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Histone Chaperone FACT Coordinates Nucleosome Interaction through Multiple Synergistic Binding Events*[S]

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Background: The histone chaperone FACT binds and reorganizes nucleosomes during critical cellular processes.
Results: FACT binds histones, DNA, and mono- and tri-nucleosomes with high affinity. FACT reduces non-nucleosomal histone/DNA interactions.
Conclusion: Multiple regions of FACT strategically bind target sites on nucleosomes to coordinate (dis)assembly.
Significance: The thermodynamic parameters guiding multiple FACT/nucleosome interaction(s) coincide with reorganization events.

In eukaryotic cells, DNA maintenance requires ordered disassembly and re-assembly of chromatin templates. These processes are highly regulated and require extrinsic factors such as chromatin remodelers and histone chaperones. The histone chaperone FACT (facilitates chromatin transcription) is a large heterodimeric complex with roles in transcription, replication, and repair. FACT promotes and subsequently restricts access to DNA as a result of dynamic nucleosome reorganization. However, until now, there lacked a truly quantitative assessment of the critical contacts mediating FACT function. Here, we demonstrate that FACT binds histones, DNA, and intact nucleosomes at nanomolar concentrations. We also determine roles for the histone tails in free histone and nucleosome binding by FACT. Furthermore, we propose that the conserved acidic C-terminal domain of the FACT subunit Spt16 actively displaces nucleosomal DNA to provide access to the histone octamer. Experiments with tri-nucleosome arrays indicate a possible mode for FACT binding within chromatin. Together, the data reveal that specific FACT subunits synchronize interactions with various target sites on individual nucleosomes to generate a high affinity binding event and promote reorganization.

Chromatin is a multitiered assembly of cellular genetic material (DNA) and the macromolecular protein complexes (histone and non-histone) that coordinate its compaction. Nucleosomes represent the primary level of chromatin and include 147 bp of DNA wrapped around a core histone octamer (1). The histone $(H3\text{-}H4)_2$ tetramer must first bind DNA in position to allow deposition of adjacent H2A-H2B dimers and completion of the canonical nucleosome (2–4). The linker histone H1 and post-translational modifications to the histones orchestrate condensation into higher order chromatin (5–7). Histone chaperones are among a large group of proteins that modulate chromatin architecture through recognition of specific nucleosome attributes and modifications.

A prominent member of the histone chaperone family, FACT is thought to reorganize nucleosomes through the destabilization of multiple intra-nucleosome contacts (8–10). FACT was discovered as a factor that allows passage of the transcribing RNA polymerase through chromatinized DNA templates *in vitro* (11). FACT shows a preference for H2A-H2B over H3-H4 *in vitro*, yet, under certain conditions, it promotes binding of both species to free DNA (10). Separate work indicates additional roles for FACT in transcriptional regulation (12, 13), DNA repair (14), and histone variant exchange (15). The complexity of FACT function is underscored by relevant interactions with a wide array of chromatin-associated proteins including but not limited to Chd1 (Chromatin organization modifier, helicase, and DNA-binding domains 1) (16, 17), PARP1 (poly-ADP-ribose polymerase 1) (18), and CK2 (casein kinase 2) (19).

The human FACT complex is composed of two polypeptides identified as Spt16 (Suppressor of ty 16) and SSRP1 (structure-specific recognition protein 1) that are both essential for nucleosome reorganization (9, 10). The Spt16 protein is divided into three distinct structural domains and a highly acidic intrinsically disordered C-terminal domain required for alleviation of nucleosome blockage during polymerase II elongation (supplemental Fig. S1) (10, 20). The N-terminal domain of Spt16 has recently been shown to stably bind H3-H4 but not H2A-H2B *in vitro* (21). However, genetic analysis has demonstrated a functional relationship between the Spt16 N-terminal domain and a conserved region of H2A (22). The SSRP1 component of FACT is divided into five well defined domains, which includes a C-terminal HMG-1 DNA binding domain (supplemental Fig. S1) (14, 23). Genetic experiments in yeast have uncovered partially redundant roles for the SSRP1 middle domain and the Spt16 N-terminal domain, although the crystal structures of both domains display unrelated folds (21, 22, 24, 25). The multitude of interactions directing FACT-nucleosome complex

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formation has stymied the establishment of a complete model for FACT function.

Here, we quantitatively evaluate the multitude of interactions that promote nucleosome binding by FACT. Outside of the limited qualitative information obtained through gel shifts, pulldown assays, and density gradient fractionation experiments, no definitive data exist on the contribution of individual components within FACT and intact nucleosomes. Precise thermodynamic evaluation of the interactions guiding FACT-nucleosome complex formation is critical for developing an unambiguous mechanism for FACT function. Our laboratory previously established a thermodynamic framework for the histone chaperone Nap1 with various histone substrates (26). Our current data demonstrate that several elements of FACT form nonoverlapping synergistic interactions with nucleosomes to create a singular high affinity binding event. In-depth analysis of the conserved Spt16 CTD[4] reveals a key role in promoting access to the nucleosome core. Work with tri-nucleosome arrays demonstrates the influence of linker DNA on FACT occupancy. Collectively, we present significant insight into the thermodynamics of nucleosome binding and the mechanism of reorganization by the histone chaperone FACT.

MATERIALS AND METHODS

Reagents—All histones used in this study were *Xenopus laevis* constructs prepared via techniques described previously (27). Tail-less versions of the histone complexes contain the following residues: H2A-H2B (H2A residues 14–118 and H2B residues 24–122) and H3-H4 (H3 residues 27–135 and H4 residues 20–102). All DNA sequences are based on the "601" nucleosome positioning sequence (28). Nucleosome particles were then constructed from these recombinant histone substrates using salt dialysis techniques (29, 30). Tri-nucleosomes were made in a similar manner but utilized 621-bp (LE) or 561-bp (NLE) DNA that accommodates three nucleosome-binding sites connected by 60 bp of linker DNA, either with (LE) or without (NLE) outer 30-bp linkers. The reconstituted tri-nucleosomes were checked for saturation by EcoRI digestion following a protocol given in Ref. 31 (supplemental Fig. S6*A*). The sedimentation coefficients were then determined for the saturated tri-nucleosomes via analytical ultracentrifugation in an An60Ti rotor using a Beckman XL-A centrifuge and compared against values determined previously for analogous tri-nucleosome constructs (supplemental Fig. S6*B*) (32). Labeling mutations to H2B (T112C) and H4 (E63C) provided the means for adding fluorescent tags to the histone complexes of interest (33). Alexa-488 C5-maleimide (Invitrogen) was used to label free histones, and Alexa-647 C2-maleimide (Invitrogen) was used to label nucleosome constructs on H4 (E63C). Clear bottom 384-well Sensoplate Plus microplates (Greiner Bio-One) were used for the fluorescence titration assays. Hellmanex II (Fisher) was used for plate washes, and Sigmacote (Sigma) was applied to the microplates after cleaning. Repel polymer (RPT) micro-pipette tips (USA Scientific) were used to avoid sticking by labeled histones and nucleosomes during preparation and experimentation transfer.

