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Modulation of chromatin conformation by linker histone H1 and histone chaperone NAP1

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Publications

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“Complete the motion if you stumble“

(RHCP)

Table of content

1. Chromatin organization and dynamics

1.1 Chromatin conformation regulates cell function

Nearly every cell of an individual organism carries the full DNA sequence information. Differentiation into one of the numerous specialized cells within an eukaryotic organism is accomplished by translating only a certain subset of genes. The genetic information is translated from the DNA to proteins. Thus, the cells in the nervous system, the epidermal tissue or in the liver differ not in their DNA sequence composition but in the spatial and temporal divergent gene expression patterns during embryogenesis and development. Fully differentiated cells maintain a distinct and well defined gene expression pattern that assures the survival of the organism. Disruption of the underlying regulatory network can result in severe diseases. Malignant cancer tissues divide continuously and are capable of invading other healthy organs.

One of the regulative elements that ensure proper gene expression is the dynamic organization of the DNA into the so called chromatin within the cell nucleus. Chromatin is constituted by the DNA in the cell nucleus as well as its associated proteins (van Holde, 1989). Its composition and structure is highly dynamic and reflects the functional differences in biological activity. On microscopic images different chromatin conformations are visible. Highly condensed regions of chromatin persist during all cell cycle stages whereas other chromatin regions become decondensed during interphase (Craig, 2005; Cremer and Cremer, 2001). These compacted chromatin segments referred to as heterochromatin, are established and maintained by a variety of enzymatic and structural factors. For example genes remaining silent in the nervous system can be shut down by the dense compaction of the encoding DNA sequences into heterochromatin, whereas transcriptionally active genes are more often found in the more decondensed chromatin environment called euchromatin (Gilbert et al., 2004). The different chromatin environments can be interchangeable and are then termed facultative hetero- and euchromatin. If specific chromatin segments are persistently condensed, which often occurs at centromers and telomers (the midst and ends of the chromosomes) these domains are termed constitutive heterochromatin, often characterized by highly repetitive DNA elements.

However, not only gene expression is regulated by different chromatin structures. The compaction of the chromatin into the mitotic chromosomes for example, a highly condensed state during mitosis, is a prerequisite for the correct segregation of the DNA into the two daughter cells. Specialized chromatin structures are involved in the

interaction of the chromosomes with the spindle apparatus during mitosis and direct specific proteins to form the correct attachments. The compaction state of gene encoding DNA sequences is regulated by a wide variety of protein factors. The mechanism by which these factors alter chromatin conformation and thereby influence gene expression is therefore of fundamental interest. Before evaluating the changes in chromatin conformation it is inevitably necessary to understand the main principles that determine chromatin structure and govern the dynamic properties of chromatin organization.

1.1.1 The organization of DNA into chromatin

The fundamental step in condensing DNA into chromatin is achieved by the formation of the nucleosome. This complex consists of ~147 bp of DNA that are wound about ~1.7 times around an octameric histone protein complex (Germond et al., 1976; Kornberg, 1974). Two copies of the histone proteins H2A, H2B, H3 and H4 form this octameric core. The nucleosome is the main building block of chromatin and almost all DNA in the cell nucleus is associated with core histones into a chain of nucleosomes. An additional fifth histone protein, the linker histone, binds to the nucleosome at the DNA entry-exit sites, forms the chromatosome unit and thereby contributes to the formation of higher order chromatin structure (Bednar et al., 1998; Bellard et al., 1976; Carruthers et al., 1998; Kepert et al., 2003; Kepert et al., submitted; Thoma et al., 1979). While the four basic histones are highly conserved among organisms the linker histones form a more heterogeneous protein family (Cole, 1984).The core nucleosome complex has been resolved in high resolution x-ray studies and the path of the DNA around the histone octamer is well known (Arents et al., 1991; Harp et al., 2000; Harp et al., 1996; Luger et al., 1997a). However the exact binding sites for the linker histones and the further condensation of the nucleosomal chain are still controversial and unresolved (Travers, 1999; Vignali and Workman, 1998).

From microscopic observations it was concluded that the chain of nucleosomes folds back into a fiber like structure with an apparent diameter of 30 nm (Felsenfeld and McGhee, 1986; Finch and Klug, 1976; McGhee et al., 1983; Thoma et al., 1979). The exact structure of this fiber is not known in detail and different models exist that mainly differ in the geometry and path of the DNA between the nucleosomes (Fig. 1.1) (Finch and Klug, 1976; Williams et al., 1986; Woodcock et al., 1993). Recent electron microscopy data hint at a zig-zag model in which the linker DNA is straight (Dorigo et al., 2004). In addition, a crystal structure of a tetranucleosome has been published recently, showing straight linker DNA, indicative of a fiber geometry similar to the zig-zag model (Schalch et al., 2005). The 30 nm fiber folds back into higher-order structures with a diameter of 100-200 nm as

proposed by the chromonema model (Belmont and Bruce, 1994; Sedat and Manuelidis, 1978)[Horn, 2002 #8997. The organization in the nucleus further condenses the chromatin. At its highest condensation state during mitosis the DNA is then compacted by a factor of ~10000.

The transitions in chromatin conformation, being catalyzed by a wide variety of protein factors with significant differences in function and complexity, are tightly regulated. They range from the small chromatin binding HMG-proteins (high mobility group-proteins) to larger structural determinants as MeCP and HP1 (methyl CpG binding protein, and heterochromatin protein) and multi-component chromatin remodelling machineries with molecular weights in the mega dalton range [Bustin, 1996 #4965](Georgel et al., 2003; Wade et al., 1998). These factors can change the protein composition of chromatin and lead to conformation changes of the chromatin fiber (Becker, 2005; Langst and Becker, 2004). The rearrangements are also inevitable during processes like replication, DNA repair and transcription, as shown for the RNA polymerase, which has to transcribe through a nucleosomal template (Belotserkovskaya et al., 2003; Studitsky et al., 2004). Some of the underlying modifications and factors involved in these processes will be discussed in the following in relation with their putative involvement in gene expression.

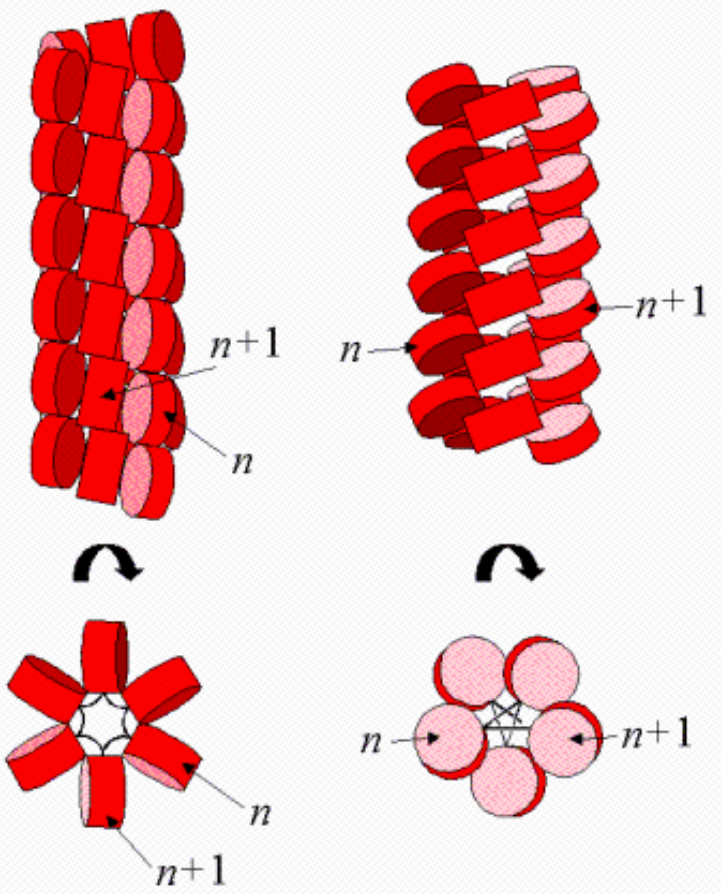

Figure 1.1 Two different models for the 30 nm chromatin fiber. (A) Schematic illustration of a 30 nm fiber according to the solenoid model. The chain of nucleosomes forms a regular helix in which successive nucleosomes interact with each other. The linker DNA between nucleosomes is tightly bent. (B) Fiber organization after the zig-zag model. The resulting fiber resembles the fiber structure from the solenoid model. Instead the geometry is very different. The linker DNA is straight and successive nucleosomes do not interact. From (Schiessel et al., 2001).

1.1.2 The Linker histones

Linker histones are not related in their sequence to core histones and many isoforms exist (Harvey and Downs, 2004; Kasinsky et al., 2001). In human for example seven linker histones have been described. However, most of the linker histones of higher eukaryotes show a similar tripartite structural composition (Harvey and Downs, 2004). The common motifs are highly positively charged N- and C-terminal domains which flank a compact globular domain. These N- and C-terminal domains are rich in the amino acids lysine and arginine. They are believed to be mainly unstructured in solution while the globular domain has been crystallized and analyzed in high resolution x-ray studies (Ramakrishnan et al., 1993). The structural data and in vitro DNA binding studies hint at the presence of two DNA binding sites at the globular domain (Goytisolo et al., 1996; Varga-Weisz et al., 1994). This domain comprises a winged helix domain, which is found in a number of DNA binding proteins (Gajiwala and Burley, 2000). The C-terminal domain has also been proposed to carry a DNA binding motif (Mamoon et al., 2002; Vila et al., 2000). Micrococcus nuclease digestions on chromatin templates show that linker histones are capable of protecting 20 bp of DNA additional to the 146 bp in the nucleosome core complex (Noll and Kornberg, 1977; Simpson, 1978). These additional 20 bp appeared to be asymmetrically protected in a study with the 5 S RNA sequence of *Xenopus laevis* and *Lytechinus variegatus* (An et al., 1998; Hayes and Wolffe, 1993). Roughly five base pairs on one side and 15 one the other were protected against Mnase digestion. But this asymmetric protection pattern was suspected to arise from differences on the 5` and 3` flanking sequences in the templates or be due to a translational shift of the nucleosome position upon linker histone binding. Thus, the occurrence of symmetric protection could not be ruled out completely and might depend on the flanking DNA sequences. The binding position at the nucleosomal surface and the exact interactions of the linker histone with the nucleosomal DNA has also been challenged in several studies. These studies conclude in divergent models concerning the position and the DNA contacts formed by the linker histone and are reviewed in the following reviews (Crane-Robinson, 1997; Travers, 1999; Vignali and Workman, 1998).

One of the key differences in these models is the rather symmetric or asymmetric binding of the linker histone at the nucleosome core (which is not necessarily correlated with the symmetric or asymmetric DNA protection pattern, see also Figure 1.2). Previous analysis with the 5 S RNA gene proposed an asymmetric binding pattern, formed on two translationally different positions of the nucleosome complex on the studied nucleotide sequence (Hayes, 1996; Pruss et al., 1996). Studies of bulk sequences and a detailed analysis on the translational nucleosome positions on the 5 S rDNA argue rather for a

symmetric position of the linker histones (Panetta et al., 1998; Zhou et al., 1998). The linker histone and its binding properties to free DNA duplexes along with the implications in chromatosome structure were therefore investigated (Kepert et al., submitted). In this study an atomic model for the chromatosome has been build with molecular modeling techniques, based on the recently published x-ray structure of a tetranucleosome (Schalch et al., 2005).

