A New Fluorescence Resonance Energy Transfer Approach Demonstrates That the Histone Variant H2AZ Stabilizes the Histone Octamer within the Nucleosome

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Nucleosomes are highly dynamic macromolecular complexes that are assembled and disassembled in a modular fashion. One important way in which this dynamic process can be modulated is by the replacement of major histones with their variants, thereby affecting nucleosome structure and function. Here we use fluorescence resonance energy transfer between fluorophores attached to various defined locations within the nucleosome to dissect and compare the structural transitions of a H2A.Z containing and a canonical nucleosome in response to increasing ionic strength. We show that the peripheral regions of the DNA dissociate from the surface of the histone octamer at relatively low ionic strength, under conditions where the dimer-tetramer interaction remains unaffected. At around 550 mM NaCl, the (H2A-H2B) dimer dissociates from the (H3-H4)2 tetramer-DNA complex. Significantly, this latter transition is stabilized in nucleosomes that have been reconstituted with the essential histone variant H2A.Z. Our studies firmly establish fluorescence resonance energy transfer as a valid method to study nucleosome stability, and shed new light on the biological function of H2A.Z.

Chromatin is built from nucleosomes, the universally repeating protein-DNA complexes in all eukaryotic cells. The crystal structure of the nucleosome core particle (NCP) reveals an octameric histone core around which 147 base pairs of DNA are wrapped in 1.65 tight superhelical turns. The histone octamer itself is a modular assembly of two copies each of the four histone proteins H2A, H2B, H3, and H4. Two histone pairs, composed either of H2A and H2B, or H3 and H4, form stable heterodimers. In solution, two H3-H4 dimers form a tetramer in the shape of a flat, twisted horseshoe that binds the central 60 base pairs of the nucleosomal DNA around its outside (1, 2). One (H2A-H2B) dimer is tethered to each face of the (H3-H4)2 tetramer-DNA complex, to complete a "helical ramp" for continued DNA binding (reviewed in Ref. 3).

The interaction between the two histone subcomplexes occurs via two spatially distinct interaction interfaces of quite different character (Fig. 1). A four-helix bundle structure (formed by H2B and H4) is characterized mainly by hydrophobic interactions, and buries 1000 Å² (1). The second, larger interface (1600 Å²) is characterized mostly by direct and solvent-mediated hydrogen bonds between the "docking domain" of H2A, and the histone fold extensions of H3 and H4 (4). In addition, a small interface is formed between the L1 loops of the two H2A molecules, which may contribute to holding together the two gyres of the DNA superhelix at the back of the nucleosome (Fig. 1) (5). Under physiological conditions, the (H2A-H2B) dimer associates stably with the (H3-H4)2 tetramer only in the presence of DNA (6). Each (H2A-H2B) dimer organizes 30 base pairs toward either end of the DNA. The penultimate 10 base pairs of nucleosomal DNA on either side are organized by a region of H3 that does not form an integral part of the (H3-H4)2 tetramer, and that is most likely not positioned to bind DNA in the absence of the (H2A-H2B) dimer (7). The interactions between protein and DNA in this region are weaker than anywhere else in the NCP (7).

Received for publication, December 2, 2003, and in revised form, March 12, 2004
Published, JBC Papers in Press, March 13, 2004, DOI 10.1074/jbc.M313152200

The on-line version of this article (available at http://www.jbc.org) contains Fig. S1.
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§ The abbreviations used are: NCP, nucleosome core particle; FRET, fluorescence resonance energy transfer; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; CPyM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; FM, fluorescein-5-maleimide.

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2 1The work was supported by Human Frontiers Science Program Grant RG00059 (to K. L. and D. J. T.) and National Institutes of Health Grant GM61909 (to K. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3 The on-line version of this article (available at http://www.jbc.org) contains Fig. S1.
nently disassembled in vivo, it seems obvious that disassembly in vivo occurs in a reversal of the assembly pathway, with the possible involvement of histone chaperones and chromatin assembly factors.

Nucleosomes and chromatin regulate all processes that utilize DNA as a template. One way to regulate these processes, in addition to post-translational histone modifications and ATP-dependent chromatin remodeling, is to change interactions within the nucleosome, or between nucleosomes, by the incorporation of histone variants (16). Interestingly, most variants belong to the H2A class that probably reflects the unique role H2A plays in the nucleosome. H2A, more than any other histone, makes significant contributions to the surface and stability of the nucleosome by forming an acidic patch on the surface of the histone octamer, and by generating a large interaction interface with the (H3-H4)2 tetramer (the H2A docking domain) (17). In addition, the L1 loop of H2A is the only area of the interface between the two (H2A-H2B) dimers within the NCP, and it is the only histone variant with a flexible N- and C-terminal tail (18).

