Studies on roles of histone H1 in nucleosome condensation

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**STUDIES ON ROLES OF HISTONE H1 IN
NUCLEOSOME CONDENSATION**

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2015

**STUDIES ON ROLES OF HISTONE H1 IN
NUCLEOSOME CONDENSATION**

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in fulfillment of the requirement for the degree of
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Table of Contents

Acknowledgments.....	I
Table of Contents.....	II
Summary	VII
List of Tables.....	IX
List of Figures	X
List of Abbreviation.....	XVII

Chapter 1 - Introduction

1.1 DNA Structure and Topology	1
1.1.1 Molecular structure of DNA	1
1.1.2 DNA conformation	2
1.1.3 DNA supercoiling	4
1.1.4 DNA topological changes	7
1.2 Histone, Nucleosome, Chromatin, and Chromosome.....	10
1.2.1 Chromosome packaging.....	10
1.2.2 Core histones and nucleosome core particle	12
1.2.3 Linker histone H1 and the 30-nm chromatin fibre.....	15
1.3 Nucleic Acid Gel Electrophoresis.....	19
1.3.1 Fundamentals of electrophoresis.....	19
1.3.2 Electrophoretic analysis of DNA supercoiling	21
1.4 Atomic Force Microscopy of Biological Samples.....	23

1.4.1	Fundamentals of atomic force microscopy	23
1.4.2	Advantages of AFM in biological applications	26
1.5	Principles of Fluorescence Resonance Energy Transfer (FRET)	27
1.6	References.....	29

Chapter 2 - Atomic Force Microscopy Verification of Chromatosome Aggregation with the Aid of Linker Histone H1

2.1	Background Information	44
2.2	Materials and Methods.....	49
2.2.1	Oligodeoxyribonucleotides, enzymes, chemicals and equipments.....	49
2.2.2	Nucleosome assembly.....	50
2.2.3	Histone H1-DNA binding assay	50
2.2.4	Chromatosome assembly	51
2.2.5	Preparation of open-circular DNA.....	51
2.2.6	Topoisomerase I relaxation of supercoiled DNA	51
2.2.7	DNase I digestion assay	52
2.2.8	Synthesis of APS (1-(3-aminopropyl)silatrane).....	52
2.2.9	Adsorption of DNA to mica mediated by counterions	53
2.2.10	Adsorption of DNA to APS-modified mica.....	53
2.2.11	AFM imaging of DNA and DNA-histone complexes	54
2.3	Results and Discussion	54
2.3.1	Visualization of topologically-different forms of double-stranded DNA with atomic force microscopy.....	54
2.3.2	Verification and characterization of the complexes formed by circular DNA and histone proteins	67

2.3.3	Investigation on compaction of nucleosomal arrays by the action of linker histone H1	79
2.3.4	Disaggregation of the compact chromosomal structures by relieving the torsional tension in circular DNA.....	87
2.4	Conclusion	93
2.5	References.....	94

Chapter 3 - Gel Electrophoretic Determination of Linker DNA Supercoiling Induced by DNA-Histone Interactions

3.1	Background Information	103
3.2	Materials and Methods.....	106
3.2.1	Oligodeoxyribonucleotides, enzymes, chemicals and equipments.....	106
3.2.2	Nucleosome assembly.....	107
3.2.3	Histone H1-DNA binding assay	107
3.2.4	Chromatosome assembly	108
3.2.5	Preparation of open-circular DNA.....	108
3.2.6	Ligation of nicked circular DNA	108
3.2.7	Removal of proteins from DNA samples.....	109
3.2.8	Agarose gel electrophoresis	109
3.3	Results and Discussion	110
3.3.1	Gel electrophoretic analysis of DNA topological properties	110
3.3.2	Determination of DNA supercoiling generated by the formation of nucleosome core particle	120
3.3.3	Determination of DNA supercoiling generated by the action of histone H1	131

3.3.4	Examination of DNA topological changes in the closed circular nucleosomal arrays by linker histone H1	143
3.4	Conclusion	150
3.5	References	152

Chapter 4 - FRET-based Studies of Linker DNA's Path on Cylindrical Histone Octamer

4.1	Background Information	158
4.2	Materials and Methods	161
4.2.1	Oligodeoxyribonucleotides, enzymes, chemicals and equipments	161
4.2.2	Nucleosome assembly	162
4.2.3	Histone H1-DNA binding assay	162
4.2.4	Chromatosome assembly	163
4.2.5	Annealing of oligonucleotides	163
4.2.6	Ligation of duplex DNA fragments	163
4.2.7	Agarose gel electrophoresis	164
4.2.8	Polyacrylamide gel electrophoresis	164
4.2.9	AFM imaging of DNA and DNA-histone complexes	165
4.2.10	Fluorescence measurements	165
4.3	Results and Discussion	166
4.3.1	Construction of DNA-based FRET systems for determination of nanoscale distances	166
4.3.2	Fluorescence studies of histone H1-induced inter-molecular DNA aggregation	174
4.3.3	FRET-based determination of the path of linker DNA under physiological conditions	181
4.3.4	FRET-based investigation of the topological roles of	

histone H1 in nucleosomal DNA condensation.....	192
4.4 Conclusion	199
4.5 References.....	200
List of Publications	206

Summary

Genomic DNA in eukaryotic cells is packaged with associated proteins into a set of compact chromosomes. The first level of DNA packaging involves the formation of nucleosomes in which chromosomal DNA wraps around core histone proteins. The binding of linker histone H1 organizes the nucleosomal arrays into a more condensed chromatin fibre. However, the exact location and binding mode of histone H1 as well as its fundamental roles in nucleosome condensation still remain controversial up till this day.

In this study we investigate the effect of histone H1 on DNA topological properties, and attempt to elucidate the inherent relationship between the DNA superhelical path in the nucleosome and the supercoiling in the linker DNA segments, which may change the traditional view of linker histone action and chromatin fibre organization. In the first part, atomic force microscopy was used to directly visualize and characterize DNA-histone complexes. It has been demonstrated that cooperative binding of linker histone H1 onto the topologically closed nucleosomal arrays remarkably triggers the formation of DNA-histone higher order structures. Furthermore, disintegration of the histone H1-induced nucleosome macroaggregates was observed upon removal of the negative supercoils by DNA topoisomerase, which suggests the existence of negative DNA supercoiling within the linker DNA and its possible roles in nucleosome compaction.

Based on these remarkable findings, additional electrophoretic studies were carried out to quantitatively determine the number of DNA supercoils generated by the binding of histone H1. A rough estimate of the degree of supercoiling was

presented and discussed in the second part. Accordingly, we propose here a model depicting the effect of linker histone H1 on chromosomal DNA topology. It is our belief that the histone H1-induced DNA supercoiling is likely to serve as the triggering event for DNA condensation.

In order to disclose the structural origin of these DNA supercoils, in the third part, a series of DNA-based fluorescence resonance energy transfer systems were designed and developed for studying the path of internal linker DNA on cylindrical histone octamer. Our results showed that upon the binding of histone H1, two linker DNA segments orient themselves towards each other, which is opposite to the directions of nucleosomal DNA paths in the original 1.67 turns. From a topological point of view, this right-handed solenoidal wrap of linker DNA on histone octamer was expected to constrain local positive supercoiling inside the chromatosome particle, and will be theoretically compensated with the global negative supercoils introduced in the overall chromatin fibres.

It is therefore our hope that the discoveries presented in this thesis could have certain implications in our understanding of the roles of linker histones in the higher-order organization of eukaryotic chromosomes.

List of Tables

Table 2-1	Comparison of the DNA visible superhelical densities obtained from three different sample deposition methods.	65
Table 2-2	Correlation between the number of nucleosome core particles formed on each plasmid DNA molecule and the molar ratio of DNA to histone octamer.	72
Table 2-3	Correlation between the average height of DNA-histone aggregates and the molar ratio of DNA to histone H1.	75
Table 2-4	Correlation between the number of nucleosome core particles formed on plasmid DNA and the concentration of NaCl.	76
Table 2-5	Correlation between the average height of DNA-histone aggregates and the concentration of NaCl.	78
Table 2-6	Correlations between the average height and diameter of the histone H1-induced DNA-histone aggregates and the molar ratio of DNA to histone proteins.	83
Table 2-7	Correlations between the average height and diameter of the nucleosome particles and the molar ratio of DNA to histone proteins.	86
Table 4-1	Sequence of oligonucleotides used for the construction of our newly designed duplex DNA standards.	169
Table 4-2	Comparison of the experimentally-determined distances (R_e) between fluorophores and the computational-determined distances (R_c) between the corresponding fluorophore-linked phosphate groups.	172
Table 4-3	Sequence of oligonucleotides used for the construction of our newly designed duplex DNA standards.	176
Table 4-4	Sequence of oligonucleotides used for the construction of our fluorophore-labeled 601-element-containing DNA duplexes.	184

List of Figures

Figure 1-1	Illustration of the double helix structure for DNA.	1
Figure 1-2	Chemical structures of four principal bases in DNA.	2
Figure 1-3	Structural parameters of B-form DNA double helix.	3
Figure 1-4	Illustration of relaxed and supercoiled circular DNA molecules.	5
Figure 1-5	Schematic illustration of plectonemically interwound and solenoidal forms of supercoiled DNA.	6
Figure 1-6	Generalized mechanism for the action of DNA topoisomerases.	8
Figure 1-7	Schematic illustration of the DNA supercoiling locally generated by the formation of nucleosome core particle, and PNA invasion into duplex DNA.	10
Figure 1-8	Schematic illustration of chromosome packaging in eukaryotic cells.	11
Figure 1-9	Crystal structure of the nucleosome core particle.	15
Figure 1-10	Schematic illustration of the association of linker histones with nucleosomal DNA and the proposed models of the 30-nm chromatin fibre.	18
Figure 1-11	Schematic illustration of conventional gel electrophoresis of DNA in a horizontal agarose gel.	20
Figure 1-12	Schematic illustration of a typical atomic force microscopy.	24
Figure 1-13	Jablonski diagram illustrating the fluorescence resonance energy transfer (FRET) process.	28
Figure 2-1	Schematic illustration of linker histone H1-induced 30-nm chromatin fibres.	45
Figure 2-2	Schematic illustration of DNA topological domains in prokaryotic and eukaryotic genomes.	46
Figure 2-3	Schematic illustration of four topological states of	55
		X

duplex DNA.

Figure 2-4	AFM visualization of different forms of plasmid DNA.	57
Figure 2-5	Quantitative height measurement of double-stranded DNA by section analysis using the Nanoscope Analysis software.	58
Figure 2-6	Determination of the straight line distance between two points on a DNA contour map of digitized AFM image.	59
Figure 2-7	Schematic illustration of divalent counterions mediated absorption of duplex DNA on mica surface, and absorption of duplex DNA on APS-modified mica surface.	60
Figure 2-8	AFM visualization of highly supercoiled DNA molecules mediated by magnesium cations.	61
Figure 2-9	AFM visualization of highly supercoiled DNA molecules on spermidine pre-treated mica.	62
Figure 2-10	AFM visualization of highly supercoiled DNA molecules immobilized on APS-modified mica.	64
Figure 2-11	AFM visualization of ethidium bromide-induced topological changes of circular DNA.	66
Figure 2-12	Schematic illustration of the formation of nucleosomal arrays on plasmid DNA and the histone H1-induced DNA aggregates.	69
Figure 2-13	AFM images of the reconstituted nucleosomal arrays on pBR322 plasmid DNA.	70
Figure 2-14	Illustration of height, and size measurement of the nucleosome core particles by section analysis using the Nanoscope Analysis software.	72
Figure 2-15	AFM images of DNA-histone H1 aggregates on the mica mediated by magnesium cations or modified by APS.	74
Figure 2-16	AFM studies of salt effects on the stability of	76

nucleosome core particles.

Figure 2-17	AFM studies of salt effects on formation and dissociation of DNA-histone H1 aggregates.	78
Figure 2-18	Illustration of chromosome structure and the previously proposed location of linker histone globular domain.	80
Figure 2-19	AFM visualization of histone H1-induced aggregation of nucleosomal arrays.	82
Figure 2-20	Height measurements of DNA-histone aggregates by section analysis using the Nanoscope Analysis software.	83
Figure 2-21	AFM visualization of interactions between linker histone H1 and nucleosomal arrays on the nicked plasmid DNA.	85
Figure 2-22	Schematic illustration of DNA superhelical tension that may be stored in the linker DNA between chromosomes.	88
Figure 2-23	AFM examination of DNase I accessibility of the histone H1-induced nucleosome aggregates.	89
Figure 2-24	Schematic illustration of the relaxation of DNA supercoils that may be involved in the histone H1-induced nucleosome aggregates catalyzed by DNA nicking endonuclease or E.coli DNA topoisomerase I.	90
Figure 2-25	AFM examination of the histone H1-induced nucleosome aggregates relaxed by DNA nicking endonuclease.	91
Figure 2-26	AFM examination of the histone H1-induced nucleosome aggregates relaxed by E.coli DNA topoisomerase I.	92
Figure 3-1	Agarose gel electrophoretic analysis of four topological forms of double-stranded DNA.	111
Figure 3-2	Schematic illustration of negatively supercoiled double-stranded circular DNA with different superhelical turns (writhing numbers).	112

Figure 3-3	Electrophoretic separation of negatively supercoiled pUC19 topoisomers on standard agarose gel.	113
Figure 3-4	Electrophoretic separation of supercoiled pUC19 topoisomers on chloroquine-containing agarose gel.	114
Figure 3-5	Electrophoretic separation of negatively supercoiled pBR322 topoisomers on agarose gel in the absence or presence of chloroquine.	117
Figure 3-6	Quantitative gel electrophoretic analysis of the equilibrium distribution of circular DNA topoisomers by Syngene gel imaging system using GeneTools software.	118
Figure 3-7	Determination of the Gaussian center of DNA topoisomers.	119
Figure 3-8	Schematic illustration of the topological changes occurring in relaxed circular DNA by the formation of nucleosome core particles.	121
Figure 3-9	Schematic illustration of our designed experimental route for determining circular DNA supercoiling changes generated by the formation of nucleosome core particles (Route 1).	123
Figure 3-10	Schematic illustration of our designed experimental route for determining circular DNA supercoiling changes generated by the formation of nucleosome core particles (Route 2).	124
Figure 3-11	Electrophoretic analysis of DNA supercoiling changes generated by the formation of nucleosome core particles via Route 1 and Route 2.	125
Figure 3-12	Quantitative analysis and determination of the supercoiling levels of DNA topoisomer distributions produced via Route 1 or Route 2.	126
Figure 3-13	Electrophoretic analysis of DNA supercoiling changes generated by the formation of nucleosome core particles with increasing concentration of histone octamer.	128

Figure 3-14	Electrophoretic analysis of DNA supercoiling changes generated by the formation of nucleosome core particles with increasing concentration of spermidine.	130
Figure 3-15	Schematic illustration of the topological changes occurring in relaxed circular DNA by non-specific binding of linker histone H1.	133
Figure 3-16	Schematic illustration of our designed experimental route for determining circular DNA supercoiling changes generated by the binding of linker histone H1.	135
Figure 3-17	Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 via Route 1 and Route 2.	136
Figure 3-18	Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 to naked pBR322 DNA with increasing concentration of histone H1.	138
Figure 3-19	Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 to naked pUC19 DNA with increasing concentration of histone H1.	140
Figure 3-20	Electrophoretic analysis of the effect of salt on the level of DNA supercoiling generated by linker histone H1.	142
Figure 3-21	Schematic illustration of our designed experimental route for determining the changes in chromosomal DNA supercoiling induced by linker histone H1 binding to nucleosomes.	145
Figure 3-22	Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 to the nucleosomal arrays constructed on pBR322 DNA.	147
Figure 3-23	Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 to the nucleosomal arrays constructed on pUC19 DNA.	149
Figure 3-24	Illustration of our proposed model of linker histone-	152
		XIV

induced nucleosome condensation.

