Inverted Factor Access and Slow Reversion Characterize SWI/SNF-altered Nucleosome Dimers*

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Human SWI/SNF (hSWI/SNF) is an ATP-dependent chromatin remodeling complex that can use the energy of ATP hydrolysis to structurally alter nucleosomes or change their positions on DNA (for reviews, see Refs. 1 and 2). hSWI/SNF functions as a transcriptional coactivator for a large number of human transcription factors, including most nuclear hormone receptors, p53, AP-1, EKLF, MyoD, and NeuroD (for recent review, see Ref. 3). hSWI/SNF functions as a tumor suppressor, because it promotes cell growth arrest, and because several subunits of the complex are mutated in human cancers (for review, see Ref. 4). This tumor suppressor function may arise from a hSWI/SNF requirement for the expression of the p21 cdk/cyclin inhibitor (5, 6) and for the repression of E2F-activated genes by Rb (7, 8).

In vitro studies have shown that human SWI/SNF remodels chromatin in an ATP-dependent manner (for reviews, see Refs. 2 and 9). Some of the effects of hSWI/SNF (such as restriction enzyme accessibility) are primarily transient, requiring continued action of the complex. Other hSWI/SNF effects, including changes in nucleosome positions and structures, are stable after removal of hSWI/SNF or ATP from the reaction. First, we and others have shown that hSWI/SNF stably alters nucleosome positions on polynucleosomal arrays and mononucleosomes (10–13). Nucleosome repositioning could influence transcription by moving nucleosomes over or away from regulatory sequences. Second, hSWI/SNF acts in a catalytic manner to generate structurally altered nucleosomes. On mononucleosomal templates hSWI/SNF can connect two mononucleosomes together in an altered noncovalent dimer, which can be readily isolated and characterized (13, 14). A conserved function for altered dimers in eukaryotic transcriptional regulation is highlighted by the ability of the related yeast RSC complex to form indistinguishable dimeric products (15). Altered dimers formed from mononucleosomes bearing a central binding site for the Gal4 transcription factor or RAG recombination proteins had a higher affinity for these factors than normal nucleosomes (13, 16), suggesting that dimers or similar structures might function to facilitate factor access in chromatin. It was unclear from these studies, however, whether altered dimers were more accessible only in the centers of the nucleosomal templates used to form them, or if they represented a generally more accessible nucleosomal species.

Each normal nucleosome constrains one negative supercoil of DNA, and hSWI/SNF action stably reduces the number of constrained negative supercoils held by closed-circular plasmid chromatin (14, 17). This reduced supercoiling is not due to histone loss, but to the formation of metastable altered nucleosomal structures, because it reverts to normal on a time scale of several hours even in the presence of competitor DNA that would sequester any histones that might have been removed during remodeling (18). Evidence that hSWI/SNF forms structurally altered nucleosomes on polynucleosomes also comes from our atomic force microscopy experiments, where hSWI/SNF-remodeled polynucleosomes were less stable to surface deposition when unfixed than control polynucleosomes (13). More recently, we found that hSWI/SNF converts ~30% of adjacent pairs of nucleosomes on polynucleosomal arrays into altered dinucleosomes called “altosomes” (19). These products appear to be responsible for the reduced negative supercoiling observed on plasmid chromatin, because both effects are of similar magnitude and revert to normal at the same rate. Unfortunately, because altosomes only form on polynucleosomal DNA we have been unable to isolate a pure population of altosomes associated with specific DNA sequences. As a result the potential function of hSWI/SNF-generated altosomes in regulating transcription factor accessibility is unknown.
Here we further examine the properties of altered mononucleosome dimers formed by hSWI/SNF action. We find that the accessibility of the DNA in altered dimers for Gal4 binding is the opposite of that for normal mononucleosome cores: highest in the middle of the template and lowest at the ends. We also find that altered dimers revert to normal mononucleosomes similarly to altosomes formed on polynucleosomes. This observation, together with other shared properties, suggests that dimers and altosomes have similar structures. These results help inform models for altered dimer as well as altosome structures and suggest potential functional properties of altered hSWI/SNF product nucleosomes.

EXPERIMENTAL PROCEDURES

Proteins—Human SWI/SNF was isolated by affinity chromatography from HeLa cells expressing a FLAG-tagged Ini1/hSNF5 hSWI/SNF subunit (14). Note that this purification method results in hSWI/SNF complexes that contain one of two possible catalytic ATPase subunits, BRG1 (the most abundant) or hBRM. In general, BRG1 and hBRM complexes have similar in vivo functions and in vitro activities, although some differences have been noted (for review, see Ref. 20). In some cases the hSWI/SNF used for initial dimer generation was purified by conventional chromatography (14). In prior studies we found that dimers have identical properties when formed from conventionally purified hSWI/SNF, affinity purified hSWI/SNF, or the isolated BRG1 ATPase (14, 21). The Gal4 DNA binding domain (Gal4–1–94) and Gal4-AH fusion between the Gal4 DNA binding domain (amino acids 1–146) and an amphipathic α-helix activation domain (22) were isolated as described in Refs. 14 and 17. Gal4–1–94 was 80% pure and 40% active. Gal4-AH was 95% pure and 100% active. Gal4 binds its site as a dimer, and Gal4 concentrations are given as the concentration of active Gal4 dimers.