The contribution of linker histones in maintaining higher-order chromatin structure has been investigated in detail (Bednar et al., 1998; Carruthers et al., 1998; Huang and Cole, 1984; Leuba et al., 1998). Since not a complete compaction of chromatin templates without linker histones could be accomplished it has been concluded that linker histones maintain a higher-order conformation and facilitate the compaction of the chromatin fiber (Ausio, 2000b; Carruthers et al., 1998; Leuba et al., 1998). The implications of linker histones on gene expression have also been investigated in several studies. It has been shown that over-expression of the histone H1.0 in cultured mouse cells reduced steady-state transcription of nearly all Pol-II dependant genes (Zlatanova and Doenecke, 1994). Binding of linker histones to the nucleosomes has been shown to inhibit in vitro transcription of a Pol III transcribed gene in vitro (Hayes et al., 1996; Ura et al., 1996). Generally linker histones are considered as closing factors that repress transcription (Zlatanova et al., 2000).

The mechanisms by which linker histone content modulates transcriptional activity of specific genes might differ from case to case. The idea that condensing the chromatin per se leads to specific gene repression is likely not to be true. It has been shown for some genes that transcription factors could displace linker histones from enhancer/promotors of specific genes thereby stimulating transcription (Bresnick et al., 1992; Cirillo et al., 1998; Kermekchiev et al., 1997; Lee and Archer, 1998). One of the most intensively studied model system again are the oocytic and somatic 5 S RNA genes. Both coding sequences comprise 120 bp and differ only in seven base pairs. The 5’ and 3’ flanking sequences lead to different nucleosome positioning signals resulting in changed expression patterns (Panetta et al., 1998). The two genes require TFIIIA binding for expression. In the somatic gene the binding sites for TFIIIA is mainly exposed at the outside of the nucleosome while most of the oocytic nucleosome positions bury the TFIIIA binding site in the nucleosome. Additionally in the somatic version the TFIIIA binding site overlaps with the linker histone binding site and TFIIIA binding displaces H1 resulting in insensitivity to linker histone dependant repression. In contrast, in the oocytic gene the TFIIIA binding sites do not exclude H1 binding and therefore can be repressed by linker histone binding.

Recently a new group of highly abundant proteins in the nucleus, the histone chaperones, have been implicated with linker histone displacement from chromatin, which was also

investigated in my PhD work (Kepert et al., 2005; Ramos et al., 2005; Shintomi et al., 2005). It has been proposed that linker histones bound to the nucleosome abolish binding of factors needed for transcription initiation. Nucleosomes depleted of linker histones display a temporally unpeeling of DNA, whereby transcription factors can gain access to their target sequences (Mizuguchi et al., 2004; Yang et al., 1994). The decondensation of the chromatin fiber by linker histone displacement may further facilitate the access of the transcription machineries to the nucleosomes during transcription elongation. Consequently, factors that bind more tightly to the nucleosome as the linker histones and induce a chromatin condensation could act as transcriptional repressors. Posttranslational modifications of linker histones, as phosphorylation and ADP-ribosylation have been reported and extend the possibilities of regulation (Dou et al., 2002; Sweet et al., 1997; Sweet et al., 1996).

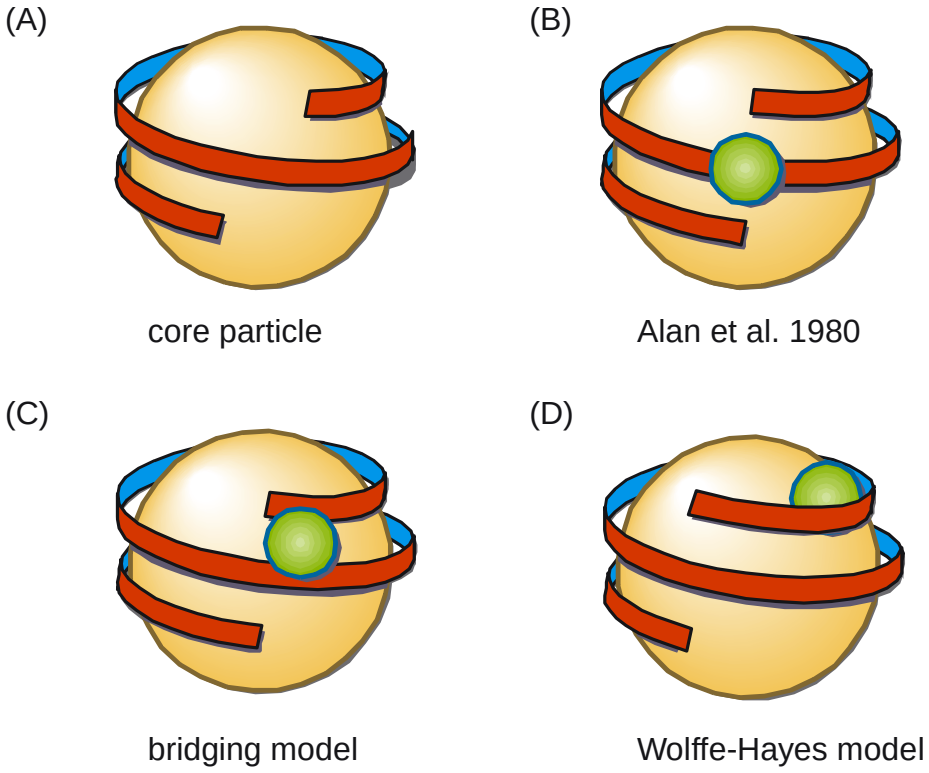

Figure 1.2 Different models for linker histone position at the nucleosome. (A) Nucleosome core particle, detailed known from x-ray studies. (B) Symmetric binding of the globular domain of the linker histone at the inner DNA gyre in the nucleosome. The protein contacts only one DNA duplex (Allan et al., 1980). (C) A model similar to the Alan model but the protein contacts two DNA strands at the entry/exit site (Lambert et al., 1991). (D) The Wolffe-Hayes model that proposes the asymmetric binding of the globular domain. These results stem form the analysis with the 5 S RNA positioning sequence (Pruss et al., 1996) (see Text for detailed overview) (Scheme adopted from Travers et al. (Travers, 1999)).

1.1.3 Histone variants

Another mechanism in regulating chromatin conformation arises from the possibility to exchange the core histone proteins against the so-called histone variants which include H2A.X, H2A.Z, macroH2A, H2A-Bbd, H2AvD, H3.3 and CENP-A (centromer protein A) (Henikoff and Ahmad, 2005; Kamakaka and Biggins, 2005). These histone variants can be incorporated both in a replication dependant and independent pathway and contribute to the epigenetic histone code, which is inherited to ancestor cells. Up to date mostly variants for the histones H2A and H3 were found. Since the two H2A histones (and the two H3 histones) contact each other in the nucleosome this specificity in variance could be a mechanism to impede chimerical nucleosome formation (Suto et al., 2000).

The histone variants for H2A differ mainly in sequence and length of their C-terminal tails. Some variants are restricted to individual chromosomes, as macroH2A to the inactive x-chromosome and H2A-Bbd to the active one (Chadwick and Willard, 2001; Costanzi and Pehrson, 1998). H2A.X and H2A.Z are constitutively expressed and found throughout the whole genome (Kamakaka and Biggins, 2005). There exist reports about both transcriptional repression and activation by the variant H2A.Z (Farris et al., 2005; Larochelle and Gaudreau, 2003). It also seems to interact with HP-1 linking it to heterochromatin formation as well as it has been implicated in formation of boundary elements that impede facultative heterochromatin spreading throughout the chromosome (Meneghini et al., 2003; Rangasamy et al., 2004).

For the histone H3 the main variants are H3.3 and CENP-A. The incorporation of histone H3.3, which differs only in four AA from the main variant, seems to activate transcription (Ahmad and Henikoff, 2002; Janicki et al., 2004). CENP-A is involved in establishing proper centromer formation. It has been proposed as an epigenetic marker that directs other factors essential for kinetochor formation (Collins et al., 2005; Sullivan, 2001; Sullivan et al., 1994; Van Hooser et al., 2001). However CENP-A alone seems not be sufficient in centromer assembly.

These examples show that incorporation of histone variants appear to be an important determinant of the chromatin conformation. The histone variants can recruit other factors that facilitate structural rearrangements leading to different functions. The structure for some of such altered nucleosomes has been now studied and their influence on chromatin folding is being investigated. For the histone variant H2A.Z it has been shown that compact fiber folding is stabilized, but intermolecular contacts that probably enhance further folding are decreased (Fan et al., 2004). The assembly reaction of H2A.Z containing mononucleosomes and their stability seems to be essentially the same as for the canonical ones (Mazurkiewicz et al., submitted). Thus, it remains to be established for

other histone variants whether incorporation per se leads to an altered chromatin structure and if fibers consisting with nucleosome containing core and variant histones behave similar. Additionally it will be of interest to identify specific exchange complexes that catalyze the substitution of core histones with histone variants (Mizuguchi et al., 2004).

1.1.4 Histone modifications

The chemical modifications of histones have been observed back in the mid 1960s but the identification of specific enzymes catalyzing these changes and their implications in gene regulation has only begun to be discovered years later (Allfrey et al., 1964; Brownell and Allis, 1996). Up to now enzymes have been characterized that catalyze the acetylation, phosporylation, methylation, ubiquitination and ADP-ribosylation of histones and thereby regulating transcription, replication and DNA repair (Cheung et al., 2000; Fischle et al., 2003; Grunstein, 1997; Zhang and Reinberg, 2001). Numerous amino acid residues in the core histone proteins have been identified that are the targets for different chemical modifications. The acetylation occurs at specific lysine residues, lysine and arginines are being methylated, threonine and serine get phosphorylated and distinct lysine residues can be ubiquitinated. The combinatory modifications at different histone tails have at least in part overlapping, redundant and complementary effects (Turner, 2002).

Histone acetylation occurs at lysine and arginine residues in all core histone tails. The acetylation of specific residues in H3 and H4 have been associated with transcription and highly transcribed chromatin is found to be enriched in hyperacetylated histones (Braunstein et al., 1993; Grunstein, 1997). Consistent with these findings enzymes catalyzing the acetylation act generally as transcriptional activators whereas histone deacetylases function in the opposite manner. The inhibition of histone deacetylation in vivo changes chromatin structure and increases the chromatin accessibility (Fejes Tóth et al., 2004; Görisch et al., 2005).

Histone methylation has been found to have different effects on gene expression. The histone tail of H3 can be methylated at lys 4 or lys 9. The methylation of H3K9 is correlated with gene repression while the methylation at H3K4 activates transcription (Richards and Elgin, 2002; Zhang and Reinberg, 2001). The lysine residues can become either mono-, di- or trimethylated. It could be shown that dimethylated H3K4 is found more or less uniformly throughout the genome while trimethylated H3K4 is enriched in the 5’ end regions of transcribed genes (Ng et al., 2003). The methylated H3K9 functions as an anchor and binds HP-1 and thereby facilitates chromatin condensation (Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002). In embryonic stem cells the trimethylated H3K9 is enriched in pericentromeric heterochromatin and affects HP-1 localization (Peters et al., 2003). Interestingly methylation at K4 prevents deacetylation and methylation of K9

indicating an inhibitory interplay between these two lysine residues in the same histone tail (Zegerman et al., 2002). Moreover, the interplay of histone methylation becomes more complex since it has been shown that methylation at H3K4/9 and H4K20 in combination recruits the activator BRAHMA and inhibits binding of repressors like HP-1 for example (Beisel et al., 2002). Hence, it seems that methylation of H3K9 in combination with other modifications might not be a marker for gene silencing but for gene activation.
Besides this, there also exist further interactions between different chemical core histone modifications. The C-terminal domain of H2B gets ubiquitinated at K123 and seems to be essential for the methylation of H3K4 and 79 (Sun and Allis, 2002). Since nucleosomes are not found to be ubiquitinated and methylated extensively at the same time, the interplay between the two modifications is up-to-date unclear. The differences in the modification state could arise from different turn-over rates, as histone methylation is believed to be persistent for longer times (Zhang and Reinberg, 2001). Another explanation may be that H2B ubiquitination decondenses a longer stretch of chromatin thereby increasing accessibility for H3K4 methylation enzymes.
An interesting exception of histone modification is the methylation of H3K79, which is a modification site not in the histone tail but in the histone fold domain. This residue is located at the surface of the octamer complex, thus being accessible in the nucleosome. Over 90% of wild-type H3K79 is methylated which occurs in a H2BK123 ubiquitination dependant fashion (Briggs et al., 2002; van Leeuwen et al., 2002). However, it remains elusive how H3K79 methylation might act in chromatin condensation and gene silencing as such a huge extent is methylated.
Another modification, histone phosphorylation seems to play a role in chromosome condensation and segregation. The histone tail of H3 gets phosphorylated at Ser10 during chromosome condensation although this seems not to be the essential step. Again, the phosphorylation of H3S10 impedes methylation at H3K9 and vice versa, indicating interplay between both. As mentioned above it is also possible that the histone modifications act synergistically and a whole subset of modifications is needed to accomplish full activation or repression. The presented processes get even more complicated as the histone modifying enzymes can have additional targets and may exert multiple functions in different pathways and cellular compartments (Schreiber and Bernstein, 2002).

