5% expected by chance ($p < 10^{-12}$) (Figures 4B and S2B and illustrated as the "√" configuration in Figure 4C). In contrast, for 27% of all divergently transcribed genes, the +1 nucleosome of one gene is the −1 nucleosome of the other gene (illustrated as the "X" configuration in Figure 4C). None of these genes harbored a Rap1-bound nucleosome ($p < 10^{-6}$). Thus, Rap1 may place an evolutionary constraint on the spacing between two divergent Rap1-regulated promoters, such that promoter Rap1-nucleosomal interactions are restricted to configurations in which the bound −1 nucleosome does not also serve as a +1 nucleosome for a divergently transcribed gene.

Rap1 Binds to the First and Second Rotationally Exposed Major Groove inside Either Nucleosome Border

We further examined the distribution of the 13 bp bipartite directionally oriented Rap1-binding site (ACACCCRYACAYM) on the mapped Rap1-nucleosome positions at −1. The midpoint of the Rap1 sites peaked 14 bp from either nucleosome border (Figure 4D, black-filled plot) and was independent of site orientation (data not shown). This places the bipartite Rap1 DNA-binding domain and the bipartite DNA recognition site on the first and second turn from the nucleosome border of the rotationally exposed major groove (Figure 4E), which biochemical studies have shown to be the preferred location for Rap1 binding (Rossetti et al., 2001). Together, these findings provide near base-pair resolution for the placement of Rap1-nucleosomal interactions in the yeast genome.

Reb1 Selectively Binds to the NFR-Proximal Border of the −1 Nucleosomal DNA

As a second representative of type 2 nucleosome-interacting proteins, the sequence-specific DNA-binding transcription factor Reb1 is thought to bind promoter regions and promote NFR formation (Angermayr and Bandlow, 1997; Hartley and Madhani, 2009; Raisner et al., 2005), although NFR formation may be Reb1 independent at some sites (Erkine et al., 1996; Moreira et al., 2002; Reagan and Majors, 1998). Conceivably, Reb1 might promote NFR formation, in part, by creating a boundary to which a nucleosome may not encroach. In such situations, Reb1 might reside at or near the NFR-proximal nucleosome border. Alternatively, instead of a boundary, Reb1 might position a nucleosome by engaging in specific contacts with histones at some position along the nucleosomal DNA.

The genomic locations of Reb1-crosslinked nucleosomes were determined by sequencing 7,004,145 of these nucleosomes (Figure 5A). Approximately 0.5% (281) of all detectable 54,753 nucleosomes in the yeast genome were significantly crosslinked to Reb1 ($p < 0.05$, Figure S3A and listed in Table S1). The genes associated with the top 150 Reb1-crosslinked nucleosomes were selected for further study (listed in Table S2).

Remarkably, 82% of the Reb1-bound nucleosomes were at the −1 position ($p < 10^{-257}$), and 94% of the associated genes were divergently transcribed (top panels in Figures 5B, 5C, S3B, and S3C). Thus, like Rap1, Reb1 strongly favors the −1 nucleosome of divergently transcribed genes. However, unlike Rap1, Reb1-bound nucleosomal DNAs were ~12 bp shorter than the expected length (Figure 5D), suggesting that Reb1 binding might promote MNase invasion by enhancing the breathing of DNA at the nucleosome border in accordance with the site exposure model (Polach and Widom, 1995).

When the distribution of Reb1-binding sites was examined around Reb1-bound nucleosomes at the −1 position, the Reb1 sites were found to be enriched at the border (Figure 5E) and were independent of recognition motif orientation (data not shown). Strikingly, they were particularly enriched at the NFR-proximal border. The increased nuclease accessibility of the borders of Reb1-bound nucleosomes, which could be particular to the Reb1-bound border, precluded an accurate determination of their position, so we were less certain as to the rotational setting of the Reb1-binding site. Nonetheless, the NFR-proximal location of Reb1 binding is in accord with the notion of Reb1 setting a boundary for nucleosome positioning adjacent to an NFR (Hartley and Madhani, 2009; Raisner et al., 2005). Because we do not see enrichment of Reb1 at the +1 nucleosome, some other factor may be responsible for establishing the downstream border of the NFR.