Nucleosomal Templates and Assembly—The MLT 3, pHGal41, TPT, TPGal41-AH, and TPGal41-XX templates have been described previously (14, 17). The TPGal41-HXh, TPGal42-SH, and TPGal42-HXb templates are kind gifts of Drs. A. Imbalzano and R. E. Kingston. They were generated as described previously (14, 17). The TPGal41-HXh, TPGal42-SH, and TPGal42-HXb templates are kind gifts of Drs. A. Imbalzano and R. E. Kingston. They were generated as described in Refs. 14 and 17. The pHGal41-AH, TPGal41-AH, and TPGal41-XX templates have been described previously (14, 17). The TPGal41-HXh, TPGal42-SH, and TPGal42-HXb templates are kind gifts of Drs. A. Imbalzano and R. E. Kingston. They were generated as described in Refs. 14 and 17. These sequences were PCR-generated and cloned into appropriate plasmid or of a PCR-generated fragment made between HindIII and XbaI were changed to read AAGCTTCAGCTGCGGAGTACTGTCCTCACTCGAGCTCGAG. Similarly, for TPGal42-SH the sequences between SpeI and HindIII were changed to read ACTAGTCCATGGGCGGAGTACTCTCCGAGGCAGGACTGTCCTCCGAAGCCT, and for TPGal42-HXb the sequences between HindIII and XhoI were changed to read AAGCTTCAGTCTCCGAGGACTCTCTCCGAGCTGGAGGACTCTCCGCAGCTCTAGA. 155-bp mononucleosome templates were generated by restriction digestion of the appropriate plasmid or of a PCR-generated fragment from the plasmid using flanking primers. These templates were end-labeled by Klenow fill in of their 5′ overhanging ends using [α-32P]dATP and cold dT, G, and CTP, assembled with HeLa cell core histones by salt dilution, and purified by glycerol gradient ultracentrifugation as described previously (23).Templates of varied lengths were generated using a forward PCR primer beginning at the CGCGT of the MluI site of pTPT and various reverse primers to generate 135-, 145-, 155-, and 195-bp products. These were end-labeled using polynucleotide kinase and [γ-32P]ATP, assembled by octamer transfer from HeLa cell polynucleosomes as described in Ref. 10, followed by glycerol gradient centrifugation. Mononucleosome cores were isolated from HeLa cell nuclear pellets as described in Ref. 14.

hSWI/SNF Remodeling Assays and Dimer Isolation—Typically, dimers were generated in preparative hSWI/SNF reactions containing 10 ng of labeled cores, 2 μg of affinity purified hSWI/SNF, and 700 ng of unlabeled HeLa cores in 125 μl of buffer that was 60 mM KCl, 5 mM MgCl2, 2 mM MgCl2/ATP equimolar mixture, 0.1% Nonidet P-40, 10 mM Tris, pH 7.5, 12 mM HEPES, pH 7.9, 0.25 mM EDTA, ~10% glycerol, and 20 ng/μl of bovine serum albumin. Given the ratio of labeled and unlabeled input cores, almost all labeled dimers will contain one labeled template DNA and one heterogeneous nucleosome-length stretch of human genomic DNA. This means, for instance, that dimers formed from TPGal41-AH will contain only one Gal4 site. After 1–2 h at 30 °C the reactions were removed to ice and the concentration of KCl was raised to 180 or 240 mM to reduce the amount of hSWI/SNF binding to cores and dimers, and products were separated by either 4.5% PAGE at 4 °C, or by glycerol gradient ultracentrifugation, as above but with glycerol gradient buffer supplemented with 180 mM KCl. Note that, whereas this high concentration of salt can accelerate dimer reversion at 30 °C, no appreciable reversion was seen for gradient fractions maintained at 4 °C. For purification from PAGE, bands containing cores and dimers were eluted from gel slices at 4 °C into 2 volumes of 20 mM HEPES, pH 7.9, 1 mM EDTA, and 150 μg/ml bovine serum albumin.

Diagnostic hSWI/SNF remodeling assays were typically performed in 25-μl reactions containing 0.3 ng (0.15 nM) of labeled cores or dimers, 100–200 ng of affinity purified hSWI/SNF (2–4 nM), and buffer conditions as described for the preparative reactions, except that reactions from experiments examining the effects of Gal4 also contained 1 μM ZnCl2. For control reactions, hSWI/SNF or ATP/MgCl2 were omitted. Reactions were incubated for 1 h at 30 °C, and then 2 μg of plasmid DNA and 0.5 μg of HeLa cell polynucleosomes were added to stop the reaction and remove hSWI/SNF from the labeled template before PAGE to resolve nucleosomal hSWI/SNF products. Any significant departures from these conditions are noted in the figure legends.

Restriction Enzyme Accessibility Assays—50 units of HindIII or PstI were added to diagnostic hSWI/SNF remodeling reactions. After 3 h at 30 °C, reactions were stopped by addition of SDS to 1%, EDTA to 33 mM, and Proteinase K to 0.67 μg/ml followed by incubation at 37 °C for 3 h and 5% PAGE.

