Investigating effects of histone tail modification on chromatin compaction in nucleosome arrays

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INVESTIGATING EFFECTS OF HISTONE TAIL MODIFICATION ON CHROMATIN COMPACTION IN NUCLEOSOME ARRAYS

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**'Investigating Effects of Histone Tail
Modifications on Chromatin Compaction in
Nucleosome Arrays'**

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Abbreviations

APAGE	Agarose-polyacrylamide gel electrophoresis
APS	ammonium peroxydisulfate
CIA	chloroform-Isoamyl alcohol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylene diamine tetra acetic acid
H1, H2A, H2B, H3, H4	histone proteins
HAT	histone acetyl transferase
HDAC	histone deacetylase
HO	histone octamer
IPTG	isopropyl-thiogalactopyranoside
KD	kilodalton
MW	molecular weight
MWCO	molecular weight cut off
NCL	native chemical ligation
NCP	nucleosome core particle
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
RNA	ribonucleic acid
rpm	round per minute
RT	room temperature
SDS	sodium dodecyl sulfate
TB	terrific broth
TBE	tris borate EDTA

ABSTRACT

All eukaryotic organisms have elaborated ways of packaging DNA into chromosomes. Genetic processes, both vital such as transcription and replication and pathological such as cancer and viral infection depend on DNA in the context of chromatin. Chromatin consists of a linear array of uniform units called nucleosomes. The nucleosomes are made of DNA which carries a high negative charge and highly conserved and basic proteins, the histones. Unstructured N-termini of the histones called “histone tails” are believed to mediate inter-nucleosomal interactions leading to condensed chromatin. Histone tails are subject to a range of post-translational modifications, the most common of which, acetylation of the lysine ϵ -amino group, neutralizes the positive charge will induce decondensation of chromatin. A detailed characterization of the molecular interactions of histone tail mediated NCP interactions is of relevance for understanding the physical mechanisms underlying the epigenetic control of gene regulation.

In this project, large amount of stoichiometrically saturated 12-mer nucleosomal arrays feasible for extensive biophysical studies has been prepared and purified using recombinantly prepared individual histones and DNA template of a 12-mer repeat of a 177 bp strong positioning DNA sequence.

To examine the role of histone tails acetylation in nucleosomal array folding and condensation, variants of the H4 histone tail with acetylated lysine amino acids in positions K16, K5.8.12 and K5.8.12.16 have been prepared using a semisynthetic method. In this procedure a histone N-terminal peptide with acetyl lysine at a desired position was synthesized using solid phase peptide synthesis and the C-terminal domain of the histone with an N-terminal cysteine was expressed and purified, then two fragments were ligated together via native chemical ligation. To determine whether the glutamine substitution and lysine acetylation within the H4 tail domain have identical effects on nucleosomal array decondensation, constructs containing K $\rightarrow$ Q mutation in positions of K16, K5.8.12 and K5.8.12.16 have been prepared. Nucleosomal array containing acetylated or K $\rightarrow$ Q mutated variants of the H4 histones were prepared and purified.

Salt induced folding and oligomerization of the arrays was studied using analytical ultracentrifugation sedimentation velocity measurements, precipitation assay and dynamic light scattering. Increase in salt concentration leads to folding of the array from extended conformation with sedimentation velocity coefficient value $S_{20,w}=34-35$ S to compact conformation with characteristic $S_{20,w}=53-55$ S. Maximum compaction is reached in the presence of 100 mM NaCl, 1 mM $MgCl_2$, 0.8 mM $CaCl_2$, 20 μ M $Co(NH_3)_6Cl_3$, 60 μ M spermidine and 2 μ M spermine. The compacting strength of cations follows the trend: spermine⁴⁺ > $Co(NH_3)_6^{3+}$ > spermidine³⁺ > $CaCl_2$ > $MgCl_2$ > NaCl > KCl. These folding dependences on cation charge and concentration indicate a polyelectrolyte nature of chromatin.

Acetylation of lysine 16 in histone H4 decreases the folding of the array from 53 S to 44 S in presence of 1 mM MgCl₂, 1 mM CaCl₂, 20 μM Co(NH₃)₆Cl₃ and 100 mM NaCl, therefore H4-K16Ac disrupts intramolecular nucleosomal array folding. Acetylation of lysine in positions 5, 8 and 12 in histone H4 tail does not affect nucleosomal array folding significantly. We found that the acetylation of lysine in positions 5, 8, 12 and 16 has a synergistic effect on nucleosomal array unfolding and the S_{20,w} shifted from 53 S to 40-41. Similar sedimentation velocity measurements carried out on the array having K→Q mutations at the H4 histone positions K16, K5.8.12 and K5.8.12.16 demonstrated that unlike H4-K16 acetylation, K→Q mutations produced a much smaller disruptive effect on the salt-induced array folding. A novel finding was that the ubiquitous natural ion, K⁺, has an abrogating influence on the array folding almost as large as the effect of H4-K16Ac. Combining our biophysical and recent NCP crystal structure data, a novel structural mechanism behind the disruptive effect of H4-K16Ac and K⁺ ions have been proposed.

The effect of H4 histone tail acetylation on inter-array self-association (investigated by precipitation assay using the same cations) was studied by comparison of EC₅₀ (the concentration that cause 50% precipitation) for wild type, single (K16), triple and quadruple acetylation with corresponding K→Q mutated array systems. Based on our observations, we found that precipitation behavior (measured by EC₅₀) will increase with the number of acetylation in the H4 N-terminal tail, from single to quadruple, and is almost identical to EC₅₀ obtained from K→Q mutant arrays, indicating an electrostatically controlled mechanism of self-association due to charge quenching of the N-terminal lysines.

1. Introduction

1.1 Chromatin overview

DNA in eukaryotic organisms is organized in a nucleoprotein complex called chromatin (1). In recent years it has been shown that chromatin has a key role in regulation of gene expression. Understanding chromatin structure and dynamics is of fundamental importance for life science and contribute decisively to our knowledge of cell functioning, organism development and origin and treatment of diseases.

In the eukaryotic nucleus, the proteins which bind to DNA to form chromosomes are divided into two groups: the histone and the non-histone proteins. The complex of both groups of protein with the nuclear DNA is known as chromatin. The total histone mass in chromatin is approximately equal to that of the DNA. Histone proteins are responsible for the first and most basic level of chromosome organization, consisting of nucleosome, which was discovered in 1884 by Kossel et al. (2). The time line of discoveries in the field of chromatin is shown in Fig. 1.1 (2).

Chromatin in the cell is believed to be in the form of a 30-nm filament throughout most of the cell cycle, and at interphase appears to be looped onto the nuclear matrix (3). However, the extent of chromatin compaction during interphase goes beyond the 30 nm fibre. At mitosis, the 30-nm filament is subjected to further levels of folding to give the highly dense metaphase chromosome in which the overall length compaction of the DNA is about 10,000-fold. The 30-nm filament is not a suitable template for transcription by the large eukaryotic RNA polymerases and clearly has to be unfolded first; formation of an “open” (“transcriptional competent”) chromatin state is an essential prerequisite for transcription. Compactions levels of chromatin are schematically shown in Fig. 1.2 (4).

The nucleosome is the fundamental repeating unit of chromatin and is the first level of compaction of DNA in eukaryotes. Each nucleosome consists of 145-147 bp DNA molecule wrapped around a histone octamer which is comprised of two copies of four histone proteins, H2A, H2B, H3 and H4. (Fig. 1.3) The nucleosomes are joined by linker DNA and histone H1 to form chromatin (5). All histones have similar structure of the globular domain and unstructured N-terminal “tail”. Two dimers of H3/H4 associate together, and then with two H2A/H2B dimers make the histone octamer. In physiological salt condition, the H3/H4 tetramer is the core of histone octamer, and the H2A/H2B is symmetrically placed on each side of the tetramer.

Core histone proteins contain approximately 75% of the total histone protein mass and are highly conserved proteins among the eukaryotes even from yeast to human. The remaining ~ 25 of the histones mass form the evolutionarily conserved

“tail” domains. (Fig. 1.4). These domains have gene regulatory function and are located at the N-terminal end of all four histones and generally defined by their sensitivity to proteases.

Figure 1-1. History of chromatin (2)

Figure 1-2. Types of compaction levels of DNA in Eukaryotic nuclei (4).

Figure 1-3. Schematic view of the nucleosome core particle (NCP) (6)

Octameric histones H3 (blue), H4 (green), H2A (orange) and H2B (red) and DNA (green) in the nucleosome. The histone tails are shown. 147-bp DNA wrapped around histone proteins.

On the basis of X-ray crystal studies of the nucleosome core particle, Zheng et al observed that (7) “the tail domains follow the minor grooves of DNA to the exterior of the nucleosome. The tails of histone H3 and H2B follow aligned minor grooves between adjacent DNA super-helical gyres, while those of H2A and H4 follow minor grooves over or under the top /bottom superhelical turns such that the bulk of the tail domains exit to the exterior of the nucleosome”. Therefore, they cannot be structurally located in the NCP crystal (7).

The recent investigations show that the histone tails do not contribute in establishing the structure of the nucleosomes significantly, but they play an essential role in regulating the condensation degree of chromatin into higher order structure (8). *In vitro*, removal of the histone tails results in nucleosomal arrays which cannot further condense to 30 nm fiber. Although histone tails have highly positively charge and generally interact with DNA- binding regions, however, their essential roles in tail- mediated chromatin folding might involve in histone-histone interactions and subsequently inter-nucleosomal aggregation (8). The tail and core of the histones are shown in Fig. 1.4 (adapted from (7)).

1.2 Post-translational modifications of histones

In eukaryotes, transcription works in the chromatin context. Some genes are active in all cell types, whereas others are active only in specific cell types. Active genes in higher eukaryotes have a chromatin structure characterized by a greater sensitivity (7- to 10-fold) to the endonuclease DNase I than the bulk of the chromatin

in the nucleus. This state precedes active transcription and marks “transcriptional competence” (7); it is the first step in the control of transcriptional activity. DNase I sensitivity is probably due to (partial) relaxation of the 30-nm filament toward the 10-nm filament state, which is probably facilitated by partial loss of linker histones and acetylation of the core histone *N*-terminal tails.

Histone proteins undergo a large number of post-translational modifications, such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP-ribosylation of lysine (K), arginines (R), serines (S), and threonines (T) (8). In the most cases, post-translational modifications have been seen in the N-terminal tails, but recently some modifications was found in histone-core domain as well (8). Histone modification is likely to control the function of the chromatin fiber, with different modifications yielding distinct functional consequences (8).

Figure 1-4. Schematic representation of the histone structural domains (7)

Tail domains of the core histone proteins are indicated. α -Helical regions found within the crystal structure of the nucleosome core are indicated as column and the three α -helices comprising the histone fold in each protein are indicated by the gray box. The red T's indicates the peptide bond closest to the histone fold domain susceptible to trypsin proteolysis in the nucleosome core. The vertical blue arrows indicate the approximate point where the tails exit either through (H2B and H3) or (H2A /H4) the DNA superhelical gyres to the exterior of the nucleosome.

1.2.1 Histone acetylation and the enzymes involved

One of the first-studied types of histone modification is acetylation. It was initially linked to transcriptional activation, and subsequently linked to such diverse processes as gene silencing, DNA repair, and cell – cycle progression (9). This particular modification is carried out by enzymes called histone acetyltransferases (HATs), which catalyze the transfer of an acetyl group from acetyl Co-A to the lysine

ϵ - amino groups on the N-terminal tails of histones (Fig. 1.5) (9). The enzymes that act to reverse this activity are known as histone deacetylase (HDAC).

Two main super-families of HATs have been identified: the GNAT and MYST families (9). In addition, a number of HATs were also identified which do not belong to the GNAT or MYST families. A list of the identified histone acetyltransferase are summarized in Table 1.1 (10). In mammals, the Gcn5 subclass of acetyltransferase is represented by two closely related proteins: GCN5 and P300/CREB associated factor (PCAF). The role of human GCN5 has been studied both, *in vitro* and *in vivo*, and it was found that Gcn5 can act as a transcriptional adaptor analogous to that of yeast Gcn5.

Figure 1-5. Representation of histone tail acetylation and enzyme involved (11)

The removal of acetyl groups from the lysine residue induces compaction of chromatin: their addition shows transcription. Abbreviations: HAT, Histone acetyltransferases; HDAC, histone deacetylase.

Table 1.1. List of identified histone acetyltransferases (10)

Group family	HAT	Organism	Complex	Possible function
Gcn5 family	Gcn5	Yeast	SAGA, SILK, SALSA, HAT-A2	Transcriptional activation
	GCNSL	Mammal/fly	STAGA, TFTC	Transcriptional activation
	PCAF	Mammal	PCAF complex	Transcriptional activation
MYST family	Esal	Yeast	NuA4	Transcriptional activation
	Sas2	Yeast	SAS-1	Anti-silencing
	Sas3	Yeast	NuA3	Transcriptional activation
	Tip60	Mammal	Tip60 complex	Transcriptional activation /DNA repair
	HBOI	Mammal	HBOI complex	Transcriptional activation
	MORF	Mammal	?	Transcriptional activation
	MOZ	Mammal	?	Transcriptional activation
	CLOCK	Mammal	?	Transcriptional activation
	NCOAT	Mammal	MSL	Transcriptional activation
	MOF	Mammal/fly	?	Dosage compensation
Others	HAT1	Yeast	Hat1/2 complex	Histone deposition
	ELP3	Yeast	Elangator	Transcriptional elongation
	Hpa2	Yeast	?	?
	Nut1	Yeast	Mediator	RNA Pol II transcription
	TAFa250(TAF1)	Yeast/mammal	TFIID	RNA Pol II transcription
	P300/CBBP	Mammal	?	Transcriptional activation
	hTFIIIC110	Mammal	TFIIC	Transcriptional activation
	hTFIIIC90	Mammal	TFIIC	Transcriptional activation
	ACTR/SRC-1	Mammal	?	Transcriptional activation
	ATF-2	Mammal	?	Transcriptional activation

The MYST family includes MOZ, Ybf2/Sas3, Sas2 and Tip60 proteins, and it is another group of evolutionary – related proteins with HAT activity. Although the genetic regions of MYST are similar in sequence, the sub-classes of the MYST family are observed in a wide range of regulatory functions in various eukaryotes. Tip60 (60 KDa protein) was the first human MYST protein to be discovered (9). The Tip60 histone acetyltransferase shares many properties with the tumor suppressor p53. Both proteins are involved in the cellular response to DNA damage. Overexpression of a dominant negative HAT-defective Tip60 mutant decrease both DNA repair and

apoptosis upon induction of DNA double – breaks. Tip60 seems to participate in transcriptional activation of specific genes through local histone acetylation, particularly H4 acetylation (9). Tip60 might interact with cell –cycle checkpoint proteins to activate an apoptotic pathway. Although recombinant expressed Tip60 can acetylate free histone proteins, but it is not observed that it has HAT activity *in vitro* with nucleosomal histones. Whereas *in vivo* Tip60 is able to acetylate both substrates (free and nucleosomal histones). Interestingly the Tip60 complex and its yeast homolog Esa1 acetylate *in vivo* histone H4 and H2A most efficiently. TRRAP is the largest subunit in Tip60 complex. It is a transcriptional regulatory protein, which was found in human GCN5 and PCAF complexes (9). MOZ (monocytic leukemia zinc finger protein) is a member of MYST family that participates in human leukemia disease (9).

MORF (monocytic leukemia zinc finger protein- related factor or MYST4) is a member of the MYST family of histone acetyltransferases. Recombinant full-length MORF protein can act as HAT to acetylate free histones especially H3 and H4. Furthermore, the insect-extracted protein was able to acetylate histone proteins in nucleosomes with preference for histone H4.

Also in human cells, two other complexes, TFIIC and TFIIC90, have been discovered that displays HAT activity. Purified extracted TFIIC protein can alleviate chromatin-mediated transcriptional repression *in vitro*. The TFIIC90 is a subunit of TFIIC that interacts with the RNA Polymerase III machinery and contains a histone-specific acetyltransferase activity (9).

1.2.2 Role of histone acetylation in transcription

The correlation between histone acetylation and transcriptional activity was proposed 40 years ago by Allfrey et al (12). In addition, transcriptional activation is generally correlated with histone acetylation, and repression is often associated with histone deacetylation. Several studies have proven that this correlation is not exclusive. Still, histone acetylation emerges as a central switch that allows inter-conversion between permissive and repressive chromatin structures and domains. (9). One major point which is still not clear is related to the role of histone acetylation in controlling gene expression (9). There are two possible (not mutually exclusive) explanations for the molecular mechanism underlying the effects exerted by this particular modification: histone acetylation changes the structure of the nucleosome and chromatin or histone acetylation provides a signal for protein binding (9). According to the first explanation, changes in the structure of the nucleosome, the addition of an acetyl group to the lysine ϵ - amino groups of histone N- terminal tails

neutralizes the positive charge of the lysine side chain. This modification may affect the interaction between the lysine residue and DNA, leading to weaker contact with the negatively charged DNA and subsequently loosening chromatin compaction (9). According to the second mechanism, the addition of an acetyl group to lysine residue creates a new surface for protein association, thus exerting its effects through gain of function mechanisms. Lysine acetylation creates docking sites for a protein module known as bromodomain, which has been found to be present in many transcription and chromatin regulators. For example in *S.cerevisiae* there are 10 bromodomain proteins; in drosophila about 16; and in humans more than 30 bromodomains are recognized (9). Several studies have led to the identification of at least 5 functions for bromodomains. However, whether all bromodomains are able to recognize acetyllysine is currently unknown. In addition, evidence has been reported of a bromodomain- containing protein, P300 that binds to histone in an acetylation-independent manner. One of the functions of bromodomains in gene regulation is chromatin acetylation by HATs. In *S.cerevisiae* the bromodomain of the acetyltransferase Gcn5 is required for the coordination of nucleosome remodeling. Another function of bromodomains is to link the activity of ATP-dependent chromatin remodelers to the acetylation of specific lysine. Selective recognition of acetylated histones by bromodomain containing proteins have been recently established in the intact nuclei of living cell by FRET analysis (9).

1.2.3 Histone deacetylases (HDACs)

Histone deacetylase (HDACs) in yeast cells have five groups including Rpd3, Hda1, Hos1, Hos2 and Hos3, but in higher eukaryotes, two families of proteins with HDAC activity have been identified which are histone deacetylase HDAC and the sirtuins. (13). The HDAC enzymes can be divided into classes I, II, III, and IV (Table 1.2) based on their homology. Mechanistically, they behave similarly and require an active zinc to mediate deacetylation catalysis (13). HDAC 1, 2, 3 and 8 belong to class I and are similar to Rpd3; this class of HDAC are localized in the nucleus. It has been found that members of class I (in human especially) are expressed in many cell lines and tissues.

The class II has homology with yeast Hda1. According to sequence homology and domain organization, class II is further divided into class IIa, encompassing HDAC 4, 5, 6, 7 and 9 and class IIb. Class II of HDACs acts as shuttle between nucleus and cytoplasm to transfer certain cellular signals and are expressed in a few number of human tissues.

Sirtuins proteins are recognized as class III HDACs, which are different from other classes both structurally and mechanistically. As for the mechanism regulating their activity, these enzymes have an absolute requirement for cofactor NAD⁺ (13). Whereas the HDAC enzymes are not able to bind to DNA directly, the classes I and II associated with multiprotein complexes like Sin3, NuRD and CoREST. Some of the histone deacetylases are summarized in Table 1.2 (10).

Table 1.2. A list of identified histone deacetylase (10)

Group Class	HDAC	Organism	Complex	Possible function
Class I	Hos1	Yeast	?	Transcriptional repression/activation
	Hos2	Yeast	Set3C	Transcriptional repression
	Rpd3	Yeast	Rpd3S, L	Transcriptional repression/activation
	HDAC1	Mammal	mSin3-HDAC, NuRD, Co-REST	Transcriptional repression
	HDAC2	Mammal	mSin3-HDAC, NuRD, Co-REST	Transcriptional repression
	HDAC3	Mammal	N-CoR-1	Transcriptional repression
	HDAC8	Mammal	?	Transcriptional repression
Class II	Hda1	Yeast	Hda1-associated complex	Transcriptional repression
	Hos3	Yeast	Homo dimer	?
	HDAC4	Mammal		Transcriptional repression
	HDAC5	Mammal		Transcriptional repression
	HDAC6	Mammal		Transcriptional activation/cell motility
	HDAC7	Mammal		Transcriptional repression/activation
	HDAC9	Mammal		Transcriptional repression
	HDAC10	Mammal		Transcriptional repression
Class III	Hst1	Yeast	Hat1/2 complex	Silencing
	Est2	Yeast	Elangator	Silencing
	Hst3	Yeast	?	Silencing
	Nst41	Yeast	Mediator	Silencing
	Sir2	Yeast	Sir4, RENT	Silencing
	SIRT1	Mammal	?	Transcriptional repression
	SIRT2	Mammal	?	Mitotic exit
	SIRT3	Mammal	?	Metabolic regulation
	SIRT4	Mammal	?	?
	SIRT5	Mammal	?	?
	SIRT6	Mammal	?	DNA repair
	SIRT7	Mammal	?	Pol I transcriptional activation
Class IV	HDAC II	Mammal	?	?

