Reconstitution of Yeast Silent Chromatin: Multiple Contact Sites and O-AADPR Binding Load SIR Complexes onto Nucleosomes In Vitro

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SUMMARY

At yeast telomeres and silent mating-type loci, chromatin assumes a higher-order structure that represses transcription by means of the histone deacetylase Sir2 and structural proteins Sir3 and Sir4. Here, we present a fully reconstituted system to analyze SIR holocomplex binding to nucleosomal arrays. Purified Sir2-3-4 heterotrimers bind chromatin, cooperatively yielding a stable complex of homogeneous molecular weight. Remarkably, Sir2-3-4 also binds naked DNA, reflecting the strong, albeit nonspecific, DNA-binding activity of Sir4. The binding of Sir3 to nucleosomes is sensitive to histone H4 N-terminal tail removal, while that of Sir2-4 is not. Dot1-mediated methylation of histone H3K79 reduces the binding of both Sir3 and Sir2-3-4. Additionally, a byproduct of Sir2-mediated NAD hydrolysis, O-acetyl-ADP-ribose, increases the efficiency with which Sir3 and Sir2-3-4 bind nucleosomes. Thus, in small cumulative steps, each Sir protein, unmodified histone domains, and contacts with DNA contribute to the stability of the silent chromatin complex.

INTRODUCTION

Eukaryotic chromosomal DNA is organized into a nucleosomal fiber that can be compacted into higher-order structures by the binding of linker histones and/or nonhistone proteins (Woodcock, 2006). Special regions of eukaryotic chromosomes, notably centromeres and telomeres, assume a folded heterochromatic state that limits enzyme accessibility and silences genes located in these regions (reviewed in Grewal and Jia, 2007). Budding yeast have three heterochromatin-like regions containing heritable silent chromatin: subtelomeric DNA; the silent homothallic mating-type loci, HML and HMR; and regions within the rDNA (reviewed by Rusche et al., 2003). Transcriptional silencing in yeast is mediated by the Silent Information Regulatory proteins 1–4, and deletion of SIR2, SIR3, or SIR4 abolishes both HM repression (Rine and Herskowitz, 1987) and telomeric position effect (TPE) (Aparicio et al., 1991). The Sir factors are structurally unrelated to each other (reviewed in Gasser and Cockell, 2001). Their recruitment to HM loci requires the Origin Recognition Complex (ORC) and sequence-specific transcription factors Abf1 and Rap1, which bind flanking silencer elements. Sir1 bridges between ORC and Sir4 (Triolo and Sternglanz, 1996), while Sir4 recruits Sir2, an NAD-dependent deacetylase, and Sir3 (Hoppe et al., 2002; Luo et al., 2002; Rusche et al., 2002). At telomeres, Sir4 is recruited by Rap1 (Cockell et al., 1995) and Ku70/Ku80, a heterodimer that binds the single strand-double strand DNA junction (reviewed by Fisher and Zakian, 2005). Once nucleated, Sir2, Sir3, and Sir4 appear to spread along nucleosomes to silence promoters up to 3 kb from the telomeric repeat (Strahl-Bolsinger et al., 1997). Whereas Sir3 and Sir4 are assumed to be structural components of silent chromatin, Sir2’s histone deacetylase activity is essential for repression (Tanny et al., 1999).

The tails of H3 and H4 are primary targets for deacetylation by Sir2 (Imai et al., 2000), which presumably generates high-affinity binding sites for Sir3 and Sir4 (Hecht et al., 1995). Deletion analysis of the N-terminal tail of H4 identified a basic stretch from lysine (K) 16 to K20 that is critical for repression (Kayne et al., 1998). Any basic-to-neutral amino acid change in this region, or any substitution at K16, disrupts both HM and telomeric repression (Johnson et al., 1990; Aparicio et al., 1991). Surprisingly, however, Sir3 and Sir4 still bind recombinant H4 tails bearing the derepressing H4K16Q mutation (Hecht et al., 1995). This raised the possibility that H4K16 does more than attract Sir proteins. In higher eukaryotes, unmodified H4K16 appears to mediate interactions between neighboring nucleosomes (Dorigo et al., 2003). Alternatively, given that the conservative H4K16R substitution also impairs silencing (Johnson et al., 1990), active deacetylation of H4K16 by Sir2 may be a necessary step in assembling silent chromatin. Indeed, O-acetyl-ADP-ribose (O-AADPR), a product of Sir2-mediated NAD hydrolysis, has been proposed, but not proven, to contribute to silent chromatin assembly (Jou et al., 2005).
Not only histone tail mutations, but changes near and around histone H3K79, disrupt Sir-mediated repression in vivo (Ng et al., 2002; Park et al., 2002; Thompson et al., 2003; Xu et al., 2005). Moreover, two single amino acid changes within the conserved N-terminal BAH domain of Sir3 (Connelly et al., 2006) were able to suppress derepressive mutations at H4K16 and H3K79 (Johnson et al., 1990; Thompson et al., 2003). Consistently, the interaction of the Sir3-BAH domain with histones required unmodified H4K16 and H3K79 residues (Onishi et al., 2007). However, other reports argued that the Sir3 C terminus contacts the H4 tail (Hecht et al., 1995; Carmen et al., 2002), and Sir3C, like full-length Sir3, can bind unmethylated histone H3K79-containing peptides (Altaf et al., 2007). Although these data revealed several contacts between Sir3 and histones, it remained unclear which were relevant in the context of Sir holocomplex-nucleosome interaction.

