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Mechanisms of Nucleosome Assembly and Remodeling

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Contributions of Jacek Mazurkiewicz to the enclosed publications

Publication 1: All experiments except for the one shown in supplementary Fig. 4 as well as the fit of the kinetic data with the GEPASI software were conducted by Jacek Mazurkiewicz (JM). The publication was written by JM, and JM participated in the revision of the manuscript.

Publication 2: JM prepared the fluorescent histone complexes used in the paper. In addition, he carried out the gel-electrophoretic analysis of the NAP1 and histone interaction stoichiometry depicted in Figure 3.

Publication 3: JM prepared the cysteine-labeled histones used in this study, contributed to the design and interpretation of the experiments and participated in conducting the experiments in Fig. 3 and Fig. 6.

Publication 4: The fluorescence correlation and cross-correlation spectroscopy experiments as well as the assessment of the ACF subunit stoichiometry by quantitative PAGE were carried out by JM.

Publication 5: The analytical ultracentrifugation experiments and their data analysis were carried out by JM.

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Karsten Rippe

(PD Dr. Karsten Rippe)

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“The voyage of discovery lies not in seeking new horizons,
but in seeing with new eyes.“

Marcel Proust

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Chromatin: Structure, Assembly and Function

1. Introduction

The past 15 years mark a period of significant progress in chromatin research, as it has become evident that chromatin represents more than a mere static, condensed state of eukaryotic DNA. Most notably, local chromatin structure and dynamics (co-)govern many nuclear processes such as transcription, recombination, DNA replication and repair. Furthermore modifications and alterations of chromatin components were recognized to serve as an unique way of inheriting information in addition to the genetic code.
The focus of this thesis lies on the assembly, dynamics and stability of nucleosomal structures investigated with *in vitro* methods. Key questions that were experimentally addressed include how the nucleosome structure is established by physiological relevant factors, and how its stability can be modulated by intrinsic and extrinsic mechanisms. Therefore, the introductory part of this thesis emphasizes structural, dynamic and energetic aspects of nucleosomes and higher-order structures. Processes that serve in the assembly and modulation of chromatin structure are discussed within the context of other related topics such as epigenetic modifications.

2. Structure of chromatin

2.1 Nucleosomes are the building blocks of chromatin

The DNA of a human cell has a total length of around two meters and is arranged in well-organized fashion into a nucleus of about 10 μm diameter. This packaging is in apparent contradiction to the access of genetic information by numerous nuclear complexes and regulating factors. These opposing functions are accomplished by the assembly of dynamic multi-subunit protein complexes with the nuclear DNA, namely nucleosomes. These constitute basic building blocks that can further organize the DNA into higher-order structures and thereby form fiber like assemblies with high compaction ratios (up to ~10000 fold). The protein core of the nucleosome is the histone octamer, a roughly disc-shaped complex that has the remarkable capability to wrap 147 bp of the DNA around its surface in 1.65 turns (Fig. 1.1). The histone octamer is made up by two copies each of the core histones H2A, H2B, H3 and H4. These core histones are characterized by a three-helix “histone-fold” which heterodimerizes forming a handshake motif between H3 and H4 as well as between H2A and H2B in the octameric structure (Arents and Moudrianakis, 1995). Whereas each copy of the four core histones contacts the DNA in the nucleosome, only H3 and H2A interact with the other homotypic histone (Luger et al., 1997a).

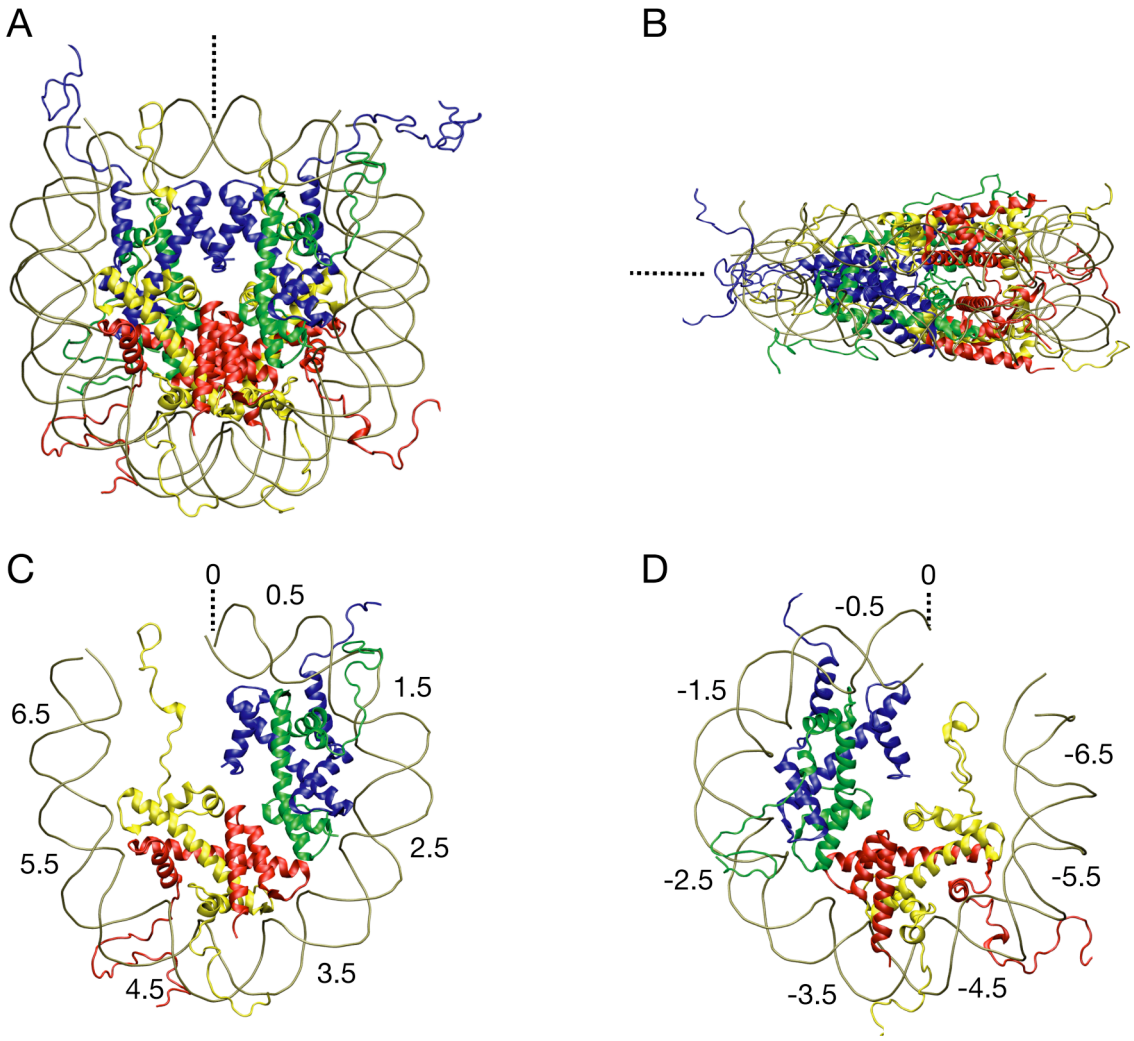

Figure 1.1 Molecular structure of the core nucleosome. The DNA is depicted as backbone line, the histones as ribbons. Histone proteins are colored blue for H3, green for H4, yellow for H2A and red for H2B. The dyad axis is depicted as broken line. **(A)** A top view of the nucleosome with vertical alignment of the dyad axis is shown. **(B)** The side view of the nucleosome is depicted. **(C)** depicts the upper half portion of the nucleosomal structure. **(D)** shows the corresponding lower half. The positions of superhelical locations are referenced by numbers. The structures were generated from the 147 bp X-ray nucleosome structure (Davey et al., 2002).

The N-termini of the core histones appear conformationally variable, which holds also true for the C-terminus of histone H2A. The highly cationic tails are the main targets of histone posttranscriptional modifications (Felsenfeld and Groudine, 2003; Goll and Bestor, 2002; Nightingale et al., 2006) and serve as recognition motif for chromatin-binding proteins such as HMGN-1, SIR 3-4 and others. They constitute important regions for interaction with chromatin remodeling factors (Clapier et al., 2001; Hamiche et al., 2001). Also, the tails play a vital role in the higher-order assembly of chromatin via internucleosomal tail-tail interactions (Dorigo et al., 2003; Fan et al., 2004; Schwarz et al., 1996). It is noted, that the histone tails are not the only regions of interaction with additional factors in the protein part of the nucleosome. Recent studies demonstrate the recognition of histone-fold regions in the nucleosome by chromatin-associating factors and covalent modifications that lie on the surface of the nucleosome core structure (Barbera et al., 2006; Mersfelder and Parthun, 2006).

The DNA in the core nucleosomal structure interacts with the histone octamer in 14 contact regions that are distributed over the inward facing side of the DNA. These can be referenced by their superhelical location (SHL) from -6.5 to 6.5 and appear periodically with a distance of one full turn of the DNA where the DNA minor groove is faced towards the nucleosomal inner surface (see Fig. 1.1 C,D). The SHL references the distance of a DNA residue to the central nucleosomal basepair. Thus, an SHL of 1 refers to a location one DNA turn from the dyad axis away. Of the 14 protein-DNA contacts in the nucleosome, 12 are in the inner, highly bent 121 bp of core DNA. These interactions consist of water-mediated or direct hydrogen bonds between peptide groups or side chains with the phosphate-backbone of the DNA minor groove. In the crystal structure the histone tails partially locate in DNA minor grooves and follow them outwards of the core particle (Davey et al., 2002). It has to be noted that the tails are only partially resolved in the current X-ray structures, possibly due to a high structural flexibility and thus the presence of multiple conformations in the crystals.

Around 80% of the nucleosomes harbor a linker histone H1 or one of its variants, which sits near the entry/exit site of DNA in the nucleosome, organizing ~20 bp DNA flanking the nucleosomal core in a stem loop like manner (Bednar et al., 1998; Hamiche et al., 1996; Simpson, 1978). The members of the H1 group of proteins show a rather conserved organization, consisting of a central globular winged-helix domain surrounded by a short N-terminus and a longer C-terminus, the latter ones both poorly structured. To date only the globular structure of the H1-like avian H5 could be solved at molecular level (Ramakrishnan et al., 1993), and there is no consensus regarding the exact integration of H1 in the nucleosomal structure (Brown et al., 2006; Kepert et al., submitted; Travers, 1999; Vignali and Workman, 1998). A number of models exist for the “chromatosome”

(core nucleosome + H1) structure which differ in regards to the positioning of the linker histone and the number of interacting sites of the globular domain with the nucleosomal DNA and core histones. Given these discrepancies it appears conceivable that multiple positions can be adopted by linker histones explaining the divergent findings of several groups (Fan and Roberts, 2006). However, a structural constraint has to exist as only one H1 can be bound per nucleosome. *In vivo* especially the longer, positively charged C-terminal domain is essential for binding to chromatin (Catez et al., 2006; Kepert et al., submitted). This binding seems to be mediated by charge based interactions and also relies on a repeating S/TPXK motif (X refers to any amino acid).

The linker histone H1 stabilizes the nucleosomal structure, reduces mobility of the nucleosome and guides higher-order chromatin folding (Pennings et al., 1994; Shen et al., 1995; Thoma et al., 1979). Accordingly, the linker histone is found to accumulate in transcriptionally inactive regions (Kim and Dean, 2003), whereas transcriptionally active regions appear depleted of linker histone. Furthermore, the histone was shown to impede transcription *in vitro* (Shimamoto et al., 1981). However, knockout studies demonstrate that H1 appears to affect transcription at the local level, as only the expression of a small set of selected genes appears affected. These genes are also closely regulated by DNA methylation pointing at a connection between linker histone function and DNA modification. Knock-out of a number of the apparently synergistically acting H1 variants leads to severe embryonic defects, possibly due to the importance of the genes under H1 control – a subset of which for instance plays a critical role in imprinting (Fan et al., 2003; Fan et al., 2005).

2.2 Higher-order structures

In vivo adjacent nucleosomes are positioned to each other with a spacing "linker" DNA segment. The spacers have a length of roughly 20-100 bp, yielding a total nucleosomal repeat length (NRL) of around 165-220 bp per nucleosome. This length varies with the species examined, the cell type and the specific chromatin context. Intriguingly, the distribution of spacer length is not random but follows a ~ 10 bp periodicity (Widom, 1992), which closely resembles a helical turn of DNA (10.4 bp) and therefore points at a sterical requirement of nucleosome arrangement in higher-order folding. Furthermore, calculations based mainly on data of recent knock-out studies demonstrate a linear relationship between the ratio of H1 per nucleosome and the NRL. These results show that the presence of the linker histone leads to a lengthening of the NRL by 37 bp (Woodcock et al., 2006). Furthermore, nucleosomes in special positions such as promoters appear precisely positioned (van Holde, 1989; Yuan et al., 2005).