Micrococcal Nuclease (MNase) Digestion Analysis—Gradient-isolated TPT cores or dimers were digested with a titration of MNase (Roche unit definition) for 30 min at 30 °C in 50 μl standard remodeling reactions lacking ATP but containing 3.2 mM CaCl2. 12.5 μl of each reaction was stoped by addition of 2 μg of plasmid DNA and immediately resolved by 5% native PAGE. The remainder was adjusted to 100 μl with 15 mM EDTA, extracted with phenol, EtOH precipitated, and separated by 8% denaturing PAGE (14).
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Gal4 Binding Assays—20 μl of electrophoretic mobility shift analysis (EMSA) (gel shift) reactions for Gal4 binding typically contained no more than 0.3 ng of purified labeled cores or dimers (≤0.15 nm), the indicated concentration of Gal4 (1–94) or Gal4-AH, 50 ng of cold HeLa cell mononucleosome cores, and 500 ng of poly(dl-dC) as nonspecific competitors in a buffer composed of 25 mM Tris, pH 7.5, 7 mM HEPES, pH 7.9, 0.5 mM EDTA, 60 mM KCl, 5 mM MgCl₂, 0.1% Nonidet P-40, 150 ng/μl of bovine serum albumin, 1 μM ZnCl₂, 1 mM β-mercaptoethanol, and 0.4 mM dithiothreitol. Binding was allowed to proceed for 30 min at 30 °C before 4.5% PAGE. Departures from these standard conditions are noted in the text and figure legends. Footprinting reactions were done under the same conditions, but in 100-μl reactions containing only 0.8 ng/μl of cold mononucleosome cores as competitor and 3 mM MgCl₂. DNase digestion, sample purification, and denaturing electrophoresis were done as described in Ref. 14. Dissociation constants (Kd value) were determined as follows. Unbound and bound species were quantitated from PhosphorImager exposures using ImageQuant, and the ratio of bound/free plotted against the logarithm of the sample lane, the no Gal4 control lane for either cores or bound was calculated as being 1/(free DNA counts in control dimer/core lane) × (fraction of counts at the same gel shift position in the core DNA lane at [Gal4]). For footprinting reactions, the fraction bound was calculated as being 1 – (i_bkg)/i_bkg, where i_bkg is the signal intensities in the footprinted region of the sample lane, the no Gal4 control lane for either cores or dimers, and the fully bound core DNA control. Because the concentration of Gal4-site template in these reactions is 0.15 nm or less (0.3 ng/20 μl of a 100-kDa labeled DNA), Gal4 binding to the template does not significantly reduce the amount of free Gal4 in reactions containing ≥1 nm Gal4. This simplifies the analysis allowing apparent Kd to be measured as the inverse slope of the best fit line when [Gal4]a bound is plotted against [Gal4-bound template]/[unbound template]. Standard deviations in Kd values were calculated from the standard deviation in the slope of the line.

Results

Gal4 binds more poorly to an end-located site in an altered mononucleosome dimer—Our previous studies showed a 3–10-fold preference for dimers over cores in Gal4 binding to a site located at the center of two 155-bp mononucleosome templates (14), pGAL41 and TPGAL41-AH, see Fig. 1A for template maps). To determine whether dimers were generally more accessible than cores, we compared three 155-bp nucleosomal templates, each with a single 17-bp high affinity dimeric Gal4 site centered over a position 18, 45, or 72 bp from the right edge of the DNA (templates TPGAL41-XX, -HXh, and -AH, respectively, see Fig. 1A). Each template was assembled into mononucleosome cores using HeLa core histones and step dilution, followed by glycerol gradient isolation (23). Because the histone octamer covers ~146 bp of DNA, each of these Gal4 sites will be inside the nucleosome (although the exact distance from each Gal4 site to a nucleosome entry/exit site may vary by up to 9 bp). For instance, on TPGAL41-XX with an octamer formed as far to the right as possible, the center of the Gal4 site will be 18 bp in from the entry/exit site. If the octamer formed as far to the left as possible, the Gal4 site center would instead be 9 bp inside the nucleosome.

After assembly, each template was remodeled by hSWI/SNF, and the normal mononucleosome cores as well as altered dimer products were separated by glycerol gradient ultracentrifugation (14). We then incubated nucleosome cores or altered dimers of each template with recombinant Gal4 DNA binding domain (Gal4-(1–94)) and/or the Gal4 DNA binding domain linked to an artificial amphipathic α-helix weak activation domain (Gal4-AH), followed by polyacrylamide gel EMSA. A representative example is shown in Fig. 1B. Consistent with our previous results, Gal4-AH binds poorly to the centrally located site on TPGAL41-AH cores, but binds significantly better to the 1-AH altered dimer (7% to cores versus 33% to dimers at 50 nm, compare Fig. 1B, lanes 5 and 8). To our surprise, the opposite trend was seen for binding to the end-positioned site in TPGAL41-XX (57% binding to cores at 50 nm versus 22% to dimers, compare Fig. 1B, lanes 13 and 16). Scatchard analysis to measure apparent Kd values indicated that Gal4 binds with an ~6-fold higher affinity to AH dimers relative to AH cores but binds with an ~4-fold lower affinity to XX dimers relative to XX cores (see Table 1, entries for Fig. 1B).