The genetic interdependence of Sir genes and extensive chromatin immunoprecipitation (ChIP) studies argue that Sir2, Sir3, and Sir4 interact and spread along chromatin as a complex (reviewed in Gasser and Cockell, 2001; Rusche et al., 2003). However, early attempts to isolate intact Sir complexes from yeast met with little success, as Sir2-4 complexes isolated from yeast did not contain Sir3 (Ghidelli et al., 2001; Hoppe et al., 2002). Recently, Baculoviral coexpression allowed for purification of a stable Sir holocomplex of 365 kDa that contains Sir2, Sir3, and Sir4 in a 1:1:1 molar ratio (Cubizolles et al., 2006). Nonetheless, most biochemical analyses performed to date used recombinant subdomains or Sir3 alone.

Here, we have carried out a comprehensive biochemical analysis of the binding of the Sir2-3-4 complex (or Sir holocomplex) and subcomponents to recombinant oligonucleosomes. We find that Sir2-3-4 binding to unmodified chromatin is only weakly sensitive to the removal of histone tails, probably due to Sir4’s high, albeit nonspecific, affinity for DNA. Sir3 alone has a lower affinity than the holocomplex for the nucleosomal template, yet shows selectivity for H3 and H4 tails. By methylation of H3K79 with Dot1 methyltransferase, we find that the presence of one K79me per nucleosome is sufficient to reduce the binding of either Sir3 or Sir2-3-4. Moreover, O-AADPR is shown to increase the affinity of Sir3 or Sir holocomplexes for chromatin. Finally, using sedimentation analysis, we determined a stoichiometry of one Sir holocomplex per nucleosomal linker in purified Sir-chromatin complexes.

RESULTS

Purification of Sir Proteins and Their Subcomplexes

To investigate the molecular basis of repressed yeast chromatin, we developed a system to reconstitute silent chromatin in vitro from purified components. Sir2, Sir3, and Sir4 were expressed singly, pair-wise, or as a Sir2-3-4 holocomplex by using the Baculovirus expression system, whereas Xenopus histones, histone variants, and the DNA template for nucleosome assembly were produced in bacteria. The epitope-tagged Sir constructs complement corresponding null alleles when expressed from endogenous promoters in yeast (Cubizolles et al., 2006). We confirmed that Baculoviral-expressed Sir3 carries an N-terminal acetylation (R. Sack, personal communication) and that Sir2 is catalytically active (Cubizolles et al., 2006), both prerequisites for silencing in vivo.

The expression and purification of Sir3 resulted in a stable monomer of >90% purity (Figure 1B; Figure S1A, available online, for comparison with Sir2-3-4). Under physiological salt conditions and low protein concentration (10−7 M), we do not detect multimers of Sir3, consistent with a report from the Hansen laboratory (McBryant et al., 2006). In contrast, Sir4 expressed alone sedimented in sucrose gradients as a nonglobal multimer, consistent with two-hybrid studies (Chien et al., 1991;
Figure S1C). Irregularly sized multimers were also detected for coexpressed Sir3 and Sir4 (Figure S1D). However, when Sir4 was expressed together with Sir2, or with both Sir2 and Sir3, its stability increased and the complexes formed a homogeneous, compact structure (Figures S1A and S1B). Sizing analysis indicates that Sir2-4 is a 250 kDa heterodimer with 1:1 stoichiometry (Figure S1B). The specific activity of Sir2-4 for NAD-dependent histone tail deacetylation was 5-fold higher than that of Sir2-3-4 and was 10-fold higher than purified Sir2 alone (Cubizolles et al., 2006).

Reconstitution of Nucleosomes and the Binding of the Sir Complex
Chromatin was reconstituted by loading histone octamers assembled from recombinant X. laevis histones (Luger et al., 1997) on tandem arrays of the Widom 601 nucleosome positioning DNA sequence as previously described (Huynh et al., 2005; Lowary and Widom, 1998). In our studies, we exploit a nucleosome trimer that is amenable to analysis by native agarose gel electrophoresis and represents the smallest array of the SIR Complex (Cubizolles et al., 2006).

