

Dissection of the unusual structural and functional properties of the variant

H2A.Bbd nucleosome

Cécile-Marie Doyen^{1,2}, Fabien Montel^{2,3}, Thierry Gautier¹, Hervé Ménoni^{2,4}, Cyril Claudet⁵,
Marlène Delacour-Larose¹, Dimitri Angelov^{2,4}, Ali Hamiche⁶, Jan Bednar⁵, Cendrine Favre-
Moskalenko^{2,3}, Philippe Bouvet^{2,4*} and Stefan Dimitrov^{1, 2*}.

¹Institut Albert Bonniot, INSERM U309, 38706 La Tronche cedex, France

²Laboratoire Joliot-Curie, ³Laboratoire de Physique, CNRS UMR 5672, ⁴Laboratoire de Biologie Moléculaire de la Cellule, CNRS-UMR 5161/INRA 1237/IFR128 Biosciences, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69007 Lyon, France

⁵CNRS, Laboratoire de Spectrométrie Physique, UMR 5588, BP87, 140 Av. de la Physique, 38402 St. Martin d'Hères Cedex, France

⁶Institut André Lwoff, CNRS UPR 9079, 7 rue Guy Moquet, 94800 Villejuif, France

*Corresponding authors

e-mail : stefan.dimitrov@ujf-grenoble.fr

Fax: (33) 4 76 54 95 95 Tel: (33) 4 76 54 94 73

e-mail: pbouvet@ens-lyon.fr

Tel/Fax: (33) 4 72728016

SUMMARY

The histone variant H2A.Bbd appeared to be associated with active chromatin, but how it functions is unknown. We have dissected the properties of nucleosome containing H2A.Bbd. Atomic force microscopy (AFM) and electron cryo-microscopy (EC-M) showed that the H2A.Bbd histone octamer organizes only ~130 bp of DNA, suggesting that ~10 bp of each end of nucleosomal DNA are released from the octamer. In agreement with this, the entry/exit angle of the nucleosomal DNA ends formed an angle close to 180° and the physico-chemical analysis pointed to a lower stability of the variant particle. Reconstitution of nucleosomes with swapped-tail mutants demonstrated that the N-terminus of H2A.Bbd has no impact on the nucleosome properties. AFM, EC-M, FRAP and chromatin remodeling experiments showed that the overall structure and stability of the particle, but not its properties to interfere with the SWI/SNF induced remodeling, were determined to a considerable extent by the H2A.Bbd docking domain. These data show that the whole H2A.Bbd histone fold domain is responsible for the unusual properties of the H2A.Bbd nucleosome.

INTRODUCTION

DNA is packaged into chromatin in the cell nucleus. The chromatin exhibits repeating structure and the repeating unit, the nucleosome, consists of an octamer of the core histones (two each of H2A, H2B, H3 and H4) around which two superhelical turns of DNA are wrapped (van Holde, 1988). The nucleosome is an obstacle for the protein factors to bind to their cognate DNA sequences and it interferes with several vital cellular processes (Beato and Eisefeld, 1997). Histone modifications, ATP-remodeling machines and the incorporation of histone variants within chromatin are used by the cell to overcome the nucleosomal obstacle (Becker, 2002; Henikoff and Ahmad, 2005; Henikoff et al., 2004; Strahl and Allis, 2000).

Histone variants are nonallelic isoforms of the conventional histones, which mRNA, in contrast to this of conventional histones, is polyadenylated (Tsanev et al., 1993; van Holde, 1988). The function of the different histone variants is poorly understood, but the emerging general picture suggests that the incorporation of histone variants within the nucleosome results in a particle with novel structural and functional properties (Abbott et al., 2001; Angelov et al., 2003; Bao et al., 2004; Gautier et al., 2004; Fan, 2004 #231; Suto et al., 2000). The presence of histone variants in the nucleosome has serious impacts on several processes, including transcription, repair, cell division and meiosis and may result in important epigenetic consequences (Ahmad and Henikoff, 2002a; Ausio and Abbott, 2002; Kamakaka and Biggins, 2005; Sarma and Reinberg, 2005). CENP-A, a histone H3 variant, is specifically associated with the centromeric DNA and it is essential for mitosis. Its presence is crucial for the assembly and maintenance of the kinetochores (for a recent review see (Henikoff and Dalal, 2005)). H3.3, another variant belonging to the H3 family, marks active chromatin (Ahmad and Henikoff,

2002b). Genome-scale distribution of H3.3 showed that transcription units, from upstream to downstream, are enriched in this histone variant (Mito et al., 2005). The recently identified variant of H2B, H2BFWT, seemed to be localized specifically on the telomeric sequences and might be involved in mitotic chromosome condensation (Boulard et al., 2006; Churikov et al., 2004).

The histone H2A has the largest family of identified variants (Redon et al., 2002; Sarma and Reinberg, 2005). This could reflect both the more labile interaction of the H2A-H2B dimer with the remaining histones and DNA (van Holde, 1988) and its strategic position within the core particle (Luger et al., 1997). The variants of the H2A family include H2A.X, H2A.Z, macroH2A and H2A.Bbd. H2A.Z and H2A.X are the best studied H2A histone variants to date. H2A.Z is highly conserved, suggesting an important function of this protein (Redon et al., 2002). Indeed, the mammalian H2A.Z is encoded by an essential gene, since its knockout results in embryonal lethality (Faast et al., 2001). H2A.Z is involved in both gene activation (Santisteban et al., 2000) and gene silencing (Dhillon and Kamakaka, 2000). In yeast, H2A.Z is found at nearly all promoters (Guillemette et al., 2005; Li et al., 2005; Raisner et al., 2005; Zhang et al., 2005), while hyperacetylated H2A.Z is concentrated at the 5' ends of active genes in higher eukaryotes (Bruce et al., 2005). It was also reported that H2A.Z is important for chromosome segregation (Rangasamy et al., 2004).

