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Comparison of the Isw1a, Isw1b, and Isw2 Nucleosome Disrupting Activities

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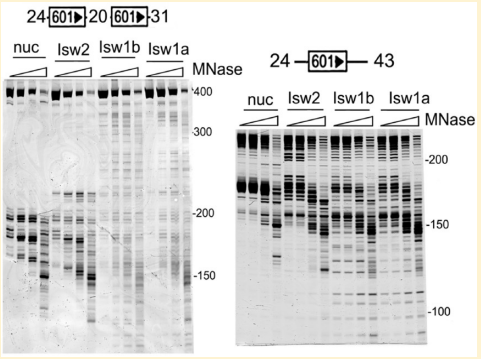
Supporting Information

ABSTRACT: The three *Saccharomyces cerevisiae* ISWI chromatin remodeling complexes, Isw1a, Isw1b, and Isw2, are implicated in the regularization of arrayed nucleosomes and regulation of gene activity. Although Isw1a and Isw1b are based on the same catalytic unit, in general, their functions in vivo do not overlap. To better understand the structural consequences of these complexes, we compared the putative nucleosome disrupting activities of the purified Isw1a, Isw1b, and Isw2. To account for the putative effects of nucleosomal environment, we employed reconstituted dinucleosomes in which the histone octamers were specifically positioned by the 146 base pair high-affinity nucleosome sequence "601". We have compared the MNase and deoxyribonuclease I protection patterns of remodeled nucleosome templates and evaluated the nucleosome destabilizing abilities of the Isw1a/b and Isw2 using restriction endonucleases. Although the Isw2 showed little evidence of nucleosome disassembly, the Isw1b remodeled dinucleosomes exhibited some common features with the ySwi−Snf remodeling products. The nuclease digestion data suggest that Isw1a can also promote ATP-dependent distortion of nucleosome structure, although less efficiently than the Isw1b complex.



Eukaryotic DNA functions in the context of chromatin, a hierarchical nucleoprotein structure, the initial level of which is based on the regular arrays of the universally repeating units, the nucleosomes, which comprise of a nucleosome core ∼146 base pairs (bp) of DNA wrapped in ∼1.7 turns around an octamer of histone proteins and the entry−exit DNA linkers of variable length.[1,2] The repressive effects of nucleosomes on DNA accessibility can be relieved by chromatin remodeling enzymes, which can reposition or disrupt nucleosomes using the energy of ATP. On the basis of the relationship of ATPase subunits, chromatin remodelers can be divided into the SWI/SNF, ISWI, Mi2/CHD, and INO80/SWR1 families.[3,4] The ATPase subunits can stably associate with other proteins, which diversifies the specialization of the resulting remodeling complexes.[3,5] ISWI chromatin remodelers are implicated in the regularization of nucleosome spacing and regulation of gene activity.[3,4,6,7] ISWI family members were identified in a wide range of eukaryotes that reflect the importance of their gene regulatory functions. Budding yeast has only two ISWI homologues, the Isw1p and Isw2p proteins. Due to the advanced yeast genetic techniques and the ease of large-scale protein purification, yeast versions of enzymes are widely used to study chromatin remodeling mechanisms.

The Isw2p ATPase forms only one complex, the Isw2, which contains Itc1, Dpb4p, and Dls1p subunits. The Isw1p, in addition to being present as a monomer, was found in two separable complexes, the Isw1a and Isw1b, which contain the Ioc3p and Ioc2p/Ioc4p proteins, respectively.[8] In vivo, Isw1a, Isw1b, and Isw2 exhibit similar regulatory effects on some genes; however, in general, their regulatory functions do not coincide. In vitro, these complexes also possess different abilities to reposition and disrupt nucleosomes.[5,8−10] Isw2 exhibits a moderate ability to regularize internucleosomal spacing in polynucleosomes. Isw1a and Isw1b, despite the same catalytic subunit they share, exhibit opposing nucleosome regularization preferences. Isw1a can efficiently generate uniformly spaced nucleosomes, whereas Isw1b has little spacing activity[8,11] and rather tends to randomize specifically positioned dinucleosomes (unpublished observation). Isw1a remodeling depends on the length of extranucleosomal DNA, which determines whether Isw1a binds to only one or both extranucleosomal linkers, and thus regulates the Isw1a catalytic activity.[9,12] In contrast, Isw1b has no requirements for the minimal and maximal length of the nucleosome linkers and its activity does not depend on the mode of interactions with DNA linkers.[5,9,12] Isw2 also has a requirement for the minimal DNA linker lengths but, unlike Isw1a, there is no limitation on the maximal length of extranucleosomal DNA.[9,12−14]

It has been shown that the Isw1 complex (copurified Isw1a and Isw1b) exhibits an ATP-dependent ability to disrupt nucleosomes in vitro at the promoter region of hsp70; however, chromatin disruption depended on GAGA transcription factor.[11] In contrast, in these experiments, Isw2 exhibited no appreciable nucleosome disruption despite its nucleosome-stimulated ATPase activity. It has also been reported that

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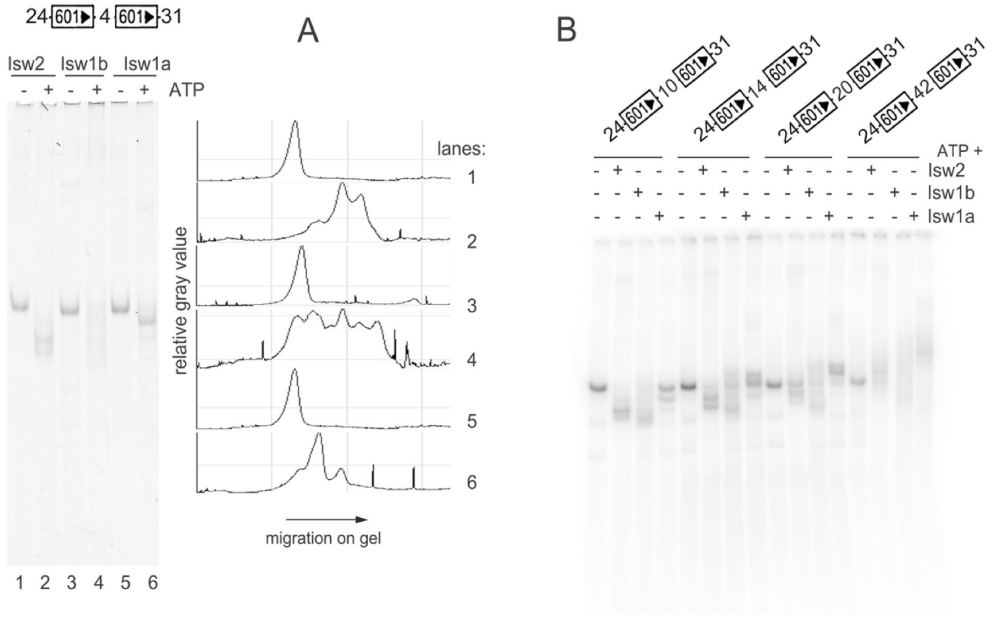

