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Remodeling of Nucleosome-Dimer Particles with yIsw2 Promotes Their Association with ALL-1 SET Domain in Vitro

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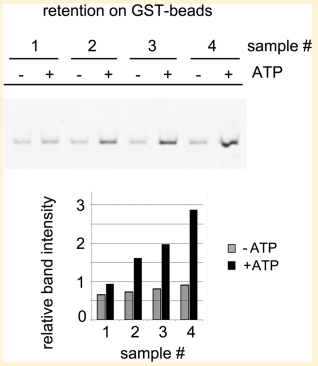
Supporting Information

ABSTRACT: Functioning of histone lysine methyltransferases (HKMTs) involves interactions of their catalytic domain "SET" with the N-termini of histone H3. However, these interactions are restricted in canonical nucleosomes due to the limited accessibility of H3 termini. Here we investigated whether nucleosome remodeling with the yeast Isw2 affects nucleosome affinity to the SET domain of ALL-1 HKMT. Reconstitution of mononucleosomes by salt dilutions also produces some nucleosome-dimer particles (self-associated mononucleosomes, described by: Tatchell and van Holde (1977) *Biochemistry*, *16*, 5295−5303). The GST-tagged SET-domain polypeptide of ALL-1 was assayed for binding to assembled mononucleosomes and nucleosome-dimer particles, either intact or remodeled with purified yeast Isw2. Remodeling of mononucleosomes does not noticeably affect their affinity to SET domain; however, yIsw2 remodeling of nucleosome-dimer particles facilitated their association with GST-SET polypeptide. Therefore, it is conceivable that nucleosome interactions in trans could be implicated in the maintenance of chromatin methylation patterns in vivo.



Chromatin activity depends on specific methylation of certain amino acids in the N-termini of histones H3/H4.[1,2] The somatically inheritable methylation of histone lysines is largely controlled by a family of proteins, sharing a conserved 130-amino acid methyltransferase domain "SET".[3] It has been shown that binding of SET domains to histone H3 N-termini constitutes an essential step during the in vivo control of chromatin methylation.[4] However, H3 N-termini in nucleosomes are poorly accessible to SET,[5] suggesting that nucleosome structure must be altered for association of SET domains with histones H3 (ref 6 and references therein).

Nucleosome packaging can be altered by ATP-utilizing chromatin remodeling complexes,[7] acetylation[8,9] and other histone modifications,[10,11] internucleosome interactions,[12−14] passage of RNA polymerases,[15] etc. It is likely that many of such processes could increase histone accessibility and alleviate nucleosome−SET association. For example, the GST-SET polypeptide of ALL-1 shows increased affinity to nucleosomes reconstituted from hyperacetylated histones,[6] presumably due to the more exposed histone H3 termini in acetylated nucleosomes.[16−18]

The interactions between arrayed nucleosomes could also modulate accessibility of nucleosomes to SET domains. It has been shown that cis interactions of nucleosome in di- and oligonucleosomes, as well as remodeling of oligonucleosome substrates by incorporation of histone H1, significantly stimulated methyltransferase activity of the EZH2 SET domain, which exhibits only weak activity on intact or H1-containing mononucleosomes.[19] An increase in nucleosome interactions with SET7 and ALL-1 SET-domain polypeptides was observed on Isw1/Isw2-remodeled dinucleosome templates.[20] Here we have shown that trans interaction between mononucleosomes in nucleosome-dimer particles could facilitate association of ALL-1 SET-domain polypeptide with histones H3 in yIsw2-remodeled nucleosome dimers.

EXPERIMENTAL PROCEDURES

Nucleic Acid and Protein Procedures. The expression vector for ALL-1 GST-SET polypeptide was prepared by cloning PCR fragment encoding ALL-1 amino acids 3819−3969 between the NdeI/EcoRI sites of the pGEX-2TX-derived plasmid pGEX-2TKN.[4] The full-size (292 bp) 601 DNA template for nucleosome assembly was the large BamH1-BamH1 fragment of plasmid pGEM3Z601R.[21] The 297 bp DNA template containing a minimal 601 sequence at the end of DNA fragment—a NotI/Tsp45I fragment of pGEM3Z601R—was prepared by digestion of large PvuII/PstI fragment of pGEM3Z601R with Tsp45I, then fragment termini were filled with dNTPs/Klenow exo$^-$, and the fragment was redigested with NotI.

To prepare shorter 601 DNA templates, the NotI/NlaIII fragment of pGEM3Z601R, containing the minimal 601 sequence, was cloned between the NotI and XbaI sites of pBluescript II (the NlaIII and XbaI termini were preblunted with Klenow) to give plasmid pBS601. 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 derived from pBS601 or pBS601N using pairs of restriction enzymes that leave a desirable amount of flanking DNA on the ends of the 601 sequence. To prepare DNA templates for dinucleosomes, an additional 601 fragment was cloned in the polylinker of pBS601 or pBS601N; the dimer

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of the 601 sequences was derived using appropriate pairs of restriction enzymes, usually SacI and SacII or *Eco*RI and *Hin*dIII (see also the Supporting Information in ref 22 for the sequences and preparation of DNA templates and construction of the plasmids).

Recombinant GST-fusion proteins were expressed in *E. coli* Origami B strain (Novagen) and purified through immobilization on glutathion-Sepharose (GE Life Sciences).[6] Purified HeLa histones and nucleosomes were prepared as described in ref 23. The yIsw2 complex was isolated from *S. cerevisiae* strain YTT966 through FLAG immunoaffinity chromatography, followed by a 1 mL Source Q-anionic-exchange column similar as described in ref 24, except that after washings of Flag M2 Sepharose (Sigma) with buffer containing 100 mM NaCl and 1 mg/mL of 3x-Flag Peptide, the yIsw2 complex was eluted in a buffer containing 400 mM NaCl without Flag Peptide (see Results and Discussion section). Before loading on Q-Sepharose, the eluate was brought to 100 mM NaCl by dialysis. Isolated protein was examined by SDS-PAGE with subsequent staining with silver reagent (Bio-Rad). The Isw2 ATPase activity was assayed in a 5 μL reaction, containing 50 ng of core histones or DNA or nucleosomes or buffer only, 1 ng of yIsw2, 0.35 μCi of [γ-P^{32}] ATP, and 0.1 mM of cold ATP. The reactions were incubated at 25 μC for 40 min, and 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 Typhoon Scanner (GE/Molecular Dynamics).