Gal4 has some nonspecific binding affinity (as indicated by the multimers of Gal4 bound to bare DNA at high concentrations, e.g. Fig. 1B, lanes 2 and 10). Although the presence of the histone octamer is expected to eliminate most or all nonspecific binding, we nonetheless wished to confirm that the gel shift results represented Gal4 binding to its proper site. To do so, we performed DNase footprinting studies on reactions containing increasing concentrations of Gal4-AH (Fig. 1, C and D, and data not shown). Quantitation of the degree of Gal4 site protection from DNase showed that, as was seen in the EMSA analysis, Gal4-AH bound better to TPGAL41-AH dimers than cores (~3-fold), but bound worse to 1-XX dimers than cores (~5-fold, see Table 1). Note that these EMSA and footprinting reactions were done under different nonspecific competitor DNA conditions. Because of its moderate sequence-independent binding activity, some Gal4 protein will be sequestered onto nonspecific competitor DNAs present in the reaction. Thus, as the concentration of nonspecific competitor DNA increases, the concentration of Gal4 needed for 50% binding to the specific template also increases, resulting in a higher apparent Kd. For instance, the apparent Kd values measured in the presence of 0.8 ng/μl of unlabeled bulk HeLa nucleosomes (e.g. Fig. 1, C and D) were ~10-fold lower than those measured in the presence of 20 ng/μl of poly(dl-dC) (e.g. Fig. 1B, see Table 1). When no nonspecific competitor was used at all, the apparent Kd values were another ~20-fold lower still (Table 1 and data not shown).

Importantly, the same relative affinities were seen regardless of the competitor conditions or assay method: dimers were always preferred over cores ~3–6-fold for the AH template, whereas cores were preferred over dimers ~4–5-fold for the XX template. In addition, we found that Gal4-(1–94) and Gal4-AH always gave very similar results when compared under the same conditions, indicating that the affinity of Gal4 proteins for their sites on nucleosome cores and dimers is not influenced by
FIGURE 1. Inverted accessibility of dimers for Gal4. A, alignment of templates used. B, EMSA analysis of TPGal41-AH or -XX bare, core or dimer templates with the indicated concentrations of G4-AH. 20 ng/μl of poly(dI-dC) was used as a nonspecific competitor. Other conditions are as described under “Experimental Procedures.” Arrows indicate the position of unbound and bound gel-shifted species. C and D, footprint analysis of Gal4-AH binding to bare, cores, and dimers of TPGal41-AH (C) or TPGal41-XX (D). The footprints due to Gal4 binding to its site are marked by brackets. E, plot of \( K_d \) versus Gal4 site position on dimers or cores using 25 ng/μl of poly(dI-dC) and 2.5 ng/μl of HeLa mononucleosome cores as nonspecific competitors. F, EMSA of TPGal42-SH, as in B, but with 100 ng of cold cores and the indicated concentrations of Gal4-(1–94).
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TABLE 1

| Template/G4 site | Competitor | Gal4 | K_d core | K_d dimer | Preference | Notes |
|-----------------|------------|------|----------|----------|------------|-------|
| TPGal41-AH/72 bp| 2.5 Nucleosomes, 25 poly(dl-dC) | 1–94 | 1126 ± 94 nM | 431 ± 39 nM | 2.6 × dimer | Fig.1E |
| 20 IC | AH | 676 ± 102 nM | 102 ± 7 nM | 6.6 × dimer | Fig.1B |
| 0.8 Nucleosomes | None | AH | 2.00 ± 0.15 nM | 0.68 ± 0.04 nM | 2.9 × dimer | |
| TPGal41-HXh/45 bp| 2.5 Nucleosomes, 25 poly(dl-dC) | 1–94 | 827 ± 116 nM | 924 ± 124 nM | ~Equal | Fig.1E |
| 20 IC | AH | 155 ± 32 nM | 848 ± 223 nM | 5.4 × core | Fig.1F |
| 0.8 Nucleosomes | None | AH | 3.03 ± 0.65 nM | 17.2 ± 1.8 nM | 5.7 × core | |
| TPGal42-HXb/45 and 18| 2.5 Nucleosomes, 25 poly(dl-dC) | 1–94 | 78 ± 16 nM | 770 ± 49 nM | 9.9 × core | |
| 5 Nucleosomes | None | AH | 6.23 ± 0.37 nM | 27.9 ± 3.2 nM | 4.5 × core | |
| TPGal42-SH/72 and 91| 2.5 Nucleosomes, 25 poly(dl-dC) | 1–94 | 497 ± 16 nM | 145 ± 7 nM | 3.4 × dimer | —
| 2.5 Nucleosomes, 25 poly(dl-dC) | AH | 275 ± 38 nM | 37 ± 2 nM | 7.4 × dimer | |
| 5 Nucleosomes | None | AH | 13.4 ± 0.5 nM | 4.2 ± 0.3 nM | 3.2 × dimer | |

* a G4 site location is given for the center of the dimeric Gal4 binding site relative to the EcoRI end of each template.
* b K_d derived from compiled data from 3 independent titration experiments.
* c Footprint assay results.
* d K_d derived from compiled data from 2 independent titration experiments.