Nucleosomal arrays, e.g. DNA with multiple nucleosomes in ordered orientation, adopt a "beads on a string" conformation when incubated at low salt concentrations. At physiological salt concentration, longer fragments can reversibly fold into a fiber characterized by a diameter of approximately 30 nm (Hansen, 2002; Hansen et al., 1989). This higher-order structure confers a compactation ratio of around 50 fold. Even though elaborate efforts have been made to solve the structure of this assembly, two conflicting main models for the fiber geometry are under current discussion, the solenoid and the zig-zag model. Both are supported by experimental evidence, however a majority of studies is in favor of the latter conformation (Adkins et al., 2004b).

The solenoid model proposes an one-start organization of the fiber (Finch and Klug, 1976; McGhee et al., 1983; Robinson and Rhodes, 2006; Thoma et al., 1979; Widom and Klug, 1985). Adjacent nucleosomes are separated by bent linker DNA which follows the spiral form of the fiber (see Fig. 1.2 A). Bending of the linker DNA is thought to be facilitated by association with linker histone H1 and overall the folding of the fiber appears guided by protein-protein interactions between adjacent nucleosomes (Widom, 1989). A modulation of the model has been proposed based on electron microscopic studies (Daban and Bermudez, 1998; Robinson and Rhodes, 2006). In this structure the nucleosomes from one gyre interdigitate into the linker DNA space of the adjacent gyres. The result is a highly compacted structure that can accommodate moderate changes in the length of the linker DNA without a change in the fiber diameter.

In contrast, the zig-zag model proposes a conformation in which neighboring nucleosomes on the DNA are oriented on opposite sides of the fiber with their connecting linker DNA crossing the inner section of the fiber (Woodcock et al., 1993). The resulting two-start fiber has been proposed based on X-ray structures, high-resolution electron microscopy work, crosslinking and *in vivo* mapping (Dorigo et al., 2004; Rydberg et al., 1998; Schalch et al., 2005) (see Fig. 1.2 B). In this structure the fiber diameter would appear linearly dependent on length of the linker DNA.

In isolated nuclei, the existence of 30 nm chromatin fibers is difficult to detect and these structures were only shown to exist in short stretches and with varying diameters. Moreover the 30 nm fiber undergoes higher-order folding itself by forming structures on the hundred nanometer scale with increased packing ratios (Belmont et al., 1989). The exact organization of these assemblies is poorly understood and different conformational states are proposed, based on various studies. Electron-microscopic investigations on isolated nuclei hinted at the folding of the 30 nm fibers in superstructures of roughly 100 nm size, named chromonemas (Belmont et al., 1989), which were also detectable by light-microscopic inquiries in living cells (Tumbar et al., 1999).

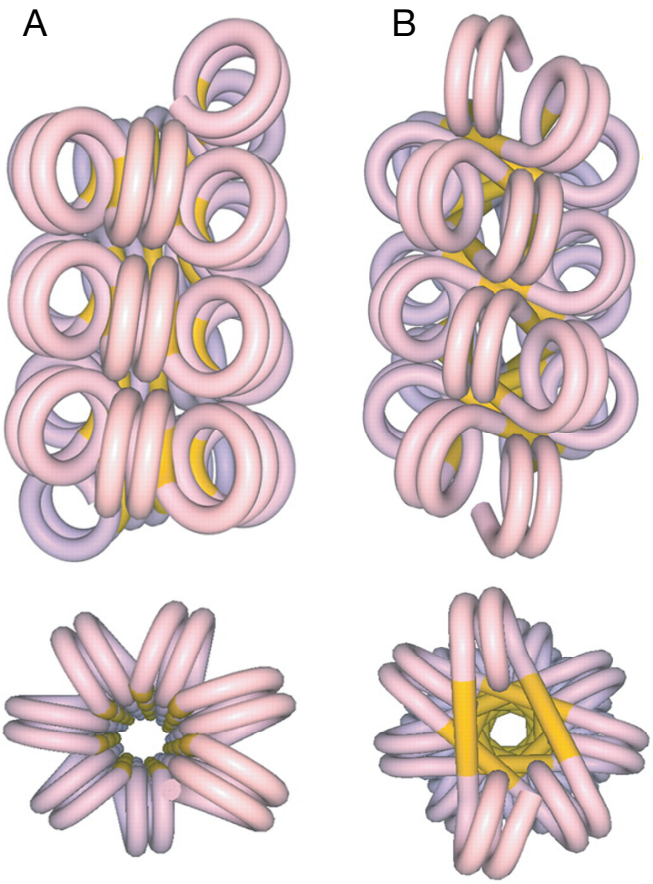

Figure 1.2 A schematic representation of the DNA path in different chromatin fiber foldings. The nucleosomal DNA is represented in red/blue, the linker DNA in yellow. **(A)** represents the conformation predicted by the solenoid model **(B)** the two start twisted conformation. In the lower part of the figures a cross section of both conformations is shown. Taken from (Dorigo et al., 2004).

In contrast, radial-loop models propose the folding of the 30 nm fiber into loops of around 150 kbps that associate to rosette-shaped assemblies (Münkel et al., 1999; Paul and Ferl, 1999). The random-walk/giant-loop (RW/GL) models suppose the looping of large regions of chromatin (3 Mbp) and tethering of these structures to a backbone (Sachs et al., 1995).
The chromosomal organization appears to take place by the occupation of distinct territories in the nucleus (Cremer and Cremer, 2001). These territories are irregularly shaped and appear localized dependent on their content of genes, with transcriptionally active territories placed more towards the nuclear interior and gene-poor regions at the nuclear periphery. The surfaces of the territories are thought to be more accessible than their interior, granting access for factors binding chromatin by a putative compartment between the territories, the interchromosomal space (Cremer et al., 1993). In fact, the localization of growing filaments and passively diffusing particles appears restricted to a subspace of the nucleus as shown by different experimental strategies (Brigder et al., 1998; Görisch et al., 2005). The same holds true for endogenous promeolytical leukemia (PML) and cayal bodies which are mostly excluded from chromosomal territories (Brigder et al., 1998; Görisch et al., 2004; Zirbel et al., 1993).

On the micrometer scale, a distinction between euchromatic and heterochromatic regions can be made for the interphase nucleus. Euchromatic regions can be generally seen as gene-rich with high transcriptional activity. Often the chromatin structure in these regions appears with an irregular nucleosomal spacing and a general depletion of nucleosomes. Based on early microscopic investigations, heterochromatin constitutes nuclear regions with dense staining patterns during meta- to interphase progression (Heitz, 1928). It is characterized by an accumulation of repetitive DNA-elements, such as satellite-DNA, endoretroviral sites and transposable elements but a low amount of active genes. On the chromosomal level, heterochromatin mostly consists of telomeric and pericentromeric regions as well as the inactive X-chromosome, whose microscopic structure is referred to as Barr body (Grewal and Elgin, 2002). Heterochromatin shows a dense packing of nucleosomes into regular structures, as well as the presence of specific histone modifications and architectural proteins (Dillon, 2004). For some regions with developmentally controlled gene activity such as the inactive X-chromosome, heterochromatin formation is facultative whereas for the telomeric and pericentric regions it is constitutive. At this highest level of organization, subnuclear compartments with a specialized set of associated factors are well described, including nuclear bodies and the nucleolus, the site of transcription of ribosomal factors.

A variety of modifications and structural variations in chromatin exist that confer its adaptability to diverse tasks. These variations include the establishment of special architectural states like the centromeric chromatin or the organization of the silenced X-chromosome. Changes in chromatin structure also serve to facilitate and guide nuclear processes which can be observed from the level of single gene transcription up to the reflection of cell differentiation states. For instance, modifications on the level of histones/nucleosomes like histone acetylation appear to induce a transition from dense heterochromatin to the more open euchromatin (Fejes Tóth et al., 2004; Görisch et al., 2005). Based on these findings it was proposed that the mentioned alterations form an „epigenetic“ code, inheritable information that is not encoded by the DNA base sequence but by patterns of histone variants, histone modifications and DNA methylation. Whereas genetic information appears (near-) universal, epigenetic information serves in the specific response to environmental or intrinsic signals and as an adaptable means to inherit information regarding for instance differentiation states to progeny cells (Nightingale et al., 2006).

3. Dynamic organization and stability of nucleosomes

3.1 *In vivo* dynamics of nucleosomal assemblies

Chromatin assemblies *in vivo* appear as dynamic entities. Whereas the H3·H4 tetramer is stably bound to the DNA on the hour scale, H2A·H2B dimers are somewhat more mobile (Kimura and Cook, 2001). In fact, different populations of H2A·H2B are detected in fluorescence recovery after photobleaching (FRAP) experiments with a small fraction of a few percent exchanging on the minute scale. H1 appears as very dynamic factor with residence times of around 250 seconds (Misteli et al., 2000). Furthermore, other chromatin associated factors exchange readily on the low second scale as shown for instance for HP1 (heterochromatin protein 1), HMGN proteins, the glucocorticoid receptor and other transcription factors (reviewed in (Hager et al., 2006)). Intriguingly, the dynamics of chromatin is greatly enhanced for pluripotent embryonic stem cells. This holds true for chromatin associated factors such as HP1 as well as the linker and core histones which appear to exist partially in a hyperdynamic fraction (Meshorer et al., 2006). How the enhanced dynamics can be linked to the pluripotency of the cells is an intriguing question. It is conceivable that the ability of the cell to differentiate is maintained by keeping the chromatin dynamic and undetermined, which might be easier than the rearrangement of preformed stable chromatin states upon differentiation (Meshorer et al., 2006).

3.2 Energetics of nucleosomes

Given the dynamics and the impact of local chromatin structure on a variety of nuclear processes, the stability of nucleosomes at a given DNA location has attracted longstanding research interest. In this context two of the key question are how the stability of nucleosomes is determined by the bound DNA sequence and by which means it can be modulated by additional factors and processes (Giresi et al., 2006). In the following the basis for specific DNA recognition by the nucleosome is reflected and research methodology to examine nucleosome energetics and their results are critically discussed. It might be argued that the modulation of nucleosome stability (and positioning) by chromatin remodeling complexes and epigenetic modifications are the main determinants of chromatin structure *in vivo*. However it is noted that recent evidence shows that a previously mapped *in vivo* positioning pattern can be mimicked for some systems in *vitro* in the absence of extrinsic factors, outlining the importance of DNA sequence on nucleosome positioning (Sekinger et al., 2005).

The high-resolution X-ray nucleosome structure provides detailed insight into the interaction between the DNA and the histone octamer. The previously discussed 14 DNA-protein contacts in the nucleosome structure appear to contribute simultaneously to the recognition of specific DNA sequences with a benefit of ~ 2 kJ per contact (Table 1). Base specific contacts between the protein content and the DNA are scarce (Davey et al., 2002). In fact the preference for DNA sequences is rather guided by geometric parameters, which allow spatially close association of the DNA with the histones at the contact points. In this way bridging water-molecules can be replaced by direct interactions between the nucleic acid and the protein which in consequence is entropically favorable due to solvent liberation (Davey et al., 2002).

Given the distortion of the DNA at the interaction sites with the histones, it appears little surprising that mainly flexible AA, TA or TT tracts are positioned at these sites (Cohanim et al., 2006; Ioshikhes et al., 1996; Thastrom et al., 2004b). It has to be noted that bendability of specific DNA conformations is also sequence-specific, but the DNA sequence is not directly "read out" by the protein part in this instance. In fact, high-affinity nucleosome binding sequences are more flexible than average DNAs (Cloutier and Widom, 2004).

The experimental assessment of binding energies for histone octamers is far more challenging as for other nucleic acid binding components due to the multi-step assembly path and kinetic competition with aggregation. The relative affinity differences for the DNA sequences tested up to date for nucleosome binding have to be regarded as rather small, as they cover a roughly 180 000 fold range for the strongest synthetic binding sequence and the weakest known DNA binding sequence (Davey et al., 2002). The differences between bulk and high-affinity naturally occupied binding sites appear much lower and span a ~ 40 fold range (Gottesfeld et al., 2001). In contrast, a range between 10 000 and 10 million fold for unspecific and specific binding of other DNA-binding proteins has been described (Jen-Jacobson et al., 2000). The lack in specificity is reflected by the ability of the histone octamer to bind to virtually every DNA sequence of sufficient length, highlighting its general role as a universal compactation factor of eukaryotic DNA.