Of all histone variants, H2A.Z is probably the one which is best characterized biophysically and biologically (4, 19–22). The structure of an NCP in which major H2A has been replaced by H2A.Z (H2A.Z-NCP) has been determined previously (4). The structure suggests that subtle localized changes in the stability of the nucleosome by forming an acidic patch on the surface of the histone octamer, and by generating a large interaction interface with the (H3-H4)2 tetramer (the H2A docking domain) (17). In addition, the L1 loop of H2A is the only area of contact between the two (H2A-H2B) dimers within the NCP, and it is the only histone variant with a flexible N- and C-terminal tail (18).

Recently, we found that H2A.Z was enriched in constitutive heterochromatin in early mouse embryos (22) and that the loss of H2A.Z protein by RNAi produced chromosome segregation defects in mammalian cell lines.2 To gain insight into this function of H2A.Z, and the in vivo function of histone variants in general, it is important to compare not only the overall stability of variant and major-type nucleosomes, but to investigate which of the many intersubunit interactions are affected by the sequence changes. Because of the complicated architecture of the nucleosome, data derived from previously used methods such as fluorescence (24–27) and CD spectroscopy (15, 28), analytical ultracentrifugation (29–31), hydroxy radical cleavage (32), and gel shift assays (33), cannot be directly linked to individual molecular events of nucleosome disassembly and assembly. With a growing awareness for the dynamic nature of chromatin, and with the realization that histone variants and post-translational modifications may exert part of their function by modulating nucleosome structure and stability, the need for a precise analysis of nucleosome stability in vitro has become obvious.

We have devised a fluorescence-based assay to dissect the stepwise disassembly of nucleosomes, and to test the hypothesis that the incorporation of histone variants alters nucleosome stability in vitro. Here, we describe a comprehensive analysis of the salt-induced dissociation of two types of nucleosome core particles, either containing “major” (replication-dependent) H2A (major-NCP), or the essential histone variant H2A.Z (H2A.Z-NCP), using fluorescence resonance energy transfer (FRET) (34). By attaching fluorescence donor-acceptor pairs to (i) either end of the DNA, (ii) to H4 and the DNA, (iii) to H4 and H2B within the NCP, we can monitor distinct events in nucleosome dissociation. Earlier fluorescence resonance energy transfer experiments were limited by available attachment sites for fluorophores (24, 35), and by the highly buried nature of the only available cysteine group in the middle of the H3-H4’ 4-helix bundle structure (Fig. 1). We have overcome this limitation by developing a system to prepare highly defined “recombinant nucleosomes” (36). This allows us to use histone mutants in which selected residues have been mutated to cysteines, which are readily reacted with commercially available maleimide derivatives of most fluorophores. The presence of the fluorophores does not affect the overall stability of the modified nucleosomes.

We demonstrate that FRET is a versatile method to dissect the multistate dissociation of nucleosomes. We show that the peripheral regions of the DNA dissociate from the histone octamer in a fast equilibrium at relatively low ionic strength (~320 mM NaCl). At this ionic strength, the dimer-tetramer interaction remains unaffected. At a midpoint of 530–570 mM NaCl, the (H2A-H2B) dimer dissociates from the (H3-H4)2 tetramer-DNA complex, followed by the dissociation of the DNA from the tetramer at even higher salt concentrations. Only one of these transitions, the dissociation of the (H2A-H2B) dimer from the (H3-H4)2 tetramer, is subtly stabilized in H2A.Z-NCP compared with major NCP. This stabilization could potentially attenuate the transcription process by impeding the displacement of a dimer, and is consistent with the emerging role of H2A.Z in silenced heterochromatin.

EXPERIMENTAL PROCEDURES Purification of 5 S 146-bp DNA—24 repeats of a 146-bp 5 S rRNA gene have been cloned into a plasmid (37). The plasmid was propagated in Escherichia coli HB101 cells. Purified and inserts were purified from 6-liter DNA preparations as described (36).