Note that conditions in which there is a large molar excess of competitor DNA are similar in some ways to conditions in nuclei, where only a small fraction of available DNA sequences will be high affinity binding sites. Accordingly, the observation of a preference for centrally positioned sites in dimers and end-positioned sites in cores under a variety of competitor conditions suggests that this effect could be relevant for hSWI/SNF function in vivo.

Using a standardized set of competitor conditions (2.5 ng/μl of unlabeled nucleosomes and 25 ng/μl of poly(dl-dC)), we compared the affinities of the Gal4-DNA binding domain (Gal4-(1–94)) for cores and dimers formed from templates containing Gal4 sites at 18, 45, or 72 bp from the DNA edge. In Fig. 1E, the K_d measurements for Gal4-(1–94) binding were plotted against the distance from the center of the Gal4 site to the end of the template (also see Table 1). Prior studies have shown that transcription factors have the highest affinity if their sites are located near the edge of a nucleosome core, but have greatly lowered affinity for sites near the nucleosomal center/pseudodyad (e.g. Refs. 24–26). Accordingly, the K_d values for Gal4-(1–94) binding to nucleosome cores got progressively higher as the center of the Gal4 site was moved from 18 bp away from the DNA edge (TPGal41-XX) to 45 bp (TPGal41-HXh) and 72 bp (TPGal41-AH, Fig. 1E, squares). Strikingly, the exact opposite trend was observed for binding to dimers, with K_d values progressively decreasing as the Gal4 site was moved from 18 to 45 to 72 bp (Fig. 1E, diamonds). This inverted accessibility profile shows that altered dimers are not generally more accessible to transcription factors than cores. Instead, altered dimers appear to hold more strongly onto the ends of the nucleosomal DNA templates, while providing greater accessibility to the middle of these templates.

This inverse accessibility profile is consistent with, and helps to explain, several previous observations, including the relative protection of DNA ends in dimers from either Exonuclease III or MNase digestion, and the subnucleosomal DNA fragments released from MNase digestion of dimers (13, 14). In addition, our early studies noted qualitative differences in restriction enzyme accessibility to unique sites on TPGal41-XX dimers versus cores (Fig. 3D in Ref. 14). Quantitation of those same results suggests that the overall accessibility of restriction sites in dimers is inverted relative to cores: for the three sites showing the greatest difference between dimers and cores, there was 2.2-fold less digestion of dimers at the end-proximal XhoI site, but 2.0- and 1.7-fold greater digestion of dimers at the more centrally located PstI and HindIII sites.

Inverse Accessibility of Dimers Is Also Evident on Templates with Two Gal4 Sites—To determine whether the presence of more than one Gal4 binding site would overcome or alter the inverted accessibility profile of Gal4 binding to altered dimers, we examined two additional templates: TPGal42-HXb, containing two Gal4 sites 18 and 45 bp from the right edge (the same as the sites in 1-XX and 1-HXh templates), and TPGal42-SH, containing two centrally located Gal4 sites, at 72 bp (the same site as on TPGal41-AH) and 91 bp from the right edge (see Fig. 1A). A representative gel showing Gal4-(1–94) binding to TPGal42-SH cores and dimers is shown in Fig. 1F. Two gel-shifted species corresponding to one or two bound Gal4 sites could usually be resolved (arrows indicating 1xG4 and 2xG4 in Fig. 1F). For the initial analysis, we did not distinguish between these two species and used the overall fraction bound to estimate the overall affinity of Gal4 for the template. The results showed that the 4-fold preference for dimers for a single centrally located site also extended to two centrally located sites, under various competitor conditions and with Gal4-(1–94) or Gal4-AH (Table 1, compare 1-AH and 2-SH templates). Similarly, the 4-fold preference for cores over dimers for a single site at the edge of the DNA also extended to two end-positioned sites (Table 1, compare 1-XX and 2-HXb). Thus, the relative accessibilities of different locations in cores and dimers was not overridden by the presence of two Gal4 sites.

Previous studies showed that transcription factor binding to two or more sites near the edge of a nucleosome core was inherently cooperative (e.g. Refs. 24–26). This is because binding of a first factor to a site near the edge of the nucleosome helps reduce the number of DNA:histone contacts that need to be broken for binding of a second factor to a more internal site. For instance, Gal4-AH binding to a site centered 20 bp from the edge of a 152-bp nucleosomal template facilitated NF-κB bind-
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Gal4 Binding Can Affect Dimer Formation and Reconversion—hSWI/SNF action on mononucleosomes generates altered dimers. In addition, hSWI/SNF can act on altered dimers, causing them to be converted back to normal nucleosome cores (14). The combination of both effects typically results in a dynamic equilibrium in which ∼10–20% of the templates exist as dimers and ∼80–90% as cores. We hypothesized that Gal4 binding might influence this equilibrium by promoting either dimer formation or reversion. In one model hSWI/SNF action might result in a disrupted intermediate that would rapidly resolve itself into either dimers or cores. Gal4 binding to this intermediate would tend to tip the balance toward the form with the highest affinity for Gal4 (lowest overall energy when Gal4 was bound). In a second model, Gal4 binding might inhibit subsequent SWI/SNF remodeling, again tipping the balance of hSWI/SNF products toward the species with the highest Gal4 affinity.