Figure 1. Native PAGE of variably spaced dinucleosomes after incubation with Isw1a, Isw1b, and Isw2 with or without ATP. (A) The gel was stained with EtBr; gel scan profiles are shown at the right. (B) Nucleosomes assembled on [γ-^{32}P] prelabeled DNA were visualized by autoradiography.

purified Isw1b can slide nucleosomes beyond the edge of short DNA fragment that destabilizes the H2A–H2B dimer.[12,15]

Here we tried to evaluate the putative nucleosome disrupting activities of the purified Isw1a, Isw1b, and Isw2. It has been shown that nucleosome disruption by some of the remodeling enzymes can be impacted by the presence of another nucleosome such as human BAF/PBAF that can generate in vitro structurally altered pairs of adjacent nucleosomes ("altosomes")[16] or connect two mononucleosomes together to form an altered noncovalent mononucleosome dimer.[17−19] The related yeast RSC[20] and ySwi–Snf[21] also possess the same or similar activities. In addition, the Swi–Snf translocating nucleosome complex can promote dissociation of an adjacent nucleosome in vitro,[22,23] resembling how activation domains can promote eviction of adjoining nucleosome.[24] To account for the putative effects of nucleosomal environment, we employed reconstituted dinucleosomes in which the histone octamers were positioned to defined locations by the 146 bp nucleosome high-affinity sequence "601".[25]

We have compared the micrococcal nuclease (MNase) and deoxyribonuclease I (DNase I) protection patterns of remodeled nucleosome templates and evaluated the nucleosome destabilizing abilities of Isw1a/b and Isw2 using restriction endonucleases (RE). Isw2 showed little evidence of nucleosome disassembly whereas Isw1b remodeled dinucleosomes exhibited some common features with the ySwi–Snf remodeling products. Nuclease digestion data suggest that Isw1a can also promote ATP-dependent distortion of nucleosome structure, although less efficiently than Isw1b.

EXPERIMENTAL PROCEDURES

Preparation of DNA Templates for Nucleosome Assembly. To prepare plasmid pBS601, the NotI/NlaIII fragment of pGEM3Z601R,[26] containing the 146 bp 601 sequence, was cloned between the NotI and XbaI sites of pBluescript II (the NlaIII and XbaI termini were preblunted with Klenow). To prepare pBS601N, pBS601 was cleaved with NotI, and the termini were filled in with dNTPs/Klenow Exo$^-$ and religated. DNA templates for mononucleosomes were prepared from pBS601 or pBS601N with pairs of restriction enzymes that leave a desirable amount of extranucleosomal DNA flanking the 601 sequence. To prepare dinucleosome DNA templates, an additional 601 fragment was cloned in the polylinker of pBS601 or pBS601N; the dimer of the 601 sequences was prepared using appropriate pairs of restriction enzymes, usually SacI and SacII or Eco RI and HindIII (see also the Supporting Information[21] for the sequences of DNA templates and construction of the plasmids).

Purification and ATPase Activity Assays of Remodeling Complexes. The yIsw2 complex was isolated from *Saccharomyces cerevisiae* strain YTT966 by FLAG immunoaffinity chromatography, followed by a 1 mL Source Q-anion-exchange column as described before.[8,11] The Isw1a and Isw1b complexes were purified from yeast extracts through a TAP-tagged Ioc3p and Ioc2p, respectively, as described elsewhere.[8,11] The yeast Swi–Snf complex, used in some experiments, was purified through the TAP-tagged catalytic subunit, Swi2p protein. Isolated protein was assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) with subsequent staining with silver reagent (Bio-Rad). The ATPase activity of the Isw1a/b and Isw2 was assayed in a 5 μL reaction containing 50 ng of core histones or DNA or nucleosomes, 1 ng of yIsw2, 0.35 μCi of [γ-P^{32}] ATP, and 0.1 mM cold ATP. The reactions were incubated at 25 °C for 40 min then 1 μL was spotted onto PEI Cellulose F plates (EMD Chemicals) and resolved in 0.15 M LiCl/0.15 M formic acid. The plates were exposed to a Phosphorimage screen and quantified on a Typhoon scanner (GE/Molecular Dynamics).

Nucleosome Reconstitution. Nucleosomes were reconstituted by sequential dilutions of a 2 M NaCl mixture of purified histones and DNA (at starting concentrations of 0.25 μg/μL each) as described,[27] except the dilution buffer contained 50 μg/μL of bovine serum albumin (BSA) and glycerol was omitted from the final dilution buffer. To remove possible aggregates, reconstituted nucleosomes were centrifuged for 5 min in an Eppendorf centrifuge, and the