Nucleosome Reconstitutions. Nucleosomes were reconstituted by stepwise dilution of a 2 M NaCl mixture of purified histones and DNA (at starting concentrations of 0.25 μg/μL each) as described in ref 23, except the dilution buffer contained 50 μg/μL BSA and glycerol was omitted from the final dilution buffer. The BSA was added in the dilution buffer to prevent adsorption of histones on the tube and to prevent nucleosome aggregation. The addition of BSA has some positive (although small) effect on the efficiency of nucleosome assembly. No major differences were observed between nucleosome reconstitutions in the presence or absence of BSA or glycerol. To remove possible aggregates, reconstituted nucleosomes were centrifuged for 5 min in an Eppendorf centrifuge, and the supernatant was filtered by centrifugation through the top portion of the "blue" 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 1/5th volume of 60% sucrose/0.01% xylene cyanol in 1× TE (pH 7.6) and resolved on 5.5% native PAGE (29:1 acrylamide:bis(acrylamide) 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.

Where indicated, mononucleosomes and nucleosome-dimer particles were purified via native PAGE as below. Usually, the pair of mono and dimer particles was isolated at the same time in parallel. Histone−DNA mixture in 2 M NaCl was gradually diluted to 0.5−0.6 M NaCl as above and then dialyzed against 1× TE buffer, containing 1 mM DTT, 0.5 mM PMSF, and 10% glycerol. (It is important to adjust 80% glycerol stock to pH 7−7.5 with NaOH, as the buffering capacity of dialysis solution is low while even commercial "ultra-pure" glycerol often has acid pH because of accumulation of acrolein.) After electrophoresis (using a comb with a single well), narrow strips were cut from both sides of the gel and stained with ethidium bromide to locate the bands of mononucleosomes and dimer particles. The bands were excised from the gel; gel slices were placed in plastic tubes, crashed on ice with plastic pestle, and then mixed with two volumes of elution buffer [15 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 150 mM NaCl, 3.5 mM $MgCl_2$, 3.5 mM DTT, 0.35 mM PMSF, 75 μg/mL BSA (acetylated BSA was routinely used for extraction as a more inert carrier)] and left overnight at 4 °C on a rotator. Then, the crashed gel was removed by centrifuged for 10 min in an Eppendorf centrifuge, and the supernatant was additionally clarified by centrifugation through aerosol-tight tips, as above. The samples were concentrated with Millipore Ultracel-100 filter devices and adjusted to similar protein content. To cross-compare histone concentration, sample aliquots were extracted with 0.75% TCA (or 0.35 M HCl and 0.75% TCA) and titrated with Bradford reagent according to Bio-Rad microassay for microtiter plates (PCR microtubes were often used instead of microplates, and absorbency was measured in 25 μL cuvette). For a rapid comparison of protein content, a modified procedure of Bradford microplate assay was also used: 1−3 μL aliquots of acid-extracted samples were spotted on Whatman 3MM paper, paper strips were dried at 65 °C, sequentially rinsed in 80% ethanol and acetone, and dried; then 10−15 μL of Bradford reagent was spotted on top of each protein spot. After few minutes' incubation, paper strips were washed in 5% ethanol−7% acetic acid at 65 °C. This assay allows detecting up to 10 ng of protein in a spot with at least 20% accuracy of such estimate.

Nucleosome Remodeling and GST-SET Pull-Down Assays. 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 MgCl2, 2 mM DTT, 0.025% NP-40, 50 μg/mL BSA, and 0.5 mM PMSF], supplemented with 1 mM ATP and additional 2.5 mM $MgCl_2$. To avoid possible interference with the carrier oligo- or mononucleosomes, the carrier nucleosomes were omitted. Instead, the nucleosome probe of interest comprised the total chromatin content in the reaction (about 10 ng/μL of DNA). Higher concentrations of nucleosomes, smaller amounts of Isw2, and NaCl concentrations of 100−110 mM should emphasize a catalytic function of yIsw2. Typically, 10 μL remodeling reactions contained 100 ng of assembled DNA templates and about 4 ng of yIsw2 complex or 8 ng of ySwi/Snf complex. After incubations at 25 °C for 1.5 h, a 5−7 μL reaction aliquots were resolved on native gel. Nucleosome-binding assays were performed similar as described in ref 6. Remodeling reactions were scaled to 50 μL, 6 μL aliquots of remodeled samples were resolved on gel, and the rest of reactions were incubated with 10 μg of immobilized GST-SET for an additional 1 h. After 3× washings in a 500 mM NaCl buffer,[6] the beads were extracted with phenol−chloroform, DNA was ethanol-precipitated, resolved on 5.5% native PAGE, and stained with ethidium bromide.

To examine nucleosome structure with MNase (Worthington Biochemical), remodeling reactions were scaled up 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 amounts of MNase. DNA was isolated and resolved in 6% native PAGE (19:1 acrylamide:bis(acrylamide) ratio), and the gels were stained with ethidium bromide.

RESULTS AND DISCUSSION

Purification and Analysis of the Yeast Isw2 Complex. Yeast version of the enzyme is easier to purify in significant

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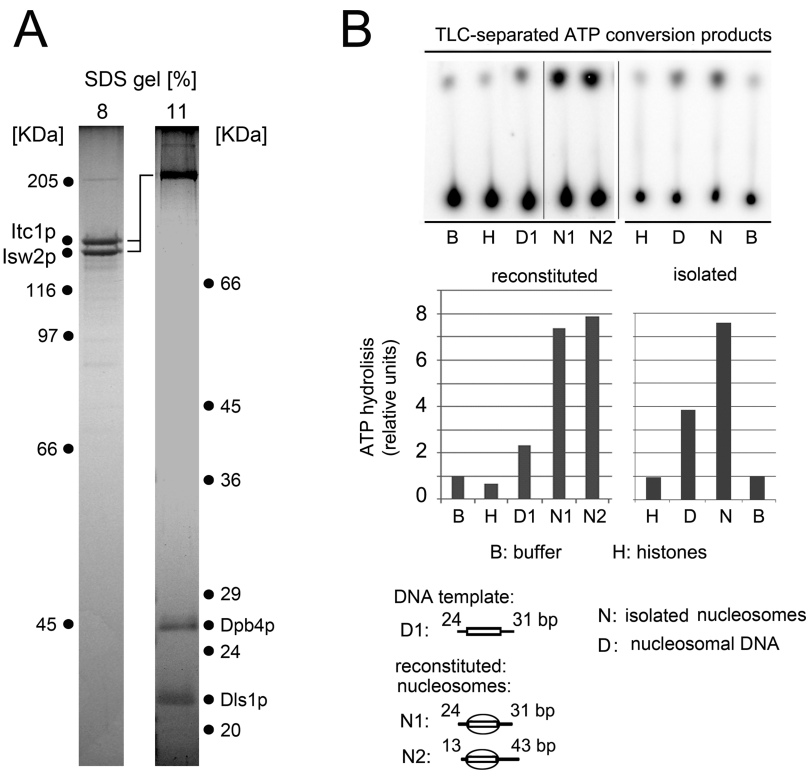