Separation of 5 S 146-mer Double Strand DNA into Its Single Strands—Complementary single strands were separated on a DEAE 5PW-HR column in an alkaline (10 mM NaOH) salt gradient (10 mM to 0.5 mM NaCl). 5 S DNA was incubated with the appropriate amounts of NaOH/NaCl at room temperature for 15 min. The DNA was loaded onto the DEAE 5PW-HR column equilibrated with 10 mM NaCl, and eluted with increasing NaCl concentrations in the presence of 10 mM NaOH, resulting in two clearly separated peaks. The pH of the DNA fractions was neutralized by adding 3 mM sodium acetate (pH 5.2). Each single strand of DNA was precipitated with 2.5 volumes of ethanol.

Modification of 5’ Ends of the 146-bp DNA (Synthesis of the 5’-Cystamine Adduct)—Each DNA strand was resuspended in TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Both DNA strands require modification at a thiol group (in this case, a cystamine) at the 5’ phosphate end to react with the maleimide group of the chromophore. The 5’-cystamine-P-146-bp oligonucleotide was prepared in a one-step synthesis in the presence of carbodiimide, 1-methylimidazole, and cystamine. A reaction mixture containing freshly prepared 3 mg/ml 1-ethyl-3,3-dimethylaminopropylcarbodiimide (EDC), 0.1 mM 1-methylimidazole, single-stranded DNA, and 0.5 mM cystamine dihydrochloride was incubated at 50 °C for 5 h (38–40). The reaction was cooled slowly to room temperature, and diluted 10-fold by the addition of TE before purifying onto a DEAE 5PW-HR column (Rohm/Haas). Both modified single strands were ethanol precipitated, and analyzed by PAGE on a 10% TBE gel in 7 mM urea to ensure the presence of cystamine.

Labeling of the DNA with Fluorescent Dyes—The thiol group donated by cystamine at the 5’ end of the 5 S 146-mer single-stranded DNA was reduced in 0.4 mM TCEP (Tris-2-carboxyethylphosphine hydrochloride in TE 10, 0.1; pH 7.0). The corresponding fluorescent dye (dissolved in solvent (N,N-dimethylformamide or dimethyl sulfoxide) was immedi-

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ficient sensitivity of the instrument to vertically and horizontally polarized light.

Steady-state Fluorescence Experiments—All samples were subjected to constant stirring during readout. Bandpass slits for excitation and emission were maintained at 2 nm. The excitation wavelength used in all FRET experiments was 385 nm and CPM intensities were measured at the emission maximum wavelength. All samples contained a final concentration of 0.25 μM for NCP in Tris buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). The buffer was scanned in the same range used in all experiments for background contributions to the readings and was corrected for in each spectrum. In all experiments, samples were incubated for 5 min at each salt concentration prior to readout. Scans of samples incubated for greater than 5 min (up to 30 min) showed no change in CPM emission intensity, indicating that an apparent equilibrium had been reached within 5 min. Control samples with donor (CPM)-only labeled NCP, and acceptor (FM)-only labeled NCP were analyzed likewise to discount salt effects on fluorescence.

Experimental data were normalized using the upper and the lower plateau values as baselines, obtained by least squares fit. We used at least five data points to calculate the baselines. To calculate the fraction of the dissociation of the (H2A-H2B) dimer (or the dissociation of DNA ends), we adopted the simple two-state dissociation model to compare the stability of NCPs. The curves were divided into three regions: the pre-transition (lower base line), the transition, and the post-transition (upper base line) (42). The fraction of “unfolded” was calculated using the equation, \( \frac{F_y}{F_0} = \frac{y - y_a}{y_b - y_a} \), where \( F_y \) is the observed variable parameter (intensity of fluorescence), and \( y_b \) and \( y_a \) are obtained by extrapolation of the linear portions of the pre- and post-transition regions of the unfolding curve. From normalized data (5 data sets for labeling combination I, 3 data sets for combination II, and 3 data sets for combination III), we calculated the mean values (average), S.D. (σ), and S.E. (error on the mean = (σ/√n) where n is the total number of the data points).

RESULTS

The Presence of Fluorophores on Histones and DNA Does Not Compromise Nucleosome Structure—FRET requires the presence of a donor and an acceptor fluorophore within the critical distance of transfer ( Förster distance or \( R_0 \)). A prerequisite for energy transfer is an overlap of the emission spectrum of the fluorescence donor with the excitation spectrum of the fluorescence acceptor. In this study, we used FM as an acceptor and CPM as a donor. This pair has an \( R_0 \) of 52 Å (41), a value that is well suited for the overall dimensions of the NCP (110 × 55 Å, Fig. 1, Table I). Attachment sites for the fluorophores were chosen with the requirement in mind that both of the fluorophores should have considerable rotational freedom to enable efficient energy transfer, and that fluorescence donor and acceptor are within \( R_0 \) for the CPM-FM pair. Amino acid residues to be changed to cysteines were selected after careful inspection of the structure, and we are confident that both sites in either histones H4 or H2B fulfill these criteria.