H2AX is intimately related to repair. Double strands breaks (DSB) induce the phosphorylation of H2AX at its C-terminus and this is mediated by members of the PIKK protein family (Rogakou et al., 1998). Experiments with H2AX^{-/-} deficient mice showed that H2AX assists the prevention of aberrant repair of DSB and functions as suppressor of genomic

instability and tumors (Bassing et al., 2003; Celeste et al., 2003). H2AX is specifically recognized by the mammalian MDC1 and the interaction of MDC1 with H2AX is crucial for the cell response to DNA damage (Stucki et al., 2005).

MacroH2A (mH2A) is an unusual histone variant with a size threefold this of the conventional H2A (Pehrson and Fried, 1992). mH2A consists of a histone H2A-like domain fused to a large non-histone region, termed the macro-domain (Allen et al., 2003; Ladurner, 2003; Pehrson and Fried, 1992). Immunofluorescence data suggested that the inactive chromosome X is enriched of mH2A (Chadwick et al., 2001; Costanzi and Pehrson, 1998; Costanzi and Pehrson, 2001; Mermoud et al., 1999), but this view was, however, challenged and it was proposed that the higher concentrations of mH2A in the inactive X chromosome reflected higher nucleosome density (Perche et al., 2000). The incorporation of mH2A within the nucleosome interfered with both transcription factor binding and nucleosome remodeling (Angelov et al., 2003). Both *in vitro* and transient transfection experiments showed that mH2A inhibited initiation of transcription and histone acetylation (Doyen et al., 2006; Perche et al., 2000). The available data suggest that mH2A is a major stopper of transcription (Doyen et al., 2006). It was recently reported that the macro-domain is an ADP-ribose binding module, suggesting that mH2A might be also involved in the biology of ADP-ribose (Karras et al., 2005; Kustatscher et al., 2005).

H2A.Bbd (Barr body deficient) is the least studied histone variant and microscopy data showed that it is largely excluded from the inactive X chromosome (Chadwick and Willard, 2001). This histone variant is quite divergent and its primary sequence showed only 48% identity compared to its conventional H2A counterpart (Chadwick and Willard, 2001). The N-terminus of

H2A.Bbd exhibits a row of six arginines, which could be important for its function. In addition, H2A.Bbd is relatively shorter and lacks both C-terminus and the very last sequence of the docking domain (Chadwick and Willard, 2001). Micrococcal nuclease digestion suggested that the H2A.Bbd octamer organized only 118 bp of DNA (Bao et al., 2004). FRAP, FRET and sedimentation measurements point to a less stable structure of the variant H2A.Bbd nucleosome (Angelov et al., 2004b; Bao et al., 2004; Gautier et al., 2004). The current view is that H2A.Bbd is enriched in nucleosomes associated with transcriptionally active regions of the genome (Chadwick and Willard, 2001) and *in vitro* experiments demonstrated that nucleosomal arrays containing H2A.Bbd are easier transcribed and their histones easier acetylated (Angelov et al., 2004b). The role of the different domains of H2A.Bbd in these processes is not known.

In this work we report a detailed analysis of the properties of the H2A.Bbd nucleosomes in solution. We have dissected the role of the different domains of H2A.Bbd in the structure and function of H2A.Bbd nucleosomes using a number of physical methods including Atomic Force Microscopy (AFM), Electron cryomicroscopy (EC-M), optical tweezers and FRET combined with molecular biology approaches. We showed that the H2A.Bbd octamer organizes ~130 bp of DNA and that its structural and functional properties are determined by the whole histone fold domain of H2A.Bbd.

RESULTS

130 base pairs of DNA are wrapped around the histone variant H2A.Bbd octamer

First we analyzed the structure in solution of the H2A.Bbd particle using micrococcal nuclease. Micrococcal nuclease digestion measures the accessibility of nucleosomal DNA that is not protected by the histone octamer. To better characterize this accessibility, we performed kinetics studies of the micrococcal nuclease digestion of conventional H2A and H2A.Bbd particles reconstituted on the 601 positioning sequence (Figure 1 A). The digestion pattern of the conventional particle showed discrete kinetics intermediates at about 208, 166, 146, and 128 bp, while the kinetics intermediates of the H2A.Bbd particle were observed mainly at 146, 128, and around 118 bp. The band at 166 bp, generated upon digestion of the conventional nucleosome, was attributed to the interaction of the N-terminal histone tails with nucleosomal DNA (Van Holde et al., 1980), the 146 bp band represents the core particle, while the 128 bp band reflects a subnucleosomal digestion (Van Holde et al., 1980). The origin of the 208 bp band is unknown, but it might be associated with the presence of histones on DNA, since upon digestion of naked 601 DNA, no such band was observed (data not shown). The presence of very faint 166 bp band in the digestion pattern of the H2A.Bbd nucleosome suggests that the interaction of the histone N-termini with DNA were perturbed. The relatively fast disappearance of the 128 bp band in the kinetics of micrococcal nuclease digestion and the presence of a stable 118 bp intermediate (Figure 1A, see the scans of the micrococcal nuclease digestion pattern) indicates that the protection against micrococcal nuclease digestion is weaker in the variant H2A.Bbd particle, suggesting a more relaxed structure.