Figure 1. (A) Purified yIsw2 complex, resolved on SDS-PAGE and stained with silver reagent. (B) ATPase activity of isolated yIsw2 complex with: N1, N2 = reconstituted mononucleosomes, D1 = template DNA only, N = mononucleosomes isolated from HeLa cells, D = DNA, purified from isolated nucleosomes. The rectangles and rectangles in ovals indicate the minimal 601 positioning sequence and the 601 positioned nucleosomes, respectively; the numbers show the sizes (base pairs) of extranucleosomal DNA.

amounts, and others have shown that it works similarly to its mammalian counterparts.[7] The yIsw2[24,25] was isolated from *S. cerevisiae* cell extracts through the Flag-tagged catalytic subunit, Isw2p protein, with subsequent purification on Q-Sepharose.[24] In our experience, elution of Flag M2 beads with 3xFlag peptide in 100 mM NaCl buffer (as in ref 24) liberates only 10% of beads-associated complex; in addition, eluted fractions contain significant amounts of contaminating proteins and Isw2 degradation products. However, additional washings of M2 beads with 350−400 mM NaCl buffer with no Flag peptide elutes most of the Isw2 with stoichiometric ratio of subunits—this Isw2 fraction is relatively pure and in most cases does not require further purification. SDS gel analysis of the purified complex revealed that it contains both high-molecular-weight subunits Isw2p and Itc1p and faster migrating "histone-fold" components Dpb4p and Dls1p (Figure 1A). The ATPase activity of the yIsw2 complex was assayed in the presence of free core histones, naked DNA and mononucleosomes, both reconstituted by salt dilution or isolated from HeLa cells (Figure 1B). ATPase hydrolysis was not stimulated significantly by free histones but was strongly stimulated by nucleosomes, implying that purified Isw2 complex was able to recognize special structural features of nucleosomes. Our Isw2 preparations exhibited relatively high DNA-dependent ATPase activity, consistent with previous observations,[24,26] and is likely explained by ATP hydrolysis when transiently bound Isw2 complex dissociates from DNA.

Formation of Noncovalent Nucleosome-Dimer Particles during Nucleosome Reconstitution by Gradual Salt Dilution. Nucleosomes were assembled using purified HeLa cell histones and DNA of the full-size "601" high-affinity nucleosome positioning sequence.[27] The full-size 601 DNA consists of a "minimal" 146 bp nucleosome positioning sequence flanked by 65 and 73 bp DNA segments, which further enhance the ability of the core 601 DNA to position a histone octamer. Nucleosomes were reconstituted by stepwise dilution of 2 M NaCl mixture of histones and DNA to a final concentration of 100−110 mM NaCl. The assembled mononucleosomes migrate largely as a single species on a native PAGE, indicative of a histone octamer strictly positioned nearly in the center of DNA template (Figure 2A, lane 1). For comparison, lane 2 in Figure 2A shows nucleosomes assembled on a similar-size DNA, in which the core 601 sequence is located on the end of DNA fragment. Such "asymmetric" nucleosomes migrate on a gel faster than the above "symmetric" nucleosomes, consistent with the observation that nucleosome electrophoretic mobility is greater when a histone octamer is located close to the end, than to the center of a DNA[28] due to the bend the DNA forms at the entry−exit point of the nucleosome.

When nucleosomes were assembled using an elevated histone:DNA ratio (starting from 0.55 to 0.7 μg of histones per 1 μg of DNA template or ~0.5 μg of minimal 601 DNA), the electrophoretic pattern of resulting nucleosomes was resolved into two major bands: one that had the same mobility as the regular mononucleosomes (N1) and a second, slower migrating band N2 (Figures 2B). A further increase of histone−DNA ratio leads to formation of bands N3 and D. Band D was formed at more than 2-fold (mass) excess of histones over the amount of core 601 DNA in the template (Figure 2B, lane 3). We assume the species marked D is a dinucleosome: the template is long enough to accommodate two histone octamers, providing that the initial centered octamer will be relocated to