Histone-DNA affinity values have almost exclusively been determined *in vitro* by the "competitive reconstitution" assay. Originally, the underlying protocol was developed to facilitate effective assembly of radioactive mononucleosomes (Drew and Travers, 1985). It uses a gradual decrease of ionic strength via dialysis to assemble the particles in an ordered way. This widespread "salt"-reconstitution method circumvents aggregation that would occur by simply mixing histone octamer and DNA. During the dialysis, which starts at typically 2 M monovalent salt concentration, the histone octamer dissociates (Eickbush and Moudrianakis, 1978) and the H3·H4 tetramer starts to interact with the DNA at a lower

ionic strength of 1.2 M salt, forming a tetrasome particle. The H2A·H2B dimers specifically associate with this subnucleosomal assembly at 0.6-0.8 M salt, thereby completing the nucleosome structure (Richmond et al., 1988; Tatchell and Van Holde, 1977). This assembly order is similar to the one of native factors that assemble nucleosomes (see below), with the fundamental difference that the ionic strength of the medium is drastically changed.

To measure the affinity of a sequence via competition, the reconstitution is carried out in the presence of an excess of an unspecific DNA, yielding a distribution between the two DNAs which reflects their relative affinity during the assembly process. The approach allowed the screening for (artificial) high-affinity sequences (Lowary and Widom, 1998), which circumvented the problem of multiplicity in nucleosome positioning. Even on the strongest natural positioning sequences, salt-reconstituted nucleosomes distribute along a variety of positions, often in 10 bp steps (Dong and van Holde, 1991) which hampers generation of evenly spaced nucleosome arrays *in vitro*. The artificial sequences (most notably the "Widom 601" sequence) display a single positioning, and thus have been successfully used for the *in vitro* generation of 30 nm fibers in the absence of chromatin remodeling factors (Dorigo et al., 2004; Robinson et al., 2006). Despite their widespread acceptance, the results of the "competitive reconsititution" method have been subjected to well-taken criticism (Drew, 1991). Most importantly, the recognition of the DNA sequence happens at high salt concentrations, where only some interactions between the histone H3·H4 tetramer and the DNA exist. Only at this stage in the assembly process "at near dissociating conditions" an equilibrium between the H3·H4 tetramer and different DNA sequences exists (Drew, 1991). At lower salt concentrations a significant exchange of histones between the competing DNAs does not take place and affinity differences that arise only at physiological conditions are not reflected. Notably the contribution of the two H2A·H2B dimers to nucleosome stability is not assessed as they join the subnucleosomal structure at ionic strengths at which the tetrasome is firmly positioned. This is demonstrated by the fact that a similar relative binding energy is derived for H3·H4 tetramer on 71 bp DNA, as for the complete nucleosome on the corresponding total 147 bp sequence (Thastrom et al., 2004a). The results seem to be also dependent on temperature, histone/DNA ratio and used competing DNA, which cannot be easily explained (Wu and Travers, 2005).

A second method to assess nucleosome free energies has been proposed a few years ago. It uses a stepwise dilution of mononucleosomes down to concentrations at which the histone octamer readily dissociates from the DNA. The fraction bound DNA is plotted against the employed nucleosome concentration and the data points are fitted to an equilibrium binding model (Gottesfeld and Luger, 2001). Even though the approach appears

straightforward, and the data are in good agreement with other results, it has to be critically assessed. This is because "specific" nucleosome assembly at physiological salt concentrations competes kinetically with the formation of nonspecific aggregates which cannot maturate into nucleosomes (Daban and Cantor, 1982a; Daban and Cantor, 1982b). In particular, unspecific complexes between H2A·H2B and DNA can form. Thus, once the nucleosome dissociates in the absence of specific histone chaperones, no true equilibrium between free histones and the nucleosomal counterpart is reached (Thastrom et al., 2004b).

Another area of interest linked to the energetics of nucleosomal assemblies is the accessibility of nucleosomal DNA to DNA-binding factors. In fact the highly distorted, partially buried DNA can not be accessed readily in its bound state by most DNA binding proteins. This barrier can be broken by histone eviction or sliding of nucleosomes via ATP-dependent mechanisms. However nucleosomal assemblies show interesting dynamic properties that faciliate access to their DNA, without the need for additional energy-dependent mechanisms. Recent studies demonstrate that nucleosomes spontaneously and transiently unwrap their DNA making nucleosomal DNA accessible. This site exposure of DNA can either happen partially or fully leaving only a few contacts of interactions between the DNA and the histone octamer in a process named breathing *in vitro* (Anderson and Widom, 2000; Polach and Widom, 1995; Tomschik et al., 2005) Unwrapping appears pronounced for the DNA at the entry and exit sites of the nucleosome and decreases in probability towards the dyad axis. This agrees with fluorescence resonance energy transfer (FRET) data showing that the unwrapping proceeds from the ends of nucleosomal DNA to internal sites (Li et al., 2005b). Quantitative analysis of single molecule measurements demonstrates that mononucleosomes on average exist for 250 ms in a compacted state and dwell for 10-50 ms in an unwrapped state (Li et al., 2005b). It is noted that the ability for spontaneous unwrapping might be hampered by internucleosomal contacts. In fact the accessibility of histones appears reduced in a fiber context, as compared to mononucleosomes (Kepert et al., 2005).

Table 1. Nucleosomal interaction energies

Energetic Contribution	*Value* (kJ mol^{-1})	*Source*	*References*
Total energy of single nucleosome assembly for a) highest affinity b) non-selected natural nucleosomal DNA c) lowest affinity sequences	 a) 63 b) 50 c) 33.8	Dilution-driven dissociation, competition experiments	(Cao et al., 1998; Filesi et al., 2000; Gottesfeld and Luger, 2001; Thastrom et al., 2004a)
Total difference between highest/ lowest affinity DNA binding	29.2	Competition experiments	(Filesi et al., 2000; Thastrom et al., 1999)
Sequence-specific histone/DNA contacts	2.1 (per contact)	Calculated from total ΔΔG° and the number of DNA/histone contacts	(Davey et al., 2002)
Internucleosomal contacts	3.2 – 8.5	Computer based fiber simulations/ Force-extension curves	(Cui and Bustamante, 2000; Wedemann and Langowski, 2002)

3.3 Histone Variants

Histone variants provide one of the possibilities to generate a specialized chromatin environment for nuclear processes. The growing group of histone variants includes variants in H2A (H2A.Z, H2A.X, macroH2A, H2ABbd), H2B (H2BFWT) and H3 (H3.3, CENP-A) (see Table 2). Their involvement in the modulation of chromatin structure is reflected here exemplarily for the histone variants H3.3 and H2.Z, the latter studied in this thesis.

H3.3 resembles the canonical H3 in the amino-acid sequence almost perfectly as only single residues are changed (Ahmad and Henikoff, 2002). However, both incorporation into chromatin and localization appear severely different from H3, which can be attributed

to the subtle changes in amino acid sequence (Ahmad and Henikoff, 2002). Unlike its canonical counterpart, H3.3 is not deposited by the chaperone complex CAF1 on the DNA but by the HIRA complex (Tagami et al., 2004). H3.3 is divergently incorporated in a replication-independent manner and appears at transcriptionally active gene regions, thereby demonstrating that histone eviction during transcription can also take place on the H3·H4 level.

The H2A variant H2A.Z is found to be associated with regions of transcriptionally active chromatin (Stargell et al., 1993). Its recruitment is conducted by the ATP-dependent chromatin remodeling complex SWR1 in yeast, which replaces H2A with the variant histones in nucleosomes *in vivo* and *in vitro* (Kobor et al., 2004; Mizuguchi et al., 2004). A study on the human SRCAP (Snf-2-related CREB binding protein activator protein) complex that has homologous function to SWR1 shows that the replacement is mediated by an exchange of H2A·H2B for H2A.Z·H2B dimers and not by exchange of individual histones (Ruhl et al., 2006). Thus, a transient formation of hexasomic or tetrasomic structures appears likely. In conclusion, unlike the canonical histones, H2A.Z is deposited via a cell replication-independent pathway. The specialized functions of H2A.Z appear numerous and occasionally contradictory, as it acts in a bifunctional manner in transcriptionally active and inactive chromosomal regions. For instance, H2A.Z is a mandatory factor for the recruitment of RNA polymerase II and TATA-binding proteins to the GAL1-10 promotors (Adam et al., 2001). However, it is also necessary for establishment of pericentric heterochromatin in mouse embryo cells (Rangasamy et al., 2003). The functions of H2A.Z appear influenced by posttranslational modifications of the histone as exemplified recently for acetylation. In the non acetylated state H2A.Z is found to be not associated with chromatin in chicken cells. In contrast a triacetylated form is found enriched in the 5' region of active genes likely acting as barrier element (Bruce et al., 2005).

The overall structure of the H2A.Z nucleosome displays high similarity to the canonical nucleosome (Suto et al., 2000). This accounts for the histone-fold domains, as well as the DNA path on the nucleosome surface. The essential region for H2A.Z activity seems to lie in its acidic C-Terminal amino acid stretch (Adam et al., 2001), which might to function as protein binding site (Suto et al., 2003). This region confers localization of H2A.Z to transcriptional active regions and its function as transcriptional activator (Larochelle and Gaudreau, 2003). *In vitro* characterizations show that the H2A.Z·H2B dimer is slightly more resilient to extraction from the nucleosome by treatment with high ionic strength buffers (Mazurkiewicz et al., 2006; Park et al., 2004). Also the formation of higher-order chromatin structures appears different for H2A.Z as the folding of nucleosome arrays

seems enhanced, whereas interfiber interactions are inhibited (Fan et al., 2002; Hayes, 2002).

Table 2. Histone variants

Canonical Histone	*Histone Variant*	*Localization and Function*	*References*
H2A	H2A.Z	associated with transcriptionally active chromatin, both activator and repressor of transcription; prevents spreading of heterochromatin into euchromatic regions	(Dhillon and Kamakaka, 2000; Meneghini et al., 2003; Santisteban et al., 2000; Stargell et al., 1993)
	H2A.X	promotion of DNA repair via recruitment of Double Strand Break repair complexes upon phosphorylation of the histone variant	(Celeste et al., 2002; Franco et al., 2006; Paull et al., 2000)
	H2ABbd	excluded from the inactivated X-chromosome, association with regions of H4 acetylation (euchromatic), reduces nucleosome stability	(Chadwick and Willard, 2001; Gautier et al., 2004)
	MacroH2A	accumulated at the inactive X-Chromosome; contains a large C-terminal macrodomain	(Costanzi and Pehrson, 1998; Pehrson and Fried, 1992)
H2B	H2BFWT	located at telomeric sequences; inhibition of association with chromatin condensation factors	(Boulard et al., 2006)
	hTSH2B	testis specific, possibly telomere-associated functions	(Churikov et al., 2004; Li et al., 2005a)
H3	H3.3	replacement with this variant confers transcriptionally active chromatin; derepression of genes	(Ahmad and Henikoff, 2002)
	CENP-A	associated with centromeric DNA; essential for assembly and preservation of kinetochores	(Henikoff and Dalal, 2005; Palmer et al., 1991)

3.4 Posttranslational modifications of histones

A variety of covalent modifications has been assigned to histones including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP-ribosylation. Even though most of these modifications have been identified in the early years of chromatin research (reviewed in (van Holde, 1989)) their impact on chromatin structure has only been revealed in the past years, leading to the "histone code" hypothesis (Strahl and Allis, 2000). Histone modifications can be thought to act in a simultaneous manner either synergistically, complementary or antagonistically, thus allowing for a rich variety of regulatory events.

Acetylation of lysine residues is possibly the best understood covalent modification of histones. Acetylation is conferred by histone acetyltransferase (HAT) domain containing proteins. These appear often in large, multiprotein assemblies such as SAGA for the GCN5 acetyltransferase (Timmers and Tora, 2005). Histone acetylation of certain residues in H3 and H4 coincidences with transcriptional activation or DNA replication (H4K5 and K12) and is absent from heterochromatic structures (Kuo et al., 1996; Sobel et al., 1995). Inhibition of the antagonistically acting histone deacetylases (HDACs) via trichostatin A treatment leads to a decrease in higher-order chromatin structure, pointing at the importance of the epigenetic mark in chromatin structure regulation (Fejes Tóth et al., 2004). From a biophysical perspective, acetylation of histone tails has attracted quite some interest as it neutralizes the positive lysine charge. In a long standing hypothesis it is proposed that the (electrostatic) interaction of the tail with the DNA can thereby be hampered, destabilizing the nucleosome and respective higher-order structures. Thus, additionally to acting as a recruitment (or eviction) signal to extrinsic factors, acetylation might have a direct structural impact on nucleosomes. In agreement with this view *in vitro* studies show that acetylation causes a decrease in folding of 30 nm structures, thereby facilitating transcription (Annunziato et al., 1988; Shogren-Knaak et al., 2006; Solis et al., 2004; Tse et al., 1998). Also, acetylation appears to facilitate the transfer of H2A·H2B dimers from nucleosomes to histone chaperone NAP1 (Ito et al., 2000).