Next we used microscopy techniques to measure the length of DNA, which is wrapped around the histone octamer. Briefly, using purified recombinant histones we have reconstituted both conventional and variant H2A.Bbd centrally positioned nucleosomes on a 255 bp DNA

fragment containing the 601 positioning sequence. Initially, AFM was used to visualize the reconstituted particles (Figure 1B-D). The large free DNA sequences present at each end of the nucleosomes allowed the precise measurement of the length of DNA which is non-wrapped around the histone octamer, and therefore the determination of the length of the DNA organized by the histone octamer. The measurements were carried out on a large number of particles (458 conventional and 290 H2A.Bbd particles), which made the experiment statistically relevant. The mean of distribution of the length of DNA, organized by conventional octamer (Figure 1D) peaked at 146 ± 1.3 bp, in perfect agreement with the crystal structure of the nucleosome (Luger et al., 1997). In contrast, the mean length of DNA organized by the octamer containing H2A.Bbd (Figure 1D) peaked at 127 ± 2.2 bp.

The AFM experiment could be affected by the deposition of the material on the functionalized mica surface and by the fact that the measurements were carried out in air. To overcome these potential problems the same types of experiments were repeated, but using electron cryomicroscopy (EC-M). Indeed, EC-M measurements carried out in solution provide high resolution images of the conformation of the studied samples and were successfully used to study both nucleosome and 30 nm chromatin fiber structures (Bednar et al., 1995; Bednar and Woodcock, 1999). The electron cryomicrographs clearly showed that the conventional and the variant H2A.Bbd particles exhibit different conformations (Figure 2A and B). The majority of the entry and exit DNA ends of the conventional nucleosomes formed a V-type structure with the nucleosome particle located at the middle of the structure (Figure 2A). In contrast, only a small number of the H2A.Bbd particles exhibited such structure, while the majority of the DNA nucleosomal ends run close to parallel to each other (Figure 2B). This suggests that the H2A.Bbd octamer interacts weakly with the entry/exit nucleosomal DNA and it is unable to generate a stable V-type orientation to the free DNA ends. The length of the DNA wrapped around the

conventional histone octamer was 148 ± 1.9 bp and 132 ± 2.6 bp for the variant H2A.Bbd octamer (Figure 2C), which is in complete agreement with the AFM measurements. Thus, using two different microscopy techniques we found that H2A.Bbd octamer organizes ~ 130 bp of DNA. Taken together, these results point to a distinct structure of the H2A.Bbd nucleosome with altered interactions of the variant octamer with the nucleosomal DNA.

Force-extension measurements of a single H2A.Bbd nucleosomal array

These as well as previous experiments (Bao et al., 2004; Gautier et al., 2004) suggest that the H2A.Bbd nucleosome exhibits lower stability compared to the conventional nucleosomes, but no direct measurements of the forces maintaining the structure of the H2A.Bbd particle were reported. We have addressed this problem using optical tweezers and measured the force necessary for the unfolding of a single H2A.Bbd nucleosome. A fragment of DNA, containing twelve 208 bp repeats comprising the 5S rRNA sea urchin gene nucleosome positioning sequence, was used to reconstitute H2A.Bbd nucleosome arrays. Upon digestion of the reconstituted arrays a clear 200 bp repeat was observed evidencing for a proper organization of the reconstituted samples (data not shown). A single array was then attached between two polystyrene beads and subjected to traction using optical tweezers (for detail see Materials and Methods and ref. (Claudet et al., 2005)). Each peak in the “saw tooth” profile of the force-extension curve (Figure 3A) reflects the unfolding of a single H2A.Bbd particle (Claudet et al., 2005). The same experiment was carried out, but using conventional H2A arrays (results not shown and (Claudet et al., 2005)). The measurements showed that the force necessary for the disruption of a single H2A.Bbd nucleosome was 16.1 ± 4.8 pN, which was very close to the force required for the disruption of a single conventional H2A particle (17.8 ± 5.3 pN) (Figure 3B).

The two sets of values of threshold disruption forces were compared using standard t-test for two independent populations. The resulting values of p and t factors were 0.00243 and -3.04722, respectively, indicating that both means cannot be viewed as different.

We have also studied the stability of the H2A.Bbd nucleosome variant at very low particle concentrations. Under these conditions, a selective release of the H2A-H2B dimer occurred, which reflects the disruption of the H2A-H2B dimer interactions with both the (H3-H4)₂ tetramer and DNA (Claudet et al., 2005). If the H2A.Bbd-H2B variant dimer exhibited lower stability relative to the conventional one, a release of H2A.Bbd-H2B dimer at higher nucleosome concentration would be expected. Figure 3C showed that this is indeed the case. At the lowest particle concentration studied (3.8 nM) the vast majority of the H2A.Bbd-H2B dimer was released from the variant H2A.Bbd particle (Figure 3C, the last two panels and the scan nuc Bbd-H3*) in contrast to conventional particle (Figure 3C, the first panel and the nuc H2A-H3* scan). This showed that the interactions between the H2A.Bbd-H2B dimer with both the (H3-H4)₂ tetramer and DNA within the H2A.Bbd particle were altered, which conferred lower stability of the H2A.Bbd variant nucleosomal particle.