FACT Expression and Purification—The baculovirus-driven expression of the FACT complex (co-expression of FLAG-Spt16 and His_6-SSRP1) in Sf21 insect cells essentially follows the protocols presented previously (34) with a few modifications. We found that 72-h expressions in 1-liter stirrer flasks at a concentration of $1–1.5 \times 10^6$ (keeping the multiplicity of infection at 10) worked well for expression of FACT. We have also found that the addition of 0.01% octyl glucoside and 0.01% CHAPS detergents inhibited nonspecific interactions between FACT and other proteins during purification without damaging the sensitive M2-resin used to bind the FLAG-Spt16. The FACT complex is de-phosphorylated by 2-h incubation with calf intestine phosphatase (New England Biosciences) on the nickel-agarose column (23). FACT binding to the nickel-agarose enables purification from the phosphatase. Depending on the purity of the FACT complex after the nickel-agarose column, the protein was run over a Superdex 200 10/300 GL size-exclusion column (GE Healthcare). Singular expression and purification of the Spt16 and SSRP1 subunits similarly grown in baculovirus-infected Sf21 insect cells were then bound and eluted from the appropriate affinity resin and further isolated by size-exclusion chromatography (Superdex 200).

Fluorescence-based Microplate Titration Assay—The microplates were prepared by a sequential wash protocol as follows: 1) acid wash (1 M HCl), 2) 1% Hellmanex wash, and 3) Sigmacote application. The wash steps were soaked for 30 min and followed by thorough rinsing with distilled water. The plates were then air-dried under an exhaust hood overnight. The titration experiments were set up by dilution of a high concentration stock of the unlabeled FACT construct into a series of increasing concentrations ranging from 1 to 1000 nM. The reactions conditions were as follows: 20 mM Tris, pH 7.5, 150 mM KCl, 5% glycerol, 1 mM tris(2-carboxyethyl)phosphine, 0.01% CHAPS, and 0.01% octyl glucoside. The labeled histones, nucleosomes, etc. were then added to the wells at a constant concentration between 0.5 and 1 nM with a final volume of 40 μl. The titrations mixtures were allowed to equilibrate at room temperature for 20 min and then scanned in the plate using a Typhoon 8600 variable mode fluorimager. Binding events were measured as a function of the fluorescence change across the titration series. The fluorescence change must be >10% of the total fluorescence signal to be considered for further evaluation. The actual fluorescence change was quantified using the program ImageQuant TL, and the data were analyzed, fit with a nonlinear regression curve with Hill coefficient, and displayed using Graphpad Prism. All experiments were performed in replicative quadruplicates. For a more detailed explanation of the equations and reactions schemes, refer to our previous work (26).

To ensure that the high affinity binding events are not due to the presence of the fluorescent labels attached to the histones or DNA, we also nonspecifically labeled the full-length FACT complex through native cysteines in the Spt16 and SSRP1 sequences and ran converse experiments in which unlabeled nucleosomes or components were titrated into a constant concentration of labeled FACT. We found that both FACT sub-

[4] The abbreviations used are: CTD, C-terminal domain; LE, linker ended; NLE, non-linker ended; TL, tail-less.

units were consistently labeled but with two or fewer labels per peptide. The control experiments were consistently within error of the experimental results presented in Table 1, which rules out significant non-native effects from the attached fluorophores.

Direct Competition Assays—Optimal ratios between fluorescently labeled H2A-H2B and H3-H4 complexes were pre-determined by titration of histone into DNA. The ratio of histone to DNA that gave the least amount of free DNA on 5% native PAGE was chosen for use in the competition assay (7:1 for H2A-H2B and 3:1 for H3-H4). The tetrasomes in this study were generated through titration(s) of $(H3\text{-}H4)_2$ tetramers into 207-bp DNA (601 sequence) held at 250 mM NaCl. The mixtures were then incubated at room temperature for 30 min. This "quick" method for tetrasome assembly has been verified in our laboratory through native gel electrophoresis and size-exclusion chromatography comparisons with "reconstituted" tetrasomes generated through salt dialysis. The DNA was kept at 0.5 μM. The purified FACT complex was spared de-phosphorylation to deter free DNA binding (23). The reaction conditions were kept the same as the microplate titration assays except the KCl concentration was raised to 250 mM to help impede nonspecific binding events at high concentrations. FACT was titrated into a constant concentration histone/DNA mixture and incubated at room temperature for 30 min. The titrations and relevant controls were then run out on a 5% native PAGE at 150 V, 4 °C for 60 min. After electrophoresis, the gels were scanned for fluorescent histone visualization and then soaked in ethidium bromide to visualize complexes containing DNA under UV light.

RESULTS

Development of High Throughput Method to Measure Thermodynamic Parameters Guiding Chromatin Binding by FACT—We have recently designed a fluorescence (de)quenching microplate assay that permits the precise quantification of apparent dissociation constants ($K_{d(app)}$), Hill coefficients (n_H), and stoichiometries for a near complete set of FACT/nucleosome and FACT/histone interactions. Measurements with individual FACT subunits and an Spt16 truncation construct allowed us to dissect their relative contributions to the different binding events. 384-Well clear bottom microplates were utilized for titration of FACT, or any other chromatin binding protein, into a fluorescently labeled chromatin component near physiological conditions. The measured fluorescence change upon increasing FACT titration indicated a direct interaction between FACT and the chromatin component present. The physical interaction can then be confirmed by running the well contents on 5% polyacrylamide gels and scanning for EMSA shift at the emission wavelength of the fluorescent label. Table 1 groups these measured parameters into categories based upon the FACT construct used. The values listed were derived from the titration of FACT, FACTΔCTD (Spt16 acidic C-terminal domain truncation + SSRP1), Spt16, or SSRP1 into fluorescently labeled histone complexes, nucleosomes, or DNA kept between 0.5 and 1 nM. For a more detailed explanation of the techniques, see under "Materials and Methods."

TABLE 1

The calculated dissociation constants ($K_{d(app)}$), Hill coefficients (n_H), and overall nonlinear fit of the data (R^2) for FACT, FACTΔCTD, Spt16, and SSRP1 binding various histone/nucleosome constructs

Stoichiometries are shown for all high affinity FACT, Spt16, and SSRP1 interactions ($K_{d(app)}$ <100 nM) with histones and nucleosomes.