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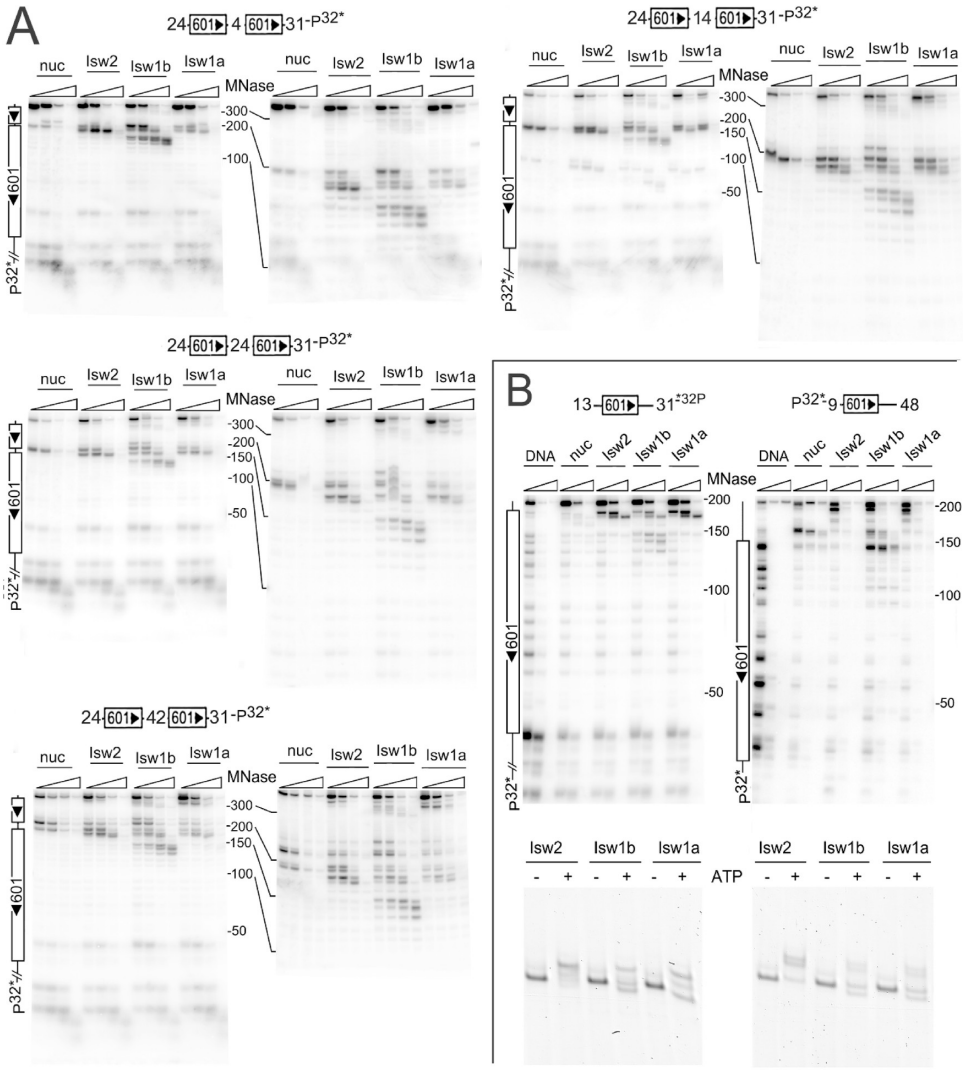

Figure 2. Autoradiograms of 6 M urea−PAGE of DNA from [γ-P^{32}]-labeled dinucleosomes (A) or mononucleosomes (B). Each was remodeled with Isw2, Isw1a, or Isw1b and then digested with increasing concentrations of MNase. (A) The left and right subpanels for each template show PAGE of lower and higher density, respectively. The schemes at the left of the panels indicate the position(s) of the 601 DNA within the templates. The orientation of the 601 sequences and the sizes (bp) of the 601 flanking DNA are shown on the schemes at the top of panels. The bottom panels in (B) show gel-mobility assays of remodeled 13N31 and 9N48 mononucleosomes.

supernatant was filtered by centrifugation through the top portion of the 1 mL aerosol-tight tip (which was cut just below the aerosol filter) inserted in the Eppendorf tube. To verify reconstitutions, 5−7 μL nucleosome aliquots were mixed with one-fifth volume of 60% sucrose/0.01% xylene cyanol in 1 × TE (pH 7.6) and resolved on 5.5% native PAGE (29:1 acrylamide:bisacrylamide ratio) in 0.5E [1 × E = 40 mM Tris−OH, 20.6 mM acetic acid, 5 mM NaOAc, 2 mM EDTA] or 0.5 TBE buffers and stained with ethidium bromide (EtBr).

Nucleosome Remodeling. Nucleosome remodeling was performed directly in the nucleosome assembly buffer [10 mM Tris−HCl (pH 7.4), 1 mM EDTA, 100−110 mM NaCl, 2.5 mM $MgCl_2$, 2 mM DTT, 0.025% NP-40, 50 μg/mL BSA, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] supplemented with 1 mM ATP and additional 2.5 mM $MgCl_2$. To avoid possible interference with the carrier oligonucleosomes or mononucleosomes, the carrier nucleosomes were omitted. Typically, 10 μL remodeling reactions contained 100 ng of assembled DNA templates and about 4 ng of yIsw1a/yIsw2 complex. After incubations at 25 °C for 1.5 h (the extended reaction times were chosen to reach final equilibrium positions of the nucleosomes), 5−7 μL reaction aliquots were resolved on native gel and stained with EtBr. In some cases (Figure 1B), the DNA template for nucleosome assembly contained indicator amounts of [γ-P^{32}]-labeled DNA and the gels in this case were exposed to a Phosphorimage screen and scanned on a Typhoon scanner.

Analysis of Nucleosomes with MNase, DNase I, and Restriction Endonucleases. To examine nucleosome structure with DNase I (Sigma) or MNase (Sigma), nucleosomes were assembled on [γ-P^{32}]-labeled DNA. The positions of the labeled nucleotide are indicated in the figures (typically, the 3′ end of the DNA template). Remodeling reactions were scaled up 10 times (to 100 μL). Remodeling was terminated by 20 min incubation with 50 milliunits of apyrase (New England Biolabs). Nucleosome aliquots (15−20 μL) were digested for 2 min with increasing concentrations of the nuclease, and then DNA was isolated and resolved on urea-containing PAGE (6 M urea, 19:1 acrylamide:bisacrylamide ratio). Gels were dried and exposed to a Phosphorimage screen.

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To examine MNase digestion of native unremodeled and remodeled nucleosomes, four things were done: (1) remodeling reactions were terminated by apyrase, (2) nucleosome aliquots were digested for 2 min with increasing concentrations of MNase, (3) digestion was stopped by 1 mM EGTA, and then (4) samples were resolved in the nucleoprotein gel and stained with EtBr. To examine nucleosome accessibility to restriction endonucleases, 20 μL remodeling reactions were terminated by incubation with 10 milliunits of apyrase for 15 min at room temperature. Then nucleosomes were digested with 10U of restriction enzyme (New England Biolabs) for 30 min at room temperature. DNA was isolated and resolved on 7% polyacrylamide gels (acrylamide to bisacrylamide = 29:1) and stained with ethidium bromide.

RESULTS AND DISCUSSION

We sought to compare the abilities of related yeast Isw1a and Isw1b complexes to alter nucleosome structure. The nucleosome "nondestructive" Isw2 was used as a reference. The SDS–PAGE and ATPase assays of isolated complexes are shown in the Figure S1A of the Supporting Information. The ATPase activity of Isw1a/b and Isw2 was stimulated by mononucleosomes and dinucleosomes over histones and bare DNA, indicative that purified complexes can specifically recognize nucleosomes. As a simple yet defined model of a nucleosome array, we used a range of differently positioned dinucleosome templates. The two (asymmetric) 146 bp 601 positioning sequences[25] were placed in the same orientation (indicated in the figures), also defined as the 5′ to 3′ orientation of the entire template. Templates were designated as "*x*Ny" or "*x*Nz*N*y" for mononucleosomes and dinucleosomes, respectively, where the "*x*", "*y*", and "*z*" show the lengths (bp) of double-stranded DNA adjoining the 601 DNA. The amount of histone octamers required to nucleosomize both 601 sequences was determined by titrations (Figure S1B of the Supporting Information).