SWI/SNF is unable to remodel swapped tail H2A-H2A.Bbd mutants containing the H2A.Bbd histone fold domain

The H2A.Bbd particle exhibits distinct structural and functional properties, including an inability to be remodeled by SWI/SNF (Angelov et al., 2004b). The mechanism and role of the different H2A.Bbd domains in this interference in the remodeling are not known. We approached this problem by studying the properties of nucleosomes containing H2A.Bbd-H2A swapped domain mutants. Initially we have concentrated on the N-ter and C-ter swapped-tail mutants (schematically depicted in Figure 4A). The different mutant proteins were purified (Figure 4B)

and efficiently incorporated into nucleosomes (Figure 4C-D). The DNase I footprinting analysis showed that nucleosomes which contain the histone fold domain of H2A.Bbd, exhibited the same alterations in the DNase I digestion pattern as the H2A.Bbd nucleosome (Figure 4E, compare lanes 1, 2 and 4 with lanes 3 and 5), suggesting that this domain alone determines the perturbations observed in the structure of the H2A.Bbd nucleosome. To test if SWI/SNF was able to remodel the swapped-tail mutants which contain the histone-fold domain of H2A.Bbd, we used DNase I footprinting (Figure 5). The DNase I footprinting evidences that SWI/SNF was able to remodel chimeric nucleosomes containing the histone fold domain of conventional H2A (lanes 1-4 and 13-16), but not the chimeric nucleosomes containing the histone fold domain of H2A.Bbd (lanes 5-12 and 17-20). We conclude that the presence of the histone fold domain of H2A.Bbd in a nucleosomal particle was sufficient to generate structural properties that prevent the remodeling by SWI/SNF.

Involvement of the H2A.Bbd docking domain in the wrapping of nucleosomal DNA

The major difference between the conventional H2A and the histone variant H2A.Bbd is observed in the C-terminal domain of the two proteins. H2A.Bbd does not possess a C-terminal tail and its docking domain lacks the very last segment, which is involved in interactions with histone H3. The fusion of the C-terminal tail of H2A to H2A.Bbd was not sufficient to generate a nucleosomal particle which could be remodeled by SWI/SNF (Figure 5, lanes 9-12) indicating that the absence of a C-terminal tail in H2A.Bbd is probably not responsible for the particular properties of this variant towards SWI/SNF remodeling. To test if the divergent docking domain of H2A.Bbd could be involved in the generation of the specific properties of the H2A.Bbd nucleosome, we have produced and purified to homogeneity a H2A.Bbd mutant containing both the docking domain and the C-terminal tail of conventional H2A (Bbd-ddH2A). This

recombinant protein was used to reconstitute nucleosomes (Figure 6A), which were analyzed by DNase I footprinting (Figure 6B). Interestingly, the DNase I digestion pattern of the Bbd-ddH2A nucleosomes was almost the same as this of H2A.Bbd nucleosomes, with characteristic alterations in DNase I sensitivity compared to conventional nucleosomes. This indicates that the presence of the docking domain of H2A was not sufficient to restore the DNase I digestion pattern of conventional nucleosomes.

We next measured the length of DNA wrapped around the Bbd-ddH2A histone octamer by AFM. Interestingly, the length of the DNA organized by the Bbd-ddH2A octamer was essentially the same (143 ± 2.2 bp) as for the conventional octamer (146 ± 1.3 bp) (Figure 6C). These measurements were statistically relevant, since a very significant number of nucleosomes (465 conventional and 208 Bbd-ddH2A) were used in these experiments. Therefore, the presence of the docking domain and the C-terminal tail of H2A in the Bbd-ddH2A nucleosome led to the wrapping of an additional 20 bp of DNA around the histone octamer.

The conformation of the Bbd-ddH2A nucleosome in solution was then studied by EC-M (Figure 6D). The electron cryo-micrographs showed that the proportion of the Bbd-ddH2A nucleosomes exhibiting V-type structure (typical for the conventional H2A nucleosome), is definitely higher compared to the parental H2A.Bbd nucleosomes (compare Figure 6D and figure 2A with figure 2B). We conclude that the docking and C-terminus domains of H2A were able to rescue to considerable extent the specific orientation of the entry/exit of nucleosomal DNA, possibly by reducing the breathing of the nucleosomal DNA ends.

The docking domain of H2A.Bbd determined the lower *in vivo* stability of the H2A.Bbd nucleosome

FRAP (Fluorescence Recovery After Photobleaching) is a suitable technique for measuring the core histone exchange *in vivo* and thus, for the determination of the stability of the nucleosome (Kimura and Cook, 2001). Previous experiments have shown that GFP-H2A.Bbd exchanges much more rapidly than GFP-H2A, which is compatible with a lower stability of the variant H2A.Bbd nucleosome compared to the conventional H2A particle (Gautier et al., 2004). To determine the importance of the docking domain of H2A.Bbd in this process we have compared the exchange of Bbd-ddH2A with that of wild type H2A.Bbd and H2A. Stable cell lines expressing either the fusion GFP-H2A.Bbd or GFP-Bbd-ddH2A or GFP-H2A proteins have been established. In these cell lines the GFP-fusions were assembled into nucleosomes (data not shown, but see (Gautier et al., 2004)). For the FRAP experiments, a small rectangular area in the nucleus was photobleached with the laser light and images were recorded at different times post-bleaching (Figure 7A). A very clear difference in the fluorescence recovery kinetics was observed for each sample (Figure 7 A and B). For GFP-H2A.Bbd a complete recovery was found after 10-15 minutes, a result in complete agreement with our previous report (Gautier et al., 2004)). The recovery of photobleaching of the fusion GFP-Bbd-ddH2A was, however, quite slow and even after 30 minutes of recovery less than 40% of the initial fluorescence was measured (Figure 7B). Remarkably, the photobleaching recovery curve of the GFP-Bbd-ddH2A was identical (within the margin of the experimental error) to the one of the GFP-H2A (Figure 7B). This shows that the exchange of the docking domain of H2A.Bbd by the docking domain and the C-terminal tail of H2A was sufficient to create a chimeric protein with the same exchange properties as the conventional H2A histone. This suggests that not only *in vitro*, but also *in vivo*, the docking domain of H2A.Bbd and the absence of C-terminal tail are essential for the lower stability of the H2A.Bbd nucleosome.