FACT	$K_{d(app)}$ ($\times 10^{-9}$ M)	Hill Coefficient	Overall Fit (R^2)	Stoichiometry (FACT : Ligand)
WT H2A-H2B	31.3 ± 8.45	1.1 ± 0.3	0.89	1.2 : 1
Tailless H2A-H2B	211 ± 47.9	0.8 ± 0.3	0.95	-
WT H3-H4	685 ± 111	1.6 ± 0.3	0.96	-
Tailless H3-H4	>1 μM	1.4 ± 0.4	0.87	-
147 bp Nucleosome	64.3 ± 15.7	0.9 ± 0.2	0.95	1.2 : 1
Tailless 146-Nucleosome	81.9 ± 28.9	0.9 ± 0.2	0.92	1.0 : 1
207 bp Nucleosome	22.4 ± 5.18	1.1 ± 0.2	0.91	1.7 : 1
Tailless 207-Nucleosome	77.2 ± 23.2	0.9 ± 0.3	0.88	1.5 : 1
NLE Tri-Nucleosome	35.6 ± 7.33	1.4 ± 0.4	0.94	2.4 : 1
LE Tri-Nucleosome	25.5 ± 5.01	1.6 ± 0.5	0.90	3.3 : 1
30 bp Linker DNA	7.16 ± 0.85	2.1 ± 0.5	0.92	-

FACTΔCTD	$K_{d(app)}$ ($\times 10^{-9}$ M)	Hill Coefficient	Overall Fit (R^2)	Stoichiometry (FACT : Ligand)
WT H2A-H2B	170 ± 72.9	2.2 ± 0.8	0.88	-
Tailless H2A-H2B	243 ± 39.3	1.7 ± 0.2	0.98	-
WT H3-H4	>1 μM	1.4 ± 0.4	0.88	-
Tailless H3-H4	>1 μM	1.4 ± 0.2	0.94	-
147 bp Nucleosome	>1 μM	0.8 ± 0.1	0.92	-
207 bp Nucleosome	501 ± 116	1.2 ± 0.2	0.96	-
30 bp Linker DNA	8.75 ± 1.01	1.8 ± 0.3	0.93	-

Spt16	$K_{d(app)}$ ($\times 10^{-9}$ M)	Hill Coefficient	Overall Fit (R^2)	Stoichiometry (Spt16 : Ligand)
WT H2A-H2B	84.7 ± 15.7	0.8 ± 0.2	0.98	1.3 : 1
Tailless H2A-H2B	140 ± 24.7	1.2 ± 0.4	0.89	-
WT H3-H4	>1 μM	1.5 ± 0.6	0.91	-
Tailless H3-H4	No Binding	-	-	-
147 bp Nucleosome	85.9 ± 25.6	1.3 ± 0.4	0.93	2.0 : 1
207 bp Nucleosome	73.5 ± 21.5	1.6 ± 0.3	0.89	1.9 : 1
30 bp Linker DNA	No Binding	-	-	-

SSRP1	$K_{d(app)}$ ($\times 10^{-9}$ M)	Hill Coefficient	Overall Fit (R^2)	Stoichiometry (SSRP1 : Ligand)
WT H2A-H2B	No Binding	-	-	-
Tailless H2A-H2B	No Binding	-	-	-
WT H3-H4	762 ± 222	2.1 ± 0.7	0.88	-
Tailless H3-H4	> 1 μM	1.3 ± 0.1	0.98	-
147 bp Nucleosome	> 1 μM	0.7 ± 0.3	0.93	-
207 bp Nucleosome	67.4 ± 14.8	1.2 ± 0.4	0.85	2.2 : 1
30 bp Linker DNA	9.89 ± 1.16	1.5 ± 0.3	0.91	-

FACT Is Highly Specific for Histones H2A-H2B and This Interaction Is Promoted by the Histone Tails—We first set out to quantify FACT binding to the two histone complexes, namely H2A-H2B and H3-H4, and to investigate the contribution of histone tails to these interactions. The full-length FACT complex binds H2A-H2B at low nanomolar concentrations, which is in the same range as previously determined dissociation constants for the histone chaperone Nap1, measured under similar conditions (26). In contrast to Nap1, FACT shows a clear preference for H2A-H2B over H3-H4 (Fig. 1 and Table 1). A quantitative ~20-fold binding preference for H2A-H2B establishes a clear thermodynamic basis for the reported H2A-H2B-specific chaperone activity of FACT (9, 10, 15).

Histone tails are highly basic and intrinsically disordered N- and C-terminal regions of all four core histones. Substantial binding defects were observed when titrating full-length FACT into completely tail-less versions of the histone complexes H2A-H2B and H3-H4. Removal of these basic extensions lowers the binding affinity of FACT for H2A-H2B ~7-fold (Fig. 1*A* and Table 1), and although FACT can bind wild-type H3-H4 within the high nanomolar range, the dissociation constant for FACT and tail-less H3-H4 measures well above 1 μM (Fig. 1*B*). Histone tail deletion does not completely eliminate FACT

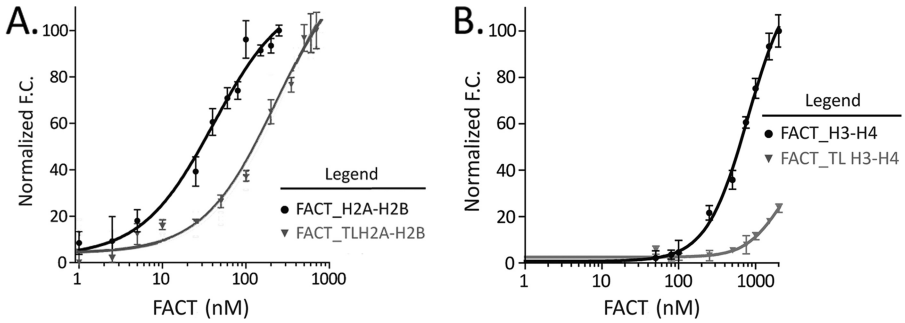

FIGURE 1. **FACT preferentially binds H2A-H2B over H3-H4, yet both interactions are enhanced by the histone tails.** *A* shows the normalized fluorescence change upon titration of FACT (log [FACT]) into fluorescently labeled H2A-H2B and TL H2A-H2B. Fluorescence change (*F.C.*) occurs as a result of direct protein/protein interaction. A 7-fold decrease in affinity between FACT and H2A-H2B is observed when these basic extensions are removed from the histones (binding curve shifts right to higher FACT concentrations). *B* displays the binding curves for FACT with H3-H4 and TL H3-H4. FACT·H3-H4 binding occurs at high nanomolar concentrations, yet deletion of the N- and C-terminal tails pushes binding out of the nanomolar range. The *error bars* represent the standard error within individual data points. The total data points (*N*) for a single experiment are 12.