Figure 4-1	FRET-based distance determination on the nanometre scale.	160
Figure 4-2	Schematic illustration of fluorescence resonance energy transfer (FRET) occurring between Cy3 and Cy5 fluorescent moieties covalently linked to the phosphate groups on DNA.	167
Figure 4-3	Illustration of our newly designed duplex DNA standards with Cy3 and Cy5 fluorophores labeled terminally or internally.	168
Figure 4-4	Fluorescence absorption and emission spectra of the Cy3-labeled oligonucleotide and the Cy5-labeled oligonucleotide.	170
Figure 4-5	Fluorescence spectra of our newly designed duplex DNA standards with Cy3 and Cy5 fluorophores labeled terminally or internally excited at the wavelength of 500 nm.	171
Figure 4-6	The standard curve showing the correlation between experimentally-determined distances (R_e) and computational-determined distances (R_c).	173
Figure 4-7	Illustration of our proposed model of the histone H1-induced intermolecular aggregation of fluorophore-labeled DNA fragments.	175
Figure 4-8	Illustration of our newly designed 206 base pairs fluorophore-labeled DNA duplex prepared from ten pieces of single-stranded oligonucleotides.	176
Figure 4-9	Fluorescence examination of our newly designed 206 base pairs fluorophore-labeled DNA duplex excited at 500 nm, or 600 nm.	177
Figure 4-10	FRET studies on the interaction between duplex DNA and linker histone H1.	178
Figure 4-11	Conformation of the histone H1-induced DNA duplex aggregation.	179
Figure 4-12	FRET studies on the effect of salt concentrations on	180

the formation of histone H1-DNA aggregates.

Figure 4-13	Illustration of our FRET-based determination of linker DNA's path on cylindrical histone octamer.	183
Figure 4-14	Conformation of our newly prepared fluorophore-labeled 601-element-containing DNA duplex.	186
Figure 4-15	FRET studies on the distance between the ends of linker DNA in the histone H1-deficient mononucleosome particle.	188
Figure 4-16	FRET studies on the effect of temperature on the conformation of nucleosomal DNA.	189
Figure 4-17	FRET studies on the effect of salt concentrations on the conformation of nucleosomal DNA.	190
Figure 4-18	FRET studies on the effect of spermidine on the conformation of nucleosomal DNA.	191
Figure 4-19	Our proposed model of histone H1-induced alteration in the linker DNA path and the positive supercoils constrained within the chromatosome.	194
Figure 4-20	FRET studies on the distance between the ends of linker DNA in the mononucleosome and the monochromatosome particles.	195
Figure 4-21	Our FRET-based spatial distance measurements between the entering and exiting linker DNA segments in the histone H1-deficient mononucleosome and the histone H1-bound monochromatosome particles.	196
Figure 4-22	AFM conformation of the inexistence of the unspecific histone-DNA inter-nucleosomal aggregates.	197
Figure 4-23	FRET studies on the effect of salt concentrations on the path of linker DNA in the histone H1-bound monochromatosome particle.	198

List of Abbreviations

AFM	atomic force microscopy
APS	1-(3-aminopropyl)silatrane
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
°C	degree Celsius
cccDNA	covalently closed circular DNA
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
FRET	fluorescence resonance energy transfer
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Lk	linking number
TAE	tris, ammonium acetate, EDTA buffer
TBE	tris, boric acid, EDTA buffer
TEMED	N, N, N', N'-tetramethylenediamine
topo I	topoisomerase I
Tris	tris(hydroxymethyl)aminomethane
Tw	twist number
Wr	writhe number

Chapter 1

Introduction

1.1 DNA Structure and Topology

1.1.1 Molecular structure of DNA

Nucleic acids, which are major biological macromolecules essential for all forms of life, were first discovered by Friedrich Miescher in 1869¹. On the basis of different monomeric unit, nucleic acids are divided into two groups, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), while DNA is found in almost all living organisms and is known as hereditary material to carry genetic information between generations. As illustrated in Figure 1-1, DNA is a chain-like polymeric macromolecule which is built from individual nucleotides linked through phosphodiester bonds.

Figure 1-1. Illustration of the double helix structure for DNA.

Each nucleotide is composed of (i) a five carbon sugar molecule, (ii) a nitrogen-containing base, and (iii) a phosphate molecule. The sugar in DNA is deoxyribose, which is a pentose sugar ribose lacking a hydroxyl group at its 2'-position when compared to ribose (sugar molecule found in RNA). In DNA, there are four different nitrogenous bases: Adenine (A), Guanine (G), Cytosine (C) and Thymine (T), chemical structures of which are shown in Figure 1-2. Of these four bases, A and G which have a double-ringed structure are known as purines, whereas T and C containing single six-member ring are pyrimidines. One of the nitrogenous bases covalently attached to the 1'-position of deoxyribose sugar to form a nucleoside as indicated in Figure 1-1. The free hydroxyl on the 3'-position of the sugar is connected to the 5' carbon of the next sugar through a phosphate group. In this way, individual nucleotides are linked one by one by 3'-5' phosphodiester bonds to form the DNA polynucleotide chain. Consequently, this sequence of nucleotide bases gives the primary structure of DNA.

Figure 1-2. Chemical structures of four principal bases in DNA.

1.1.2 DNA conformation

The interactions between bases give the secondary structure of DNA. The original DNA model is first presented by James Watson and Francis Crick in 1953 in their scientific paper², and proved by Rosalind Franklin using X-ray

crystallography³⁻⁴. In their proposed model, B-form DNA exists as a duplex structure, in which two DNA strands run in opposite directions, each in a right-handed helix, and coiled around a common axis to form an antiparallel double helix as shown in Figure 1-3^{2, 4}. The two strands of DNA are held together by hydrogen bonds that occur between the complementary nucleotide bases in anti conformation. According to Chargaff's rules, the two strands will pair adenine with thymine while guanine with cytosine⁵. Moreover, three hydrogen bonds form between guanine and cytosine, but only two hydrogen bonds can form between adenine and thymine. As a result, double-stranded DNA with a high GC content is, in most cases, more stable than DNA with a low GC content.

As indicated in Figure 1-3, B-form DNA is ~20 Angstroms (Å) in diameter with about 10.5 base pairs per helical turn. The axial rise (distance between two adjacent base pairs) of B-DNA is 3.4 Å and the overall helical pitch is 35.7 Å⁶⁻⁸. As the two strands of DNA are not symmetrically located around the center of the helix, a major groove, 22 Å in width, occurs where the phosphate backbones are far apart. On the other side, the minor groove, 22 Å in width, appears where they are close together⁹.

Figure 1-3. Structural parameters of B-form DNA double helix. In the right-handed double helix of B-form DNA, the base pairs are separated by about 3.4 angstroms with a helical repeat of 10.5 bp (adapted from "DNA Structure and Function", by Sinden, R. R., 1994⁸).

Other than the B-form helix, DNA can assume a wide range of structural forms that include A-form¹⁰ (a thicker right-handed duplex with a shorter axial rise), Z-form¹¹ (a left-handed duplex with a larger axial rise), C-form¹², etc. Although most of DNA molecules have been observed as B-form in naturally occurring biological systems¹³⁻¹⁴, DNA may undergo conformational changes at particular conditions of temperature, pH, solvent, salt concentrations and the nature or sequence of nucleotides¹⁵⁻²⁰.

1.1.3 DNA supercoiling

As introduced above, under physiological conditions, duplex DNA is usually present as B-form helix, in which the right-handed double helix makes a turn every 10.5 base pairs. In linear DNA molecules, this kind of arrangement could be achieved by winding of two DNA strands around each other. However, in the covalently closed circular DNA (cccDNA), both strands of a double-stranded DNA molecule are topologically constrained as shown in Figure 1-4. In this kind of structure, the number of times one strand crosses over the other remains constant, which is called the "linking number" (*Lk*). Since two DNA strands must be wound around each other an integral number of times to form a covalently closed circle. The linking number of circular DNA, therefore, is always an integer. Depending on the orientation of the two strands, the linking number can be either

positive or negative. By convention, the linker number of the right-handed B-form circular DNA is positive²¹.

Figure 1-4. Illustration of relaxed and supercoiled circular DNA molecules.

Topologically, the linking number can be resolved into the other two components in closed circular DNA: twist (Tw) and writhe (Wr). The twist is a property of DNA double helix, which represents the number of helical turns in the DNA. In B-form DNA, the double helix makes one full turn every 10.4-10.5 base pairs without torsional strain⁶⁻⁸. The number of twist can be therefore calculated based on the overall length of DNA. Different from that of linking number, the twist is generally not an integer, but remains constant in a given segment of DNA free of torsional strain. Unlike the linking number and twist, the writhe of circular DNA stands for the total number of turns of the duplex axis coiled about itself in three-dimensional space. The writhe depends on the spatial path of DNA helix axis. The number of writhe will be zero if the overall DNA molecule lies in one plane. However, if the DNA double-helix twists around itself like a circular rubber band, forming a superhelix structure, the obtained writhing turns are termed

plectonemically interwound coiling as shown in Figure 1-5A. On the other hand, if the DNA molecule wraps around proteins or other molecules, forming a spring-like structure, this type of writhe is named solenoidal (or toroidal) form as shown in Figure 1-5B. Both these types of coiling are known as DNA supercoiling, and the circular DNA molecules with either type of writhes are called supercoiled DNA.

Figure 1-5. Schematic illustration of (A) plectonemically interwound and (B) solenoidal forms of supercoiled DNA (adapted from "DNA structure: alphabet soup for the cellular soul", by Ho, P. S. and Carter, M., 2011²²).

DNA supercoiling is essential for DNA packaging in both prokaryotic and eukaryotic cells. Supercoiling allows for compact packing of cellular DNA into tiny spaces. In prokaryotic cellular DNA, interwound supercoils are known to be predominant, while in eukaryotic chromosome, DNA is wound into both solenoidal and interwound configurations. Characterization of DNA supercoiling is based on all of the three components, linking number (Lk), twist (Tw) and writhe (Wr). As described by Fuller in 1971²³, the linking number of a covalently closed circular DNA is always the sum of the Twist and the Writhe. The relationship of these three components can be shown by the equation below:

$$Lk = Tw + Wr \quad (1.1)$$

where writhe (Wr) in this equation represents the number of superhelical turns in the DNA. It is a characteristic of circular DNA that describes the shape of DNA

molecule as a whole. As shown in Figure 1-4, the partially unwound circular DNA (left) has $Tw = 20$ and $Wr = 0$, whereas the supercoiled DNA (right) has $Tw = 21$ and $Wr = -1$. The two forms of supercoiled DNA have the same linking number, which is 20, and thus can be interconverted without breaking of DNA strands. In order to compare the extent of supercoiling between different DNA molecules, the term superhelical density (σ) is introduced, which is defined as the number of superhelical turns relative to the total number of linking number:

$$\sigma = \tau / Lk_0 \quad (1.2)$$

in which τ indicates the superhelical turns that is equivalent to the linking difference between the linking number of certain DNA and the linking number (Lk_0) of the DNA in its relaxed state.

1.1.4 DNA topological changes

DNA topology, as a fundamental property of DNA double helix, is of great importance in the process of DNA replication, segregation, recombination, and transcription²⁴⁻²⁹. According to topological rules³⁰, the global topological state of DNA can be altered by (i) the change in linking number, and (ii) the change in twisting number. As we know, the linking number of DNA represents the number of crosses that a single strand wrap around the other. Therefore, it cannot be changed without breaking the DNA strand, unwinding or rewinding, then rejoining it. In all cellular organisms, this complex function can be achieved by a class of ubiquitous enzymes, namely DNA topoisomerases. These kind of enzymes are further classified into type I and type II according to their different structure and mechanism^{21, 31-32}. As illustrated in Figure 1-6A, type I topoisomerases break one strand of the duplex DNA, allow this strand to rotate around the other strand, and

then reseal it. In this way, the type I topoisomerase changes the linking number by intervals of one³³. Unlike type I topoisomerases, type II topoisomerases bind and cut both strands of one duplex DNA, facilitate the passage of another unbroken DNA through the break, and re-ligate the cutting site. As illustrated in Figure 1-6B, type II topoisomerases require the energy from ATP hydrolysis and change the linking number by two³³⁻³⁴.

Figure 1-6. Generalized mechanism for the action of DNA topoisomerases. (A) Type I topoisomerase introduces a single-stranded break in the DNA allowing the rotation of one strand around the other strand. (B) Type II topoisomerase cleaves both strands of duplex DNA allowing the passage of another DNA duplex through the break with the help of ATP (redrawn from "DNA Topology", by Andrew D. Bates and Anthony Maxwell, 2005²⁴).

Other than the linking number, the changes in DNA twist can result in the topological change in circular DNA as well. Goldstein and Drlica reported their findings that circular plasmid DNA could undergo a temperature-dependent change in helical twist³⁵. In addition, previous investigations of the effects of salt

concentrations on DNA topological properties have suggested that an increase in the ionic strength (*e.g.* Na^+ , Mg^{2+}) may cause an increase of the twist angle, and further lead to negative supercoiling of the DNA axis³⁶⁻⁴². Besides these environmental factors, a number of small organic molecules, once interact with duplex DNA, are demonstrated to affect the twist and change the physical properties of the double helix. Among them, DNA intercalating agents (*e.g.* ethidium bromide, berberine, chloroquine) can insert themselves into the two adjacent base pairs and thus reduce the twist of the closed circular DNA^{24, 43-46}. On the contrary, DNA minor groove binding agents (*e.g.* netropsin) have been proved to increase the helical twist of DNA⁴⁷⁻⁴⁸.

Different from the above mentioned factors that may change the DNA superhelicity globally, there are also a number of strategies that can introduce local changes in DNA topology. These local variations in DNA supercoiling are known to have significant biological and functional consequences for the living cell, especially during the nucleosomal packaging of eukaryotic DNA. It has been found in all eukaryotic chromosomes that the DNA is wrapped in a left-handed superhelix around histone proteins producing the nucleosome particle. In a covalently closed circular DNA, each nucleosome formed is proved to constrain one negative solenoidal supercoil in the complex, and thereby induces one positive plectonemic supercoil in the histone-free region of the same DNA molecule^{7, 49} (illustrated in Figure 1-7A). Compensatory accumulation of these locally-generated positive supercoils in eukaryotic chromatin can be recognized and further relaxed by cellular topoisomerases, leaving the net negative supercoiling in chromosomal DNA^{8, 50}. Additionally, some of bacterial proteins (*e.g.* HU protein) have been reported to affect DNA supercoiling in a dose-dependent fashion⁵¹.

These proteins have been proved to have similar effects as histone cores, generating localized supercoils upon binding to topological constrained DNA molecules.