Assembled dinucleosomes migrated as single bands in native PAGE, indicative of uniquely positioned histone octamers. Typically, remodeling products migrated faster or slower than the original nucleosomes, depending on the initial nucleosome positioning (Figure 1). It could be supposed that the mobility of dinucleosomes is lower when both histone octamers are located closer to the center than to the ends of the DNA, analogous to the position of a mononucleosome on a DNA fragment causing its retardation[28,29] (as illustrated in the Figure S1C,D of the Supporting Information). In general, Isw1a and Isw2 remodeling species formed single or well-resolved multiple bands on the gel, whereas the Isw1b products formed more diffused patterns, which are characteristic for randomly positioned nucleosomes (Figure 1A,B). This is consistent with the reported abilities of these complexes to space arrayed nucleosomes.[8,11] Increasing the length of internucleosomal DNA linker had different effects on the three remodelers. The Isw1b remodeling pattern was not significantly affected by variations of the nucleosome spacer (Figure 1B). In general, Isw1a and Isw2 exhibited similar nucleosome repositioning preferences due to the tendency to regularize nucleosomes on the DNA. These complexes move apart closely spaced nucleosomes (24N4N31) and move the distant nucleosomes closer to each other (24N42N31) resulting in higher or lower electrophoretic mobility of the dinucleosome template, respectively (Figure 1B and unpublished). However, Isw1a showed a tendency to place nucleosomes at shorter distances

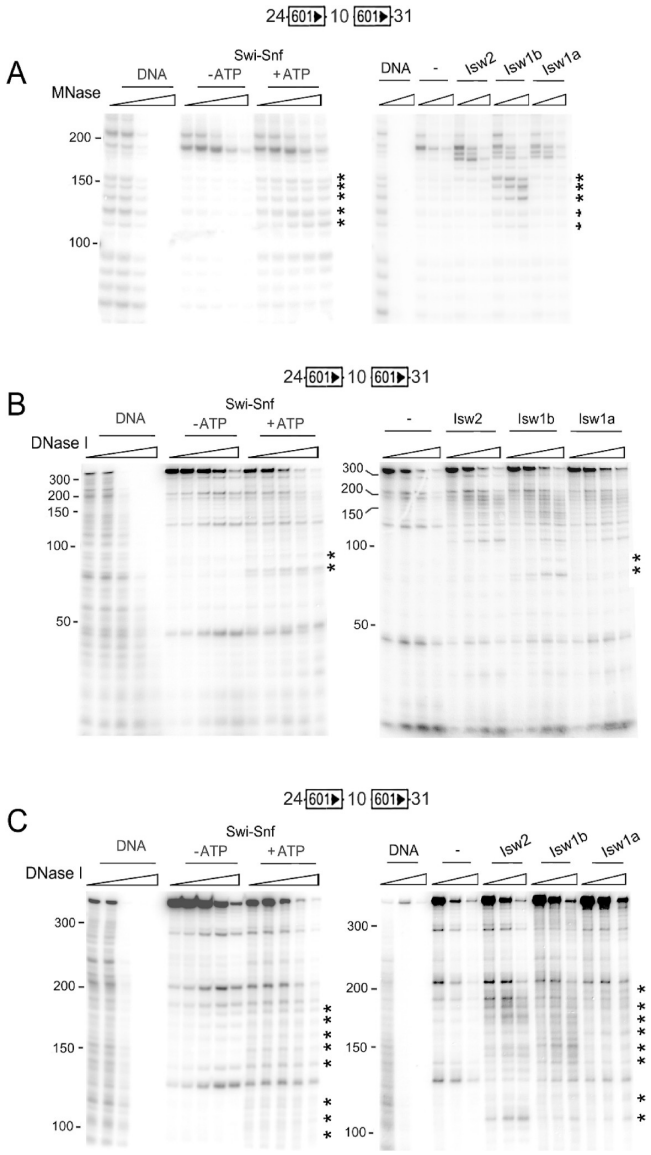

Figure 3. Autoradiograms of 6 M urea–PAGE of DNA from [γ-P^{32}]-labeled 24N10N31 dinucleosomes. Each was remodeled with Isw1a/b, Isw2 or ySwi–Snf (as indicated) and probed with MNase (A) or DNase I (B, C). The asterisks indicate the similarities in the nuclease cutting patterns between the Swi–Snf and Isw1b (A, B) or Isw1a (C).

than that for Isw2 (e.g., in the 24N20N31 template, Isw1a moves two nucleosomes closer together, whereas Isw2 still tends to move nucleosomes away from each other).

To evaluate the alterations in the dinucleosome structure, we compared MNase sensitivities of remodeling products. A number of different templates were tested to estimate the effects of nucleosome spacing (Figure 2A). In unremodeled templates, the nucleosomal DNA was protected from digestion and only linker DNA was accessible to cleavage. Compared with nuclease susceptibility of more widely spaced dinucleosomes, in 24N4N31, the nuclease susceptibility of internucleosomal linkers was significantly reduced due to tight nucleosome spacing. The digestion patterns of remodeled dinucleosomes were generally consistent with the gel-mobility data. Deprotection of DNA between nucleosomes and at the ends of templates likely reflects nucleosome repositioning.