1.2.4 Role of histone deacetylation

Histone acetylation and deacetylation are dynamic events, and transcriptional activation can be considered a cyclic process that requires both activation and repressive epigenetic events (10).

The acetylation level in various promoters is regulated by specific combinations of HATs and HDAC; analyses performed over large chromosomal domains indicate that the state of acetylation is in a continuous genome- wide flux. It has not been clarified if the function of histone acetylation is based on the charge effects, or because of the acetyl group (as well as other epigenetic modifications) creates signals that start the binding of proteins responsible in chromatin remodeling and/or transcription initiation. Most probably both mechanisms are used *in vivo*.

Yeast and some multicellular organisms use similar molecular approaches to connect histone acetylation and genes transcriptional regulation but in multicellular organisms some difference was observed between permissive and restrictive chromatin (13).

One of the best characterized genes in terms of histone acetylation and promoter accessibility is *Saccharomyces cerevisiae* *adh2*. This gene, coding for the enzyme alcohol dehydrogenase II, is tightly regulated by glucose and becomes active when the glucose concentration of the medium is lowered or in the presence of Rpd3 and Hda1 histone deacetylase, the structure of the TATA box- containing nucleosome is destabilized. This acetylation-dependent chromatin remodeling facilitates the recruitment of both transcriptional coactivator Adr1 and faster kinetic parameters for ADH2 mRNA accumulation, but it is not the only factor that determined the binding of the TATA box-binding protein. On the contrary, in the absence of Gcn5, chromatin remodeling and mRNA accumulation occurring after depression is less efficient (13).

1.2.5 Model for how histone acetylase and deacetylase selectivity affect gene expression

Although histones are associated with virtually all eukaryotic genomic DNA sequence, histone acetylase and deacetylases do not universally affect the transcription of all genes. In yeast cell, Gcn5 and Rpd3 are important for the transcription of a small subset of genes; expression of most genes is unaffected in Gcn5- or Rpd3- deletion strains (14). These observations are in accord with the fact that histone acetylation patterns are not uniform in eukaryotic chromosomes. There are three basic models to account for how histone modifying activities can affect the transcriptional activity in a gene-specific manner (Fig. 1.6).

In the untargeted model (model 1) the histone modifying activities operate on a genome – wide basis with minimal specificity for particular chromosomal regions. In this case, selective gene expression would arise from promoter-specific responses to a generally altered chromatin structure. For example, promoters whose DNA sequences inherently cause nucleosomes to be tightly packed or preferentially positioned over binding sites for transcription factor might be particularly sensitive to acetylation status (14).

Thus, histone acetylases that function by general promoter targeting might be part of the mechanisms that accounts for the long – standing and general correlation between an active chromatin structure and gene transcription.

Figure 1-6. Models for how histone acetylation selectively affects transcriptional activity (14)

For each model, sites of acetylation (green Ac) are indicated with respect to five promoters (arrows) that either are (blue) or not (red) affected by acetylation. In model 1, histone acetylases are not targeted, and histone acetylation occurs at promoter and nonpromoter regions. In model 2, histone acetylases are generally targeted to promoters (arrows), because of their association with a general component of the Pol II transcription machinery. For both model 1 and 2 selective effects on transcription are attributable to inherent differences in the promoters with respect to the state of histone acetylation. In model 3, histone acetylase is targeted to specific promoters by gene-specific activator proteins, leading to selective effects on transcription.

In model 2, the histone modifying activity is selectively targeted to promoter regions, in a manner that is relatively nonspecific for individual genes (Fig. 1.6).

The gene-specific targeting model (model 3) involves recruitment of a histone modifying activity to particular promoters by sequence-specific, DNA-binding proteins (Fig. 1.6). The best-studied for such gene-specific targeting is transcriptional repression by Sin3-HDAC/Rpd3 histone deacetylase complexes (14).

This repression mechanism appears to be conserved from yeast to mammals, and the supporting arguments for this mechanism derive and benefit from complementary experimental approaches and observations. First, the yeast and mammalian histone deacetylase complexes specifically interact with DNA-binding repressor proteins (e.g. Mad, YY1, Ume6) or their associated corepressors (NCor, SMRT). Second, mutational analysis indicates that the ability of these proteins to interact physically with histone deacetylase complexes is strictly correlated with the ability to repress transcription target genes *in vivo*. Third, artificial recruitment of histone deacetylase complexes to promoters via heterologous DNA-binding domains is sufficient to mediate transcriptional repression; in mammalian cells, this repression is sensitive to histone deacetylase inhibitors. Fourth, yeast Rpd3 deacetylates histone *in vivo* and histone deacetylase activity is important for repression.

1.2.6 Histone methylation

Histone methylation was discovered about 40 years ago (15). Histone methylation is a transfer of methyl group to lysine or arginine residues of histones results in transcriptional activation or silencing. Lysine can be methylated with one, two, or three methyl group. Rea et al. found that many (but not all) mammalian proteins that contain a SET (suppressor of variegation, Enhancer of Zeste, and Trithorax) domain are histone lysine methyltransferases (16). The closely – related SET domain proteins Suv 39h1 and Suv 39h2 were shown to be essential for normal histone methylation within heterochromatin, deletion of Suv 39 h1 and Suv 39h2 genes reduced net methylation of histone H3 lysine (H3 K9) by approximately one-half, and in with- type cells Suv 39h1 was seen to colocalize with the polycomb group protein HP1 at heterochromatic regions of interphase nuclei (17).

Phenotype of mice that lack a second H3 K9 methyltransferase that acts in the euchromatic compartment. Loss of this histone methyltransferase (G9a) reduced total levels of H3-K9 methylation to approximately one-eighth of wild type and residual H3-K9 methylation was localized to pericentric heterochromatin, where histones are under methylated in the Suv 39h null mutant. G9a – deficient mice showed defects in Lys9 methylation at euchromatic sites and suffered a more severe phenotype than did suv39h null mutants. In drosophila, H3K9 methylation demonstrates an intimate association both with cytological heterochromatin and the heterochromatin binding

protein HP1. It has been shown recently that the HP1 chromodomain specially binds histone H3 N-terminal tails when those tails are methylated at H3 Lys9. (15)

It has been suggested previously that two distinct modification states were interchanged by means of a switch between H3K9 methylation and H3 Ser10 phosphorylation. It may be that H3 Lys9 methylation mediates transcriptional modulation in euchromatin regions, whereas its role in heterochromatin is related to chromosome structure. An interesting question that stems from this distinction in function is how two different H3 Lys 9 methylation events might be distinguished. One potential difference might be the ability of G9a to also methylase H3 Lys 27 *in vitro* (17).

1.2.7 Lysine methylation and acetylation of histones

The studies showed that enzymatic acetylation of the ϵ -amino group in conserved lysine amino acids in the N- terminal tail of H3 and H4 histones reduce the positive charge. Moreover, the recent discoveries suggest that histone acetylation may change the structure of histone tails via increasing their alpha-helical content (15). In contrast, methylation does not alter the overall charge of the histone tails; however, addition of methyl group (mono-, di- or tri-) increases its basicity and hydrophobicity. (Fig. 1.7) (15).

In contrast to acetylation, it has been found that the methylation has an irreversible effect on chromatin structure and function. Some investigations show that histone methylation to be biochemically stable and irreversible. In other words, methylation could be a dead-end modification (18,19). In some organisms LSD1 was reported as nuclear homolog of amine oxidases functions as a histone demethylase (20). However in recent studies, it has been observed a mechanism for methylation (similar to acetylation) that this process occurs on specific residue at specific promoters (15).

Conserved lysine residues located in N-terminal of histone proteins H3 and H4 are the major targets for post-translational modifications in both acetylation and methylation; these modifications can also happen on the H2A and H2B tails. Acetylation of specific lysine amino acid in a desired residue is only catalyzed by specific histone acetyl transferase (HAT). Histone methylation is a non-random event *in vivo*, which is similar to acetylation. In this process specific lysines are methylated by specialized histone methyl transferase (HMT). Histone methyl transferases are similar to HATs, have their own kinetic parameters, catalytic site, as well as histone and lysine as substrate (15).

Figure 1-7. Histone methylation versus histone acetylation (15)

(a) Molecular structure of lysine, acetyl lysine and methyl lysine. Conserved lysine amino acid within histone N-terminal can be modified by acetylation or methylation. Acetylation is catalyzed by HAT and the removing of acetyl group from histone tail residue is catalyzed and regulated by histone deacetylase (HDACs). As long as binding of acetyl to the ϵ - amino group of lysine results in more acidic, hydrophobic residue. Addition of methyl group is catalyzed by HMTs but the demethylase enzymes which are responsible for removing of methyl group has not been completely identified (b) The acetyl and methyl post-translational modifications of lysine residues on N-terminal tails of human histone H3 and H4. In the picture conserved lysines are indicated in bold and their amino-acid position in the histone tail indicated by the numbers. The *in vivo* positioning sites for lysine acetylation and methylation are shown in the figure.

Histone acetylation takes place in whole of the cell cycle, but histone methylation is observed mostly in G2 phase. This phenomenon may be because of DNA replication and histone synthesis in S phase that chromatin required to be more opened. An example for synergistic effect of histones acetylation and methylation on chromatin unfolding is that hyper acetylated H4 is a preferential target of H3 methylation, that suggest these modification may act synergistically to increase transcription level in a unknown mechanism. Therefore it can be summarized that combinations of acetyl and methyl modifications of lysine in histone tail residue may have synergistic or antagonistic effects (15).

1.2.8 DNA methylation

DNA methylation is a transfer of a methyl group from cofactor, S-adenosyl methionine, to the 5-carbon of cytosine or 6-nitrogen of the adenine group. DNA methylation is a crucial part of X- chromosome and transposone inactivation (21,22), genomic imprinting (23) and chromosome condensation (24). It has been realized that DNA methylation changes the gene expression pattern in cell and suppresses the expression of viral and cancer genes. In addition, it was found that DNA methylation

prevents the expression from chromatin elements which are near to methylated region. (25). Methylation of cytosine at position C5 is the only well-defined epigenetic modification of DNA in mammals that its importance has been emphasized by increasing number of human diseases correlated to epigenetic abnormalities (26). A correlation between DNA methylation and human cancer was first demonstrated in 1983, when it was found that the genomes of cancer cells are hypomethylated compared to their normal cells (27). Hypomethylation in tumor cells is due to the loss of methylation from special regions of the genome, and this state results in genomic instability of tumor cells (27).

Molecular mechanisms for transcriptional repression by DNA methylation

The principle mechanism by which DNA methylation could interact with transcription regulation can be explained to prevent the binding of the transcriptional machinery and transcription factors to promoters (28). The second possibility is that specific transcriptional repressors are available that recognize methyl-CpG; either independently or together with other components of chromatin, and binding of these elements turns off transcription. It is believed that this mechanism is independent of DNA sequence itself. (28). Bird et al discovered two repressors MeCP1 and MeCP2 that bind to methyl-CpG without sequence specificity (29).

DNA methylation influences chromatin structure

A role for methylated DNA in transcriptional silencing has been suggested by several investigators. Keshet et al used the transfection of the methylated DNA into mammalian cells and observed a nuclease-resistant structure containing unusual nucleosomal particle complexes (30). These unusual nucleosomes migrate as large nucleoprotein on the agarose gel. These complexes are characterized by higher-order protein-DNA interaction and absence of abundant micrococcal nuclease cleavage points within the DNA. Felsenfeld et al reported DNA methylation does not influence the association of core histones with the vast majority of DNA sequences in the genome (31).

1.2.9 Histone phosphorylation

Phosphorylation is an addition of a phosphate group (PO_4^{2-}) to a protein molecule. In 1906, Phoebus et al. identified phosphate in the protein Vitellin (or phosvitin) (32) and in 1933, Lipmann et al. detected the phosphoserine in casein (33).

In contrast to the acetyltransferase that there is enough knowledge and large number of HAT enzymes, relatively little is known about the histone phosphorylation

and enzymes involved (34). The mechanism of contribution of phosphorylation to transcriptional activation is not well clarified. One of the possible proposed mechanisms for relationship between phosphorylation and open chromatin is the addition of negatively charged phosphate groups to histone tails neutralizes their positive charge and the result is reducing their affinity for DNA, but phosphorylation is not always correlated to chromatin de-compaction. For example, phosphorylation of H2A has also been observed to correlate with mitotic chromosome condensation, but it has been found that in histone H3 Ser-10 phosphorylation plays a key role to make open chromatin. The recent discovery is that mutation of H3 Ser-10 in *Tetrahymena* histones causes some abnormality in chromosomal condensation and defective chromosome separation during anaphase. Also some kinases have been found that they are similar to Ipl1/aurora kinases in yeast and nematodes, the NIMA kinase in *Aspergillus nidulans*, have been reported to regulate H3 Ser-10 phosphorylation (34).

Phosphorylation of H3 Ser-10 became interesting subject of research when it was found that this modification can be associated with chromosome condensation and segregation during meiosis. Recently, it was found that phosphorylation at Ser -28 and at threonine 11 also occurs in histone H3 that are associated with mitosis. The development of specific antibodies for phosphorylated histone H3 facilitated the discovery of mechanism of correlation between histone H3 phosphorylation and chromosome condensation which happens in the pericentric heterochromatin and spreads throughout the genome during the G2-M phase transition. Further analysis of histone H3 phosphorylation during mitosis shows that in general phosphorylation in mammalian and thermophila cells is relevant with gene activation, but in yeast phosphorylation of histone H2B may have a redundant role. Moreover in yeast, phosphorylation of H3 Ser 10 is not necessary for cell-cycle progression (35).

1.2.10 Correlation between phosphorylation and histone H3 acetylation

The Ser-10 amino acid in the N-terminal tail of histone H3 has been located in a region that also is subject to other post-translational modifications. Moreover, Lys-9 and Lys-14 acetylation has been linked with transcriptional activation, while methylation of Lys-9 amino acid leads to silencing genome and formation of heterochromatin. Thus, these kinds of modifications may affect the phosphorylation of Ser-10, Recent studies from *in vitro* and *in vivo* experiments approves this conclusion (35). In addition, recent investigation supported a relationship between acetylation and phosphorylation in histone H3 by a wealth of experimental data. Although acetylation and phosphorylation act as two modifications which had been found to be associated with active transcription, it has not been cleared whether they

occurred on the same N-terminal tail of histone H3 or they occurred in H3 molecules in different nucleosomes.

Using specific antibodies generated against phosphorylated and acetylated histone H3 tails has been proven that both phosphorylated and acetylated histone H3 can be observed *in vivo*. Also di-modified H3 isoform was observed in both *in vivo* and *in vitro*. Further experiments revealed that the yeast GCN5 histone acetyltransferase displayed a preference for binding to a portion of the histone H3 tail that is pre-phosphorylated at the Ser-10 position (35).

Taken together, these results suggest a synergistic mechanism of addition of each modifications to the N-terminal tails of histone H3 in the nucleosome particles at these promoters, whereby MAP-kinase signaling results in the phosphorylation of histone H3 at Ser 10 (35). This relationship between acetylation and phosphorylation is shown in Fig. 1.8

Figure 1-8. A schematic representation of the two pathways of histone H3 N-terminal modification during gene transcription activation (35)

(a) The synergistic effect between histone H3 phosphorylation and acetylation for gene induction. It was found that Lys-9 in histone H3 to be acetylated via GCN5 histone acetyltransferase (HAT) activity before phosphorylation at the Ser-10 position. (i) Result of MAPK signaling in the phosphorylation of histone H3 at Ser-10. (ii) Phosphorylation of Ser-10 in histone H3 N-terminal tail increases binding affinity for HAT activity of GCN5 that results in acetylation of H3 at the lysine 14 amino acid. (iii) The acetylation of H3 at lysine 14 position led to enhance in transcription of the associated genes. (b) The parallel independent pathway of histone modification. (i) Acetylation of histone histone H3 at lysine 9 and 14 is regulated by a equilibrium between endogenous HAT and histone deacetylase (HDAC) activity. (ii) Kinase activity coming from either MAPK-signaling or gene induction by phosphorylation of pre-acetylated lysine 9 and 14 in histone H3 tail. (iii) Transcription from the genes which associated with nucleosomes having di-acetylated, Ser-10 phosphorylated histone H3 N-terminal tail. Abbreviations: K, lysine; MAPK, mitogen-activated protein-threonine kinase; Ser, serine.

1.3 Histone tail importance in chromatin folding

The histone tails have a key role in regulation of chromatin compaction and transcription. The positive charge of histone provides the necessary force for internucleosomal interactions and screening DNA repulsion. Gene activation requires that related regions of chromatin undergo some changes like unfolding to allow access to transcription machinery. Acetylation of lysine amino acids of histone tails unfolds chromatin, most probably through a partial neutralization of charges on tail. Acetylation of histone tail, along with other types of post-translational modifications, provides a network for function of histone tail in regulating chromatin. Moreover, the histone tails play important roles in the higher order structure of chromatin into heterochromatin through fiber/fiber interactions.

To explain the role of the tail in chromatin folding Gaurav Arya et al. use the flexible -tail model of oligonucleosomes (36). The authors compared tail interactions /extensions for three folded states of the oligonucleosome: an extended oligonucleosome at 0.01 M monovalent salts, a moderately folded oligonucleosome at 0.02 M monovalent salts, and a highly compact fiber at 0.2 M monovalent salt. At low salt (0.01 M) the histone tail either remain free or interact exclusively with nucleosomal DNA or linker DNA. In this case internucleosomal interactions almost are nonexistent. At higher (up to 0.2M), electrostatic screening of linker DNA causes the tails to mediate internucleosomal interactions with linker DNA of adjacent nucleosomes (mostly through their ends) at the expense of internucleosomal interactions (36). With increasing the salt concentrations, the histone tail extends out from the nucleosome as a result of the electrostatic attraction between tail and DNA of neighboring nucleosomes. Although this model describes the mechanism of tail-mediated chromatin condensation (by tail bridging), it has recently been questioned by our laboratory since it cannot account for the attraction effects of multivalent ions in the electrostatics of chromatin folding (37). The presence of multivalent ions like Mg^{2+} or polyamines leads to folding of chromatin at low mM (Mg^{2+}) or μM (spermine⁴⁺) concentrations due to ion-correlation and ion competition effects not taken into account in the Debye-Hückel based ion screening model used by Araya et al. Instead, modeling the folding of chromatin necessitates the explicit presence of the mobile ions, due to added salt (37). Like-charged polyelectrolytes would be expected to repel each other, following simple electrostatics. Ion correlations (or charge fluctuations) is now well established as the mechanism, which induces DNA-DNA attraction when multivalent ions are added to DNA solution containing only monovalent salt. This mechanism of fluctuation induced dynamic correlation in the positions of the cations condensed on each polyanion, contributes to an attractive electrostatic force, which explains experimental condensation of DNA (38-40). This

mechanism is reminiscent of the well-known London dispersion interaction (the attractive van der Waals force) between two atoms, caused by fluctuation in the positions of the electron ion clouds. This effect is general and attraction between negative like-charged biopolyelectrolytes such as DNA, f-actin and filamentous virus particles, induced by the presence of multivalent ions (e.g. Mg^{2+} , Ca^{2+} , $\text{Co}(\text{NH}_3)_6^{3+}$ and polyamines) has been frequently documented (38-40). In the presence of multivalent ions, this mechanism is consequently expected to be an important driving force in attraction between DNA segments in chromatin folding and self-association. In older theories of chromatin condensation (see e.g. (41) the effects of increased salt on folding is simply described as an effect of increased screening (monovalent ions) or charge of the DNA being reduced by counterion condensation on DNA by multivalent ions like Mg^{2+} .

1.4 Recombinant nucleosomal array system as a model for chromatin

The histone tails are essential for maintenance of the higher-order compact folded structures of chromatin and for regulation of transcription and replication. The fact that the condensed structure of chromatin is induced by high monovalent salt or by divalent ions is indicative of an electrostatic mechanism (37,42). Experiments using small angle X-ray scattering have shown that isolated linker free NCPs display aggregation as a result of increased concentration of mono- and divalent salt present in solution and this compaction seems to be induced by presence of the charged histone tails, suggested to be due to tail bridging between different molecules. Mononucleosome is an incomplete model for chromatin, which contains extended periodic arrays of nucleosomes. The result of studies on mononucleosome may not always show the same behavior as in chromatin. In addition, the interactions of the core histone tails may be different in mononucleosomes compared to that in extended nucleosome arrays (43). For instance, the histone H2A C-terminal tail interacts differently with nucleosomes in the presence and in the absence of linker DNA. This has been motivated research on the compaction of chromatin using nucleosomal arrays.