Srm1 Abundantly but Nonselectively Occupies Nucleosomes Genome-wide

Srm1 (RCC1 in human) is a guanine nucleotide exchange factor that is thought to regulate chromatin condensation and nucleocytoplasmic shuffling (Aebi et al., 1990; Hadjebi et al., 2008). Importantly, Srm1 is nuclear and binds nucleosomes (Nemergut

(D) Illustration of how the model in (C) might allow Pol II to traverse the region and maintain histone modification states.
(E and F) Distribution of Pol II (produced by standard sonication-based ChIP) (Venters and Pugh, 2009) and Pol II-bound nucleosomes around the TSS. The Bdf1-bound filled trace is from Figure 2C. (B) and (C) are for the same genes as in Figure 2C.
(G) Nucleosome exchange rate of Bdf1-bound nucleosomes. Nucleosome exchange rates were from Dion et al. (2007) and Rufiange et al. (2007). Exchange rates for the top and bottom 5 percentile at the +1/+2 nucleosomal positions, along with the median value for the top 150 Bdf1-bound genes' +1/+2 nucleosomes, were divided by the genome-wide median for +1/+2 and then $\log_{10}$ transformed and plotted.

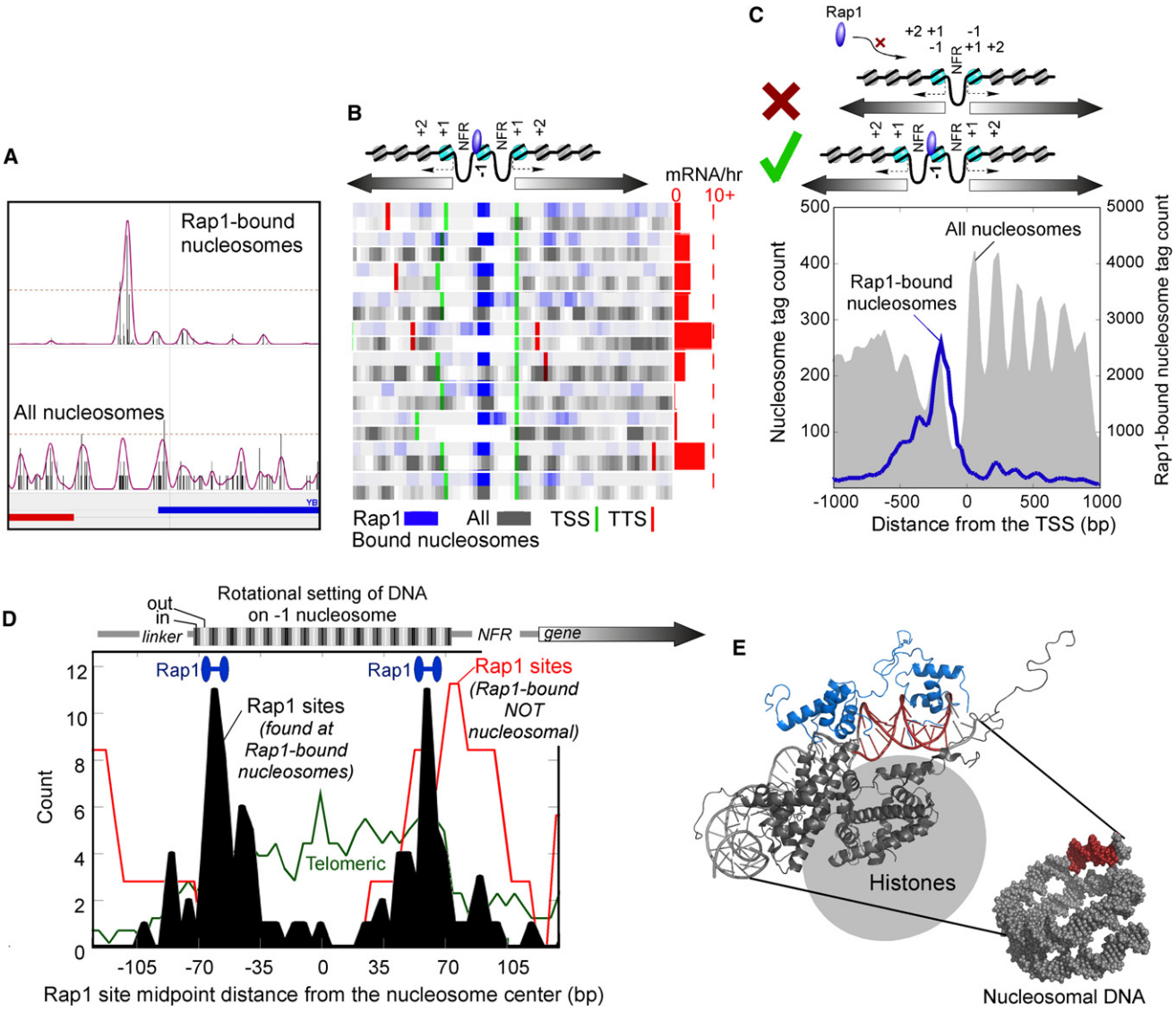

Figure 4. Rap1 Associates with a Specific Rotational Setting on the −1 Nucleosome

(A–C) Distribution of sequence tags for Rap1-bound nucleosomes. The panels show (A) individual loci, (B) a collection of genes, (C) and a composite profile of tags, as described in Figure 2. Also illustrated in (C) is a Rap1-unallowable (X) and -allowable (√) promoter nucleosome configuration.