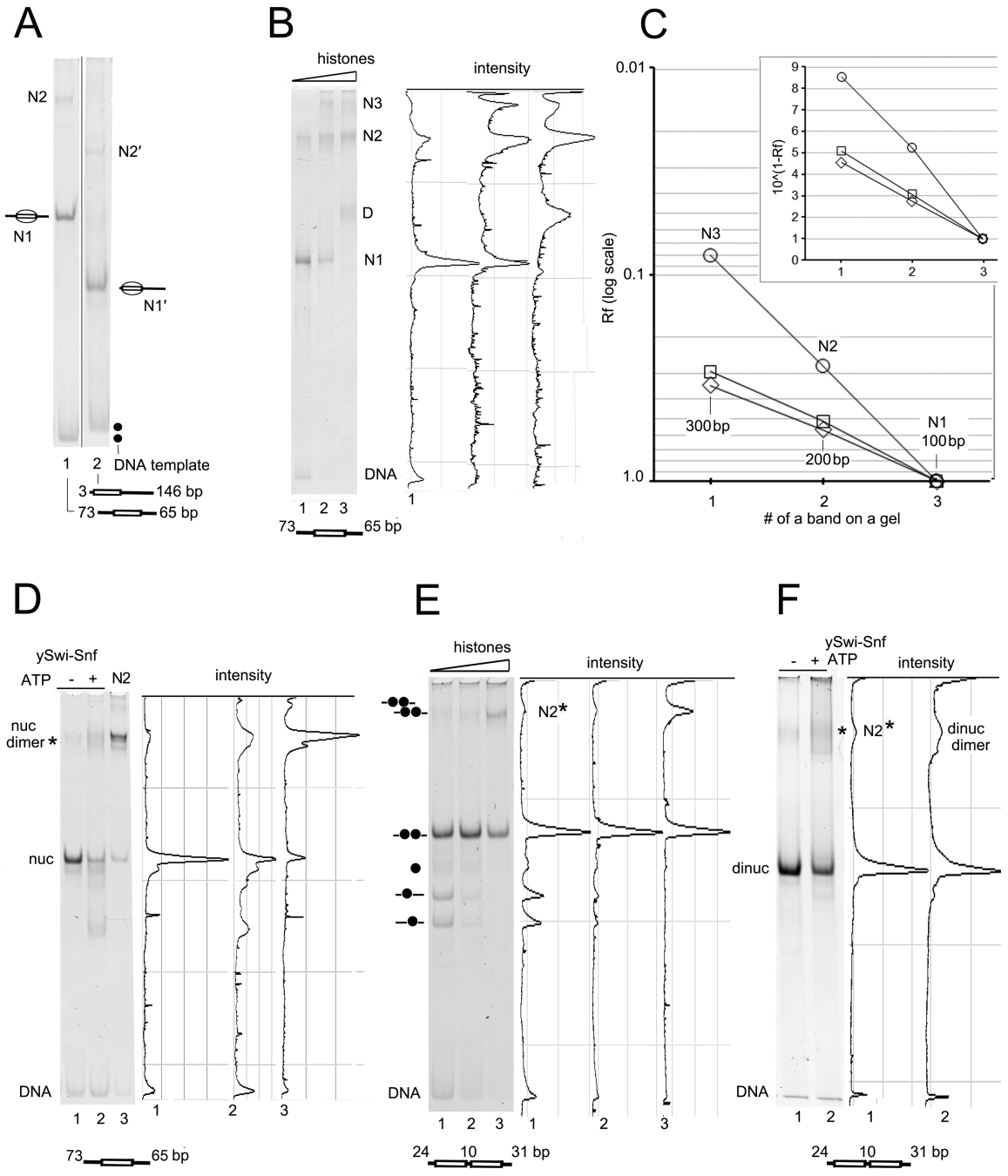

Figure 2. (A) Ethidium-stained native PAGE of nucleosomes, assembled on DNA templates with the minimal 601 sequence in the center or at the edge of the DNA fragment (depicted at the bottom). The rectangles and rectangles in ovals indicate the minimal 601 positioning sequence and the 601 positioned nucleosomes, respectively; the numbers show the base pairs of extranucleosomal DNA. N1, N1′ and N2, N2′ indicate the nucleosomes and nucleosome-dimer particles, respectively. (B) Ethidium-stained native PAGE of nucleosomes assembled using 0.25 μg of full-size 601 DNA and 0.16, 0.2, 0.24 μg of HeLa histones (lanes 1−3, respectively). Resulting nucleosome species: D = dinucleosomes, N1 = mononucleosomes, N2 and N3 = nucleosome multimers. Gel scan profiles are shown at right. (C) Main panel: relative electrophoretic mobilities (Rf) of nucleosome species plotted in a logarithmic scale versus band number (in the order of migration); it is presumed that these bands are integral multiples of monomeric precursor. Graph in the inset shows calculated values $10^{(1-Rf)}$, which are proportional to the relative MW (number of monomers) of the complexes. (○) shows nucleosome species; for comparison (□) and (◇) show the mobilities of 100, 200, and 300 bp bare DNA on 5% PAGE and sequencing gels, respectively. (D) Gel mobility-shift assay of ySwi/Snf-remodeled mononucleosomes (lanes 1, 2) and gel-purified species N2 (lane 3). (E) Ethidium-stained PAGE of dinucleosomes assembled using 2.5 μg of 2× 601 DNA template (depicted at bottom) and 1.38, 1.63, 1.88 μg of HeLa histones. (-●●-) and (-●−/−●-) indicate nucleosomes assembled on both or one or the other 601 sequence, respectively; (●) = unpositioned mononucleosomes. (F) Gel mobility-shift assay of ySwi/Snf-remodeled dinucleosomes (lanes 1, 2).

the end of the template. Alternatively, the second octamer might be half-covered by each of the DNA tails surrounding the 601 core sequence. This may explain the relatively inefficient formation of dinucleosomes on the full-size 601 DNA when compared to the analogous formation of dinucleosomes (D) on similar-size DNA with an eccentrically positioned 601 core sequence (Figure S1A of the Supporting Information). The left panel on Figure S1B shows native PAGE of DNA fragments,

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purified from the MNase-digested species D formed on the asymmetric 601 template. The electrophoretic pattern is suggestive of two closely packed nucleosomes on one piece of DNA: predominantly, the entire DNA template (indicated by the filled circle) is protected at lower MNase concentrations, and the nucleosome core-/subcore-size DNA (blank circle) is released at higher nuclease concentrations. For comparison, the right panel on Figure S1B shows the MNase digestion profile of mononucleosomal DNA: in this case shorter DNA is liberated at lower MNase concentration and the mononucleosome/histone termini protected DNA is also visible (indicated by asterisks). The electrophoretic patterns of DNA purified from the MNase-digested mononucleosomes and species N2, formed on the full-size 601 DNA, are similar (Figure 4C), suggesting that species N2 are not dinucleosomes.

It has been shown that at elevated (0.5−0.6 M) NaCl concentration nucleosome particles compete for an extra histone octamer, such that the core particle can co-operatively associate with an additional histone octamer[16,29−32] or an additional nucleosome.[16,30,33] These nucleosome-dimer/octamer particles are likely formed by similar mechanism ("histone octamer migration"[29]), which involves trans interactions between histone tetramers. At low histone−DNA ratios during nucleosome assembly, an excess of bare DNA efficiently competes with nucleosomes for histone octamers and mononucleosomes are mostly formed. At higher histone−DNA ratios, when template DNA is limiting, mononucleosomes start to associate with an extra histone octamer or another nucleosome. A nucleosome particle can bind more than one histone octamer or a nucleosome with a weaker association constant[29,31]—this may also result in formation of nucleosome multimers.[33] In view of the above, it would be more correct to postulate that formation of nucleosome-dimer particles is stimulated by the absence of free template DNA, but not the presence of excess histone octamers as such, as one can think of no reason why a higher concentration of free octamers would promote the interaction of two normal mononucleosomes.

Thus, the low-mobility bands N2 and N3 might be multimers of the nucleosomes or nucleosomes to which additional histone octamers have bound. These bands do in fact behave electrophoretically like multimers of some precursor. This can be demonstrated by assuming the bands have integral multiples of a monomer and then plotting the relative multimer mobility (Rf) in logarithmic scale versus the band number (in the order of band migration). A linear plot is obtained which suggests that the low-mobility bands are multimers either of a nucleosome or of some structure derived from it (Figure 2C). The linear relationship suggests a constant charge/mass/shape ratio, arguing against the proposition that these structures are simply nucleosome particles with added histones. If the latter were the case, one would expect a varying charge mass ratio and thus a nonlinear behavior in the plot. In addition, the amount of DNA associated with gel-purified mononucleosomes N1 and particles N2, estimated via ethidium bromide staining, is close in these two species (Figure 4A and Figure S1C). The somewhat less efficient EtBr staining of nucleosome dimers could be a result of a higher protected of DNA in dimer particles.