The sequence identity between Xenopus and budding yeast histones is high (88% for H3; 92% for H4), and the overall structure of the nucleosome core particle from S. cerevisiae is very similar to that of X. laevis (White et al., 2001). However, yeast octamers have fewer stabilizing interactions between the two H2A–H2B dimers and thus yield less stable assemblies. We therefore exploited Xenopus histones to create a stable and uniform binding substrate and, where indicated, introduced yeast H4 or Htz1 to create hybrid octamers (Figure 1D).

The saturation point for nucleosome assembly was determined for each preparation of chromatin substrate by titrating the 601 DNA array and an equimolar concentration of 147 bp competitor DNA with increasing concentrations of histone octamers (Figure 1E). On the 601-167 trimer, nucleosomal monomers, dimers, and trimers form sequentially (Figure 1E, lanes 2–5). The presence of competitor DNA contributes to chromatin solubility by sequestering excess histones while promoting full occupancy of the 601 template (Huynh et al., 2005). The homogeneous nucleosome trimer was then used for Sir binding (Figure 1E, lane 7).

To analyze the association of Sir protein with chromatin, increasing concentrations of Sir2-3-4 complex were titrated into a fixed amount of 32P-labeled chromatin template at ~10⁻⁸ M. The resulting complexes were analyzed by native agarose gel electrophoresis. In lane 1 of Figure 2A, one sees, from top to bottom, the 601 nucleosomal trimer, mononucleosomes formed on the competitor, and naked competitor DNA (“147” in figure). At a molar ratio of 0.5 Sir2-3-4 complex per nucleosome, the trimer shifted completely to a broad, slow-migrating band (Figure 2A, lane 4). Increasing Sir protein concentration caused a further retardation of the complex, suggesting that higher molecular weight complexes also form (discussed below).

From the native gel in Figure 2A, it appeared that Sir2-3-4 shifted naked DNA even more efficiently than the nucleosomal trimer. However, quantitation in the linear range of exposure (Figure 2A) and competition with naked DNA (Figure S2A) indicated that the binding affinities of Sir2-3-4 for naked DNA versus the nucleosomal trimer are very similar, with a slight preference for nucleosomes under some conditions (Figure S2A). Consistently, variation in linker length by 10 bp did not significantly alter the affinity of Sir2-3-4 for nucleosomes (data not shown).

Importantly, a 2-fold increase in the molar ratio of Sir complex to nucleosomes (from 0.25 to 0.5) induced a reproducible shift from weak to strong binding, suggesting that the interaction of Sir2-3-4 complexes with chromatin at 10⁻⁸ M is cooperative. Cooperative binding was also observed when the Sir holocomplex was incubated with a hexamer of 601 nucleosomes, in both the absence and presence of DNA competitor (Figure 2D; data not shown). On the other hand, when nucleosomal trimers were at 10⁻⁷ M, complex formation increased linearly with increasing Sir concentration (Figure S2B). This suggests that the SIR-nucleosome interaction has a K_D around 10⁻⁸ M. In all subsequent experiments, we kept the chromatin concentration at 10⁻⁸ M, to allow for the detection of small differences in binding affinities. These in vitro data demonstrate cooperativity in Sir holocomplex loading onto unmodified chromatin. Besides its high affinity for nucleosomal trimers, the Sir holocomplex showed lower-affinity binding to mononucleosomes, and a surprisingly robust interaction with free DNA (Figure 2A).

The Sir2-3-4 Complex Has Poor Specificity for the H4 Tail
The histone H4 N-terminal tail has been shown to play a major role in silencing in vivo. We therefore examined the effect of mutations within the H4 tail on the Sir holocomplex-chromatin interaction. We first incorporated into the chromatin template H4 bearing the K16Q mutation, and we then tested this mutation alone and in combination with the yeast histone variant Htz1. Htz1 seems to prevent SIR complex spreading (Raisner et al., 2005), although there is no evidence that it directly interferes with the Sir-nucleosome interaction. Indeed, neither substitution impaired the loading of the Sir complex onto the nucleosomal template (Figure S2C). This is consistent with in vitro binding data showing that Sir3 and Sir4 can still efficiently bind an H4 N-terminal peptide containing K16Q (Hecht et al., 1995).

Since targeted mutation of H4K16 had no effect, we created nucleosomes lacking the entire histone H4 tail (gH4; Δ1–20 amino acids [aa]; Figure 1D). The binding affinity of Sir complexes to these gH4-containing nucleosomes was mildly, but reproducibly, decreased (compare titration point 0.5:1, Figure 2A). This reduction in binding was not significantly affected by additionally removing the H3 N-terminal tail (gH3; Δ1–19 aa; data not shown), allowing us to conclude that the H4 N-terminal tail contributes to Sir2-3-4 complex binding.