Moreover, recent studies on nucleic acid analogues suggested that some particular synthetic nucleic acid oligomers (*e.g.* PNA) can bind with sequence specificity to complementary DNA target, partially unwind the DNA double helix, and form a triplex invasion complex while leaving the other DNA strand unbound. In the case of closed circular DNA, this DNA strand-invasion can subsequently create compensatory positive supercoils into (or relieve negative supercoils from) the rest region of DNA⁵²⁻⁵³ (illustrated in Figure 1-7B).

Figure 1-7. Schematic illustration of the DNA supercoiling locally generated by (A) the formation of nucleosome core particle, and (B) PNA invasion into duplex DNA.

1.2 Histone, Nucleosome, Chromatin, and Chromosome

1.2.1 Chromosome packaging

A chromosome is an orderly packaged structure in which the DNA of organisms is organized in a compact manner. The structure of chromosomes varies widely between different types of organisms. Prokaryotic cells are thought to contain only one small size chromosome, in which $\sim 10^6$ - 10^7 base paired DNA are

usually presented in circular form. As these cells lack a defined nucleus, typically, their chromosomal DNA is located in a clear region of cell known as the nucleoid which is not surrounded by a nuclear membrane. In contrast, eukaryotic cells store their genomic DNA in multiple, linear chromosomes, which are fundamentally different from the circular structures found in prokaryotes. These eukaryotic thread-like chromosomes appear in a cell nucleus surrounded with a nuclear membrane.

In both prokaryotic and eukaryotic cells, their genomic DNA must be folded and coiled so as to fit into the tiny nucleus. Chromosomal compaction in most of prokaryotic cells involves proteins and polyamines that help to organize chromosomal DNA into a DNA-protein complex. Eukaryotes, however, undergo a more complex process of DNA condensation. Human genomic DNA, for example, totals about two meters in length have to be packed into a compact form to fit in the nucleus whose diameter is around 6 μm . To achieve such an extremely high packing ratio, the very long genomic DNA is not packaged into final structure of chromosome directly. Instead, as shown in Figure 1-8, chromosome packaging occurs on several levels in eukaryotic cells.

Figure 1-8. Schematic illustration of chromosome packaging in eukaryotic cells (adapted from "Essential Cell Biology, Fourth Edition", by Bruce Alberts *et al.*, 2013⁵⁴).

The basic level of packaging is achieved by the wrapping of DNA around histone core proteins to form nucleosomes. Nucleosomes are connected by stretches of "linker DNA" to produce the 10 nm-width "beads-on-a-string" appearance, which gives a DNA packing ratio of about 7⁵⁵. The resulting complexes between DNA and proteins are called chromatin. In the next level of packaging, with the help of linker histone, the chromatin is further coiled into a fibre approximately 30 nm in diameter, which increases the packing ratio to about 40⁵⁵. The higher levels of compaction are still not clearly understood. A new protein-based structure named as chromosome scaffold has been reported and believed to be involved in the further condensation of chromosomal DNA⁵⁶⁻⁶⁰. Recent studies suggested that the chromosome scaffold is largely composed of the enzyme topoisomerase II and ScII, which is a structural maintenance of chromosomes family member⁶¹⁻⁶⁵. In their model, the 30 nm filament is formed into loops and anchored to the chromosome scaffold at specific sites (scaffold attachment regions), giving a final packing ratio of about one thousand in metaphase chromosomes⁶⁶⁻⁶⁷. In this way, the double-stranded genomic DNA of eukaryotic cell can be tightly packaged to fit into the small space of the cell's nucleus.

1.2.2 Core histones and nucleosome core particle

As we have discussed above, eukaryotic chromosomal DNA is wrapped around proteins to form the repeated unit of chromatin named nucleosome. In

1970's, the nucleosome was first identified by Roger Kornberg in nuclease digestion experiments⁶⁸. Micrococcal nuclease (MNase) which is an enzyme that degrades DNA, was utilized to perform the partial digestion of chromatin, and was found to cut DNA in approximately 200 base pairs intervals. Additionally, further digestion of chromatin resulted in a particle called chromatosome which consists of 166 base pairs of DNA wrapped around the histone core and held in place by linker histone⁶⁹, while prolonged digestion gradually cleaves additional 20 nucleotides, generating the nucleosome particle with 146 base pairs of protected DNA⁷⁰. Subsequent studies on the nucleosome crystal structures revealed that the nucleosome core particle consists of the nucleosomal DNA and an protein core which is formed by eight core histone proteins, two molecules each of four different types of histone called H2A, H2B, H3 and H4⁷¹⁻⁷⁵. All of these core histones are remarkably conserved and extremely abundant in most of eukaryotic cells.

During nucleosome assembly, histone H3 and H4 form a heterodimer in solution firstly, and then the two copies of H3-H4 dimer associate to form a H3₂-H4₂ heterotetramer. This tetramer recognizes and wraps DNA around itself. Subsequently, two H2A-H2B dimers further bind onto the H3₂-H4₂ tetramer from each side to form a nucleosome core particle⁷⁴⁻⁷⁷. Furthermore, recent studies on the detailed structures of histone revealed that the N-terminal region of eight core histones are rich in basic amino acids such as lysine and arginine, and thus have a higher positive charge density. These positively charged tails are known to protrude from nucleosome particle and interact with the negatively charged DNA on neighboring nucleosomes, and they are believed to play important roles in the formation of higher-order chromosome structures⁷⁸⁻⁸².

DNA is wrapped around the imaginary cylindrical surface of the histone octamer. In the recent years, high resolution crystal structures of the DNA-containing nucleosome core particle has been reported^{71, 73, 75}, in which new levels of detailed information about the interactions between nucleosomal DNA and histone octamers have been provided. X-ray diffraction patterns revealed that nucleosome core particle consists of 146-147 base pairs of DNA wrapped around the histone octamer in 1.67 left-handed superhelical turns. As shown in Figure 1-9, this superhelix has a radius of 41.9 Å and a pitch of 25.9 Å. Most importantly, the 1.67 left-handed turns of the nucleosomal DNA's path appear to generate only one negative supercoil when histones are removed. This contradiction, known as "linking number paradox", was described by J.C. Wang and has received considerable attention over the past thirty years^{7, 49, 83-84}. As the high resolution structure of nucleosome has been experimentally determined, the nucleosomal DNA is found to be overtwisted, to 10.2 average base pairs per turn presumably. Comparing to the 10.5 base pairs per turn of the free B-form DNA, the change in the twist of nucleosomal DNA results in an absorption of 0.6 negative superhelical turns, and this finding was therefore considered to explain the linking number paradox⁸⁵⁻⁸⁹.

Figure 1-9. Crystal structure of the nucleosome core particle (adapted from "Crystal structure of the nucleosome core particle at 2.8 angstrom resolution", by Luger, K. *et al.*, 1997⁷⁵).

1.2.3 Linker histone H1 and the 30-nm chromatin fibre

It has been introduced in the previous section that DNA is arranged in the cell nucleus with the help of histones. In addition to the core histones, there is an additional type of histone protein called "linker histone" which is also one of the major components of eukaryotic chromosomes. Histone H1 (a member of linker histones) was indentified in calf thymus in 1951⁹⁰ and was subsequently shown to be present in most eukaryotes⁹¹.

The structure of histone H1 consists of an evolutionarily conserved central globular domain flanked by a relative long C-terminal domain as well as a short N-terminal domain. Unlike core histones, histone H1 does not involved in constructing nucleosome core particle. Instead, it has been suggested that histone H1 molecules may be associated with the outside of nucleosome core and protect additional 10 base pairs of linker DNA on either side of the core particle from

micrococcal nuclease digestion^{69, 92}. It is widely accepted that the structured globular domain of the linker histone located in the region of the exit and entry points of the nucleosome core particle⁹³⁻⁹⁶ and thereby seal two full turns of DNA around histone octamer⁹⁷⁻⁹⁹ as illustrated in Figure 1-10. The nucleosome core particle together with histone H1 and the ~20 bp of internucleosomal linker DNA is called a chromatosome^{98, 100-101}. Although several models have been proposed to describe the position and explain the binding mechanism of histone H1 in the past decades^{93, 98, 102-113}, the role and the precise location of the linker histone still remain elusive^{95, 114}.

Other than the globular domain, the carboxyl-terminal tail of histone H1 was carefully investigated and is believed to play key roles during chromatin condensation. The C-terminal domain (CTD), rich in lysine, proline and alanine residues¹¹⁵⁻¹¹⁶, has been demonstrated to have extensive interaction with the linker DNA beyond the ~10 bp internucleosomal region, adopt a folded conformation, and facilitate the formation of linker DNA into higher-order specific structures^{115, 117-125}. On the other hand, the amino terminus of histone H1 (NTD) is composed of a hydrophobic terminus and a highly basic cluster that close to the globular domain¹²⁶. Despite the structure and function of NTD have yet to be determined and characterized, a number of experimental results suggest that the N-terminal domain of histone H1 may contribute to the DNA-binding affinity and help to correctly position the linker histone to the nucleosome particle¹²⁷⁻¹²⁸.

As previously discussed, the third level of chromosome packaging involves the coiling of 10-nm beads-on-a-string nucleosomal arrays into a filament approximately 30 nm in diameter. Although some studies proposed that linker histone may not be necessary for the compaction of nucleosomal arrays and cell

survival¹²⁹⁻¹³¹, based on the experimental evidence provided in recent studies, the cooperative binding of histone H1 to linker DNA is believed to facilitate and stabilize the formation of this 30-nm condensed structure *in vivo*¹³²⁻¹³⁵. Various models have been proposed to illustrate the interactions between linker histone H1, nucleosome core particle and linker DNA, among them the solenoid model and the zigzag model are the two major architectures to describe the arrangement of nucleosomes. The solenoid model was first proposed by Finch and Klug in 1976¹³⁶ and further developed in the following years¹³⁷, in which the 10-nm chromatin is coiled continuously to form a simple one-start helix with about six nucleosomes per turn. On the other hand, Bednar *et al.* described their three-dimensional zigzag model in 1998¹⁰⁷, in which the linker DNA is constrained within a stem-like structure and forms a straight path between adjacent nucleosomes. These nucleosomal arrays are then folded as a zigzag pattern, and further form a coiled or twisted structure with two opposing rows which named "two-start helix". The two-start zigzag model was supported by lots of substantial experimental evidence especially the crystallographic and Cryo-EM studies performed in recent years¹³⁸⁻¹⁴⁰. Furthermore, Grigoryev *et al.* reported their interesting findings that indicate the solenoid and zigzag conformations may exist simultaneously in a structurally heteromorphic 30-nm chromatin fibre¹⁴¹.

Figure 1-10. Schematic illustration of the association of linker histones with nucleosomal DNA and the proposed models of the 30-nm chromatin fibre (redrawn from "DNA Packaging: Nucleosomes and Chromatin. Nature Education", by Annunziato, A., 2008¹⁴²).

Studies over the past few decades have provided valuable insight into the structures of linker histones and their interactions with nucleosomes. In most of eukaryotic cells, linker histones are found in stoichiometric amounts with nucleosomes, and have been considered to be deeply involved in the formation of higher order chromatin structure. However, due to technical difficulty, it is hard to obtain high-resolution crystal structure of the 30-nm chromatin fibre. As such, the exact spatial location of the linker histone remains controversial, while the path of the linker DNA is still unsolved to date.

1.3 Nucleic Acid Gel Electrophoresis

1.3.1 Fundamentals of electrophoresis

Gel electrophoresis was first reported in the 1930s by Arne Tiselius¹⁴³, and developed over the following decades. Since that time, it has become a standard technique in biochemistry and molecular biology used to separate biological macromolecules, such as DNA, RNA and proteins¹⁴⁴⁻¹⁴⁷. In gel electrophoresis, separation is achieved by moving the charged biomolecules in an electrical field through a porous matrix. In general, the rates of migration of these molecules are based on (i) their molecular weights, (ii) their overall charges, and (iii) their three-dimensional shapes or conformations^{144, 147}. Depending on the sizes of molecules to be analyzed, the choice of separation matrix can be agarose^{146, 148}, polyacrylamide¹⁴⁹⁻¹⁵³, agar¹⁵⁴⁻¹⁵⁷, and other substances¹⁵⁸⁻¹⁵⁹. In the case of nucleic acids, gels of polyacrylamide and agarose are commonly used due to their appropriate pore sizes for efficiently separating DNA molecules. Agarose gels have larger pore sizes than polyacrylamide gels, and thus can be used to separate large DNA fragments with ~50-20,000 base pairs in length. Polyacrylamide gels, which have smaller pore sizes, are suitable for separating molecules of 5-500 nucleotides because of their relative high resolution.

Conventional methods of gel electrophoresis are generally described below and illustrated in Figure 1-11. Before electrophoresis, the gel is positioned in a tank between separate anode and cathode compartments, and submerged in a buffer solution that carries the electric charge and helps maintain the pH value. Once the gel wells are loaded with samples, an electrical field is applied via the power supplier. The DNA molecules with negative charges (carried by their

sugar-phosphate backbone) will then begin to migrate through the slab gel, moving towards the positive electrode. As time goes on, the difference in the migration rate of DNA fragments results in different location of the gel bands. In order to visualize nucleic acids, the gel should be treated with a DNA intercalating dye which will generate fluorescence when exposed to ultraviolet light. In 1977, Karsten and Wollenberger reported their ethidium bromide (EtBr) method for fluorometric determination of nucleic acids in tissue homogenates¹⁶⁰. Since then, EtBr has been commonly used as a sensitive fluorescent dye for visualizing DNA during electrophoresis experiments¹⁴⁷. Recently, due to the reported genotoxic of ethidium bromide¹⁶¹⁻¹⁶³, several alternative dyes have been designed and developed to provide researchers with a safe replacement for EtBr¹⁶⁴⁻¹⁶⁵. After exposure under UV light, the bands of nucleic acid are visible. Their sizes and quantities can be therefore deduced by comparing their relative positions and staining intensities to a given set of standards, which is named DNA ladder.

Figure 1-11. Schematic illustration of conventional gel electrophoresis of DNA in a horizontal agarose gel. DNA samples are loaded into the wells and will migrate into the gel toward the anode in an applied electrical field. During the gel run, the DNA fragments are separated according to their size and structure. DNA can be

visualized after staining and exposure of the gel to UV light.

1.3.2 Electrophoretic analysis of DNA supercoiling

DNA supercoiling has been known as an important and intrinsic property of topologically-constrained DNA molecules. Supercoiling occurs most commonly when DNA double helix is further twisted around its own axis in a helical manner. Mathematically, writhe (Wr) represents the turning of the global helix axis in space and quantitatively describes the DNA supercoiling. Over the past forty years, a number of methods has been developed and demonstrated to measure the degree of supercoiling, including (i) band counting in conventional gel electrophoresis, (ii) two-dimensional gel electrophoresis, (iii) gradient sedimentation, (iv) crossover counting under microscopy, and other approaches.