Reactions using TPGal41-AH cores as template, and containing or lacking 10 nM Gal4-AH, were incubated with hSWI/SNF and ATP for the indicated times. This concentration of Gal4-AH is expected to be saturating, because the apparent $K_d$ values for Gal4 binding to TPGal41-AH cores and dimers in the absence of competitors were 1.2 and 0.48 nM, respectively. At the indicated times hSWI/SNF and Gal4 were removed from the template by addition of 3 μg of unlabeled plasmid DNA, and the nucleosomal products were resolved by PAGE (Fig. 2A). When the percentage of dimers observed in three separate experiments was plotted versus remodeling time, there was no significant effect of Gal4 on the rate of dimer formation (Fig. 2B, compare + Gal4 (squares), to −Gal4 (diamonds)).

By contrast, addition of Gal4 to reactions containing TPGal41-XX cores at time 0 decreased the percentage of dimers resulting from hSWI/SNF action (Fig. 2C, lanes 1–3). When remodeling was stopped after 1 h by addition of apyrase (which hydrolyzes all of the ATP needed for remodeling), and Gal4 added for an additional hour, no decrease in dimers was seen (lanes 8 and 9). This suggested that Gal4 binding did not, by itself, cause dimers to be converted back to mononucleosome cores, but that it inhibited dimer formation by hSWI/SNF and/or helped hSWI/SNF reconvert dimers to cores. One possible explanation for this effect is that Gal4 binding near the edge of a nucleosome might inhibit all hSWI/SNF action on 1XX cores. To test this possibility, we examined a hallmark of ongoing hSWI/SNF remodeling on cores; increased accessibility of restriction enzymes to sites that are normally covered by a nucleosome (27). This increased restriction enzyme accessibility in the presence of hSWI/SNF, ATP, and restriction enzyme can be readily measured by resolving purified template DNA by PAGE and quantitating the percentage of cut templates (27). As expected, hSWI/SNF action on 1XX resulted in an ∼2-fold increase in concurrent digestion at HindIII and PstI sites located 53 and 92 bp from the left edge of the template (Fig. 2D).

Note that this increased digestion was not blocked by the inclusion of Gal4 in the reaction (Fig. 2D, +Gal4 bars), indicating that Gal4 did not generally inhibit hSWI/SNF remodeling of 1XX, but specifically interfered with altered dimer formation.

The low percentage of dimers formed from 1XX cores in the presence of Gal4 could result from inhibition of dimer formation and/or stimulation of dimer reversion. To examine the contribution from stimulated reversion, 1XX dimers were generated by hSWI/SNF over 1 h, followed by Gal4 addition for 1 more hour. This resulted in a percentage of dimers below that seen for a 1-h SWI/SNF reaction in the absence of Gal4, suggesting that Gal4 promotes 1XX dimer reversion by hSWI/SNF (Fig. 2C, compare lane 6 to lane 8). To examine this further, we used purified dimers, and found that the hSWI/SNF-dependent conversion of TPGal41-XX dimers back to cores was faster in the presence of Gal4 (Fig. 2E, lane 10). Interestingly, this was also true for TPGal41-AH dimers (Fig. 2E, lane 5).

Because Gal4 has a higher affinity for 1XX cores than dimers, the observation that Gal4 reduces the dimer percentage after hSWI/SNF remodeling of 1XX cores or dimers is consistent with both of the models presented above. However, on 1AH templates (on which Gal4 binds best to dimers), Gal4 did not significantly increase the percentage of dimers formed from cores and also increased the reconversion of dimers to cores. This suggests an alternative model in which Gal4 binding generally inhibits dimer formation and promotes dimer reversion, but this effect can be weakened (perhaps as per model 1 or 2) when Gal4 prefers binding to dimers. As described below, dimers share many characteristics with altosomes formed by hSWI/SNF on polynucleosomes. Accordingly, these results indicate that the abundance or precise location of altosomes on
polynucleosomal DNAs might be regulated by transcription factor binding.