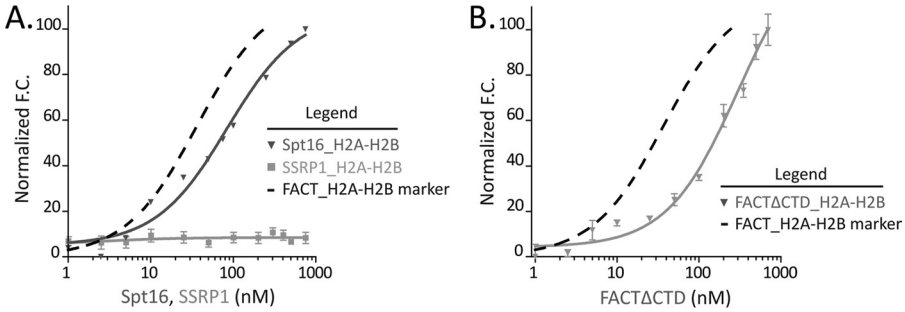

FIGURE 2. **Spt16 subunit and its acidic C-terminal domain coordinate high affinity FACT interaction(s) with H2A-H2B.** *A* identifies the FACT subunit (Spt16 or SSRP1) responsible for the interaction with H2A-H2B. The lack of discernible fluorescence change for the SSRP1·H2A-H2B titration indicates a lack of binding within this concentration range. The Spt16 subunit binds H2A-H2B with slightly lower affinity than full-length FACT. The FACT·H2A-H2B binding curve from Fig. 1 is displayed as a *dashed line* to provide a clear comparison. *B* shows the binding curve for an Spt16 CTD-deleted FACT construct (FACTΔCTD) and H2A-H2B. Without the Spt16 CTD, the binding affinity for H2A-H2B decreases ~6-fold. The *error bars* represent the standard error within individual data points. The total data points (*N*) for a single experiment are 12.

binding, which supports a complex mode of interaction through multiple contact points on histones. The facilitating roles that histone tails play in FACT binding is striking in light of previous results regarding other histone chaperones that demonstrate the histone tails have no tangible effect on CAF-1 action (35) and negatively affect Nap1 binding (26). The data also suggest a regulatory mechanism induced by the various post-translational modifications known to occupy these tail extensions (7).

Spt16 and Specifically the Acidic CTD Coordinates FACT Interaction(s) with H2A-H2B—The fundamental FACT subunits (Spt16 and SSRP1) are both essential to FACT function within the cell or as a component of an *in vitro* transcription system (10, 36–38). Fig. 2*A* delves deeper into the FACT/H2A-H2B interaction by isolating the subunit principally responsible for the high affinity interaction. The Spt16 subunit binds H2A-H2B with only slightly lower affinity than the full-length FACT complex (85 and 31 nM, respectively). Under the same conditions, we do not observe any binding between SSRP1 and H2A-H2B through fluorescence change (Fig. 2*A*) or EMSA shift on 5% native polyacrylamide gels. Thus, the Spt16 subunit of FACT provides the major interaction interface for H2A-H2B binding, whereas the presence of SSRP1 in FACT may contribute to the low affinity contacts or stabilization of the Spt16/H2A-H2B interaction.

Numerous histone-binding proteins contain acidic regions thought to be critical for histone binding (39). Analysis with FACTΔCTD exposes a direct relationship between the CTD of Spt16 and FACT/histone interactions (Table 1). Deletion of the CTD from Spt16 negatively affects H2A-H2B and H3-H4 binding by the remaining FACT molecule. The FACTΔCTD·H2A-H2B $K_{d(\mathrm{app})}$ increases ~6-fold compared with full-length FACT, and FACTΔCTD fails to bind H3-H4 below 1 μM (Fig. 2*B* and Table 1). It is not difficult to envision a scenario where the positively charged histones form an attractive platform for binding by the flexible negatively charged CTD of Spt16. Again, removal of the CTD from Spt16 negatively impacted histone binding, yet complete interaction was not abolished indicating additional contact sites remain unaffected.

HMG-1 Domain Containing SSRP1 Subunit (Alone or Bound in the FACT Complex) Binds Free DNA with High Affinity—FACT is unusual among histone chaperones in that it binds DNA as well as both free histone and intact nucleosomes. To quantify the DNA binding affinity, we measured FACT binding to a short 30-bp DNA fragment that is identical in sequence to the linker regions of the 601 sequence 207-bp nucleosomal DNA (28). Titration of a fluorescently end-labeled 30-bp DNA fragment with FACT, FACTΔCTD, or SSRP1 results in binding affinities below 10 nM (7.16, 8.75, and 9.89 nM respectively) (supplemental Fig. S2 and Table 1). The Hill coefficients for each binding event show some level of cooperativity ($n_{\mathrm{H}} > 1$), yet the values decrease upon removal of the Spt16 CTD or the entire Spt16 subunit. Thus, although the Spt16 subunit cannot directly bind DNA (Table 1), the presence of Spt16 notably

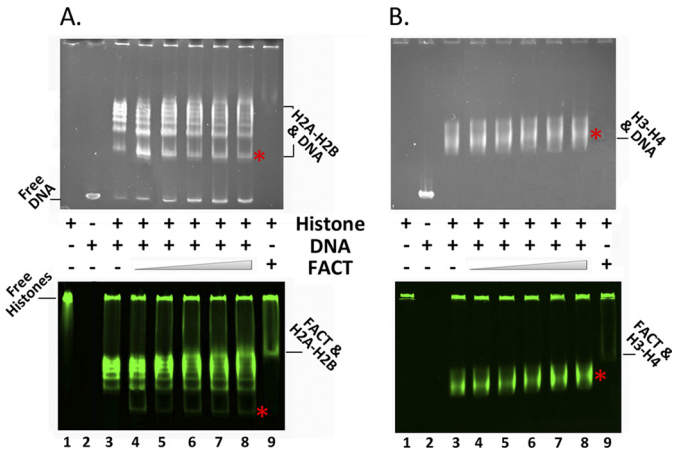

FIGURE 3. **FACT competes with DNA for a shared interaction interface on H2A-H2B but cannot compete H3-H4 from DNA.** Histones can readily bind DNA at physiological salt concentrations *in vitro*, as seen by native PAGE. The use of fluorescently labeled histones allows visualization by two distinct means (UV, *top panels*, to monitor DNA; and fluorescence scanning, *bottom panels*, to monitor histones). The H2A-H2B dimer forms many stable complexes with a 207-bp 601 sequence DNA construct (*A*). Incubation with H3-H4 will form a stable tetrasome where DNA is wrapped around a single $(H3\text{-}H4)_2$ tetramer (*B*). The appearance of accumulating free DNA (*top left panel, lanes 4–8*) and a higher order band running similar to a preformed FACT·H2A-H2B complex (*bottom left panel, lanes 6–8*) confirms that FACT can compete H2A-H2B from DNA but cannot form a ternary complex with DNA-bound H2A-H2B. *Right panels* show that, in contrast, FACT cannot remove H3-H4 from DNA once bound. No evidence for free DNA, a FACT·H3-H4 complex, or ternary complex is observed upon FACT titration (*B, lanes 4–8*). *Red asterisks* are displayed next to the furthest running histone·DNA species to aid specific band comparisons between the UV and fluorescence gel images.

enhances the overall cooperative nature of the interaction. Further investigation is necessary to appreciate the role of Spt16 in DNA binding by the HMG-1 domain of SSRP1 (see below).