One of the widely used methods is the conventional gel electrophoresis¹⁶⁶⁻¹⁶⁸. This approach is based on the fact that supercoiled DNA with different writhing number vary in their overall shapes. The circular DNA molecules with high supercoiling densities are more compact, and thus migrate faster through the gel than their relaxed counterparts. As a result of this, a distribution of DNA topoisomers can be observed, where the DNA superhelical turns between adjacent bands are differ by one. By counting the bands on the gel, we can therefore estimate the mean writhing number (or the τ value) in a given set of DNA topoisomers. However, due to the limit of gel resolution, highly supercoiled DNA topoisomers will co-migrate as a single band. In this case, electrophoresis can be performed on the gel containing a DNA intercalator to resolve these topoisomers. DNA intercalators, such as chloroquine or ethidium bromide, have strong effects on the DNA conformation by unwinding their double helix, convert highly

supercoiled DNA molecules into less supercoiled ones, and thus reduce their mobility to obtain better resolution in the gel.

The limitation of conventional gel electrophoresis is that it has difficulty analyzing a wide distribution of DNA topoisomers, especially in a mixture where both negatively and positively supercoiled DNA are present. In order to overcome this shortcoming, two-dimensional electrophoresis has been proposed by Lee *et al.* in 1981¹⁶⁹ and developed in the following years for identifying DNA topoisomers¹⁷⁰⁻¹⁷². In the two-dimensional electrophoresis, DNA topoisomers was first separated in the first direction in a standard gel slab. This gel was then equilibrated in a buffer containing certain amount of chloroquine, and electrophoresed in the second direction perpendicular to the first one. As a result of chloroquine intercalation, the negatively supercoiled DNA will be pulled towards relaxed form and migrate slowly, whereas the DNA which contains positive supercoils will gain extra supercoils and migrate rapidly on the gel. Consequently, the DNA topoisomers having the same superhelical density but opposite signs of supercoiling can be separated according to their different mobilities in the second direction.

Other than gel electrophoresis, the superhelical density of circular DNA can be precisely determined by sedimentation in ethidium bromide-containing sucrose gradients as reported previously¹⁷³⁻¹⁷⁶. Similarly, this method is also based on the principle that DNA molecules with more supercoils are more compact in their overall shape, and will sediment more rapidly through a sucrose density gradient. Different from the electric field in gel electrophoresis, migration of DNA during density-gradient sedimentation is driven by gravity force provided by a centrifuge. In the presence of ethidium bromide, the negatively supercoiled DNA

will be unwound and result in a decrease in superhelical density. Thus, at certain concentration of ethidium bromide (the critical dye concentration), all the negative supercoils will be removed and the completely relaxed circular DNA will achieve a minimum rate of sedimentation. In this case, the value of the critical dye concentration can be used to precisely determine the superhelical density of circular DNA.

In addition to the above mentioned methods, visualization tools can provide us more information about the actual shape of the supercoiled DNA. With the help of microscopy, measurement of the writhing number of circular DNA can be conducted by counting the crossover points in each molecule flattened on a grid. Recently, besides the conventional electron microscopy (EM)¹⁷⁷⁻¹⁸¹, cryo-electron microscopy (cryo-EM)¹⁸²⁻¹⁸⁴ and atomic force microscopy (AFM)^{52, 185-189} have been used by a number of researchers to determine the sign (left-handed or right-handed) of the DNA crossing and obtain the high-resolution three-dimensional images of supercoiled DNA molecules. Although the DNA supercoils identified by counting the crossovers are basically consistent with the biophysical results as reviewed by McCreedy¹⁹⁰, in most cases this approach is only used to roughly estimate the value of superhelicity owing to the DNA conformational changes during sample preparation process.

1.4 Atomic Force Microscopy of Biological Samples

1.4.1 Fundamentals of atomic force microscopy

Atomic force microscopy (AFM) is one of the high-resolution scanning probe microscopes (SPM) that proposed by Binnig *et al.* in 1986¹⁹¹ and further

developed by Wickramasinghe *et al.* in 1987¹⁹². Principally, AFM uses a sharp tip attached to a flexible cantilever to scan over a sample, records the vertical deflections of the cantilever, and accordingly provides three-dimensional topographical information of the sample surface.

Physically, an AFM mainly consists of (i) a silicon or silicon nitride cantilever, (ii) a tip fixed to the end of the cantilever, which is used to scan the sample surface, (iii) a detector configured to sense the cantilever deflection, (iv) a piezo-electric element which drives the probe as a mechanical oscillator, (v) a xyz-drive used to control the scanning motion of the sample in x/y and z direction, and (vii) a controlled implemented by a computer for data processing and visualization, as shown in Figure 1-12.

Figure 1-12. Schematic illustration of a typical atomic force microscopy (redrawn from Roduit, C. "AFM figures" 2010, www.freesbi.ch, Creative Commons Attribution¹⁹³).

Based on the nature of the tip motion, atomic force microscopes can be operated in a number of modes. Contact mode AFM is most commonly used for

obtaining topographical information on hard surface. In this mode, the tip is in direct contact with the sample surface and maintains its position by the feedback signals as the scanning proceeds. However, the high dragging force between the tip and the surface during a contact scanning may damage the soft sample, which limits its usage for biomolecules such as nucleic acids.

Different from the operation of contact mode, the AFM cantilever in tapping mode is driven to oscillate vertically at its resonance frequency (normally around hundreds of kHz) by a piezoelectric element. As the tip is brought into proximity of a sample surface, the oscillation amplitude will be reduced due to the interaction between the tip and the sample. During scanning over the surface, the changes in oscillation amplitude are collected to form surface images. Because of the lower lateral forces in its tip-sample interaction, tapping mode is usually preferred to measure topography of the soft samples with complex structures, such as polymers or biological macromolecules.

Unlike the above mentioned modes, in non-contact AFM mode, the tip never makes contact with the sample. Instead, the cantilever is oscillated above the sample surface (from 1 nm to 10 nm) at a lower amplitude (normally <10 nm) than that of tapping mode. In this case, the long-range forces acting between the sample and AFM tip can be sensitively detected according to recorded variation of the cantilever deflection signal. Although the non-contact mode provides the lowest resolution compared to the other approaches, it is still one of the most promising tools for imaging fragile biological samples, especially for microscopic investigations in liquid phase.

Moreover, in recent years, a number of advanced modes have been developed by scientists according to the principle of AFM, which allow us to

characterize the electrical properties (CAFM)¹⁹⁴⁻¹⁹⁵, electric fields (EFM)¹⁹⁶, magnetic fields (MFM)¹⁹⁷⁻¹⁹⁸, and surface potential (HD KFM)¹⁹⁹ of our samples, and undoubtedly expand the area of employment of atomic force microscopy.

1.4.2 Advantages of AFM in biological applications

In the past few years, atomic force microscopy has emerged as a powerful tool for imaging biological structures, such as nucleic acids²⁰⁰⁻²⁰¹, proteins²⁰²⁻²⁰³, cells²⁰⁴⁻²⁰⁶, tissues²⁰⁷⁻²⁰⁸ and biomaterials²⁰⁹⁻²¹¹. As stated, AFM provides topographical information by moving a metallic tip over a sample and recording the interaction forces between the sample surface and the tip. Compared with other microscopes, AFM has a number of advantages in the study of biological samples.

Firstly, atomic force microscopy provides very high resolution images, which is comparable to those obtained by transmission electron microscopy (TEM) and scanning tunneling microscopy (STM). This is due to the extremely sharp AFM probe, the radius of which is about 5-15 nm typically. Thus, the optimum resolution of the tapping mode AFM can be 50 Å lateral and 1 Å vertical²¹².

In addition, AFM offers researchers a unique opportunity to visualize three-dimensional images of biological and nanomaterial samples. As reported, structural analysis of macromolecules and determination of the surface roughness can be easily achieved in nanometer scale by atomic force microscopy²¹³⁻²¹⁵.

Furthermore, the sample preparation procedures for biological AFM are relative simple without any complicated treatments. However, since the samples for TEM and SEM analysis have to be conductive, additional metal coating process is required during their sample preparation processes which may cause damage of conformational changes to biological molecules.

Most importantly, the principle of AFM visualization make it possible to conduct biological studies under biocompatible conditions. Almost all kinds of AFM probes work perfectly well in air under ambient temperature and pressure, and even particular types of probes have been developed for imaging in liquid environment where nucleic acids and proteins will maintain their original conformation²¹⁶⁻²¹⁸. In general, these advantages of AFM over other microscopes make it a powerful and versatile technique for biological investigations.

1.5 Principles of Fluorescence Resonance Energy Transfer (FRET)

Fluorescence is a molecular phenomenon in which a substance absorbs light or other electromagnetic radiation, promoting its electrons from the ground state to the excited state. After a short period of time the electrons relax back to the ground state and subsequently emit light of longer wavelength²¹⁹. This process can be described by the following equation:

$$\text{excitation: } S_0 + h\nu_{ex} = S_1$$

$$\text{fluorescence emission: } S_1 \longrightarrow S_0 + h\nu_{em} + \text{heat}$$

where S_0 and S_1 represent the ground state and the first excited state respectively, and $h\nu$ is the energy of photon.

The fluorescence lifetime, which refers to the average time a fluorophore spends in its excited state before returning to the ground state, ranges from picoseconds to hundreds of nanoseconds depending on the chemical structure and environment of the fluorescent molecule²¹⁹. In addition, the efficiency of the fluorescence process can be quantified by the fluorescence quantum yield (Φ), which defined as the ratio of the number of photons emitted to the number of

photons absorbed per time unit²¹⁹. The fluorescence quantum yield is one of the most important spectroscopic properties of a fluorescent dye, and most of efficient fluorescent dyes have quantum yield lying in the range from 0.3 to 0.8.

On the basis of fluorescence principles, the technique of fluorescence resonance energy transfer (FRET), also named as Förster resonance energy transfer, was initially described by Förster in the 1940's²²⁰. Over the past decades, a number of excellent FRET-based studies have been conducted to indentify molecular interactions and determine nanoscale distances in many areas of biological research²²¹⁻²²⁵. The mechanism of fluorescence resonance energy transfer involves a pair of fluorophores, which are called the donor and the acceptor. When these two molecules are within close proximity, energy can transfer from the excited donor fluorophore to the nearby acceptor fluorophore though dipole–dipole coupling as illustrated in Figure 1-13.

Figure 1-13. Jablonski diagram illustrating the fluorescence resonance energy transfer (FRET) process (adapted from " Förster Resonance Energy Transfer (FRET) as a Tool for Dissecting the Molecular Mechanisms for Maturation of the

Shigella Type III Secretion Needle Tip Complex", by Dickenson, N. E. *et al.*, 2012²²⁶).

In order to achieve the energy transfer, the emission spectrum of the donor must overlap the absorption spectrum of the acceptor. In general, higher degrees of overlap between the spectra will result in an increased FRET efficiency. Since the efficiency of energy transfer depends on the inverse of the sixth power of the distance between donor and acceptor (experimentally confirmed by Edelhoch *et al.* in 1967²²⁷), the range of distances for most FRET studies is limited from 1 nm to 10 nm. On the other hand, this distance dependence of the energy transfer process makes FRET a sensitive technique for investigating a variety of molecular interactions, and monitoring the conformational changes of macromolecules in the fields including biophysics and biochemistry.

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Chapter 2

Atomic Force Microscopy Verification of Chromatosome Aggregation with the Aid of Linker Histone H1

2.1 Background Information

As introduced in chapter 1, the genomes of eukaryotes are condensed and further packaged into chromosomes in an efficient and coordinated manner *in vivo*¹⁻². Large size cellular DNA packing occurs on several levels, among which the first and second levels play active roles in gene transcription and have crucial effect on eukaryote biological activities^{1, 3-4}. The first level of chromosome packaging is commonly known as 10-nm "beads-on-a-string" structures, in which DNA wraps around eight histone core proteins forming nucleosomes^{1, 5-10}. In addition to the beads on string appearance, the second level of chromosome packaging is described as 30-nanometer fibres, which appear as coiled structures under electron microscope¹¹⁻¹⁶. This type of structure is found to be stained more intense during interphase, which indicates its tighter organization compared to the 10-nm fibres. Finch and Klug reported for the first time in 1976¹² their observations of these tighter 30-nanometer fibres *in vitro*. Their electron microscope results revealed that the solenoidal 30-nm structure was dissociated to 10-nm fibre once all the linker histone H1 was removed without concomitant loss of the other four histones (Figure 2-1).

Their unprecedented findings, in other words, indicated that linker histone may act as a crucial factor for the chromatin higher order condensation. These encouraging discoveries in the fundamental nature of linker histone proteins have paved the way for scientists to delve deeper into the mysteries of chromosomal DNA packaging over the past forty years. As summarized by Harshman *et al.* in their review paper in 2013¹⁷, the crystal structure as well as the genetic information of linker histones were carefully characterized by numerous talent scientists and researchers in the past decades¹⁸⁻²⁴. However, their fundamental roles in chromosome condensation and decondensation still remains a mystery up till now.

Figure 2-1. Schematic illustration of linker histone H1-induced 30-nm chromatin fibres (redrawn from "Controlling the double helix", by Felsenfeld, G. *et al.*, 2003⁸).

In order to unravel these mysteries, the deep understanding of DNA topological differences between eukaryotic and prokaryotic cells is needed. The typical structure of DNA molecule consists of two interwound complementary

nucleotide chains, forming a double helix, also known as B-form DNA. Topological aspects of DNA arise in constrained DNA segment, where the free rotation of DNA strands is impossible. These topologically constrained DNA segments are called topological domains²⁵⁻²⁹. It is therefore easy to imagine that the circular structure is a typical topological domain. Both strands are covalently closed in a circular DNA which prevents the rotation of one DNA strand over the other. This kind of structure can be found in most prokaryotic DNA^{26, 30-31}. Things are somehow different in eukaryotic cell. Unlike prokaryotic DNA, the genomic DNA in a eukaryotic cell is linear overall. However, most of these linear DNA forms numerous large loops that partially attached to the nuclear matrix^{25, 29, 32}. In this case, the entire eukaryotic DNA can be considered to be many separated local structures, each of which is one topological domain (Figure 2-2). All these domains are topologically equivalent to a circular DNA molecule.

Figure 2-2. Schematic illustration of DNA topological domains in prokaryotic and eukaryotic genomes. (A) A prokaryotic circular DNA molecule as the paradigm of one topological domain. (B) Locally compacted topological domains in eukaryotic chromosomal DNA.

As to prokaryotic cells, their DNA topology is precisely regulated by a particular kind of enzymes called DNA topoisomerases. These enzymes can introduce or release torsional tension in DNA duplex by breaking, rotating and rejoining either single or double strands of DNA^{27, 33-35}. Once the DNA molecule under torsion, it will twist around itself like a circular rubber band, which is called DNA supercoiling. DNA supercoiling is important for DNA packaging within all cells. If linearized, the length of bacterial DNA would be measured about 1.5 millimeters long which is over 500 times the length of the cell. Supercoiling of DNA, in this case, could help reduce the space and allows for DNA packing into a little space.