It has been reported that the Swi/Snf chromatin-remodeling complexes, human BAF/PBAF, yeast RSC, and Swi/Snf, can catalyze formation of noncovalent dimers from mononucleosomes in vitro.[22,34,35] Figure 2D shows that the gel-purified species N2 comigrates with the dimeric nucleosomes generated from mononucleosomes by ySwi/Snf and ATP (see also ref 22). Figure S1D shows a similar band comparison when some assembly reaction dimer N2 was present before ySwi/Snf remodeling—the same band increased after remodeling, indicating that N2 species and ySwi/Snf-generated dimers have similar electrophoretic mobility. This suggests that reconstituted nucleosome-dimer particles have similar shape and charge/mass ratio as two dimerized mononucleosomes. This conclusion, though, might be complicated by the fact that Swi/Snf complexes can also transfer histone octamers from one nucleosome to other DNA, thus potentially forming dinucleosomes by adding an octamer to a mononucleosome. However, if the latter were the case under the conditions employed, one would expect an increase in the amount of bare template DNA in the Swi/Snf-remodeled samples, as histone octamers from a portion of assembled mononucleosomes will be transferred to other nucleosomal templates (forming two histone octamers on one piece of DNA and bare DNA template). However, the described Swi/Snf remodeling reactions were not accompanied by significant liberation of bare template DNA (Figure 2D and Figure S1D; see also ref 22) that argues against the ySwi/Snf-directed histone octamer transfer reaction.

When nucleosomes were assembled on DNA with two 601 core sequences, the electrophoretic pattern resulted in a set of bands: the major bands, which correspond to nucleosomes occupying only one (-●−/−●-) or both (-●●-) positioning sequences, and some lower-intensity bands (●), corresponding to unpositioned mononucleosomes (Figure 2E). If, under the conditions employed, assembled nucleosomes would adsorb excessive histone octamers upon increase of histone:DNA ratio, one would expect formation of several extra bands of lower mobility, corresponding to the association of histone octamers with only one or both assembled nucleosomes, respectively. However, further increase of histone:DNA ratio results in formation of only one slowly migrating species N2*, which likely corresponds to two dimerized dinucleosomes (associated via nucleosome−nucleosome link, as schematically depicted), since the band N2* comigrates with ySwi/Snf-catalyzed altered dinucleosome dimers (Figure 2F; see also ref 22).

The above data suggest that the slowly migrating band N2 corresponds to nucleosome-dimer particle. It has been reported[29,31] that nucleosome-octamer particles tend to aggregate at physiological salt and the experiments with nucleosome-octamer particles require the presence of more than 0.45 M NaCl. That could be one of the reasons why the reconstitution/reassembly reactions terminating at physiological salt, as in the experiments described, provide mostly dimerized nucleosomes instead of nucleosome octamers.[30,33] The stronger association of histone octamer with the high-affinity 601 sequence could also favor formation of nucleosome-dimer particles instead of nucleosome octamers. The previous studies reporting insolubility of nucleosome-octamer particles in 100 mM salt had used different DNA sequences and different template lengths and/or assembly conditions (the 601 DNA was not discovered at that time) which complicates direct comparison between this and previous studies. However, nucleosome-dimer particles could also be formed with nucleosomes assembled on shorter 601 DNA templates, as well on the sea urchin "5S"[36] or MMTV "nucleosome B"[37] positioning sequences (Figure S1E). The leftmost panel in Figure S1E shows that nucleosome-dimer particles, formed on shorter 601 template, also comigrate with the Swi/Snf-generated mononucleosomes dimers (ref 22 includes more data on the comparison of electrophoretic mobility of the ySwi/Snf-generated dimers and the "assembly-reaction" dimers,

formed on various 601 DNA templates). The above data suggest that the "assembly-reaction" nucleosome-dimer particles are not very specific to the employed "full-size" 601 DNA template and provide some support to justify the comparison of nucleosome dimer formation in this and earlier studies. Although Yager et al.[33] concluded that nucleosome multimerization is more efficient when the salt is removed rapidly from the initial mixture of histones and DNA in high salt ("jump-dilution" technique), it had also been shown that nucleosome-dimer particles are formed efficiently when nucleosomes are reconstituted by gradual dialysis in the presence of excess of histones.[30]

Assembled mononucleosomes do not spontaneously dimerize in physiological salt, so nucleosome-dimer particles are likely to represent more than two pieces of DNA that each bind part of the surface of two octamers. Although little is yet known about the precise structure of nucleosome-dimer/octamer particles formed during nucleosome assembly/refolding, it can be presumed that the basic nucleosomal organization is not significantly altered in these structures. As such, neither the nuclease digestion kinetics nor the nuclease protection pattern is considerably altered[29,38] (see also the MNase-protection patterns in Figure 4C). In addition, the difference in the relative electrophoretic mobility of mononucleosomes with symmetrically and asymmetrically positioned histone octamer (Figure 2A; bands N1 and N1′, respectively) is preserved in nucleosome dimers (bands N2 and N2′, respectively), suggesting that the shape of constituent nucleosomes is not significantly disturbed.

SET Domain of ALL-1 Methyltransferase Possesses Increased Affinity to Isw2-remodeled Nucleosome-Dimer Particles. Human ALL-1/MLL protein is a Trithorax group HKMT[39] which functions as a transcriptional maintenance factor for developmentally regulated genes. The SET-domain polypeptide of ALL-1 can efficiently bind bare histone H3−H4 tetramers, but not histones in nucleosomes,[20] suggesting that nucleosomes must be structurally altered to associate with SET domain.