Sir3 Confers H4 Tail Specificity
Given that both Sir3 and Sir4 can bind the H3 and H4 tails (Hecht et al., 1995), we examined the contributions of individual Sir proteins to histone tail specificity. We compared the loading of Sir3 alone and of the Sir2-4 subcomplex onto nucleosomes with histone tails or without. When Sir3 alone was titrated into
wild-type chromatin, it bound with lower affinity than the Sir2-3-4 complex, requiring a 5- to 10-fold molar excess over nucleosomes to shift the array completely (Figure 2B). Moreover, the Sir3-chromatin complex was less stable, splitting into multiple bands during electrophoresis. Sir3 nonetheless bound preferentially to chromatin with intact histone tails (Figure 2B, lanes 3 and 9). A similar preference was also detected on the H4 N-terminal tail contributes to the Sir3-nucleosomal interaction.

To test whether Sir4 also contributes to selective binding, we titrated the stable Sir2-4 complex into wild-type and gH4 chromatin (Figure 2C). Sir2-4 bound chromatin with slightly lower affinity than Sir2-3-4 yet showed no preference, and possibly a slight aversion, for chromatin containing H4 tails (Figure 2C, see quantitation). Instead, Sir2-4 showed a pronounced nonspecific interaction with naked DNA, shifting the competitor DNA efficiently (Figure 2C). This is examined in detail below. From our comparative analysis of Sir3, Sir2-4, and Sir2-3-4 binding, we conclude that Sir3 confers a modest preference for histone tail-containing chromatin, whereas Sir2-4 contributes to the affinity of the holocomplex in another way.

### SIR Binding Confers Protection from Exogenous Nuclease Digestion

The association of SIR complexes with chromatin is thought to reduce the accessibility of linker DNA to enzymes, such as micrococcal nuclease (MNsase) (reviewed by Rusche et al., 2003). We tested this hypothesis in vitro by using a hexamer of the Widom 601-167 nucleosome array reconstituted with yeast H4 and Xenopus H3, H2A, and H2B. In this experiment, Sir2-3-4 or Sir2-4 complexes were loaded onto purified nucleosomal arrays (Figure 2D), which were then challenged by MNase digestion. We saw a significant protection of linker DNA from MNase cleavage when Sir proteins were bound (Figure 2D), similar to the protection observed in vivo (Weiss and Simpson, 1998). Indeed, this assay shows that our SIR-nucleosomal complex recapitulates an important feature of silent chromatin. However,
whereas MNase mapping in vivo indicates closely spaced dinucleosomes at HM loci (Weiss and Simpson, 1998), the spacing of nucleosomes on the Sir-bound 601-167 template remained uniform (Figure 2D).

Methylation at H3K79 Reduces the Binding of Sir2-3-4 to Chromatin

Recent studies suggest that Sir3 domains contact not only the H4 tail, but also the nucleosomal core, in a manner sensitive to methylation of H3K79, which sits on the face of the nucleosomal disk (van Leeuwen et al., 2002). The presence of mono-, di-, or trimethylated H3K79 is correlated with the impaired spreading of SIR-mediated repression in living cells (Frederiks et al., 2008). It was proposed that H3K79me antagonizes Sir2-3-4 binding in euchromatic regions, although this had never been tested directly. To test this, we first incubated reconstituted chromatin with recombinant Dot1 enzyme, and we monitored the efficiency of H3K79 methylation by western blots by using H3K79me-specific antibodies. The degree of H3K79 methylation was determined by comparison with native yeast nucleosomes, whose methylation status was determined by quantitative mass spectrometry (Frederiks et al., 2008). We routinely generate recombinant substrate bearing at least one mono- or dimethyl H3K79 per nucleosome (50% efficiency). Tri-methylation was only rarely detected, possibly because our recombinant substrate lacks H4K16ac, which is known to stimulate Dot1 activity (Altaf et al., 2007).

Increasing amounts of Sir2-3-4 were then bound either to methylated template or to chromatin that was incubated with Dot1 in the absence of the methyl donor SAM. Twice as much Sir2-3-4 complex was needed to shift H3K79-methylated trimers as compared to unmethylated substrate (Figure 3C). This effect appears to be mediated at least in part by Sir3, as it also showed a reduced affinity for Dot1-modified chromatin (Figure 3D). Neither Dot1 enzyme nor SAM alone caused a chromatin shift at the concentrations used (0.1 molecule of Dot1 per nucleosome; Figures 3C and S3A). Nor did either reagent independently influence the binding of Sir2-3-4 or Sir3 to chromatin.
Thus, we conclude that the methylation of H3K79 impairs binding of Sir3 and of Sir2-3-4 directly, implying that Sir3 probably contacts the face of the nucleosome even when part of a SIR holocomplex.