FACT Competes for H2A-H2B Binding with DNA but Cannot Compete H3-H4 Away from DNA—The histone complexes H2A-H2B and H3-H4 form stable associations with DNA at near physiological conditions. Regulation of non-nucleosomal histone/DNA interactions was previously proposed and later confirmed to be the principal function of select histone chaperones (40–42). Does FACT function under these same principles? To test the ability of FACT to eliminate non-nucleosomal histone/DNA interactions, we established a direct competition assay performed by incubation of fluorescently labeled histones with DNA at a constant concentration and ratio, which is then titrated with FACT (final FACT/histone ratio = 1:1) (Fig. 3). Fig. 3*A* shows the UV visualized (*top left*) and fluorescently scanned (*bottom left*) native gel for the H2A-H2B competition assay. H2A-H2B forms multiple interactions with 207-bp DNA creating a ladder of visible bands (non-nucleosomal interactions). The UV visualized gel shows the depletion of histone·DNA binding and free DNA accumulation upon increasing FACT concentrations. The fluorescently scanned gel confirms the location of labeled H2A-H2B within the bands. An additional higher order band appears with increasing FACT concentrations and migrates similar to a preformed FACT·H2A-H2B complex, which suggests that FACT can compete with DNA for H2A-H2B binding. Also, FACT and DNA likely contend for an overlapping interaction interface on H2A-H2B because no evidence of a stable ternary complex (FACT·H2A-H2B·DNA) was observed. Fig. 3*B* demonstrates that, in contrast to H2A-H2B, FACT cannot compete H3-H4 away from DNA. Incubation of H3-H4 with 207-bp DNA gives rise to a single band on the native gel (tetrasome), unlike the ladder seen for H2A-H2B. DNA binding to H3-H4 is unaffected by FACT titration with no appearance of free DNA or a FACT·H3-H4 complex. The notion that FACT can modulate H2A-H2B/DNA interactions but cannot alter tetrasomes fits well with our measured binding affinities and alludes to the mechanistic details of FACT-mediated nucleosome assembly.

The ability of FACT to remove H2A-H2B from DNA depends on the CTD of Spt16. In a parallel competition experiment, FACTΔCTD cannot compete with DNA for binding to H2A-H2B, yet it forms a FACTΔCTD·H2A-H2B·DNA ternary complex. Unlike with FACT, titrating FACTΔCTD into preformed H2A-H2B·DNA complexes fails to give rise to increasing amounts of free DNA (supplemental Fig. S3*A*). However, at higher FACTΔCTD concentrations, a slower migrating species appears that can be observed through both UV visualization (*A, top panel*) and fluorescence scanning (*A, bottom panel*) indicative of a ternary complex. The inability of a FACTΔCTD·H2A-H2B complex (*A, bottom panel, lane 9*) to enter the gel provides additional evidence for characterizing the slower migrating species in *lanes 7* and *8* as a ternary complex (the negatively charged DNA of the ternary complex facilitates gel entry). The results signal that the CTD of Spt16 is essential for competing DNA away from H2A-H2B. Other regions/domains of FACT must tether to distinct H2A-H2B interfaces accessible during H2A-H2B/DNA interaction to form a stable ternary complex.

Spt16 and SSRP1 Collaborate in the FACT Complex to Create a Single High Affinity Binding Event with Nucleosomes—We have now shown high affinity binding by FACT and the two subunits Spt16 and SSRP1 to various nucleosomal components. However, within the nucleosome, these components are restricted in accessibility and conformational freedom (1). Two separate mono-nucleosome constructs were used to interpret the individual contributions of Spt16 and SSRP1 in nucleosome binding by FACT. Fig. 4, *A* and *B*, shows the binding curves for the titration of FACT, Spt16, and SSRP1 into either the 147-bp nucleosome, which contains no linker DNA (Nuc147), or the 207-bp linker DNA-containing nucleosome (Nuc207). The full-length FACT complex binds both forms of the nucleosome with high affinity (22 nM for Nuc207 and 64 nM for Nuc147). The presence of two symmetrical 30-bp linker "arms" enhances binding ~3-fold. Previous studies with yeast FACT have estimated a FACT·nucleosome $K_{d(\mathrm{app})}$ of around 10–20 nM based upon low concentration EMSA shifts (43). The Spt16 subunit binds both nucleosome constructs with slightly lower affinity compared with FACT (Table 1). Linker DNA has little consequence on nucleosome binding affinity by Spt16. In contrast, the SSRP1 subunit requires linker DNA to interact stably with the nucleosome, as seen from a comparison of the $K_{d(\mathrm{app})}$ values for Nuc147 and Nuc207 (>1 μM and 67 nM, respectively) (Fig. 4*B* and Table 1). The result that neither Spt16 nor SSRP1 can bind nucleosomes as tightly as the full-length FACT complex reveals synergistic binding by the two FACT subunits.

Basic Histone Tail Extensions Enhance Nucleosome Binding by FACT—The tail extensions of nucleosomal histones intercalate through the DNA and are the targets of extrinsic modula-

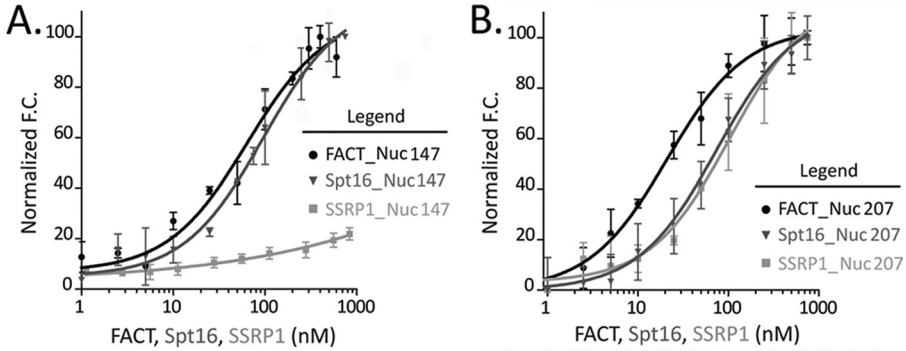

FIGURE 4. **Linker DNA is required for high affinity interaction between SSRP1 and nucleosomes.** Nuc147 lacks the symmetrical 30-bp linker DNA extensions of Nuc207. FACT and Spt16 can bind either form with high affinity ($K_{d(app)}$ <100 nM). However, as seen in *A*, the SSRP1 subunit cannot bind the 147-bp nucleosome in the nanomolar range. However, SSRP1 binds Nuc207 with high affinity (*B*), which suggests that linker DNA is a target of SSRP1 binding. *B* also shows that both Spt16 and SSRP1 subunits together in complex are essential for achieving the highest affinity binding event with Nuc207. The *error bars* represent the standard error within individual data points. The total data points (*N*) for a single experiment are 12.

tors (histone modifiers). Removal of the histone tails significantly affects free H2A-H2B and H3-H4 binding by FACT (Fig. 1 and Table 1). Does the deletion effect extend to the nucleosome? To answer this, we prepared completely TL versions of both Nuc147 and Nuc207, and surprisingly, we found that histone tail deletion leads to very similar $K_{d(app)}$ values for both TL nucleosome constructs when titrated with FACT (82 nM for TL Nuc147 and 77 nM for TL Nuc207) (supplemental Fig. S4 and Table 1). This noticeably contrasts with FACT titrations into tail-containing Nuc147 and Nuc207 where longer DNA lengths correlate with higher affinity binding. Removal of all histone tails from the nucleosome essentially eliminates the positive effect of linker DNA on FACT binding. Thus, regions of FACT may directly bind histone tails (21, 22) to coordinate the chaperone for interaction with and/or promote accessibility to linker DNA.