In general, histone tail hyperacetylation is correlated with transcriptional activation while deacetylation is correlated with repression. Recently, much interest has been directed to the chromatin remodeling functions and misregulation of HAT and HDAC activity is implicated in cancer. Thus, a detailed investigation of the molecular interaction of histone tail mediated chromatin interaction is important for

relevance for understanding the mechanism of epigenetic control of normal gene regulation as well as pathological condition.

The first experiments on chromatin were done on extracted chromatin from chicken blood by Thomas and Butler (44). The authors purified chromatin from chicken blood, and then digested by micrococcal nuclease and exonuclease. The DNA and histone octamer was characterized. Seminal work, characterizing array folding, using analytical ultracentrifugation sedimentation velocity measurements was done by Hansen et al (45). In that work, the authors used chicken extracted histone octamer, and used 5S 12-172, 5S 12-190, 5S 12-207 DNA templates. Recent work by Hansen and co-workers, using recombinantly prepared nucleosome arrays, studied by sedimentation measurements, has demonstrated the importance of the histone tails in facilitating the folding and self-association of chromatin (46,47).

The effects of polyamines on array folding and self-association was first studied by Peterson and co-workers, who found that spermine⁴⁺ promoted chromatin condensation at submillimolar concentration in a manner dependent on the histone tails (48).

Using recombinant DNA technology, Richmond et al. constructed a plasmid which contains 25 tandem copies of a 154 bp fragment from the 5 S RNA gene of *L. variegates* in pXRC24 vector, then transformed into HB101 and grown the cells in 2×TY medium (49).

Huynh et al. used 12 and 19 copies of 200 bp repeats of the 601 high-affinity positioning sequences (50). The authors constructed 12×200-601 DNA and 19×200-601 DNA using the *AvaI* asymmetric restriction site between each repeat to achieve a tandem, a head-to tail arrangement. (“601” is the name of clone amplified high affinity DNA positioning sequence by Widom et al (51)). The authors reconstituted the DNA array using isolated chicken erythrocyte nuclei histones. The authors studied sedimentation velocity with the analytical ultracentrifuge. They measured sedimentation velocity at 58.8 S and 79.7 S respectively for 12×200-601 and 19 × 200-601 nucleosomal arrays in 1 mM MgCl₂. In 100 mM NaCl the authors got 55.6 S and 73.0 S for the 12-mer and 19-mer nucleosomal array respectively (50).

Dorigo et al. Studied the 12×177-601 DNA and reconstituted with recombinant histone octamer. The authors investigated the behavior of salt induced compaction by magnesium chloride on 12-mer nucleosomal arrays. A sedimentation velocity coefficient 53 S was reported in 1 mM MgCl₂ (1).

Shogren-Knaak et al. used 12×177-601 DNA reconstituted it with recombinant histone octamer. The authors determined the compaction degree of wild type nucleosomal array and determined the sedimentation coefficient at 53 S in 1mM MgCl₂ (52). The authors used histone H4 where the lysine at position 16 of the tail has been acetylated and at position 23 the arginine has been changed to cysteine. They used this modified histone for array reconstitution and found that it cannot form the 30 nm fiber, and the sedimentation coefficient decreased to 44 S at 1 mM MgCl₂. This demonstrates that the acetylation at lysine 16 of H4 abrogates the folding. The author used the K16Ac array in oligomerization experiment to compare with wild type nucleosomal array and found that acetylation on H4-K16 disrupts array oligomerization. They measured EC₅₀ for MgCl₂ in wild type and acetylated H4K16 at 1.75 and 2.75 mM respectively (52).

Hayes and co-workers studied 12×177-601, 12×207-601 and 12×208-5S arrays in both wild type and different K→Q mutations at lysines 5 and 9 for H2A; 5,12,15,24 and 27 for H2B and 27 for H3 and 5, 8, 12 and 16 for H4 as mimics of acetylation (53). The authors investigated the role of acetylation of specific histone tail domains on the stability of tertiary nucleosomal array structures by comparison of EC₅₀ induced by Mg²⁺ for wild type and various K→Q mutations. The authors assumed that glutamine resembles acetylated lysine in chemical structure and charge, so it can functionally replace this modification (53). This assumption was corroborated by showing that enzymatically acetylated samples (tetraacetylated on H4) behaved identically to the tetramutated H4 analogue. They observed that EC₅₀ increases with introducing K→Q substitution in nucleosomal array compared with wild type. However, the position of the K→Q mutation is also important, e.g. addition of six K→Q changes in H3 did not increase EC₅₀ induced by Mg²⁺ as much as addition of six K→Q mutations in H2B or four K→Q mutations in H4 (53).

Tremethick and co-workers worked with 12×208-5S and 12×200-601 arrays in wild type and with H2A-STT mutation (54). (neutralizing the amino acid residues D90, E91 and D92 of the negatively charged acidic patch on the core surface to STT). The authors determined the folding of arrays by sedimentation velocity experiments and measured the S-value for both wild type and H2A-STT arrays as 32S at low salt conditions, corresponding to extended ‘beads-on-a-string’ form. They observed that with increasing the Mg²⁺ concentration up to 0.9 mM, the H2A-STT is inhibited from full folding. They hypothesized that the H2A acidic patch is necessary for nucleosomal array folding. To investigate the role of this acidic patch in 30-nm fiber formation, Tremethick et al. assembled nucleosomal array in an *in vitro* model system using recombinant mammalian histones whose acidic patch residues were changed

(H2A.1, H2A-STT, H2A.Bbd, H2A.Bbd-EE, H2A.Bbd-EE/E). Either the above acidic patch of H2A was partially neutralized by mutation of acidic residue. The authors observed that H2A-STT arrays have a considerably less folded conformation at each concentration of MgCl_2 . They concluded that mutations in the acidic patch of H2A prevent the fully folding formation and the 30-nm fiber is not formed (54).

Robinson et al used 61×202-601 arrays to study the compaction behavior induced by MgCl_2 . The author used 30% acetylated H4 in K16 and H4-K16Q to investigate the effect of acetylation on nucleosomal array decondensation. The authors observed that, arrays containing 30% H4-K16Ac appears to disrupt folding to the same extent as the complete removal of H4 tail. The author found also that arrays containing the H4-K16Q did not give a profile like H4-K16Ac and it reached a level of compaction equivalent to the wild type array and proposed that acetylation of K16 modifies the secondary structure of the H4 N-terminal tail, causing a disruption of nucleosome-nucleosome interactions (55).

Jason et al studied the effect of ubiquitinated H2A on magnesium induced folding of nucleosomal array (56). The authors reconstituted 12-208-5S DNA with wild type and ubiquitinated H2A and measured sedimentation velocity coefficient induced by MgCl_2 in both wild type and ubiquitinated arrays. They observed that adding of ubiquitin to H2A had little effect on the ability of nucleosomal arrays to make higher-order structures in presence of 2M MgCl_2 . In other words, uH2A arrays oligomerize at lower MgCl_2 concentration than wild type arrays. The authors suggested that histone ubiquitination might increase nucleosomal fiber association.

Luger and co-workers investigated 12×208-5S nucleosomal array to study the salt-dependence of folding of wild type and H3-K56Q (57). The reason for selection of H3-K56Q is that lysine 56 acetylation is an abundant modification of newly characterized histone H3 molecules that participate during S-phase and DNA damage repair (58). Because the acetylation of H3K56 is associated with chromatin assembly and mostly occurred on free genomic regions, the authors investigated H3-K56Q for the condensation properties in both saturated and subsaturated nucleosomal arrays (57). The authors observed that in saturated nucleosomal array there is no distinguishable change in the oligomerization curve and EC_{50} for Mg^{2+} between wild type and H3-K56→Q, but in subsaturated array, the EC_{50} for Mg^{2+} of H3-K56Q array is significantly larger than wild type array (57). They also studied the folding of wild type and H3-K56Q nucleosomal array in both saturated and subsaturated forms and found that in saturated level, wild type and H3-K56Q in low salt condition have identical S-values, also observed that both wild type and H3-K56Q arrays at 1.75 mM MgCl_2 folded robustly as indicated by sedimentation velocity coefficient at 55 S.

With these data, the authors suggest that H3K56Q does not affect folding of nucleosomal arrays.

The work by Dorigo et al. advocates a two-start models in 30-nm fiber chromatin (59): the first and most common model is solenoid, which the nucleosomes coil around a central cavity with $\approx$ six to eight nucleosomes per turn, the second model is supercoiled and the third model is crossed-linker model (59). The authors constructed mutant versions of histone H2A, H2B, and H4 by site-direct mutagenesis in selected amino acid with cysteine for H2A, E56C, E61C and E64C; for H2B Q44C, V45C and E110C; and for H4 K20C, V21C, R23C and D24C. The authors prepared nucleosomal array by using of mutant histones and 12-mer DNA (59). The authors observed that the disulfide formation in this system requires array compaction, and found that from above mentioned mutant histones only H4-V21C/H2A- E64C can make cross-link. Sedimentation velocity showed that 12-mer array containing H4-V21C/H2A-E64C histones have a similar sedimentation velocity compared to wild type array (59). Also, the authors observed the cross linked arrays maintain as fully folded in lower divalent cation concentration as compared wild type arrays (0.5 mM v.s. 1.0 mM MgCl_2), indicates that the disulfide bonds making stabilize the folded state. With these data, the authors proposed that H4-V21 and H2A-E64 contribute to internucleosomal interaction in the folded fiber (59). Notably, H4-V21 and H2A-E64 belong to the H4-K16-R23 domain of one nucleosome and to acidic patch of the other and this contact is observed in crystal structure defined by Luger et al (6).

1.5 Biophysical methods

1.5.1 Dynamic light scattering (DLS)

The absorption of ultraviolet, visible, infrared, and microwave radiation provides detailed information about electronic, vibration and rotational energy levels of molecules and has in some instances enabled the chemist and to determine the structure of complex molecules. Recent advance in laser techniques have made possible the measurement of very small frequency shifts in the light scattered from gases, liquids, and solids. Moreover, because of the light intensities of laser sources, it is possible to measured even weakly scattered light. When light beam shines on particles, the electric field of the light induces an oscillating polarization of the electrons in the molecules. The molecules then act as secondary sources of light and subsequently scatter the light. The frequency of scattered light changes depends on size, shape structures. Some parameters like angular distribution, polarization, and the intensity of the scattered light can be in the scattering material (60).

Dynamic Light Scattering (DLS), is also known as Photon Correlation Spectroscopy. This technique is one of the most popular methods to determine the size of particles. Commercial particle size determining systems mostly work at only one scattering angle (90°) and use red light (600 nm). Although commercial DLS is able to distinguish whether a protein is monomer or dimer, but commercial light scattering has less accuracy compared to sedimentation velocity for distinguishing small oligomers. Dynamic light scattering is able to analyze solution containing broad distributions of widely differing molecular masses (for example a native protein and various sizes of aggregates), and also able to detect very small amounts of the higher mass species. The theory of light scattering can be developed on the basis of quantum field theory. The major results of this theory differ little from a classical theory of light scattering.

According to the semi-classical light scattering theory the first assumption is that the particles are in Brownian motion. In this situation the probability density functions is given by the formula:

$$P(r,t|0,0)=(4\pi Dt)^{-3/2} \exp (- r^2 / 4Dt)$$

Where D is the diffusion constant

The second assumption is that the particles used in the experiment, are spherical particles. If it is so, then it is possible to apply the Stoke-Einstein relation and hence have a formula that easily gives the diffusion constant:

$$D=k_b T/6\pi\eta a$$

Where a is the radius of the particles, k_b is the Boltzmann constant, T is the temperature in Kelvin degrees and η is the viscosity of the solvent.

DLS set up is illustrated below. The laser light passes through a collimator lens and transmits into the cell having the solution. The scattered light is detected and analyzed by a photomultiplier which transforms a variation of intensity into a variation of voltage.

Because in solution the particles are in Brownian motion and move independently one from the other, the frequency spectrum of the intensity of the scattered light will have the form of a Lorentzian shaped line whose width depends on D and the scattering angle:

$$S(\omega) = \Gamma(\Theta) / [\omega^2 + \Gamma^2(\Theta)]$$

Where $\omega = 2\pi f$ with f the roll-off frequency and

$$\Gamma(\Theta) = 2D(1 - \cos\Theta)$$

Where λ is the wavelength of the incident light, n is the index of refraction of the solution and Θ is the scattering angle.

1.5.2 Analytical ultracentrifugation (AUC)

Sedimentation velocity analytical ultracentrifugation has a long history for the characterization of synthetic polymers, biological macromolecules, and viral particles and can give rich information about the purity, molar mass, state of association, protein interactions, hydrodynamic shapes, conformational changes, size-distributions, among other properties of proteins. It is based on a conceptually very simple principle of applying a gravitational force to the protein solution and observing the resulting changes in the concentration distribution. The hydrodynamic resolution is typically significantly higher than that of diffusion-based techniques, due to the mass dependent gravitational driving force of migration. Diffusion spreading of the sedimentation boundary makes both the hydrodynamic frictional coefficient and the

molar mass experimentally accessible quantities. The coupled diffusion and sedimentation process is described by the Lamm equation, and significant progress in SV was stimulated in the last decade by the ability to efficiently solve this partial differential equation and model experimental data for non interacting and interacting macromolecular mixtures (61).

Theory

A particle of mass M in a centrifugal field generated by a spinning rotor is subjected to a centrifugal force. If a field (say, a centripetal field caused by spinning the sample at an angular frequency, ω) is applied, the molecule quickly accelerates and very soon reaches its terminal velocity. The situation in the sector-shaped analytical ultracentrifuge cell looks like this:

Attainment of terminal velocity signifies a balance of forces (a molecule feeling a net force would accelerate). The balance between centripetal, drag and buoyant forces is expressed:

$$F_{total} = F_d + F_b + F_c = 0$$

$$F_c = \text{centripetal force} = \omega^2 r m$$

Where m is the particle mass and $-\omega^2 r$ represents the centripetal acceleration.

$$F_d = \text{viscous drag force} = -f \cdot v$$

where f is the translational friction coefficient and v the linear velocity

$$F_b = \text{buoyancy force} = -\omega^2 r m_o$$

Where m_o is the mass of solvent displaced by the particle

Thus:

$$\omega^2 r(m - m_o) - f \cdot v = 0$$

The displaced volume could be taken as the product of the mass and the partial specific volume: $m_o = m \tilde{v}_2 \rho$. The force balance equation becomes:

$$\omega^2 r m (1 - \rho \tilde{v}_2) - f \cdot v = 0$$

The particle mass is the molar mass divided by Avogadro's number, $m = M/N_a$, and we define the sedimentation coefficient, S , to be the velocity obtained divided by the angular acceleration applied, v is the volume in mL and r is the density of the solvent in grams per mL (62).

$$S = \frac{v}{\omega^2 r} = \frac{M (1 - \rho \tilde{v}_2)}{N_a f}$$

- S has units of s/radian². One doesn't usually worry about the radian²
- 1×10^{-13} s is called a *Svedberg*, and given the symbol S
- Many biological components are identified by their sedimentation coefficients. Example: 4S RNA has $S = 4 \times 10^{-13}$.
- S measurements are often quoted as if they were made at 20°C in water, even if they were not. The correction recognizes that f is proportional to viscosity, η , and the $1 - \rho \tilde{v}_2$ term also has to be adjusted (62).

$$S_{20,water}^o = S_{T,solvent}^o \frac{\eta_{T,solvent}}{\eta_{20,water}} \frac{1 - \rho_{20,water} \tilde{v}_{20,water}}{1 - \rho_{T,solvent} \tilde{v}_{T,solvent}}$$

In studies of nucleosome array compaction induced by multivalent cations, an increase in the S value is observed. Since the molecular weight of the array is not changed (assuming no aggregation), it means that the frictional coefficient decreases due to the compaction of the particle.

Measurement of sedimentation velocity coefficient, S-value

Centrifuge and rotor

The Optima XL-I ultracentrifuge is readily convertible between analytical and preparative configurations. The induction drive is equipped with a dynamic damping capability that reduces rotor precession to near zero. Rotor temperature is monitored by radiometry and regulated by thermoelectric modules. This system provides rotor temperature stability to within 0.13°C at equilibrium. The titanium rotor is designed to run double-sector sample cells, and includes a counterbalance with reference holes for radial calibration (62).

Optical absorption system

A xenon flash lamp serves as the light source, providing a usable wavelength range of 190-800 nm. The lamp is fired as the sector of interest passes over the detector, with the timing regulated by monitoring the passage of a reference magnet in the bottom of the rotor. The maximum firing rate is 100 flashes per second, corresponding to once per ten revolutions at 60,000 rpm. The optical system has been shown in Fig. 1.9

Figure 1-9. Schematic representation of the absorbance optical system of the Optima XL-I.

An experimental data set (Fig. 1.10) consists of radial scans (Absorbance vs. radius) acquired at several times during the sedimentation (62). Sedimentation velocity experiments are normally of a type called *boundary sedimentation*. The experiment begins with the sample mixed uniformly throughout the cell, so that a plot

of concentration vs. radius is a horizontal line ($C(r) = \text{constant}$). As sedimentation proceeds, molecules are depleted from the top of the solution column. This results in the formation of a trailing boundary for the concentration distribution. Analysis of the boundary curves with appropriate software enables the extraction of the sedimentation distribution curves of the boundary (see below).

Figure 1-10. Typical data set for sedimentation velocity.

The figure shows typical data from a boundary sedimentation experiment in the Optima XL-I. The data presents the absorbance in the sample sector compared to the reference sector of the double-sector cell. Sharp peaks result from the refraction of light away from the photomultiplier by the menisci in each sector. The sedimentation coefficient is determined from the movement of the boundary region with time

The XL-I is a dual-beam instrument; incredibly, it can distinguish absorbance from the reference half of its dual cell and the sample half, even though both are within 1 cm of each other and spinning at up to 60,000 RPM. The sharp, spikes (around 6 cm) are due to the menisci of reference and sample compartments.

Several key features of the data are pointed out in the Fig. 1.10 The sample compartment of the cell is normally filled with slightly less liquid than the reference compartment, so the inner meniscus is that of the solvent compartment and the outer meniscus is that of the sample compartment (62). Normally, the sample's concentration can be varied from $0.05 \text{ OD} < c < 1.5 \text{ OD}$. Obviously, the solvent component should interfere with the optical detection, and the reference buffer must be matched in composition with the sample.

1.6 Objectives and research design

The general objective of my thesis is to obtain a principal understanding of the interactions of counterions, multivalent ions and charged ligands with nucleosomal array in higher structural order. We would like to study the electrostatic and molecular mechanism for inducing and stabilizing nucleosomal array folding and self-association and how it is affected by posttranslational modifications in the histone tails.

Within the project the following issues are addressed:

i) To study the relationship between charge neutralization by multivalent ions (K^+ , Na^+ , Mg^{2+} , Ca^{2+} , cobalt(III)hexammine $^{3+}$, spermidine $^{3+}$ and spermine $^{4+}$) and nucleosomal array inter- and intra- molecular interactions. Studies *in vitro* suggests that the folding of the array of nucleosomes to a condensed 30-nm secondary structure chromatin fibre, as well as further inter-array oligomerization to tertiary chromatin structure, occurs as salt dependant condensation (63,64) indicative of an electrostatic mechanism (41,65). Sedimentation velocity studies of model nucleosomal arrays have been focused on investigating the effects of removal of tails on the condensation of these model chromatin fibres. In particular, H4 tail internucleosomal interactions have been implicated (1,46,66) and a work pointed to the acetylation of the H4 lysine 16 as being crucial for inducing array folding (52). The study of the effect of different cations of varying charge is relevant for two reasons. Firstly by establishing the general sensitivity of these condensation processes to cation charge, the polyelectrolyte nature of of chromatin condensation can be investigated (i.e. the effects due to non-specific electrostatic interactions). Secondly, the ions Na^+ , K^+ , Mg^{2+} and Ca^{2+} are prevalent in the cell and it is of great interest to investigate whether ion specific effects are at hand. Polyamines are ubiquitous in the cell and their effects on chromatin structure are not well established. In the living cells, K^+ and Na^+ are the most dominant cations (67). The cells usually use energy to keep the gradient of Na^+ and K^+ between inside and outside the membrane. The intra- and extracellular concentrations of mono and divalent cations is shown in the table below.