The interactions of nucleosomes with the ALL-1 SET domain were examined in a pull-down assay using the GST-tagged 151-amino acid C-terminal polypeptide of ALL-1, immobilized on Glutathione-Sepharose. To compare GST-SET affinity to Isw2-remodeled mono- and dimeric nucleosomes, we evaluated binding of GST-SET to nucleosome samples with increasing amounts of assembled nucleosome-dimer particles. Figure 3A shows native PAGE of nucleosomes samples and a diagram of the relative content of mononucleosomes and nucleosome-dimer particles. Nucleosome samples were incubated for 1.5 h with purified Isw2 and ATP and then mixed on a rotator with GST-SET beads for 1 h. Reacted beads were washed in a buffer containing 0.5 M NaCl/0.2% NP-40. Bound nucleosomes were extracted with phenol−chloroform; deproteinized DNA was ethanol-precipitated, resolved on gel, and stained with ethidium bromide. The amount of pulled-down nucleosomes increased in proportion to the relative content of nucleosome dimers in the sample (Figure 3B), suggesting that Isw2-remodeled nucleosome dimers have higher affinity to the GST-SET polypeptide than Isw2-remodeled mononucleosomes or unremodeled nucleosome species. Immobilized GST or the GST-SETΔ polypeptide, which lacks the histone-binding region, binds nucleosomes inefficiently (Figures 3C and 3D, respectively).

Reconstituted nucleosome-dimer particles are relatively stable in 0.1 M NaCl and could be purified from the assembly mixture via native PAGE (Figure 4A). However, some amount of mono- and dimerized nucleosomes were present in gel-purified samples of dimeric nucleosomes and mononucleosomes, respectively. This could be due to dimerization of mononucleosomes and dissociation of nucleosome dimers during storage and processing. In our experience, dissociation of nucleosome-dimer particles was noticeably stimulated by freezing−thawing of the samples; therefore, the samples were routinely stored on ice.

Incubation of gel-purified mononucleosomes with yIsw2 and ATP generated a set of species which migrated on a gel faster than the original nucleosomes (Figure 4B, lanes 1, 2). These bands likely correspond to nucleosomes in which a histone octamer has slid from its original central position to the ends of DNA fragment.[28] For comparison, lanes 6, 7 in Figure 4B show nucleosomes assembled on a template with the 601 sequence at the end of DNA fragment. In this case yIsw2 generated a set of slowly migrating bands, extending up to the band of nucleosomes with a centrally positioned histone octamer (lane 5); i.e., in this case histone octamers sliding occurs in the opposite direction: to the center of DNA template. Incubation of nucleosome-dimer particles with Isw2 and ATP did not significantly affect their migration on a native gel, except that the band of remodeled dimers became somewhat more diffuse (Figure 4B, lanes 3, 4). This could be explained by more restricted sliding of histone octamers in dimeric nucleosomes. In addition, the electrophoretic patterns of DNA purified from the MNase-digested nucleosome species, remodeled by yIsw2 and ATP (Figure 4C), revealed no significant remodeling-related alterations, except the MNase protection pattern of Isw2 remodeled mononucleosomes appeared somewhat more diffuse. This, could also be explained by increased sliding of histone octamers in mononucleosomes compared to nucleosome-dimer particles.

Gel-purified mononucleosomes and nucleosome-dimer particles were incubated with yIsw2 with or without ATP for 1.5 h at 25 °C in a total volume of 50 μL. Then 6 μL aliquots were resolved on native PAGE and stained with ethidium bromide (Figure 4D, left panel). The rest of the reactions were incubated with GST-SET beads for an additional 1 h, and then the beads were washed in 0.5 M NaCl/0.2% NP-40 buffer and extracted with phenol−chloroform. Extracted nucleosomal DNA was resolved on gel and stained with ethidium bromide (Figure 4D, at right). The amount of bound nucleosomes was appreciably higher in case of remodeled nucleosome dimers than in case of remodeled mononucleosomes or non-remodeled mono- and dimeric nucleosomes. In addition, nucleosome-dimer particles per se bind GST-SET somewhat more efficiently than mononucleosomes (lanes 1 vs 3, respectively), possibly the result of structural alterations of mononucleosomes upon dimerization. To address whether ongoing Isw2 remodeling is required to facilitate association of nucleosome dimers with GST-SET, the remodeling reaction was stopped by depleting ATP with apyrase before binding to the GST-SET (Figure 4E). However, this did not significantly change binding of remodeled nucleosome dimers to GST-SET, indicating that Isw2-remodeled nucleosome-dimer particles could preserve their altered structure in the absence of the active remodeling complex.

The ySwi/Snf-remodeled nucleosome-dimer particles also exhibit an increased affinity to the GST-SET polypeptide of ALL-1 protein (Figure 5); thus, the experiment with yeast Swi/Snf shows that this effect can also be created by other remodeling complexes. It is notable, that the ySwi/Snf-generated altered nucleosome dimers, as such, do not possess an increased affinity to ALL-1 GST-SET polypeptide, when compared to