1.1.5 Other chromosomal proteins

Besides the diverse enzymes that mediate the covalent modification of core histones other factors bind to chromatin and influence the conformation of the fiber. Among these factors are HMG-proteins, MeCP2, HP-1 and others. The small HMG-proteins are very abundant

factors in the cell and act antagonistic to linker histones (Bustin, 2001; Zlatanova and van Holde, 1998). Despite the fact that their binding sites at the nucleosome seem not to be the same as the ones for the linker histones, HMG-protein and linker histone binding is exclusive (Alfonso et al., 1994). If HMG-proteins displace linker histones from the nucleosome they propagate a more open chromatin conformation (Catez et al., 2002).

MeCP2 has been found to function in two ways on chromatin conformation (one enzymatic and one structural). The protein consists of two functional domains. One is a methyl-CpG-binding domain (MBD) and the other confers transcriptional repression (TRD) (Hendrich and Bird, 1998; Nan et al., 1993). Highly methylated CpG-islands are found in gene promotors and enhancer elements (Bird and Wolffe, 1999). It was previously shown that MeCP2 binds methylated DNA and is enriched in heterochromatin foci and pericentromeric heterochromatin (Nan et al., 1997). In addition, MeCP2 has been shown to interact with histone methylases and deacetylases via the TRD domain linking it to transcription repression (Jones et al., 1998; Nan et al., 1998). Recently, it was shown that MeCP2 is also capable of binding to unmethylated DNA and chromatin templates. Using analytical ultracentrifugation and electron microscopy it was demonstrated that MeCP2 condenses a defined chromatin template (Georgel et al., 2003). This condensed structure which is formed also under low salt conditions seems to exhibit a special conformation maintained by additional MeCP2-nucleosome or MeCP2-MeCP2 contacts. Even cleavage in the linker DNA did not resolve the condensed chromatin conformation. By comparing the methyl-CpG-binding domain with sequence databases four proteins with closely related domains have been identified (MBDs 1-4) (Hendrich and Bird, 1998). MBD 3 resides in the Mi2/NuRD deacetylase complex, one of the most abundant macromolecular complexes found in Xenonus eggs and mammalian cells (Tyler and Kadonaga, 1999).

Besides MeCP2, other factors have been identified such as heterochromatin binding protein (HP) or polycomb proteins that bind to methylated histone tails and promote heterochromatin formation. In human three isoforms HP1-alpha, beta and gamma have been discovered. HP1 comprises a chromodomain (CD) and a chromo-shadow domain (CSD) (Lechner et al., 2005). The chromodomain has been shown to interact specifically with methylated H3K9 residues (Bannister et al., 2001; Lachner et al., 2001) (Nakayama et al., 2001). HP1 may therefore bind to one of the epigenetic markers that specify gene repression and propagate heterochromatin formation. This evokes a general mechanism by which gene expression is repressed through factors that bind to methylated DNA sequences recruit an enzymatic machinery and induce chromatin conformation rearrangements.

Additional factors that modulate chromatin conformation are small RNA transcripts, the so-called RNAi molecules, which are also involved in the maintenance of heterochromatic structures. The Xist-RNA is one of the key regulators of x-chromosome inactivation in female mammals (Okamoto et al., 2004). It coats the chromosome and triggers gene silencing in cis.

1.2 Chromatin assembly/remodeling

1.2.1 Histone chaperones

The assembly of nucleosomes from DNA and core histones is strongly dependant on chaperone proteins since solely mixing of the components at physiological salt concentrations in vitro leads to the formation of insoluble protein-DNA aggregates (Daban and Cantor, 1982a; Daban and Cantor, 1982b). Thus the histones have to be kept in the cell nucleus tightly associated with histone chaperones preventing spontaneous and premature association with chromatin. The histone chaperones are proteins that guide the assembly of chromatin in an enzymatic manner as they facilitate the reaction without being part of the final product. Unlike “real” enzymes they do not act in catalytic amounts but are very abundant proteins in the cell. They comprise a protein family of different factors with distinct functions in vivo (Loyola and Almouzni, 2004).

Table 1.1 summarises known histone chaperones and their putative functions in the cell. One of the first characterized was nucleoplasmin which was mainly studied in the Xenopus oocyte nucleus (Dutta et al., 2001; Laskey et al., 1978). It is associated with the histone H2A·H2B dimer and allows the progressive release of histones after fertilization, thereby ensuring nucleosome assembly during the ongoing cell divisions in early development (Earnshaw et al., 1980). It has been shown that nucleoplasmin participates in the chromatin decondensation processes probably by acting as a histone acceptor during histone exchange reactions (Philpott and Leno, 1992). Recently, it has been shown that it induces linker histone displacement from somatic and sperm Xenopus chromatin, leading to decondensation of chromatin (Ramos et al., 2005). The counterpart to nucleoplasmin in the Xenopus egg are the N1/N2 proteins which store the H3·H4 tetramers in the oocyte (Kleinschmidt et al., 1986). In combination with nucleoplasmin they can propagate the proper assembly of nucleosome complexes (Kleinschmidt et al., 1990).

Another chaperone that has been investigated in detail is CAF-1 which consists of three different subunits (Smith and Stillman, 1989). CAF-1 is the major binding partner for newly synthesized histones H3 and H4. Like almost all other chaperones CAF-1 has nucleosome assembly activity in vitro. It has been shown to play a crucial role in the maintenance of heterochromatin silencing at telomeres (Kaufman et al., 1997). Moreover

CAF-1 interacts with PCNA which links it intimately to DNA replication (Gaillard et al., 1996; Shibahara and Stillman, 1999). It has been proposed to play a crucial role during replication by de-novo assembly of nucleosomes on the newly synthesized DNA (Tagami et al., 2004; Verreault et al., 1996). However, paradoxically CAF-1 is completely dispensable for cell viability in *Saccharomyces cerevisiae* suggesting at least additional factors that could complement for CAF-1 function in vivo (Enomoto et al., 1997; Kaufman et al., 1997). The histone chaperone HIRA is also linked to nucleosome assembly in vivo. Unlike CAF-1 it seems to act in chromatin assembly and remodeling events independent of DNA replication and specific to the histone variant H3.3 (Loyola and Almouzni, 2004; Tagami et al., 2004).

The major chaperone for the histone H2A·H2B dimer is NAP1, albeit it also interacts with the H3·H4 tetramer and can catalyze complete nucleosome formation (Ishimi et al., 1984) (Fejes Tóth et al., 2005; McQuibban et al., 1998);(Mazurkiewicz et al., submitted). It is the main transporter for histone H2A·H2B dimer from the cytoplasm to the nucleus (Mosammaparast et al., 2002). NAP1, which is found in the cytoplasm in the G1 and G2-phase relocates into the nucleus during S-phase suggesting a supportive role during DNA replication (Ito et al., 1996a). Recently it has been linked to transcription and histone exchange. It has been proposed to shuttle histone H2A·H2B dimer back and forth from the chromatin to the RNA allowing the polymerase to progress through the nucleosomal substrate and proper re-assembly in the wake of transcription (Levchenko and Jackson, 2004). NAP1 was also found as histone carrier in a sub complex of SWR1 in yeast, which catalyzes the exchange of the histone H2A·H2B dimer against the histone dimer variant H2A.Z·H2B (Mizuguchi et al., 2004). In yeast, loss of NAP1 leads to an altered gene expression of about 10% of the genome indicating a fundamental role also in vivo. To some extent it has been uncertain how NAP1 influences nucleosome structure since it has been shown to be sufficient in extracting and exchanging mainly a single histone H2A·H2B dimer at a mononucleosomal template (Kepert et al., 2005; Park et al., 2005). The effect on nucleosome and fiber structure on longer chromatin templates and in the presence of linker histones has not been clarified in detail and was therefore investigated in this work (Kepert et al., 2005). Surprisingly, NAP1 did not extract histone H2A·H2B dimer from a chromatin template but induced linker histone displacement and chromatin decondensation.

Histone chaperone	Target histone	Chaperone association state	Proposed functions in the cell	Refs
Nucleoplasmin	Linker histones, H2A·H2B dimer	pentamer-decamer	Storage of H2A·H2B in Xenopus oocyte for chromatin assembly. Sperm chromatin decondensation via linker histone depletion.	(1), (2), (3), (4)
N1/N2	H3·H4 tetramer	unknown	Storage of H3·H4 in the Xenopus oocyte for chromatin assembly.	(5), (6), (7)
NAP-1	Linker histones, Core histones	dimer-octamer-hexadecamer equilibrium	Nuclear transport of H2A·H2B, cell cycle regulation, shuttling of histones between chromatin and RNA during transcription, decondensation of chromatin through linker histone depletion, nucleosome assembly during replication	(8), (9), (10), (11), (12), (13), (14)
CAF-1	H3·H4 tetramer	hetero-trimer	replication dependant nucleosome assembly	(15), (16), (17)
HIRA	H3.3·H4 tetramer	2 Mda, heteromer	replication independent chromatin assembly, essential for proper embryonic development	(18), (19)
FACT	H2A·H2B dimer	hetero-dimer	Extraction of H2A·H2B dimer from chromatin, facilitates transcription through chromatin and restores nucleosomes	(20), (21) (22)
Rsf-1	H3·H4 tetramer	hetero-dimer	Chromatin assembly replication independent, ATPase, nucleosome spacing	(23), (24)

Table 1.1. Overview of histone chaperone functions.

References: (1):(Laskey et al., 1978), (2):(Earnshaw et al., 1980), (3):(Dutta et al., 2001), (4):(Ramos et al., 2005), (5):(Bonner, 1975), (6): (Kleinschmidt et al., 1986), (7):(Kleinschmidt et al., 1990), (8):(Ishimi et al., 1984),(9):(Ito et al., 1996a), (10):(Mosammaparast et al., 2001),(11):(Levchenko and Jackson, 2004),(12):(Kepert et al., 2005),(13):(Fejes Tóth et al., 2005),(14):(Ito et al., 2000), (15):(Smith and Stillman, 1989), (16):(Gaillard et al., 1996),(17):(Mello and Almouzni, 2001),(18): (Sherwood and Osley, 1991),(19):(Ray-Gallet et al., 2002), (20):(Orphanides et al., 1998),(21):(Orphanides et al., 1999),(22):(Belotserkovskaya and Reinberg, 2004),(23):(LeRoy et al., 1998),(24):(Loyola et al., 2001)

1.2.2 Chromatin assembly

During S-phase the DNA content of the cell is doubled and shortly after the DNA synthesis newly made core histones are deposited and form nucleosome complexes (Worcel et al., 1978). It has been shown that during replication the pre-existing nucleosomes are transferred to the sister chromatids (Krude, 1999). Along with the replication dependent de-novo nucleosome pathway a replication independent nucleosome pathway exist in other cell stages (Kim et al., 1988). These pathways are likely to be fundamental for maintenance of chromatin states after processes like DNA repair, transcription or other processes that initially alter or dissolve chromatin structure.