Dimers Are Metastable Structures That Revert Slowly to Cores without hSWI/SNF Action—Our studies of polynucleosomal templates identified an altered dinucleosomal product of hSWI/SNF called the altosome. Like altered dimers, altosomes are composed to two intact histone octamers and give rise to subnucleosomal MNase digestion fragments (14, 19), suggesting that altered dimers are structurally related to altosomes. Altosomes have the additional property of reverting to nucleosomes with normal MNase footprints over time (19). To determine whether altered dimers have this same property, hSWI/SNF-altered dimers were gradient isolated and incubated for increasing times under different conditions. Increasing incubation times at 30 °C in reactions containing 60 mM monovalent salt resulted in progressive loss of dimers with a corresponding increase in mononucleosomes (Fig. 3A, lanes 1, 3, and 5). From this and other experiments we calculate the half-life for dimers at 60 mM KCl and 30 °C to be between 19 and 25 h, which is quite similar to the reversion rate of altosomes under similar conditions (19). In addition, as was seen for altosomes, incubation in 180 mM KCl accelerated reversion (Fig. 3A, lanes 2, 4, and 6). Incubation at 300 or 400 mM KCl resulted in rapid conversion of dimers to mononucleosomes, but, as for control
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A Model for Altered Dimers—These results, combined with those of prior studies, suggest the following model for dimer structure. hSWI/SNF action on linear mononucleosome templates has been shown to result in movement of histone octamers from centrally located regions to DNA edges, and often up to 60 bp beyond DNA edges (10–12). Similar results were also seen for yeast SWI/SNF complexes (28, 29). Thus, SWI/SNF action could result in mononucleosome cores on which up to half the DNA length has been spoiled off the edge of the DNA (Fig. 5, A and B). If two such “off the edge” molecules interact, the unoccupied DNA on each one can interact with the unoccupied histone surface on the other, forming a moderately stable nucleosome dimer (Fig. 5C). This model for dimers would explain the increased accessibility of Gal4, RAG recombination factors, and nucleases to DNA sequences in the center of a nucleosomal template (white bars in Fig. 5, A and C, data from Figs. 1 and 4, and Refs. 13, 14, and 16). By contrast, the DNA ends in this model would be far less accessible than normal, consistent with reduced Gal4 affinity (Fig. 1) and decreased accessibility of the DNA ends to nucleases (Fig. 4, A and B, and Refs. 13 and 14). Reduced accessibility of ends may arise, in part, from steric occlusion due to the proximity of the adjacent nucleosome in the dimer. In addition, the strongest DNA:histone-fold contacts do not extend to the last ~12 bp of DNA at the edges of a normal nucleosome (30), which is thought to facilitate factor access to sites at the edges of the nucleosome by allowing dynamic DNA unpeeling (25). In dimers, however, the DNA ends would be relatively inaccessible because they are held by stronger histone-fold contacts near the pseudodyad of the H3/H4 tetramer.

According to this model, stable dimer formation will require a DNA template long enough to accommodate the additional length of the DNA bridges formed between each octamer. This is consistent with the relatively poor dimer formation observed increased. Interestingly, species with the mobility of cores in the dimer lanes were more resistant to MNase than control cores (e.g. the highest MNase concentration decreased the core band for control mononucleosomes by 7-fold, lane 5, but decreased the core band in dimers by only 1.6-fold, lane 10). This could occur if digestion of dimers (e.g. at internucleosomal bridges in the model in Fig. 5C) separated the two halves of the dimer, giving rise to a species with the same mobility as a normal nucleosome core, but containing two subnucleosomal DNA fragments.

If dimers are held together by DNA bridges, dimer formation might be efficient only when the DNA template has sufficient DNA length to occupy normal histone-DNA contact points and also span the gaps between octamers. To test this, we assembled mononucleosomes on shorter DNA fragments, of 145 and 135 bp. hSWI/SNF remodeling of each of these templates resulted in some dimer formation, although this was less efficient than on the 155-bp template (Fig. 4C, compare lanes 3 and 6 to lane 9). We also examined a longer, 195-bp template and found that dimer formation was also reduced relative to the 155-bp template (Fig. 4C, lanes 10–12). The implications of these results for dimer structure are discussed below.

**DISCUSSION**

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According to this model, stable dimer formation will require a DNA template long enough to accommodate the additional length of the DNA bridges formed between each octamer. This is consistent with the relatively poor dimer formation observed
for templates shorter than 155 bp (Fig. 4C). We also observed, however, that dimers form more poorly on a 195-bp TPT-based template (Fig. 4C). Our previous studies also showed that dimers were often hard to detect on 265 or 315 bp as compared with 215-bp mononucleosomal 5 S rDNA templates (10). One possible explanation is that longer DNA sequences allow for an intramolecular stabilization of the off the edge product in Fig. 5B, in which the free DNA end loops around to contact the exposed histone surface on the same octamer, creating a “loop mononucleosome” (Fig. 5D). Indeed, recent studies support the idea that mononucleosomes with a loop of DNA on the histone octamer surface can be generated as a result of human and yeast SWI/SNF action on linear mononucleosome templates (12, 29, 31). Together, these results suggest that altered dimers will form best on templates that are long enough to allow bridges between octamers, but too short to allow stable formation of a loop mononucleosome (which given the bending properties of DNA might be expected to require an additional ~60 bp or more (32)).