If this is correct, the exchange of the docking domain and C-terminal tail of H2A with the docking domain of H2A.Bbd should create a H2A chimeric fusion protein (H2A-ddBbd) with mobility similar to that of H2A.Bbd. Stable cell lines expressing H2A-ddBbd were established and analyzed by FRAP. As seen (Figure 7 A and B), the recovery of photobleaching of GFP-H2A-ddBbd was the same as this of GFP-H2A.Bbd. All these data clearly demonstrated that the *in vivo* stability of H2A.Bbd is determined by its docking domain.

The docking domain and C-terminal tail of H2A partially restore the ability of SWI/SNF to remodel Bbd-ddH2A nucleosomes. .

The data described above show that nucleosome particle containing the Bbd-ddH2A chimeric protein exhibited physico-chemical properties similar, but not identical to nucleosome containing the conventional H2A particle. This raises the question whether Bbd-ddH2A nucleosomes exhibit functional properties similar to H2A nucleosomes and in particular towards SWI/SNF induced mobilization. We used AFM to monitor nucleosome mobilization (Figure 8). Briefly, centrally positioned conventional, H2A.Bbd and Bbd-ddH2A nucleosomes were incubated with SWI/SNF in the presence or absence of ATP and then visualized by AFM. The number of centrally and end- positioned nucleosomes before and after the mobilization reaction were counted and the efficiency of mobilization (the net percentage of the slid nucleosomes relative to the proportion of the initially centrally positioned nucleosomes) was then calculated. Under the experimental conditions 52% of conventional H2A, 14% of H2A.Bbd and 31% of Bbd-ddH2A particles were mobilized by SWI/SNF in the presence of ATP, evidencing that the presence of the docking domain and C-terminal tail of H2A in H2A.Bbd is able to rescue partially the ability of SWI/SNF to mobilize the nucleosomes.

DISCUSSION

In this paper we have carried out a detailed analysis on the structural and functional properties of the H2A.Bbd nucleosome and dissected the role of its different domains using a combination of physical, biochemical and cell biology approaches. Our micrococcal digestion data (Figure 1) showed a strong kinetics intermediate at 118 bp, a result in agreement with (Bao et al., 2004). However, micrococcal nuclease digestion reveals the length of the DNA which is protected against the enzyme, and not the length of DNA which is wrapped around the histone octamer. This is why AFM and electron cryo-microscopy were used to directly measure the length of the DNA wrapped around the variant histone octamer. Remarkably, both methods showed that ~130 bp of DNA were wrapped around the H2A.Bbd histone octamer.

The EMSA experiments of nucleosomes at very low concentrations showed directly that the H2A.Bbd nucleosome was less stable compared to the conventional nucleosome and that the interaction of the H2A.Bbd-H2B dimer with the (H3-H4)₂ tetramer and nucleosomal DNA was weaker than that of the conventional H2A-H2B dimer, which agrees with the data on the measurements of the sedimentation coefficient of the H2A.Bbd particle (Gautier et al., 2004). Recent FRET experiments suggested that the nucleosomal ends of the H2A.Bbd particle were weakly bound to the histone octamer (Bao et al., 2004). Our data confirmed these results and in addition showed that the DNA ends are released from the surface of the H2A.Bbd octamer and no longer formed, as in the case of conventional nucleosome, a V-type structure. We hypothesized that 10 bp of each end of nucleosomal DNA are released from their interactions with the histone octamer in the H2A.Bbd nucleosome. The optical tweezers elasticity measurements of the force, necessary for the unfolding of single H2A.Bbd nucleosome gave, however, value very close

value to the force required for the unfolding of a conventional H2A nucleosome (16.1 ± 4.8 pN versus 17.8 ± 5.3 pN). This could be explained by the thermodynamical considerations of Kulic and Schiessel (Kulic and Schiessel, 2004), which showed that in the stretching experiments the major part of the energy necessary for the nucleosome unfolding appeared to be absorbed by the flipping of the nucleosome around its dyad and only a small part is consumed by the unwrapping of the DNA from the histone octamer.

SWI/SNF was not able to remodel the N- and C-terminal swapped tail H2A and H2A.Bbd mutants, indicating that the histone fold domain of H2A.Bbd, but not its peculiar N-terminus or the complete absence of C-terminal tail, is determining this property of the H2A.Bbd particle (Figure 5). This could reflect the alterations in the structure of nucleosomes containing the histone fold domain of H2A.Bbd (Figure 4). The replacement of the docking domain of H2A.Bbd with this of H2A (Bbd-ddH2A) makes a fusion protein, which incorporation into the histone octamer was able to generate a particle with some properties characteristics for the conventional H2A particle. For example, the length of the DNA organized by the histone octamer and the *in vivo* stability of the nucleosome particle studied by FRAP of the nucleosomal particle containing the Bbd-ddH2A were very similar to the particle containing the H2A protein. However, the DNase I footprinting assay revealed that the Bbd-ddH2A nucleosomal particles exhibited the same structural alterations as parental H2A.Bbd nucleosomes. In addition, incorporation of the fusion protein Bbd-ddH2A in nucleosomal particles led to an assembly of only a subset of particles with the V type conformation characteristic for the conventional nucleosomes (Figure 2A and 6D) and to a partial rescue of the efficiency of nucleosome mobilization by SWI/SNF (Figure 8). All together, the data reported in this manuscript allowed the conclusion that the

whole histone fold domain of H2A.Bbd determines its unusual structural and functional properties.