(D) Distribution of Rap1 binding motifs on the −1 nucleosome. Plotted is the midpoint of the 13 bp motif (ACACCCRYACAYM for the top 150 Rap1-bound nucleosomes), which is composed of two separate half-sites as illustrated by the blue barbells. The rotational setting of DNA on the histone surface is indicated above the plot, where black indicates that the major groove faces inward. Motifs that existed as tandem repeats (found near telomeres) were removed and plotted separately (green trace). Also shown is the distribution of Rap1 sites that have previously been shown to be bound by Rap1 (Buck and Lieb, 2006) but were detected here as having insignificant ($p > 0.3$) Rap1-nucleosomal interactions (red trace).

(E) Model depicting the interaction of Rap1 with the nucleosome core particle in the rotational and translational setting defined in vivo. The model represents a merge of the Rap1/DNA structure (blue/red, 1IGN) with a portion of the nucleosome core particle structure (gray, 1KX5).

et al., 2001). In our in vivo factor-nucleosome interaction assay, Srm1 generated the strongest interaction ratio (Table 1).

Genome mapping of Srm1-nucleosome interactions revealed a distribution pattern around genes that was essentially indistinguishable from bulk nucleosomes (Figures 6A–6C and S4). Thus, whereas Srm1 binds abundantly to nucleosomes, it does not appear to bind specifically. This is in accord with a general role of Srm1/RCC1 in maintaining chromatin structure, particularly in light of the fact that an *srm1-1* mutant displays gross chromosomal structural abnormalities (Aebi et al., 1990).

Pol II-Bound Nucleosomes Are Delocalized

As a representative of type 3 nucleosome-interacting proteins, Pol II is not expected to stably bind to an intact nucleosome. However, due to the fact that it must translocate along DNA, Pol II might collide with nucleosomes, and this could present