Spt16 CTD Promotes Nucleosomal DNA Binding by the SSRP1 Subunit—We measured the dissociation constants for all HMG-1 domains containing FACT constructs (FACT, FACTΔCTD, and SSRP1) with free "linker" DNA consistently below 10 nM. These values are ~7-fold lower than the $K_{d(app)}$ for Nuc207 titrated with SSRP1. The inability of SSRP1 to bind linker DNA-containing nucleosomes with similar affinities to that of free linker DNA signals interference from the histone octamer itself and/or from an octamer-induced conformation of the linker DNA. This inhibition can be largely alleviated by full-length FACT. We find that removal of the Spt16 CTD from FACT (FACTΔCTD), which still contains an active SSRP1 subunit, binds Nuc207 with ~20-fold less affinity than FACT and ~7-fold less affinity than SSRP1 alone (supplemental Fig. S5 and Table 1). Indeed, removal of the Spt16 CTD not only diminishes the interaction with H2A-H2B (Fig. 2) but also indirectly hinders nucleosomal DNA binding by the SSRP1 subunit. The role of the CTD of Spt16 may be to help the HMG-1 domain of SSRP1 bind stably to nucleosomal DNA through loosening of H2A-H2B/DNA interactions.

Multiple FACT Molecules Bind to Saturated Tri-nucleosome Arrays with Similar Affinities to Mono-nucleosomes—Highly defined tri-nucleosome arrays provide a minimal system to evaluate the FACT/nucleosome interaction in a chromatin context. In the cell, FACT must navigate through chromatin assemblies to bind and reorganize individual nucleosomes. To better understand the effects of inter-nucleosomal connections on FACT function, we constructed two distinct tri-nucleosome arrays that can be fluorescently labeled at the same locations as their mono-nucleosome counterparts. The first construct, termed LE (linker-ended), includes 30-bp linker DNA arms extending from the outer nucleosomes (Fig. 5*C*). The second construct, termed NLE (nonlinker-ended), lacks peripheral linker DNA on the outer nucleosomes (Fig. 5*D*). FACT binds either array with comparable affinities to Nuc207 (26 nM for LE and 36 nM for NLE) suggesting that existing nucleosome-nucleosome contacts do not deter FACT binding (Table 1).

Results from another laboratory have previously shown that maximal transcription rates *in vitro* occur when the FACT/nucleosome ratio is near 1:1 (9, 11). To assess whether this ratio is the preferred binding status between FACT and nucleosomal arrays, we modified our fluorescence (de)quenching microplate assay to determine binding stoichiometries for FACT and mono-nucleosomes as well as tri-nucleosomes. Here, the labeled (tri)nucleosome concentrations are kept constant 5–10-fold above the measured $K_{d(app)}$, and FACT is titrated in increasing ratios (0:1–4:1 [FACT/(tri)nucleosome]). Thus, fluorescence change (binding) will begin immediately with FACT titration and plateau upon binding site saturation (stoichiometric ratio). Fig. 5, *A–D*, compares the FACT·mono-nucleosome stoichiometry to the results for FACT and tri-nucleosome arrays. 1–2 FACT molecules can stably bind an individual nucleosome, whether mono or as part of an array. In general, for all FACT/(tri)nucleosome interactions, Hill coefficients (n_H), and stoichiometry increase as a function of linker DNA (Table 1). Our data clearly indicates that, in addition to mono-nucleosomes, FACT can bind at high density to unmodified nucleosomal arrays in the absence of additional chromatin modulators or recruiting factors.

DISCUSSION

The FACT complex has been classified as a histone H2A-H2B-specific chaperone (9, 10) with direct roles in transcription (11), replication (12, 13, 44), histone variant exchange (15), and DNA repair (14). Our present results definitively and quantitatively assign the histone selectivity of FACT. FACT is shown to compete with DNA for binding to H2A-H2B (non-nucleo-

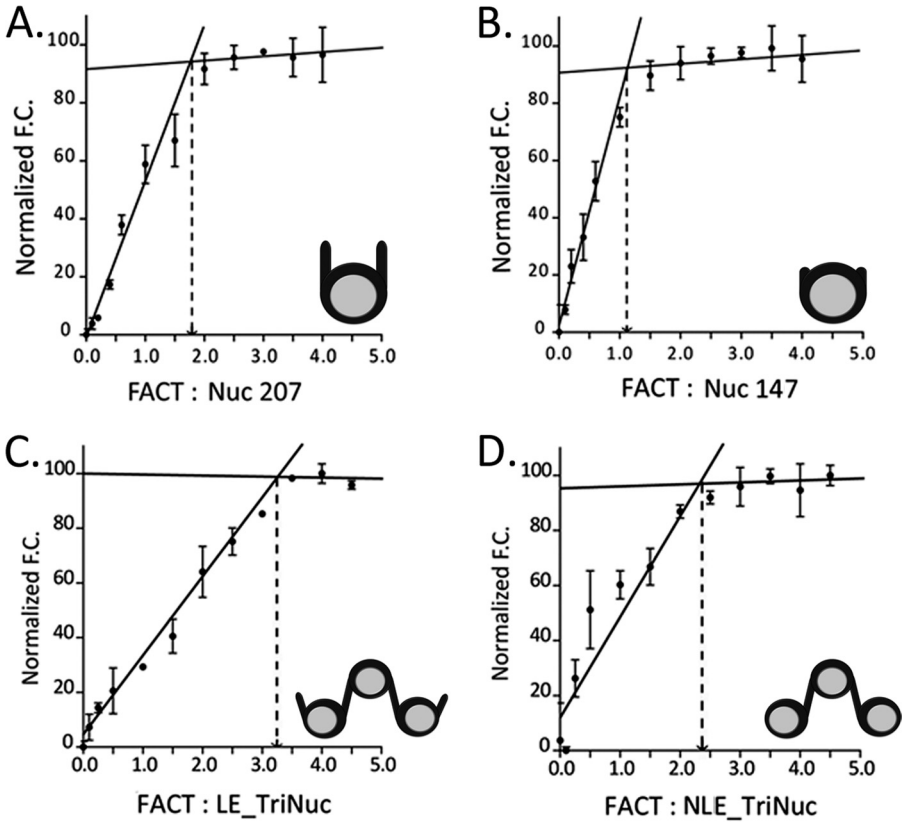

FIGURE 5. **Stoichiometry and positions of FACT within tri-nucleosome arrays.** *A–D* show stoichiometry measurements for FACT with the Nuc207, Nuc147, and two distinct tri-nucleosome constructs. The tri-nucleosomes are labeled LE (linker ended), which contain 30-bp linker arms extending from the terminal nucleosomes, and NLE (non-linker ended), which lacks terminal linkers. To measure stoichiometries, the labeled probe ((tri)nucleosomes) was kept constant 5–10-fold over the measured $K_{d(app)}$ so that any addition of FACT results in direct interaction. The fluorescence change deviates from linearity at the point of saturation. The presence of terminal linker DNA on the (tri)nucleosome (*A* and *C*) increases the stoichiometry compared with the Nuc147 and NLE tri-nucleosome. The *error bars* represent the standard error within individual data points. The total data points (*N*) for a single experiment are 12.