Table 1.3. Biological concentration of cation in the cell (67)

Ion	Extracellular concentration, mM	Intracellular concentration, mM		Separation factor
		total	free	
Na ⁺	140-150	5-15	5-10	D ^K Na>600
K ⁺	4-5	140-160	100-120	
Ca ²⁺	1-2	1-2	0.0001	D ^{Mg} Ca>20-40
Mg ²⁺	1-2	10-400	0.5	
Cl ⁻	110	5-15	5-10	

ii) We take advantage of a novel approach to the native chemical ligation (NCL) method for preparation of histones with site- specific modifications at the N-terminal tails, which can be applied to design any tail modification at any position, to prepare large amounts (tens of mg) of nucleosomal array with desired modified histone for biophysical studies. In the project, we use peptide synthesis of the H4 histone tail with specific acetylation of one or more lysines, e.g. fragment of amino acid 1-20 of the H4 tail with H4-K16 acetylated was synthesized followed by ligation to the core histone H4 (68).

iii) To study the relationship between post-translational modifications of amino acids in the histone H4 N-terminal tail on chromatin condensation. We aim to characterize the effects of modifications that alter the histone tail charge (e.g. lysine acetylation), on the condensation of reconstituted nucleosomal array and how this is affected by salt solution conditions. We use reconstituted nucleosomal array and intramolecular aggregation determined by analytical ultracentrifugation velocity measurement. Intermolecular aggregation was measured by precipitation assay (PA). Such investigations enable the characterization of the electrostatic and molecular aspects of chromatin condensation.

The experimental part of the project consists of the following steps:

1. Preparation of wild type nucleosomal array.

a) Preparation of DNA template. 12-mer DNA template of high affinity 601 sequence (1,51) amplified and purified using protocols described in (6,69,70).

b) Preparation of recombinant wild type histone proteins. The H2A, H2B, H3 and H4 histones were overexpressed, separated and purified by gel filtration and ion exchange

chromatography (69). Histone octamer was refolded from pure individual histone proteins.

c) Reconstitution of wild type nucleosomal array. Step-wise salt dialysis method was used to reconstitute of nucleosomal array using pure DNA and histone octamer (1).

2. Study intra/inter molecular aggregation of the wild type nucleosomal array.

Compaction (intramolecular aggregation) of the nucleosomal array induced by different ions and charged ligands (Na^+ , K^+ , Mg^{2+} , Ca^{2+} , CoHex^{3+} , Spd^{3+} and Spm^{4+}) is studied by analytical ultracentrifugation sedimentation velocity (AUC) measurement. Inter-array association is studied by precipitation assay monitoring aggregation of the arrays as a function of solution conditions.

3. Preparation of nucleosomal array containing K→Q mutation in the H4 histone

Large quantities of the histone H4 with K→Q mutations at the positions K16, K5+K8+K12 and KK5+K8+K12+K16 are prepared by site-directed mutagenesis. Arrays with mutated H4 histone are prepared and studied in a way similar to the wild type array (See points 1-2 above).

4. Preparation of nucleosomal array containing acetylated lysine in H4 tail

Peptides containing the residues 1 to 19 of H4 with selected acetylation(s) were synthesized by Yang Renliang in the laboratory of Dr Liu Chuan-Fa, using manual solid-phase peptide synthesis employing Boc/TFA strategy. Truncated H4 with residues 20 to 102 and lysine 20 → Cys “(H4 (20-102) K20C)” has been transformed into *E. coli* BL 21 (DE 3), expressed and purified. Finally truncated H4 (20-102) K20C was ligated to the synthesized peptide using native chemical ligation reaction and the products were purified by HPLC. Acetylated histone octamer was refolded from acetylated H4 and wild type H2A, H2B and H3. Nucleosomal array was reconstituted from acetylated histone octamer and DNA. Compaction and aggregation of nucleosomal array containing acetylated histone have been studied by AUC and precipitation assay.

2. Materials and Methods

Materials

2.1.1 Chemicals

All the chemicals used in the current study were of at least analytical grade.

Chemical	Supplier
Acetic acid	Merck
Acrylamide	Bio-Rad
Agar granulated	Difco
Amberlite	Merck
Ampicillin, Sodium salt	USB
Bacto tryptone	Difco
Boric acid	Sigma
Bromphenol blue sodium salt	RdH
Calcium chlororide anhydrous	Sigma
Chloramphenicol	Sigma
Chloroform	Fluka
Cobalt hexammine	Sigma
Coomassie brilliant blue R250	Bio-Rad
D(+)-Glucose	Sigma
D(+)-Sucrose	Sigma
DTT	Gold Biotechnology
Ethanol	Merck
Ethidiumbromide	Bio-Rad
EDTA	USB
Glycerol	USB
Glycine	Bio-Rad
Guanidine hydrochloride	Sigma
Hydrochloric acid	Merck
Isoamyl alcohol	Sigma
Isopropanol	Merck
IPTG	Gold biotechnology
Magnesium chloride	USB
Phenol	USB
PEG 6000	Fluka
Potassium acetate	Sigma
Potassium chloride	Merck
Sodium chloride	USB
Sodium acetate	Sigma
Spermidine	Fluka
Spermine	Sigma
TEMED	Bio-Rad
Urea	USB
Yeast extract	Difco

2.1.2 Buffer recipes

Buffer	Composition
Alk. Lysis buffer I	50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0
Alk. Lysis buffer II	200 mM sodium hydroxide, 1% SDS
Alk. Lysis buffer III	4 M potassium acetate, 2 M acetic acid
HO unfolding buffer	7 M guanidinium HCl, 10 mM Tris-HCl (pH7.5), 10 mM DDT
Refolding buffer	2 M NaCl, 10 mM Tris-HCl (pH7.5), 1 mM EDTA, 5 mM beta- mercapto-ethanol
SAUDE 1000	7 M Urea, 20 mM sodium acetate, 1 M sodium chloride, 5 mM 2-mercaptoethanol, 1 mM EDTA,
SAUDE 200	7 M Urea, 20 mM sodium acetate, 200 mM sodium chloride, 5 mM 2-mercaptoethanol, 1 mM EDTA,
S ₂₀₀ unfolding buffer	7 M guanidinium HCl, 20 mM sodium acetate (pH 5.2)
SDS-PAGE (10X)	Tris-base 30 g, glycine 145 g, SDS 10 g and water up to one litter. pH: 8.0
TAE (50x)	Tris base 242 g, 57.1 mL acetic acid, 100 mL EDTA (0.5 M), and water up to one litter. pH: 8.0
TBE (10x)	Tris base 108 g, Boric acid 55 g, 40mL EDTA (0.5 M), water up to one litter. pH: 8.0
TEK2000	2 M KCl, 50 mM Tris pH 8.0, 1 mM EDTA
TEK	10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 10 mM KCl
TK	10 mM Tris-HCl (pH 7.5), 10 mM KCl
TES100	100 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA
TE10/50	10 mM Tris pH 8.0, 50 mM EDTA
TE10/0.1	10 mM Tris pH 8.0, 0.1 mM EDTA
Washing buffer	50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM 2-mercaptoethanol

2.1.3 Gel recipes

Gel	Recipe
APAG (for 12 mL)	Agarose 0.12 g, 60 μ L TAE (50x), 390 μ L Acrylamide(40%), 390 μ L Bisacrylamide (2%), 60 μ L APS 10%, 11.08 mL deionized water
10% DNA-PAGE (for 5 mL)	1 mL TBE (5x), 1.25 mL Acrylamide (40%), 425 μ L Bisacrylamide (2%), 50 μ L APS 10%, 5 μ L TEMED, 3.86 mL deionized water
5% DNA-PAGE (for 5 mL)	250 μ L TBE (5x), 625 μ L Acrylamide (40%), 210 μ L Bisacrylamide (2%), 50 μ L APS 10%, 5 μ L TEMED, 2.27 mL deionized water
18% SDS-PAGE 3 mL for resolving 2 mL for stacking	Resolving gel: 750 μ L Tris-HCl 1.5 M (pH 8.8), 1350 μ L Acrylamid (40%), 450 μ L Bisacrylamide (2%), 30 μ L SDS 10%, 30 μ L APS 10%, 3 μ L TEMED, 387 μ L deionized water Stacking gel: 500 μ L Tris-HCl 0.5 M (pH 6.8), 250 μ L Acrylamid (40%), 250 μ L Bisacrylamide (2%), 20 μ L SDS 10%, 20 μ L APS 10%, 2 μ L TEMED, 958 μ L deionized water

2.1.4 Media recipes

Media	Recipe
TB	12 g/L bacto tryptone, 24 g/L yeast extract, 8.5 mL/L KH_2PO_4^* (1M), 36 mL/L K_2HPO_4^* (1M) and 4 mL/L glycerol* (50%) *: must be autoclaved separately and add into the flask media over a laminar hood
TYE Agar	10 g/L bacto tryptone, 5 g/L yeast extract, 8 g/L sodium chloride and 15g/L Agar
2x TY	16 g/L bacto tryptone, 10 g/L yeast extract, 5 g/L sodium chloride and 10 mL/L filter-sterlized glucose (10%)

2.2 Methods

The schematic procedure for preparation of nucleosomal array that is comprised of DNA preparation and histone proteins preparation is shown in Fig. 2.1.

Figure 2-1. Diagram of preparation of the 12-mer nucleosomal array

2.2.1 DNA preparation

Plasmid with template of 12-mer DNA (12×177-601) was gift from Prof. Richmond, ETH Switzerland, and has been transformed into the *E. coli* HB101 competent cells. (The sequence of 601 construct is shown in appendix I) The cells were plated and incubated at 37°C for 10-18 hours on TYE plates containing ampicillin to select the presence of pWM530 plasmid. 50 mL of 2x TY (100 µg/mL ampicillin) was taken as pre-culture and was inoculated with a transformed HB101 colony from the TYE transformation plate and grown at 37°C until the medium become turbid. Subsequently 15 flasks each containing 500 mL of TB medium having ampicillin was inoculated 3-4 mL of pre-culture and incubated in a shaking incubator at 37°C, 200 rpm for overnight. The cells were centrifuged for 7 min at 6,000 g and 4°C. The pellet was resuspended in 60 mL lysis buffer I. After resuspension, 120 mL of lysis buffer II was added and solution was vigorously shaken for two min followed incubation on ice for 20 min. 210 mL of cold lysis buffer III was added and mixed with inverting for 2 min and incubated in ice for 20 min. Then the lysed cells have been centrifuged at 10,000 g for 20 min at 4°C. The supernatant has been filtered through sterile gauze. DNA was precipitated by adding 0.52 volume of isopropanol to the supernatant and incubated for 30 min at room temperature. Lysed cell solution was centrifuged at 16,000 g for 30 min at 4°C. The supernatant was discarded and the pellet was dissolved in 20 mL of TE 10/50 and thereafter 120 µL RNase A (10 mg/mL) was added to digest the RNA contamination and incubated overnight at 37°C, 200 rpm.

After RNA digestion, DNA solution was centrifuged in 10,000 g at 20 °C to remove insoluble components. The supernatant was washed with 10 mL equilibrated phenol and centrifuged at 27,000 g at 20°C. The phenol extraction was repeated, and then the aqueous phase was washed with chloroform-isoamyl alcohol (CIA) to remove traces of phenol and centrifuged at 12,000 g at 20°C.

DNA was precipitated by 0.2 volume of 4 M sodium chloride and 0.4 volume of 40% PEG6000 was added to aqueous phase and incubated for 30 min on ice, then centrifuged at 4°C for 10 min at 27,000 g. The pellet was dissolved in TE 10/0.1 and washed with CIA as described above. DNA was precipitated again by addition of 0.1 volume of 4M sodium chloride and 2.5 volumes of cold ethanol to the aqueous phase, incubation on ice for 30 min, spinning down in 27,000g for 30 min at 4°C. After this, the pellet was dissolved in TE10/0.1 and the DNA concentration was measured using UV spectrophotometer at 260 nm (71).

Gel filtration chromatography was used to remove RNA and other impurities from the plasmid DNA, (ÄKTA FPLC, Uppsala, Sweden) Sepharose 6 column was equilibrated in TEK2000 and the plasmid DNA (dissolved in TE10/0.1) was loaded into the column. The first peak containing pure plasmid was collected. First peak was pooled and precipitated by 0.5 volume of isopropanole, then centrifuged at 27,000 g and 4°C for 30 min.

2.2.2 EcoRV digestion and purification of 12-mer DNA

The EcoRV reaction mixture consists of 1 mg/mL plasmid DNA, 50 mM Tris-HCl (pH 7.5), 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM DDT and EcoRV enzyme at 400 units per each mg of plasmid. First small scale of digestion has been done at 10 µg of plasmid DNA to check the quality of digestion on 10% DNA-PAGE gel. Then digestion was repeated on a large scale in batches of 30 mg. Reaction mixture for EcoRV digestion was incubated overnight at 37°C and then digestion was checked for completeness on 10% DNA-PAGE gel.

After EcoRV digestion, DNA was precipitated by 2.5 volume cold ethanol and 0.1 volume of 3 M potassium acetate (pH: 5.2). After centrifugation, the pellet was dissolved in TE10/0.1. 12-mer DNA and undigested plasmid were separated from short admixture fragments of DNA (200 -700 bp) using fractional PEG precipitation.

Concentration of PEG6000 was increased in DNA solution stepwise by 0.5% adding stock PEG6000 35% solution, 0.5 M sodium chloride (1). When onset of precipitation becomes visible (slight turbidity of the DNA solution is observed), the mixture was incubated on ice for 30 min and then spun down in 10,000 g for 30 min at 4°C. The supernatant was collected and stepwise addition of the PEG6000 solution and centrifugation were repeated again until no precipitation was observed. The pellet was dissolved in TE10/0.1 and composition of precipitated DNA was checked by 10% DNA- PAGE. If the supernatant did not contain 12-mer DNA, it was discarded. If it had 12-mer DNA, PEG precipitation was repeated.

DNA purification was completed by using gel filtration chromatography on Sephacryl SF1000 column which has been equilibrated in TES 100 buffer and DNA solution was loaded to the column at max 30 mg in each run. The purity of the fractions was checked by 10% DNA- PAGE. Typically the first peak was undigested vector which was discarded and the first 8-10 fractions of second peak were pure 12-mer DNA. The last fractions were used for further purification and were concentrated using Amicon Ultra - 15 concentrator 30 KDa cut off.

2.2.3 ScaI digestion of 12-mer DNA to produce the monomer 177-DNA

The ScaI reaction mixture consists of 1 mg/mL 12-mer DNA of high purity, 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, ScaI enzyme at 100 U/mg of pure 12-mer DNA.

First, small scale of digestion has been done at 25 µg of 12-mer DNA to check the quality of digestion on 10% DNA-PAGE gel. Then digestion was repeated on a large scale in batches of 10 mg. Reaction mixture for ScaI digestion was incubated overnight at 25°C and then digestion was checked for completeness on 10% DNA-PAGE gel. After complete digestion by ScaI, DNA was precipitated by 2.5 volume cold ethanol and 0.1 volume of 3 M potassium acetate pH: 5.2. After centrifugation, the pellet was dissolved in TE10/0.1.

2.2.4 Wild type histone expression

Four different pET-3a plasmids each containing the gene for individual *Xenopus laevis* wild type histones, H2A, H2B, H3 and H4, were transformed into competent *E. coli* BL21 DE3 pLysS cells. The cells were plated and incubated at 37°C for 10-18 hours on TYE plates containing ampicillin and chloramphenicol to select the presence of the pET-3a plasmid. 50 mL of 2x TY (100 µg/mL ampicillin, 25 µg/mL chloramphenicol) was taken as starter culture and was inoculated with six colonies from a transformation plate and grown at 37°C until the medium become turbid. Subsequently 15 flasks each containing 500 mL 2x TY (100 µg/mL ampicillin, 25 µg/mL chloramphenicol) were inoculated with 3-4 mL of the starter culture and grown until the A₆₀₀ reached 0.6–0.8. Then protein expression was induced by 0.4 mM IPTG. The flasks have been incubated for 3 h at 220 rpm and 37 °C, and then the cells were spun down for 7 min at 20 °C in 6,000 g. The pellet was resuspended in 100 mL washing buffer and frozen in liquid nitrogen (69).

2.2.5 Preparation of inclusion bodies

The frozen cells were lysed by thawing in 37 °C water bath. The freeze thawing disrupts the inner cell membrane and releases the T7 lysozyme, which can digest outer cell membrane. The freeze thawed cells were sonicated to break the genomic DNA and remove the spongy form of lysed cells. After sonication, the inclusion bodies containing expressed histone protein were collected by spinning down at 14,000 g for 20 min at 4°C, the pellet was resuspended in 50 mL wash buffer plus 1% triton x-100 to remove membrane proteins, then spun down at above mentioned

condition. The washing of the pellet was repeated one time with 1% triton in wash buffer and once with wash buffer only.

2.2.6 Wild type histone purification

Inclusion bodies containing expressed protein were dissolved in 1-2 mL DMSO, then 30-40 mL S₂₀₀ unfolding buffer has been added, mixed for 5-6 h and spun down at 18,000 g for three times. Supernatant was loaded in Sephacryl 200 gel filtration column pre-equilibrated with SAUDE 1000 buffer and eluted with this buffer. Typically the first peak is histone-DNA garbage peak and second peak which is related to histone protein was collected 6 mL volume per tube. The purity was checked on 18% SDS-PAGE. Fractions of the semi pure histone were collected, dialyzed against water containing 5 mM β -mercaptoethanol, frozen in liquid nitrogen, lyophilized and stored at -20 °C. (69).

To further purify the histone proteins by cation exchange chromatography, the lyophilized histone was dissolved in SAUDE 200 and spun down for 10 min at 10,000 g. A maximum 50 mg protein was loaded onto a 6 mL Resource S column. The column equilibrated with SAUDE 200 and run with following parameters: Flow rate: 5 mL/min, buffer A: SAUDE 200, buffer B: SAUDE 1000, after injection 3 min run with buffer A, gradient of buffer B was applied as 65%/ 1.0 min, then wash with buffer at 100%/ 0 min for three min, then switch back to buffer A to regenerate the column for next run. In this purification method, DNA and histone impurity was eluted in 3 min run with buffer A, therefore highly pure histone protein eluted in the gradient of 65% buffer B at one min and finally low molecular weight contaminations were removed by using 100% buffer B. The quality of fractions of pure histone was checked on 18% SDS-PAGE. The fractions with single band on SDS-PAGE were pooled, dialyzed against water containing 5 mM β -mercaptoethanol, frozen in liquid nitrogen, lyophilized and stored at -80 °C.

2.2.7 Mutant histone expression and purification

Site direct mutagenesis has been done by PhD student Liu Ying at School of Biological sciences, NTU. Mutagenesis for exchanging the lysine amino acid to glutamine in histone H4 tail has been selected. Mutant clones were used in this study obtained by two different approaches. Mutant clones of the H4 histone K16Q was directly purchase from EZbiolab, where insertion sequence were designed in our lab. Gene codon AAA at K16 was substituted with CAG. Mutant clones of the H4 histone K5.8.12Q and K5.8.12.16Q were generated from site-directed mutagenesis using QuikChange® Site-Directed Mutagenesis kit from Stratagene. Mutagenic primer P1 [ATGTCTG GTCGTGGTCAAGGTGGTCAGGGTCTGGG] and p1

[CCCAGACCCTGACCACCTTGACCACG ACCAGACAT] were designed accordingly to the primer design guidelines. Wild type H4 and proper H4 mutant clones were used as mutagenic templates. The annealing temperature was optimized at 55°C. PCR reactions were carried out for 18 cycles with the extension time of 6 minutes according to the user's manual. Parental strands were then digested with Dpn1. 6.5 µL of each of the reaction mixture containing mutagenic products was transformed into 50 µL of XL-1 blue super competent cell following the procedure in the user's manual. The nick was thus ligated and the mutagenesis products were amplified. Extracted using mini-prep, the identities of the mutagenesis products were confirmed by DNA sequencing.

The mutants histone protein K16Q, K5.8.12Q, and K5.8.12.16Q were expressed individually followed by section 2.2.4, purified followed by sections 2.2.5, 2.2.6 and lyophilized.

2.2.8 Modified histone preparation

Preparation of modified histone proteins has been done as collaborative project with the laboratory of Dr. Chuan-Fa Liu with synthesis done by Renliang Yang.

Synthesis of H4 N-terminal peptide thioesters.