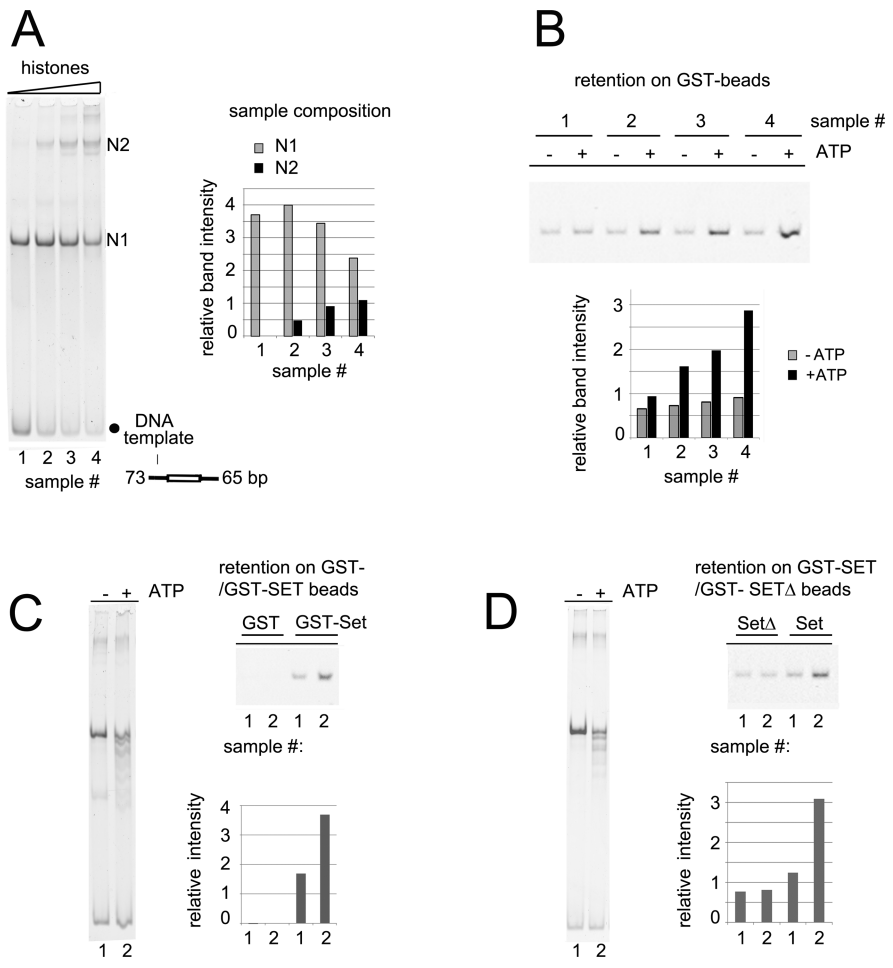

Figure 3. (A) Ethidium-stained native PAGE of nucleosomes assembled using 1 μg of full-size 601 DNA and 0.45, 0.53, 0.6, 0.7, and 0.8 μg of histones, lanes 1−5, respectively. The graph at right shows relative amounts of mononucleosomes and nucleosome-dimer particles in the samples. (B) Samples with equalized total amounts of nucleosomes (monomeric plus dimeric) were incubated for 1.5 h with yIsw2 with or without ATP; then samples were additionally incubated for 1 h with immobilized GST-SET polypeptide. Next, GST-SET beads were washed, bound nucleosomes were phenol-extracted, deproteinized DNA was ethanol-precipitated, resolved on gel and stained with ethidium bromide. (C, D) Isw2-remodeled nucleosome samples (lanes 1 and 2) were incubated for 1 h with (C) immobilized GST-SET and immobilized GST or (D) GST-SET polypeptide, lacking most of the histone-binding region (SETΔ, contains amino acids 3819−3850 of ALL-1). After washings, beads were phenol-extracted; deproteinized DNA was resolved on gel and stained with ethidium bromide.

mononucleosomes.[20] This suggests that despite the apparent similarity between the nucleosome-dimer particles and Swi/Snf-generated dimers—in both cases these are two associated mononucleosomes—these structures are not exactly the same. This is consistent with the observations that the footprint of DNA fragments protected from MNase in Swi/Snf-altered nucleosome dimers is distinct from that of normal mononucleosomes.[22,34,35] However, some amount of the ∼100 bp fragment resulting from MNase digestion of nucleosome dimer products (Figure 4C) is in fact diagnostic of SWI/SNF-generated dimers.[33,34] This suggests that these two species might have some similar structural elements. For now, we cannot conclude with certainty why the Swi/Snf-remodeled nucleosome-dimer particles exhibit an increased affinity to ALL-1 GST-SET, while the altered dimers, generated by Swi/Snf from mononucleosomes, do not. It might be presumed that by some reason ySwi/Snf cannot convert nucleosome-dimer particles to altered dimers.

The observed increase in the SET-domain affinity to yIsw2-remodeled nucleosome dimers was only incremental. However, the fact that Isw2 remodeling of nucleosome dimers increases ALL-1 accessibility without grossly altering the mobility of the dimers or causing them to fall apart to mononucleosomes suggests that Isw2 does something to the surface of nucleosome dimers that it cannot do to mononucleosomes that makes the H3 tails accessible to ALL-1. For example, this could be because the more "open" (but unstable) remodeling intermediates of individual nucleosomes are stabilized by the interaction of histone octamers in a dimer particle.

Although only incremental in vitro, this effect could play a role in the targeting of SET domains to chromatin in vivo. The nucleosome-octamer or nucleosome-dimer particles may be considered as artifacts of in vitro nucleosome reassembly in high salt solutions. However, these structures form very efficiently. Stein has estimated that ∼25% of nucleosomes during assembly/refolding are involved in the histone octamer migration mechanism.[29] Therefore, it may be proposed that these interactions could be of biological significance and nucleosome dimer-particles could function as intermediates in biological processes. For example, these structures may play a role in processes involving reassembly of chromatin structure, such as DNA replication or transcription.

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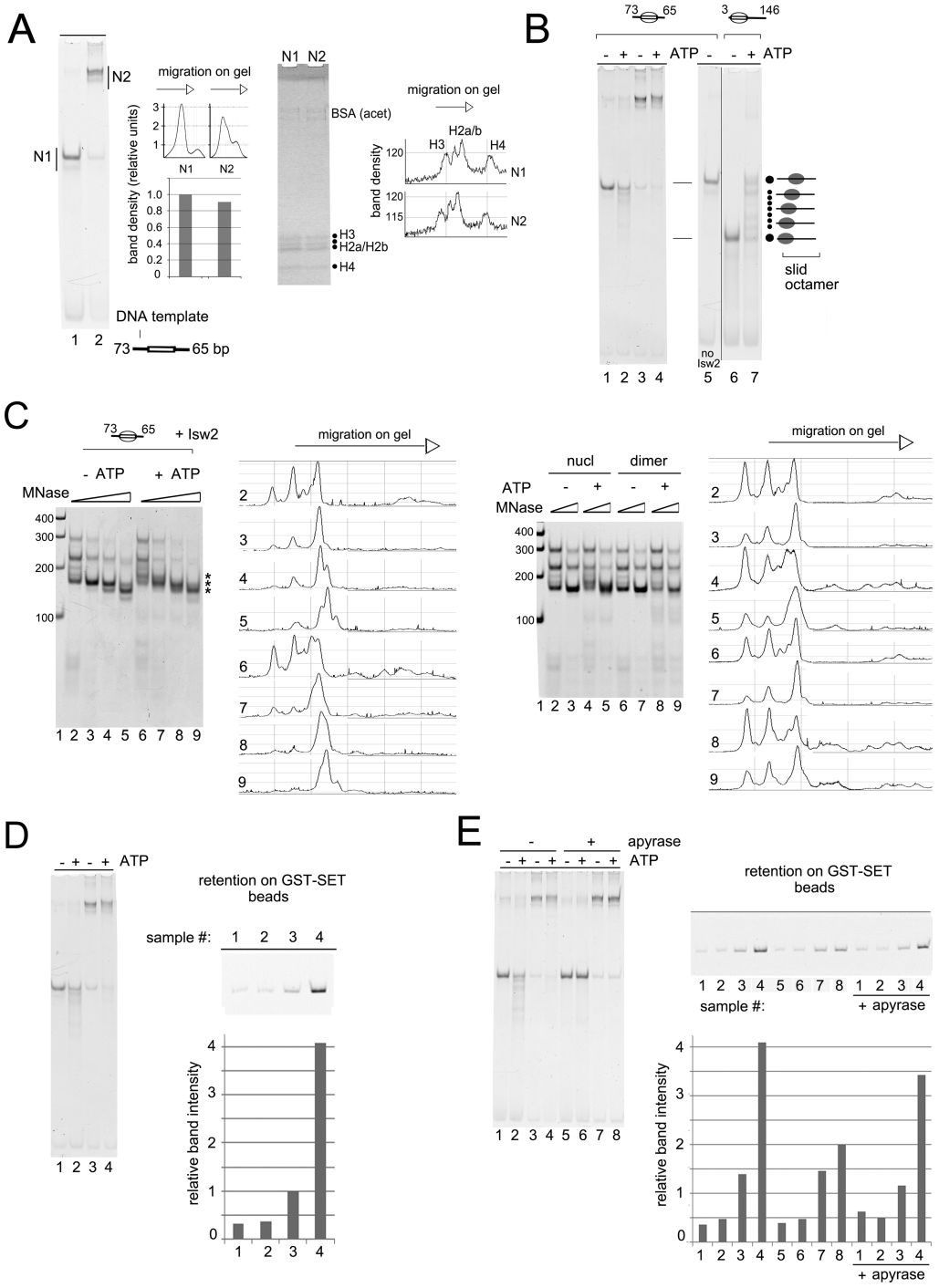