Similar to prokaryotic DNA, there is also supercoiling in eukaryotic DNA topological domain. In eukaryotes, a supercoiled chromosome has been twisted and wound around itself very tightly. However, different from that of prokaryotic circular DNA, the supercoiling in eukaryotic chromosomal DNA is not only generated (or released) by DNA topoisomerases, but also introduced by a group of structural protein called histone. As discussed in chapter 1, histone proteins play essential structural and functional roles in chromatin formation and chromosome packaging. During chromatin formation, eukaryotic genomic DNA wraps around histone octamer core and simultaneously introduce negative supercoiling into chromosomal DNA^{6, 9, 36-37}. The solenoidal supercoiling that achieved with histone octamer core help eukaryotic DNA to form the 10-nm fibre structure^{1, 5-10}. As mentioned above, this fibre is further coiled into 30-nm fibre with the aid of linker histone¹¹⁻¹⁶. However, after several decades' pursuit of the 30-nm chromatin fiber structures and properties, precise nucleosomes and linker DNA arrangement have remained elusive. The driving force that directs the nucleosomes orderly

arrangement upon addition of linker histone has never been found. It is these mysteries that lead us into doing our current research on the very exciting frontier of eukaryotes chromosome packaging study. In this chapter, our investigation is an attempt to show whether the linker DNA undergoes bending, supercoiling, zigzag arrangement or other architecture during the formation of 30-nm chromatin fiber. We have also made efforts to investigate and clarify where the driving force comes from in the following chapters.

Atomic force microscopy (AFM), a versatile tool for imaging, measuring and monitoring matter at the nanoscale, was invented in 1986³⁸ and developed in the followed years³⁹⁻⁴⁵. Different from other optical or electron microscopes, this type of microscopy collects information by direct touching the surface with a mechanical microprobe. Fundamental principles and theories of atomic force microscopy have been summarized in chapter 1. Since the measurement of an AFM could be made in three dimensions, it can be used to gather topological information for various biomolecules⁴⁶⁻⁴⁹ and biomaterials⁵⁰⁻⁵². In our research, AFM is being used to quantitatively study single DNA molecules' topological properties and their interactions with histone proteins. It is our hope that our atomic force microscopy studies of linker histone-induced DNA aggregation will help investigators gain a better understanding of linker histone's roles in eukaryotic DNA condensation.

2.2 Materials and Methods

2.2.1 Oligodeoxyribonucleotides, enzymes, chemicals and equipments

Product(s)	Manufacturer
Oligodeoxyribonucleotides	Sangon Biotech (Shanghai, China)
pUC 19 Vector, pBR322 Vector	New England Biolabs (Singapore)
pAdEasy-1	Addgene (Cambridge, US)
1 kb DNA ladder	New England Biolabs (Singapore)
T4 DNA ligase	New England Biolabs (Singapore)
Topoisomerase I (E. coli)	New England Biolabs (Singapore)
DNase I	New England Biolabs (Singapore)
EcoRI restriction endonuclease	New England Biolabs (Singapore)
Nt. BspQI nicking endonuclease	New England Biolabs (Singapore)
Nucleosome Assembly Kit	New England Biolabs (Singapore)
Histone H1 ⁰ (human, recombinant)	New England Biolabs (Singapore)
QIAquick PCR Purification Kit	Qiagen (Singapore)
Agarose	Invitrogen
Ethidium bromide	Bio-Rad Laboratories (Singapore)
TAE, Tris, HEPES	1st Base (Singapore)
Eppendorf tubes, Pipette tips	Greiner (Singapore)
Atomic force microscopy	Veeco, (Santa Barbara, US)
AFM probes	Bruker (Camarillo, US)
Gel documentation system	Syngene (Cambridge, UK)

Most all the chemicals used in this research were listed above otherwise were obtained from Sigma-Aldrich with analytical grade or molecular biology grade.

2.2.2 Nucleosome assembly

Nucleosomes were prepared from the Epimark Nucleosome Assembly Kit (New England Biolabs #E5350S) following the dilution assembly protocol for 50 pmol DNA substrate. A 20 μ l mixture containing 5 μ g DNA, 2 M NaCl, 5 μ M Histone H2A/H2B Dimer and 2.5 μ M Histone H3.1/H4 Tetramer (dosages of core histones should be adjusted according to the required molar ratio of nucleosome to DNA) was incubated at room temperature (25 $^{\circ}$ C) for 30 minutes. Subsequently, 7 μ l dilution buffer (10 mM Tris, pH 8.0) was added into the mixture to bring the sodium concentration to 1.48 M. After incubation at room temperature (25 $^{\circ}$ C) for 30 minutes, the mixture was diluted by adding 13 μ l dilution buffer (10 mM Tris, pH 8.0) to bring the sodium concentration to 1.0 M. Afterwards, the mixture was incubated at room temperature (25 $^{\circ}$ C) for additional 30 minutes and further diluted by adding 27 μ l dilution buffer (10 mM Tris, pH 8.0) to bring the sodium concentration to 0.6 M. Finally, the mixture was incubated at room temperature (25 $^{\circ}$ C) for 30 minutes again and diluted by adding 93 μ l dilution buffer (10 mM Tris, pH 8.0) to bring the final concentration of NaCl to 0.25 M. The obtained mixture was further incubated at room temperature (25 $^{\circ}$ C) for 30 minutes and stored at 4 $^{\circ}$ C.

2.2.3 Histone H1-DNA binding assay

A 20 μ l aqueous solution containing 5 μ g DNA, 50 mM NaCl (concentration of NaCl should be adjusted according to experimental requirements)

and 2.5 μ M recombinant human histone H1⁰ (New England Biolabs #M2501S, dosage of histone H1 should be adjusted according to the required molar ratio of histone H1 to DNA) was incubated at 37 °C for 30 minutes.

2.2.4 Chromatosome assembly

Nucleosomes were prepared from the Epimark Nucleosome Assembly Kit (New England Biolabs #E5350S) following the dilution assembly protocol as described before. A 20 μ l solution containing nucleosomal arrays (nucleosome concentration of 2.5 μ M), 200 mM NaCl and 2.5 μ M recombinant human histone H1⁰ (New England Biolabs #M2501S, dosage of histone H1 should be adjusted according to the required molar ratio of histone H1 to DNA) was incubated at 37 °C for 30 minutes.

2.2.5 Preparation of open-circular DNA

A 30 μ l solution containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 μ g of supercoiled pUC19 (New England Biolabs #N3041S), pBR322 (New England Biolabs #N3033S) or pAdEasy-1 (gift from Addgene #16400) vector DNA and 2 U of Nt.BspQI nicking endonuclease (New England Biolabs #R0644S) was incubated at 50 °C for 1 hour. The obtained open-circular DNA product was then purified by QIAquick PCR Purification Kit (Qiagen), and further examined by agarose gel electrophoresis.

2.2.6 Topoisomerase I relaxation of supercoiled DNA

A 30 μ l solution containing 20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 1 μ g of

supercoiled DNA or DNA-histone complex and 2 U of E.coli topoisomerase I (New England Biolabs #M0301S) was incubated at at 37 °C for 1 hour. The obtained relaxed DNA product was then purified by QIAquick PCR Purification Kit (Qiagen), and further examined by agarose gel electrophoresis.

2.2.7 DNase I digestion assay

A 30 µl solution containing 10 mM Tris-HCl (pH 7.6), 2.5 mM MgCl₂, 0.5 mM CaCl₂, 1 µg of DNA-histone complex and 2 U of DNase I (New England Biolabs #M0303S) was incubated at at 37 °C for 30 minutes. The obtained product was deposited onto mica surface and visualized in air by atomic force microscopy.

2.2.8 Synthesis of APS (1-(3-aminopropyl)silatrane)

The synthesis of APS (1-(3-aminopropyl)silatrane) was accomplished through adaptation of the previously reported procedures⁵³⁻⁵⁴. A solution containing 14.92 g of triethanolamine (0.1 M, from Sigma-Aldrich) and 2 ml of sodium hydroxide solution in methanol (2 mg/mL) was prepared in a 250 ml round-bottom flask under nitrogen atmosphere. An equivalent amount of (3-aminopropyl) triethoxysilane (22.14 g, 0.1 M) is then added to the mixture. Then, a rotary evaporator was applied in 60 °C water bath to remove the ethanol formed in the reaction. At the end of the reaction, crude solids were obtained and recrystallized from the mixture of methanol and toluene to yield white crystals. The APS reagent was stored at -80 °C refrigerator over a desiccant.

2.2.9 Adsorption of DNA to mica mediated by counterions

Divalent counterions (*e.g.* Mg^{2+} , Ni^{2+})⁵⁵⁻⁵⁶ and polyamines (*e.g.* spermidine, spermine)⁵⁷ can be used to absorb negatively charged DNA molecules onto mica surface. In the divalent counterion-mediated method, a solution containing 10 ng DNA sample (amount of DNA should be adjusted according to the size of DNA molecules), 10 mM MgCl_2 and 40 mM HEPES (PH = 7.0) was deposited onto freshly cleaved mica and incubated at room temperature for 5 minutes. The mica was then rinsed with deionized water and dried with nitrogen gas before AFM analysis. In the polyamine-mediated method, a freshly cleaved mica was treated with 50 μM spermidine for 5 min, followed by washing with deionized water and flowing nitrogen gas. Then, a solution containing 10 ng DNA sample (amount of DNA should be adjusted according to the size of DNA molecules) and 40 mM HEPES (PH = 7.0) was deposited onto the spermidine-treated mica and incubated at room temperature for 5 minutes. The mica was then rinsed with deionized water and dried with nitrogen gas before AFM analysis.

2.2.10 Adsorption of DNA to APS-modified mica

As an alternative, a reported sample preparation method for AFM imaging with the use of APS-modified mica was used in our studies^{54, 58}. A 50 mM stock solution of APS was prepared and stored at -20 °C. A working solution was obtained by dissolving the stock solution in 1:300 ratio in water. Fifty microliters of the working solution was then deposited onto a freshly cleaved mica and incubated at room temperature for 30 min, followed by washing with deionized water and flowing nitrogen gas. Then, a solution containing 10 ng DNA sample

(amount of DNA should be adjusted according to the size of DNA molecules), 10 mM Tris-EDTA (PH = 7.0) and 50 mM NaCl was deposited onto the APS-modified mica and incubated at room temperature for 5 minutes. The mica was subsequently rinsed with deionized water and dried with nitrogen gas before AFM analysis.

2.2.11 AFM imaging of DNA and DNA-histone complexes

AFM images were obtained in Tapping ModeTM on a MultimodeTM AFM (Veeco) in connection with a Nanoscope VTM controller. Antimony (n)-doped Si cantilevers with nominal spring constants between 20 and 80 N/m were selected. Scan frequency was 2.0 Hz per line and the modulation amplitude was in a nanometre range. All DNA sample determinations were carried out in air at room temperature. The AFM images were further analyzed by NanoScope Analysis Version 1.50 program.

2.3 Results and Discussion

2.3.1 Visualization of topologically-different forms of double-stranded DNA with atomic force microscopy

Organization of DNA involves primary, secondary and tertiary structures. The sequence of bases in the nucleic acid chain gives the primary structure of DNA. DNA's secondary structure is determined by base-pairing between nucleotides on the anti-parallel strands of DNA to form a twisted double helix structure. The tertiary structure of a nucleic acid refers to the arrangement of nucleic acid in three-dimensional space. As a kind of tertiary structure, DNA

supercoiling plays an important role in maintaining DNA three-dimensional conformation and is essential for cell survival^{32, 59-60}. Naturally-occurring DNA is negatively supercoiled either in prokaryotic or eukaryotic cells. Cleavage of the supercoiled DNA (SC form) by restriction enzyme will turn it into a linear piece (L form). Nicking endonucleases which introduce a nick on one strand of DNA circle convert the covalently closed circular DNA (cccDNA) to a nicked open-circular DNA (OC form). Unlike nicking enzymes, type I topoisomerases generate a single-strand break on duplex DNA and allow rotation of the cleaved strand around the other strand. In this way, the superhelical tension within supercoiled DNA will be relieved, and the DNA will be given in its lowest energy state, which is named relaxed circular form (RC form). It is possible for these four types of DNA structures coexist in a single cell, and the interchanges of them occur during different phase of DNA replication. Therefore, it is important for us to identify these topological states of DNA (linear DNA, open-circular DNA, relaxed circular DNA, and supercoiled circular DNA as illustrated in Figure 2-3) in our following studies of DNA topological properties.

Figure 2-3. Schematic illustration of four topological states of duplex DNA.

Technically, DNA with different topological forms can be separated by electrophoretic mobility shift assay (EMSA), which is a traditional technique to

analyze nucleic acid molecules^{32, 59, 61-63}. In addition to gel electrophoresis, atomic force microscopy (AFM) provides us an alternative way to distinguish single DNA molecules with different topological properties^{57, 64-67}. Nucleic acids molecules were fixed onto a flat mica surface followed by drying with nitrogen gas and vacuum pump. Then, the DNA-loaded mica disc could be imaged in air under ambient conditions. Different from gel electrophoresis, AFM is a wonderful tool for biological studies, since it gives us new possibilities for direct visualization of single DNA molecule. With the help of atomic force microscopy, the surface morphology and three-dimensional structure of DNA molecules can be obtained at an atomic resolution. As shown in Figure 2-4, four types of DNA molecules were visualized by AFM under the same environmental condition. All of the DNA substrates used are pBR322 plasmid DNA which direct purchased from New England Biolabs Ltd. Prokaryotic plasmid DNA is known to be negatively supercoiled (shown in Figure 2-4D) when it has been extracted from bacteria cells. The linear DNA molecules shown in Figure 2-4A were restriction endonuclease (EcoRI) digestion products of the circular plasmids. The open-circular DNA molecules (Figure 2-4B) were obtained by nicking endonuclease (Nt.BspQI) digestion. The relaxed circular DNA molecules (Figure 2-4C) were E.coli topoisomerases I relaxation products of the supercoiled plasmids. Linear DNA and highly supercoiled circular DNA can be easily identified, while open-circular and relaxed circular DNA molecules share the similar shape under this condition. In order to further differentiate these two forms of DNA molecules, other methods will be applied and further discussed in the text below.

Figure 2-4. AFM visualization of different forms of plasmid DNA. An aqueous solution containing 2 fmol of (A) linear, (B) open-circular, (C) relaxed circular, and (D) supercoiled circular pBR322 DNA were deposited onto the APS-modified mica surface and visualized in air with the tapping mode AFM.

For quantitative characterization of biological molecules in our following studies, we have to develop a reasonable and precise methodology to describe the size, height and other parameters of these molecules. While scanning, the AFM probe is being used to touch the surface of the sample so as to measure slightly frequency variation. Once the whole picture has been captured, all the information gathered while scanning will be converted to digital information and can be further analyzed by NanoScope Analysis Version 1.50 program. That is, the altitude variation of the sample was veritably recorded by AFM and easily readable by build-in function of the AFM modular program. As shown in Figure 2-5, the height measurement of a double-stranded DNA sample was carried out. The pixel size in

these images was set to 5 nm which is closed to the physical radius of the AFM probe. The height value of the DNA duplex can be read from the section analysis diagram as indicated in Figure 2-5 (right).

Figure 2-5. Quantitative height measurement of double-stranded DNA by section analysis using the Nanoscope Analysis software. Left, AFM image of a double-stranded DNA molecule with a resolution of 512×512 pixels; right, section analysis of the corresponding line indicated by the white arrow in the left image.