O-AADPR Increases the Affinity of Sir2-3-4 for Binding Chromatin

In a previous study with reconstituted SIR complexes, the amount of Sir3 bound to Sir2-4 increased when the incubation was carried out in the presence of tetra-acetylated histone H4 tails and NAD (Liou et al., 2005). These authors argued that Sir2-catalyzed deacetylation might alter SIR holocomplex conformation, possibly through the generation of O-AADPR (Figure 4A). Indeed, EM micrographs indicated a conformational change in SIR particle structure upon the addition of O-AADPR (Liou et al., 2005).

To test whether this product of NAD hydrolysis has an impact on the association of the Sir2-3-4 complex with nucleosomes, we purified O-AADPR as well as its unacetylated form, ADPR, by HPLC (Figure S4). We added O-AADPR at 50 µM to the Sir2-3-4 complex for 10 min and then titrated it into recombinant chromatin template. After binding, the samples were fixed briefly with glutaraldehyde prior to electrophoresis to stabilize the effect of O-AADPR binding. Even without O-AADPR, the fixation step stabilized the complex formed by SIR holocomplex and nucleosomes (Figure 4B), while slightly increasing the material that failed to enter the gel. On top of this, incubation with O-AADPR had a pronounced effect on the affinity of the SIR holocomplex for chromatin. Roughly 4-fold less Sir2-3-4 was sufficient to fully shift chromatin into the slower-migrating band (Figure 4B). This effect was not observed after incubation of Sir2-3-4 complex with ADPR (Figure S3C), nor was it provoked by the addition of ATP, γATP-S, or NAD in the absence of acetylated substrate (data not shown).

We and others have speculated that the AAA⁺ motif found in the C-terminal domain of Sir3 might bind O-AADPR (Gasser and Cockell, 2001; Liou et al., 2005). To test if Sir3 mediates its effects on the holocomplex, we preincubated Sir3 alone with O-AADPR prior to the chromatin binding assay. The significant and highly reproducible increase in the affinity of O-AADPR-treated Sir3 for chromatin was comparable to the change observed for the SIR holocomplex (Figure 4C). Quantitation of
three separate experiments confirmed a consistent increase in the affinity with which both Sir3 and the holocomplex bound unmodified chromatin.

Sir4 Binds dsDNA and Mononucleosomes, whereas Sir2 Binds Neither

We observed a high affinity of Sir2-3-4 and Sir2-4 complexes for naked competitor DNA (Figures 2A and 2C). We next asked whether this is attributable to Sir4 or Sir2, by testing their DNA-binding activities. As a DNA substrate, we used the well-characterized 200 bp sea urchin 5S rDNA fragment (Simpson and Staf ford, 1983). At 5- to 10-fold molar excess, Sir4 bound both naked end-labeled DNA and the 5S mononucleosome, whereas Sir2 bound neither appreciably (Figure 5A). Sir4 bound all dsDNAs tested, and it was able to shift a 270 bp stretch of telomeric end-labeled DNA and the 5S mononucleosome, whereas Sir2 bound neither appreciably (Figure 5A). Sir4 bound all dsDNAs tested, and it was able to shift a 270 bp stretch of telomeric end-labeled DNA and the 5S mononucleosome, whereas Sir2 bound neither appreciably (Figure 5A). Sir4 bound all dsDNAs tested, and it was able to shift a 270 bp stretch of telomeric end-labeled DNA and the 5S mononucleosome, whereas Sir2 bound neither appreciably (Figure 5A).

We asked whether Sir4N or the coiled-coil domain of Sir4 (Sir4cc, aa 1271–1358) disrupts silencing by binding DNA or chromatin. Titrations show that the recombinant Sir4N domain bound naked DNA almost as efficiently as full-length Sir4 (Figure 6B, cf. Figure 5A), although its affinity for chromatin was ~30-fold lower than Sir2-3-4 (Figure 6C). Sir4cc, in contrast, bound neither DNA nor nucleosomes, even at high molar excess (Figures SSA and SSB).

To recapitulate the effect of Sir4N in vitro, we examined whether increasing amounts of the DNA-binding Sir4N domain would interfere in the association of SIR holocomplex with chromatin. Increasing amounts of Sir4N were added to a saturated assembly of SIR holocomplex on nucleosomes (Figure 6C); at 4-fold molar excess, the presence of Sir4N generated free nucleosomal trimer (Figure 6C, lane 7), presumably due to a partial disruption of the SIR-nucleosomal assembly. At higher concentrations, Sir4N itself bound to nucleosomes, apparently replacing Sir2-3-4, which we could monitor by supershifting the Sir2-3-4-chromatin complex with purified antibodies against Sir2, thereby separating it from the Sir4N-chromatin complex (Figure S5C).