It has been shown that the replication dependent process involves the activity of histone chaperone proteins. Most likely the newly synthesized core histones are associated with histone chaperones, get transferred from the cytoplasm into the nucleus and are deposited onto the DNA by the action of the histone chaperones. The main carriers for the core histones, CAF-1 and NAP1 have been introduced above. The initial step in nucleosome formation at the replication fork is suspected to be the deposition of a H3·H4 tetramer by CAF-1 (Polo and Almouzni, 2005). CAF-1 which is associated with the newly synthesized H3 and H4 histones in the cell and interacts with PCNA has been shown to efficiently assemble nucleosomes in vitro (Smith and Stillman, 1989). Despite this strong relation to replication and nucleosome assembly CAF-1 is completely dispensable for cell viability suggesting additional pathways that may substitute for CAF-1 function (Enomoto et al., 1997; Kaufman et al., 1997; Verreault, 2000). Accordingly other factors like HIRA and Asaf1 have been discovered that could complement CAF-1 in nucleosome formation. From several studies a model that favors the initial deposition of the H3·H4 tetramer on the DNA followed by addition of the histone H2A·H2B dimer has been proposed (Cremisi et al., 1977; Mazurkiewicz et al., submitted; Senshu et al., 1978; Worcel et al., 1978).

Yet, it has not been shown, how and guided by which histone chaperone the H2A·H2B dimer is incorporated into nucleosomes during replication. As NAP1 has been characterized as the main carrier for the H2A·H2B dimer *in vivo* and has been shown to also interact with the H3·H4 tetramer it is likely to be involved in processes that depend on nucleosome assembly or reorganization (Fejes Tóth et al., 2005; McQuibban et al., 1998; Mosammaparast et al., 2002). It was therefore investigated, how NAP1 facilitates nucleosome formation *in vitro* and the association kinetics of intermediate products as the hexasome complex during nucleosome assembly were determined (Mazurkiewicz et al., submitted).

1.2.3 Chromatin remodelers

The *in vitro* reconstitution of chromatin with DNA, histones and histone chaperones generates templates with unregulary and short spaced nucleosomes (Ito et al., 1996b). Hence, the action of another class of chromatin modifying enzymes with diverse functions is needed to generate evenly spaced nucleosomal templates: the chromatin remodelers (Ito et al., 1997).

Chromatin remodelers are compositionally and functionally diverse protein complexes which all share an ATP-driven motor subunit as a common motif. This subunit belongs to the superfamily of Snf-2 like ATPases (Eisen et al., 1995). As mentioned above chromatin remodelers play a crucial role in mobilizing nucleosomes and can generate well defined chromatin templates in *in vitro* reconstitution systems. They can be classified into mainly four subfamilies according to different structural features outside their ATPase domain (Becker and Horz, 2002; Lusser and Kadonaga, 2003). These are the SNF2, ISWI, CHD1 and INO 80 subfamilies. The members of these families can be large multi-subunit complexes as for example the SWF-SNF complex belonging to the SNF2 subfamily or rather small ones like the complexes of the ISWI family. Despite carrying a common ATPase subunit the activity of this domain is modulated by the presence of other subunits and different subfamilies perform different biological functions (Fyodorov and Kadonaga, 2002; Ito et al., 1999; Phelan et al., 1999; Xiao et al., 2001). While the remodelers of the SNF2 subfamily (SWF-SNF etc.) for example appear to rather disassemble chromatin templates and can transfer histone octamer onto a donor DNA template, the ISWI family form stable chromatin templates and catalyze only modest repositioning of nucleosomes (Aalfs et al., 2001; Imbalzano et al., 1996; Ito et al., 1997) (see details in Figure 1.3).

The mechanism by which these enzymes can move nucleosomes along the DNA template has been clarified in a recent study (Strohner et al., 2005). An initially formed DNA loop at the entry/exit site is propagated around the nucleosome and drives the repositioning. The mobilization of nucleosomes is most likely a key regulative step in the initiation of transcription. Binding sites buried within a positioned nucleosome complex may be exposed to the transcription factors thereby facilitating efficient factor binding and transcription initiation (Korber et al., 2004; Reinke and Horz, 2003). Therefore chromatin remodeling at the promotor region of genes is often regarded as a permissive step during gene expression. Nucleosome mobilization of course is not only restricted to promotor regions and nearly all processes that deal with chromatin rearrangements involve the recruitment of chromatin remodeling complexes. As mentioned above, chromatin remodelers act during replication generating an even spacing of the newly assembled nucleosomes shortly after synthesis which seems to be essential for the successive proper

folding of the chromatin fiber. The remodeler Ino80.com for example comprises a DNA helicase activity and most probably functions in DNA repair reactions (Lusser and Kadonaga, 2003).

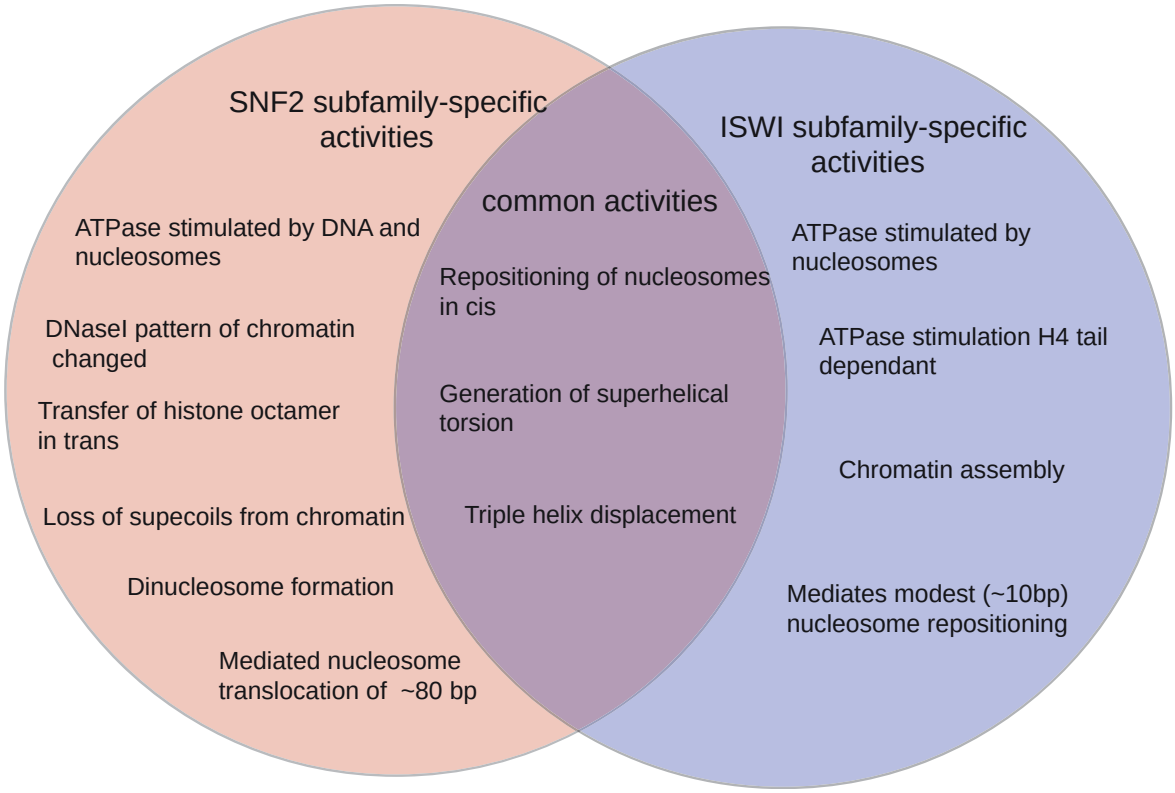

Figure 1.3 Overview of different chromatin remodeling specifities. Activities of the SNF2 and ISWI subfamily of chromatin remodelers. Different functions of these families are listed and compared. Adopted from Lusser and Kadonaga 2003 (Lusser and Kadonaga, 2003).

1.2.4 Transcription through chromatin

One of the most important processes in the cell nucleus is the process of gene expression which initially starts with transcription. Consequently transcription has been one of the most widely and intensively studied subjects in the last decades. There have been identified a lot of factors that initiate, facilitate elongation and terminate the processive ribonucleotide polymerization catalyzed by RNA polymerases (Belotserkovskaya et al., 2004; Hahn, 2004; Ng et al., 2003).

The mechanisms that modulate chromatin conformation have important roles in the regulation of transcription. All steps during transcription require specific rearrangements of chromatin conformation to allow proper function of the diverse factors (Hampsey and

Reinberg, 1999; Hartzog, 2003; Orphanides and Reinberg, 2000). Basal transcription factors have to gain access to their target sequences in the promotor regions, while during elongation the RNA polymerases have to be facilitated to transcribe through the nucleosomal barrier (Reinberg et al., 1998). Here the contributions of two factors that are involved in gene regulation by modulation of chromatin conformation were investigated: the linker histone H1 and the histone chaperone NAP1.

The effect of linker histone binding and its implications to transcriptional regulation has been reviewed already in a preceding chapter. A new feature investigated about H1 in this study is its interaction with the histone chaperone NAP1 and their potential implications in transcription. As the binding of H1 has been attributed to gene repression any alterations in the binding properties of H1 to chromatin may influence gene expression patterns. The RNA polymerase has been shown to effectively disrupt a nucleosomal template while polymerizing the RNA from the DNA template (ten Heggeler-Bordier et al., 1995). It has been proposed that histone chaperones assists the transcriptional machinery during elongation and retain nucleosome integrity after transcription. Early studies reported a depletion of histone H2A·H2B dimer in transcriptional active regions (Baer and Rhodes, 1983; Bazett-Jones et al., 1996). Recently, the FACT complex was found in HeLa cells that mediated the extraction of H2A·H2B dimer from chromatin during transcription elongation thereby allowing the RNA polymerase to proceed through the nucleosomal template (Belotserkovskaya et al., 2003; Orphanides et al., 1998; Orphanides et al., 1999). Additionally FACT reorganizes intact nucleosomes after transcription restoring proper epigenetic information. As NAP1 also has high affinity to the H2A·H2B dimer and has been proposed to shuttle it back and forth between chromatin and RNA it could also facilitate transcription through chromatin (Figure 1.4) (Levchenko and Jackson, 2004). The question has been raised if NAP1 alone is sufficient in extracting histone dimer from a chromatin template in a fashion similar to FACT (Park et al., 2005). In the here presented study it was found that NAP1 is indeed not capable of extracting histone H2A·H2B dimer from chromatin but induces the displacement of linker histone H1 (Kepert et al., 2005). Thus the extraction and exchange of histone H2A·H2B dimer from a chromatin template is dependant on additional nucleosome destabilizing events, induced by chromatin remodeling events as for example through transcription by the RNA polymerase itself.