To allow a direct correlation of our results with the protein-protein interactions reported in the previously determined crystal structures (1, 4), we used recombinant \( X. laevis \) histones in combination with recombinant mouse H2A.Z. Because we were particularly interested in comparing the strength of the interaction between the (H3-H4)2 tetramer and either the (H2A-H2B) or the (H2A-Z-H2B) dimer within the nucleosome, we designed experiments that would allow us to monitor (I) changes in distance between the ends of the DNA; (II) changes in distance between both ends of the DNA and the (H3-H4)2 tetramer; and (III) changes in distance between the (H3-H4)2 tetramer and the (H2A-Z-H2B) dimer. Fig. 1 shows the location of fluorophores on the nucleosome, and the corresponding distances are shown in Table I. Because of the inherent symmetry of the NCP, two copies of each fluorophore are present in combinations II and III. Site-directed mutagenesis was performed to introduce cysteine residues into H2B and H4 (H2B T112C and H4 T71C). Both mutated residues are surface exposed and are not engaged in any intramolecular interac-
ions. To avoid difficulties in the interpretation of results, we also mutated the only native cysteine present in any of the four histones, H3 C110, to alanine (H3 C110A). This cysteine is buried within the 4-helix bundle that forms the H3-H3' interface, and has no discernable structural function. It is an alanine in yeast, and the structure of the yeast NCP in this particular region is virtually identical (5).

Maleimide derivatives of each fluorophore were reacted with the unfolded histone proteins, and unbound fluorophore was removed by gel filtration. Mass spectrometry confirmed the covalent linkage of only one fluorophore per cysteine residue (not shown). Histones were refolded to histone octamers using previously described protocols (36). A comparison of the elution profiles of refolded histone octamers from a size exclusion column, and analysis of refolded histone octamers by SDS-PAGE, and after and before staining with Coomassie Blue, clearly demonstrates the correct folding and stoichiometry of the histone octamers, a high efficiency of labeling of H4 and H2B, and the absence of fluorescence label on histones H3 and H2A (compare upper and lower panel in Supplemental Materials Fig. 1A).

Because of the necessity to attach donors and acceptors to the Watson and Crick strand of a 146-bp DNA fragment, respectively (chromophore combination group I, Table I), we used an asymmetric 146-bp DNA fragment derived from the 5 S rRNA gene in all experiments (27, 37). Strands were separated by ion exchange chromatography under highly basic conditions (Supplemental Materials Fig. 1B), and 5' DNA phosphates were modified with a cystamine group. For chromophore combination groups II and III, the double-stranded DNA was labeled without strand separation. The reactive SH groups were reacted with the maleimide forms of fluorescence donor and acceptor, as indicated in Table I, and fluorescence of the individual strands was demonstrated by PAGE (Supplemental Materials Fig. 1B).

NCPs with three different combinations of donor and acceptor fluorophores (as listed in Table I) were prepared by salt gradient dialysis. The quality of the assemblies was analyzed by native gel electrophoresis, which is a very sensitive measure for the structural integrity of nucleosomes (43) (Fig. 2, A–C). None of the fluorophores compromised reconstitution efficiency and the ability of the histone octamer to reposition in a temperature-dependent manner on the DNA (a process referred to as heat shifting (43); for example, Fig. 2A, compare lanes 1 and 2 with lanes 3–6). The new band that appears for the unlabeled X-NCP control is aggregate that we usually observe with older nucleosome preparations, and is not apparent in the species that were used for the actual FRET experiments. Note that the samples contain no traces of free DNA (Fig. 2, A–C, middle panels). This is important because residual free DNA could serve as a sink for the (H2A-H2B) dimer during dissociation, and could thus shift the equilibrium of dissociation toward lower salt concentration. Control NCPs labeled only with fluorescence donor at the relevant sites were prepared and analyzed similarly (data not shown).

We wanted to test whether the overall stability of the NCP toward salt-induced dissociation was in any way altered by the presence of the fluorophores. In a folded nucleosome, histone tyrosine fluorescence is quenched by the presence of the nearby DNA bases, and the loss of quenching is concomitant with nucleosome dissociation (27, 51). We monitored tyrosine fluorescence in response to increased ionic strength for unlabeled NCPs, and for NCPs in which DNA, H2B, or H4 has been analyzed similarly (data not shown).