Different from the height measurement, the horizontal distance between points cannot be determined directly by AFM modular program. As indicated in Figure 2-6B, the visible width of duplex DNA is around 10 nm, which is much larger than its true value (diameter of DNA double helix is ~ 2 nm). The broadening of DNA double helix could be attributed to the physical size of the AFM tip. In tapping-mode AFM, a metal tip is used to scan the surface features of the samples. Thus, the natural size of this tip should be taken into account when measure the horizontal distance. Here, we have developed a mathematical way to precisely determine the size of biological molecules for our following AFM studies⁶⁸. Additional mathematical computing software, MATLAB, was used to perform the conversion from the RGB color image to the binary matrix. As shown in Figure 2-6, circular plasmid DNA was first captured as a 512×512 pixels bitmap image by AFM (Figure 2-6A). This two-dimensional image was then

imported into MATLAB program and converted to logical matrix format. After that, several morphological operations (skeleton, shrink, thin, etc.) were performed for drawing up the contour line of the circular structure. In this way, the DNA double helix axis could be traced out as continuous binary points in skeletonised binary images (Figure 2-6B). In these binary images, the pixels were labeled as 1 (“on” pixel) and 0 (“off” pixel) separately. Thus, the horizontal distance between two pixels d can be calculated based on the following equation:

$$d = \alpha \times \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2} \quad (2.1)$$

In this equation, d and α represent the distance between two pixels and the length per pixel in the binary image, respectively, while x_1, x_2, y_1 and y_2 are the two-dimensional Cartesian coordinates of the two pixels.

Figure 2-6. Determination of the straight line distance between two points on a DNA contour map of digitized AFM image. (A) A 512×512 pixels bitmap image of duplex plasmid DNA obtained by tapping-mode AFM. (B) A skeletonised contour plot of the DNA molecule achieved by MATLAB software. The distance between the two pixel points (indicated by white arrows) is determined.

Although atomic force microscopy is known as a reliable tool for imaging DNA molecules, it should be pointed out that the overall appearance of circular DNA molecules can be altered by the sample preparation processes. As mentioned before, nucleic acid samples have to be absorbed onto mica surface before AFM visualization. Mica is a group of silicate minerals, the surface of which is covered with siloxy groups. In aqueous solution, these siloxy groups possess negative charges under neutral pH condition. Since the duplex DNA molecule is also composed of negatively charged phosphodiester groups on its backbone, direct immobilization of DNA on mica surface is incapable due to the intermolecular repulsion force. Instead, some chemical treatments should be made in order to introduce positive charges upon them. Typically, two kinds of approaches were developed for binding DNA onto mica substrate. In the first method, divalent counterions are used as bridges to mediate the electrostatic interactions between the negatively charged phosphate groups of the DNA backbone and the siloxy groups on mica surface as illustrated in Figure 2-7A. This approach was first presented by Vesenka *et al.*⁶⁹ and has been successfully applied for many AFM imaging applications^{47, 70-76}. The cations used in this approach should be soluble in water and possess a high binding affinity to both DNA backbone and mica substrate.

Figure 2-7. Schematic illustration of (A) divalent counterions mediated absorption of duplex DNA on mica surface, and (B) absorption of duplex DNA on APS-modified mica surface.

In our following experiments, circular DNA molecules with different topological conformations were immobilized by the divalent cation bridges and visualized using AFM. DNA substrates used in these studies were negatively supercoiled pUC19 (2,686 bp in length) and pBR322 (4,361 bp in length) plasmids. The typical divalent metal cation Mg^{2+} was introduced into DNA-containing aqueous solution with a concentration of 10 mM. The pH value was carefully adjusted to 7.0-7.5 with 50 mM HEPES buffer. These neutral solution were then deposited onto several pieces of freshly cleaved mica sheets respectively, and permitted to interact with the mica substrates for 5 minutes. Subsequently, the mica discs were washed with deionized water and dried thoroughly by applying to nitrogen gas and vacuum desiccators before imaging. As shown in Figure 2-8, the supercoiled DNA molecules in these images form irregular winding instead of plectonemic superhelical appearances. Especially, smaller circular DNA molecules even assume open circular conformations.

Figure 2-8. AFM visualization of highly supercoiled DNA molecules mediated by magnesium cations. A mixture containing 5 ng of (A) pUC19 (2686 bp) or (B) pBR322 (4361 bp) plasmid DNA, 10 mM MgCl₂ and 50 mM HEPES was deposited onto freshly cleaved mica, and imaged by tapping-mode AFM in air. The height images are obtained with a resolution 512 × 512 pixels of retrace collecting data.

In addition to the divalent cations, spermidine which is one of the typical multivalent cations was utilized to increase the binding affinity of nucleic acids to mica. In this approach, the freshly cleaved mica was pre-treated with 1 mM spermidine according to the procedure described by Sato *et al.*⁷⁷ before loading the DNA sample. After the plasmids were applied onto the mica surface, visualization was carried out on a tapping-mode AFM at room temperature in air. As shown in Figure 2-9, similarly, conformational changes of supercoiled DNA molecules have been observed in these images. The supercoils in circular DNA tend to be lost during these two deposition procedures for atomic force microscopy.

Figure 2-9. AFM visualization of highly supercoiled DNA molecules on

spermidine pre-treated mica. A mixture containing 5 ng of (A) pUC19 (2686 bp) or (B) pBR322 (4361 bp) plasmid DNA and 50 mM HEPES was deposited onto spermidine pre-treated mica, and imaged by tapping-mode AFM in air. The height images are obtained with a resolution 512×512 pixels of retrace collecting data.

Different from the above mentioned approaches in which cations were used to mediate electrostatic interactions between mica and DNA backbones, covalent chemical modification can also be used to functionalize the mica surface. 1-(3-aminopropyl)silatrane (APS) is a hydrolytically stable silatrane reagent that is commonly used to modify the mica surface with amino groups (APS-mica). As previously reported, the surface of APS-mica remains positively charged in aqueous solution over a broad pH range and works reliably in various topographic studies involving DNA as illustrated in Figure 2-7B⁷⁸⁻⁸⁰. Synthesis of 1-(3-aminopropyl)silatrane and preparation for APS-mica were followed the protocol reported by Shlyakhtenko *et al.*⁸¹. In our following investigation, pUC19 and pBR322 DNA molecules were deposited onto the APS-modified mica and visualized by AFM. As shown in Figure 2-10, the supercoiled DNA molecules display a tightly twisted geometry on APS-mica, which looks quite different from those in Figure 2-8 and Figure 2-9. Therefore, these results indicated that the geometry of supercoiled DNA depends highly on the sample preparation techniques. The supercoiled DNA images obtained using APS-functionalized mica are found to be closer to their conventional plectonemic superhelical structure. Similar results were reported by Lyubchenko at different ionic conditions in their studies⁸²⁻⁸³.

Figure 2-10. AFM visualization of highly supercoiled DNA molecules immobilized on APS-modified mica. A mixture containing 5 ng of (A) pUC19 (2686 bp) or (B) pBR322 (4361 bp) plasmid DNA and 100 mM NaCl was deposited onto freshly prepared APS-mica, and imaged by tapping-mode AFM in air. The height images are obtained with a resolution 512×512 pixels of retrace collecting data.

In order to further evaluate the reality of the superhelicity of these obtained DNA images, the number of visible DNA superhelical turns was determined by counting the crossover number in each molecule from the skeletonised binary image. Average numbers were obtained in our bulk measurements of fifty molecules. Consequently, their visible superhelical densities (σ) can be calculated according to the following equation:

$$\sigma = \tau / Lk_0 \quad (2.2)$$

in which linking difference (τ), also denoted as ΔLk , represents the changes from the number in the relaxed DNA molecule (Lk_0). Here in our study, the visible linking difference is the average number of superhelical turns obtained from AFM images. Superhelical density (σ) is known as a useful characteristic of supercoiled

DNA for evaluation of the supercoiling level between different DNAs. The theoretical value of the superhelical density (σ_t) for plasmid DNA molecules isolated from eukaryotes should be around -0.06 under physiological condition⁸⁴⁻⁸⁷. The theoretical superhelical turns (τ) were calculated accordingly and listed in Table 2-1 along with their visible values obtained in our AFM images. By comparing various sample preparation methods, we could therefore conclude that the DNA molecules absorbed onto APS-modified mica surface mostly retain their supercoiling geometry and their superhelicities during the deposition procedure for AFM imaging.

Table 2-1. Comparison of the DNA visible superhelical densities obtained from three different sample deposition methods.

Deposition methods	pUC19		pBR322		Theoretical superhelical density (σ_0)
	Visible superhelical turns (τ)	Visible superhelical density (σ)	Visible superhelical turns (τ)	Visible superhelical density (σ)	
Magnesium-mediated mica	1.3 ± 0.5	-0.01	2.7 ± 0.6	-0.01	-0.06
Spermidine pre-treated mica	1.5 ± 0.4	-0.01	7.2 ± 1.7	-0.02	-0.06
APS-modified mica	13.1 ± 1.9	-0.05	22.9 ± 3.9	-0.05	-0.06

In addition, it has been reported that DNA intercalating agents, which bind to duplex DNA by insertion between the bases, are capable of altering the topological structure of circular DNA significantly^{59, 88-90}. Among DNA intercalator compounds, ethidium bromide (EtBr) and chloroquine are typically

used to relax negative supercoils (or introduce positive supercoils) into covalently closed circular DNA. Unlike DNA topoisomerases, these DNA intercalators greatly change the tertiary structure of circular DNA without altering its linking number. In order to explore the topological alteration of circular DNA in the presence of DNA intercalator, negative supercoiled DNA was treated with increasing amount of ethidium bromide. Ethidium bromide-treated products as well as the original circular DNA were then deposited onto APS-mica and further visualized by AFM.

As shown in Figure 2-11A, the negatively supercoiled plasmids preferentially adopt interwound conformations on APS-modified mica. As the concentration of ethidium bromide increased, the negative supercoils were accordingly released until fully relaxed circles were observed in Figure 2-11B. When the ethidium bromide concentration kept growing, these closed circular DNA would be converted to positively supercoiled form as shown in Figure 2-11C. These conformational changes in closed circular DNA are due to the ethidium bromide-induced untwisting of DNA double helix.

Figure 2-11. AFM visualization of ethidium bromide-induced topological changes of circular DNA. 5 ng pBR322 DNA (left) was incubated with 50 μ M (middle) or 200 μ M (right) ethidium bromide at 25 $^{\circ}$ C for 30 min, followed by AFM imaging on APS-mica in air.

In summary, visualization of DNA molecules was achieved in different approaches in our AFM investigations. Among them, APS-modified mica which accurately and truthfully records DNA tertiary structure was believed to be the most reliable and reproducible technique for imaging of supercoiled DNA molecules. As a consequence, it will be primarily used in our subsequent visualization of protein-DNA complexes and supercoiling-induced DNA conjugates.

2.3.2 Verification and characterization of the complexes formed by circular DNA and histone proteins

Unlike prokaryotic genomic DNA, eukaryotic chromosome is organized by duplex DNA binding to small basic proteins called histones. For example, in human cell, the shortest chromosomal DNA contains 40 million base pairs, which can be condensed into 2 μm long chromosome during mitosis. To achieve such a high packing ratio, prokaryotic DNA is packaged into final condensed chromosome through several hierarchies of organization. Histones, which are among the most highly conserved eukaryotic proteins, are known to play important roles in these stages. The double-stranded DNA first wraps around the histone core to form a nucleosome, which is the basic unit of the 10 nm fibre structures. The histone proteins involved in this stage were found to be histone H2A, histone H2B, histone H3 and histone H4. Salt bridges and hydrogen bonds between the DNA backbone and amino acids, as the main interaction, help DNA stay tightly bound to the nucleosome^{1, 6-7, 9}.

Different from the core histones, histone H1 and histone H5 are known as the linker histones. The linker histones are not involved in the formation of 10-nm-

fibre structures. Instead, they binds the nucleosome at the linker DNA entry/exit region. As a result of the linker histone binding, linker DNA was tightly locked on the surface of histone core, which facilitates the formation of higher order structure^{17-21, 23}. The linker histone-induced 30-nm fibre structure was first observed by Paulson and Laemmli in 1970s using Electron Microscopy⁹¹. However, this technique lacks high resolution to distinguish the detailed conformation of such nucleosome fibres. During the past forty years, several molecular models have been built up to elucidate the internal packing arrangement of nucleosomes^{12-16, 20, 92-100}. In the solenoid model suggested by Rhodes and colleagues, the 10-nm fibre coils around a central axis forming a regular helical structure, in which six to seven nucleosomes are packed every helical turn⁹³. On the other hand, the two-start zigzag model has been proposed by Richmond *et al.* in 2004, in which the zigzagging of two nucleosomes has been predicted to form a two-stranded helical conformation⁹⁴⁻⁹⁵. Furthermore, cross-linker model⁹⁶⁻⁹⁷, supranucleosome model⁹⁸⁻⁹⁹, 3D-zigzag model^{20, 100} and many other patterns were described in recent years. Although scientists could hardly draw a convincing conclusion based on the existing evidence at this point, scientific accomplishment, as a path, will open our minds in our further investigation of chromosome high-order structures.

In the following sections, circular plasmids were used to model the conformational changes within nucleosomal DNA in association with nucleosome core particles and/or linker histone H1. Topologically closed structures of these plasmid DNA molecules perfectly mimic the individual topological domain of eukaryotic chromosomal DNA, while their well-controlled sizes allow direct observation by atomic force microscopy. Similar to eukaryotic chromatin, as

illustrated in Figure 2-12A, circular plasmid DNA molecules are considered to form “bead-on-a-strand” nucleosomal arrays after wrapped around nucleosome core particles. On the other hand, as shown in Figure 2-12B, linker histone H1 are known to bind to naked (without nucleosome core particles) duplex DNA, and further facilitate the formation of intermolecular DNA-histone aggregates¹⁰¹⁻¹⁰⁴.

Figure 2-12. Schematic illustration of the formation of nucleosomal arrays on plasmid DNA and the histone H1-induced DNA aggregates.

With increasing the ratio of histone/DNA, the circular nucleosomal arrays formed experimentally as expected (Figure 2-13). The nucleosome assembly assay was carried out following the protocol provided by New England Biolabs Ltd. For linker histone H1 binding experiments, recombinant human histone H1⁰ was mixed with 10 μ M plasmid DNA in the presence of 50 mM NaCl. The mixture was then incubated at 37 $^{\circ}$ C for 2 hours. As to the sample deposition method, both the magnesium-mediated approach and the APS-modified mica discussed in the last section were tested respectively.

Figure 2-13. AFM images of the reconstituted nucleosomal arrays on pBR322 plasmid DNA. Nucleosome assembly reactions are performed with 10 ng negatively supercoiled pBR322 and core histones at the molar ratios of 1:10, 1:20 and 1:40, respectively. All the images were captured at 512×512 pixels under tapping mode in air.

As shown in Figure 2-13, formation of nucleosomal arrays on plasmid DNA can be observed with the use of both sample deposition methods. All of these

height images shown were obtained on tapping mode AFM in air. The colors in these images represent the height of molecules as indicated in the height bar. Due to the resolution limit of AFM, nucleosome core particles that bind to DNA backbones appear as visible dots under microcopy. The number of nucleosome cores on each plasmid DNA molecule be counted directly from these 512×512 pixels images, as given in Table 2-2. It can be seen that the number of particles binding to each plasmid DNA increased at a steady rate with the rising of histone/DNA ratio. To further quantitatively characterize these particles, their height and horizontal sizes were carefully examined by section analysis (Figure 2-14). The average height of measured in our AFM studies is 4.3 nm, which is well within acceptable limits of the nucleosome diameter 55 Å obtained from the high-resolution X-ray structure^{37, 105-106}. The average diameter of these particles was 17.2 nm, much longer than 84 Å as reported^{37, 105-106}, which is due to the horizontal broadening effect by AFM metal tip as discussed in our previous sections. In summary, the orderly-arranged particles that bind to the circular DNA were verified to be the nucleosome cores in our AFM studies.