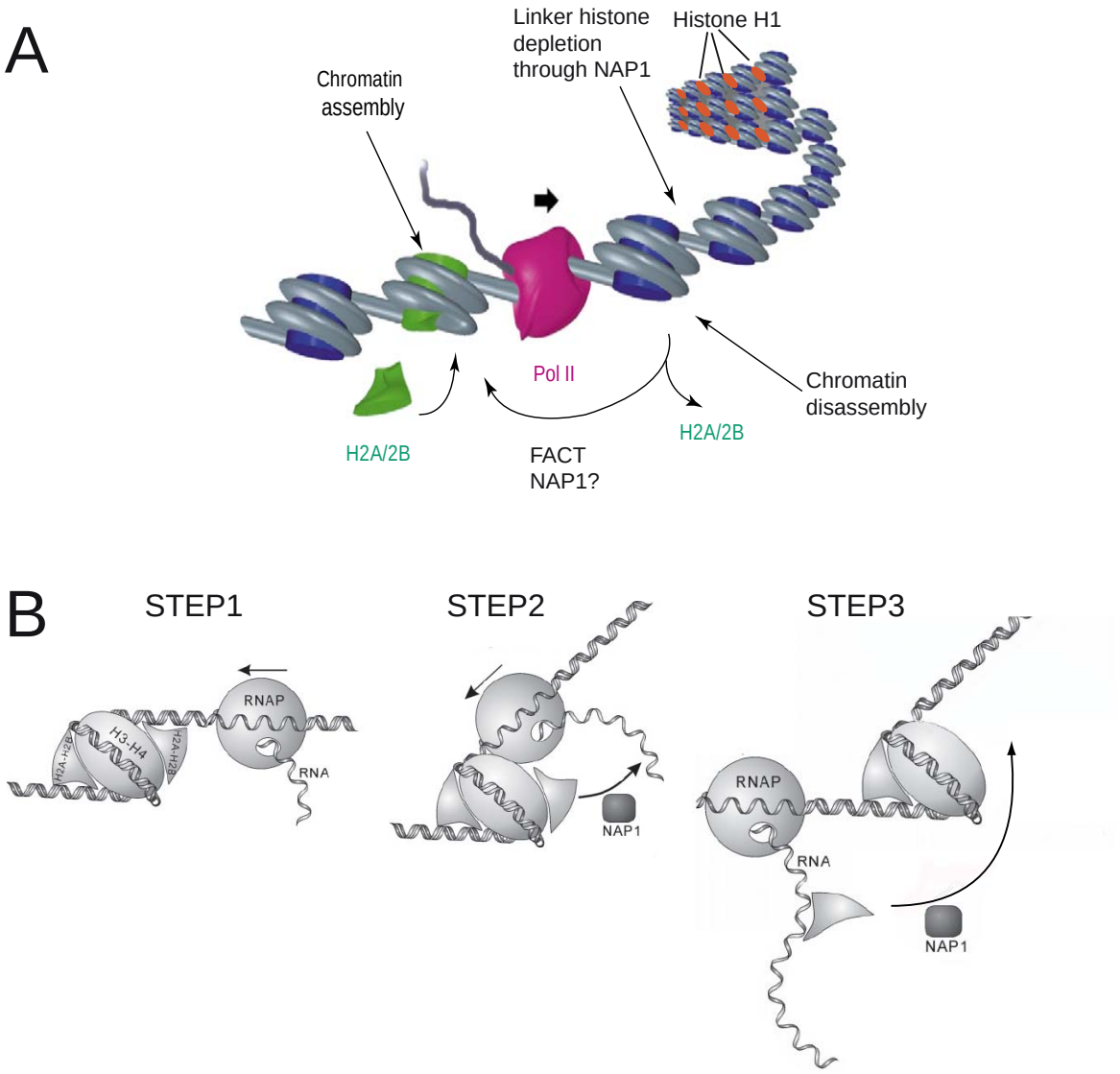

Figure 1.4 Transcription through chromatin. (A) The RNA polymerase proceeds via transcribing through a nucleosomal template. In front of the polymerase the chromatin structure opens up also facilitated through NAP1 induced linker histone depletion. Nucleosomal structure is altered during transcription and one histone H2A·H2B dimer is transferred from the octameric core complex. In the wake of the polymerase intact octameric nucleosome complexes are reformed. Scheme adopted and amended from Studitsky et al. (Studitsky et al., 2004) (B) Detailed scheme for NAP1 induced shuttling of H2A·H2B dimer between chromatin and the nascent RNA chain during transcription. NAP1 facilitates in extracting histone H2A·H2B dimer from the nucleosome. Once the dimer has been extracted it can be transferred to the RNA allowing NAP1 to act in substoichiometric amounts. After the polymerase has passed through the hexasome the H2A·H2B dimer is transferred to chromatin. Adopted from Levchenko et al. (Levchenko and Jackson, 2004).

1.3 Objectives of this work

The organization and maintenance of chromatin structure in the cell nucleus is a central parameter for the proper regulation of gene expression. Linker histones and histone chaperones are among the key components that define higher order chromatin structure and its dynamic reorganization. In this work these components and their implications on chromatin conformation was characterized in detail.

In a first set of experiments important structural parameters for the chromatin fiber folding were determined with high resolution SFM studies on recombinant mononucleosome complexes. In particular, the effects of linker histone H1 binding on the DNA geometry at the nucleosome complex was addressed. The linker histones were proposed to constrain the DNA at the entry/exit site of the nucleosome, thereby facilitating the condensation of the chromatin fiber. The results provided a low resolution model for the interaction of H1 with the linker DNA.

Hence, in a second step the DNA binding behaviour of H1 was further characterized and a molecular model for the interaction with the linker DNA was developed. To derive such a model, hydrodynamic measurements were combined with computer based modelling techniques. Based on an previous structure of the chromatosome complex the experimental results were combined with a recent X-ray structure of a tetranucleosomal particle to develop an improved model with atomic resolution for the chromatosome.

The third part of the work evaluated the dynamic interaction of the histone chaperone NAP1 with chromatin. It has been proposed that NAP1 is the main carrier for the H2A·H2B dimer and facilitates processes like transcription and histone exchange. However, also interactions with the linker histone H1 had been postulated. Therefore, it was analyzed how this chaperone can influence the chromatin fiber conformation and its interaction partners were identified. By determining the effects of NAP1 on chromatin composition and structure it was assessed how NAP1 contributes to the processes of transcription and histone exchange.

In summary, a detailed model for the action of H1 in the condensation of the chromatin fiber was established and the dynamic interaction of the histone proteins with the chaperone NAP1 were elucidated. The work provides further insight into the role of NAP1 mediated chromatin organization in relation to important processes like transcription and histone exchange.

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2. Visualization and quantification of chromatin conformation changes

In this chapter a short summary of biochemical and biophysical methods applied in my work is briefly introduced.

2.1 Biochemical approaches to examine chromatin/nucleosome structure

When interpreting studies on chromatin structure one first has to consider which experimental set-up in the individual studies has been used. Earlier investigations mostly examined chromatin and histone components from "native" sources as for example chicken blood or thymus glands. In the recent years recombinant histones have been available, which lack any post translational modifications typically found in native core histones (Luger et al., 1997b). These recombinant histones offer the possibility to study the influence of histone modifications or additional factors on the nucleosome structure in a defined system.

Besides the source of histone proteins, the choice of DNA substrates used for the analysis of chromatin structure is also important. Chromatin folding has been often exemplary studied with nucleosomal array systems carrying strong nucleosome positioning signals and confer even and discrete nucleosome spacing. These array systems can constitute a very defined system, which mimics the basic principles in chromatin folding. However, these systems are restricted to only short template lengths (up to 12 nucleosomes mostly) and the strong positioning signals confer very stable nucleosome complexes, which prevents the analysis of folding steps only occurring on longer chromatin templates. The *in vitro* reconstitution of longer chromatin templates with recombinant histones results in the formation of highly condensed chromatin templates different from native fibers (Fejes Tóth, 2004). Another possibility is therefore the purification of chromatin fibers from cell culture tissue. These fibers are not well defined in composition and display structural heterogeneity. Their advantage lies in the presence of native sequences, no artificial strong positioning signals and their length which far exceeds the 12-mer arrays normally available. Additionally, these fibers contain more of the modifications and components needed to maintain native chromatin structure. In this study, a combination of these systems has been used. Recombinant mononucleosomes were investigated to clarify the contribution of linker histone binding on the nucleosome structure. Native chromatin fragments from HeLa cells were purified to elucidate the effect of linker histones and NAP1 on longer "natural" chromatin templates.

2.1.1 *In vitro* mononucleosome reconstitution

Recombinant histone proteins can be over-expressed in E. coli cells and further purified (Kepert et al., 2003; Luger et al., 1997b). To analyze the effect of linker histone binding, mononucleosome complexes were reconstituted with a salt dialysis method. Core histones and DNA were mixed at 2 M monovalent salt and subsequently slowly dialyzed against a low salt buffer, typically containing a 50 mM monovalent salt concentration. This procedure reconstitutes intact nucleosome complexes and prevents the formation of insoluble DNA-histone aggregates which occur when the components are directly mixed at physiological salt conditions (Daban and Cantor, 1982a; Daban and Cantor, 1982b).

2.1.2 Isolation of native chromatin fibers

To investigate the effects of the linker histone and NAP1 on longer chromatin templates, fibers from HeLa cells were purified. First, cell nuclei were extracted and by using an appropriate concentration of MNase during further digestion reactions defined chromatin fragments can be obtained from these nuclei. Besides the core histones, linker histones and many other additional factors are present in such a chromatin preparation. Linker histone depleted fibers were obtained from these substrates by raising the ionic strength and subsequent anion exchange chromatography. The integrity of the prepared chromatin was checked by MNase digestions revealing the regular spacing of nucleosomes characteristic for native chromatin.

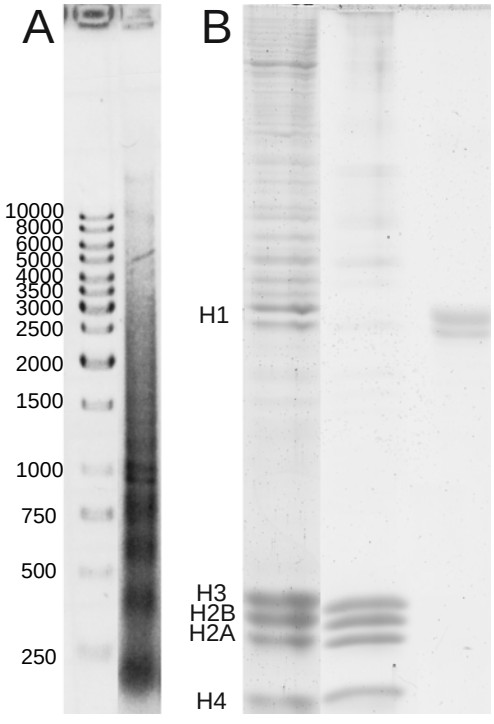

Figure 2.1 Biochemical characterization of isolated HeLa chromatin. (A) Partial MNase digestion of isolated chromatin showing regular nucleosome spacing. A repeat length of ~200 bp is apparent according to earlier observations (van Holde, 1989). (B) SDS-Page of native and linker histone stripped HeLa chromatin. Besides the core and linker histones additional not determined protein bands are visible in the prepared samples.

2.1.3 Fluorescence labeling of histone proteins and analysis of nucleosome structure

Fluorescence labeling of histone proteins allows to selectively monitor the distribution of these tagged proteins during nucleosome assembly and their binding to diverse interaction partners (RNA, NAP1 etc.). In this study either fluorescently tagged H2A or H1 proteins were used. By labeling with a tetrafluorophenyl ester at pH 8.0 it is possible to selectively label proteins at the N-terminal amino group of the protein. This has been applied here for histone H1 preventing functional deletions through labeling randomly internal amino groups. Nucleosome complexes with fluorescently labeled H2A were reconstituted and the redistribution of the H2A·H2B dimer was visualized on agarose gels (Kepert et al., 2005). Thus, it was possible to monitor the extraction of histone H2A·H2B dimer from a nucleosome complex upon incubation with NAP1 or RNA. The relative affinities of NAP1 for histone H2A·H2B dimer and histone H1 were initially compared on standard agarose gels (Fig 2.2). Further AUC experiments allowed the analysis of the association states of NAP1 and linker histone H1.