However, the intrinsic effect of acetylation on mononucleosomes appears controversial. One study shows that it might even be a slightly stabilizing one (~ -4 kJ) (Widlund et al., 2000). Furthermore, recent results demonstrate that the outcome of histone acetylation in terms of altering linker DNA conformation is critically dependent on the sites of modification (Toth et al., 2006), pointing at the complicated structural consequences of histone acetylation.

Histone methylation can take place at either lysine or arginine residues. Lysine methylation ranges from mono- to trimethylation of the lysine ε-nitrogen and is conferred by histone lysine methyltransferases (KHMTase). These can be distinguished in two classes dependent on the presence of a SET-domain which is named after its founding members from Drosophila (Su(var)3-9, Enhancer of zeste (E(z)) and trithorax). Well described residues for histone methylation are H3 K9, K4 and K79. The effect of histone methylation herein on chromatin state is critically dependent on the residue modified and the number of methyl-groups attached (for review see (Shilatifard, 2006).

(Tri-)methylation of H3K4 for instance is an euchromatic marker. It is set by complexes containing the MLL (mixed lineage leukemina) protein in humans and the SET1 protein in yeast. The recruitment of the complexes takes place via elongating factors associated with the transcribing RNAP II, such as the Paf1 (polymerase II associated factor 1) complex in yeast. Methylation of H3K4 seems to also depend on H2BK123 monoubiquitination set by Rad6/Bre1 (Wood et al., 2003), demonstrating the synergistic interplay between two histone marks. The details of this interactions remain to be determined. The modification is recognized by the Mi2 family remodeler CHD1 which further destabilizes nucleosomes, possibly to grant access for the transcription machinery. Binding of CHD1 is conferred by its tandem chromodomains, that specifically recognize the methylated histone mark (Flanagan et al., 2005).

In contrast to H3K4 methylation, higher-order H3K9 methylation marks a step in a cascade of events that are necessary in the establishment and spreading of heterochromatin. The methylation reaction is carried out by Su(var)3-9 in Drosophila and its homologues in humans and yeast (Rea et al., 2000). Su(var)3-9 was identified as an effector of position effect variegation (PEV) (Tschiersch et al., 1994) The term PEV refers to a process in which an euchromatic gene becomes silenced due to its positioning into a heterochromatic region. Hence, it is a prime example for modification of gene activity by chromatin structure. Its modulators can be divided into suppresors of variegation Su(var)s and enhancers of variegations E(var)s.

Su(var)3-9 itself is probably recruited to heterochromatic regions by its interactions with HDACs. The resulting H3K9 methylation recruits HP1 family proteins to the modified nucleosomes. The HP1 group of proteins consists of HP1 α, β and γ and the first two can be seen as a heterochromatic marker (Minc et al., 2000). All isoforms reflect a tripartite structure of a chromo- and chromoshadow domain connected by a poorly conserved hinge region. HP1 directly interacts with the methylated H3 tail via its chromodomain (Jacobs and Khorasanizadeh, 2002; Jacobs et al., 2001). The binding appears enhanced for the higher methylated forms of H3K9 (Fischle et al., 2003). The ability of HP1 to dimerize has lead to the proposition that this protein serves in the spreading of heterochromatin via

cooperative binding. It is noted that the HP1 binding itself is unlikely to solely depend on the methylated H3 residue. A variety of binding partners have been demonstrated for HP1, including the histone chaperone CAF1, the linker histone H1b, the lamin B receptor, DNA methyltransferases, chromatin remodeling factor BRG1, as well as an apparent RNA binding activity (Daujat et al., 2005; Hediger and Gasser, 2006; Polioudaki et al., 2001). Possibly these interactions serve in guiding HP1 to its site of action or in the recruitment of additional factors and point at activities of the protein also in gene activation and nuclear assembly. Especially the interaction with the histone chaperone is interesting, since it might reflect a recruitment of a heterochromatin marker via a player in DNA replication to confer the reestablishment of heterochromatic state.

Intriguingly, another histone modification appears to control the association of HP1 with trimethylated H3 in a cell cycle dependent manner. H3 serine 10 phosphorylation through Aurora B kinase strongly antagonizes the association of HP1 with the methylated histone (Fischle et al., 2005; Hirota et al., 2005). This mark is set in pericentric heterochromatin only during mitosis and appears critical for proper chromosome seggregation and condensation (Hendzel et al., 1997; Mellone et al., 2003; Wei et al., 1999). Hence, phosphorylation allows transient release of HP1 during mitotic events while ensuring that the epigenetic information for reestablishment of constitutive heterochromatin (the methyl mark) can be spread to the progeny cells (Fischle et al., 2005).

It is noted that histone methylation has been long thought to be removable only by histone eviction, as no corresponding demethylase was described until recently. The discovery of a series of these enzymes shows the dynamic nature of this modification (Cloos et al., 2006; Klose et al., 2006; Shi et al., 2004; Tsukiyama et al., 1994). Three ways of bypassing the mark can therefore be described, e.g. histone eviction and demethylation as well as the transient compensation through serine phosphorylation (Eissenberg and Elgin, 2005).

4. Establishing chromatin

4.1 Chaperone guided nucleosome assembly

As discussed above, *in vitro* experiments show that under physiological salt concentrations the assembly of free histones into nucleosomes competes with the formation of nonspecific DNA-histone aggregates. *In vivo* this problem is circumvented by complexation of not DNA-bound histones to specific chaperones. These histone chaperones essentially have a bimodal function. On the one hand they prevent non-specific DNA association leading to aggregation. On the other they 'guide' the specific, nucleosomal assembly path. This is achieved by thee thermodynamic balance between binding of the histones either to the chaperone, sub(nucleosomal) structures or nonspecific association on DNA (Mazurkiewicz et al., 2006). The affinity of the chaperone protein for the histones is chosen such, that the formation of DNA/histone aggregates is avoided, while the specific assembly path can take place. To a limited extent the activity of histone chaperones can be mimicked by polyanions pointing at the simplicity of the interaction. However, the variety of histone chaperones points at their diverse additional functions. Histone chaperones include Nucleosome Assembly Protein 1 (NAP1) (Ishimi et al., 1984; Ito et al., 1996), Chromatin Assembly Factor 1 (CAF1) (Bonner, 1975; Kleinschmidt et al., 1986) , N1/N2 (Bonner, 1975; Kleinschmidt et al., 1986), Nucleoplasmin (Arnan et al., 2003; Laskey et al., 1978; Prado et al., 2004), HIRA (Ray-Gallet et al., 2002) and ASF1 (Tyler et al., 1999; Tyler et al., 2001).

The heterotrimeric CAF1 interacts directly via its largest subunit with PCNA (proliferating cell nuclear antigen, a component of the DNA polymerase η machinery) so that it is directly targeted to the replication fork. It serves in the assembly of $(H3{\cdot}H4)_2$ tetrasomes on the newly replicated template (Shibahara and Stillman, 1999). In human cells, CAF1 is essential for nascent chromatin assembly and efficient S-phase progression (Hoek and Stillman, 2003). Nucleoplasmin and N1/N2 appear to function as histone sinks during early development in Xenopus oocytes (Dingwall and Laskey, 1990), whereas HIRA mediates the deposition of the variant H3.3 histone (Ray-Gallet et al., 2002; Tagami et al., 2004). ASF1 is involved in the assembly of silent chromatin (Sharp et al., 2001), as well as in the dis- and reassembly of nucleosomes during RNA polymerase II transcription (Schwabish and Struhl, 2006). It can interact with subunits of CAF1 and HIRA possibly acting as histone donor for these complexes (Loyola and Almouzni, 2004; Tagami et al., 2004). ASF1 is thought to function as chromatin disassembly factor in yeast, as suggested by asf1 mutant cells analysis which show decreased accessibility to micrococcal nuclease and DNAseI (Adkins et al., 2004a; Adkins and Tyler, 2004).

NAP1 is a histone chaperone with versatile functions in regulatory processes. *In vitro* the chaperone is capable of promoting complete nucleosome assembly as sole histone carrier at physiological ionic strength (Ishimi and Kikuchi, 1991; Ito et al., 1996). It has been assigned as a carrier for H2A·H2B *in vivo*, as it interacts with H2A·H2B dimers in *Drosophila* embryo extracts and with newly synthesized H2A in HeLa cell extracts (Chang et al., 1997; Ito et al., 1996). NAP1 is also involved in the shuttling of the histones from cytoplasm to nucleoplasm via interaction with karyopherin 114, a factor that also represses histone deposition by NAP1, thereby possibly regulating chromatin assembly (Mosammaparast et al., 2005; Mosammaparast et al., 2001).
NAP1 copurifies with HTZ1-Flag in yeast, pointing at its association with H2A.Z *in vivo* and a role in the generation of transcriptionally active chromatin by acting as histone donor for the SWR1 complex (Mizuguchi et al., 2004). NAP1 was also shown to be involved in transcription control processes mediated by p300/CREB (Asahara et al., 2002; Kawase et al., 1996; Rehtanz et al., 2004; Shikama et al., 2000). Besides its interaction with the H2A·H2B dimer, NAP1 functions as a linker chaperone in *Xenopus* oocytes (Shintomi et al., 2005) and can also fulfil this role *in vitro* (Saeki et al., 2005). On isolated chromatin fibers the chaperone regulates the H1 content in a concentration-dependent manner (Kepert et al., 2005). NAP1 is capable of disassembling nucleosomes completely *in vitro*, which is facilitated by the chromatin remodeling complex RSC (Lorch et al., 2006). Furthermore, NAP1 participates in the regulation of cell cycle progression via direct interaction with B-Type cyclins and other cellular proteins such as SDA4 and the GIN4 kinase (Kellogg and Murray, 1995; Miyaji-Yamaguchi et al., 2003; Zimmerman and Kellogg, 2001)

4.2 Chromatin remodeling complexes

Histone chaperones deposit nucleosomes in a rather random, unordered orientation to each other (Ito et al., 1996). Thus, even though the folding of a chromatin fiber is energetically favourable, with an estimated benefit of 3.2 to 8.5 kJ/mol per internucleosomal contact (Cui and Bustamante, 2000; Wedemann and Langowski, 2002), this structure will not be formed spontaneously. The corresponding interactions can only be established after deposition of the histone octamers, as a competition with misaligning binding sites on the DNA takes place, preventing regular nucleosome spacing which is necessary for ordered assembly. Also, the high energetic barrier to nucleosomal mobility along the DNA has to be considered, as the spontaneous, thermal mobility of nucleosomes appears rather low (Flaus and Owen-Hughes, 2003). In conclusion, an unmediated assembly into higher order chromatin structures does no take place.

The translocation of nucleosomes along DNA and the formation of regular higher order chromatin structures are catalyzed by the so called chromatin remodeling complexes. *In vivo*, these complexes mediate a proper chromatin context for diverse, central nuclear processes such as replication, DNA repair and gene expression. They eject or move nucleosomes to grant access to DNA for regulatory proteins, that otherwise would be impeded by their binding to the nucleosomal DNA (Reinke and Horz, 2003), and confer the exchange of canonical histones for their variant counterparts, as shown for H2A.Z. This broad involvement is reflected by the severe phenotypes arising from knock-out of these complexes (Cairns et al., 1996; Deuring et al., 2000).

Chromatin remodeling complexes can be classified by their molecular composition, which evolves around the central DNA translocating and ATP consuming unit. Following this nomenclature, families of remodeling complexes such as SWI/SNF, INO80/SWR, NURD/Mi2/CHD and the imitation switch (ISWI) protein are distinguished (reviewed in (Cairns, 2005; Eberharter and Becker, 2004; Saha et al., 2006)). The central units are generally characterized by partial homology to the ATPase region of the snf2 protein from yeast (Eisen et al., 1995; Laurent et al., 1992; Lusser and Kadonaga, 2003). However, each protein contains additional domains such as SLIDE, SANT and bromodomains which confer the specialized function.