somal interactions), but it is unable to out-compete DNA for H3-H4 (nucleosomal pathway intermediate). We also determined the individual contributions of the FACT subunits (Spt16 and SSRP1) and relevant domains (Spt16 CTD and SSRP1 HMG-1 domain) to synergistic nucleosome binding. A picture emerges in which multiple domains in FACT interact with distinct target regions on the nucleosome. We illustrate a contemporary model for the role of the Spt16 CTD in promoting nucleosomal DNA accessibility. Finally, work with tri-nucleosome arrays provides insight into FACT·nucleosome binding in a chromatin context.

Preliminary Thermodynamic Model for FACT-mediated Nucleosome (Dis)assembly—Previous work from our laboratory indicates that the histone chaperone Nap1 promotes nucleosome formation through the elimination of non-nucleosomal histone/DNA interactions (42). Through this related work, critical thermodynamic parameters regarding nucleosome (dis)assembly have been enumerated, including the dissociation constants for the H2A-H2B·DNA and H2A-H2B·tetrasome complexes ($K_{d(app)}$ for H2A-H2B·DNA = 44 nM and H2A-H2B·tetrasome = 13 nM) (42). Remarkably, the $K_{d(app)}$ for the binding of free H2A-H2B by FACT ($K_{d(app)}$ = 31 nM), measured under similar conditions, lies between the aforementioned values. The proximity of the measured dissociation constants signals that thermodynamics provide a means for FACT to exchange H2A-H2B with DNA and tetrasome in the absence of ATP hydrolysis. For instance, FACT could readily bind newly translated, evicted, or non-nucleosomal DNA bound H2A-H2B for purposes of nucleosome (re)assembly or H2A-H2B variant exchange. Direct competition assays shown in Fig. 3 demonstrate that FACT can actively remove H2A-H2B from DNA. In contrast, FACT binds H3-H4 with ~20-fold lower affinity than H2A-H2B and cannot compete with DNA for H3-H4 binding given that the dissociation constant for tetrasome formation reliably measures ~1 nM (42). This explains why FACT can promote H3-H4 binding to DNA (10) but cannot remove H3-H4 from DNA once bound (Fig. 3*B*).

FACT Coordinates Interaction with Nucleosome through a Multitude of Synergistic Binding Events—Unlike several members of the histone chaperone family, FACT interacts with both H2A-H2B and H3-H4 in addition to DNA and nucleosomes (Table 1) (39). We have shown here that collaborative interactions by several conserved FACT domains create a singular high affinity binding event with the nucleosome. Consequently, neither Spt16 nor SSRP1 alone bind nucleosomes as tightly as the complete FACT complex. The FACT complex binds H3-H4 in the high nanomolar range through sites on both the Spt16 and SSRP1 subunits. This fits well with previous biophysical data demonstrating that SSRP1 and the N-terminal domain of

Spt16 stably bind intact H3-H4 and N-terminal tail peptides of both H3 and H4 *in vitro* (9, 21). Nevertheless, FACT vastly prefers H2A-H2B, which it binds with ~20-fold higher affinity than H3-H4. Removal of the N- and C-terminal tails from H2A-H2B noticeably impedes FACT binding. The high affinity FACT/H2A-H2B and FACT/nucleosome interactions rely greatly upon the acidic C-terminal domain of Spt16, which is essential for transcription through chromatin *in vitro* and yeast viability (10, 45). Interestingly, removal of the H2A-H2B tails does not significantly deter FACTΔCTD binding (Table 1). Therefore, the results suggest that the Spt16 CTD and the histone tails facilitate FACT·H2A-H2B binding through direct interaction. The DNA binding utility of FACT is directed by the HMG-1 domain containing the SSRP1 subunit (9). Related HMG-1 domain-containing proteins are purported to bind nucleosomal DNA near the entry/exit sites where the DNA is most accessible (46). We have shown that SSRP1 binds nucleosomes with accessible linker DNA (Nuc207) significantly tighter than nucleosomes lacking linker DNA (Nuc147) (Fig. 4). Stoichiometric assessment of the FACT/(tri)nucleosome interaction illustrates that the presence of linker DNA positively influences FACT occupancy. Ultimately, the FACT·nucleosome binding event is highly involved and requires coordination of multiple nucleosome target sites through collaborating FACT domains (Fig. 6, *A*–*C*), which is unlike other known examples of nucleosome/protein interactions (47).

FACT, but Not FACTΔCTD, Can Compete with DNA for Non-nucleosomal Bound H2A-H2B—Direct competition assays performed at concentrations above 1 μM confirm that FACT can compete with DNA for H2A-H2B binding but together cannot produce a stable ternary FACT·H2A-H2B·DNA complex. In agreement with our findings, recent work utilizing *in vitro* transcription assays illustrate that polymerase II pausing within nucleosomes can be alleviated through histone mutations that weaken DNA binding (48–50). FACT may serve to relieve these blockages in the absence of specific histone mutations. Deletion of the Spt16 CTD from FACT (FACTΔCTD) prevents removal of H2A-H2B from DNA. The formation of a ternary complex (FACTΔCTD·H2A-H2B·DNA) at higher FACTΔCTD concentrations, but not for FACT, implies a reversible two-step mechanism for FACT-mediated H2A-H2B exchange with DNA. 1: FACT binds to an available site on H2A-H2B not involved in DNA binding. 2: The CTD of Spt16 out-competes DNA for H2A-H2B, which produces free DNA and a FACT·H2A-H2B complex. Without the Spt16 CTD, the mechanism is "trapped" in step 1, resulting in a stable ternary complex.

C-terminal Domain of Spt16 Can Effectively Dislodge DNA from Interactions with Histone Octamer—The CTD of Spt16 is the most conserved region of FACT across all eukaryotic species and is analogous to acidic extensions preserved in other members of the histone chaperone family (20, 51). Additionally, this region of FACT has been shown to be essential for stable nucleosome binding, histone binding to DNA (after heating), and FACT-mediated nucleosome reorganization and disassembly generating a hexasome (10). Deletion of this acidic domain from FACT (FACTΔCTD) severely diminishes nucleosome binding despite the presence of the SSRP1 subunit that can stably bind DNA and nucleosomes with linker DNA (Nuc207) (Fig. 6*D* and Table 1). High affinity nucleosome binding by the entire FACT complex may require dislodging of DNA from the histone octamer by the Spt16 CTD.