The *in vitro* transcription assays have shown that arrays containing H2A.Bbd nucleosomes were more easily transcribed and acetylated than conventional H2A arrays (Angelov et al., 2004b). In addition, H2A.Bbd was found to colocalize with acetylated H4 within the nucleus (Chadwick and Willard, 2001). These data suggest that, *in vivo*, the presence of H2A.Bbd could be viewed as a mark of active chromatin. The structural properties of H2A.Bbd nucleosomes described in this study as well as the previously reported data (Angelov et al., 2004b; Gautier et al., 2004) point to a possible mechanism of the H2A.Bbd nucleosomes in the assembly and maintenance of transcriptionally active chromatin. The exchange of conventional H2A with H2A.Bbd would create a more relaxed structure, which, in turn, would help the interactions of transcription factors and polymerases with H2A.Bbd chromatin and the passage of RNA polymerase through the H2A.Bbd nucleosomes. Since the H2A.Bbd nucleosomes cannot be mobilized, the cell would not be able to use chromatin remodelers to slide the histone octamers and to create nucleosome-free promoter regions. Instead, eviction of the histone octamers should be used, a process which would be facilitated by the weaker stability of the H2A.Bbd nucleosomes.

Materials and Methods

Preparation of DNA fragments

The 255 bp and 241 bp DNA fragments, containing the 601 nucleosome positioning sequence at the middle or at the end of the fragment, were obtained by PCR amplification from plasmid pGem-3Z-601 and p199-1, respectively (kindly provided by B. Bartholomew and J. Widom). Labeling was performed by adding 30 μCi of [α - ^{32}P] CTP to the PCR reaction. The

207 bp 5S DNA fragment was obtained by PCR amplification from plasmid pXP-10. PCR product was digested with *EcoRI* and *RsaI* and the resulting 154 bp fragment was labeled at the 3' end by filling with dATP and [α - 32 P]dTTP. All labeled DNA fragments used for nucleosome reconstitution were gel purified.

For the stretching experiment, plasmid p2085S-G5E4, (kind gift from J. L. Workman) was digested with *Acc65I*, *CatI*, *SspI*, and *ClaI*. The restriction fragment containing the ~ 400 bp E4 promoter DNA, flanked by two DNA sequences, each composed of five 208 bp tandem repeats of the 5S rRNA sea urchin gene (Neely et al., 1999), was purified by 4% native PAGE and end-labeled at the *ClaI* side by biotin-dCTP. A fraction of biotin-dCTP labeled fragments was labeled at the *Acc65I* end by digoxigenin-dUTP. The uniquely and both ends labeled DNA fragment were mixed at a ratio 1 :10 and used for reconstitution of conventional and H2A.Bbd nucleosomal arrays by salt dialysis (Claudet et al., 2005). The attachment of the reconstituted arrays to the polystyrene beads was performed as described earlier (Claudet et al., 2005).

Protein purification, nucleosome reconstitution, remodeling and stability

Recombinant *Xenopus laevis* full-length histone proteins were produced in bacteria and purified as described (Luger et al., 1999). For the H2A.Bbd protein and its mutants the coding sequences for the H2A and for H2A.Bbd were amplified by PCR and introduced in the pET3a vector. NtBbd-H2A (H2A.Bbd domain from M1 to S21 in frame to H2A domain from R18 to K130); Bbd-CtH2A (H2A.Bbd domain from M1 to D115 fused to H2A domain from T121 to K130); NtH2A-Bbd (H2A domain from M1 to T17 fused to H2A.Bbd domain from R22 to D115); Bbd-ddH2A (H2ABbd domain from M1 to I83 fused to H2A domain from P81 to K130); H2A-ddBbd (H2A domain from M1 to I80 fused to H2A.Bbd domain from T84 to D115). Recombinant

proteins were purified as previously described (Angelov et al., 2004b). For the FRAP experiments, the coding sequences were amplified by PCR and cloned in the pEGFP-C1 vector.

Yeast SWI/SNF complex was purified as described previously (Cote et al., 1994) and its activity was normalized by measuring its effect on the sliding of conventional nucleosomes: 1 unit being defined as the amount of SWI/SNF required to mobilize 50% of input nucleosomes (50 ng) at 30°C during 45 min. Nucleosome reconstitution was performed by the salt dialysis procedure (Mutskov et al., 1998). Carrier DNA (150-200 bp, 2 µg) and 50 ng of ³²P-labeled DNA were mixed with equimolar amount of histone octamer in nucleosome reconstitution buffer NRB (2M NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 5 mM β-MeEtOH). DNase I footprinting was performed as described previously (Angelov et al., 2003). Nucleosomes reconstituted on a 154 bp 5S DNA fragment (50 ng) were incubated with SWI/SNF as indicated in remodeling buffer (RB) containing 10 mM Tris-HCl, pH 7.4, 5% glycerol, 100 µg/ml BSA, 1 mM DTT, 0.02% NP40 40 mM NaCl, 2.5 mM MgCl₂, and 1 mM ATP for 45 min. The reaction was stopped by adding 1 µg of plasmid DNA, 0.02 U of apyrase, 10 mM EDTA.

Mobilization experiments were carried out using centrally-positioned nucleosomes, reconstituted on a 255 bp DNA fragment containing the 601 positioning sequence. The nucleosome samples (~20 ng/µl) were incubated in a solution of 10 mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, and 1 mM ATP for 60 minutes at 29°C.

Micrococcal nuclease digestion was performed at 8 units/ml at room temperature for the times indicated in 10 mM Tris, pH 7.4, 1 mM DTT, 25 mM NaCl, 5% glycerol, 100 µg/ml BSA, 1.5 mM CaCl₂ and 100 µg/ml of plasmid carrier DNA. The digestion was stopped by adding 20 mM EDTA, 0.1% SDS, 200 µg/ml Proteinase K (30 min at 45 °C). DNA was then extracted and run on a 10% native acrylamide-bisacrylamide (1/29 w/w) gel.