Titrations of Sir4cc did not have the same effect on the chromatin association of the SIR holocomplex. As shown in Figure 6D, despite 16- or 32-fold molar excess of Sir4cc, the Sir2-3-4 complex remained nucleosome bound. However, quantitation of the disappearance of free nucleosomal trimer indicated that SIR-holocomplexes bound less cooperatively when challenged by Sir4cc (Figure 6E). This argues that exogenously added Sir4cc may block the protein-protein interactions that promote cooperative loading onto nucleosomes. In contrast, the affinity of Sir4N for DNA seems to directly stabilize SIR-chromatin interactions.

The Sir4 N Terminus Binds Naked DNA and Competes for Sir2-3-4-Nucleosome Binding

We next examined the domain of Sir4 that might be implicated in DNA binding and asked whether overexpression of the domain disrupts silencing in vivo. Previous studies showed that overexpression of either full-length Sir4 or a coiled-coil C-terminal domain derepressed TPE and HM silencing (Cockell et al., 1995; Maill et et al., 1996; Marshall et al., 1987). Intriguingly, TPE and Sir protein foci could be restored by co-overexpression of Sir3 with Sir4C (aa 731–1358) or full-length Sir4, suggesting that the interaction mapped between Sir3 and the Sir4cc is of crucial importance for repression (Chang et al., 2003; Gotta et al., 1998; Murphy et al., 2003). Here, overexpression of the N-terminal 271 aa of Sir4 (Sir4N) is also shown to impair TPE (Figure 6A). This effect could not be suppressed by balanced expression with Sir3 (data not shown), arguing that Sir4N, unlike Sir4cc (Murphy et al., 2003), does not act by binding Sir3.

Sir2-3-4 Complex Binds with a Stoichiometry of Two Complexes per Three Nucleosomes

Titrations of the Sir2-3-4 complex showed that all free chromatin substrate was reproducibly bound by SIR holocomplex when Sir2-3-4 was added at a molar ratio of ~1 per 2 nucleosomes.
At higher concentrations of Sir2-3-4, even larger-molecular weight complexes form. The potential association of free DNA and mononucleosomes with the complex rendered estimation of the SIR:nucleosomal ratio difficult. However, we found that a brief glutaraldehyde fixation after assembly released free DNA from the SIR-nucleosomal particle without altering the latter (Figure 7A, arrowheads), allowing us to monitor complex size.

Assemblies of nucleosome trimers with the SIR holocomplex formed at molar ratios of 0.5 and 1 Sir2-3-4 complex per nucleosome (Figure 7A, red A or B) were fixed and analyzed by sucrose gradient sedimentation (Figure 7B). To calibrate the gradients, reconstituted nucleosomal arrays of various sizes were sedimented in parallel (Figures 7A and 7D). The A complex sedimented as a symmetrical peak with an estimated mass of 1.38 MDa (Figures 7B and 7C, fractions 13–15). The two smaller peaks in the gradient represent naked competitor DNA and mononucleosomes. Doubling the molar ratio of Sir2-3-4 in the binding mixture led to the formation of particles with an estimated mass of 2.76 MDa (Figure 7C, labeled B, fraction 19).

The B peak would be consistent with either six molecules of Sir2-3-4 per trimer, or a stable interaction of two 1.38 MDa complexes (Figures 7C–7E). Besides the major peak at fraction 19, there is a shoulder of larger size in fraction 22, which could arise from interactions between complexes. Although gel electrophoresis experiments did not detect dimerization of a differentially tagged trimer and hexamer in the presence of SIR complexes (data not shown), we cannot exclude that these form in solution.

From the SIR-chromatin complex molecular weight (1.38 MDa), we can estimate the stoichiometry of SIR holocomplex per nucleosome. Subtraction of the molecular weight of the three nucleosomes (3 \times 210 \text{ kDa}) leaves a mass of 750 \text{kDa}. Since the molecular weight of the Sir2-3-4 complex is 360 \text{kDa}, we conclude that the remaining 750 \text{kDa of ligand corresponds to two SIR holocomplexes. Recovery of the SIR-nucleosomal complex after assembly confirmed that Sir2, Sir3, and Sir4 maintain their original 1:1:1 ratio (data not shown). Thus, our results argue that SIR holocomplexes sit between neighboring nucleosomes, with two per nucleosomal trimer or five per hexamer. Thus, the basic unit of silent chromatin comprises one SIR complex per linker, and not per H3 or H4 tail (Figure 7E).