Three peptide thioesters containing the residues 1 to 19 of H4 as well as acetylation(s) at Lys16, Lys(5, 8, 12) and Lys(5, 8, 12, 16), respectively, were synthesized by manual solid-phase peptide synthesis (SPPS) using Boc/trifluoroacetic acid (TFA) strategy. S-trityl mercaptopropanoic acid was coupled to MBHA resin and served as the thiol linker. The side-chain protected *N*_α-Boc amino acids used were Boc-Ser(Bzl)-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Lys(Ac)-OH, Boc-His(Dnp)-OH and Boc-Arg(Tos)-OH. After sequence assembly, Dnp group was removed by treating the peptide resins with a solution of excess *p*-thiocresol in *N*-ethyl-diisopropylamine/DMF until no yellow drain. After HF/*p*-cresol/anisole (9:0.5/0.5) cleavage, the peptide thioesters were purified with Vydac C18semi-preparative HPLC. The identity of the thioesters was confirmed with ESI-MS.

Expression of truncated H4 with residues 20 to 102 and Lys20 mutated to Cys (H4 (20-102) K20C)

Xenopus laevis histone H4 gene was amplified by PCR using forward primer 5'-GGAATTCCATATGTGCGGTCTGCGTGACAAC-3' and reverse primer 5'-CCCGGATCCTTAACCAACCGAAACCGTACAGGGT-3'. The amplified segment was digested with NdeI and BamHI and incorporated into similarly digested plasmid pET-3a. The constructed vector was transformed into BL21 DE3 pLysS competent cells. The expression and purification of the protein was similar to the wild type H4. The identity of the purified protein was confirmed with SDS-PAGE and MALDI-TOF MS and the protein was lyophilized.

NCL reaction and S-alkylation reaction.

In a typical ligation reaction, 10 to 15 mg of H4 N-terminal thioester and 20 to 25 mg of truncated H4 H4(20-102)K20C were dissolved in 1.5 mL ligation buffer (6 M GdnHCl, 0.2 M phosphate, 20 mM TCEP, 1.5% benzyl mercaptan, pH 8.0). The reaction was monitored with Vydac C4 analytical HPLC or SDS-PAGE and was stopped after an over night reaction. The ligation product was purified with Vydac C4 semi-preparative HPLC. The molecular weight of the product was confirmed with MALDI-TOF MS and lyophilized. For the s-alkylation reaction, lyophilized ligation product (final concentration 0.5 mM) was dissolved in alkylation buffer (4 M GdnHCl, 1 M HEPES, 10 mM D/L methionine, 5 mM TCEP, pH 7.8). 20 mM DTT and 160 mM 2-bromoethylamine (final concentration) were then added into the solution. DTT and 2-bromoethylamine were dissolved immediately before use. The alkylation was completed in dark after 12 h. The final product was isolated either with C4 semi-preparative HPLC or dialysis against 2-mercaptoethanol containing water and lyophilized. The schematic diagram for preparation of acetylated H4 preparation is shown in Fig. 2.2.

Figure 2-2. General scheme for the semi-synthesis of histones with methyl- and/or acetyl lysine at N-terminal tails via NCL in combination with S-alkylation.

2.2.9 Wild type histone octamer refolding

Purified and lyophilized wild type histone proteins H2A, H2B, H3 and H4 were separately dissolved in histone unfolding buffer containing 7 M guanidine chloride (30 min at 37°C). The concentration of histone was determined by measuring the A_{276} of the undiluted sample against unfolding buffer. The four histones were mixed at molar ratio 1:1:1.2:1.2 for H2A, H2B, H3 and H4 respectively and then dialyzed in 6-8kDa MWCO dialysis tubes against 2 L refolding buffer at room temperature for 1 h. The buffer was changed 3 times, finally for overnight, and then the sample was spun down for 10 min at 18,000 g and 20 °C. The supernatant containing histone octamer was concentrated in an Amicon Ultra 15 concentrator at 20 °C in 3000 g to get a final volume of 5-8 mL. The sample was spun down at 20 °C for 5 min at 18,000 g and then loaded onto the Sephacryl S200 column (pre-equilibrated with refolding buffer). After the histone octamer peak came out, the fractions have been run on 18% SDS-PAGE to check the quality of histone octamer. The fractions with histone octamer (three bands for H2A&B, H3 and H4) were pooled together and concentrated by Amicon Ultra 15 concentrator to get final volume approximately 1 mL. Concentration was measured as absorbance 276 nm against refolding buffer. Then histone octamer has been mixed with equal volume of autoclaved glacial glycerol to get 50% glycerol concentration in histone octamer and stored at -20°C (71).

2.2.10 Wild type nucleosomal array reconstitution

The molecular weight of 12-mer DNA is 1,312,419 Da. For reconstitution of nucleosomal array, 5 μ moles of 12-mer DNA was mixed with 4 M KCl and water to get a final concentration of 2 M KCl in a total volume of 200 μ L (1). Then histone octamer was added to get the desired molar ratio of octamer/DNA. Ratios 12:1 (12 histone octamer per 1 array DNA), 12.5:1, 13:1, 13.5:1 and 14:1 were tested to get optimum saturation by histone octamer. This optimal ratio can be slightly different for each batch of histone octamer. To prevent excessive binding of the histone to the 12-mer DNA, 0.5 molecule of core length DNA per one array repeat was added to reconstitution mixture as octamer buffer. The reconstitution mixture was put on the dialysis membrane (Spectra/Por, MWCO 6-8 kDa). The reconstitution started in 200 mL of TEK1.4 (the number shows the molar concentration of KCl). The steps (in time and KCl concentration) were 90 min TEK 1.4, 90 min TEK1.2, 420 min TEK1.0, 90 min TEK0.8, 90 min TEK 0.6 and twice in TEK for 180 min and repeated with same time in TEK (1).

The reconstituted array was recovered from the dialysis bag and checked for transparency. In case of visible turbidity or precipitation, the solution was centrifuged

for 10 min with 13,000 g at 4 °C to remove precipitated material. Stock solutions of the nucleosomal array were kept at 4 °C. Quality of reconstituted array was tested on 5% Poly Acrylamide Gel Electrophoresis (PAGE) (1).

2.2.11 Determination saturation of 12-mer nucleosomal array

The saturation of nucleosomal array was assayed by digesting the reconstituted arrays with ScaI in digestion buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT). Each repeat of the 12-mer DNA array contains a ScaI restriction site. The ratio of free 177 bp DNA to nucleosomal DNA was analyzed on 5% DNA-PAGE or APAGE. In fully saturated array, the free DNA should not be observed. However, sometimes we observed the presence of traces of free DNA when full saturation of the array was expected. Special experiments were carried out to demonstrate that this effect was caused by dissociation of nucleosomal array in the presence of the ScaI enzyme (1).

2.2.12 Purification of nucleosomal arrays

To remove the core length DNA and other impurities from nucleosomal array, the array was precipitated by addition of MgCl₂ to the array solution to final concentration of 4 mM. The array was turbid and it was incubated for 15 min at room temperature (RT) and then centrifuged at RT for 10 min with 20,000 g. The pellet was dissolved in TEK and the concentration of array was measured by optical density of DNA using UV spectrophotometer in 259 nm multiply by 1.9981 (conversion factor of DNA to nucleosomal array). Purity and saturation of the array was checked by 5% DNA-PAGE (1).

2.2.13 Wild type 177-NCP reconstitution

For reconstitution of 177-NCP, 5 μmoles of 1×177-601 DNA was also mixed with 4 M KCl and water to get a final concentration of 2 M KCl in a total volume of 200 μL. The optimum molar ratio of histone octamer to DNA can be used from previous step (section 2.2.10.). The stepwise dialysis for 177-NCP reconstitution has been carried out with the protocol similar to nucleosomal array. The reconstituted NCP was recovered from the dialysis bag and checked for quality of reconstituted NCP on 5% PAGE. If free 177 DNA was observed in NCP solution, the NCP was purified by precipitation at 4-5 mM MgCl₂. Stock solutions of the NCP were kept at 4 °C.

2.2.14 Analytical ultracentrifugation

Sedimentation velocity experiments were carried out using a Beckman XL-I analytical ultracentrifuge equipped with AN-50T rotor. The stock solutions of array were diluted to get A^{259} 0.80 (concentration of DNA phosphate 121 μ M) in TEK buffer, 420 μ L per each sedimentation velocity experiment was needed. All nucleosomal array and NCP concentration in our biophysical experiments will expressed as moles/liter DNA phosphate. We found that reproducible sedimentation curves can be obtained if concentration of monovalent salts was bellow 100 mM, divalent salt up to 1 mM and for cobalt hexamine as trivalent salt up to 20 μ M. Higher concentration of salt in the solution resulted in intermolecular aggregation of the array and loss of data reproducibility (1).

The reference cell contained the components same as sample solution excluding nucleosomal array. Sample and reference were equilibrated in the analytical ultracentrifuge chamber under vacuum for 30 min at 20°C. The sedimentation velocity measurement was carried out at 12,000 rpm. 40 scans were collected at 259 nm with interval of 10 min between scans. Boundaries were analyzed by the method of van Holde and Weischet using program UltraScan version 9.9. The results were verified by using distribution method (program Sedfit). Sedimentation coefficients were corrected to $S_{20,w}$ using a partial specific volume of 0.622 mL/g for saturated chromatin arrays and density and viscosity specific to the buffer solution (1). The average of sedimentation velocity from 20% to 80% of boundary was reported as S-value.

2.2.15 Precipitation assay

The stock solution of 12-mer nucleosomal array has been diluted to concentration 200 μ g/mL ($A^{259} = 2$) and mixed with equal volume of buffer solutions (10 mM Tris HCl pH 7.5) containing twice the final salt concentration. Mixture was incubated for 15 min and centrifuged at 20,000 g for 15 min at room temperature. The absorbance at 259 nm of the supernatant was measured by NanoDrop® ND-1000 spectrophotometer. The following ranges concentration of salt concentration were applied: monovalent salts 0-400 mM, divalent salts 0-20 mM, trivalent salts 0-400 μ M and tetravalent salt 0-100 μ M.

2.2.16 Dynamic light scattering titration

The light-scattering spectrometer was Brookhaven model BI-9000AT, using He-Ne laser operating at 600 nm and 35 mW. The other specifications of this

instrument are: size range is 1 nm to 6 μm , sample volume: 1 to 3 mL. To study intermolecular aggregation, the stock solution of nucleosomal array (1.125 mg/mL, 1.71 mM in DNA phosphate) was diluted to get DNA-phosphate 8.56 μM (200 times dilution). The scattering intensity and array particle size were measured using DLS during the aggregation of nucleosomal array that was induced by stepwise addition of the salts/ligands into the array solution. Various concentrations of chloride salt of sodium, potassium, magnesium, calcium, cobalt hexammine, sperimidine and spermine were used.

3. Results

3.1 Large scale amplification and purification of 12×177-601 DNA

The 12×177-601 DNA array used for this work was produced in *E.coli* HB101 cultures and extracted by alkaline lysis buffer according to the protocol described above (section 2.2.1). After phenol extraction and washing with CIA, the DNA plasmid was separated from digested RNA by PEG precipitation. Still there are impurities in DNA which may reduce the EcoRV efficiency. To remove the impurities, Sepharose 6 column was equilibrated in TEK2000, and plasmid DNA has been loaded to the column (about 30 mg) and the column was eluted at 8 mL/min by TEK2000. The first peak was pooled and precipitated by the addition of 0.5 volumes of isopropanol. The chromatogram of Sepharose 6 column is shown in Fig. 3.1. After resuspension in TE10/0.1, the DNA was subject to EcoRV digestion.

Figure 3-1. Purification of plasmid DNA on Sepharose 6 column.
Fractions A1-B11 containing purified DNA were pooled

After digestion of plasmid with EcoRV, the short DNA fragments were separated by PEG fractionation from 12-mer DNA as described in section 2.2.2.

EcoRV-digested DNA fragments and PEG-treated supernatant and precipitate were checked on 10% DNA-PAGE. (Fig. 3.2)

Figure 3-2. Separation of the products of the Eco-RV digestion of plasmid by PEG recipitation.

The pellet of 12-mer DNA after PEG precipitation was dissolved in TE10/0.1. Purification was carried out on Sephacryl SF-1000 gel filtration column to separate 12-mer DNA from undigested vector and short DNA fragments. The chromatogram of this purification is shown in Fig. 3.3. Gel electrophoresis analysis of the fractions A1-A9 is shown in Fig. 3.4.

Figure 3-3. Separation of 12-mer DNA from undigested vector and short DNA fragments by Sephacryl SF1000 column.

Fractions A1-A12 were analyzed by 10% DNA-PAGE and fractions A1-A9 were pooled.

Figure 3-4. Gel electrophoresis of the fractions A1-A9 after separation on Sephacryl SF1000 gel filtration column.

3.2.1 Wild type histone expression and purification

Expression of the individual histones, H2A, H2B, H3, H4, were carried out according to the protocol described in section 2.2.4. After purification using Sephacryl S200 gel filtration, followed by section 2.2.6; the pure histone were collected and lyophilized. The chromatograms of histones gel filtration are shown in Fig. 3.5.

Figure 3-5. Elution profile of individual histone proteins (indicated in figure) on Sephacryl 200 column.

Fractions which are corresponding to the histones were run on 18% SDS-PAGE to check the purity.

Ion exchange chromatography has been carried out to further purify the histone proteins. Histones have positive charge so they bind to cation exchange resin. But DNA and contaminated proteins which have a negative charges or neutral cannot bind to the negative beads of the column and eluted with buffer. Absorbed histones are washed from resin by increasing of salt gradient. Chromatograms of histones cation exchange are shown in Fig. 3.6.

Figure 3-6. Separation profile of individual histone proteins (indicated in figure) on Resource S cation exchange column.

Fraction which is corresponding to the histone was dialyzed and lyophilized.

3.2.2 Mutant histone proteins expression and purification

The site direct mutagenesis has been done by PhD student Liu Ying (section 2.2.7). Expression of the individual mutant H4 histones, K16Q, K5.8.12Q and K5.8.12.16Q were carried out according to the protocol described in section 2.2.6. After purification using Sephacryl S200 gel filtration, the pure histone proteins were collected and lyophilized. The chromatograms of gel filtration are shown in Fig. 3.7.

Figure 3-7. Elution profile of individual mutant H4 histone proteins on Sephacryl S200 gel filtration column. (Figures A, B, and C are related to K16Q, K5.8.12Q, and K5.8.12.16Q mutant H4 proteins)

Mutant H4 proteins were further purified on a resource S cation exchange column followed by the procedure described in 2.2.6 section. The chromatograms of cation exchange chromatography are shown in Fig. 3.8.

Figure 3-8. Separation profile of individual mutant H4 proteins (type of mutant is indicated in figure) on Resource S cation exchange column. (Figures A, B, and C are related to K16Q, K5.8.12Q, and K5.8.12.16Q mutant H4 proteins)

Fraction which is corresponding to the histone was dialyzed and lyophilized.

3.2.3 Acetylated histone proteins preparation and purification

Selectively acetylated H4 has been prepared by Yang Renliang as a collaborative project with Dr. Chuan-Fa Liu (described in section 2.2.8). H4 tail-less protein (H4_C20-g102) has been expressed and purified in our laboratory and the tail with desired modification has been synthesized by PhD student Yang Reliang using solid phase peptide synthesis and ligated to H4-C20 via Native Chemical Ligation (NCL) method, finally the cysteine residue at the ligation junction was then changed to pseudolysine (sK) via S-alkylation using 2-bromodomaine (see Fig. 2.2). The chromatogram of gel filtration of H4 C20-g102 with SDS-PAGE gel image are shown in Fig. 3.9 and the mass spectrometry graphs of final products H4K16Ac,

H4K5.8.12Ac and H4 K5.8.12.16Ac indicate the accuracy of synthesized components is shown in Fig 3.10.

Figure 3-9. (A) Elution profile of H4 tailless proteins (H4 C20_g102, K20C) on Sephacryl 200 gel filtration column. (B) 18% SDS- PAGE (denaturing conditions) of H4 tailless proteins (H4 C20_g102, K20C)

Figure 3-10. MS profile of final product of H4-K16Ac with MW: calculated $[M+H]^+ = 11297$, found: 11297.9; H4-K5.8.12Ac with MW: calculated $[M+H]^+ = 11381$, found: 11386 and H4-K5.8.12.16Ac: MW: calculated $[M+H]^+ = 11423$, found: 11433.39.

(Figures A, B, and C are related to K16Ac, K5.8.12Ac, and K5.8.12.16Ac mutant H4 proteins)

3.3.1 Wild type histone octamer refolding and purification

Histone octamer has been refolded according to the protocol described in section 2.2.9. The histone octamer was concentrated by centrifugation on Amicon concentrator, loaded on Sephacryl S200 gel filtration column and eluted with refolding buffer. The octamer peak was collected and the other peaks were discarded.

The chromatogram of histone octamer refolding and SDS-PAGE of the histones as well as histone octamer is shown in Fig.3.11.

Figure 3-11. (A) Gel filtration of the histone octamer. Fractions A1-A7 containing octamer were pooled. (B) 18% SDS- PAGE (denaturing conditions) of individual histones and histone octamer. (SDS-PAGE picture was taken from Wu Bin)

3.3.2 Histone octamer containing K→Q mutations in H4 histone refolding and purification

Three different mutant histone octamer K16Q, K5.8.12Q and K5.8.12.16Q have been refolded in a same procedure as wild type according to the protocol described in section 2.2.9. After refolding the mutant histone octamers were concentrated by centrifugation on Amicon concentrator, loaded on Sephacryl 200 gel filtration column and eluted with refolding buffer. The histone octamer peak was collected and the other peaks were discarded. The chromatograms of the mutant histone octamer refolding are shown in Fig.3.12.

Figure 3-12. Gel filtration of the mutant histone octamer.

(Figures A, B, and C are related to K16Q, K5.8.12Q, and K5.8.12.16Q mutant H4 proteins)

3.3.3 Histone octamer with selectively acetylated in H4 histone tail refolding and purification

The modified histone octamers K16Ac, K5.8.12Ac and K5.8.12.16Ac have been refolded in same procedure to wild type according to the protocol described in section 2.2.9. After completeness of refolding process, the refolded modified octamers

were recovered from dialysis bag, and then the histone octamer were concentrated by centrifugation on Amicon concentrator, loaded on Sephacryl 200 gel filtration column and eluted with refolding buffer. The modified histone octamer peaks were collected and the other peaks were discarded. The chromatograms of octamer refolding are shown in Fig.3.13.

Figure 3-13. Gel filtration of the modified histone octamer.

(Figures A, B, and C are related to K16Ac, K5.8.12Ac, and K5.8.12.16Ac acetylated H4 proteins)

3.4.1 Reconstitution of the wild type 12-mer nucleosomal array

In the first trials for reconstitution of 12-mer nucleosomal array, 12-mer DNA after EcoRV digestion was used. After several $MgCl_2$ purifications, we got a nucleosomal array, but the array was unsaturated. Unsaturations were proven by observation of the free DNA band on 5% native polyacrylamide gel (PAGE) after ScaI digestion and by low $S_{20,w}$ values of the array in 1 mM $MgCl_2$. Most probable reason

for the absence of saturation is the presence of excessive amount of short DNA fragments. Therefore we decided to purify the 12-mer DNA after EcoRV digestion.

Nucleosomal array was reconstituted by mixing 12-mer DNA and histone octamer in 2 M KCl followed by stepwise decrease of salt concentration (see section 2.2.10) For reconstitution of the arrays with high affinity of DNA sequences, a short DNA fragment was added as octamer buffer to prevent oversaturation. Core length DNA (about 145 bp) from nuclease digestion of calf thymus chromatin was used for this purpose.

The optimum ratio of histone octamer and DNA must be determining empirically and can be different for each batch of histone octamer. Five ratios, 12:1, 12.5:1, 13:1, 13.5:1 and 14:1 were reconstituted and digested by ScaI and run on 5% DNA-PAGE (Fig. 3.14)

Figure 3-14. Result of the analysis of nucleosomal array saturation before (lanes 1, 3, 5, 7, 9) and after (lanes 2, 4, 6, 8, 10) ScaI digestion on 5% DNA-PAGE. Optimal octamer to DNA ratio for the given batch of the histone octamer refolding was chosen 12.8:1

3.4.2 Reconstitution of the nucleosomal arrays containing K→Q mutations in H4 histone

Mutant nucleosomal arrays also were reconstituted by mixing 12-mer DNA and mutant histone octamer proteins in 2 M KCl followed by stepwise decrease of salt concentration (see section 2.2.10). For reconstitution of mutant nucleosomal arrays with high affinity of DNA sequences, a short DNA fragment was also added as octamer buffer to prevent oversaturation. Core length DNA (about 145bp) from nuclease digestion of calf thymus chromatin was used for this purpose.

The optimum ratio of histone octamer and DNA must be determining empirically and can be different for each batch of histone octamer for this purpose, three ratios 12:1, 12.5:1 and 13:1 were tested. The saturation of the nucleosomal array was checked through digestion by ScaI and run on 5% DNA-PAGE. The gel picture for each mutant reconstituted array has been shown in Fig. 3.15.