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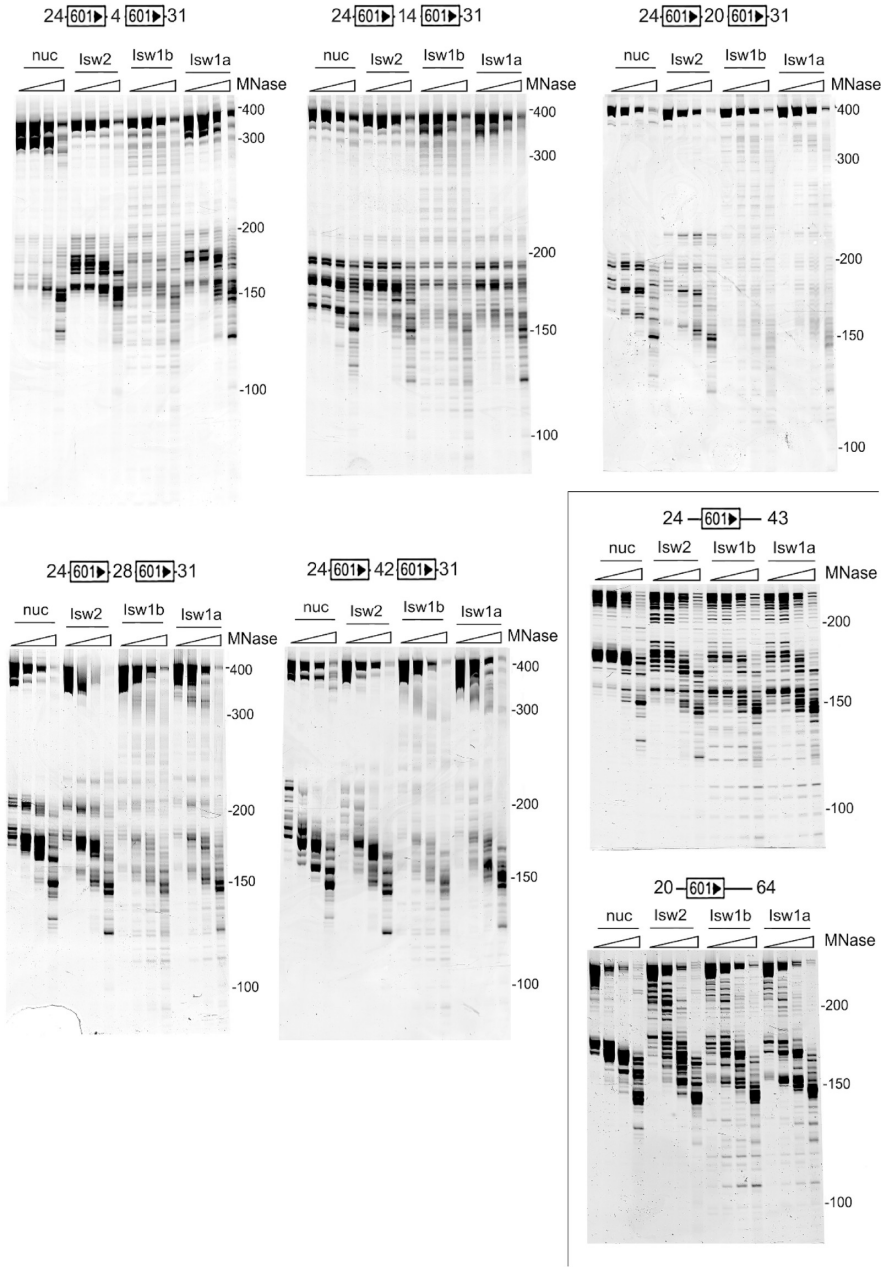

Figure 4. EtBr-stained PAGE of total DNA from dinucleosome and mononucleosome templates. Each was remodeled with Isw2, Isw1a, or Isw1b and then digested with increasing concentration of MNase. The schemes of the templates are shown at the top of the panels and indicate the orientation of the 601 sequence(s) within the templates and the sizes (bp) of the 601 flanking DNA.

In contrast to Isw1a and Isw2, Isw1b generated an additional MNase sensitive region over the 601 positioning sequence, spanning ~25−35 bp around the edge of the nucleosome near the linker region. Isw1b disrupting activity was not specific for dinucleosomes and was also observed with mononucleosomes (Figure 2B). The MNase cutting patterns by Isw1b had certain similarity to those by the yeast Swi−Snf (Figure 3A). Both remodelers caused a similar deprotection of the DNA in the upstream region of the 3′ nucleosome (indicated by asterisks), except the sensitive region by Swi−Snf was more extended to the center of the 601 sequence. Considering the location and the spread of the MNase sensitivity by Isw1b, it seems unlikely that this can be due entirely to the reported Isw1b ability to move nucleosomes off the DNA end by 5−11 bp[12] and, thus, a further increase the internucleosome distance. Such a MNase sensitivity pattern may reflect a local internal destabilization of the histone−DNA contacts without a significant displacement of the histone octamer off the edge of the DNA. It is notable that, despite the pronounced differences in MNase sensitivity, Isw1b and Isw1a remodeled mononucleosomes had very similar gel-mobility patterns (Figure 2B), which seems unlikely if their geometries would be significantly different (i.e., if in Isw1b remodeling species the nucleosomes were significantly shifted off of the DNA, compared to the Isw1a). In addition, Isw1b generates DNase I cleavage sites close to the center of the 3′ 601 sequence (indicated by asterisks), which are similar to the ones observed in the Swi−Snf remodeled dinucleosomes (Figure 3B). The similarity of nuclease digestion patterns of Isw1b and Swi−Snf remodeling products suggests that Isw1b,

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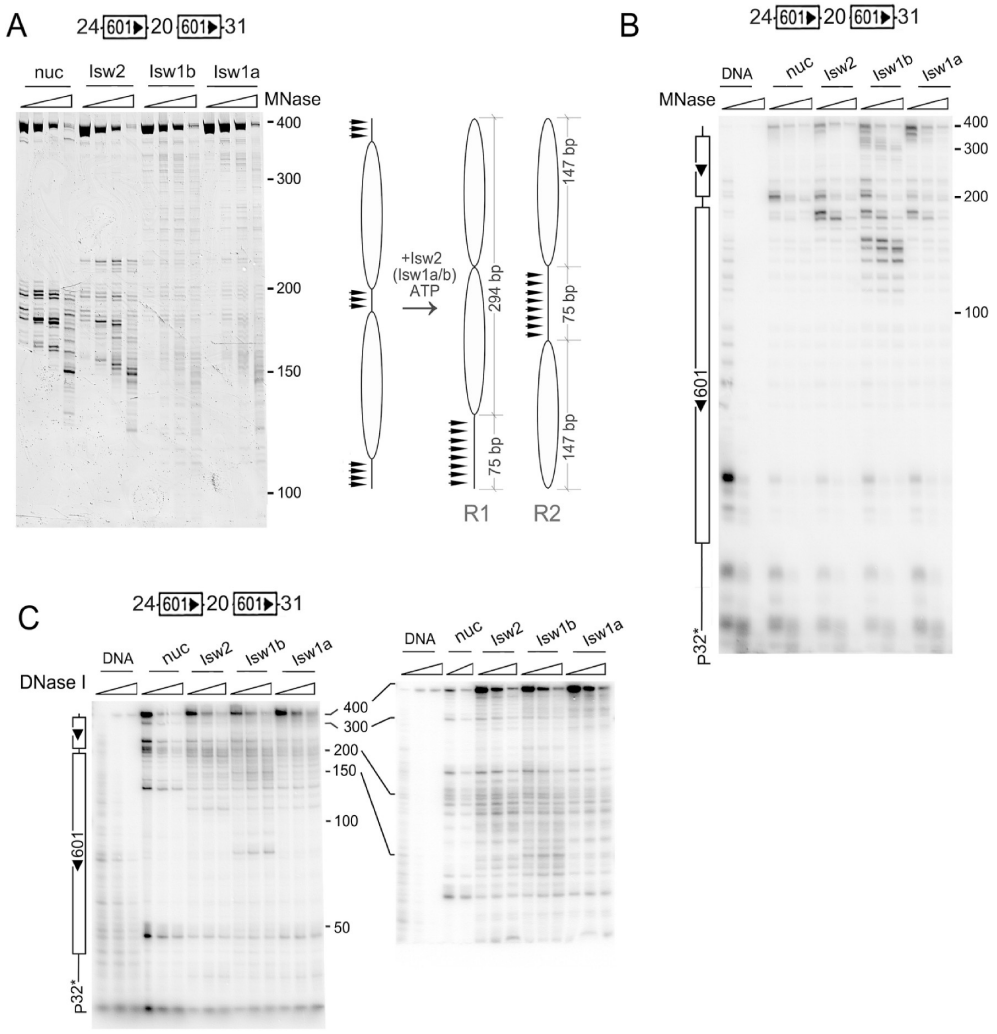