Relationship between Dimers and Altered Dinucleosomes Formed on Polynucleosomes—According to the model in Fig. 5, A–C, altered dimers can only be generated from templates

FIGURE 4. MNase digestion and template length effects suggest DNA bridges in dimers. Gradient isolated TPT cores and dimers were digested with the indicated amounts of MNase before resolution by native PAGE (A) or denaturing PAGE (B), as described under “Experimental Procedures.” C, 135-, 145-, 155-, and 195-bp templates, all sharing the MluI site on the right side of the TPT template (the same as the TPGal4 constructs, but lacking Gal4 sites) were generated. Isolated mononucleosomes formed from these templates were incubated with hSWI/SNF and/or ATP, as indicated, under standard remodeling conditions (except containing 4 mM MgCl₂ and 30 mM KCl) for 1 h at 30 °C, before addition of 1.8 μg of plasmid DNA and 0.5 μg of polynucleosomes, and resolution by 5% PAGE. Dimer percentage for each remodeled lane is indicated at the bottom of the figure. On templates longer than ~146 bp, nucleosomes with a centrally positioned octamer run more slowly than those with an end-positioned octamer. Thus, differences in octamer position give rise to the two mononucleosome bands on the 195-bp template before remodeling and two different mononucleosome bands after remodeling. Mononucleosome and dimer species were identified by comparing their relative mobilities (the distance from the well to each species divided by the distance from the well to bare DNA) to those previously observed for 215-bp template nucleosomes (10). Relative mobilities were: 215 dimer, 0.39; 195 dimer, 0.37; 215 mononucleosomes, 0.53 to 0.68; 195 mononucleosomes, 0.55 to 0.67.

FIGURE 5. Dimer and altosome structure models. hSWI/SNF repositioning activity on normal nucleosome cores (A) can result in off the edge nucleosomes (B). Two off the edge nucleosomes might be stabilized as a dimer (C), or one off the edge nucleosome with sufficient DNA length might be stabilized as a loop mononucleosome (D). Note that, in this model, dimers need not form from nucleosomes with equal unwrapped DNA lengths, allowing for different length subnucleosomal DNA fragments upon MNase digestion of dimers. On polynucleosomal DNA, hSWI/SNF activity might promote release of ~70 bp from one nucleosome (E). If this DNA is replaced by linker DNA distal to an adjacent nucleosome, a stable altered dinucleosome, altosome, can be formed (F).
bearing DNA ends. Because cellular chromatin normally has no accessible ends, dimers, per se, are unlikely to be important for hSWI/SNF regulatory function. However, we find that altered dimers revert to normal mononucleosome cores with a half-life of ~24 h at 60 mM KCl (Fig. 3A). This is very similar to the reversion times seen for the altered dinucleosome (altosome) products formed by hSWI/SNF from 30 to 40% of the nucleosomes on polynucleosomal templates without DNA ends (19). Combined with the observation that altosomes and altered dimers have similar histone composition (two intact octamers) and similar nuclease accessibility (resulting in subnucleosomal MNase product fragments), this result suggests that these two hSWI/SNF products may have very similar structures. One model we proposed for altosome structure postulates that hSWI/SNF action might displace approximately one-half of the DNA associated with one nucleosome, followed by the association of linker DNA distal to the immediate downstream nucleosome, forming an altered dinucleosome (Fig. 5, E and F, and Ref. 19). This wrapping of DNA on two octamers constrains a total of zero negative supercoils (instead of the normal −2), consistent with the reduction of supercoiling that also characterizes altosomes. Strikingly, the model for dimer structure and the model for altosome structure that fit best with our current data are essentially analogous, with the major difference being the break between the two DNA fragments on the right-most nucleosome in the dimer (compare Fig. 5, C and F).

**Factor Accessibility on hSWI/SNF Products**—In remodeling polynucleosomal templates, hSWI/SNF alters nucleosome locations at the same time as it converts normal nucleosomes to altosomes. This has, so far, made it impossible to create altosomes localized only to one specific DNA sequence within a polynucleosomal template. As a result, we have not been able to directly compare the properties of altosomes and altered dimers containing identical DNA sequences. Furthermore, the difficulty in creating altosomes encompassing defined sequences has prevented us from measuring transcription factor accessibility at different locations within altosomes. However, given their many similarities, the properties of altered mononucleosome dimers (which can be readily generated on specific sequences) may give important insights into how altosomes could influence the binding of transcription factors in vivo. If the inverted factor accessibility profile we observe for dimers also applies to altosomes, then altosomes would be expected to be most accessible at the DNA bridges corresponding to the linker DNA on either side of the right-most nucleosome in Fig. 5F, and least accessible at the DNA entering and exiting the altosome (near the pseudodyad of the leftmost nucleosome). The accessibility of any transcription factor site will depend, of course, on where hSWI/SNF places altosomes when they are created, a question that we are addressing in ongoing studies. One possibility suggested by the model in Fig. 5, E and F, is that, if the rightmost nucleosome does not move, altosome formation will free up sequences normally associated with the leftmost nucleosome while simultaneously occluding sites on the linker DNA distal to the right-most nucleosome (e.g. the white bar in Fig. 5, E and F). In this way altosome formation could act as a molecular switch, swapping the accessibility of two DNA sequences separated by ~200 bp.

The current studies also showed that the ratio of mononucleosomes to altered dimers can be modulated by Gal4 binding (e.g. dimer formation was inhibited by Gal4 binding to the end but not the middle of a template). This suggests that the locations where altosomes are formed on chromatin may be similarly regulated by the presence of bound transcription factors. Taken together these results suggest that hSWI/SNF and transcription factors will influence each other in multiple ways: e.g. altosomes may modulate the accessibility of transcription factor binding sites in chromatin, whereas previously bound transcription factors will influence the positions and/or nature of hSWI/SNF products.

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