**DISCUSSION**

The budding yeast silent chromatin in subtelomeric regions and mating-type loci shares many of the characteristics ascribed to...
heterochromatin in higher eukaryotes (Grewal and Jia, 2007). Although the powerful combination of genetics and ChIP led to detailed models for transcriptional repression (reviewed in Rüsche et al., 2003), it is difficult to differentiate direct from indirect effects by using in vivo studies. Our reconstituted in vitro system provides a unique opportunity to study the minimal interactions that allow silencing factors to assemble onto chromatin. It shows a multiplicity of contacts that contribute incrementally to the high-affinity association of SIR holocomplex with nucleosomes.

Multiple Binding Sites for Sir2-3-4 on Nucleosomes Generate High Specificity and Affinity

In yeast cells, efficient SIR-mediated transcriptional repression is sensitive to mutations in the H4 N-terminal tail and on the surface of the nucleosomal core near H3K79 (Aparicio et al., 1991; Johnson et al., 1990; Kayne et al., 1988; Park and Szostak, 1990; Ng et al., 2002; Park et al., 2002; Xu et al., 2005). Biochemical analysis of in vitro-translated Sir3 and Sir4 binding to histone tail peptides suggested that the decrease in silencing associated with the lack of H4 tail is caused by a reduced binding of the Sir2-3-4 complex to chromatin (Hecht et al., 1995). However, our reconstituted system revealed only a slight decrease in the affinity of the SIR holocomplex for nucleosomes lacking the H4 tail, and this we attribute to Sir3, consistent with earlier work (Georgel et al., 2001).

To reconcile the modest effect of the H4 tail mutation in vitro with the loss of TPE detected in vivo, we propose that the H4 N terminus has roles in silencing other than Sir recruitment. For example, unmodified H4K16 can promote nucleosome stacking (Dorigo et al., 2003; Robinson et al., 2008; Shogren-Knaak et al., 2006). In our system, which exploits the strongly positioning Widom sequence, we may bypass the need for H4 tail-mediated stacking. A further role demonstrated for acetylated H4K16 is Dot1 recruitment for H3K79 methylation (Altaf et al., 2007). Since we demonstrate that H3K79 antagonizes Sir binding (van Leeuwen et al., 2002; Frederiks et al., 2008), the loss of Dot1 recruitment and K79 methylation at euchromatic sites may indirectly titrate SIR proteins from telomeres, disrupting TPE. Indeed, the contribution of the H4 tail-Sir3 interaction is partially redundant with other Sir2-3-4 contact sites, namely H3K79 and DNA.

Sir4 Mediates SIR Complex Binding to Naked DNA

We show that both Sir2-4 and Sir2-3-4 bind chromatin with higher affinities than Sir3 alone, reflecting the high affinity of Sir4 for dsDNA. Indeed, an N-terminal domain of Sir4 binds DNA and disrupts TPE when overexpressed (Figure 6). Intriguingly, Sir4N can complement HM repression in a sir4 deletion when expressed in trans with the C-terminal 750 aa of Sir4 (Marshall et al., 1987). This argued for distinct yet essential
functions for Sir4N and Sir4C in silencing, which we are able to confirm in our reconstituted system.

The self-dimerizing coiled-coil domain in the Sir4 C-terminus binds Sir3 and is capable of forming a Sir3-Sir4-Sir4-Sir3 complex (Chang et al., 2003; Murphy et al., 2003). Consistent with a role in dimerization, we find that an excess of Sir4C impairs the cooperativity with which the Sir2-3-4 complex loads onto nucleosomes. Sir4N did not affect cooperativity but was able to compete for Sir2-3-4 binding to DNA. In conclusion, Sir complexes contact the H4 tail, a region surrounding H3K79, and DNA by Sir4N, which contribute incrementally to the high affinity of the Sir-chromatin association.

A Sir2 Byproduct Promotes a Stable Sir3-Nucleosome Complex

Our in vitro reconstitution system has allowed us to identify a role for the Sir2-generated by-product O-AADPR in the assembly of Sir holocomplex with chromatin. O-AADPR may enhance the affinity with which both Sir2-3-4 and Sir3 bind nucleosomes. Given that O-AADPR is unstable, it is unlikely to diffuse far from the site at which Sir2 catalyzes deacetylation, and thus a model in which Sir2 hands off this effector directly to Sir3 seems likely. Indeed, earlier work argued that its binding invoked a change in the conformation of the Sir2-3-4 complex (Liou et al., 2005). We note, however, that it was possible to restore silencing in a strain with nonacetylatable histones and catalytic dead Sir2 by overexpression of Sir3 (Yang and Kirchmaier, 2006). Similarly, recent data argue that the hydrolysis of NAD may not be essential for repression of a reporter gene at HMR (Chou et al., 2008). In this study as well, overexpression of Sir3 was needed for efficient repression. Although we show that O-AADPR increases the efficiency of both Sir3 and Sir holocomplex binding to nucleosomes, the presence of strong silencers or nucleation sites in vivo, and/or of high concentrations of Sir3, may obviate the effect of O-AADPR. Alternatively, differences in the efficiency of propagation or inheritance of silent chromatin may be conferred by O-AADPR.