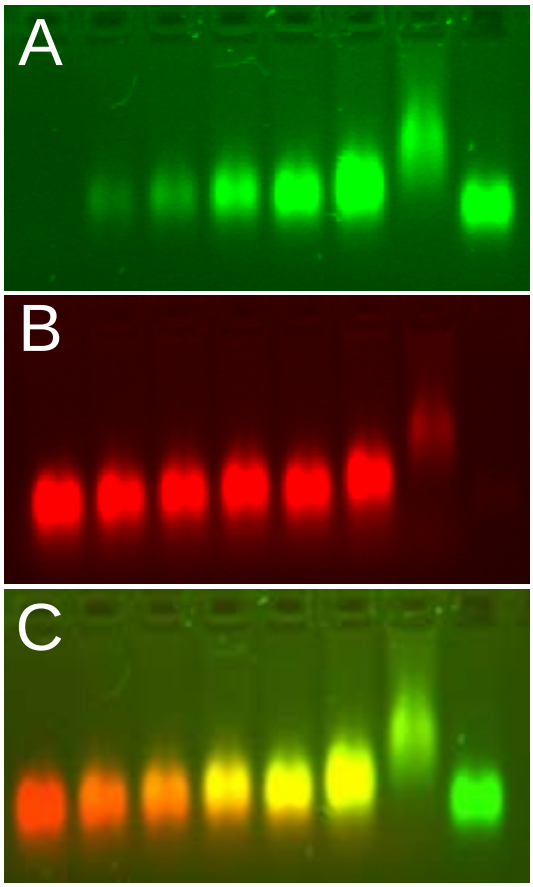

Figure 2.2 Gel shift analysis of $H1^{f}$ and $H2A^{r}$·H2B binding to NAP1. A saturated NAP1-$H2A^{r}$·H2B dimer complex was titrated with increasing amounts of labeled $H1^{f}$ (lane 2-7, r = labeled with Alexa633, f = labeled with Alexa488). (A) Fluorescence signal of histone $H1^{f}$. Increasing amounts of $H1^{f}$ are titrated to a NAP1-$H2A^{r}$·H2B dimer complex and forms a complex with either free NAP1 or NAP1 bound to the $H2A^{r}$·H2B dimer. Last lane: a saturated NAP1-$H1^{f}$ complex. (B) Fluorescence signal of the NAP1- $H2A^{r}$·H2B dimer complex. (C) Merge of the two signals

2.2 Analytical Ultracentrifugation

Analytical Ultracentrifugation (AUC) monitors the distribution of particles in a centrifugal field to determine the hydrodynamic parameters of proteins and chromatin samples. The method allows it to characterize for example protein-DNA interactions as well as the multimerisation of proteins (Demeler and van Holde, 2004; Philo, 2000; Schuck, 2003). With AUC, one can distinguish the different association states and define binding constants for protein-protein or protein-DNA interactions (Carruthers et al., 2000; Schuck et al., 2001; van Holde and Rossetti, 1967; van Holde and Weischet, 1978). Additionally the technique proved to be a valuable tool in the investigation of chromatin structure (Ausio, 2000a; Carruthers et al., 1998; Dorigo et al., 2003; Hansen, 2002; Hansen and Turgeon, 1999; Schwarz and Hansen, 1994). This is due to the sensitivity of sedimentation coefficients not only for weight but also for the shape of the particles under investigation. Thus, it is possible to compare decondensed and highly compacted chromatin conformations in a real solution based system.
The hydrodynamic parameters are derived from the sedimentation behaviour of the sample, which is monitored by the absorption profile during centrifugation. Through the use of a monochromator different wavelength measurements can be obtained during one experimental run. Thus, by tagging proteins or DNA specifically with fluorescent dyes, single components can be selectively studied in AUC experiments. The AUC also offers the possibility to quantify a whole subset of molecules in physiological buffer solutions but under defined conditions. This can refine experimental results from non-solution based gel shift analysis significantly (Kepert et al., submitted). Two different experiments can be conducted in the AUC in principle: sedimentation equilibrium and sedimentation velocity experiments, which give complementary information on the molecular shape and molecular weight of the sample.

2.2.1 Sedimentation velocity measurements

During sedimentation velocity experiments the sample is sedimented from the meniscus to the bottom of the cell at a relatively high speed (Fig 2.3A). The sedimentation of the sample to the bottom of the cell can be visualized by absorbance scans over time. A moving boundary forms during the run, which can be used to derive the hydrodynamic parameters of the sedimenting species. Due to diffusion, the sample moves not as a sharp band but gets broadened (Fig 2.3A). Thus it is possible to derive a sedimentation and diffusion coefficient during the sedimentation velocity experiments. The velocity of the boundary is directly proportional to the molecular weight of the absorbing species and

indirect proportional to the frictional coefficient which reflects the molecular shape of the particle. The sedimentation also depends on the density and viscosity of the buffer. Equation 1 gives the relationship between the sedimentation coefficient and these variables:

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

where M is the molecular weight, $\bar{\nu}$ is the partial specific volume of the sample, ρ is the buffer density, N_A the Avogadro number, f the frictional coefficient, v the velocity of the moving band, $\omega^2 \cdot r$ the centrifugal acceleration and s the sedimentation coefficient. The changes of the concentration distribution during the sedimentation process can be described by the Lamm equation:

$$\frac{dc}{dt} = \left(D\frac{d^2c}{dr^2} + \frac{1}{r}\frac{dc}{dr} \right) - s \cdot \omega^2 \left(r\frac{dc}{dr} + 2c \right) \qquad \text{(eq. 2)}$$

As no general analytical solution to this equation exists several new software packages make it possible to numerically describe the sedimentation process and allow thereby the computation of the hydrodynamic coefficients. Besides these numerically based approaches other methods of analysis describe the sedimentation behavior with an analytical approximation to the Lamm equation by simplification of some of the parameters. Hence, nowadays a combination of the methods can be used in dependence on the purpose and focus of the investigation. Two of these analysis techniques were used here to analyze the DNA binding pattern of linker histone H1 to short DNA duplexes and the effect of NAP 1 on chromatin conformation (Kepert et al., submitted; Kepert et al., 2005).

2.2.2 Sedimentation equilibrium measurements

With sedimentation equilibrium experiments the molecular weight distribution of a sample can be determined very precisely. In contrast to velocity experiments the sample is spun with moderate speeds so that it does not sediment over time to the cell bottom. When the chosen speed is appropriate a constant concentration gradient is formed which is the equilibration product of sedimentation due to the centrifugation force and diffusion against the forming concentration gradient (Figure 2.3). Once equilibrium is reached, the formed concentration gradient is determined solely by the molecular weight of the particle.
The concentration of particles increases with the distance r from the rotor midpoint. The

different positions from the rotor center can be understood as different occupied energetic states. The transitions between these states are characterized by the work that is needed to move a particle between different radial positions. The effective mass for the particle has to be taken into account which is given by $M_{eff} = M \cdot (1 - \overline{\nu} \cdot \rho)$. From this the concentration distribution, described by the absorbance, in dependance of the radial positions is calculated according to eq.3:

$$A_r = A_0 \cdot \exp\left(\frac{M \cdot (1 - \bar{\nu} \cdot \rho) \cdot \omega^2 \cdot (r^2 - r_0^2)}{2 \cdot RT} \right) + E \qquad \text{(eq. 3)}$$

A_r and A_0 are the absorption at the distances r and 0, M the molecular weight, ω the centrifugal acceleration and E the absorption of the baseline after sample depletion. This describes the absorption profile for a single species in a sedimentation equilibrium experiment. Two or more non-interacting particles expand the equation to a sum of two or more expressions as in the eq. 4:

$$\begin{aligned} A_r = {} & A_1 \cdot \exp\left(\frac{M_1 \cdot (1 - \bar{\nu}_1 \cdot \rho) \cdot \omega^2 \cdot (r^2 - r_0^2)}{2 \cdot RT} \right) \\ & + A_2 \cdot \exp\left(\frac{M_2 \cdot (1 - \bar{\nu}_2 \cdot \rho) \cdot \omega^2 \cdot (r^2 - r_0^2)}{2 \cdot RT} \right) + E \end{aligned} \qquad \text{(eq. 4)}$$

A wide variety of models can be fitted to the experimental data with the available software packages, which can be used to identify the molecular association states or binding constants for each system being investigated. The combination of sedimentation velocity and equilibrium experiments allows a reliable description of inter- and intramolecular interactions. Analytical ultracentrifugation experiments afford commonly high protein or DNA concentrations in the μM range. New advancements with fluorescence ultracentrifugation will allow measurements down to the nM concentration level, reducing the amount of material needed and thereby expanding the possibility to investigate reactions with binding constants in the nM range.

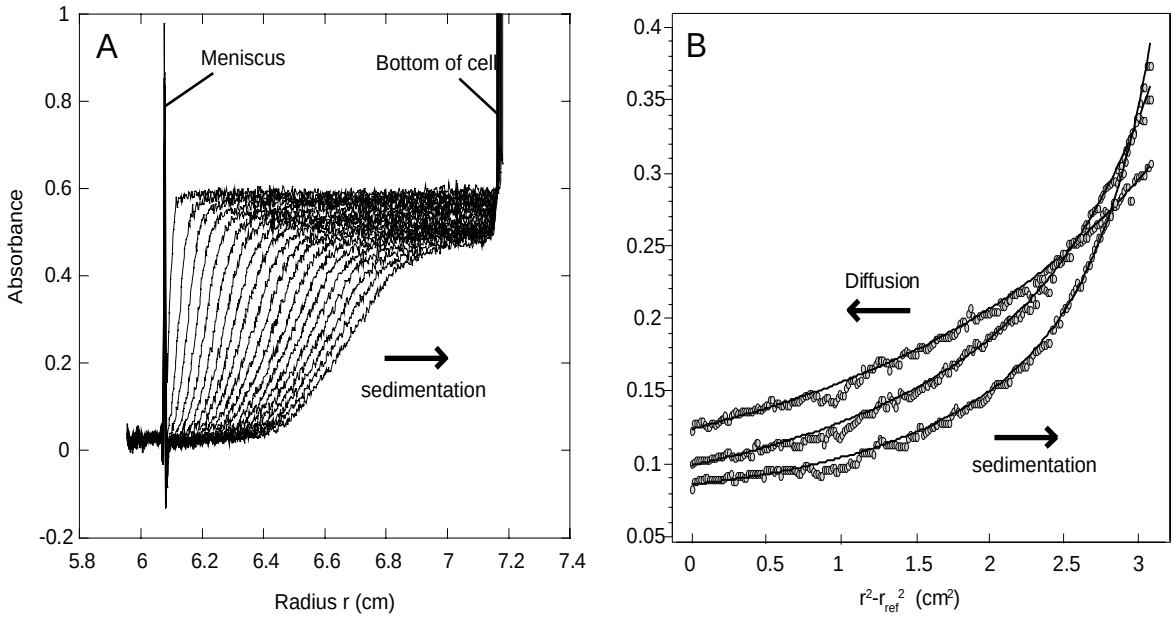

Figure 2.3 Sedimentation equilibrium and velocity experiments. (A) Sedimentation velocity experiment of NAP1-H1^f complexes. The sample was measured at 494 nm and radial scans were taken at 37000 rpm, in the continuous scan mode, at 20° C and with 0.003 cm radial step size. Every forth scan is shown. The sample/boundary moves with time from the meniscus to the bottom of the cell. The band gets also broadened at later time points reflecting the diffusion of the sample during sedimentation. (B) Sedimentation equilibrium experiment of the same sample shown in (A). Three different equilibrium scans at 5000, 7000 and 10000 rpm are shown. Each scan is taken after centrifugation for 28 h at the different speeds. A monomer-tetramer model is fitted to the data points and is shown with solid lines.