(Lanes 1, 3, 5 correspond to reconstitution at 12:1, 12.5 and 13:1 before digestion and lanes 2, 3, 6 after ScaI digestion)

(Lanes 1, 3, 5 correspond to reconstitution at 12:1, 12.5:1 and 13:1 before pdigestion and lanes 2, 4, 6 after ScaI digestion)

(Lanes 1, 3, 5 correspond to reconstitution at 12:1, 12.5:1 and 13:1 before digestion and lanes 2, 4, 6 after *ScaI* digestion) (Figures A, B, and C are related to K16Q, K5.8.12Q, and K5.8.12.16Q mutant H4 proteins)

Figure 3-15. Result of the analysis of mutant nucleosomal array saturation before and after *ScaI* digestion on 5% DNA-PAGE.

Optimal octamer to DNA ratio for the given batch of the octamer refolding was chosen 12.5:1

3.4.3 Reconstitution of nucleosomal arrays containing selectively acetylated in H4 histones

Nucleosomal arrays with selectively acetylated lysine in H4 histone tail were also reconstituted by mixing 12-mer DNA and modified histone octamers in 2 M KCl followed by stepwise decrease of salt concentration (see section 2.2.10). For reconstitution of mutant nucleosomal arrays with high affinity of DNA sequences, core length DNA (about 145bp) from nuclease digestion of calf thymus chromatin as octamer buffer was used.

The optimum ratio of histone octamer and DNA must be determining empirically and can be different for each batch of histone octamer. Two or three ratios 12:1, 12.5:1 and 13:1 were tested. The saturation of the nucleosomal array was checked through digestion by *ScaI* and run on 5% DNA-PAGE. The gel picture for each mutant reconstituted array has been shown in Fig. 3.16.

Figure 3-16. Result of the analysis of acetylated nucleosomal arrays saturation before and after *ScaI* digestion on 5% DNA-PAGE

Optimal octamer to DNA ratio for the given batch of the octamer refolding was chosen 12.5:1

3.4.4 Reconstitution of the wild type 177-NCP

The protocol for reconstitution of 177-NCP is similar to reconstitution of nucleosomal array, and it has been carried out by mixing of 177 bp DNA, obtained from cutting the 12-mer DNA by ScaI, and histone octamer in equimolar ratio in 2M KCl followed by stepwise dialysis. Saturation was proved by lack of the band of free 177-DNA in 5% native polyacrylamid gel (PAGE) (see section 2.2.13). For reconstitution of NCP, adding the short bp of DNA as octamer buffer is not necessary.

The optimum ratio of histone octamer and DNA must be determining empirically but can be similar from optimum ratio chosen from nucleosomal array reconstitution. (Fig. 3.17.)

Figure 3-17. Result of the analysis of 177-NCP saturation on 5% DNA-PAGE.

Optimal histone octamer to DNA ratio for the given batch of the octamer refolding was chosen 12.5:1

3.5 Summary and conclusions on preparations of the nucleosomal arrays

1. Histone proteins were individually overexpressed in *E.coli* BL21 DE3 PlysS. All four histone proteins are not sensitive to room temperature, so it is possible to purify them in room temperature. After sonication of cells, the four histones are concentrated in inclusion bodies; it was shown that there was no histone protein in the supernatant and denaturing condition was used to dissolve the inclusion bodies.

The purification of H2A and H2B was easier than H3 and H4. The yield of H2A and H2B was 600 mg per 7.5 L culture media, the yield for H3 was 400 mg L and the yield for H4 was 150 mg.

2. 12-mer DNA (2124 bp) was amplified in *E.coli* HB101. Purification of the plasmid on Sepharose 6 gel filtration column removes contaminations of RNA and proteins that significantly increases efficiency of EcoRV digestion. PEG precipitation was a key step to remove the short DNA fragments. The sample which interfere the next stage of DNA purification on Sephacryl SF-1000 gel filtration column should not have the short DNA fragments. The yield of plasmid DNA after extraction from the cells was 250 mg per 7.5 L and the final yield after several gel filtration steps was 70 mg (per 7.5 L).

3. Histone octamer refolding was done in 4°C, but recently it was found that the yield of refolding will be better if the histones are refolded in room temperature.

4. Preparation of the fully saturated wild type 12-mer nucleosomal arrays was successfully completed. To find the optimum ratio of histone octamer to DNA reconstitution with ratios 12:1, 12.5:1, 13:1, 13.5:1 and 14:1 was carried out and optimal ratio 12.8:1 was chosen.

5. Three different K→Q mutant histones H4 have been prepared using mutagenesis method. The mutant histone H4 protein successfully refolded into histone octamer using wild types of the other histones. Fully saturated nucleosomal arrays containing K→Q mutant histones H4 have been reconstituted and purified. The optimal ratio histone octamer to DNA 12.5:1 was chosen.

6. Three different histones H4 containing acetyl lysine in posions K16, K5.8.12 and K5.8.12.16 have been prepared using peptide synthesis and chemical ligation methods. Histone octamer was refolded using acetylated H4 and wild types of the other histones and finally saturated nucleosomal arrays have been reconstituted and purified. The optimal ratio acetyl histone octamer to DNA 12.5:1 was chosen.

3.6.1 Results of analytical ultracentrifugation of wild type array

Analytical ultracentrifugation is a traditional method of studies of the individual nucleosomal array folding (63). The sedimentation properties are indicative of intramolecular interaction of array. Typically, when solution conditions facilitate intermolecular aggregation, sedimentation profiles in AUC loose reproducibility.

The sedimentation velocity of array can also be used to estimate of array saturation. In our first trials of the array reconstitution, low values of $S_{20,w}$ in TEK and in $MgCl_2$ were indication (together with the presence of 177 DNA band in PAGE after ScaI digestion) of incomplete number of nucleosomes in the array.

For the measurement of the sedimentation coefficient, nucleosome arrays were prepared in the TEK buffer at an A^{259} of 0.8 and sedimentation velocity boundaries were scanned every 10 min at 12,000 rpm for 40 scans.

Data were analyzed by using Ultrascan software version 9.9 which integrates van Holde-Weischet method. With van Holde-Weischet methods often first and last scans were discarded until a common plateau region was observed. Data from the analysis by van Holde-Weischet method were plotted as boundary fraction (y-axis) versus $S_{20,w}$ (x-axis). Sedimentation corrected to water by using a partial specific volume of 0.622 mL/g for saturated chromatin arrays with density and viscosity of the buffer solution to get the integrated sedimentation velocity distribution (1). The average sedimentation coefficient from 20% to 80% of boundary was taken as S-value. The results from Van Holde-Weischet method was confirmed by another method using the software Sed-Fit version 94.

Figure 3.18 presents the results of Van Holde-Weischet analysis of the AUC data for the solution of wild type nucleosomal arrays in the presence of various concentrations of NaCl, KCl, $MgCl_2$, $CaCl_2$, $Spd^{3+}.3Cl$, $Co(NH_3)_6Cl_3$ and $Spm^{4+}.4Cl$. The experiment of sedimentation velocity has been repeated at least twice and the mean of the S-value with total standard division (SD) has been calculated and combined with S-value averaged over 20-80% interval and shown in Table 3.1. Figure 3.19 shows the dependence of sedimentation velocity coefficient on the concentration of the corresponding salt in various types of nucleosomal arrays.

Table 3.1. The mean of S-value with SD in wild type nucleosomal array

(Concentration of nucleosomal array 80 µg/ml; Cp=121 µM)

The S-value of wild type array only in TEK buffer (10 mM KCl, 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA) was 35.2 ± 0.2 S determined in four experiments.

Wild type array	Na ⁺ mM	K ⁺ mM	Mg ²⁺ mM	Ca ²⁺ mM	Spd ³⁺ µM	CoHex ³⁺ µM	Spm ⁴⁺ µM
Concentration S _{w,20}	15 39.4±0.3	15 36.9±0.2	0.2 38.4±0.3	0.4 41.5±0.5	15 39.5±0.3	5 42.4±0.2	0.5 38.35±0.3
	25 43.45±0.3	30 38.5±0.7	0.4 42.25±0.3	0.6 51.4±0.3	20 43.50±0.3	10 51.4±0.3	1.0 44.5±0.3
	50 46.4±0.3	45 42.15±0.5	0.6 44.95±0.3	0.8 54.5±0.3	30 47.5±0.4	15 53.4±0.5	1.5 49.6±0.3
	60 48.3±0.4	60 43.35±0.3	0.8 48.5±0.3	-	40 49.35±0.3	20 54.4±0.4	2.0 53.25±0.5
	75 53.5±0.3	75 44.5±0.3	1.0 52.5±0.7	-	50 53.95±0.4	-	-
	100 54.5±0.6	100 46.9±0.3	-	-	60 54.8±0.5	-	-

3.6.2 AUC results for 12×177-601 arrays containing K→Q mutations in H4 histone

The measurement of the sedimentation velocity coefficient in arrays containing K→Q mutated H4 histone have been carried out in same procedure to wild type array. After collecting the data, it has been analyzed by Van Holde-Weischet method using UltraScan version 9.9 program. The average of sedimentation velocity coefficient from 20% to 80% of boundary was reported as S-value. The results of AUC study are summarized in Table 3.2.

The Van Holde-Weischet graphs for nucleosomal arrays containing K→Q mutated H4 histone in the presence of various concentrations of corresponding by NaCl, KCl, MgCl₂, CaCl₂, Spd³⁺.3Cl⁻, Co(NH₃)₆Cl₃ and Spm⁴⁺4Cl⁻ are presented in appendix II.

Figure 3-18. Van Holde-Weischet curves for the wild type nucleosomal array in the presence of A: NaCl, B: KCl, C: MgCl_2 , D: CaCl_2 , E: spermidine, F: $\text{Co(NH}_3)_6\text{Cl}_3$ and G: spermine. (Concentration is indicated in figure)

Table 3.2. The mean of S-value with SD in nucleosomal arrays containing K→Q mutation in H4 histone (Concentration of nucleosomal array 80 µg/ml; Cp=121 µM)

The S- value of nucleosomal array containing K→Q mutations in H4 histone only in TEK buffer was 34.8 ± 0.42 S determined in three experiments.

K16Q array	Na ⁺ (mM)	K ⁺ (mM)	Mg ²⁺ (mM)	Ca ²⁺ (mM)	Spd ³⁺ (µM)	CoHex ³⁺ (µM)	Spm ⁴⁺ (µM)
Concentration S _{w,20}	15 37.8±0.8	15 38.9±0.8	0.2 38.8±0.8	0.2 38.5±0.6	20 40.1±0.9	5 38.9±0.5	0.5 39±0.9
	30 40.8±0.8	30 40±0.7	0.4 40.8±0.9	0.4 41.6±0.8	30 42.4±0.6	10 40±0.7	1.0 42.3±0.5
	45 44.3±0.5	45 40.8±0.9	0.6 43.1±0.8	0.6 43.1±0.6	40 0.45.6±1.2	15 44.9±1.4	1.5 47.4±0.8
	60 46±1.0	60 42.9±0.7	0.8 46.8±0.7	0.8 47.7±1.0	50 47.9±0.9	20 47.5±0.9	2.0 49.8±0.8
	75 48.4±0.8	75 44.9±1.2	1.0 48.4±0.9	1.0 49±0.7	60 49.1±0.9	-	-
	100 49.6±0.7	100 47.5±0.9	-	-	-	-	-
K5.8.12Q array	Na ⁺ mM	K ⁺ mM	Mg ²⁺ mM	Ca ²⁺ mM	Spd ³⁺ µM	CoHex ³⁺ µM	Spm ⁴⁺ µM
Concentration S _{w,20}	15 38.6±0.4	15 37.7±0.5	0.2 38.1±0.6	0.2 38.3±0.4	20 39.1±0.5	5 41.3±0.7	0.5 39.1±0.9
	30 42.1±0.7	30 38.8±0.7	0.4 40.1±0.7	0.4 42.2±0.5	30 44.8±0.9	10 49.7±1.3	1 44.1±0.5
	45 47.0±0.8	45 41.2±0.7	0.6 43.3±0.6	0.6 43.9±0.6	40 50.3±0.8	15 51.3±0.6	1.5 47.7±1.2
	60 48.2±1.0	60 43.1±0.7	0.8 46.2±0.5	0.8 48.7±0.8	50 51.0±0.6	20 51.6±0.7	2.0 51.8±1.8
	75 49.4±0.6	75 45.1±1.0	1.0 50.9±0.6	1.0 51.3±0.8	60 51.4±0.7	-	-
	100 51.2±0.9	100 47.7±0.5	-	-	-	-	-
K5.8.12.16Q array	Na ⁺ mM	K ⁺ mM	Mg ²⁺ mM	Ca ²⁺ mM	Spd ³⁺ µM	CoHex ³⁺ µM	Spm ⁴⁺ µM
Concentration S _{w,20}	15 38.5±0.8	15 38.2±0.8	0.2 37.7±1.0	0.2 38.7±0.7	20 39.6±0.9	5 39.2±1.0	0.5 40.5±1.0
	30 40.8±1	30 40.3±0.6	0.4 40.2±0.9	0.4 41.0±0.6	30 43.8±0.6	10 42.18±0.9	1.0 39.1±1.0
	45 44.9±1.2	45 41.3±1.0	0.6 43.1±0.5	0.6 43.8±0.7	40 45.8±0.7	15 44.0±0.6	1.5 43.9±1.6
	60 44.6±0.9	60 43.5±0.9	0.8 45.3±0.7	0.8 46.4±0.9	50 44.3±1	20 48.1±0.7	2.0 48.1±0.6
	75 47.8±1.0	75 44.0±1.1	1.0 47.0±0.9	1.0 48.5±0.8	60 47.8±0.8	-	-
	100 49.2±1.0	100 46.2±0.9	-	-	-	-	-

3.6.3 AUC results obtained for 12×177-601 arrays with selectively acetylated in H4 histones

The measurement of the sedimentation velocity coefficient in modified array system has been carried out in same procedure to wild type array (see section 2.2.14). The results of the AUC study are presented in table 3.3.

The Van Holde-Weischet curves for nucleosomal arrays with acetylated H4 tails in the presence of various concentrations of corresponding by NaCl, KCl, MgCl₂, CaCl₂, spermidine, Co(NH₃)₆Cl₃ and spermine are shown in Appendix III.

Table 3.3. The mean of S-value with SD in nucleosomal arrays with selectively acetylated H4 tails
(Concentration of nucleosomal array 80 µg/ml; Cp=121 µM)

The S- value of nucleosomal array containing selectively acetylated in H4 tail only in TEK buffer was 34.5 ± 0.53 S determined in three experiments

K16Ac array	Na⁺ mM	K⁺ mM	Mg²⁺ mM	Ca²⁺ mM	Spd³⁺ µM	CoHex³⁺ µM	Spm⁴⁺ µM
Concentration S_{w,20}	15 37.5±0.9	15 38.9±0.8	0.2 38.4±0.9	0.2 38.3±0.7	20 39.0±0.9	5 39.3±0.9	0.5 39.5±0.9
	30 40.3±0.8	30 40.0±1.1	0.4 40.3±1.2	0.4 39.1±0.8	30 40.0±1.1	10 42.3±0.7	1.0 41.1±1.0
	45 40.1±1	45 40.4±1	0.6 41.1±0.92	0.6 40.3±0.8	40 41.4±0.8	15 44.0±0.8	1.5 43.6±0.7
	60 41.0±1	60 41.1±0.8	0.8 42.8±0.7	0.8 41.9±1.2	50 43.9±1.0	20 44.3±0.7	2.0 44.1±0.9
	75 43.2±1	75 42.7±1	1.0 44.4±1.1	1.0 44.4±0.8	60 44.7±1.0	-	-
	100 44.2±0.8	100 44.3±0.7	-	-	-	-	-
K5.8.12Ac array	Na⁺ mM	K⁺ mM	Mg²⁺ mM	Ca²⁺ mM	Spd³⁺ µM	CoHex³⁺ µM	Spm⁴⁺ µM
Concentration S_{w,20}	15 38.3±0.84	15 38.3±1	0.2 39.1±1.1	0.2 38.1±0.7	20 38.9±1.0	5 39.7±1.0	0.5 38.9±0.8
	30 42.4±1.0	30 38.7±0.8	0.4 41.2±0.9	0.4 39.8±0.8	30 42.5±1.0	10 44.4±1.2	1.0 43.4±0.8
	45 44.9±1.1	45 41.6±1.1	0.6 43.8±0.9	0.6 43.3±1.2	40 45.7±0.9	15 48.9±0.8	1.5 46.8±0.5
	60 47.6±0.8	60 43.8±0.7	0.8 46.2±1.1	0.8 47.8±0.9	50 47.1±1.2	20 49.4±1.3	2.0 49.6±1.1
	75 48.8±1.1	75 44.4±0.8	1.0 48.8±1.3	1.0 49.0±1.1	60 49.4±0.9	-	-
	100 49.1±1.0	100 46.9±1.2	-	-	-	-	-
K5.8.12.16Ac array	Na⁺ mM	K⁺ mM	Mg²⁺ mM	Ca²⁺ mM	Spd³⁺ µM	CoHex³⁺ µM	Spm⁴⁺ µM
Concentration S_{w,20}	15 36.8±0.9	15 36.6±0.8	0.2 36.8±1.0	0.2 36.7±0.6	20 36.7±0.7	5 37.1±1.0	0.5 36.2±0.5
	30 38.1±1.2	30 36.6±0.8	0.4 38.0±1.2	0.4 37.5±1.1	30 38.0±1.0	10 38.3±0.9	1.0 37.2±1.0
	45 38.7±1.1	45 39.0±0.7	0.6 38.8±1.4	0.6 37.9±1.1	40 38.4±1.3	15 29.4±1.5	1.5 39.8±0.8
	60 38.2±1.3	60 39.3±1.2	0.8 39.5±1.4	0.8 39.3±1.4	50 39.8±1.2	20 40.4±1.0	2.0 41.0±1
	75 39.8±1.5	75 40.0±1.2	1.0 41.1±1.4	1.0 40.7±1.1	60 41.2±1.4	-	-
	100 40.8±1.4	100 40.5±1.3	-	-	-	-	-

Analytical ultracentrifugation sedimentation velocity is a sensitive method to study compaction of the chromatin fibers. However, AUC the method has a limitation that is after certain salt concentration (specific for each sort of cation) intermolecular aggregation of the array occurs that results in precipitation and loss of data reproducibility. For the wild type array, we found that the highest concentration of salts which gives reproducible AUC curves is 100 mM for KCl and NaCl , 1.0 mM MgCl_2 , 0.8 mM CaCl_2 , 15 μM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$, 60 μM spermidine and 2 μM spermine. Maximum $S_{20,w}$ for Mg^{2+} is 53-54 S in 1 mM Mg^{2+} (Appendix II-D). For Mg^{2+} our result agrees with the data of Dorigo et al. 2006 (1) and Shorgen-Knaak et al. (52). Maximum $S_{20,w}$ for Ca^{2+} is 55 in 0.8 mM Ca^{2+} (Table 3.1). This result also shows calcium is slightly stronger compaction agent than magnesium. Huynh et al. used 12 $\times$ 200-601 array to study the salt induced compaction and for sedimentation velocity and measured 58.8 S for 1 mM MgCl_2 and 55.6 S for 100 mM NaCl (50). The difference in $S_{20,w}$ obtained in our experiment (53 S for 12 $\times$ 177-601) and in this work (58.8 S for 12 $\times$ 200-601 nucleosomal array) can be explained by higher molecular weight of the array used by of Huynh et al. (50). AUC data for K^+ , Ca^{2+} , Co^{3+} , Spd^{3+} and Spm^{4+} are not available in literature. Relative effectiveness of the cations in compaction of wild type, K $\rightarrow$ Q mutation forms and acetylated in H4 nucleosomal arrays is shown and compared in Fig. 3.19.

Figure 3-19. Salt concentration dependence of $S_{20,w}$ of the nucleosomal arrays.
(Type of cation is indicated in figure G and symbols are the same in A-G)

3.7.1 Precipitation assay of wild type nucleosomal array

In precipitation assay, the absorbance of the supernatant was measured after centrifugation of the array solution containing various concentration of added salt. Initial concentration of DNA-phosphate was 152 μM ($A^{259}=1$) in 10 mM Tris-HCl (pH 7.5) buffer. Fig. 3.20 shows the dependence of A^{259} in supernatant on salt concentration, the EC_{50} value the concentration of each cation in middle of transition curve is shown in table 3.4. Interestingly, in the precipitation assay, only a fraction of the arrays aggregates upon titration with K^+ and Na^+ .