Figure 4. (A) The panel at left shows ethidium-stained native PAGE of gel-purified mononucleosomes (lane 1) and nucleosome-dimer particles (lane 2), assembled on full-size 601 sequence. Densitometry tracing of band regions and quantitative estimation of bands is shown at right (after gel extraction, the samples were adjusted to similar protein content according to estimations with Bradford reagent). The panel at right shows acid-extracted histones from isolated nucleosomes (N1) and nucleosome-dimer particles (N2). (B) Gel-purified mononucleosomes (lanes 1, 2) and nucleosome-dimer particles (lanes 3, 4) were remodeled with yIsw2 with or without ATP. Lanes 6, 7 show yIsw2-remodeled nucleosomes, assembled on similar size DNA which contain minimal 601 sequence at the end of DNA fragment. (C) Ethidium-stained PAGE of DNA fragments purified from the MNase-digested (left panel) yIsw2-remodeled vs unremodeled mononucleosomes, and (right panel) yIsw2-remodeled vs unremodeled mononucleosomes and nucleosome dimer particles. Scan profiles are shown at right of the gels. (D) Gel-purified mononucleosomes (samples 1, 2) and nucleosome-dimer particles (samples 3, 4) were remodeled for 1 h with yIsw2 and ATP. Then, reaction aliquots were resolved on native gel (left panel) and the rest of reactions were additionally incubated for 1 h with GST-SET beads. Reacted beads were washed, bound nucleosomes were deproteinized by phenol extraction, and DNA was resolved on gel (right panel). (E) Gel-purified mononucleosomes (samples 1, 2 and 5, 6) and nucleosome-dimer particles (samples 3, 4 and 7, 8) were incubated for 10 min with ATP and 50 mU of apyrase as indicated (samples 1−4 were scaled up 2-fold compared to samples 5−8). Then all samples were incubated with yIsw2 for 1.5 h. Left panel shows ethidium-stained native PAGE of reaction aliquots. Remodeled samples 1−4 were divided in half, and 50 milliunits of apyrase were added to one of the parts. After 10 min incubation, all samples were mixed with GST-SET beads and incubated for an additional 1 h; then the beads were washed, bound nucleosomes were extracted by phenol−chloroform, and DNA was resolved on gel (right panel).

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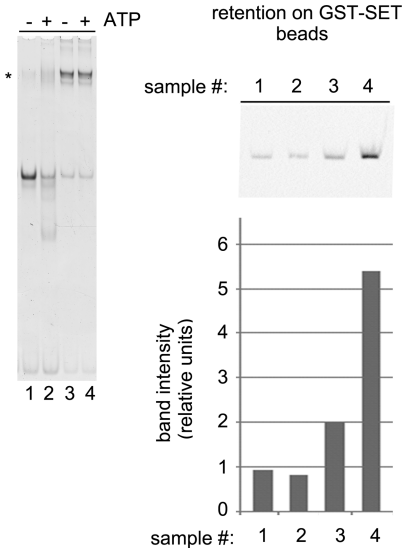

Figure 5. Gel-purified mononucleosomes (lanes 1, 2) and nucleosome-dimer particles (lanes 3, 4) were remodeled for 1.5 h with ySwi/Snf and ATP. Then, reaction aliquots were resolved on native gel (left panel), and the rest of reactions were additionally incubated for 1 h with GST-SET beads. Reacted beads were washed, bound nucleosomes were deproteinized by phenol extraction, and DNA was resolved on gel (right panel).

At present, there is no information about how the results on mononucleosome dimers might relate to the (theoretical) mononucleosome octamers formed in vivo (in the processes, where displaced histone octamer may reassociate intact with a nearby nucleosome). However, nucleosome dimers could represent a model which can reveal at least some of the properties of the nucleosome-octamer particles—a model that can be studied in physiological salt solutions.

The properties of excess histone adsorption by chromatin and octamer migration suggest an efficient mechanism for nucleosome assembly during replication. In this respect it is of interest that Isw2 has recently been shown to participate in replication fork progression.[40] In addition, these interactions could play a role in the targeting of SET domains to nucleosome in compacted nucleosome arrays in chromatin fibers, where the nucleosomes are brought into close proximity. It is notable that the published literature suggests a role for ISWI complexes in the regulation of higher-order chromatin structure and transcription in vivo, although the mechanism of this process remains not fully understood.[41]

■ ASSOCIATED CONTENT

Supporting Information

Ethidium-stained PAGE of nucleosomes assembled on "asymmetric" 601 DNA template at variable histone:DNA ratio (Figure S1A); MNase protection patterns of di- and mononucleosomes, assembled on "asymmetric" or full size 601 DNA, respectively (Figure S1B); ethidium-stained PAGE of gel-purified mono- and dimeric nucleosomes and quantitative estimation of their ethidium fluorescence (Figure S1C); native PAGE of ySwi/Snf-remodeled mononucleosomes, assembled on full-size 601 DNA (Figure S1D); gel mobility-shift assay of ySwi/Snf-remodeled mononucleosomes, assembled on the "minimal" 601 DNA, and nucleosome assembly on sea urchin "5S"[36] or MMTV "nucleosome B"[37] DNA templates (Figure S1E). 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

aa, amino acids; bp, base pairs; BSA, bovine serum albumin; EtBr, ethidium bromide; HMT, histone methyltransferase; HKMT, histone lysine methyltransferase; MNase, micrococcal nuclease; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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