2.3 Scanning force microscopy (SFM)

SFM technique has proven to be a reliable tool in the investigation of chromatin and DNA structures (Bash et al., 2003; Fritzsche et al., 1995; Kepert et al., 2003; Leuba et al., 1994; Rivetti et al., 1996; Schulz et al., 1998; Wyman et al., 1995). About ten years ago the first images of chromatin fibers have been recorded with a SFM microscope (Zlatanova et al., 1994). Along with high resolution imaging of native chromatin, conformation changes of fibers can be detected in a physiological buffer solution (Bustamante et al., 1997). The schematic function of a scanning force microscope is shown in Figure 2.4. A laser beam is focused via a cantilever, to which an ultra fine silicon or silicon nitride tip is tethered, onto a four-quadrant photo diode (Figure 2.4 and 2.5). During the initial adjustment steps the cantilever is excited through a piezo-element near the resonance frequency of the cantilever. This forces the cantilever to oscillate with an amplitude that can be regulated by the voltage that is applied to the piezo-element. Through the oscillation of the cantilever the laser beam also swings in the photo-diode. This oscillation is focused in the middle of the photo-diode during the calibration setting and marks the reference for the free amplitude. The microscope offers the possibility to measure in two different operation

modes: the tapping and contact mode. During the tapping mode the cantilever is approached to the sample surface by another piezo-element beneath the sample holder. While the sample approaches to the tip the oscillation gets damped due to interactions with the surface. These interactions can be characterized in a simple case for a two atomic system by the Lennart-Jones potential. At longer distances the tip gets attracted by van der Waals interactions, which range over the distance of hundreds of angstroms. The repulsive interactions at smaller distances are mainly due to the Pauli principle. Most biological substrates are scanned in the tapping mode minimizing the interactions of the sample with the tip. No direct contact between the tip and the sample is needed. A ~70 % reduced amplitude signal is used as the regulatory parameter at which the sample gets retracted from the tip and the original distance is restored. The tip that scans over the surface and records a topographic image. Thus DNA, chromatin or other biological samples can be visualized in a three-dimensional image when deposited on a very smooth surface (Figure 2.6). A commonly used substance for the generation of such surfaces is mica. It can be cleaved to produce very even crowns with a mean roughness below 1 nm. The resolution of the imaging mostly depends on the geometry of the scanning tip, which is limited by the opening radius (Figure 2.5). As depicted in Figure 2.5 every object gets at least broadened by twice the radius of the tip. The typical radii for commercial silicon tips range between 5-10 nm. Therefore, for example a DNA molecule deposited on mica which is in reality ~2 nm broad appears minimally as a 10 nm thick structure.

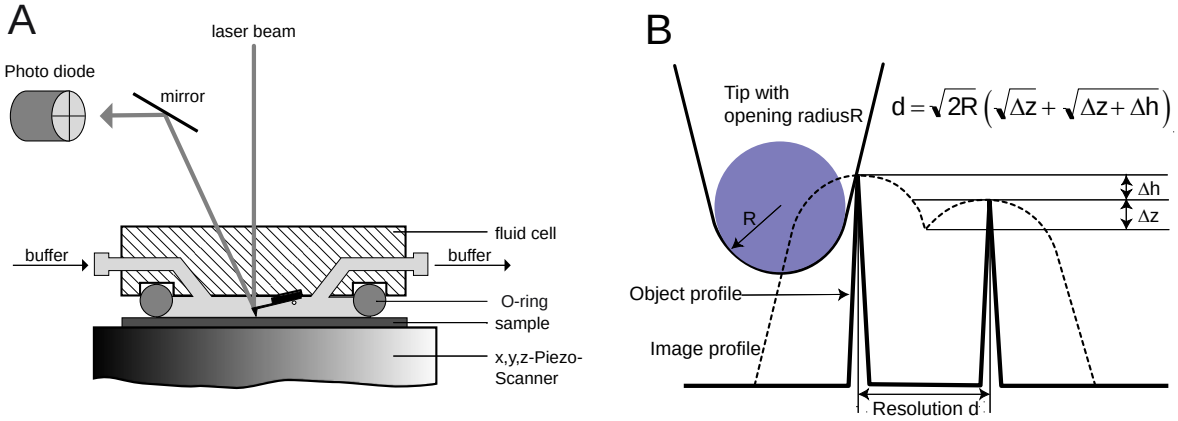

Figure 2.4 Schematic illustration of the SFM. (A) Illustration of the experimental set-up of a conventional SFM microscope. A laser beam focused on the cantilever is targeted via a mirror into the photo-diode. The cantilever is inserted in the fluid cell which is sealed with a silicon O-ring to avoid cell leakage. The buffer solution can be exchanged in a defined manner. (B) Lateral resolution of the SFM tip. The tip oscillates over the surface and “contacts” objects that protrude from the surface. The real dimensions get broadened due to the given geometry of the tip.

Figure 2.5 Commercial available etched silicon probe for AFM tapping mode. High magnification of an ultra sharp RTESP-probe from Veeco-Europe. The opening radius for the tip is typically below 10 nm. The resonance frequency is around 300 kHz.

One major advantage of the SFM technique is the possibility to image biological samples in the fluid cell under physiological buffer solution, allowing real-time visualization of fluctuations in chromatin conformation. Using this method the change in shape of a nucleosomal array due to different salt concentrations was visualized (see Figure 2.7). Additionally the effect of linker histone H1 in stabilizing a more compact chromatin structure could be determined with this approach (Figure 2.7).

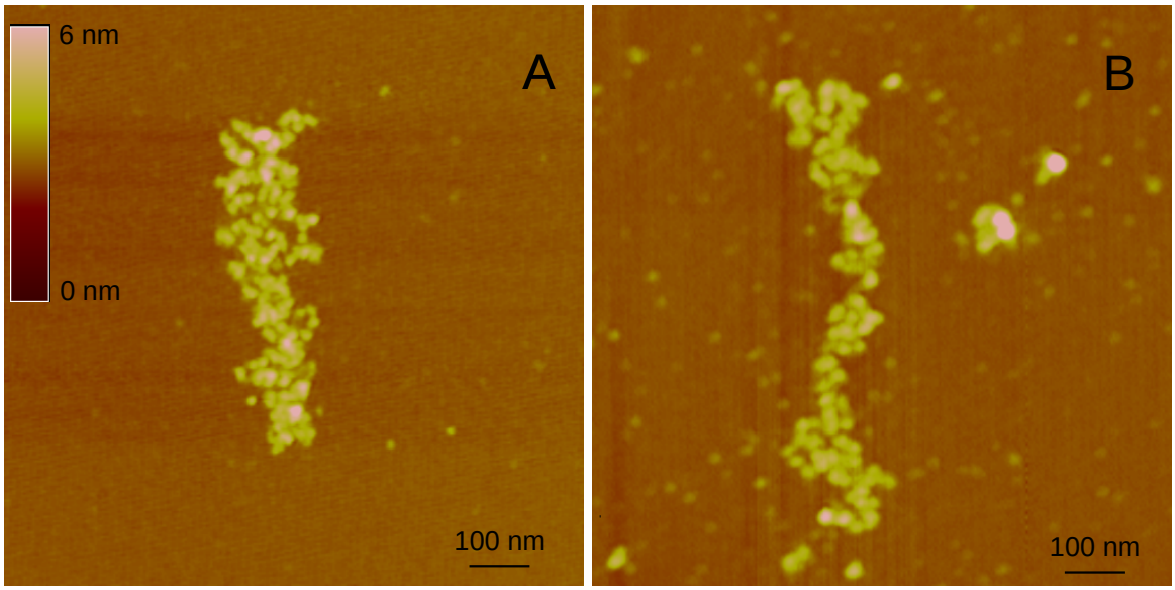

Figure 2.6 SFM imaging of HeLa chromatin. (A) Air dried sample of a chromatin fiber isolated from HeLa cells. The Nucleosomes of the fiber are arranged during the depositioning on the surface. Mostly a single layer of nucleosomes is present. (B) Image of a chromatin fragment isolated from HeLa cells that have been treated with TSA (unpublished data). This causes the hyperacetylation of core histone tails and chromatin decondensation (Heuvelman, Kepert, Rippe in preparation).

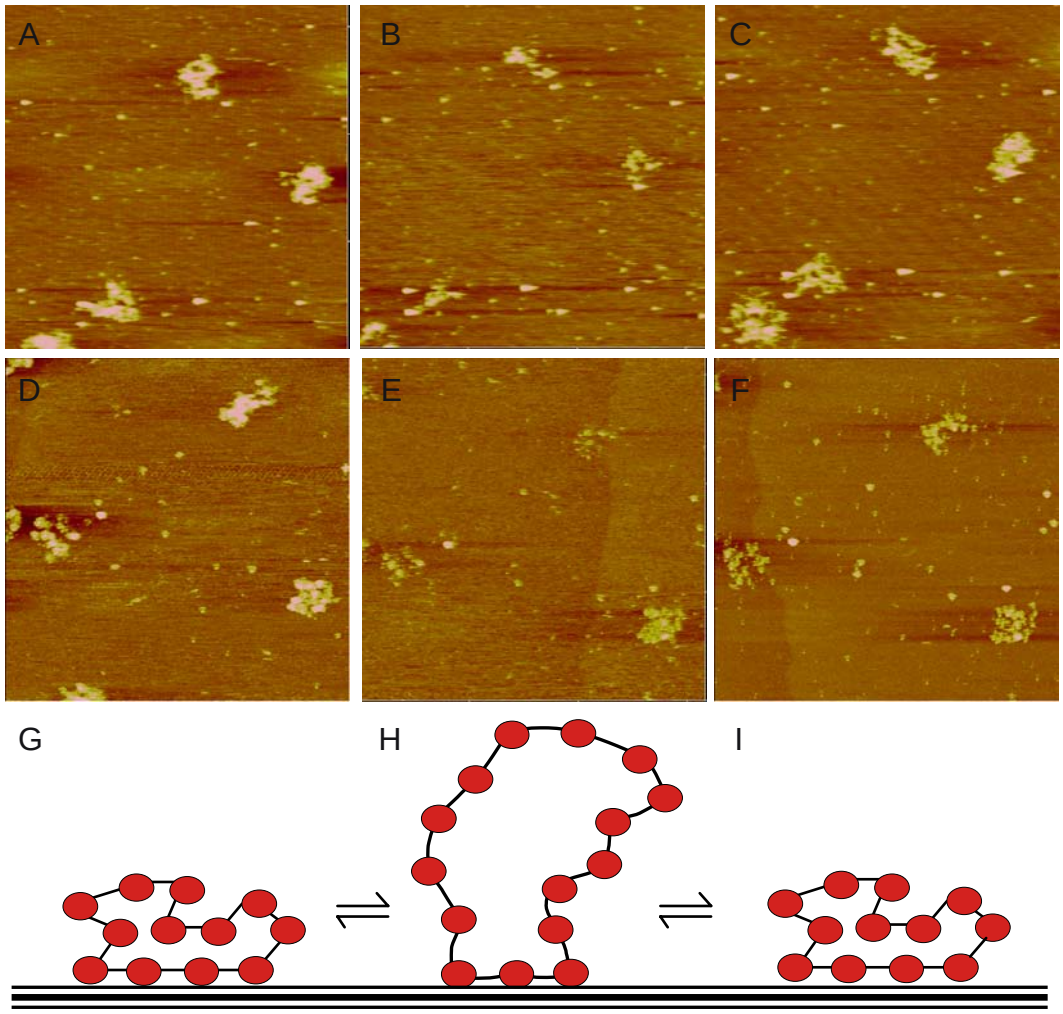

Figure 2.7 Visualization of chromatin conformation changes. (A-C) Salt dependant decondensation of a nucleosomal array. A circular plasmid was reconstituted with drosophila core histones by salt dialysis to form a chromatin template. Under starting conditions (10 mM Hepes pH 8.0, 50 mM KCl) it is bound to the surface and adopts a closed conformation (A). Upon buffer exchange against 10 mM Hepes pH 8.0 the array decondenses and adopts a highly mobile conformation that is partly detached from the surface (B). After an additional buffer exchange against 10 mM Hepes, 50 mM KCl the array condenses again and binds to the surface (C). (D-F) Linker histone H1 stabilizes a more condensed array conformation. The same nucleosomal template as in the first three panels is bound to the mica surface and detaches upon reduction of the salt concentration (D-E). A solution of linker histone H1 is added to the fluid cell and the arrays partly reassociate to the surface reflecting the condensation of the arrays (F). (G-I) Schematic drawing of changes in conformation of the nucleosomal template and its binding to the mica surface.