Figure 3-20. Measurement of absorbance at 259 nm in the supernatant of wild type nucleosomal array after titration with cation and centrifugation.

Table 3.4. EC_{50} (mM) of multivalent salts from precipitation assay in wild type nucleosomal array. The present EC_{50} is the mean of 3-4 measurements.
(Concentration of nucleosomal array 100 $\mu\text{g}/\text{ml}$; $\text{Cp}=151 \mu\text{M}$)

Magnesium	Calcium	Cobalt Hexammine	Spermidine	Spermine
2.8 ± 0.2	2.6 ± 0.1	0.027 ± 0.001	0.113 ± 0.01	0.0120 ± 0.0013

3.7.2 Precipitation assay for nucleosomal array containing K→Q mutations in H4 histone

Procedure of precipitation assay in nucleosomal array containing K→Q mutations in H4 tail is similar to wild type. For this purpose, initial stock solution of the array was diluted and mixed with desired concentration of cations and after centrifugation, the concentration of supernatant was measured. Initial concentration of DNA-phosphate was 152 μM ($A^{259}=1$) in 10 mM Tris-HCl (pH 7.5) buffer. Fig. 3.21 shows the dependence of A^{259} in supernatant on salt concentration.

Figure 3-21. Precipitation assay of nucleosomal array containing K→Q mutations in H4 histone tail. Measurement of absorbance at 259 nm in the supernatant after titration with cation and centrifugation. (Type of mutant is indicated in figure)

In precipitation assay of nucleosomal array, when the total charge of array has been neutralized by titration with salt, the array will aggregate and precipitate. Substitution of lysine with glutamine in histone tail reduces the total positive charge, so the amount of multivalent salt /charged ligand needed to neutralize the negative charge of DNA will increase. EC_{50} values are shown in table 3.5. It can be observed that the EC_{50} will increase along with increasing number of K→Q mutation in H4 tail.

Table 3.5. Comparison of EC_{50} (mM) of multivalent salts from precipitation assay in different nucleosomal array containing K→Q mutations in H4 histone tail. The present EC_{50} is the mean of 3-4 measurements. (Concentration of nucleosomal array 100 μ g/ml; Cp=151 μ M)

	Magnesium	Calcium	Cobalt Hexammine	Spermidine	Spermine
K16Q Array	3.48±0.1	3.30±0.03	0.033±0.001	0.150±0.030	0.023±0.001
K5.8.12Q Array	5.45±0.1	5.30±0.10	0.053±0.003	0.270±0.005	0.0366±0.002
K5.8.12.16Q Array	9.1±0.1	8.90±0.10	0.088±0.003	0.490±0.002	0.056±0.003

3.7.3 Precipitation assay for nucleosomal arrays with selectively acetylated in H4 tails

In precipitation assay, the absorbance of the supernatant was measured after centrifugation of the selectively acetylated nucleosomal arrays solution containing various concentration of added salt. Initial concentration of DNA-phosphate was 152 μ M ($A^{260}=1$) in 10 mM Tris-HCl (pH 7.5) buffer. Fig. 3.22 shows the dependence of A^{259} in supernatant on salt concentration. The experiment has been repeated three times and mean of EC_{50} , the concentration of each cation in middle of transition curve, along with standard division (SD) has been calculated for each cation for three different acetylated studied arrays and is shown in table 3.6.

Figure 3-22. Precipitation assay of nucleosomal array with selectively acetylated in H4 histone tail. Measurement of absorbance at 259 nm in the supernatant after titration with cation and centrifugation. (Type of acetylated arrays is indicated in figure)

The nucleosomal array folding and aggregation is mediated by charged histone tails. On the other hand, when the overall charge of array has been neutralized by titration with cation, the array will precipitate. In vivo chromatin folding is regulated by post-translational covalent modifications of the histone tail amino acids. The most frequent modification is the acetylation of the lysine amino acid that changes the distribution and net amount of charged group in the tails. Acetylation of the lysine causes in removal of positive charge. One of hypothesis here is that global and local variation of electrostatic interactions between the tail and surrounding molecules is an important component of chromatin structure and dynamics. In precipitation assay test,

with increasing the acetylation of lysine in H4 tail, in fact removes the positive charge of the tail and amount of cation to neutralize the negative charge of DNA will be increased.

Table 3.6. Comparison of EC_{50} of multivalent salts from precipitation assay in different nucleosomal array with selectively acetylated in H4 histone tail. The present EC_{50} is the mean of 3-4 measurements. (Concentration of nucleosomal array 100 $\mu\text{g/ml}$; Cp=151 μM)

	Magnesium	Calcium	Cobalt Hexamine	Spermidine	Spermine
K16Ac Array	3.52 $\pm$ 0.2	3.56 $\pm$ 0.10	0.034 $\pm$ 0.001	0.166 $\pm$ 0.009	0.02 $\pm$ 0.0009
K5.8.12Ac Array	5.43 $\pm$ 0.1	5.30 $\pm$ 0.07	0.0494 $\pm$ 0.0005	0.315 $\pm$ 0.004	0.036 $\pm$ 0.0006
K5.8.12.16Ac Array	8.90 $\pm$ 0.1	8.12 $\pm$ 0.07	0.0892 $\pm$ 0.0005	0.530 $\pm$ 0.005	0.052 $\pm$ 0.0005

3.8.1 DLS study of nucleosomal array aggregation

The stock solution of nucleosomal array was diluted to 8.56 μM DNA phosphate in buffer contains 10 mM KCl, 10 mM Tris-HCl (pH 7.5). Signal intensity and array particle size was measured during titration by NaCl, KCl, MgCl_2 , CaCl_2 , $\text{Spd}^{3+} \cdot 3\text{Cl}^-$, $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ and $\text{Spm}^{4+} \cdot 4\text{Cl}^-$. Fig 3.23 displays the dependence of the signal intensity and array particle size on concentration of the titrant in the cuvette.

Aggregation of arrays is reflected by sharp increase in scattering intensity and particle size (Fig. 3.23). The effectiveness of array condensation by cations is measured as EC_{50} , concentration of each cation in the middle of light scattering transition curve. The EC_{50} values for the cations vary in a very broad range from 15 μM for spermine to 80.7 mM for sodium. Values of EC_{50} for K^+ , Na^+ , Mg^{2+} , Ca^{2+} , Co^{3+} , Spd^{3+} , Spm^{4+} are shown in Table 3.7. The strength of intermolecular aggregation is following: $\text{Spm}^{4+} > \text{Co}^{3+} > \text{Spd}^{3+} > \text{Ca}^{2+} \geq \text{Mg}^{2+} > \text{Na}^+ \geq \text{K}^+$.

Figure 3-23. DLS titration curves of the nucleosomal array. (A) Signal intensity and (B) array particle size in different concentration of added salt.

The size of the particles is large and corresponds to the formation of aggregates of many nucleosomal arrays. Interestingly, the EC_{50} values of the tricationic $Co(NH_3)_6^{3+}$ and Spd^{3+} are very different; with $Co(NH_3)_6^{3+}$ being almost as effective as tetravalent Spm^{4+} . Ca^{2+} and Mg^{2+} , K^+ and Na^+ do not show much difference in term of aggregation of the arrays (table 3.7).

3.8.2 DLS study of 177-NCP aggregation

The stock solution of 177-NCP was diluted to get the concentration of 8.56 μM DNA phosphate in buffer contains 10 mM KCl, 10 mM Tris-HCl (pH 7.5). Signal intensity and particle size was measured during titration by NaCl, KCl, $MgCl_2$, $CaCl_2$, $Spd^{3+}.3Cl^-$, $Co(NH_3)_6Cl_3$ and $Spm^{4+}.4Cl^-$. The dependence of the signal intensity and particle size on concentration of each cation is presented in Fig 3.24 and EC_{50} values for signal intensity are shown in table 3.7.

Figure 3-24. DLS titration curves of the 177-NCP. (A) Signal intensity and (B) NCP particle size in different concentration of added salt.

The order of effectiveness causing aggregations is the same for both precipitation assay and dynamic light scattering methods. For precipitation assay, the EC₅₀ values are consistently higher than these for DLS, likely due to the higher array concentration used in precipitation assay. A similar effect has been seen for nucleosome core particle aggregation (72). The diameter of each arrays and NCP before adding any salt was detected in range of 15-30 nm and the size after adding the cation was detected in range of 800-1200 nm.

In DLS result, the average particle size for arrays is slightly higher than NCP. I have used the same concentration of array and NCP. Although in aggregated NCPs the signal induced by Na⁺ and K⁺ was much less than the signal induced by Na⁺ and K⁺ in aggregated arrays, however, the particle size of arrays and NCPs by Na⁺ and K⁺ was similar. The experiment was repeated 3-4 times.

Table 3.7. EC₅₀ (mM) values from dynamic light scattering induced by multivalent salts. The present EC₅₀ is the mean of 3-4 measurements.

	Sodium	Potassium	Magnesium	Calcium	Cobalt Hexammine	Spermidine	Spermine
Array	80.81±10.4	94.1±9.4	1.53±0.2	1.78±0.1	0.0233±0.003	0.143±0.02	0.00443±0.00008
177-NCP	103.4±12.2	103.4±14.9	2.22±0.62	2.12±0.5	0.050±0.007	0.203±0.03	0.008±0.0004

4. Discussion

4.1 Nucleosomal array reconstitution

The first level of the eukaryotic genome organization is constituted by the nucleosome core particle (NCP) which can be described as a wedge-shape cylinder 10.5 nm in diameter and 5.7 nm in height (73). Variable length (20-60 bp) of the linker DNA connects the NCPs together and makes nucleosomal array.

To reconstitute the nucleosomal array, we expressed the four above mentioned histone proteins followed by the protocol described by Luger et al. (69,74). To adjust conditions of our histone preparation to large quantities and to improve resolution during histone purification on cation exchange resins, we applied Resource S FPLC column instead of SP-SPW column used by Luger et al (74). The resource S column has higher capacity (50 mg vs 15 mg) and higher affinity to the histone proteins than SP-SPW column.

The histone octamer was refolded by protocol of Luger et al. (74). However, the following changes have been made:

- 1) At the beginning of our experiments, refolding of the histone octamer at 4°C produced a large junk peak in the purification chromatogram; therefore we have tried to run the purification procedure at room temperature. This change in conditions did result in reduction of the junk peak (it became smaller than the HO peak) even for mutant and modified forms (these forms were typically not easy to refold and purify with good yield). (Figs 3.12. and 3.13.)
- 2) In the protocol of Luger et al. (74), the authors used equimolar ratio of the four histone proteins. We optimized the stoichiometry ratio to 1:1:1.2:1.2 for the H2A, H2B, H3 and H4 respectively. When I used 1:1:1.2:1.2 ratio for four histones we observed the H2A-H2B dimer peak is negligible compared to the equimolar ratio being used, so the yield became better.

I have used the DNA template that contains 12 tandem repeats of 177bp of the high affinity 601 sequence. Knowledge of nucleosome positioning is essential for understanding mechanisms of the regulation of gene expression in eukaryotic cells; also, reliable prediction of nucleosome positioning may facilitate wide-scale annotation of the genomes (51). We have used the DNA template described by Dorigo et al for reconstitution of 12-mer nucleosomal array utilizing of wild type, K→Q mutations and selectively acetylated-types of histone octamer, except using of KCl instead of NaCl (59). The reason for changing the salt, is that K⁺ is the major cation of cell cytoplasm; moreover utilizing AUC velocity measurement, I observed the nucleosomal array reconstituted in presence of KCl are more homogenous than those arrays reconstituted in NaCl.

Three different mutant histones H4 have been prepared by site-direct mutagenesis. The reason for changing K→Q in H4 tail, is that glutamine is structurally similar to acetylated lysine (Fig 4.1), Wang and Hayes prepared a number of histones with K → Q mutations in the histone tails and used them to prepare the nucleosomal array and to model the influence of histone tail acetylation on intermolecular aggregation of the arrays (53).

Figure 4-1. Molecular structure of lysine, glutamine and acetyl lysine (respectively from left to right).

We have prepared K16Q, K5.8.12Q and K5.8.12.16Q mutant histone H4, and then reconstituted them into nucleosomal array using wild type for the other histones. I wanted to compare the effect of K→Q mutation and lysine acetylation with regard to chromatin folding. In order to do this, K16Ac, K5.8.12Ac, and K5.8.12.16Ac in H4 tail have been prepared in the collaboration with the laboratory of Dr. Chuan Fa Liu (synthesis made by Renliang Yang). The acetylated histones have been used in reconstitution into nucleosomal arrays. To make acetylated lysine in H4 tail, we used a tailless H4 where lysine 20 was substituted for cysteine. The tail with the desired modified amino acid has been synthesized utilizing solid phase peptide synthesis (SPPS) and was ligated to the purified tailless H4 utilizing native chemical ligation. Finally the cysteine which was necessary for native chemical ligation has been transferred to a pseudolysine using S-alkylation.

Acetylated lysine was prepared to reconstitute in nucleosomal array by Robinson et al. (55) and Shogren-Knaak et al (52), but the arrays used in the cited work were either not 100% acetylated (about 30% acetylation in Robinson et al. work) or contained modified H4 tail sequence (H4 R23C) in Shogren-Knaak et al. work. Robinson et al. used enzymatic partially acetylated lysine on position lysine 16 in H4 tail (55). The authors prepared 61×202-601 array and then used the array as substrate for MOF *Drosophila melanogaster* acetyltransferase, and obtained 30% acetylated K16Ac array. Shogren-Knaak et al. (52) used similar protocol to ours to prepare modified K16Ac nucleosomal array. However, the authors of the cited work

changed the arginine 23 to cysteine and expressed this truncated H4 in *E.coli*. The Author synthesized H4 peptide containing residue 1-22 using standard solid-phase peptide and they used acetyl lysine to incorporate directly during synthesis. The authors used native chemical lysine of the H4 Δ 1-22 R23C and H4 1-22K16Ac thioester fragments (52).

Comparing our method for preparation of modified H4 with the method of Shogren-Knaak et al. the following differences are observed:

1. We changed lysine 20 to cysteine to make the ligation with synthesized tail peptide but Shogren-Knaak changed arginine 23 to cysteine. This different ligation position does not change the nature of prepared modified H4 (52).
2. We converted cysteine 20 to pseudolysine using S-alkylation that is very similar to lysine, but Shogren-Knaak left cysteine in the modified product. Actually, the author used H4-K16AcR23C to study the modification effect of H4-K16Ac on nucleosomal array compaction. Although the authors showed that the changing of a single arginine 23 to cysteine does not have any effect on array folding, a synergistic effect when this charge quenching is coupled with K16 acetylation cannot be ruled out and the H4 tail sequence with K16Ac is also not native.

4.2 Nucleosomal array folding studied by AUC

As background solution in AUC experiments, we used TEK buffer which contains 10 mM K^+ and 10 mM Tris-HCl. So the total concentration of monovalent cations corresponds to low salt condition and nucleosomal arrays in this environment is expected to exist in extended “bead-on-a-string” conformation. The presence of 0.1 mM EDTA in the TEK buffer may obscure the measurements in solutions with added multivalent cations (like Mg^{2+} or Ca^{2+}) so we tried to perform AUC experiment in the TK buffer (TEK buffer without EDTA). This attempt resulted in irreproducible, unstable AUC sedimentation curves likely due to the design of the AUC cell allowed contaminations of the solution by metal cations from the cell walls and sealing, so the presence of EDTA is necessary to chelate out these metal contaminations. Data by Lu et al. (75) supports our observation: Tris-HCl, EDTA and sodium chloride buffer, TEN, in AUC experiment to investigate the effect of H3K79 dimethylation and H4K20 trimethylation on chromatin structure and found the adding of 0.1 mM EDTA is needed to get a homogenous data.

For the wild type arrays, maximal compaction is indicated by an S-value at the midpoint of the S value boundary distribution curve of 53-54 in the presence of various multivalent cations. The maximal S-values are summarized in table 4.1. At 100 mM we obtained an S-value of 54 S. Remarkably, the maximal S value in the

presence of 100 mM KCl is 46.9S. We have tried to measure sedimentation coefficient at higher concentration than that mentioned in table 4.1, but the $S_{20,w}$ value did not increase and the quality of data has been deteriorated, indicating aggregation of the arrays.

Huynh et al used 12-177-601 nucleosomal array, and got 55.6 S for compaction at 100 mM NaCl that is in a good agreement with our result (54.5 ± 0.6 S for 12-177-601 array at 100 mM Na^+) (50). Zinchenko A. et al. (76) reported that Na^+ shows a higher potential than K^+ in DNA compaction. The authors studied DNA compaction by alkali metal salts NaCl, KCl, RbCl, CsCl, and found that all of these monovalent cations promote DNA compaction (76). Among these cations Na^+ produces the greatest compaction state. The compaction strength follows by the series $\text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+$.

Satoh et al. reported that K^+ and other heavy alkali metal ions (Rb^+ and Cs^+) inhibits the coil – α helix transition of polyglutamic acid (77). It has been shown that the inhibitory effect of K^+ on α -helix conformation suggests that K^+ has disruptive effect on α -helix formation which is mediated by a specific K^+ interaction with peptide carbonyl groups (78). We carried out AUC velocity measurements for wild type nucleosomal arrays at varying amounts of RbCl and CsCl and these ions show the same behavior as K^+ (data not shown). Therefore, we propose that this K^+ (and Rb^+ or Cs^+) interaction has an inhibitory effect on the H4-K16 nucleosomal interaction that prevents maximal folding, by disruption of H4 tail interaction with the neighboring nucleosome. Support for this hypothesis comes from recent NCP crystal structures obtained in the presence of Rb^+ or Cs^+ that showed specific binding of those ions to a site containing three peptide carbonyl groups of the H2B histone (R96, L97, L99) (79). This site is located close to the H4 N-terminal tail the amino acids K16-R23 binding region (as shown in figure 4.2b, c). We propose that K^+ has the same binding site and that the H4K16 amino group, which is disrupted by acetylation, leading to inability of full folding of the array (see below and Fig 4.2b). The complete absence of K^+ -specificity on folding of the array containing H4-K16Ac is a strong indication that both K16Ac and the K^+ effects have a single common site. This phenomenon suggests a disruption of hydrogen bonding of the K16 ϵ -amino group of H4-K16 to the carbonyl groups of the H2B histone (R96-L99) by K^+ binding to this site or by H4-K16 acetylation. In fact, acetylation of the amine leaves it with only one H-bonding possibility, which may be disrupted by the presence of the steric hindrance imposed by the acetyl group blocking access to the cavity with H2B R96-L99 carbonyls (as shown in figure 4.2d). The side chain amide of Gln can form two H-bonds without any steric constraints, the difference compared to Lys is that the Gln side chain is only one atom shorter than Lys and this may explain the moderate effect of this modification on the array folding. K^+ ions as well as H4-K16Ac seem to have

an inhibitory effect on the folding and as a result prevent meshing of the NCP-NCP staking. Adding the charge-neutralizing and bulky acetyl group to the H4-K16 residues in the NCP octamer is apparently sufficient to disrupt chromatin folding. This results are in agreement with observation by Rhodes et al 30% H4-K16 acetylation can produce a structural effect comparable to that observed in our experiment with 100% single H4-K16 acetylation (55). Mutation of the negatively charged residues of the acidic patch in the H2A-H2B dimer resulted in a similar reduction in the sedimentation velocity coefficient from 47 S to 38 S for 12-200-601 arrays in the presence of 0.9 mM $MgCl_2$ as we observed for H4-K16Ac (54).

Divalent cations, Mg^{2+} and Ca^{2+} compact array from 0.2 mM to 1 mM, and we found that Ca^{2+} is slightly stronger in inducing compaction than is Mg^{2+} . Zinchenko et al. suggested that, the cations that have the same charges but smaller atomic number are more active in DNA/nucleosomal array compaction (76).

The value of $S_{20,w}$ was measured both in 0.8 and 1.0 mM Ca^{2+} was equal to 53 S. However, data obtained at 1.0 mM Ca^{2+} was of poor quality with indication of array aggregation.

For trivalent cations, cobalt (III) hexammine and spermidine, maximal folding of the array is reached in the presence of 20 μM $CoHex^{3+}$ and 60 μM Spd^{3+} . Cobalt hexammine is therefore stronger than spermidine in inducing folding. In Spd^{3+} , the charge is distributed in whole of the molecule but $CoHex^{3+}$ is only a single ion. Probably, this difference makes $CoHex^{3+}$ more potent compaction agent.