Figure 5. Comparison of different nuclease digestions assays of 24N20N31 dinucleosomes remodeled with Isw1a/b and Isw2. (A) EtBr-stained PAGE of total DNA from remodeled templates, digested with increasing concentration of MNase. The scheme on the right illustrates two extreme positions (R1/R2) of histone octamers in remodeled template. The arrows indicate the MNase accessible sites (in the assumption that the nucleosomes are structurally unaltered), the numbers show the lengths of the nucleosomal and extranucleosomal DNA. (B) and (C) Autoradiograms of the 6 M urea–PAGE of DNA from the [γ-P^{32}]-labeled remodeled dinucleosomes, which were probed with the MNase (B) or DNase I (C). The schemes at the left of the panels indicate the positions of the 601 DNA. The left and right subpanels in (C) show PAGE of lower and higher density, respectively.

similar to Swi–Snf, can unravel DNA from the nucleosome surface, although in a more limited scale.

On the other hand, recent studies have shown that the remodelers' ability to slide nucleosomes over the end of DNA can be significantly enhanced by increasing the remodelers' affinity to histone termini (e.g., by replacing the Chd1 DNA-binding domain with monomeric streptavidin (mSA)). It has been shown[30] that Chd1–mSA can efficiently slide H3 A21C biotinylated nucleosomes or H2A T120C biotinylated nucleosomes up to 40–50 bp over the ends of a DNA fragment and even eject nucleosomes from the DNA. However, if instead of histones biotin was attached to the extranucleosomal DNA, only a small population of nucleosomes were shifted off of DNA ends by 20–30 bp. This suggests that, chromatin remodelers that are insensitive to DNA (as Isw1b appears to be, on the basis of sliding across a DNA fragments) could, in general, slide nucleosomes off of DNA ends if conditions favor stable binding of remodelers to nucleosomes via histones, a possibility that should also be taken into account in the interpretation of our experiments. Thus, the altered nuclease digestion pattern of the Isw1b remodeled templates may have resulted from local distortion of histone–DNA interactions, sliding of a nucleosome off a DNA end or, most likely, some combination of the two. Indeed, in dinucleosomes the Chd1–mSA fusion remodeler can reversibly disrupt the canonical nucleosomal wrapping of DNA around the biotinylated histone core in a manner similar to that of yeast Swi–Snf and RSC.[30] However, it should be noted that, in contrast to experiments with Chd1–mSA, we never observed any appreciable ejections of nucleosomes from the DNA during remodeling of mononucleosome or dinucleosome templates. This allows for supposing that the nucleosome shift over the DNA ends by Isw1b is not as significant in terms of the distance by which the nucleosomes are shifted off of DNA, as it does not cause nucleosome ejection (which was observed with Chd1–mSA[30]). In addition, the Isw1a/b-dependent nucleosome changes are also less likely a result of a significant nucleosome disassociation. In our conditions, we were unable to

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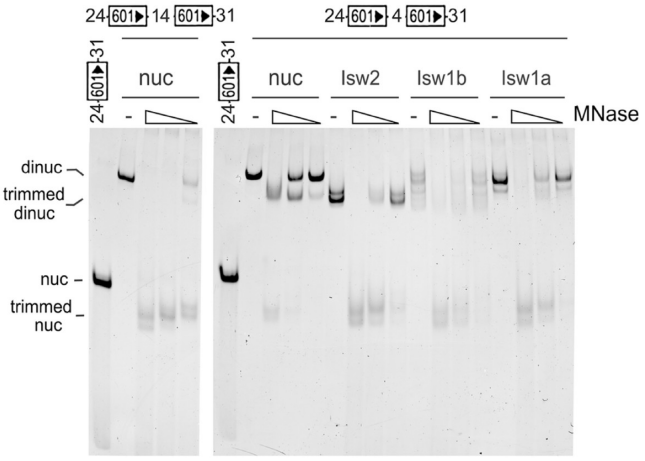

Figure 6. EtBr-stained native PAGE of 24N4N31 dinucleosomes. Each were remodeled with Isw2, Isw1a, or Isw1b and then digested with the MNase and the EGTA-stopped reactions were loaded on a gel. Undigested 24N31 dinucleosomes were loaded as the mononucleosome markers, and the MNase-digested unremodeled 24N14N31 (left) were loaded as a control for the degree of nuclease digestion.

conclusively evaluate the eviction of the H2A−H2B dimer(s) in mononucleosome or dinucleosome templates. However, in similar experiments with Chd1−biotin fusion, the efficient APB−H2B cross-linking in nucleosomes that were shifted off DNA ends[30] argues against a total H2A−H2B reorganization as such histone mapping requires proper H2B positioning. In this respect, an important conclusion has come from recent studies of DNA methyltransferase "mapping" of a number of Swi−Snf and ISWI-type remodeling products,[31] which suggestes that, at least for mononucleosome templates, nucleosome sliding off DNA ends could be a predominant structural outcome of remodeling reactions (rather than the disrupted loops on the nucleosome surface). However, in dinucleosomal templates, the collision between two remodeled nucleosomes could be inferred to give rise to structurally altered particles.[30]

It is of note that, in the Isw1a and Swi−Snf remodeled templates, the DNase I cutting patterns of internucleosomal linkers were very similar (Figure 3B,C) and distinct from those produced by Isw2 and Isw1b. This is notable given that Swi−Snf remodelers can convert two adjacent nucleosomes to structurally altered dinucleosome-like assemblies, the altosomes, which contain two intact histone octamers where a portion of DNA of one nucleosome interacts with the surface of another nucleosome that results in altered DNA accessibility and reduced negative writhe of DNA associated with canonical nucleosomal wrapping.[16,19,21] Therefore, although Isw1a does not catalyze formation of full altosomes, it could impose some of the alterations to nucleosome structure.