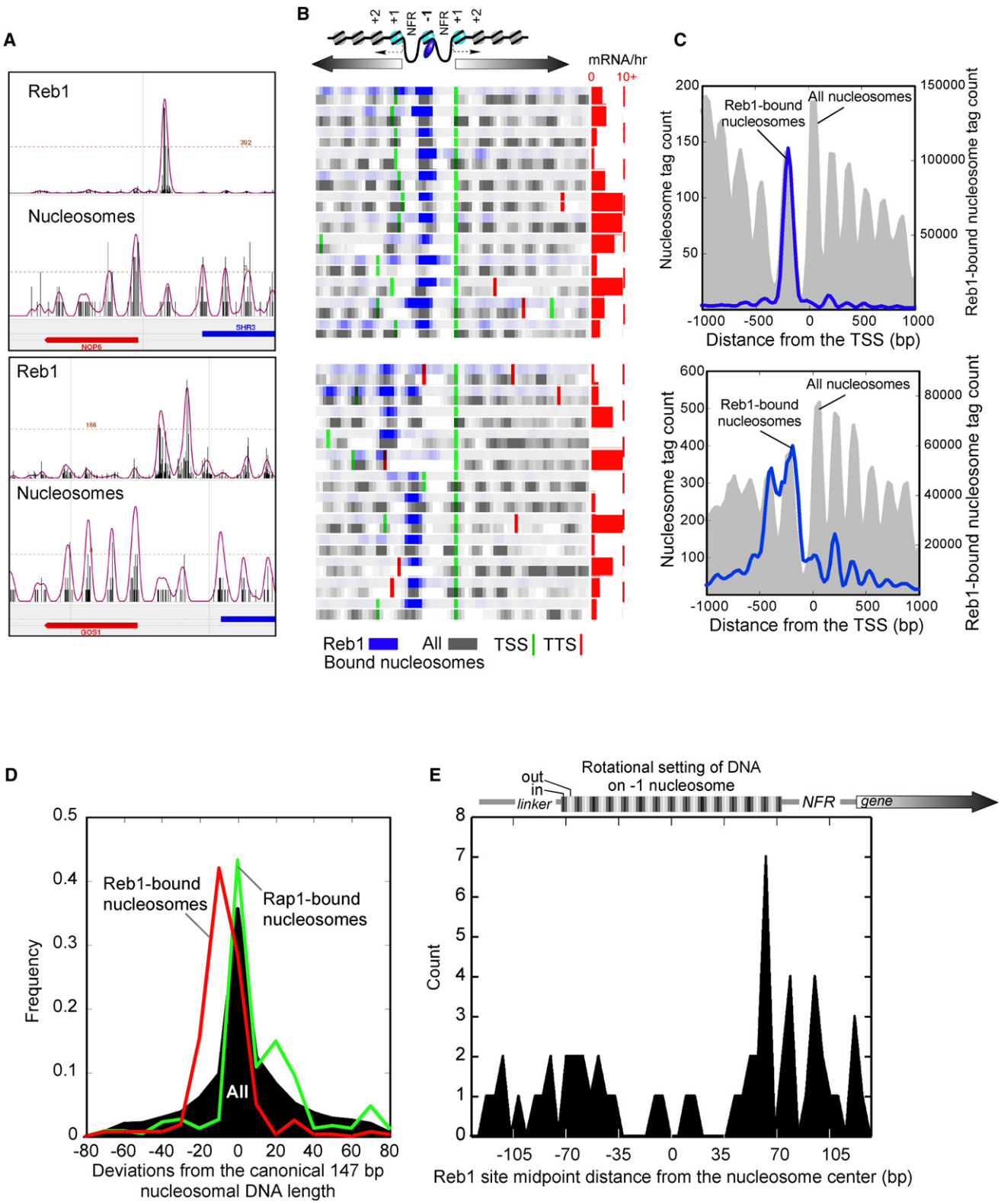

Figure 5. Reb1 Associates Specifically with the −1 Nucleosome

(A–C) Panel descriptions are as indicated in Figure 4 but for Reb1. (D) shows the frequency distribution of nucleosomal DNA lengths as deviations from the canonical 147 bp for Reb1-bound, Rap1-bound, and all nucleosomes. In (E), −1 nucleosomes shared between divergently transcribed genes were removed so as to clearly assess any asymmetry in Reb1 binding to nucleosome borders.