To verify the rotational freedom of the attached fluorophores, fluorescence anisotropy measurements were performed with NCPs and histone complexes labeled with various labeling combinations. The results are listed in Table II. In all cases, the measured anisotropy was below 0.2, indicative of significant rotational freedom of all fluorophores, especially in light of the
FRET Studies on Variant Nucleosome Dissociation

Fig. 2. Nucleosomes can be modified with fluorescent probes without effects on structure or dynamic behavior. NCPs reconstituted with either recombinant X. laevis histones (major NCP or Xla-NCP) or with recombinant X. laevis H3, H4, H2B, and recombinant H2A.Z-NCP were analyzed by native PAGE (5% polyacrylamide, 0.25× TBE). Fluorescently labeled nucleosome samples are indicated by a asterisk. Samples were analyzed prior to heat shifting (indicated with US, for unshifted), and after heat shifting (S). DNA markers (M) are 100, 200, 300, 400, 500, 700, 10,000, 15,000, and 20,000 bp. Gels were first viewed on a transilluminator without staining (upper panel), followed by staining with ethidium bromide (middle panel) and Coomassie Brilliant Blue (lower panel), as indicated. A, major NCPs (lanes 3 and 4) and H2A.Z-NCPs (lanes 5 and 6) labeled with CPM and FM at the 5′ ends of the DNA (fluorophore combination I, Table I). Unlabeled nucleosomes (lanes 1 and 2) are shown as controls. B, major NCPs (lanes 2 and 3) and H2A.Z-NCPs (lanes 3 and 4) labeled with CPM on histone H4, and with FM on the 5′ end of the DNA (combination II, Table I). C, major NCP (lane 4) and H2A.Z-NCP (lane 3), labeled with CPM and FM on H2B and H4, respectively (combination III, Table I). D, Xla-NCP is shown as a control (lanes 1 and 2). Symbols denoting unshifted and shifted nucleosome species, and an aggregate species (A) that is sometimes observed in older nucleosome preparations, are given on the right. D, salt-induced nucleosome dissociation profiles for fluorescently labeled NCPs and wild type NCP. Fluorescence intensity of tyrosine was measured at an emission of 306 nm and excitation of 275 nm, and was plotted against the salt concentration. White diamonds, unmodified wild type NCP (n = 5); black triangles, DNA-CPM NCP (n = 3); gray squares, H2B-CPM NCP (n = 2); white squares, H4-CPM NCP (n = 1). n denotes the number of measurements. Error bars are omitted for clarity.

<table>
<thead>
<tr>
<th>Labeling</th>
<th>Anisotropy</th>
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<tbody>
<tr>
<td>NCP-H2B-CPM</td>
<td>0.186</td>
</tr>
<tr>
<td>NCP-H4-CPM</td>
<td>0.190</td>
</tr>
<tr>
<td>NCP-DNA-CPM</td>
<td>0.144</td>
</tr>
<tr>
<td>DNA-CPM</td>
<td>0.071</td>
</tr>
<tr>
<td>(H2A-H2B) dimer-CPM</td>
<td>0.071</td>
</tr>
<tr>
<td>CPM alone</td>
<td>0.049</td>
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Overall size of the NCP (210 kDa). In addition, the comparison between anisotropy values obtained for NCP labeled at the DNA ends and NCPs labeled on the histone proteins (Table II) strongly suggests that the fluorophores are not buried in hydrophobic regions of the histones. A two-carbon linker was used for the former, whereas the fluorophore is linked directly to the cysteine side chain in the latter. Nevertheless, anisotropy was very similar for these two particles tested (Table II).

Based on these results, we conclude that fluorophores attached to histone proteins and DNA are likely to be rotationally flexible and solvent accessible, and thus will be suitable for FRET. Importantly, the presence of fluorophores does not affect the formation of functional nucleosomes, as shown by analysis by gel electrophoresis and tyrosine fluorescence quenching.