The ISWI remodeler family is named after its founding component, the *Drosophila* ISWI protein and extends to a variety of eukaryotic organisms, including the human SNF2h (SNF2 homologue) (Tsukiyama, 2002). All catalytic units share a C-terminal SANT domain, involved in the recognition of histone tails and nucleosome-proximate linker DNA (Grune et al., 2003). The catalytic unit itself suffices for translocating nucleosomes along DNA, however has a limited ability to create regular spaced arrays (Corona et al., 1999). Together with its subunits it accounts for an amazing variety of chromatin related maintenance and assembly functions (Bozhenok et al., 2002; Corona and Tamkun, 2004; Deuring et al., 2000).
ISWI class remodelers participate in mediation of transcription by RNA polymerase I (Zhou et al., 2002) and RNA Polymerase II (Morillon et al., 2003), as well as acting as transcriptional repressors (Goldmark et al., 2000). The catalytic protein is augmented by different subunits, providing specificity to the remodeling action. One of these units is the Acf1 protein (ATP utilizing chromatin-assembly and remodeling factor 1) which together with ISWI forms the ACF complex (Ito et al., 1999). This complex along with a histone chaperone generates ordered nucleosome arrays *in vitro* (Ito et al., 1997). Intriguingly, the

ACF complex shows an opposing directionality in mononucleosome sliding assays to ISWI, pointing at the modulation of ISWI function by Acf1 (Strohner et al., 2005).
Another ISWI class remodeler is the NoRC complex, formed by ISWI and TIP5. Noteworthy, it was recently demonstrated that the TIP5 protein directs ISWI to the rDNA promotor via recognition of a RNA element. There the remodeling complex exerts its function in changing the position of a single nucleosome which acts as a transcriptional switch. Furthermore, the histone methylation pattern changes, abolishing recruitment of HP1 (Mayer et al., 2006).

Members of the SWI/SNF remodeler class function often in apparent antagonistic ways to the ISWI class remodeler. In yeast, SWI/SNF acts mainly as a transcriptional activator, however only around 6% of the total genes underlie activation by the remodeler (Sudarsanam and Winston, 2000). Thus, SWI/SNF seems to be located precisely to single promoters rather then being randomly distributed. SWI/SNF was shown to be recruited to these promoters by transcriptional activators such as SWI5 for association with the HO gene (Neely et al., 1999). The interaction with transcriptional activators appears also required for localization of a variety of mammalian chromatin remodelers of the SWI/SNF class (Cavellan et al., 2006; Hsiao et al., 2003; Kowenz-Leutz and Leutz, 1999). It is noted that, even though SWI/SNF remodeler are therefore commonly thought of as transcriptional activators, some studies demonstrate the opposite function (Moreira and Holmberg, 1999; Trouche et al., 1997). On a molecular level, pronounced differences in the outcome of the remodeling of nucleosomes between ISWI-class remodelers and SWI/SNF exist. SWI/SNF appears to disorganize previously ordered nucleosomal arrays (Schnitzler et al., 2001). Furthermore, it is capable of sliding nucleosomes partially beyond the ends of linear DNA, leading to a loss of H2A·H2B dimer. A SWI/SNF mediated exchange of H2A·H2B was also seen for circular templates, pointing at a role in histone eviction (Bruno et al., 2003). Indeed, some evidence exists that *in vivo* remodeling by SWI/SNF has a similar outcome like depletion of H2A·H2B (Hirschhorn et al., 1992). However recent biophysical evidence suggests that SWI/SNF remodeled nucleosomes are identical to intact ones regarding their histone composition. Thus, it might be that disruption of nucleosomal structure during remodeling is only transient (Shundrovsky et al., 2006).

The molecular mechanism of nucleosome movement is still under discussion, with two conflicting main models. The first model, referred to here as “twist model”, postulates the propagation of a twist-defect along the histone-DNA surface. This leads to a base pair by base pair motion of the nucleosome along the DNA analogous to a screw nut progressing

on a thread (Saha et al., 2005). For translocation of the nucleosome a twist force has to be exerted on the DNA by the remodeling complex. This main aspect of the model has been challenged by various experimental findings. Most importantly, the incorporation of DNA nicks, gaps or bulky obstacles does not stop the activity of various remodeling complexes, in conflict with the need for a twist force (Lorch et al., 2005; Strohner et al., 2005). Furthermore the minimal step-size detected in reactions with ISWI and SWI/SNF class remodelers is ~ 9-11 up to 50 bp, inflicting with the proposed one base pair step size (Stockdale et al., 2006; Strohner et al., 2005; Zofall et al., 2006).

The second model referred to as ”loop-recapture” or “bulge-recapture” model proposes the partial detachment of DNA on the entry/exit site of the nucleosomal surface. Additional DNA is pressed into the nucleosome, resulting in the formation of DNA bulge, which migrates over the nucleosomal surface and in the end leads to translocation of the histone octamer. In a recently proposed modulation of this model the detachment of DNA could be achieved inside the nucleosomal structure near the dyad axis at SHL 2. This was deducted from the result that a concentrated array of gaps at this site actually does inhibit ISW2 nucleosome remodeling and the previously shown interaction of ISWI with this location (Kagalwala et al., 2004; Zofall et al., 2006). It was proposed that these positions might be the nucleation site of bulge formation and that here DNA torsion is required for initial detachment of the DNA.

One intriguing point in the mechanisms of chromatin remodeling complexes is the positioning specificity they provide for the nucleosome. Various results point at a translocation of nucleosomes by these complexes that is specific for the employed remodeler and varies upon its complex composition (Stockdale et al., 2006; Strohner et al., 2005). Even more, the positioning of single nucleosomes achieved by purified assembly extracts diverges from that seen with salt reconstitution in some systems albeit prolonged temperature shifts (Herrscher et al., 1995). Hence, the positioning of even single nucleosomes *in vivo* does not necessarily reflect the thermodynamically optimal distribution for an isolated histone-octamer/DNA system. Thus, it appears that specialized DNA sequences or conformations exist that are read-out by the remodeler as end positions, e.g. there is DNA recognition by the ternary complex between nucleosomes and remodeler (Rippe et al., 2006).

At the same time, little is know about the exact events during remodeling of nucleosomal arrays. Data on the dynamics of the remodeling complex ACF indicate that seven nucleosomes are remodelled on average before ACF leaves and rebinds another substrate (Fyodorov and Kadonaga, 2002). However, how the remodeling of multiple nucleosomes takes place mechanistically remains to be determined.

4.3 Chromatin assembly

Chromatin assembly processes *in vivo* can be classified in relation to the cell cycle. The majority of chromatin formation is coupled to the replication of DNA in the S phase whereas other assembly pathways have to exist to allow incorporation (and eviction) of histones due to processes such as DNA repair, transcription and recombination.

The replication of DNA opposes a stringent barrier to chromatin, since higher-order structures are disrupted as the DNA strands get separated at the replication fork. The replication of DNA a in a semiconservative fashion is well established, but details for the reassmembly of chromatin states and inheritance of epigenetic information are still under discussion (Annunziato, 2005). First, existing core histones are transferred from the parental strand to the nascent daughter strands during replication in a process called parental chromatin segregation (Krude, 1999). This includes destabilization of nucleosomes immediately in front of the replication fork and reassembly around 250 bp behind the replication machinery (Gasser et al., 1996). Early research has pointed at an exclusive transfer of histones to one daughter strand. This view has later been revised and a distribution of nucleosomes on both strands in arrays of variable size is commonly assumed (Annunziato and Seale, 1984; Jackson and Chalkley, 1985). The parental nucleosomes are disrupted into H3·H4 tetramers and dissociating H2A·H2B dimers during passage of the replication machinery (Gruss et al., 1993; Jackson, 1990). It remains unclear whether H3·H4 particles are transiently detached at this step and transferred to the DNA behind the fork or whether they can pass through the replication machinery (Krude, 1999).

Simultaneously newly synthesized histones fill the gaps on the daughter strands via *de novo* nucleosome assembly. This latter process has been found to take place in a precise order. First, acetylated histone H3·H4 is deposited through CAF1, followed by augmentation of the subnucleosomal structures by H2A·H2B dimers (Smith and Stillman, 1989). Finally, the linker histone are added to the nucleosomes (Worcel et al., 1978). The acetylation of H3·H4 appears essential for nucleosome formation *in vivo*, and is quickly removed after assembly (Jackson et al., 1976; Ling et al., 1996). The establishment of regular chromatin fibers is conferred by chromatin remodeling complexes, most likely acting after the deposition of core histones is complete, as the deposition of histones and chromatin remodeling likely are separate processes (Mazurkiewicz et al., 2006).

The fate of H3·H4 during replication is currently under discussion with the assumed transfer of complete tetrasomes onto nascent DNA being challenged by recent studies. Analysis of the association states of H3·H4 and H3.3·H4 pre-deposition complexes containing CAF1 and HIRA *in vivo* show that both harbour the histones as heterodimers, e.g. H3·H4 and not $(H3{\cdot}H4)_2$ ((Tagami et al., 2004). An analogous result was derived for

ASF1 *in vitro* which is also present in both complexes (English et al., 2005). As implication, a splitting of tetramers at the replication fork into heterodimers on both strands has been proposed. These parental H3·H4 dimers are then augmented with nascent H3·H4 from the CAF1 complex. As a result each nucleosome receives half of the parental posttranscriptional H3·H4 modifications. This mechanism provides a model for the simultaneous transfer of the epigenetic pattern to both newly synthetized strands and omitts dividing it to groups of nucleosomes on the individual strands. However, it is in contradiction with the proposed mechanism above and results, which demonstrate the presence of tetramers as non-disrupted units through repeated replication cycles (Jackson, 1990). Even more a subnucleosomal structure consisting of an H3·H4 dimer on DNA has neither been detected *in vivo* nor *in vitro* up to date. However, *in vitro* this structure appears to be at least transiently formed by the histone chaperone ASF1. ASF1 is capable of catalysing the formation of tetrasomes at a concentration at which the chaperone harbors one H3·H4 dimer. Thus, it can be derived that the assembly has to happen in a two step process yielding a tetrasome structure from two separate binding events of dimeric units (English et al., 2005).

5. Objectives of this work

The presented study addresses the elementary processes that govern the establishment of chromatin structure and its dynamic properties. Since chromatin acts as a key element that controls access to the DNA, the assembly and altering of its structure are of central importance for cellular function. The main factors that determine chromatin assembly, reconfiguration and dynamics are histone chaperones and chromatin remodeling complexes. Here, two of these factors, the chaperone NAP1 and the ISWI class remodeling complex ACF were investigated. Key questions that were addressed include the molecular mechanisms for the biological activity of both factors and their relation to the assembly and dynamics of chromatin fibers.

One part of the work aimed at the development of a quantitative, kinetic model for nucleosome assembly by physiologically relevant factors. A model system that allows for the assessment of single nucleosome assembly steps was set up. Unlike previous equilibrium determinations of the particle stability, a kinetic analysis yields far more information about the mechanisms of the underlying processes. In the well defined minimal *in vitro* system employed here, the detection of subnucleosomal species as well as the computation of rate constants for their formation was possible. In addition, factors influencing the kinetics of nucleosome assembly were studied, including replacement of canonical H2A with histone variant H2A.Z, different DNAs and the activity of ACF. In a related study, the mechanism of nucleosome translocation by ISWI class remodelers was investigated, with the results pointing at a loop-recapture mechanism.

Based on the findings for the role of NAP1 in mononucleosome assembly, its activity in modulating chromatin organization was further examined. It was therefore tested how NAP1 acts in the disassembly of single nucleosome structures as well as chromatin fibers. As *in vivo* chromatin assembly is a concerted action of histone chaperones and chromatin remodelers this interplay was investigated. For the combination of NAP1 and ACF it was examined whether the activities of both factors are separate or constitute a combined process.

Procedures for the *in vitro* analysis of chromatin assembly and remodeling processes

1. Preparation of material for *in vitro* analysis

1.1 Preparation of protein complexes

Two experimental strategies for the analysis of chromatin assembly and dynamics *in vitro* can be distinguished. On the one hand, it is possible to use nuclear extracts for instance from *Xenopus* oocytes or *Drosophila* embryos. These extracts include all relevant components needed for the assembly of chromatin structure and therefore mimic the *in vivo* situation precisely (Becker et al., 1994; Ladoux et al., 2000; Tremethick, 1999). However, an individual assignment of function to single components is difficult due to the multitude of present factors. On the other hand, it is possible to use recombinant systems that utilize single, highly-purified components. The latter approach has become feasible due to the development of procedures that allow purification of single components involved in chromatin assembly (Ito et al., 1996; Ito et al., 1997; Luger et al., 1999).

In the presented study histones and histone chaperone NAP1 were prepared from recombinant sources. Therefore, they lack posttranslational modifications, which is of significance mostly for the histones. Advantage was taken from protocols for the expression of histones in bacteria (Luger et al., 1997b). Thereby, histone complexes such as H2A·H2B dimers, H3·H4 tetramers and histone octamers could be assembled from individual histones. It was possible to obtain histone complexes with single cysteine substitutions used for fluorescent labeling or complexes with stoichometric amounts of histone variants. By the use of recombinant material, a well-defined nucleosome assembly system could be established, in which the influence of single components could be easily examined. This included activity of ACF, partial assembly in the absence of H2A·H2B, variation of DNA sequence or length, and substitution of canonical H2A with H2A.Z.