For the nucleosome dissociation experiments a wild type H3 and swapped tail H3-H2B mutant histones were used and labeled as previously described (Angelov et al., 2004b). Nucleosome dissociation experiments were carried out in either TE, 10 mM NaCl or in PBS. Briefly, aliquots of nucleosomes were diluted with the appropriate buffer in 10 μ l final volume to the indicated concentrations (in the range of 30-3.8 nM), and left for 45 minutes at room temperature. Then the samples were analyzed by Electrophoretic Mobility Shift Assay (EMSA) on a 5% (w/v) polyacrylamide gel (acrylamide to bisacrylamide, 29:1 w/w), 0.3x TBE, at 4°C.

Atomic Force Microscopy, Cryo-Electron microscopy, FRAP and optical tweezers.

The E-CM analysis, FRAP and the stretching experiments were carried out essentially as described previously (Angelov et al., 2004a; Claudet et al., 2005; Gautier et al., 2004). For the AFM imaging the conventional and variant nucleosomes were immobilized onto APTES-mica surfaces. The functionalization of freshly cleaved mica disks (muscovite mica, grade V-1, SPI) was obtained by self-assembly of a monolayer of APTES under Argon atmosphere (Lyubchenko et al., 1993). Nucleosomes (DNA concentration \sim 75 ng/ μ l) were filtered and concentrated using Microcon[®] centrifugal filters to remove free histones from the solution, and diluted 10 times in Tris-HCl 10 mM, pH=7.4, and 1mM EDTA, just prior to deposition onto APTES-Mica surfaces. A 5 μ l droplet of the nucleosome solution is applied on the surface for 1 min, rinsed with 1mL of milliQ-Ultrapure[®] water and dried by spotting with an optic paper. The samples were visualized by using a Nanoscope III AFM (Digital Instruments[™], Veeco, Santa Barbara, CA). The images were obtained in Tapping Mode in air, with silicon tips (resonant frequency 250-350 kHz) at scanning rates of 2Hz over scan areas from 0.6 to 1 μ m wide. The images (512 x 512 pixels) have been flattened using a homemade MATLAB[®] script to remove the long-term drift of the set-up. Complexed DNA length distribution was obtained by an automated analysis of AFM images

using a home written MATLAB[®] script allowing measuring the non-complexed DNA length of each nucleosome. This program uses height and area criteria to segregate mononucleosomes from other objects in the image and morphological tools (such as erosion, dilatation and skeletonization) to measure their free DNA length. This type of analysis allows to partially overcoming the tip convolution effect. Nucleosomes at the extreme position of the DNA fragment were excluded from the statistical analysis. The error on the distribution function mean value (standard error) is given by σ/\sqrt{N} , where σ is the standard deviation of the experimental distribution, and N the number of analyzed nucleosomes (central limit theorem).

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Legend Figures

Figure 1. (A) Micrococcal nuclease digestion kinetics of conventional and H2A.Bbd nucleosomes. Identical amount of conventional nucleosomes (nuc H2A) and H2A.Bbd nucleosomes (nuc Bbd) were digested (in presence of 100 μ g/ml plasmid DNA) with 8 units/ml of micrococcal nuclease for the indicated times. The reaction was stopped by addition of 20 mM EDTA and 0.1 mg/ml proteinase K, 0.1% SDS. DNA was isolated and run on a 10 % polyacrylamide gel. The lower panel shows the scans of the gel. 50 bp M, marker DNA fragments (indicated at the left part of the figure). (B, C) Atomic Force Microscopy imaging shows that the H2A.Bbd variant histone octamer organizes 127 bp of DNA. A 255 bp fragment containing the 601 positioning sequence and either conventional or H2A.Bbd variant octamers were used to reconstitute centrally positioned nucleosomes. Both types of nucleosomes were visualized by AFM. AFM images of conventional nucleosomes (nuc H2A) are presented in (B), and variant H2A.Bbd nucleosomes (nuc Bbd) are shown in (C). An enlarged view of a conventional or variant H2A.Bbd nucleosome particle is shown below the B and C panels. (D) Quantification of the data of figures (B) and (C). The measured mean length of the DNA in complex with the H2A.Bbd variant histone octamer was only 127 \pm 2.2 bp compared to 146 \pm 1.3 bp for conventional H2A nucleosomes.

Figure 2. Conventional and H2A.Bbd histone variant nucleosomes exhibit different organization of the entry and exit nucleosomal DNA ends. Centrally positioned conventional and H2A.Bbd variant nucleosomes were reconstituted on a 255 bp DNA fragment containing the 601 positioning sequence and visualized by cryoelectron microscopy. (A) Representative cryoelectron micrographs of conventional nucleosomes. The majority of the entry and exit DNA ends (black *arrows*) form a V-shaped structure with the nucleosome (designated with white arrow) localized in the center. (B) Same as (A) but for variant H2A.Bbd nucleosomes. The majority of the DNA ends (black *arrows*) form an angle close to 180 degrees. (C) Quantification of the data of figures (A) and (B). The length of DNA (132 \pm 2.2 bp) complexed with the H2A.Bbd variant octamer was essentially the same (within the margin of the experimental error) as this measured by AFM.

Figure 3. Force measurements of the disruption of a single variant H2A.Bbd nucleosome.