FRET Can Be Used Monitor Nucleosome Dissociation—The fluorescence properties of each NCP preparation were first analyzed by fluorescence emission scans. Fig. 3 shows a typical example for nucleosomes with chromophore combination I upon excitation of donor emission at 385 nm. In the folded state, acceptor fluorescence as a result of FRET is clearly evident by the appearance of an emission peak for the acceptor at 520 nm. This peak is absent if labeled free DNA is assayed, because the distance spanned by the 5′ ends of a linear 146-bp DNA fragment is far beyond the critical distance for FRET for this pair (100 Å). In 1 M NaCl, the ends of the DNA are completely unraveled from the surface of the histone octamer, extending the distance between the fluorophores attached to both ends of the DNA significantly past the critical transfer distance. Its emission spectrum thus resembles that of free DNA. The slight shift in the emission maximum for the donor chromophore upon nucleosome unfolding is an indication for...
different interactions of the chromophore with its environment. Very similar results were obtained with the other two fluorophore combinations (not shown). Note that whereas only one donor and one acceptor are present in chromophore combination group I, two donor-acceptor pairs exist for the other two combinations, because of the inherent 2-fold symmetry of the nucleosome core particle. Because none of the distances are significantly above $R_0$, each donor can excite each acceptor (Fig. 1, Table I). We verified that true FRET was observed by measuring the same spectra in the absence of acceptor, with donor-only labeled DNA and nucleosomes (see below). In addition, we measured spectra of donor-only and donor-acceptor labeled DNA at different salt concentrations, and observed no shoulder at 520 nm upon donor excitation (not shown).

**Different Steps in the Dissociation of NCPs Are Observed with Different Donor-Acceptor Pairs** — Having established loss of FRET as a signal for nucleosome dissociation, we monitored changes in donor emission quenching in response to increasing ionic strength. As the salt concentration is increased gradually, the nucleosome dissociates into its components, as is evident by an increase in donor emission intensity (or loss of FRET, see Fig. 3). Equilibrium dissociation curves are obtained by plotting the increase in donor emission against the salt concentration. Fig. 4A shows representative normalized curves for NCPs reconstituted with the three chromophore combinations listed in Table I.

We find that the release of the DNA ends (monitored by loss of FRET between fluorophores attached to the two ends of nucleosomal DNA) takes place over a relatively wide salt range (Fig. 4, triangles), and occurs via an intermediate state. No significant dissociation is observed below 200 mM NaCl. Between 200 and 400 mM NaCl, the peripheral regions of the DNA exhibit increased "breathing." This transition is fully reversible even under the dilute conditions (0.12 μM NCP) of the experiment, because FRET re-appeared immediately after dilution to lower salt concentrations (not shown). The second transition is interpreted as the loss of one or both (H2A-H2B) dimers from the nucleosome. Under the dilute conditions of the experiments, this transition is only partially reversible (not shown). This interpretation is consistent with the observation that the loss of FRET between the (H3-H4)$_2$ tetramer and the DNA end also occurs in two transitions (Fig. 4A). The midpoint for the first transition is identical with that observed for nucleosomes labeled with combination I, and signifies breathing of the DNA.
ends away from the histone octamer. Dissociation of one or both (H2A-H2B) dimers from the DNA (and (H3-H4)₂ tetramer) then allows the DNA to unravel further, resulting in complete loss of FRET between the ends of the DNA and histone H4. Similar biphasic behavior is observed if FRET between the (H2A-H2B) dimer and the DNA end is observed (not shown). Consistent with the above interpretation, the dissociation of the (H2A-H2B) dimer from the (H3-H4)₂ tetramer-DNA complex clearly occurs in a single transition, with a midpoint at 580 mM NaCl.

Several controls were performed to verify that the observed signal is not an artifact of changes in fluorophore environment as a result of increased ionic strength. First, we performed salt titration experiments for donor-only labeled NCP preparations (Fig. 4B, triangles and white squares). This experiment showed that donor fluorescence emission in the absence of acceptor was unaffected by increased ionic strength. Second, we monitored salt-dependent dissociation of an NCP (labeling combination I) by simultaneously monitoring donor and acceptor, and observe that acceptor emission decreases as donor emission increases (Fig. 4C). Finally, to exclude the possibility that FRET was because of intermolecular interactions between two nucleosomes in solution, we incubated different ratios of nucleosomes either labeled only with fluorescence donor, or only with fluorescence acceptor. No FRET was observed at any of the tested ratios (not shown). Equimolar amounts of these two nucleosomes were then incubated with increasing salt concentrations. Again, no FRET was apparent under these conditions (not shown). We can therefore exclude FRET because of intermolecular reactions or because of exchange of subunits at elevated salt concentrations. In addition, salt-induced effects on the observed signal are negligible.