The 601 DNA sequence contains several conserved regions with an inherent palindromic symmetry and exhibits a distinct symmetrical pattern of nuclease accessibility. Thus, the arising asymmetry in the MNase accessibility in the Isw1b remodeled dinucleosomes (Figures 2 and 3) may indicate formation of altered asymmetric nucleosome structures. With endonuclease that introduces double-stranded (ds) DNA breaks, such structures are thought to produce cutting patterns abundant with internucleosome-size DNA. Figure 4 shows EtBr-stained native PAGE of the total DNA from the Isw1a/b and Isw2 remodeled dinucleosomes, which were digested with increasing concentrations of MNase. This nuclease digestion assay reveals the distribution of the nuclease ds breaks. This assay is more selective for the "open" nucleosome structures, as MNase exhibits a relative preference to induce single-stranded (ss) nicks within the nucleosome itself, but only ds breaks within nucleosomal linker regions. In two steps, cleavage reaction of the enzyme first introduced ss breaks and then converted these to ds breaks by an adjacent cleavage on the opposite strand.[32] In unremodeled dinucleosomes, MNase digested the internucleosomal linker DNA to give ∼146 bp nucleosome "footprint". In underdigested templates, the mononucleosome band lengths were increased by the linker DNA protected by histone termini; overdigestion of octamer-associated DNA results in the accumulation of subnucleosomal DNA fragments (Figure 4).

In all tested variably spaced dinucleosomes, Isw1b plus ATP generated discrete MNase ds footprints, continuously extending down from the top of gels. As illustrated on the scheme in Figure 5A, the DNA ladders sized from ∼220 to 320 bp and from ∼75 to 145 bp are unlikely to result from randomization of nucleosome positioning but likely indicate nucleosome disruption (and, possibly, some nucleosome sliding off of DNA). For the structures R1 and R2 (the extreme positions of nucleosomes in remodeled templates), MNase could be inferred to produce DNA ladders sized from 294 to 369 bp and from 147 to 222 bp, respectively (and below 75 bp for both structures). It is notable that some of the Isw1a-remodeled templates also exhibited the extended discrete MNase ds cutting patterns (most prominent in the 24N20N31 and 24N28N31 dinucleosomes), which suggests that Isw1a also possesses some nucleosome disrupting activity. We cannot conclude with certainty the reason these Isw1a effects were most pronounced on the 24N20/28N31 templates (which, of note, have more "physiological" spacing than that of other tested templates). The cryo-electron microscopy (EM) structures of Isw1a with nucleosomes and photo-cross-linking data suggest that Isw1a can interact simultaneously with two adjoining nucleosomes.[33] This could influence the outcome of the remodeling reaction and result in differential accumulation of various remodeling intermediates (i.e., in nucleosome spacing-dependent manner).

The Isw1b and Isw1a nucleosome destabilization activities were not specific for dinucleosomes and were also observed with the mononucleosome templates (Figure 4, bottom right). In contrast, Isw2 did not generate the altered MNase ds footprints in any studied nucleosome templates. It is of note that the altered ds MNase cutting by Isw1a was much less evident in 4 or 14 bp spaced dinucleosomes compared to templates with longer nucleosome spacing. However, Isw1a exhibited high nucleosome mobilization activity on 24N4N31 and 24N14N31, as was evident from the native gel-shift assays (Figure 1). Therefore, the inability of Isw1a to alter the 4/14 bp spaced dinucleosomes is unlikely a result of poor Isw1a catalytic activity on such templates. It seems more likely that, due to sterical hindrances, the close apposition of two nucleosomes impairs some of the Isw1a nucleosome processing abilities.

Figure 5 shows a comparison of different nuclease protection assays of remodeled 24N20N31 templates in which the altered MNase ds footprint by Isw1a was the most evident. However, except for the altered MNase ds cutting, other nuclease footprints did not reveal conclusive distinctions of 24N20N31 from other Isw1a-remodeled templates (see footprints for 24N10N31 in Figure 3). This suggests that the structural

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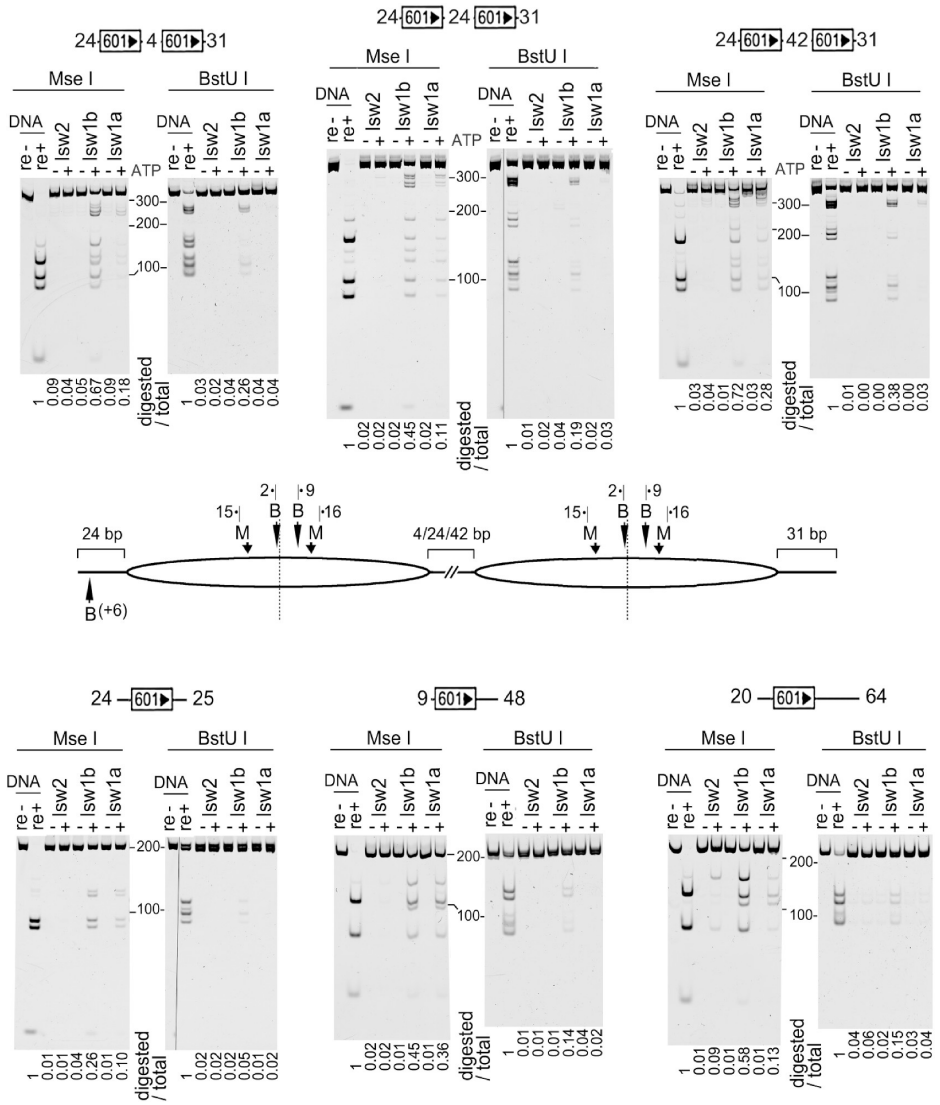