Two exceptions from the rule were made regarding the origin of material. H1 was obtained from *Bos taurus*, as no protocols for the recombinant expression of the full length linker histone are currently available. For studies on chromatin fibers, material isolated from HeLa cells was utilized. These fibers were preferred over recombinantly made ones due to native conformation (Kepert et al., 2005).

1.2 Labeling procedures

Introduced a few years ago, the site-specific labeling of histones has been successfully applied in a variety of studies (Bruno et al., 2003; Dorigo et al., 2004; Park et al., 2004). Here, such fluorescently labeled histones were used for the quantification of assembly processes, the discrimination of (sub-)nucleosomal species or chaperone-bound complexes and the quantification of binding stoichiometries. The modified histones contain single cysteine mutations that were labelled with maleimide dyes, such as Alexa Fluor 488 C5 maleimide (Molecular Probes Europe BV, Leiden, Netherlands). The labeling procedure takes advantage of the fact that except H3 the core histones contain no endogenous cysteine. Thus, a site-specific mark for labeling can be set with just one point mutation in the histone. Labeling of the endogenous H3C110 residue that might interfere with function of the histone (or experimental strategy) is omitted by mutation of this residue to alanine or serine. In the presented study an established protocol was used (Bruno et al., 2004). It was found that unlike histone octamers, H2A·H2B dimers and H3·H4 tetramers were lost in the steps that separate the free fluorophore from labeled histone complexes. Therefore, the procedure was modified for these subassemblies. An ion-exchange resin (Bio-Rex 70) was used for the separation rather than centrifugal concentrators (Fejes Tóth et al., 2005). The labeling of H1 from natural sources was conducted with tetrafluorophenyl ester that selectively reacts with the N-termini of proteins under appropriate conditions (Kepert et al., 2005).

A property of histones that was largely exploited here, is their electrophoretic mobility on agarose gels. Isolated histone complexes do not enter the gels due to their positive charge. However, the net charge can be reversed upon binding to DNA, NAP1, HP1 or other negatively charged macromolecules, leading to migration of the complexed histones into the gel. As these differ in running behavior in dependence on the binding partner, assemblies with fluorescently marked histones can easily be discriminated by gel electrophoresis (see also Fig.2.1). Subsequent staining with ethidium bromide can show which of these entities contain DNA. This is for instance important in the analysis of nucleosome assembly reactions.

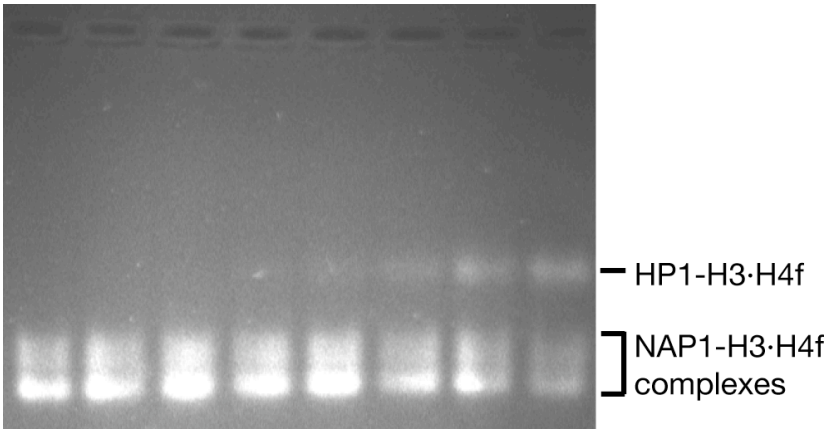

Figure 2.1 Gel-electrophoretic analysis of the competition of NAP1 and HP1 for H3·H4. Histone octamer (0.125 μM) labelled via H4C5 with Alexa Fluor 488 was bound to NAP1 (1 μM). An increasing amount of HP1 was titrated to the complex (0, 0.5, 1, 2, 4, 8, 16, 32 μM) from left to right. It is apparent that HP1 competes with NAP1 for the non methylated H3·H4, however has a lower affinity for the particle than NAP1. When the experiment was conducted with labelled H2A·H2B no formation of a fluorescent HP1-histone complex was observed (data not shown). The results point at a specific recognition of the H3·H4 complex by HP1.

2. Fluorescence Methodology

2.1 Fluorescence Anisotropy

Measurements of fluorescence anisotropy can be used to assess the rotational diffusion of biomolecules. As this parameter changes upon processes such as (self-)association of proteins and nucleic-acids or conformational changes, these dynamic events among others can readily be examined by utilizing fluorescence anisotropy (Lakowicz, 1983). The method itself takes advantage of the fact that a fluorophore emits polarized light, when excited in a polarized manner. As a result, if a fluorophore is excited with vertically polarized light and the emission has to pass an horizontally oriented polarization filter, the fluorescence will only be detected when a significant portion of the molecules rotate within the lifetime of the fluorophore by 90°. Thus, there will be a bias between fluorescence intensity measured for different alignments of the emission polarization filter depending on the rotational diffusion of the fluorescent molecule. The fluorescence anisotropy is defined by the difference in emission intensity when excitation and emission polarizers are oriented in parallel (||) to the emission intensity when the emission polarizer is perpendicular to the excitation polarizer (⊥). This is reflected by the following formula where the anisotropy is given by *r*:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_T} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad \text{(eq. 1)}$$

Herein I_T refers to the total emission intensity. Through division with this value the anisotropy *r* becomes dimensionless and independent to fluctuations in total intensity, as reflected by equation 1.

Generally, two experimental setups can by utilized for fluorescence anisotropy measurements. In the L-configuration one detection system for emitted light is present. The measurement of horizontal and vertical polarized emission is carried out by rotation of a polarization filter in front of the emission detector (see Figure 2.2). In the T-conformation two detection systems are present, one of which measures the horizontally polarized emission and one the vertically polarized emission. The advantage of the latter setup is the possibility for fast data acquisition, most important during kinetic measurements.

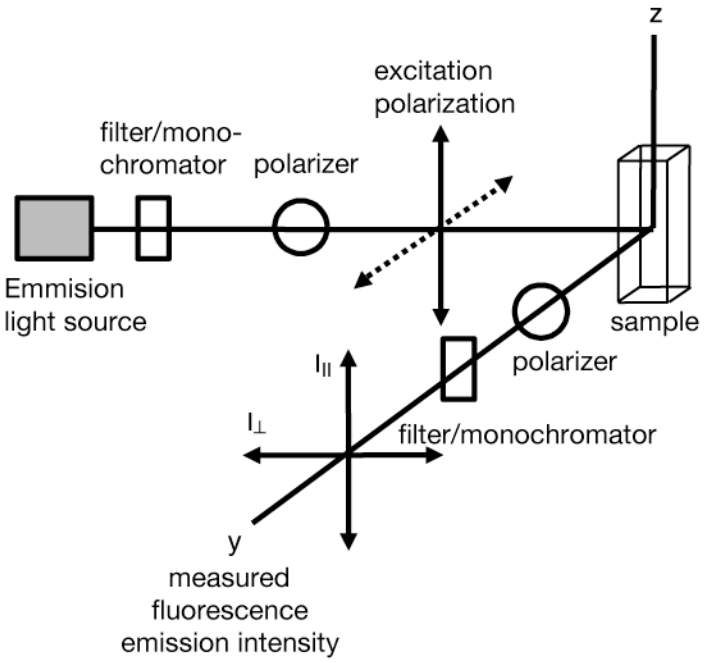

Figure 2.2 Schematic view of a single-channel fluorimeter. The excitation light is polarized vertically during measurements and horizontally (indicated by the dashed line) during determination of the G-factor. See text for details. Adapted from (Lakowicz, 1999).

The spectrofluorometer used for the presented study was a JASCO FP-6300 instrument (JASCO, Tokyo, Japan) which utilizes the L-configuration. For measurements with such a setup the anisotropy values have to be corrected by a factor dependent on the employed instrument and wavelength. This so called G-factor corrects for intrinsic differences in the detection of vertically and horizontally polarized light by the detection system. It is given by:

$$G = \frac{I_{HV}}{I_{HH}} \qquad \text{(eq. 2)}$$

HV refers to a state in which the excitation polarizer is set horizontally and the emission polarizer vertically. Analogously, *HH* describes a state in which both polarizers are set in the horizontal position. In the L-configuration horizontally polarized excitation light creates an emission that should be equal in intensity for all rotations of the emission polarizer – in a perfect detection system. Thus, a *G*-value that does not equal 1 allows for correction of instrument biases. The augmented equation 1 with *G*-factor correction in a single-channel fluorimeter can be written as follows:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \qquad \text{(eq. 3)}$$

A typical binding experiment can now be carried out by calculation of the fraction bound (f_B) of a fluorescent species in dependence on the present concentration of a ligand. This measurement of course is only conductible if the unbound species has a different anisotropy from the formed complex. The fraction bound can be expressed in this case by:

$$f_B = \frac{r - r_F}{r_B - r_F} \qquad \text{(eq. 4)}$$

The free and bound anisotropy contributions are given by r_F and r_B in the equation. The actual measured anisotropy is r. This formula is written in a form that assumes identical emission of complex and unbound species. Even though the fluorophores employed for anisotropy measurements are chosen to be environmentally insensitive, sometimes a correction for intensity changes upon binding has to be made. This can be achieved by expressing fraction bound as:

$$f_B = \frac{r - r_F}{(r - r_F) + R(r_B - r)} \qquad \text{(eq. 5)}$$

Here, the factor R corresponds to the ratio of total emission intensities between the free and bound forms, e.g. $R = I_{T,B}/I_{T,F}$.

2.2 Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) records intensity fluctuations due to diffusion of fluorescent particles through the focal volume. Through analysis of these fluctuations, information about size, association state and molecular compositions of particles can be obtained. Even though the method itself has been described around 30 years ago, its applicability for biologically relevant topics has been greatly enhanced in the last years due to the development of increasingly optimized microscope optics as well as superior photodetection methods (Weidemann et al., 2002). The method is not limited to *in vitro* analysis, but can provide valuable insights into *in vivo* processes as demonstrated by a growing number of studies (Briddon et al., 2004; Dauty and Verkman, 2005; Schmiedeberg et al., 2004; Wachsmuth et al., 2000; Weidemann et al., 2003).

The standard commercial FCS setup is a Confocal Laser Scanning Microscope (CLSM) with attached avalanche photodiodes for single molecule detection (see Fig. 2.3 A). The diffusion of fluorescent particles through the focus volume is analysed by recording the overall fluorescence signal over time. Measurements at short time intervals allow the precise detection of signal fluctuations due to particles entering and leaving the focus volume. As a limit the concentration of the fluorophore has to be low enough so that fluctuations due to single molecule events can be resolved. From these data an autocorrelation function can be derived that gives information about the mean time the fluorophores dwell through the focus. This function has the following form:

$$G(\tau) = \frac{< F(t)F(t+\tau) >}{< F(t) >^2} - 1 \qquad \text{(eq. 6)}$$

$G(\tau)$ describes the (self-)similarity of fluorescence signals separated by a time interval of τ (Haustein and Schwille, 2004). $F(t)$ and $F(t+\tau)$ are the fluorescence intensities at the respective times. Plotting the autocorrelation against τ yields a graph as depicted in Fig. 2.3 B,C. The y-axis intercept is inversely proportional to the average number of particles in the focus volume, e.g.:

$$G(0) = \frac{1}{< N >} \qquad \text{(eq. 7)}$$

where <N> is the average number of fluorophores in the focus volume (Maiti et al., 1997).