Differences in Subunit Interactions within Major and Variant NCP Can Be Observed by FRET—Having established a system with which we can monitor the dissociation of individual components within a nucleosome, we compared the properties of H2A.Z-NCPs with those of major NCPs, to test the hypothesis that the observed changes in the dimer-tetramer interface may result in the altered stability of the variant nucleosomes. The same labeled DNA and histone preparations were used for assembly of major NCP and H2A.Z-NCP. Fig. 5A shows a typical experiment for chromophore combination I. Again, the multiphasic nature of the equilibrium dissociation curve is apparent. The separation is not clear enough for the two parts of the curve to be treated individually. Nevertheless, it is clear that H2A.Z-NCP is stabilized against salt-induced dissociation compared with major NCP. This is apparent in Fig. 5A, showing a linear fit for parts of the data. The midpoint for the overall transition observed with this chromophore combination is 440 mM (±15 mM) NaCl for major NCP, and 506 mM (±10 mM) NaCl for H2A.Z-NCP, respectively. The two curves exhibit the same slope, indicating that the same types of protein-DNA interactions (e.g. hydrogen bonds) are disrupted in the two types of nucleosomes.

When the salt dependence of FRET between the ends of the DNA and histone H4 is monitored (Fig. 5B), the first transition is completely superimposable for the two nucleosomes, whereas the second transition clearly occurs over a wider salt range in H2A.Z-NCPs. This can be interpreted by a stronger interaction between the (H2A.Z-H2B) dimer and the (H3-H4)₂ tetramer-DNA complex, compared with the (H2A-H2B) dimer-(H3-H4)₂ tetramer interaction. The differences in the slopes of the second dissociation curves between the two types of nucleosomes may either indicate a lesser degree of cooperativity in dimer dissociation, or may reflect differences in the nature of the affected molecular interactions. In fact, we reproducibly observe a third

![Fig. 5. H2A.Z-NCP is stabilized against salt-induced dissociation.](http://www.jbc.org/)
transition (centering around 900 mM NaCl) in the dissociation curves obtained with H2A.Z-NCP, which is absent in major NCPS. Again, there is indication of a second minor transition at high salt for H2A.Z-NCP that is absent in major NCP.

Our interpretation that the (H2A-Z-H2B) dimer-(H3-H4)$_2$ tetramer interaction may be stabilized in H2A.Z-NCPs is confirmed by monitoring the loss of FRET between the (H2A-Z-H2B) dimer and (H3-H4)$_2$ tetramer within the NCP. Using fluorophore combination III (Fig. 5C). In this system, dissociation clearly occurs in a single transition that is between the (H2A-H2B) dimer and (H3-H4)$_2$ tetramer still firmly in place. The latter transition is also observed by monitoring FRET between the (H2A-Z-H2B) dimer and (H3-H4)$_2$ tetramer, which obeys two-state behavior. We applied this experimental system to compare nucleosomes containing the histone variant H2A.Z with canonical nucleosomes containing replication-dependent major histones, and found that the dissociation of the (H2A-Z-H2B) dimer from the nucleosome occurs at higher salt concentrations, and over a broader salt range compared with the (H2A-Z-H2B) dimer.

The transient exposure of DNA sites as a result of sequential dissociation of the ends of the DNA from the surface of the histone core has been postulated and proven indirectly in an elegant series of experiments by Widom and colleagues (Ref. 50, and references therein). By monitoring salt-dependent changes in FRET between the ends of the DNA, we show that significant partial dissociation of DNA takes place with the (H2A-H2B) dimer and (H3-H4)$_2$ tetramer still firmly in place. Although partial dissociation of the DNA ends from the surface of the histone octamer has been suggested as an important mechanism in nucleosome dissociation, it has never been demonstrated directly. In a related study, we used this technique to demonstrate that transcription factor binding near the nucleosomal dyad results in transient dissociation of the DNA ends.

The observed dissociation of the (H2A-H2B) dimer at relatively low ionic strength is consistent with in vivo observations (9, 10, 44).

Several studies have addressed the structural changes in nucleosomes in response to increased ionic strength, using a variety of methods, and arriving at different conclusions (see Ref. 31, and references therein). Most studies agree that all four core histones are bound to the DNA up to ~600 mM NaCl, and that dissociation of the (H2A-Z-H2B) dimer from the DNA occurs before that of the (H3-H4)$_2$ tetramer (e.g. Refs. 15, 29, and 51). Up to ~700 mM NaCl, the nucleosomal DNA appears to maintain its rotational positioning (32). Because ends of nucleosomal DNA are notoriously difficult to map with DNase footprinting, our results are consistent with previous observations that the structure of the DNA in the nucleosome remains unaffected up to 800 mM NaCl.