2.4 Molecular modelling and hydrodynamic simulations

To derive the atomic model of a biological structure the most precise method is the structural determination by X-ray crystallography (van Holde et al., 1998). However, the crystallisation of biological samples is often not possible due to problems during the crystallisation process itself. An alternative approach is the prediction of protein folding from comparison with proteins or protein domains where the structure is known. For this purpose, nowadays powerful software tools are available that enable the user to evaluate structural properties of the complexes under investigation. As a starting point a previously resolved structure can be taken that can then be refined or amended with molecular modelling techniques.

The atomic coordinates for numerous DNA-protein complexes can be obtained from online protein data banks. Rearrangements and molecular modelling of given structures is then conducted with a variety of software packages as for example the vmd graphics viewer (Humphrey et al., 1996), pymol (http://pymol.sourceforge.net/) or the Sybyl7.1 software (http://www.tripos.com/). If a protein domain has not been experimentally resolved yet a structural homology search helps to identify related sequences for which structural data are available (Kelley et al., 2000). If the sequence identity exceeds 25% a protein structure with atomic resolution can be derived by homology alignment. Once the generated structures have been fit to the experimental data, the interactions and contacts established are optimized with an energy minimisation software package. This software computes the energy for a given molecule by calculating the internal or bonded terms, which describe the bonds, angles and bond rotation in a molecule. An additional external term accounts for the interactions between non bonded atoms. This includes electrostatic and van der Waals interactions. The energy for the sum of those numerical force fields is then minimized to generate a most favorable conformation. Limitations are given by the fact that within these force fields no bonds can be broken and rearranged. It has also to be taken into account that the potential energy minimum has to be expanded for entropic effects to assure that the thermodynamic equilibrium is reached.

Once the atomic coordinates of the minimised structure have been obtained they can be used to calculate hydrodynamic parameters like sedimentation and diffusion coefficients. To this end the atomic model is approximated by a bead model that is initially computed by the software HYDRPRO from the given pdb coordinates (Figure 2.8) (Garcia de la Torre, http://leonardo.fcu.um.es/macromol/).

The idea is that for a simple molecule like a sphere the frictional coefficient can be computed according to eq. 5:

$$f_{shell} = 6 \cdot \pi \cdot \eta \cdot r \qquad \text{eq.5}$$

In this expression f is the frictional coefficient, η the viscosity of the solvent and r the radius of the sphere. According to Kirkwood the frictional coefficient of a structure with n spheres can then be calculated with eq. 6 (ref):

$$f_N = N \cdot f_1 \cdot \left(1 + \frac{f_1}{6 \cdot \pi \cdot \eta \cdot N} \sum_{i=1}^{N} \sum_{j \neq 1}^{N} \frac{1}{R_{ij}} \right)^{-1} \qquad \text{eq. 6}$$

In equation 6, R_{ij} is the distance between the midpoints of two spheres. If the frictional coefficient is determined the hydrodynamic parameters can be calculated with the equations 7 and 8,

$$D = \frac{kT}{f} \qquad \text{eq. 7}$$

$$s = \frac{M \cdot \left(1 - \bar{\upsilon} \cdot \rho \right)}{N_A \cdot f} \qquad \text{eq.8}$$

where D is the diffusion and s the sedimentation coefficient. In this way the hydrodynamic parameters for the generated structures are obtained and help to select a model that fits best to the experimental results (Figure 2.9).

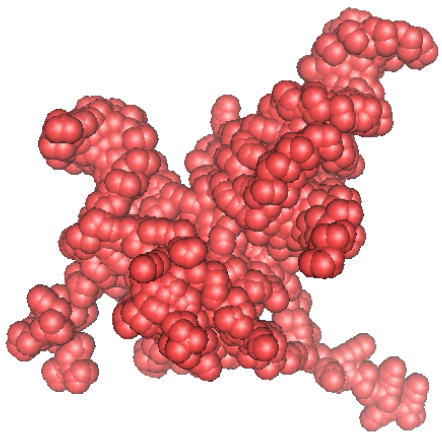

Figure 2.8. Shell model of H1 bound to two DNA duplexes. A bead structure was derived from the pdb coordinates with the Hydropro software package and used for the calculation of theoretical hydrodynamic parameter (Kepert et al., submitted).

Figure 2.9 Remodelling of a chromatosome complex. (A) Structure of the dinucleosomal particle published by Schalch et al. (Schalch et al., 2005).One linker DNA strand is straight while the other is bent approximately in the mid between the nucleosomes.(B) and (C) Energy minimized structures of a chromatosome complex. One nucleosome core complex with linker DNA was taken from the dinucleosome structure. Full length histone H1d was superimposed and linker DNA contacts were adopted from the initial proposal from Bharat et al. (Bharath et al., 2003).The linker histone is positioned symmetrically on the nucleosome core. The DNA contacts formed by the winged helix and the C-terminal domain confer asymmetric linker DNA protection. Again one DNA duplex seems to leave the nucleosome straight. The C-terminal domain of H1 introduces a sharp bending of the other DNA linker subsequently introducing a angle of roughly 70^.

Summary

The compactions of the 30 nm fiber due to linker histone binding is an important regulatory step in chromatin dependent processes like transcription, DNA repair or replication. The determination and characterization of different chromatin conformations and the dynamic changes induced by protein factors are essential to understand the regulatory mechanisms involved. In this study the dynamic effects of linker histone H1 and histone chaperone NAP1 on the nucleosome core composition and on the conformation of native chromatin fiber fragments were investigated in detail.

First, high resolution SFM imaging of recombinant mononucleosome complexes was used to determine important structural parameters that are modulated by H1. It was found that the angle between the DNA exiting and entering the nucleosome was decreased upon linker histone binding. Furthermore the ability of assembled mononucleosome complexes to change their position along the DNA strand was significantly reduced by H1 incorporation.

Second, the interaction of H1 with DNA was characterised to elucidate the above changes on the molecular level. Analytical ultracentrifugation results and biochemical data identified two strong DNA binding sites on full length histone H1. Based on these data and previous results in conjunction with computer based homology modelling a structure of the chromatosome complex was built. The linker histone was positioned symmetrically at the nucleosome core in a manner that is consistent with most of the experimental data. The resulting molecular model provides a starting point for computational modelling of longer fibers and the evaluation of structural changes in chromatin upon linker histone binding.

Third, the influence of histone chaperone NAP1 on chromatin and nucleosome structure was determined. NAP1 is involved in the assembly of proper nucleosome complexes. It formed a hexameric intermediate complex during assembly, that seems to be also important during transcription through chromatin. Furthermore, the ability of NAP1 to interact with the linker histone and its contribution on chromatin fiber conformation was evaluated. The addition of NAP1 induced a reversible extraction of linker histones in chromatin, which leads to a decondensed and more open fiber structure. In contrast to previously proposed models it was found that NAP1 alone was not sufficient to extract/exchange histone H2A·H2B dimer in the context of native chromatin fibers.

In summary, the results obtained here identify the interconnected association properties of linker histone H1 and histone chaperone NAP1 as important parameter that can induce a more accessible chromatin conformation.

Zusammenfassung

Die Kompaktierung der 30 nm Faser durch Bindung von Linkerhiston H1 ist ein wichtiger, regulatorischer Schritt in chromatinabhängigen Prozessen wie Transkription, DNA-Reparatur und Replikation. Die Bestimmung und Charakterisierung von unterschiedlichen Chromatinkonformationen und den dynamischen Veränderungen, die von Proteinfaktoren eingeleitet werden, sind zum Verständnis der regulatorischen Mechanismen essentiell. In dieser Arbeit wurden die dynamischen Effekte des Linkerhistons H1 und des Histonchaperons NAP1 auf die Nukleosomzusammensetzung, sowie auf die Konformation nativer Chromatinfasern detailliert untersucht.

Erstens wurde hochauflösende Rasterkraftmikroskopie an rekombinanten Mononukleosomenkomplexen verwendet, um wichtige Strukturparameter zu bestimmen, die durch H1 verändert werden. Es konnte gezeigt werden, dass der Winkel zwischen ein- und austretendem DNA-Strang durch die Bindung von H1 verringert wird. Außerdem wurde durch den Einbau von H1 die Fähigkeit von assemblierten Mononukleosomkomplexen stark eingeschränkt, ihre Position auf dem DNA-Strang zu verändern.

Zweitens wurde die Interaktion zwischen H1 und DNA charakterisiert, um die zuvor gefundenen Veränderungen auf molekularer Ebene zu erklären. Mit Hilfe analytischer Ultrazentrifugationsergebnisse und biochemische Daten wurden zwei starke DNA-Bindungsstellen auf dem vollständigen H1 Protein identifiziert. Anhand dieser Daten und früherer Ergebnisse konnte mittels Computer gestützten Homologiemodellierungen eine Chromatosomstruktur zusammengesetzt werden. Das Linkerhiston wurde so symmetrisch an den Nukleosomenkern gesetzt, dass die Struktur mit den meisten experimentellen Daten übereinstimmte. Das resultierende molekulare Modell schafft die Voraussetzungen für die Modellierung langer Fasern und die Bestimmung struktureller Veränderungen des Chromatins, die durch H1-Bindung vermittelt werden.

Drittens wurde der Einfluss des Histonchaperons NAP1 auf die Chromatin- und Nukleosomstruktur bestimmt. Dazu wurde zunächst die NAP1-induzierte Modellierung von Nukleosomenkomplexen über einen hexameren Histonkomplex charakterisiert, der auch während der Transkription durch Chromatin eine Rolle spielen soll. Im weiteren wurde die Fähigkeit von NAP1 mit Linkerhiston zu interagieren untersucht und dessen Einfluss auf die Chromatinkonformation zu bestimmen. Die Zugabe von NAP1 induzierte eine reversible Dissoziation von H1, die zu einer weniger kompakten und offeneren Faserstruktur führte. Im Gegensatz zu früher vorgeschlagenen Modellen war NAP1 nicht in der Lage, Histon H2A·H2B Dimer an nativen Chromatinfasern auszutauschen.

Anhand der hier erzielten Ergebnisse konnten die wechselseitigen Interaktionen zwischen Linkerhiston H1 und Histonchaperon NAP1 als wichtiger Parameter bestimmt werden, die eine offenere und zugänglichere Chromatinkonformation auslösen können.

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