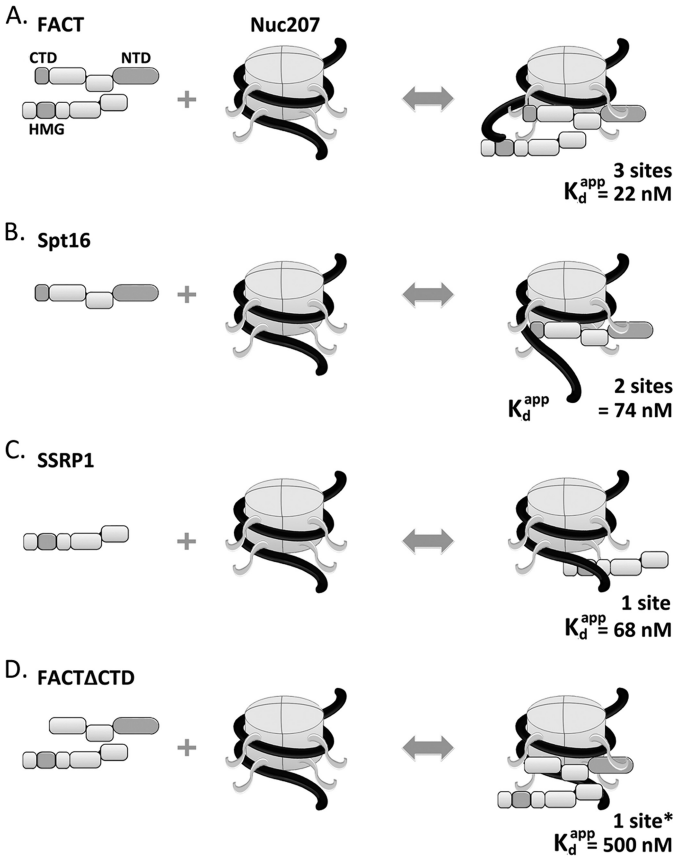

FIGURE 6. **Multiple interactions between FACT and nucleosome produce a singular high affinity binding event.** The critical nucleosome-binding sites on FACT, as identified in our study, are shaded *dark* and labeled as *CTD*, N-terminal domain (*NTD*), and *HMG* for the acidic C-terminal domain of Spt16, N-terminal domain of Spt16, and the HMG-1 DNA binding site on SSRP1, respectively. *A* illustrates the inclusion of the three binding sites in the modulation of an intact nucleosome by full-length FACT. The Spt16 N-terminal domain binds histone tails, and the CTD displaces DNA for binding by the SSRP1 HMG-1 domain. Elimination of the SSRP1 subunit reduces the number of interaction sites to two and thus decreases the overall affinity for Nuc207 (*B*). The SSRP1 subunit alone, in *C*, can only promote a single high affinity interaction with Nuc207 through accessible linker DNA. Deletion of the Spt16 CTD from FACT not only eliminates a direct interaction interface with the core histones but seemingly blocks DNA binding by the SSRP1 HMG-1 domain. The overall affinity for Nuc207 decreases ~20-fold.

The following question remains. How does the SSRP1 subunit alone bind to nucleosomes without help from the Spt16 CTD (Fig. 4*B*)? Recent work on the accessibility of DNA-binding proteins to nucleosomes uncovered evidence that rapid dissociation/re-association of nucleosomal DNA is enough for low occupancy binding near the ends of the DNA (52, 53). Disruption of terminal histone·DNA contacts as a result of protein binding promoted access for additional DNA binding events further into the nucleosome. To this end, SSRP1 alone may bind to the accessible ends of the Nuc207 linker DNA, but it needs higher concentrations than FACT to do so. The Spt16 CTD of FACT may compete away and preserve the dissociated state of the DNA. This detachment would allow greater acces-

sibility to nucleosomal DNA for SSRP1 binding (Fig. 6*A*) and is consistent with previous findings that show FACT binding creates "open" exonuclease-sensitive nucleosomes and/or H2A-H2B-evicted hexasomes (8, 10). A similar mechanism has been proposed for the H2A-H2B-specific histone chaperone nucleoplasmin, in which an extended acidic region outside of the core domain promotes exchange of the basic histone regions that bind DNA. The model supposes that nucleoplasmin works as an "intermediate carrier" that reduces the energy barrier of nucleosome disassembly (54). The role of the Spt16 CTD as a "helper" for stable high affinity DNA binding by SSRP1 could explain why yeast FACT, which functions through preliminary nucleosome binding by a separate HMGB DNA binding domain (Nhp6), requires a 10-fold excess of Nhp6 (43). An increased local concentration of Nhp6 and multiple DNA binding events may be necessary for global access to the histone octamer.

Nucleosome Binding by FACT in a Chromatin Context—FACT functions to remove primary chromatin blockages (nucleosomes) for the purposes of replication, transcription, DNA repair, and histone exchange (20, 55). Secondary chromatin structures and inter-nucleosomal interactions have the potential to influence FACT binding and function. Our work with tri-nucleosome arrays suggests that FACT binds individual nucleosomes within arrays at affinities similar to that of mono-nucleosomes. The availability of additional linker DNA on the outer nucleosomes increases both cooperativity and stoichiometry of the overall binding event. Despite the same number of histone octamers, the LE construct, which contains external linkers, bound approximately one additional FACT molecule compared with the NLE construct (no external linkers) (3.3:1 and 2.4:1, respectively). The absence of both linker arms on the outer nucleosomes within the NLE construct may hinder stable binding at these sites and decrease the overall occupancy of FACT. Our results indicate that FACT can concomitantly bind neighboring nucleosomes without apparent steric hindrance and correspond well with previous data that indicate maximal FACT activity occurs at or near equal ratios of FACT and nucleosomes (9). Also, FACT exists in high numbers within the nucleus (~25,000 per yeast cell), which could allow FACT persistent access to individual nucleosomes without recycling of the same FACT molecule to adjacent nucleosomes, essentially coating local regions of chromatin (56).

Initial quantitative binding measurements for FACT with various nucleosome components provided a framework to deconvolute the multifaceted FACT/nucleosome interaction. Data obtained with mono-nucleosome and saturated tri-nucleosome arrays provide a concept for FACT binding and occupancy within chromatin. Further experiments with Spt16, SSRP1, and a truncated form of FACT allowed postulation of a mechanism of FACT function where the N-terminal domain of Spt16 strategically tethers FACT to accessible regions on histones (including tails) allowing intercalation of the CTD of Spt16 between the histone octamer and DNA. The newly accessible DNA can then be bound by the HMG-1 domain of SSRP1. The prevalence of post-translational modifications to histone tails and the effect these terminal extensions have on FACT binding provides the means for regulating FACT function. In all, we have revealed the primary thermodynamic parameters guiding FACT/nucleosome interaction(s) and we utilized these data to develop new models for FACT function.

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