In contrast with our own findings, Eshaghpour and co-workers (24) demonstrated by FRET that the DNA-H3 C110 dimer remains constant up to 600 mM NaCl, and in the presence of moderate amounts of urea; the effect of higher salt concentrations has not been investigated. However, analysis of the nucleosome structure (which had not been available at the time these studies were performed) shows that the deeply buried H3 C110 is unlikely to sustain modification without significant structural disruption of the (H3-H4)$_2$ tetramer. The same authors also analyzed FRET between the DNA ends and a fluorophore attached to residue 73 of H4, with basically the same results.

Oohara and Wada (25) monitored CD and quenching of tyrosine fluorescence by nearby DNA bases as a measure for the degree of nucleosome folding, and identified three transitions in the salt-dependent dissociation: an unidentified structural change near 0.55 mM NaCl, dissociation of the (H2A-H2B) dimer near 0.95 mM NaCl, and dissociation of the (H3-H4)$_2$ tetramer near 1.45 mM NaCl. These transitions are at significantly higher ionic strength than those identified here. The reason for this probably lies in the tendency of basic histone complexes to associate with DNA in a random manner once they are released.

DISCUSSION

Nucleosomes within the context of higher order chromatin structures are highly complex modular assemblies that respond dynamically to ATP-dependent chromatin remodeling factors, histone tail modifications, incorporation of histone variants, binding of architectural chromatin-associated proteins and transcription factors, and to advancing DNA and RNA polymerases (10–13, 44–46). The breathing of DNA ends, and the dynamic exchange of histone subunits are likely to be key aspects in the biological function of nucleosomes (12, 47, 48). For example, the ends of the bound nucleosomal DNA are thought to dissociate and re-associate with the histone core in a rapid equilibrium, to allow access of factors to nucleosomal DNA (49). The (H2A-H2B) dimer can exchange dynamically in interphase nuclei, independent of replication-dependent chromatin assembly pathways (9, 10). The replacement of histone H2A with histone variants would be a simple and efficient way to alter the thermodynamic properties of the nucleosome, and thus change the local and global structure of chromatin (for example, see Ref. 21). The strength of the interactions between (i) the DNA and the histone octamer, and (ii) between the (H2A-H2B) dimer and the (H3-H4)$_2$ tetramer within the nucleosome will determine the stability of higher order conformations as well as the ease by which chromatinized DNA is made accessible to the cellular machinery in vivo. To investigate the effect of histone variants and mutants on these processes, it is important to study the relative strength of these interactions independently of others.

We have shown that the complex dissociation behavior of the nucleosome in response to salt can be dissected by FRET. Using fully recombinant nucleosomes (36, 43), fluorophores can be attached virtually anywhere on the histones, and on the 5′ ends of the DNA, while maintaining significant rotational freedom. The structure and stability of fluorescently labeled nucleosomes remains unaffected, as judged by native gel electrophoresis and tyrosine fluorescence experiments. Using this system, we observed that the ends of nucleosomal DNA dissociate in a reversible manner at relatively low salt concentrations, followed by dissociation of the (H2A-H2B) dimer at more elevated salt concentrations. This is apparent in the appearance of an intermediate state in the dissociation curve when loss of FRET between the ends of the DNA, and between the DNA ends and the (H3-H4)$_2$ tetramer is observed. The latter transition is also observed by monitoring FRET between the

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from the nucleosome (52), which would still result in significant fluorescence quenching. The strength of the experimental system described here lies in the rigorous quality control of labeled nucleosomes, and in the defined location of fluorophores, allowing us to independently study the dynamic behavior of histone subcomplexes.

We applied this experimental approach to a comparison between canonical nucleosomes and nucleosomes in which H2A has been replaced by the essential histone variant H2A.Z (4, 21). The increase in the strength of the (H2A.Z-H2B) dimer-(H3-H4)2 tetramer interaction on the structural transitions imparted onto chromatin during transcription and remodeling events under physiological conditions (33). Furthermore, it can easily be adapted for kinetic measurements, or to monitor the fate of the nucleosome during transcription and remodeling events under physiological conditions. Such studies will provide long overdue information on the structural transitions imparted onto chromatin during these biologically highly relevant processes.

Acknowledgments—We thank Devis W. Close and Robert K. Suto for assistance in the initial stages of this project.

REFERENCES