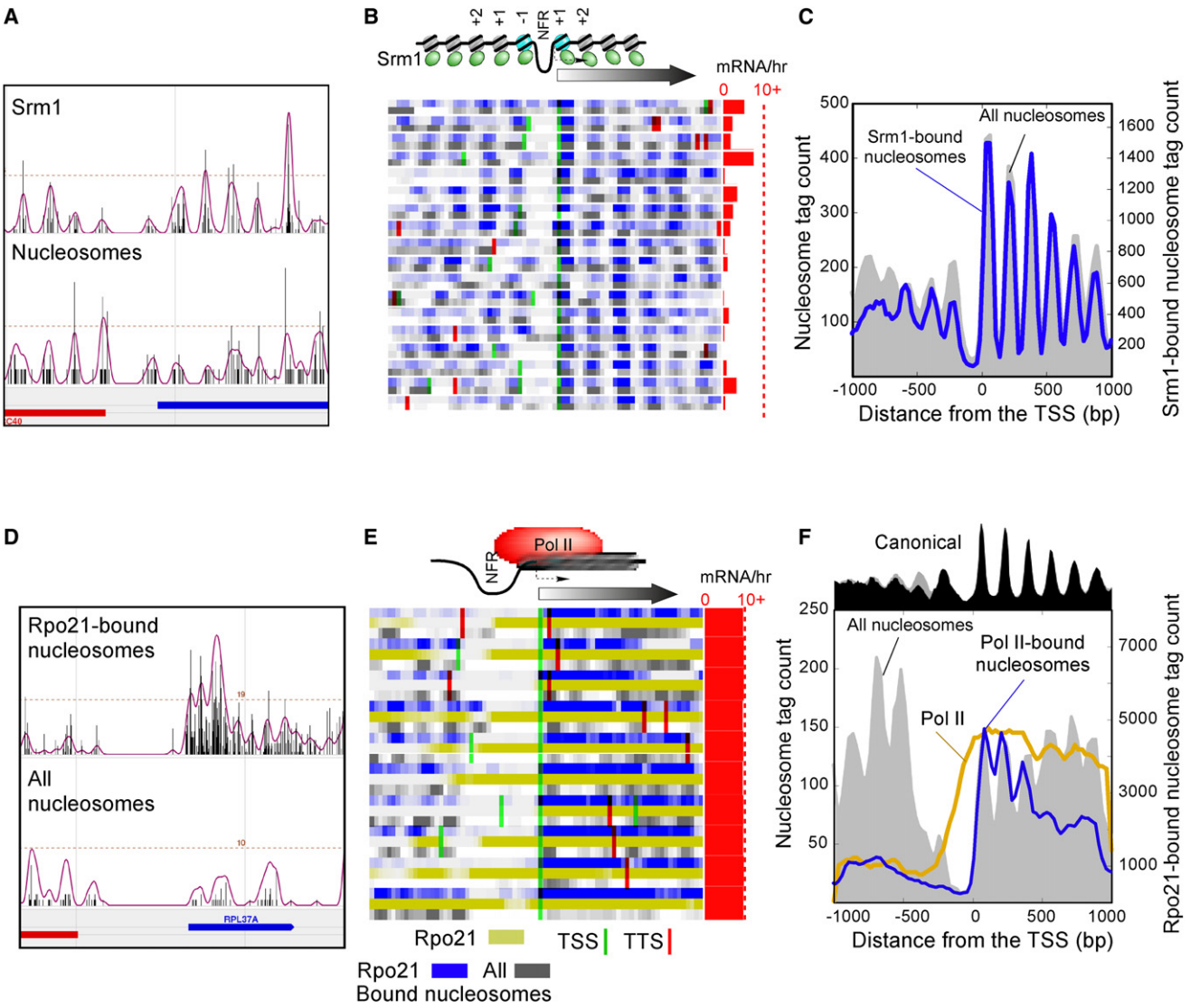

Figure 6. Srm1 Binds Nucleosomes Broadly, and Pol II-Bound Nucleosomes Are Delocalized

The panels show the distribution of sequence tags for Srm1-bound (A–C) and Pol II-bound (D–F) nucleosomes for individual loci (A and D), a collection of genes (B and E in blue), and a composite profile (C and F blue trace), as described in Figure 2. Rpo21 is the largest subunit of Pol II. The data shown in gold (E and F) represent the distribution of Pol II from standard sonication-based ChIP-chip.

a barrier to elongation (Bondarenko et al., 2006). Indeed, in *Drosophila*, Pol II initiates transcription and then pauses as it contacts the +1 nucleosome (Mavrich et al., 2008b; Muse et al., 2007; Zeitlinger et al., 2007). Continued transcription elongation requires that Pol II either eject a nucleosome barrier or traverse some remodeled state of the nucleosome.

The genomic locations of Pol II-crosslinked nucleosomes were determined by sequencing 5,097,371 of these nucleosomes (Figure 6D) and were verified by hybridization to high-density tiling arrays. Size selection after MNase digestion (Figure 1B) ensured that intact nucleosomes were being examined.

The genes associated with the top 150 peaks were analyzed further (Figure S5A and listed in Tables S1 and S2). The positions of the Pol II-crosslinked nucleosomes lacked phasing (Figures 6D, 6E, and S5B), so making consensus calls of their positions was not informative. Individual nucleosomal tags were not enriched at canonical locations, as evidenced by a lack of well-defined peaks and valleys of tags around the TSS (Figures 6F and S5C). Genes that contained relatively high levels of Pol II-crosslinked nucleosomes were generally highly transcribed (red bars in Figure 6E) and depleted of nucleosomes. We interpret these findings to suggest that, during transcription, Pol II collides with nucleosomes (detected as Pol II-crosslinked nucleosomes), and this results in their random repositioning and ultimately their eviction or partial dismantling to allow passage of Pol II (hence, nucleosome depletion). The enrichment of Pol II-nucleosomal interactions toward the 5′ end of genes might

reflect slower release or a quicker return of nucleosomes at the 5′ end of genes upon transcription.

DISCUSSION

The results presented here advance our understanding of the interplay between the transcription machinery and the highly organized chromatin structure of the yeast genome. The conservation of chromatin architecture across eukaryotes indicates that these findings are likely to be applicable to higher eukaryotes. The −1, NFR, +1, and +2 canonical positions can be thought of as providing a fixed scaffold upon which the transcription machinery assembles and where individual nucleosome positions take on specific functions. The fact that the transcription machinery and its regulators occupy nucleosomes that span from the −1 to the +2 position, a range of nearly 600 bp of DNA sequence, indicates that transcription complex assembly may encompass a much larger stretch of DNA than previously recognized.

Rap1 Binds Stereoselectively to the −1 Nucleosome

If a nucleosome resides over the core promoter, then the nucleosome must be removed prior to assembly of the transcription machinery. However, most genes have constitutively nucleosome-free core promoters (NFRs) and thus should be intrinsically accessible. Nevertheless, assembly of the transcription machinery (general transcription factors [GTFs]) at the nucleosome-free core promoter requires sequence-specific DNA-binding proteins, such as Rap1. If GTF recruitment requires other sequence-specific activators located further upstream and those binding sites are occluded by nucleosomes (specifically, the −1 nucleosome), then nucleosome disruption or displacement is necessary. In this way, the −1 nucleosome serves a regulatory function.