Gel-purified 5S rDNA tandem repeats were labeled at one end with biotin-dCTP, while the other end was labeled with digoxigenin-dUTP. The fragments were used for reconstitution of nucleosomal arrays containing either conventional H2A or variant H2A.Bbd histones. One end of the DNA of an individual nucleosomal array was “sticked” on a digoxigenin functionalized bead, while the other was “sticked” to a streptavidin functionalized bead. This tethered between the two beads individual nucleosomal array was stretched by using optical tweezers device. **(A)** Force-extension curve of the stretching cycle. The extension is shown in red, while the relaxation is in black. Note the typical ‘saw tooth’ profile of the force extension curve. Each “tooth” reflects the disruption of a single variant H2A.Bbd particle. **(B)** Distribution profile of the disruption forces of a single H2A.Bbd particle (nuc Bbd) and conventional H2A particle (nuc H2A). **(C)** Stability of conventional and H2ABbd nucleosomes as a function of nucleosome concentration. End positioned nucleosomes were reconstituted on a 241 bp DNA fragment comprising the 601 sequence using either ³²P-labeled H3 (H3*) or ³²P-labeled H2B (H2B*). The tetrameric particles contained ³²P-labeled H3. The nucleosome solutions were diluted to the indicated concentrations and run on a 5 % native polyacrylamide gel. The positions of the nucleosomes and the tetrameric (H3-H4)₂ particles are indicated on the left part of the gel. The lower panel shows the scans of the gels for the conventional and H2A.Bbd variant nucleosomes containing ³²P-labeled H3. Since the amount of the loaded material on the different wells was different, the presented measured intensities were normalized to the concentration.

Figure 4. Biochemical characterization of the swapped-tail mutant conventional H2A and H2A.Bbd nucleosomes. Conventional core histones, H2ABbd (Bbd), the swapped-tail mutant Bbd-C_tH2A (a fusion between H2A.Bbd and the C-terminal domain of conventional H2A), N_tBbd-H2A (a fusion of the N-terminus of H2A.Bbd and the histone fold and C-terminus of conventional H2A) and N_tH2A-Bbd (a fusion of the N-terminus of H2A and the H2A.Bbd histone fold domain) and fragment of DNA containing the 154 bp DNA fragment of 5S gene, were used to reconstitute nucleosomes. **(A)** Schematics of different proteins used for nucleosome reconstitution. **(B)** 18% SDS electrophoresis of the purified recombinant core histones, H2ABbd and the swapped-tail histone mutants. **(C)** Histone composition of the reconstituted nucleosomes. The histones were separated on a 18% SDS gel. The positions of the conventional core histones

are designated. (D) EMSA analysis of the reconstituted nucleosomes. The positions of the nucleosomes (nuc) and the naked DNA are indicated on the left part of the figure. (E) DNase I footprinting of the reconstituted conventional H2A, variant H2A.Bbd and the swapped-tail histone mutant nucleosomes. Note that the presence of the histone fold domain of H2A.Bbd was sufficient to induce the DNase I digestion characteristics for the H2A.Bbd alterations (indicated by stars).

Figure 5. Remodeling of conventional H2A, variant H2A.Bbd and swapped-tail H2A and H2A.Bbd histone mutant nucleosomes. A 154 bp ³²P-end labeled DNA fragment comprising the 5S *X. borealis* gene was used for reconstitution. The indicated increasing amounts of SWI/SNF were added to the nucleosome solutions and they were incubated at 30°C for 45 minutes in the presence of 1 mM ATP. The nucleosome remodeling was assessed by DNase I footprinting. Lanes 1, 5, 9, 13 and 17, DNase I footprinting of nucleosomes in the absence of SWI/SNF. Note that SWI/SNF was unable to remodel the nucleosomes, which contained the histone fold of H2ABbd. Stars indicate DNA fragments that appear after SWI/SNF remodeling.

Figure 6. Characterization of nucleosomes reconstituted with the fusion of the histone fold (without the docking domain) of H2A.Bbd and the docking domain and the C-ter of H2A (Bbd-ddH2A). (A) EMSA of reconstituted nucleosomes. (B) DNase I footprinting of the reconstituted particle shown in (A). The stars indicate the altered DNase I digestion pattern of the H2A.Bbd and Bbd-ddH2A nucleosomes. (C) Distribution of the DNA in complex with the histones in conventional and Bbd-ddH2A nucleosomes. Conventional and Bbd-ddH2A nucleosomes were reconstituted on a 255 bp 601 DNA fragment and the particles were visualized by AFM. Note that both conventional and Bbd-ddH2A octamers organize the same length of DNA within the nucleosome. (D) Electron cryo-microscopic visualization of Bbd-ddH2A particles. A higher proportion of the particles exhibit, like the conventional H2A reconstituted nucleosome (see figure 2A), a V-shaped structure; the black arrows designated the DNA ends, while the nucleosome is designated by white arrow.

Figure 7. The docking domain of H2A histone proteins is essential for the *in vivo* nucleosome stability. (A) FRAP analysis of GFP-fusions. Stable cell lines expressing either GFP-H2A, GFP-

Bbd, GFP-H2A.ddBbd, or GFP-Bbd.ddH2A proteins were imaged before photo-bleaching (pre-bleach) or at the times indicated post-bleaching. The bleached area is indicated by arrows. **(B)** Quantification of the FRAP data. The means for 8-10 nuclei from six independent experiments are presented.

Figure 8. The fusion of the docking domain of H2A to H2A.Bbd rescues partially the ability of SWI/SNF to remodel the nucleosomes. Centrally positioned nucleosomes were reconstituted on a 255 bp DNA fragment containing the 601 sequence. SWI/SNF (1 unit) was added to the nucleosome solutions and they were incubated at 29°C for 60 minutes in the presence/absence of ATP, then the samples were visualized by AFM and the efficiency of mobilization was calculated. For each case the number of analyzed centrally and end-positioned nucleosomes is indicated.