Figure 7. EtBr-stained native PAGE of DNA from variably positioned mononucleosomes (top panels) and dinucleosomes (bottom panels). Each were remodeled with the Isw1a/b or Isw2 and then digested with the MseI or BstUI restriction endonucleases. Rough estimates of the digested to total DNA ratio are shown at the bottom of each panel. The schemes of the nucleosome templates are shown at the top of the panels and indicate the orientation of the 601 sequence(s) and the sizes (bp) of the 601 flanking DNA. A template map shows positions of the RE cleavage site in dinucleosome templates. The ovals indicate the positions of the minimal 601 DNA sequences, and the numbers near the RE sites show the distance (bp) from the center of the nucleosome.

changes in the Isw1a remodeled templates could be rather moderate.

Figure 6 shows EtBr-stained nucleoprotein PAGE of remodeled 24N4N31 dinucleosomes, which were lightly cleaved with the MNase. The left panel shows that the digestion was just sufficient to liberate a single mononucleosome from 24N14N31. However, in unremodeled 24N4N31, the linker between the closely abutting nucleosomes is poorly accessible to MNase. In more widely spaced remodeled 24N4N31, such MNase treatment efficiently liberates mononucleosomes. Despite the discrete MNase ds pattern of Isw1b remodeling products (Figures 4 and 5), the gel on the right in Figure 6 shows no obvious evidence for MNase-generated subnucleosomal structures (except somewhat higher overall MNase sensitivity of Isw1b remodeled templates). This suggests that the observed discrete MNase ds footprints (Figure 5) are not a result of a total or partial nucleosome disruption/dissociation but rather indicate the restrained structural changes of nucleosomes.

Figure 7 shows restriction endonuclease analysis of the Isw1a, Isw1b, and Isw2 remodeled dinucleosomes and mononucleosomes. Two used RE enzymes, MseI and BstUI, have cleavage sites near the center of the 601 sequence. Despite high nucleosome mobilization activity of Isw2 (Figure 1 and S1C of the Supporting Information), Isw2 exhibited a poor ability to uncover the nucleosomal DNA in the studied templates. In contrast, Isw1b efficiently deprotected the RE recognition sequences. Consistently with the MNase ds assay (Figure 5), Isw1a stimulated RE cleavage in an ATP-dependent manner, albeit less efficiently than that for Isw1b. This provides more evidence for the ability of Isw1a to destabilize nucleosome structure under employed conditions. As discussed above, nucleosome sliding off DNA ends could also contribute to uncovering of nucleosomal DNA to RE. However, considering the Isw1a tendency to equally distribute

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nucleosomes rather than shifting them off of DNA, it seems likely that the observed changes in nuclease sensitivity reflect mostly nucleosome unwrapping. In 24N20N31, Isw1a generated an extended ds MNase cleavage pattern (Figures 5), whereas the gel-mobility shift data suggest that nucleosomes are repositioned closer to the center of the DNA fragment (Figure 1).

Taken together, the data here suggest that Isw1b alone can to a certain extent "unravel" the DNA from the nucleosome without significant displacement of the nucleosome over the end of the DNA fragment. The results also suggest that individual Isw1a can also distort the nucleosome structure. Although the observed in vitro nucleosome destabilizing ability of Isw1a was only incremental, this activity may be implicated in the gene regulatory mechanisms in vivo. A significant portion of accumulated data, however, suggest yeast Isw1p complexes as negative regulators,[7,34−36] which implies that these enzymes are implicated in maintaining rather than in disruption of nucleosomes (e.g., Isw1b maintain chromatin structure during transcription by preventing histone exchange,[36] Isw1a controls RNA pol II recruitment at active genes[35]). However, the repressive effects of Isw1 complexes derive from their ability to position nucleosomes on gene regulatory regions[34] and thus, could be context-dependent.[34] It has been shown that the Isw1 complex can cooperate with the GAGA factor to disrupt nucleosomes in vitro,[11] the "related" Chd1 complex can also disrupt dinucleosomes, providing it can be targeted to nucleosomal histones.[30] Isw1b and Chd1 could be implicated in the activation of *S. cerevisiae* ADH2 gene.[37] In addition, Pinskaya et al.[38] suggested that nucleosome remodeling and transcriptional repression could be distinct functions of Isw1 in *S. cerevisiae* because the Isw1 lacking SANT domain, essential for its catalytic function, still retains its ability to repress transcription initiation at the MET16.

■ ASSOCIATED CONTENT

Supporting Information

Discussion on the relation of dinucleosome positioning and their electrophoretic mobility's; SDS−PAGE of purified Isw1a, Isw1b, and Isw2 and their ATPase activity assay (Figure S1A); Ethidium-stained native PAGE of dinucleosome DNA template, titrated with increasing amounts of histone octamers (Figure S1B); Ethidium-stained native PAGE of variably spaced dinucleosomes after their remodeling with Isw2 and ATP (Figure S1C); D, EtBr-stained native PAGE of the MNase digested unremodeled and Isw2-remodeled dinucleosomes (Figure S1D). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS:

bp, base pairs; BSA, bovine serum albumin; DNase I, deoxyribonuclease I; ds, double-stranded; EtBr, ethidium bromide; MNase, micrococcal nuclease; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RE, restriction endonuclease; SDS, sodium dodecyl sulfate

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