Rap1 appears to bind to both nucleosomal and nonnucleosomal DNA. When binding to nucleosomal DNA, it is selective for the −1 nucleosome and is enriched at divergent genes that share the same −1 nucleosomes. Rap1 regulates ribosomal protein genes. However, these genes are highly expressed and tend to lack a −1 nucleosome. Indeed, we find that genes associated with Rap1-nucleosomal interactions tended to be devoid of ribosomal protein genes when compared to the set of genes having nonnucleosomal Rap1 interactions (data not shown).

Our results provide the first demonstration on a genomic scale and in vivo that Rap1 binds to the rotationally exposed first and second major grooves of DNA inside the nucleosome border, essentially as previously determined in an in vitro reconstitution experiment (Rossetti et al., 2001). Such locations are the least curved and most "breathable" of the nucleosomal DNA (Polach and Widom, 1995; Rossetti et al., 2001) and thus may be as suitable of a binding site as a nucleosome-free site. The "locking in" of Rap1 into the first and second major grooves of the −1 nucleosome might impose phasing onto this nucleosome. Indeed, we find that, unlike the situation with Reb1, Rap1-bound nucleosomes have a substantially higher degree of phasing compared to all other −1 nucleosomes (data not shown).

Interestingly, many nucleosome-free Rap1-bound sites are located immediately adjacent to the −1 nucleosome border. Thus, translational repositioning of the −1 nucleosome over a very short distance could convert nucleosomal-bound sites to nonnucleosomal and vice versa. At some genes, Rap1 might promote nucleosome displacement or eviction, with the detected Rap1-nucleosomal interactions being a consequence of a transient interaction. Consistent with this, the −1 nucleosome is relatively depleted (when all nucleosomes are examined) at sites where Rap1-nucleosome interactions are detected.

Whereas Rap1-nucleosomal interactions appear to be confined to the first and second rotationally exposed major groove from either nucleosome border, Reb1-nucleosomal interactions appear to be more limited to the NFR-proximal border. Consequently, Reb1 appears to be in position to create a boundary for positioning of the −1 nucleosome, thereby creating the upstream NFR border, in agreement with a recent study (Hartley and Madhani, 2009). Consistent with this, genes that have Reb1-bound nucleosomes have smaller NFRs in a Reb1-depleted strain (determined from analysis of Badis et al., 2008). However, Reb1-bound nucleosomes are no more phased than other −1 nucleosomes (data not shown), indicating that phasing and boundary formation may not be entirely linked at the −1 position. Within the NFR, poly dA:dT tracks appear to contribute to nucleosome exclusion. Thus, both Rap1 and Reb1 interact predominantly with −1 nucleosomes near the nucleosome border but may do so in different ways with distinct functional outcomes. At other locations, both Rap1 and Reb1 may interact with DNA without nucleosomal interactions.

In contrast to the −1 nucleosome, we see no direct evidence for either Rap1 or Reb1 being involved in establishing the position of the +1 nucleosome. Although the Bdf1/SWR1 complex binds to the +1/+2 nucleosomes and thus could stabilize binding of these nucleosomes, they are not sequence-specific DNA binding proteins and thus cannot be directly responsible for positioning the +1/+2 nucleosomes. Instead, other mechanisms may be involved, as discussed elsewhere (Albert et al., 2007; Hartley and Madhani, 2009; Zhang et al., 2009).

Repositioning of Nucleosomes in the Wake of Pol II

While the assembly of the GTFs at the NFR does not require eviction of the −1 nucleosome, it is ultimately evicted upon subsequent recruitment of Pol II (Venters and Pugh, 2009). Consistent with this, we see negligible interactions of Pol II with the −1 nucleosome, despite an abundance of Pol II in this region. As Pol II transcribes a gene, it appears to collide with nucleosomes, displacing them from their canonical positions. Those nucleosomes do not adopt new phased positions but instead are randomly positioned. This may reflect the continuity of Pol II positions along a transcribed gene and indicates that nucleosome phasing can be disrupted by a transcribing polymerase. Importantly, any mechanism to account for the traversal of Pol II through chromatin must account for nucleosome repositioning as an intermediate stage, as opposed to simple nucleosome ejection or traversal of a fixed-position nucleosome.

How Pol II Might Traverse Nucleosomes while Maintaining Histone Modification States

Given that Bdf1 is homologous to the bromodomain region of TAF1 in higher eukaryotes, we initially expected Bdf1 to bind to

the −1 nucleosome, where it could facilitate GTF assembly in the NFR (via its interaction with TFIID). Bdf1 is also part of the SWR1 complex (Kobor et al., 2004; Krogan et al., 2003), and we envisioned that an interaction with the −1 nucleosome could also position SWR1 to load H2A.Z into the −1 and +1 nucleosomes, where it is found. We were, therefore, surprised to find that Bdf1 bound to the +1 and +2 nucleosomes and that its function seemed more linked to SWR1-directed deposition of H2A.Z than with TFIID.