Toward Defining the Core Unit of Silent Chromatin

Our sedimentation analysis of reconstituted Sir-bound chromatin argues for a binding stoichiometry of two Sir complexes per nucleosomal trimer, or one Sir complex between neighboring nucleosomes. We propose this as the basic unit of yeast silent chromatin. This allows Sir3 to contact both the H4 N-terminal tail and the region around H3K79. At higher Sir concentrations, we and others detect multimerization of Sir proteins and interactions between arrays (Georganel et al., 2001; McBryant et al., 2006, 2008), although our current study is unable to address their physiological relevance.

More importantly, our data confirm in vivo CHIP experiments and indicate that Sir2, Sir3, and Sir4 bind as a complex in repressed chromatin (reviewed in Rusche et al., 2003; Gasser and Cockell, 2001). Given that Sir4 is more stable in complex with Sir2, and that Sir2 is more active when bound to Sir4 (Cubizolles et al., 2006), Sir2-4 may well be recruited independently to silencers and Rap1 (Rusche et al., 2002). The Sir2-mediated deacetylation of H3K56 or of histone H4K5, K12, and K16 is likely to contribute to a dense packing of nucleosomes. The spreading of the Sir2-3-4 complex along this template then likely requires the loading of either Sir3 or a preformed Sir2-3-4 complex. For this, the coiled-coil domain of Sir4 is implicated by several studies (Chang et al., 2003; Murphy et al., 2003; Rudner et al., 2005). Finally, the presence of H3K79\textsuperscript{ac} could attenuate the spread of the Sir holocomplex by weakening the Sir-nucleosome interaction (Frederiks et al., 2008). Our reconstitution of Sir2-3-4 binding to nucleosomes is consistent with this model, and it paves the way for electron and scanning probe microscopy studies on the structure of yeast heterochromatin.

EXPERIMENTAL PROCEDURES

Recombinant Sir Proteins, Complexes, and DNA

The expression and purification of Sir proteins and complexes are described in Cubizolles et al. (2006) and in Supplemental Experimental Procedures. Purification of 601-167-3-mers and reconstitution of nucleosomes are performed as described (Hyyn et al., 2005) (Supplemental Experimental Procedures).

Analysis of Sir2-3-4 Bound to Nucleosomes

Trimers of radionabeled 601-167 nucleosomes at 2 x 10\textsuperscript{-6}M were incubated for 20 min on ice with he indicated Sir proteins or complexes in a buffer containing 10 mM TEA (pH 8.0), 25 mM NaCl, 0.05% Tween 20. Binding was analyzed on a 0.7% agarose gel in 0.2 x TB buffer at 80V for 3 hr at 4°C, after which dried gels were exposed to Phosphorscreens. Band intensity was monitored by ImageQuant TL2005 (Amersham).

For sucrose density gradient centrifugation, nucleosomal trimers at 2 x 10\textsuperscript{-4}M were incubated with the indicated amount of Sir2-3-4 complex, and samples were fixed for 30 min with 0.05% gluteraldehyde. The equivalent of 0.2 umoles nucleosomes with or without added SIR complex was layered on 4.2 ml 10%–25% (w/v) sucrose gradients in 10 mM TEA (pH 8.0), 0.1 mM EDTA, 0.2% glycerol, 25 mM NaCl, and 0.05% Tween 20. Sedimentation was analyzed on 4°C in a SW60 Beckman rotor (16 hr, 35,000 rpm), 100 μl fractions were collected. Cerenkov scintillation counts were scored and plotted against gradient fractions. Complexes of proteins of known size were used to calibrate each gradient.

MNease digestion was performed on a 6-mer of 601-167 nucleosomes that was reconstituted with yeast H4 and Xenopus H2A, H2B, and H3 and was subsequently diluted to 5 x 10\textsuperscript{-6}M. After assembly with the indicated molar ratio of Sir2-4 or Sir2-3-4 complexes, CaCl\textsubscript{2} was added to a final concentration of 1.5 mM, and 0.25 nmol chromatin was digested with 0.025 U MNease for 5 min on ice. The digestion was stopped by 10 mM EDTA, proteins were removed by ProteinA K digestion for 15 min at 37°C, and samples were run on a 1.5% agarose gel in 0.5 x TBE.

Plasmid, Strains, and Yeast Methods

Standard culture conditions at 30°C were used for yeast cultures. pJG plasmids (2μ origin) expressing Sir4N and Sir4C were previously described (Cockell et al., 1995). Repression assays are 10-fold dilution series on media selective for the plasmid with or without uracil.

SUPPLEMENTAL DATA


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