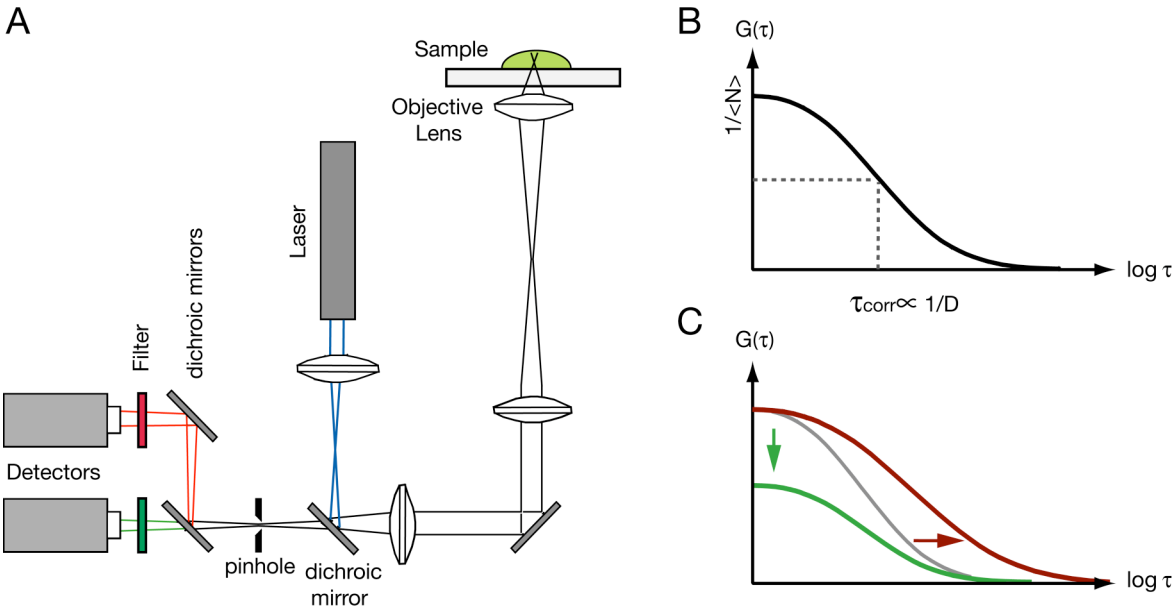

Figure 2.3 FCS setup and correlation functions. **(A)** Schematic setup of a commercial CLSM-FCS system. The excitation laser light is focused through a lens system into the sample. The fluorescence emission follows the same path backwards and is split from the laser light by a dichroic mirror. It is focused on a pinhole minimizing detection of light outside the vertical focus. The emission intensity is recorded by avalanche photodiode detectors. **(B)** Autocorrelation function plot. The correlation $G(\tau)$ is plotted against the lag time τ. The intercept with the Y-axis marks the inverse particle number. The indicated correlation time τ_{corr} equals the mean dwell time of fluorophores in the focus volume. **(C)** Influence of changes in diffusion properties on the correlation function. The green arrow and curve mark an increase in concentration. The red arrow and curve depict the effect of an increase in diffusion time.

The mean dwell-time of particles in the focus volume is shown by the width at half maximum of the autocorrelation graph, the so-called correlation time τ_{corr}. Slower particles appear in the plot with a larger correlation time, as these particles remain longer in the focus volume. For the assessment of experimental data the triplet states of the fluorophores have to be considered as these contribute to the autocorrelation function for small time scales (Bacia et al., 2006).

FCS is used to measure diffusion properties and parameters based thereon. However, for most binding reactions the method is limited by the fact that the binding of ligands creates rather insignificant changes in diffusion time. This is due to the fact that the diffusion time (τ_D) of a particle appears proportional to the cubic root of its mass (M):

$$\tau_D \propto M^{1/3} \qquad \text{(eq. 8)}$$

Thus, a homodimerization is accompanied by an 1.3 fold change in diffusion time (Bacia et al., 2006). The binding of a smaller ligands would be not resolvable.

An elegant method to circumvent this problem is the simultaneous detection of the emission from two spectrally different fluorophores (Schwille et al., 1997). If these fluorophores are attached to molecules that interact with each other, they will diffuse through the focus volume simultaneously. Thus, a parallel fluctuation of fluorescence intensity will be seen in both detection channels which is quantifiable independent of the change in diffusion times (see Fig. 2.4 B for experimental data). The detection of fluorescence correlation between more than one fluorophore is named fluorescence cross correlation spectroscopy (FCCS). It is analytically represented by:

$$G(\tau) = \frac{< F_g(t) F_r(t+\tau) >}{< F_g(t) >< F_r(t) >} - 1 \qquad \text{(eq. 9)}$$

Herein the subscripts g (green) and r (red) represent the time-dependent fluorescence intensities of the two detection channels. For a molecule being perfectly labelled with two distinct fluorophores the cross correlation could maximally reach a value of one. However, this value is experimentally not achieved, as labeling of biomolecules proceeds with a lower efficiency. For instance, an average 85% yield for DNA labeling reactions was observed in this study. Thus, the maximal cross-correlation for one double-labelled species decreases to roughly 72%. In addition, the overlap of both focus volumina is not perfect, with the red focus volume (e.g. the focus volume for higher wavelength detection) being bigger due to optical restraints. As result the experimentally maximal detection signal is about 65% cross-correlation. The minimal cross-correlation does not approach 0% for two separately labelled molecules that do not interact. This is due to crosstalk, e.g. emission of the green fluorophores that is detected in the red channel thereby creating a cross-correlated signal.

It has to be taken into account that the maximally reachable cross-correlation is also restricted by combinatorial aspects (Rippe, 2000; Weidemann et al., 2002). This can be exemplified by simple means for a homodimerizing species which was labelled to equivalent parts with red and green fluorophores. In this example three different complexes can be formed, only one of which shows cross correlation (Fig 2.4 A). These aspects have to be taken into close consideration if the binding of labelled ligands to a macromolecule carrying multiple binding sites is examined. Even though this characteristic of FCCS seems to be an experimental disadvantage, it can be used for the assessment of binding site numbers and association states (Rippe, 2000; Strohner et al.,

2005; Weidemann et al., 2002). This is due to the fact that the maximally reachable cross-correlation is dependent on the number of available binding sites.

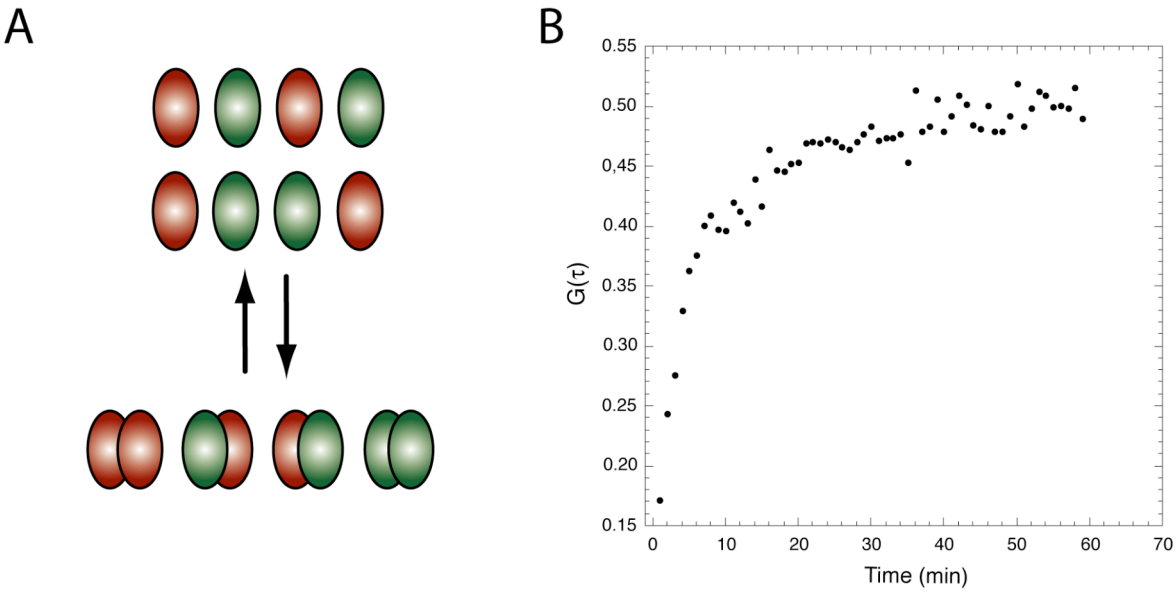

Figure 2.4 Dual color fluorescence cross correlation spectroscopy **(A)** Statistical considerations of FCCS. A dimerizing species labelled to equal parts with red and green dyes can form three complexes with different fluorescence properties, e.g. red-red, red-green, and green-green dimers are formed. As these occur with a distribution of 1:2:1, the maximal cross correlation is limited to 50%. **(B)** Demonstration of a kinetic measurement by FCCS. Here, the reaction of NAP1-bound histone octamer with 146 bp DNA was monitored. The H2A in the histone octamer was labelled with Alexa 488 and the DNA was 5' labelled with ROX. The increase in cross correlation reflects the transfer of the H2A from NAP1 to the DNA, and thereby the formation of nucleosomes and hexasomes could be monitored.

As mentioned above FCS and FCCS represent single-molecule techniques. Thus, the diffusion pattern of individual molecules can be assessed, enabling the discrimination between changes due to specific ligand binding and aggregation. The events can be distinguished, since aggregates appear as uncommonly slowly diffusing entities with high brightness. This is of central relevance to the assessment of complicated macromolecular assemblies prone to aggregation, like the chromatin remodeling complexes studied here.

3. Analytical Ultracentrifugation

3.1 Introduction

Analytical ultracentrifugation (AUC) constitutes a well-established biophysical technique for the investigation of hydrodynamic parameters of macromolecules. Indeed, it is one of the very few quantitative methods that allow for investigation of proteins and nucleic acids without modification of the samples and true in solution measurements. Even though AUC has been developed and used since the early half of the last century, major progress in instrumentation and computational analysis approaches has greatly enhanced its usability in recent years (Lebowitz et al., 2002; Schuster and Toedt, 1996). New technological innovations as the integration of fluorescence optics broaden the spectrum of application (Laue et al., 1997). The analytical centrifuge can be seen as an preparative ultracentrifuge equipped with a spectrophotometer, enabling real-time spectroscopic monitoring of sample sedimentation (see Fig. 3.1). Based on this instrumentation, two main experimental approaches can be distinguished, which are reflected as follows.

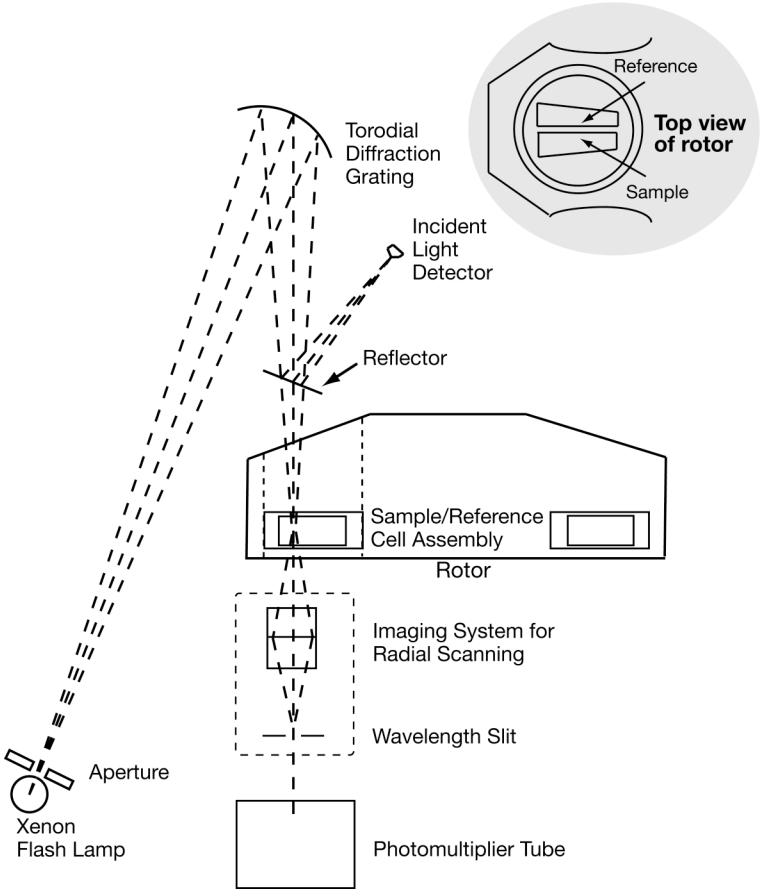

Figure 3.1 Schematic setup of an analytical ultracentrifuge. A xenon lamp briefly illuminates a sector of the sample cell when the cell passes the beam path for absorption detection. The wavelength slit is moved to different radial distances from the rotor center to derive an absorption profile for the sample and reference chambers. The incident light detector references for variations in light intensity. The inlet shows a top-view at a two-channel sample cell in the rotor. The reference cell is filled with buffer and its signal is subtracted from that of the sample cell. Adapted from (van Holde et al., 1998).