Given that the −1 nucleosome is evicted upon Pol II recruitment to promoters and that the +1 and +2 nucleosomes appear to be evicted during transcription, no nucleosomal location for Bdf1 seemed suitable. However, the data presented here provide a potential explanation, in that simultaneous binding of Bdf1 to both +1 and +2 nucleosomes allows one or the other of these nucleosome pairs to be evicted by Pol II and yet retained in the local region for reassembly after Pol II has passed. Consistent with this, Bdf1-bound nucleosomes appear to have less-dynamic histone exchange than other +1/+2 nucleosomes. The observed enrichment of Pol II immediately upstream to the Bdf1-bound nucleosome and the crosslinking of Pol II to the Bdf1-bound nucleosome support the notion of stable Bdf1-bound +1/+2 nucleosomes. The broader significance of a protein complex engaged with a +1/+2 dinucleosome is that it provides a general paradigm for an epigenetic mechanism to maintain histone modification states (e.g., methylation) at specific nucleosomal positions as an RNA or DNA polymerase passes through the region.

EXPERIMENTAL PROCEDURES

Factor-Nucleosome Interaction Assay and Genome-wide Mapping of Interactions

C-terminally TAP-tagged strains were obtained from Open Biosystems and grown in 0.5 l YPD at 25°C until OD = 0.8. For Bdf1-nuclesome ChIP in temperature-sensitive mutant strains (Y13.2), yeast strains yMD26 (untagged *taf1-2*), yMD34 (untagged *esa1-414*), yMD59 (*taf1-2* Bdf1-TAP), yMD65 (*taf1-2* Htz1-TAP), yMD67 (*esa1-414* Bdf1-TAP), and yMD73 (*esa1-414* Htz1-TAP) were used (Durant and Pugh, 2007), and crosslinking was performed after a 45 min temperature shift to 37°C. All crosslinking was performed with 1% formaldehyde at 25°C for 15 min.

Cells were harvested and disrupted, and chromatin pellets were washed extensively with FA lysis buffer (50 mM HEPES [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 0.1% sodium deoxycholate), as previously described (Albert et al., 2007). Mononucleosomes were solubilized via digestion with 160 units of MNase in 600 ul of NP-S Buffer (Yuan et al., 2005) (0.5 mM Spermidine, 0.075% IGEPAL, 50 mM NaCl, 10 mM Tris-Cl [pH 7.5], 5 mM $MgCl_2$, and 1 mM $CaCl_2$) at 37°C for 30 min. Mononucleosomes crosslinked to TAP-tagged factors were immunoprecipitated with IgG sepharose, washed with FA lysis buffer, and TEV eluted. Stringent washes were used so that nucleosome isolation depended upon the use of formaldehyde and TAP tags. Details of this procedure can be found elsewhere (Albert et al., 2007). Mononucleosomes bound to TAP-tagged factors were further purified via calmodulin sepharose. Eluate DNA was subjected to ligation-mediated PCR (LM-PCR) and electrophoresed on a 2% agarose gel. The data shown are representative of at least three biological replicates. Adaptor sequences are as follows: 5′-GCGGTGACCCGGGAGATCTGAATTC-3′ and 5′-GAATTCAGATC-3′. Several technical factors that affect the yield of factor-bound nucleosomal DNA include the number of nucleosomes bound by the factor, crosslinking efficiency of the factor, and the use of multiple purification steps.

Following gel extraction of the mononucleosomal band, samples were prepared for either DNA sequencing (Bdf1, Vps72, Rap1, Reb1, Srm1, and Rpo21) using Applied Biosystems SOLiD genome sequencer or hybridization with Affymetrix 1.0 GeneChips. Two biological replicates were used in each platform and were found to be highly correlated. When samples were to be sequenced, the adaptors were replaced with SOLiD-specific adaptors.

For measuring intrinsic ChIP efficiency of factors, the location of each of the factors listed in Table 1 was measured by ChIP-Chip using customized microarrays containing 20,000 probes (two probes per promoter and one probe internal to genes), using published and unpublished data (Venters and Pugh, 2009). The two promoter probes (~12,000 in total) were used in the rank ordering of hybridization signals (after local background subtraction and normalization to the corresponding probe intensities of the null data set).