Figure 2-14. Illustration of (A) height, and (B) size measurement of the nucleosome core particles by section analysis using the Nanoscope Analysis software.

Table 2-2. Correlation between the number of nucleosome core particles formed on each plasmid DNA molecule and the molar ratio of DNA to histone octamer.

DNA-histone octamer molar ratio	Average number of particles per DNA molecule*	
	Magnesium-mediated method	APS-modified mica
1 : 0	0	0
1 : 10	4.2 $\pm$ 2.0	6.8 $\pm$ 2.3
1 : 20	16.1 $\pm$ 3.2	16.2 $\pm$ 3.0
1 : 40	--**	28.1 $\pm$ 4.2

* All the data were collected from fifty individual DNA molecules visualized by AFM.

** The nucleosomal arrays formed aggregates under this condition.

In addition to the nucleosome structure formed by DNA wrapping on the histone octamers, linker histone can associate with naked DNA to form oligomeric “tramtrack” structures^{101, 107}. Fang *et al.* reported in their FRET studies that histone H1 can bind to naked DNA in both inter- and intra-molecular manners¹⁰⁷. In our AFM studies, the formation of massive networks occurred between naked plasmid DNA molecules and linker histones as shown in Figure 2-15. However, these DNA-histone aggregates could hardly be absorbed onto the magnesium-mediated mica surface when the molar ratio of DNA to histone H1 comes to 1:50. In other words, the divalent cations dramatically trigger the aggregation of DNA-protein complexes, but on the other hand, hinder the absorption of these aggregates onto mica surface. Similar phenomenon was observed by Frutos and colleagues in their electrophoretic mobility studies¹⁰⁸. Different from the cation-assisted method, APS functionalized mica works reliably for imaging these DNA-histone complex structures. The nucleosomes formed on plasmid DNA can be identified clearly from the unbound histone protein molecules in Figure 2-13, while the linker histone H1-induced aggregation were well recorded as seen in Figure 2-15. The detailed information of these DNA-histone aggregates were collected from a number AFM images and summarized in Table 2-3. Results of these studies suggested that AFM is capable of imaging and analyzing the nucleosome core particles formed on the plasmid DNA as well as the H1-induced DNA aggregates. In addition, the histone-containing DNA molecules deposited onto APS-modified mica are most likely to maintain their native conformation during our AFM experiments.

Figure 2-15. AFM images of DNA-histone H1 aggregates on the mica mediated by magnesium cations or modified by APS. The mixtures of 10 ng negatively supercoiled pBR322 DNA and linker histone H1 at the molar ratios of 1:20, 1:50 and 1:100 were incubated at 37 °C for 30 min before AFM imaging. All the images were captured at 512 × 512 pixels under tapping mode in air.

Table 2-3. Correlation between the average height of DNA-histone aggregates and the molar ratio of DNA to histone H1.

DNA-histone H1 molar ratio	Average height of DNA-histone aggregates (nm)*	
	Magnesium-mediated method	APS-modified mica
1 : 0	0.9 ± 0.1	0.8 ± 0.1
1 : 20	8.2 ± 1.2	3.9 ± 0.6
1 : 50	--**	7.2 ± 1.2
1 : 100	--**	10.1 ± 4.2

* All the data were collected from fifty individual DNA-histone aggregates.

** The DNA-histone aggregates may not be absorbed onto mica surface under these conditions.

Over the past forty year, a large number of studies have been conducted to elucidate the detailed structure and the stability of nucleosome¹⁰⁹⁻¹¹⁴. Based on these studies, alkali metal ions have shown remarkable effects on either formation or dissociation of nucleosomes. Their investigations, in general, are based upon free radical cleavage, electrophoretic separation or fluorescence resonance energy transfer technique that can monitor nucleosome conformational features. It would be of great interest to combine these traditional methodologies with atomic force microscopy to visualize the molecular structures and assemblies. Therefore, sodium and potassium were selected as typical monovalent cations for evaluation of their effects on nucleosome conformation using AFM in our following studies.

Circular nucleosomal arrays were prepared by the above mentioned procedures and incubated in the buffer solution containing various concentration of NaCl. The mixtures were subsequently deposited onto APS-modified mica followed by AFM examination in air, results of which are shown in Figure 2-16.

The circular DNA molecules in these images appear to be slightly different geometry due to the ionic effects on DNA supercoiling^{57, 67}. It can be seen that the nucleosome core particles remain stable over a wide range of ionic strength. Furthermore, the number of nucleosome particles formed on each plasmid DNA was counted and listed in Table 2-2. According to these quantitative measures, no significant dissociation or aggregation of these nucleosomes was observed in the physiological concentrations of NaCl in the range of 50-200 mM.

Figure 2-16. AFM studies of salt effects on the stability of nucleosome core particles. Nucleosome assembly reactions are performed with 10 ng negatively supercoiled pBR322 and core histones at the molar ratios of 1:20. These obtained nucleosomal arrays were further incubated at 37 °C for 30 min in the presence of 50 mM, 100 mM or 200 mM NaCl before deposited onto APS-modified mica for AFM imaging. All the images were captured at 512 × 512 pixels under tapping mode in air.

Table 2-4. Correlation between the number of nucleosome core particles formed on plasmid DNA and the concentration of NaCl.

NaCl concentration	Average number of nucleosome core particles*
50 mM	14.6 ± 2.4
100 mM	12.9 ± 3.1

* All the data were collected from fifty individual DNA molecules.

It has been reported that histone H1 binds strongly and cooperatively to naked DNA at the NaCl concentration of 50 mM¹⁰⁷. Moreover, Ivanchenko *et al.* demonstrated in 1996 their interesting finding that histone H1 binding unwinds DNA double helix, which will further induce positive supercoils into circular DNA¹¹⁵. The supercoiling changes have been identified by their gel electrophoresis assay. These pioneering works stimulated our thinking in the topological function of linker histone in eukaryotic genomic DNA. It can be seen from Figure 2-15 histone H1 binds to naked circular DNA molecules, and further results in massive aggregation of the DNA and histone molecules. In consideration of the overall size of these aggregates, both intra- and inter-molecular interactions most likely coexisted in these structures. As discussed above, the appearances of the nucleosomal arrays are strongly dependent on the concentration of salt in the solution. Similar to that of histone octamer, the salt effect on the formation of DNA-histone H1 aggregates has been investigated in our following studies.

As shown in Figure 2-17 and Table 2-5, with the increasing of sodium concentration from 0 mM to 100 mM, formation of DNA-histone H1 aggregates can be observed in the AFM images and further verified by the height measurements. Different from the nucleosome core particles, however, most of aggregates were dissociated when the NaCl concentration was increased to 200 mM. Since the molecular weight of human histone H1⁰ is around 20 kDa which is much lower than that of histone octamer (~110 kDa), the histone H1 proteins bound on the DNA molecules could hardly be identified after the aggregates breakdown. On the other hand, as seen in Figure 2-16, the nucleosome core

particles remain stable under such ionic conditions. These findings make it possible for us to investigate the effect of the linker histone binding to nucleosome cores on the DNA topological structure, neglecting any intermolecular interactions.

Figure 2-17. AFM studies of salt effects on formation and dissociation of DNA-histone H1 aggregates. The mixtures containing 10 ng of pBR322 DNA and 5 nM of histone H1 were incubated at 37 °C for 30 min in the absence or presence of 50 mM, 100 mM or 200 mM NaCl before deposited onto APS-modified mica for AFM imaging. All the images were captured at 512 × 512 pixels under tapping mode in air.

Table 2-5. Correlation between the average height of DNA-histone aggregates and the concentration of NaCl.

NaCl concentration	Average height of DNA-histone H1 aggregates (nm)*
0 mM	0.8 ± 0.1
50 mM	3.6 ± 0.7
100 mM	9.2 ± 2.1
200 mM	0.9 ± 0.1

* All the data were collected from fifty individual DNA-histone aggregates.

In order to better understand the relationship between the histone octamer core, linker histone and nucleosomal DNA, as well as their cooperative roles in

chromosome packaging, further investigations were carried out based on the larger plasmid substrates and AFM technique.

2.3.3 Investigation and conformation of chromosome compaction with the aid of linker histone H1

Histones, the major protein component of chromosome, are responsible for the DNA condensation and organization in the nucleus of eukaryotic cells. Based on the structural differences, histones are grouped into core histone and linker histone. Two copies of each core histone are arranged as a globular octameric core which called the histone octamer. About 146 base pairs of double-stranded DNA wrapped 1.67 left-handed superhelical turns around the histone octamer forming the nucleosome core particle. In addition to the nucleosome core, linker histone binds to the nucleosomal dyad and the entry and exit points of nucleosomal DNA to form the chromatosome. Free radical and nuclease digestion footprinting studies suggest that histone H1 protects an additional 10 base pairs of DNA localized symmetrically with respect to the nucleosomal dyad, which further facilitates the stabilization of chromatosome^{17, 21, 116-118} (Figure 2-18). Although structural information of either histone octamer or linker histones has been provided in the recent crystallographic studies^{21, 24, 37}, fundamental roles of linker histone in the chromatin arrangement remains controversial. Therefore, the purpose of this section is to study the effect of linker histone binding on the conformation of higher order chromatin structures.

Figure 2-18. Illustration of chromatosome structure and the previously proposed location of linker histone globular domain.

Eukaryotic genomic DNA associate with numerous histone proteins during chromatin condensation. In order to mimic the chromosomal DNA in our AFM studies *in vitro*, the ideal experimental model should (i) allow the accumulation of linker histone binding effects on nucleosomal DNA, and (ii) facilitate the DNA-histone complexes tightly adsorb onto the mica surface for AFM visualization. As a consequence, double-stranded circular pAdEasy-1 plasmid which is 33,477 bases pairs in length, was chosen as a suitable DNA substrate in our AFM studies. The pAdEasy-1 plasmid was a kind gift given from Addgene in the form of bacteria stabs. Preparation and purification of pAdEasy-1 were followed the procedure provided by Addgene. APS-functionalized mica was used as a flat substrate to fix the biomolecules. AFM imaging for these large-sized pAdEasy-1 DNA molecules was then conducted in order to visualize their interactions with histone proteins (detailed sample preparation and AFM visualization method have been described in Section 2.2.10 and Section 2.2.11).

According to the superhelical density equation which illustrated before, the number of negative superhelical turns within pAdEasy-1 (whose theoretical

superhelical density is also -0.06) was ~ 193 . Due to the presence of numerous superhelical turns, these plasmid DNA molecules displayed a highly twisted and tangled geometry in the AFM images as shown in Figure 2-19. After wrapped around histone octamers, although the overall geometry of these plasmids remain basically unchanged, observation of the bead-on-a-string appearance indicate the formation of nucleosomes on these circular DNA molecules. The overall size of these nucleosome particles was measured and shown in Figure 2-20 and Table 2-6 accordingly. Their horizontal diameter, 16.9 nm in average, indicated that these isolated particles are single nucleosome cores similar to those found in Figure 2-13.

As can be seen in Figure 2-19, condensation of these bead-on-string structure occurred with increasing the concentration of histone H1. The massive aggregates appeared in these images indicated the formation of a certain kind of higher-order structures induced by linker histone H1. Characterization of both height and horizontal size of these DNA-proteins complexes were methodically carried out by section analysis using the Nanoscope Analysis software. As given in Figure 2-20, in addition to the visible appearances, statistical measurements of these structures also showed significant differences after treatments with histone H1. The mean values of their height and size was collected from fifty individual aggregates from AFM images and listed in Table 2-6. Their overall size reaches ~ 200 nm in diameter when the histone H1/DNA ratio approaches 200:1. As discussed in the last section, histone H1 is able to interact with naked DNA and form aggregation under low-salt condition. In order to avoid the influence of histone H1-DNA interactions, all experiments were carried out in the presence of 200 mM NaCl.

Figure 2-19. AFM visualization of histone H1-induced aggregation of nucleosomal arrays. Nucleosome assembly reactions were performed with 2.5 μg of negatively supercoiled pAdEasy-1 plasmid DNA as described in Section 2.2.2. These obtained nucleosomal arrays were then incubated with increasing amount of linker histone H1 (molar ratios of DNA to histones octamer to histone H1 are indicated below the images) for 30 min at 37 $^{\circ}\text{C}$. 20 μl droplets of these DNA-histone mixtures were deposited onto the APS-modified mica for imaging by AFM in air.

Figure 2-20. Height measurements of DNA-histone aggregates by section analysis using the Nanoscope Analysis software.

Table 2-6. Correlations between the average height and diameter of the histone H1-induced DNA-histone aggregates and the molar ratio of DNA to histone proteins*.

DNA/octamer/H1	Average height of aggregates (nm)	Average diameter of aggregates (nm)
1 : 0 : 0	0.6 ± 0.1	--**
1 : 50 : 0	4.8 ± 0.5	$16.6 \pm 1.9^{***}$
1 : 50 : 25	8.2 ± 2.5	55.5 ± 15.9
1 : 50 : 50	10.0 ± 2.2	78.1 ± 19.2
1 : 50 : 100	14.9 ± 2.3	108.1 ± 39.0
1 : 50 : 200	16.9 ± 5.0	150.6 ± 35.8

* All the data were collected from fifty individual DNA molecules visualized by AFM.

** No aggregates formed.

*** Data were collected from the single nucleosome core particles.

These experimental results suggest that a certain kind of higher order structure was formed on the circular DNA with the aid of linker histone H1. In the absence of histone H1, the bead-on-string structure constructed by the interactions between histone octamer and the circular plasmid DNA. Subsequently, with the addition of histone H1, aggregation of these structures occurred rapidly and has been visualized directly by microscope. To some extent, these experimental observations in our modelling studies are consistent with the mitotic chromosome condensation phenomenon *in vivo*.

One step forward, in order to find out whether there was superhelical tension in these condensed DNA-histone complexes, the closed circular DNA substrate was replaced by the nicked open-circular DNA in our next experiments. At this time, nicking enzyme was used to generate a single strand break on the duplex circular DNA molecule (detailed experimental procedures provided in Section 2.2.5). The nicked plasmids were verified by gel electrophoresis assay (data not shown), and were then used as new substrates in our following studies. Similar experiments were carried out to investigate the interactions between the nicked circular DNA and histone proteins, results of which were shown in Figure 2-21 and Table 2-7.

Figure 2-21. AFM visualization of interactions between linker histone H1 and nucleosomal arrays on the nicked plasmid DNA. Nucleosome assembly reactions were performed with 2.5 μg of open-circular pAdEasy-1 plasmid DNA as described in Section 2.2.2. These obtained nucleosomal arrays were then incubated with increasing amount of linker histone H1 (molar ratios of DNA to histones octamer to histone H1 are indicated below the images) for 30 min at 37 $^{\circ}\text{C}$. 20 μl droplets of these DNA-histone mixtures were deposited onto the APS-modified mica for imaging by AFM in air.

Table 2-7. Correlations between the average height and diameter of the nucleosome particles and the molar ratio of DNA to histone proteins*.