Table 4.1. Comparison of maximum $S_{20,w}$ induced by ions in different array systems

Array Ions	Wild Type	K16Ac	K16Q	K5.8.12Ac	K5.8.12Q	K5.8.12.16Ac	K5.8.12.16Q
Na^+ (100 mM)	54.5 $\pm$ 0.6	44.2 $\pm$ 0.8	49.6 $\pm$ 0.7	49.1 $\pm$ 1	51.2 $\pm$ 0.9	40.8 $\pm$ 1.4	49.2 $\pm$ 1
K^+ (100 mM)	46.9 $\pm$ 0.3	44.3 $\pm$ 0.7	47.5 $\pm$ 0.9	46.9 $\pm$ 1.2	47.7 $\pm$ 0.5	40.5 $\pm$ 1.25	46.2 $\pm$ 0.9
Mg^{2+} (1.0 mM)	52.5 $\pm$ 0.7	44.4 $\pm$ 1.1	48.4 $\pm$ 0.9	48.8 $\pm$ 1.3	50.9 $\pm$ 0.6	41.1 $\pm$ 1.4	47.0 $\pm$ 0.9
Ca^{2+} (1.0 mM)	54.5 $\pm$ 0.3	44.4 $\pm$ 0.8	49.0 $\pm$ 0.7	49.0 $\pm$ 1.1	51.3 $\pm$ 0.8	40.7 $\pm$ 1.1	48.5 $\pm$ 0.8
Co^{3+} (20 μM)	54.4 $\pm$ 0.5	44.3 $\pm$ 0.7	47.5 $\pm$ 0.9	49.4 $\pm$ 1.3	51.6 $\pm$ 0.7	40.4 $\pm$ 1	48.1 $\pm$ 0.7
Spd^{3+} (60 μM)	54.8 $\pm$ 0.5	44.7 $\pm$ 1	49.1 $\pm$ 0.9	49.4 $\pm$ 0.9	51.4 $\pm$ 0.7	41.2 $\pm$ 1.4	47.8 $\pm$ 0.8
Spm^{4+} (2.0 μM)	53.25 $\pm$ 0.5	44.1 $\pm$ 0.9	49.8 $\pm$ 0.8	49.6 $\pm$ 1.1	51.8 $\pm$ 1.75	41.0 $\pm$ 1.1	48.1 $\pm$ 0.6

I have used nucleosomal arrays containing K $\rightarrow$ Q mutant and acetylated lysine to investigate effect of the H4 tail modification on array compaction. In single modification/mutant array K16Ac/K16Q was applied. The maximal S-value induced by different cations in K16Ac and K16Q is presented in table 4.1. Although glutamine is structurally similar to acetyl lysine, (Fig 4.1) the difference in sedimentation coefficients reflect that the compaction states are very different for the H4-K16Ac and

H4-K16Q arrays. The average of the maximal $S_{20,w}$ in H4-K16Ac array induced by multivalent cations is about 44-45S but in H4-K16Q it is in the range of 48-49S. Comparing the maximal S-value of H4-K16Q, H4-K16Ac and wild type arrays we can observe that the $S_{20,w}$ in H4-K16Q array is in between of H4-K16Ac and wild type. We found that:

1. Glutamine does not act as acetylated lysine. So the dramatic destabilization of the chromatin fiber caused by H4-K16Ac is not simply attributed to the decreasing of charge since the same effect was not observed in nucleosomal array containing the H4-K16Q mutation, which serves as a chemical mimic of the acetylated lysine group.
2. The results of measured S-values from K16Ac array is in agreement with result of Robinson et al (55) and Shogren-Knaak et al. (52,55)

Robinson et al. also reported that the S-value in H4-K16Q is similar to wild type, however, acetylation of H4-K16 significantly decreased nucleosomal array folding (see Fig 4.3 presenting the result of wild type, 30% H4-K16Ac and H4-K16Q arrays in presence of 1.6 mM $MgCl_2$ obtained by Robinson et al. (55). Shogren-Knaak et al. report that sedimentation velocity coefficient 12-mer array in wild type and K16Ac form were 53 and 44 S respectively and it is the same as our result (52).

To the best of our knowledge effect of Ca^{2+} , $CoHex^{3+}$, Spd^{3+} and Spm^{4+} on chromatin folding have not been systematically studied. The only available data on array is work by Widom (80) on $CoHex^{3+}$ influence and an investigation of the Spd^{3+} influence on the model array by Pollard et al. (48). The general behavior of cation-induced chromatin condensation suggests a contribution of electrostatic interactions to chromatin compaction (37). The constancy of condensed chromatin depends of two forces: an attractive force from the transient dipoles resulting from concentration fluctuations in the positions of the counterion in solution (ion correlation effect) and electrostatic repulsive force from the negatively charged phosphate groups (81). The $S_{20,w}$ values of the wild type array for the Mg^{2+} -titration are in agreement with results of the Dorigo et al. obtained for the same array under similar condition (1). At low concentration of monovalent salt (like 10 mM KCl) the $S_{20,w}$ measured in range of 34-36S for all types of arrays. This condition is characteristic of an extended 12-mer array or ‘beads-on-a-string’ structure. Comparing cations of different charge, a remarkably wide range in the observed concentration needed to induce maximum folding is: from 100 mM Na^+ / K^+ , 1mM Mg^{2+}/Ca^{2+} , 60 μM Spd^{3+} , 20 μM $CoHex^{3+}$, and 2 μM for Spm^{4+} . The higher efficiency of $CoHex^{3+}$ in inducing folding compared to Spd^{3+} is in agreement with the established effects of these cations on DNA condensation (82). This behavior strongly suggests an unspecific electrostatic polyelectrolyte nature of the wild type nucleosomal array folding, similar to DNA condensation (82,83). For all cations except K^+ , the wild type arrays showed the

highest degree of folding with maximal value in the range of 52-55S; the Q-Triple array folding is in the range 51-52S. A value of $S_{20,w} \approx 51-55S$ corresponds to a dense folding of the 12-mer array with gyration radius, $R_g \approx 12-15$ nm and has been confirmed by electron microscopy visualization (50). However, intermolecular array interaction (aggregation and precipitation) follows expected polyelectrolyte behavior with no significant difference between the acetylated and K→Q mutated H4 histone tails.

Figure 4-2. Structural analysis of NCP-NCP stacking (84).

(a) NCP-NCP contact in the crystal 1AOI with important domains involved in formation of the contact shown in space filling: 1 light blue, H4 K16-R23 of the NCP1; 2 dark red, acidic islet of the H2A histone (a.a. E56, E61, E64, D90, E91, E92) in the NCP2 interacting with H4 K16-R23 domain of the NCP1; 3 four red spheres, carbonyl atoms of the main chain peptide H2B R96-L99 of the NCP2; 4 light red, acidic islet of the H2A histone of the NCP2 needed to be screened in stacking contact. (b) Detailed presentation of the H4 K16-R23 contact with the acidic domain of the H2A NCP2. Suggested relocation of the H4-K16 ε-amino group to the carbonyl groups of the H2B R96-L99 domain is shown by curled arrow. (c) The same region of the NCP-NCP contact as in (b) but built using the crystal structure of the NCP saturated with Cs⁺ (3MGS (79)). The Cs⁺ ion coordinated with R96, L97, L99 carbonyl oxygens is seen at the bottom of the cartoon. In (b) and (c), domains are numbered as in (a) with charged oxygen atoms of the carboxylate group in the acidic islet of the H2A histone highlighted as red spheres. (d). Proposed hydrogen bonding of the H4-K16 binding to the crystallographically determined Rb⁺/Cs⁺ binding pocket in (c). To the left is shown the proposed native K16 ε-amine hydrogen bonding and the right demonstrates alternative hydrogen bonding upon K→Q mutation of H4-K16, which maintains this structural element. Acetylation is suggested to completely disrupt this binding due to steric constraints and reduced H-bonding capacity.

Figure 4-3. Comparison of $S_{20,w}$ in different array systems in the presence of 1.6 mM Mg^{2+} (array types are indicated in figure). (55)

We have used nucleosomal arrays containing H4 K5.8.12Q and H4 K5.8.12Ac to investigate the effect of triple mutation/modification on nucleosomal array compaction and effect of the position of modification on chromatin compaction. The maximal S-value of multivalent cations on K5.8.12Q and K5.8.12Ac nucleosomal arrays is shown in table 4.1.

Comparing the result of $S_{20,w}$ in K16Ac with K5.8.12Ac, we found that arrays containing the K5.8.12Q and K5.8.12Ac reached a level only marginally below wild type array (51S). Therefore the number of acetylations in H4 tail is not the sole determination of chromatin folding and therefore the position of acetylation is also important.

Comparing the S-value in K16Ac and K5.8.12Ac with K5.8.12.16Ac, we observed some synergistic effect when quadruple acetylated histone H4 was used. Shogren-Knaak et al. (52) reported that H4-K16Ac and H4 tailless 12-mer arrays can compact at 1 mM $MgCl_2$ till 44S, we got same result for H4-K16Ac but we do not know why the quadruple acetylated H4 has sedimentation velocity is less than the H4 tailless arrays (52). It may be caused by an interfering effect of the four acetyl group with nucleosome-nucleosome stacking.

Because Mg^{2+} is the most commonly used ion applied in nucleosomal array compaction studies, we compare the transition $S_{20,w}$ from wild type, K16Ac/Q , K5.8.12Ac/Q and K5.8.12.16Ac/Q in Fig. 4.4.

Figure 4-4. Comparison of $S_{20,w}$ in different array systems in the presence of 1 mM $MgCl_2$ ($S_{20,w}$ of wild type array in TEK buffer is shown as reference)

4.3 Nucleosomal array aggregation

Light scattering can be used to characterize macromolecule shape, molecular weight (85). Using modern instrumentation, one can readily obtain the weight average molecular weight and radius from total intensity (static) light scattering, and hydrodynamic (Stokes) radius from dynamic light scattering. Theory shows that scattering and hydrodynamic behaviors are strongly nonlinear functions of size, due to hydrodynamic interaction if the particles are composed of multiple subunits. We have used DLS to study the intermolecular aggregation, and it is reflected by increase of light scattering intensity. We used both wild type nucleosomal array and 177-NCP to study the intermolecular aggregation induced by multivalent salt and we compared them as EC_{50} , the concentration of each cation to get 50% of maximum intensity, which are shown in table 3.4. The EC_{50} of from light scattering in 177-NCP is higher than EC_{50} in nucleosomal array, although 177-NCP and nucleosomal array have same sequence but it is unclear, why the EC_{50} of 177-NCP is smaller than that of the array.

4.3.1 Precipitation assay results on the array aggregation

Precipitation assay (PA) is a method to study the nucleosomal array aggregation. This procedure is based on neutralization of array's charge by multivalent cations. Precipitation happens when the number of positive charges of cations is approximately equal to negative charges on nucleosomal array (86). We have used precipitation assay to observe aggregation of nucleosomal array upon addition of multivalent cations. We applied precipitation assay using mono-, di-, tri- and tetravalent salt/ligand to measure the intermolecular aggregation of wild type, K16Ac/Q, K5.8.12Ac/Q and K5.8.12.16Ac/Q arrays and it is compared in Fig 4.5.

Figure 4-5. Comparison of precipitation curves obtained in titrations of different nucleosomal arrays (Type of construct is indicated in figure E and the symbols are the same in figures A-D. The lines are fitting to sigmoidal curves).

Opposite to dynamic light scattering (DLS) results indicating that Na^+ and K^+ could induce the aggregation with as well-defined EC_{50} , in precipitation assay we observed only 20% decrease in absorbance in supernatant solution titrated with Na^+ and K^+ (Figs. 3. 20, 3.21 and 3.22) therefore we could not calculate EC_{50} for sodium and potassium. This apparent discrepancy may be due to differences in solution conditions (array concentration). It is also likely that the in the scattering results, incomplete aggregation occurs, as indicated by smaller intensities and particle sizes in the presence of Na^+ and K^+ as compared to the other cations of higher charge. Smaller and more loosely forming aggregates may also not be able to precipitate under the influence of the centrifugation in the PA, while such particles still contributes to the increase in scattering. We used Mg^{2+} and Ca^{2+} as divalent salts to monitor array aggregation; and observed that Ca^{2+} has a slightly higher efficiency than Mg^{2+} in formation of array aggregation. This is indicated by lower values of EC_{50} of wild type, also nucleosomal arrays containing K→Q mutations and modified histones H4. The higher efficiency of Ca^{2+} to Mg^{2+} in aggregation of nucleosomal array is in agreement with the results that Ca^{2+} has higher affinity for DNA (87,88). We applied cobalt hexamine and spermidine as trivalent cations. For these two cations $\text{Co}(\text{Hex})^{3+}$ is more powerful than Spd^{3+} in provoking aggregation. In Spd^{3+} the charge is distributed in whole molecule but in cobalt hexamine is gathered in one ion. Spermine is the strongest studied ligand for initiating aggregation in all the different array systems.

The EC_{50} of different array systems induced by multivalent cations is compared in Fig. 4.6.

Figure 4-6. Comparison of EC_{50} induced by multivalent cations in different nucleosomal arrays

We measured EC_{50} induced by Mg^{2+} at 2.75 mM for wild type array, this result is in good agreement with Schwarz et al. that measured $Mg^{2+} EC_{50} \approx 2.9$ mM for 12-208 array (89). Our result also is in agreement with result of Wang and Hayes that reported $Mg^{2+} EC_{50}$: 2.38 mM for wild type 12-177 array (53). Small differences observed between our and literature data can be explained by slightly different composition of the background solvent as well as experimental conditions (array concentration, spinning speed/ duration etc).

To investigate the role of histone tail on intermolecular aggregation of nucleosomal array, Schwarz et al (90) reported that arrays lacking all histone tails do not aggregate and arrays lacking various combination of histone tail require higher concentration of divalent cations to aggregate than the wild type array. It was observed that all four histone tails are important for array aggregation. Among these histone tails, the H3 and H4 tails providing the largest contribution to aggregation (46,91).

To investigate the effect of histone modification on intermolecular aggregation, we have prepared three modified H4-K16Ac, H4-K5.8.12Ac and H4-K5.8.12.16Ac arrays and used them in precipitation assay experiment. We have prepared three mutant H4-K16Q, H4-K5.8.12Q and H4-K5.8.12.16Q arrays to assess the difference between the glutamine substitution and lysine acetylation arrays at same position. In contrary to intramolecular compaction that acetyl lysine in position of amino acid 16 in H4 protein had a key effect on chromatin decompaction; we found that glutamine substitution and lysine acetylation have a similar effect on intermolecular aggregation of arrays (Fig. 4.5).

We found that EC_{50} enhances with increasing of number of acetylation in lysine amino acid in H4 tail, also with number of glutamine substitution (K→Q) simultaneously.

5. Conclusion and Future Work

In the first part of my PhD project, I focused on the development of methods to obtain homogeneous fully saturated precisely positional nucleosomal arrays. The most important factor in this system is the 601 sequence, so the 601-based arrays provide intrinsically very well positioned and strongly bound nucleosomes. Compared to the existing *in vitro* array system based on chicken histones and 5S rDNA repeat, the 601 array system is better defined and can be manipulated by recombinant techniques.

After preparing the various types of fully saturated array, I applied biophysical methods like analytical ultracentrifugation sedimentation (AUC) and dynamic light scattering (DLS) to investigate the chromatin condensation behavior. These methods require a well defined model system for which the 601-based array should be the best choice. Unmodified recombinant histones were used for the initial biophysical studies to visualize the compaction of chromatin fiber. Also in the present work folding and oligomerization of nucleosomal arrays induced by a wide range of cations was investigated and compared histone H4 single (K16), triple (K5.8.12) and quadruple (K5.8.12.16) acetylation with corresponding K→Q mutations. In summary work done with unmodified array system led to the following conclusions:

- i) The degree of compaction of the chromatin fiber depends strongly on the valence and concentration of salt present in the buffer.
- ii) For monovalent ions, concentration at ~ 100 mM was needed to induce compaction, whereas divalent ions in the 0.4-1.0 mM were enough to form compact array and as little as 5-20 μM for CoHex³⁺ and 0.5-2 μM for Spm⁴⁺.
- iii) Aggregation of chromatin was observed when the concentration of salt is reached above the level that is required for folding.
- iv) Both nucleosomal array folding and array aggregation are inhibited by electrostatic repulsion between the negatively charged DNA. This repulsion is a major force that drives the formation of an extended bead-on-a-string structure at low to intermediate monovalent salt concentration. Electrostatic screening, ion-ion correlations, and histone tail bridging facilitate formation of the folded and aggregated structures (37).

At the second part of my thesis the result of preparation and biophysical data accumulated in the first stage of my project opened the prospect of comparative studies using similar systems with various mutations and modifications in the arrays which are related to the chromatin control of the vital cell functions like transcription, replication and DNA repair. The results on nucleosomal arrays containing acetylated lysine as following can be summarized:

- i) Acetylation on H4-K16 tail has a strong disruptive effect on nucleosome-nucleosome interaction.
- ii) The number of acetylation in H4 tail is not only determining factor for chromatin compaction, therefore the position of acetylation is also important.
- iii) Comparisons of EC_{50} obtained from precipitation assay shows that EC_{50} increases with enhancement of acetylation in H4 tail.

In my thesis, I did not study the following aspects which are important for nucleosome array folding and aggregation: i) Influence of the linker histones. ii) Dependence of the array folding on the nucleosome repeat length. iii) Influence of a mixtures of cations and ion competition effects. These important factors which influence chromatin structure and dynamics might be addressed in my (and my colleagues in our laboratory) future work.

In the future, I am planning to investigate the following items:

- i) Study the folding behavior of H4 tailless array induced by multivalent salts and charged ligands to compare the result with quadruple acetylated array.
- ii) I am going to study the folding behavior of H4-K16Ac array induced by RbCl and CsCl.
- iii) In order to study the large array molecule behavior, I would like to amplify the long DNA template 36×177- 601 and 60 × 197- 601. After reconstitution with different types of histone octamers, I will get a long nucleosomal array and it is very good model to study the salt behavior by atomic force microscopy (AFM) also pulsed field electrophoresis.
- iv) In cooperation with the laboratory of Dr. Liu Chuan Fa using the native chemical ligation method, we are going to extend the list of histone modifications to study the influence of acetylation in the H3 histone tails as well as methylation and phosphorylation of serine in this histone tails.

6. References

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7. Appendix

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EcoRV	BpiI	Linker	CC	core	Linker	ScaI
157 5'-atcgaagacagtact				- GC		agt
162 5'-atcgaagacagtact			CCGCC	- core	GCAT	agt
167 5'-atcgaagacagtact			GGCCGCC	- core	GCATGTA	agt
169 5'-atcgaagacagtact			CGGCCGCC	- core	GCATGTAT	agt
171 5'-atcgaagacagtact			GGGCCGCC	- core	GCATGTATT	agt
172 5'-atcgaagacagtact			CGGSCCGCC	- core	GCATGTATT	agt
173 5'-atcgaagacagtact			CGGCCCGCC	- core	GCATGTATTG	agt
175 5'-atcgaagacagtact			ACGGCGCCGCC	- core	GCATGTATTGA	agt
177 5'-atcgaagacagtact			TACGGCGCCGCC	- core	GCATGTATTGAA	agt
208 5'-atcgaagacagtact	TATGTGATGGACCCCTATACGCGGCCGCC			- core	GCATGTATTGAACAGCGACCTTGCCGG	agt

ScaI	Linker	CC	core	GC	Linker	BpI	EcoRV
act			-	-			
act		CGCC	-	-		agta	ctgtcttcgat-3'
act		CGCCGC	-	-	GCAT	agta	ctgtcttcgat-3'
act		GGCCGC	-	-	GCATGA	agta	ctgtcttcgat-3'
act		CGCCGC	-	-	GCATGAT	agta	ctgtcttcgat-3'
act		GGCCGC	-	-	GCATGTATT	agta	ctgtcttcgat-3'
act		CGCCGC	-	-	GCATGTATT	agta	ctgtcttcgat-3'
act		CGCCGC	-	-	GCATGTATTG	agta	ctgtcttcgat-3'
act		ACGGCGCC	-	-	GCATGTATTGA	agta	ctgtcttcgat-3'
act		TACGGCGCC	-	-	GCATGTATTGAA	agta	ctgtcttcgat-3'
act	TATGTGATGGACCTATACGGCGCC	-	-	-	GCATGTATTGAACAGCGACCTTGCCGG	agta	ctgtcttcgat-3'

Appendix II. Van Holde-Weischet curves for the mutant nucleosomal array in the presence of A: NaCl, B: KCl, C: $MgCl_2$, D: $CaCl_2$, E: spermidine, F: $Co(NH_3)_6Cl_3$ and G: spermine. (Concentration is indicated in figure)

Appendix III. Van Holde-Weischet curves for the modified nucleosomal array in the presence of
A: NaCl, B: KCl, C: $MgCl_2$, D: $CaCl_2$, E: spermidine, F: $Co(NH_3)_6Cl_3$ and G: spermine.
 (Concentration is indicated in figure)