Bdf1 and Rap1 Coimmunoprecipitation of Nucleosomes

Bdf1-TAP- and Rap1-TAP-tagged cultures were grown and harvested as described above but without use of formaldehyde, and lysis was performed in NP-S Buffer (Yuan et al., 2005). Chromatin pellets were isolated and washed in NP-S buffer, and native nucleosomes were released using 15 units of MNase in a volume of 300 ul for 20 min. Solubilization of the MNase-digested chromatin was accomplished by washing the spun pellet with FA lysis buffer and combining the NP-S and FA lysis buffer supernatant after MNase digestion. Bdf1-bound native nucleosomes were isolated by conventional TAP tag isolation (IgG immunoprecipitation followed by TEV elution) and detected by LM-PCR and whole-genome tiling arrays as described above.

SOLiD Sequence Analysis

Sequence tags have been deposited at NCBI Trace Archives and were mapped to the yeast genome using software provide by the SOLiD system. Nucleosome calls were made using GeneTrack software (Albert et al., 2008) (Table S1). The tags and resulting nucleosome calls are displayed in a browsable and searchable form at the Penn State Genome Cartography website at http://atlas.bx.psu.edu/.

For the analysis conducted here, significant nucleosome calls were determined to be any call with a peak height value above the mean plus two standard deviations ($p < 0.05$). For all genome-wide analysis, the top 50, 150, and 450 nucleosome calls were analyzed (just top 50 and 150 for Rap1 and Reb1), which showed similar results in all cases; therefore, the top 150 nucleosome calls were chosen for further analysis (Table S1).

Gene cluster graphs represent the tag count per bin relative to the TSS (binned every 25 bp), which were smoothed on a three-bin moving average. K means analysis and hierarchical cluster analysis were performed on the data set for each factor. The H3/H4 nucleosome tag counts (Mavrich et al., 2008a) were generated and ordered based on the gene list for each factor. Transcription frequency data was then attached to the gene cluster data, based on the previously described transcription frequency (mRNA/hr) (Holstege et al., 1998). Treeview (Eisen et al., 1998) was used to visualize the cluster plots and to generate the cluster images.

Composite graphs were generated by binning nucleosome distances to the TSS for the genes to which the top 150 nucleosomes mapped (binned every 25 bp, smoothed every three bins) or for the genes in a particular cluster.

Statistical Analysis

P values reported in the text were calculated via chi-square tests in EXCEL assuming a Gaussian distribution of the population. The null hypothesis posits that the bound nucleosomes are distributed randomly among the 54,000 total nucleosomes.

Distribution of Rap1- and Reb1-Bound Nucleosomal Sites

The Rap1 and Reb1 consensus sequence was used to scan top 150 bound nucleosomal DNA sequence along with 100 bp upstream and downstream of the nucleosome borders using FIMO (http://meme.sdsc.edu). A p value output threshold of 1e-4 was used for the FIMO program.

Distribution of Rap1 Sites that Are Rap1-Bound but Were Not Detected as Having Significant Rap1-Nucleosomal Interactions

The consensus sequence of the Rap1-binding site was used to scan promoter regions (from −600 to +200 bp of TSS) of all yeast genes. A total of 435 sites were identified. A total of 892 Rap1-bound nucleosomes that have a peak

height greater than or equal to the mean plus the standard deviation of all peak heights were used as a filter. A total of 73 binding sites on these nucleosomes were removed from the set of 435 sites. Finally, 37 of the remaining 362 sites were detected in the 262 static target genes to which Rap1 bound throughout the time course in the previous study (Buck and Lieb, 2006). These 37 sites were defined as Rap1-bound sites that were not detected as Rap1-bound nucleosomes. Their distance from the −1 H3/H4 nucleosome (Mavrich et al., 2008a) was plotted in Figure 4D (red trace).

Affymetrix Array Analysis

Model-based analysis of tiling arrays (MAT) software was used to determine enrichment regions compared to background of the Affymetrix arrays, as well as cutoff parameters (Johnson et al., 2006). Significance values ($p < 0.05$) were used for Bdf1 (ChIP and CoIP), Rpo21, and Vps72; a more stringent value ($p < 0.005$) was used for the sequence-specific factor, Rap1. After cutoff parameters (derived from MAT interval analysis using significance threshold) were determined, nucleosome calls were made using GeneTrack software (Albert et al., 2008). Composite genome-wide nucleosome interaction distributions relative to the TSS were generated as described elsewhere (Mavrich et al., 2008a).

ACCESSION NUMBERS

The ArrayExpress accession number for the microarray data is E-MEXP-1847, and the NCBI accession number for the sequencing data is SRA008256.1.

SUPPLEMENTAL DATA

Supplemental data include three tables and five figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00639-X.

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