3.2 Sedimentation velocity

In sedimentation velocity experiments the macromolecule under analysis is centrifuged at high rotor speeds, leading to sedimentation of the sample to the bottom of the cell in typically a few hours. The sedimentation of a particle is dependent on its molecular weight and diffusion coefficient. Therefore parameters that rely on size and shape, such as (self-) association, conformational changes and aggregation can be quantified (Demeler and van Holde, 2004; Schuck, 2003). The sedimentation properties are summarized by the Svedberg coefficient (s), which can be expressed by:

$$s = \frac{M \cdot (1 - \bar{v} \cdot \rho)}{N_A \cdot f} = \frac{MD \cdot (1 - \bar{v} \cdot \rho)}{RT} = \frac{v}{\omega^2 \cdot r} \qquad \text{(eq. 10)}$$

In eq. 10 M reflects the molecular weight, D the diffusion coefficient, f the frictional coefficient and $\bar{v}$ the partial specific volume of the sample. The density of the solvent is given by ρ, N_A is the Avogadro number, T is the temperature and R is the universal gas constant. The observed radial velocity of the macromolecule is v, and r is its radial position. ω refers to the angular velocity of the rotor and the centrifugation field is given by $\omega^2 \cdot r$. The frictional coefficient f and the diffusion coefficient reflect the shape-dependent part of the svedberg coefficient. Often the frictional coefficient is given as f/f_0 in reference to a smooth sphere, which has the highest possible s-value for a given molecular weight (Lebowitz et al., 2002). It might be helpful to precalculate the frictional coefficient from am a given molecular structure, which can be done with the program HYDROPRO (Garcia de la Torre et al., 1994). The program fits a number of spheres into the molecular structure and thereby approximates the diffusion properties of the particle. This allows to analyse molecular weight distributions more precisely, as the shape parameters are omitted from fitting. Furthermore, the partial specific volume of the biomolecule as well as the viscosity and density of the medium can be computed from buffer composition and amino acid sequence with the program SEDNTERP by J. Philo, D. Hayes and T. Laue (www.jphilo.mailway.com/download.htm).

Two phenomena can be observed in a typical sedimentation velocity experiment. The boundary moves to the bottom of the cell in time-dependent manner due to sedimentation of the particle and it broadens over time due to back-diffusion. Different methodologies for analysing such data have been developed, such as van Holde-Weischet analysis and g*(s) analysis (Philo, 2000; van Holde and Weischet, 1978). The possibly most sophisticated and numerically most demanding approach is given by the c(s) analysis. The c(s) analysis is based on direct fitting to the Lamm-equation, which describes the concentration distribution of a species in dependence on the run time (t) (Lamm, 1929; Schuck, 2003):

$$\frac{\partial c}{\partial t} = -\frac{1}{r}\left(\frac{\partial}{\partial r}\left(s\omega^2 r^2 c - Dr\frac{\partial c}{\partial r}\right)\right) \qquad \text{(eq. 11)}$$

The experimental data *a(r,t)* is fitted with least-squares algorithms to a large set of these equations with incremental variations between the s-values:

$$a(r,t) \cong \int_{s\min}^{s\max} c(s)\chi(s, D(s), r, t)ds \qquad \text{(eq. 12)}$$

Herin $\chi(s,D(s),r,t)$ is the concentration distribution of one single species with a certain s coefficent given from eq. 10 (Schuck, 2003). From this a global concentration distribution is derived for a range of sedimentation coefficients. An example is given in (Fig. 3.2 A). If the frictional coefficients are well defined, such distributions can be converted to c(M) distributions which depend on molecular weight.

3.3 Sedimentation equilibrium

In sedimentation equilibrium experiments the sample is centrifuged at moderate angular rotor speed. An exponential concentration gradient of the sample forms in the centrifugation cell parallel to the radial rotor axis. The gradient relies on the balance of diffusion and sedimentation transport and reflects a time-independent equilibrium between both, where the net transport is zero. The concentration distribution is solely dependent on weight of the particle and can thereby be used for the precise determination of complex compositions (Laue and Stafford, 1999). For the case of *n* multiple species the equilibrium distribution can be described as a sum of exponentials (Lebowitz et al., 2002):

$$A_r = \sum_n A_0 \cdot \exp\left[\frac{M_n(1-\bar{v}_n\rho)\omega^2}{2RT}(r^2 - {r_0}^2)\right] + \delta \qquad \text{(eq. 13)}$$

A_r reflects the absorption at a radial distance r from the rotor center, A_0 is the absorption at a reference distance. δ represents a baseline offset for example due to buffer absorption. The data can easily be fitted to diverse models with different complexity such as self- and heteroassociation. For one discrete species the function can simply be transformed to a linear form, allowing the determination of the component's molecular weight from the slope (Schuster and Toedt, 1996). Current software packages such as Ultrascan (www.ultrascan.uthscsa.edu) can globally fit multiple datasets acquired at different speeds

and concentrations (Fig. 3.2 B). In this way not only molecular weights, but also association constants can be derived.

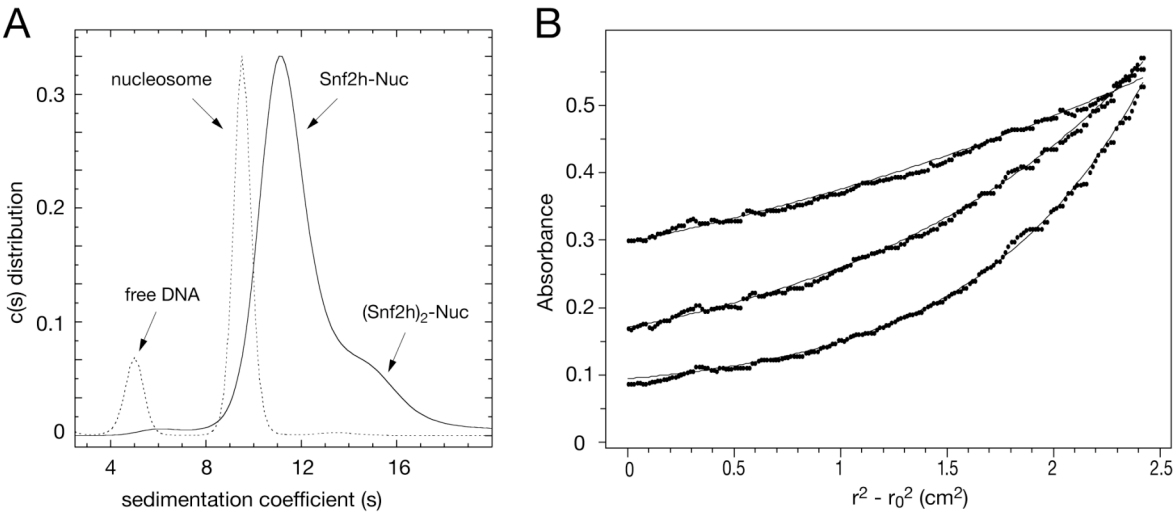

Fig 3.2 Sedimentation velocity and equilibrium analysis. **(A)** Two c(s) distributions are shown. The dotted line shows the distribution for salt reconstituted mononucleosomes. The data were collected at 260 nm where the DNA absorption is dominant over protein absorption. Two peaks can be discriminated, which correspond to free DNA and the nucleosome particle. The solid line shows a c(s) distribution of mononucleosomes that were incubated with the ISWI class remodeler SNF2h. The formation of a stoichiometric SNF2h-nucleosome complex and a higher-order form most likely corresponding to a species where two remodeler bind one nucleosome are seen. **(B)** Concentration profile of HP1 from a sedimentation equilibrium experiment. The recombinant protein was centrifuged at speeds of 10000, 15000 and 20000 rpm and the respective curves are shown. The solid lines represent a global fit to a one-component model with a fitted molecular weight of ~ 49 kDa. This value is in very good agreement with a dimeric state of the 24 kDa protein. As no improvement of the fit was seen, when fitting was conducted with a monomer-dimer equilibrium model, the dissociation constant for the self-association appears well below the employed protein concentration of 14 μM.

Summary

Chromatin assembly and remodeling resemble the two elementary processes of chromatin formation. The presented work aimed at the investigation of the underlying mechanisms. The conducted experiments center around the histone chaperone NAP1 and the chromatin remodeling complex ACF, both involved in the respective *in vivo* processes.

The role of NAP1 as histone carrier and assembly factor was examined in a set of *in vitro* experiments. Using fluorescently labeled histones, it was shown that the histone chaperone can specifically interact with H2A·H2B, H3·H4 as well as H1 with a stoichiometry of one NAP1 dimer per one of the mentioned histone entities. The specified relationship was used to set up a well defined model system that allowed to monitor nucleosome assembly. It was demonstrated that the process follows a clear stepwise mechanism that yields the nucleosomal structure, possibly augmented by a linker histone. Intriguingly, the process includes a previously poorly characterized intermediate step – the formation of an hexasome. This structure is a nucleosome that lacks one of the two H2A·H2B dimers, and has been proposed to be involved in transcription processes. Unlike the nucleosome structure, the hexasome appears more stable towards removal of its H2A·H2B dimer by histone chaperones or ribonucleotides, pointing at a reorganized histone-DNA complex. Comparison with the histone H2A.Z showed that the variant containing nucleosomes were assembled mechanistically similar, yet with an enhanced stability.

The effect of NAP1 on pre-existing nucleosomes and chromatin fibers was investigated. It was found that both structures can be at least partially disassembled in a concentration dependent manner by the chaperone, however to different extends. Whereas the fiber is only affected by its linker histone content, a single nucleosome can be disassembled, with the dominant intermediate being an hexasome particle.

The interplay between the actions of histone chaperone NAP1 and an ISWI-class chromatin remodeler was examined. It was shown that for the combination of NAP1 and ACF both processes constitute separate activities that however are dependent from each other.

Comparison with *in vivo* chromatin formation showed that the transfer of histones onto accessible DNA per se would not limit the kinetics of the assembly process. Thus, it appears that the limiting step in chromatin formation might be the generation of accessible DNA for the assembly of nucleosomes. In this regard chromatin formation could be restricted by the activity of chromatin remodeling machineries which render irregularly deposited nucleosomes into regular higher-order structures, thus allowing higher compactation and enhanced deposition of nucleosomes.

Zusammenfassung

Chromatin Assemblierung und Remodellierung sind die beiden grundlegenden Prozesse der Chromatinbildung. Die hier dargelegte Arbeit zielt auf eine Erforschung der dabei wirkenden Mechanismen. Die durchgeführten Experimente waren auf das Histonchaperon NAP1 und den Chromatin Remodellierungskomplex ACF fokussiert, die beide in den entsprechenden *in vivo* Prozessen eingebunden sind.

Die Rolle von NAP1 als Histontransporter und Assemblierungsfaktor wurde in einer Reihe von *in vitro* Experimenten untersucht. Mittels fluoreszenzmarkierter Histone konnte gezeigt werden, dass NAP1 mit H2A·H2B, H3·H4 und H1 reagiert, jeweils mit einer Stöchiometrie von einem NAP1 Dimer pro Histoneinheit. Dieses Verhältnis wurde in der Entwicklung eines Modellsystems eingesetzt, welches es erlaubt die Nukleosomassemblierung zu verfolgen. Es konnte gezeigt werden, dass der Prozess einem schrittweisen Mechanismus folgt, an dessen Ende das möglicherweise um ein Linkerhiston erweiterte Nukleosom steht.

Interessanterweise enthält der Prozess ein bislang nur ungenügend charakterisiertes Intermediat, das Hexasom. Diese Struktur ist ein Nukleosom, welchem eines der beiden H2A·H2B Dimere fehlt. Es wurde bereits vorgeschlagen, dass diese Form eine Rolle in der Transkription spielt. Im Gegensatz zum Nukleosom scheint das Hexasom stabiler in Bezug auf die Entfernung des H2A·H2B Dimers durch ein Histonchaperon oder Ribonukleotide zu sein. Dieses weist auf eine Reorganisation des Histon-DNA Komplexes hin. Experimente mit der Histonvariante H2A.Z zeigten, dass Nukleosomen, die diese Variante enthalten mechanistisch ähnlich assembliert werden. Jedoch scheint die Stabilität des entstehenden Komplexes erhöht.

Der Effekt von NAP1 auf bereits bestehende Nukleosomen und Chromatin Fibern wurde untersucht. Beide Strukturen können zumindest teilweise in einer konzentrationsabhängigen Weise durch das Chaperon dissassembliert werden. Jedoch ist das Ausmass der Dissassemblierung für beide Strukturen unterschiedlich. Die Fibern werden nur in ihrem Anteil an Linkerhiston variiert, während ein isoliertes Nukleosom abgebaut werden kann, wobei das dominante Intermediat das Hexasom Partikel ist.

Das Zusammenspiel zwischen Histonchaperon NAP1 und einem Chromatin Remodeller der ISWI Klasse wurde untersucht. Es wurde gefunden, dass die Aktivität des ACF Komplexes den Transfer einzelner Histone durch das Chaperon nicht direkt beeinflusst. Der Vergleich mit Daten der *in vivo* Chromatinbildung zeigte, das der Transfer von Histonen auf zugängliche DNS nicht der limitierende Schritt im Bildungsprozess sein kann. Vielmehr scheint es, als ob die Generierung freier Bindungsstellen auf der DNS

limitierend ist. Die Chromatinbildung könnte durch die Aktivität der Chromatin Remodellierungskomplexe begrenzt sein, welche die unregelmäßig abgesetzten Nukleosomen auf der DNS anordnen. Dadurch wird eine höhere Kompaktierung und gleichzeitig das Absetzen von mehr Nukleosomen durch die Chaperone ermöglicht.

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