DNA/octamer/H1	Average height of particles (nm)	Average diameter of particles (nm)
1 : 0 : 0	0.6 ± 0.1	--**
1 : 50 : 0	5.2 ± 0.2	16.5 ± 3.3
1 : 50 : 25	4.9 ± 0.5	17.8 ± 3.4
1 : 50 : 50	4.7 ± 0.3	15.2 ± 2.0
1 : 50 : 100	5.1 ± 0.7	15.0 ± 3.9
1 : 50 : 200	5.2 ± 0.5	17.2 ± 4.0

* All the data were collected from fifty individual DNA molecules visualized by AFM.

** No particles formed.

Similarly, histone octamer bound to nicked circular DNA molecules gave a bead-on-string appearance under AFM. The height (~5 nm) and diameter (~16 nm) of these nucleosome particles are approximately the same as those particles formed on closed circular DNA. Most importantly, no obvious aggregation of these nucleosomal arrays was observed upon the addition of linker histone H1. As can be seen in the images in Figure 2-21, the overall features of these nucleosome-containing DNA molecules remain the same before and after incubation with histone H1. That is to say, the histone H1-induced higher order structure of nucleosomal arrays may not be formed on the open-circular DNA molecules. It is therefore our expectation that the supercoiling that existed in circular DNA could be of great importance in the linker histone-triggered condensation of nucleosomes.

Based on the new understanding of nucleosome condensation phenomenon, a number of experiments were accordingly designed and performed in our

following studies, in order to investigate the role of DNA supercoiling in the process of nucleosome condensation.

2.3.4 Disaggregation of the compact chromosomal structures by relieving the torsional tension in circular DNA

As discussed in the previous sections, reconstituted nucleosomal arrays remain stable on plasmids (nucleosome core particles binding tightly to DNA) in the presence of 0.2 M NaCl. Under such condition, on the other hand, linker histone H1 may not interact with naked DNA molecules to form DNA-histone H1 aggregates. These give us the possibility to investigate the binding effects of linker histone H1 on these nucleosome-containing circular DNA, and further elucidate the roles of linker histones on chromosomal DNA condensation in eukaryotic cells. In this case, our subsequent studies were performed and revealed that linker histone H1 dramatically facilitate (or trigger) the aggregation of the nucleosomal arrays on the topologically closed circular DNA, whereas such phenomenon was not observed in the parallel experiments in which open-circular DNA were used to construct nucleosomal arrays. These interesting findings suggest that the DNA supercoiling constrained in these nucleosome-containing structures may be a crucial factor in the process of nucleosomal DNA condensation as illustrated in Figure 2-22. Thus, in this section we discuss our studies on the correlation between the presence of superhelical tension in the linker DNA and the disaggregation of histone H1-induced higher-order DNA structures.

Figure 2-22. Schematic illustration of DNA superhelical tension that may be stored in the linker DNA between chromatosomes.

With the help of histone H1, a remarkable aggregation was observed on the nucleosome-containing closed circular DNA as shown in Figure 2-19. Firstly, in order to confirm these aggregates are accessible to DNA-related enzymes, Deoxyribonuclease I (DNase I) digestion was carried out in our following experiments followed by AFM examination. Nucleosomal arrays were reconstituted on 2.5 μg pAdEasy-1 plasmids with a DNA/histone octamer molar ratio of 1:0.2 by stepwise dilution as described in Section 2.2.2. As shown on the left of Figure 2-23, the nucleosome cores particles formed on plasmid DNA can be clearly seen on the image. These nucleosome-containing plasmids were then incubated with linker histone H1 at a 1:1 molar ratio to nucleosomes in the presence of 200 mM NaCl at 37 $^{\circ}\text{C}$ for 30 min. As expected, large DNA-histone aggregates formed under such condition, which are shown in the middle image. DNase I was subsequently used to disintegrate these higher-order aggregates in our next experiments. The DNase digestion assay was performed in a reaction

containing 10 mM Tris-HCl (pH 7.6), 2.5 mM MgCl₂, 0.5 mM CaCl₂ and 5U of DNase I at 37 °C for 60 min. The digestion products were deposited onto APS-modified mica and imaged in air. As shown on the right of Figure 2-23, after nuclease treatment, the exposed linker DNA between nucleosome particles was cleaved, while the DNA-histone aggregates were broken into smaller ones or free nucleosomal units as bright blobs on the AFM image.

Figure 2-23. AFM examination of DNase I accessibility of the histone H1-induced nucleosome aggregates. Left, nucleosome core particles (the molar ratio of DNA to histone octamer was 1:50). formed on pAdEasy-1 DNA (33,477 bp); middle, nucleosome aggregates formed in the presence of linker histone H1 (the molar ratio of DNA to histone octamer to histone H1 was 1:50:50) and 200 mM NaCl; right, digestion of the histone H1-induced nucleosome aggregates by DNase I treatment.

Although the histone H1-induced nucleosome aggregates were observed as highly compact structures under the microscope, our experimental results proved that these structures are accessible to nuclease and can be disintegrated under certain conditions. In order to figure out the driving force for the formation of nucleosome aggregates, DNA nicking endonuclease and DNA topoisomerase were used in our subsequent studies to completely release the torsional stress that may be stored in the linker DNA between chromatosomes as illustrated in Figure 2-24.

Figure 2-24. Schematic illustration of the relaxation of DNA supercoils that may be involved in the histone H1-induced nucleosome aggregates catalyzed by (i) DNA nicking endonuclease, or (ii) E.coli DNA topoisomerase I.

As shown in the left and middle images of Figure 2-25, with the addition of linker histone H1, the nucleosomal arrays formed aggregates as previously described. One of the DNA nicking enzymes Nt.BspQI was then added into the DNA-histone mixture to generate single-strand DNA breaks in the internucleosomal linker region. This enzyme recognize a specific sequence of nucleotides (5'-GCTCTTC[↓]N-3') which appears six times within the entire pAdEasy-1 DNA sequence, and hydrolyze the phosphodiester bond between the nucleotide C and N as indicated by arrow. Theoretically, the breaking of DNA strand at any of the six recognition sites will result in the free rotation of DNA strands, and further leads to the release of superhelical tension within the entire ~33 kbp circular DNA. As shown on the right of Figure 2-25, after nicking enzyme

digestion, the histone H1-induced nucleosome macroaggregates were disintegrated into relaxed nucleosomal arrays whose appearance is similar to that of nucleosome core particle arrays before addition of histone H1. These remarkable results suggest that the superhelical tension within the linker DNA may serve as the driving force for nucleosome condensation. Moreover, removal of these supercoils will result in an unfolding of higher-order DNA structures.

Figure 2-25. AFM examination of the histone H1-induced nucleosome aggregates relaxed by DNA nicking endonuclease. Left, nucleosome core particles (the molar ratio of DNA to histone octamer was 1:50). formed on pAdEasy-1 DNA (33,477 bp); middle, nucleosome aggregates formed in the presence of linker histone H1 (the molar ratio of DNA to histone octamer to histone H1 was 1:50:50) and 200 mM NaCl; right, digestion of the histone H1-induced nucleosome aggregates by nicking endonuclease treatment. The aggregates were digested with 10 U of Nt.BspQI in the presence of 50 mM Tris-HCl (pH 7.9), 100 mM NaCl and 10 mM MgCl_2 at 37 °C for 60 min.

On the basis of our previous experimental results, we learned that the DNA supercoiling stored within the linker DNA between chromatosomes plays crucial roles in the condensation of nucleosomes. In addition, it is our speculation that the sign (or direction) of the supercoils in these higher-order structures should be negative, the same as those constrained in the 10-nm nucleosome fibre. Therefore,

the following experiments was accordingly designed and conducted to figure out the sign of DNA supercoiling in the histone H1-induced nucleosome aggregates.

E. coli DNA topoisomerases I is a kind of type I topoisomerases that catalyzes the relaxation of negatively, but not positively, supercoiled DNA. In our investigation, the nucleosome aggregates were incubated with 5 U topo I at 37 °C for 60 min. Both the control (Figure 2-26, left) and topo I-treated (Figure 2-26, right) samples were then deposited onto APS-modified mica followed by AFM examination. As shown in Figure 2-26, relaxation of the macroaggregates by *E. coli* topo I has been observed, which indicates the existence of negative supercoils in these higher-order structures and suggests a possible role of DNA supercoiling in eukaryotic chromosome packaging.

Figure 2-26. AFM examination of the histone H1-induced nucleosome aggregates relaxed by *E. coli* DNA topoisomerase I. Left, nucleosome core particles (the molar ratio of DNA to histone octamer was 1:50). formed on pAdEasy-1 DNA (33,477 bp); middle, nucleosome aggregates formed in the presence of linker histone H1 (the molar ratio of DNA to histone octamer to histone H1 was 1:50:50) and 200 mM NaCl; right, relaxation of the histone H1-induced nucleosome aggregates by *E. coli* topoisomerase I treatment.

In this section, we examined the accessibility of the histone H1-induced nucleosome aggregates to nuclease cleavage, and found that the constrained

negative DNA supercoils may be involved in forming and maintaining these structures. Our findings, to some extent, may provide us a new understanding for the high-order chromosomal structure and the roles of linker histone H1 in eukaryotic DNA organization.

2.4 Conclusion

In this chapter, atomic force microscopy was utilized to investigate the interactions between DNA and histone proteins. As discussed above, topographical information of the regular nucleosomal arrays and the irregular macroaggregates formed *in vitro* can be faithfully recorded by AFM with the sample absorbed on APS-modified mica. We found that the nucleosomal arrays reconstituted with core histones and circular DNA appear as a "beads-on-a-string" structure under microscope, and remain stable in a wide range of ionic strengths. On the other hand, our results showed that linker histone H1 binds to naked DNA molecules and facilitates the formation of DNA intermolecular aggregates at a NaCl concentration of ~50-100 mM.

As a result of these findings, a series of experiments were subsequently designed and carefully conducted under high-salt conditions in order to clarify the binding effects of linker histone H1 on these nucleosome-containing circular DNA, and further elucidate the roles of linker histones on eukaryotic DNA condensation. Our experimental results demonstrated that cooperative binding of linker histone H1 onto the topologically closed nucleosomal arrays remarkably triggers the aggregation of these DNA-histone complexes into higher order structures. However, such structures was not formed in the parallel experiment in which nicked circular DNA was used to construct nucleosomal arrays. As a consequence,

it is our expectation that additional DNA supercoils may be generated by the binding of linker histone H1 into the nucleosomal arrays and further involved in forming and maintaining the nucleosome aggregates.

In addition, in order to further investigate the role of DNA supercoiling in nucleosome condensation, nuclease and topoisomerase were used in our subsequent studies to relieve superhelical tension in the constrained topological domains. Disintegration of the histone H1-induced nucleosome macroaggregates was observed upon removal of the negative supercoils by certain DNA topoisomerase, which suggests that the negative DNA supercoiling presents within the linker DNA may serve as the driving force for compaction of the nucleosomal arrays. It is therefore our hope that the discoveries reported in this chapter could have certain implications in our understanding of the roles of linker histone H1 in eukaryotic DNA higher-order organization.

2.5 References

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Chapter 3

Gel Electrophoretic Determination of Linker DNA

Supercoiling Induced by DNA-Histone Interactions

3.1 Background Information

In the preceding chapters, we have discussed the conformational changes on circular DNA upon association with histone proteins. The double-stranded DNA could wrap around the histone octamer core, forming a 5.5 nm height nucleosome particle. Formation of numerous nucleosomes on plasmid DNA would produce the supercoiled 10 nm fibre structure, resembling beads on a string under atomic force microscopy. Additionally, the linker histone H1 bound to the nucleosomal linker DNA, leading to the remarkable condensation of these chromosome structures. To further investigate the topological properties of such aggregates, several DNA-related enzymes were used to relieve the torsional tension that may exist in these compacted structures. As a result, our AFM experimental results demonstrate that dissociation of these aggregates occurred as expected after the negative DNA supercoiling was dissipated by DNA topoisomerase action. In other words, our new findings indicate that additional DNA supercoils may be generated into the linker DNA between chromatosomes upon cooperative binding of the linker histone H1 to the nucleosome core particles. On the basis of our previous studies, quantitative measurements of the linker DNA

supercoiling changes by the binding of histone H1 to nucleosome core particles were performed and presented in this chapter.

On the one hand, formation of each nucleosome core particle appears to generate one negative superhelical turn into the nucleosomal DNA. However, the 146 bp of DNA are wrapped around the histone octamer in 1.67 turns. This contradiction, known as the “linking number paradox”, is now believed as DNA overtwisting on the surface of nucleosome¹⁻⁵. This type of DNA supercoiling generated by nucleosome cores was systematically studied in section 3.3.2. On the other hand, binding of linker histone H1 to the naked double-stranded DNA were proved to unwind DNA double helix, and introduce supercoils into closed circular DNA molecules. This observation was first reported by Ivanchenko *et al.* in 1996⁶ and was further proved in our electrophoretic studies. Quantitative measurements of linking number changes that generated by linker histone H1 were accordingly conducted and discussed in section 3.3.3.

Different from the interactions between DNA and histone octamer/histone H1, binding of the linker histone H1 to nucleosome cores would "lock" the DNA in place and facilitate the formation of chromatosomes. As a results of this cooperative binding process, additional supercoils were found to be introduced into linker DNA in our investigations, and was further elaborated in section 3.3.4. This complicated relationship between linker histone and nucleosomal DNA supercoiling was thoroughly studied in this chapter, while the obtained experimental results provided us new insights into the role of linker histone and the topological structure of chromosomal DNA.

Although atomic force microscope has been shown to be useful for imaging DNA-histone complexes in Chapter 2, it has remained limited to provide a

quantitative analysis of DNA supercoiling. In this case, for the purpose of determining the sign and degree of DNA supercoiling constrained in the nucleosome-containing circular DNA, precise measurement of DNA superhelical density should be conducted in our subsequent studies. Gel electrophoresis is a traditional analytical technique used by scientists to separate biomolecules on the basis of their size and conformation⁷⁻¹⁰. In conventional gel electrophoresis, an electric field is applied to the gel by the power supply. The nucleic acid molecules to be analyzed, due to the negative charges on their phosphate backbone, are forced through the pores of the gel moving toward the positive anode. In this case, the separation is based on the mobility of these DNA fragments. Normally, the nucleic acid molecules with high molecular weight migrate slowly through the gel, while the smaller molecules move faster. In addition, three-dimensional conformation of DNA molecules affects the migration rate significantly. Circular forms of DNA have mobilities that differ from linear molecules of the same mass. As previously reported, even all the DNA molecules are in circular form and have the same number of base pairs, the DNA molecules that differ in linking number (in different topological states) can be separated by gel electrophoresis¹¹, which is due to the supercoiled DNA molecule has a more compact structure than its relaxed form. As a consequence, gel electrophoresis has been used in laboratories around the world for more than thirty years to separate the mixture of DNA topoisomers¹¹⁻¹⁴. Here in our studies, an electrophoresis-based method has been developed to quantitatively determine the DNA supercoiling stored within the linker region of closed circular nucleosomal arrays.

3.2 Materials and Methods

3.2.1 Oligodeoxyribonucleotides, enzymes, chemicals and equipments

Product(s)	Manufacturer
Oligodeoxyribonucleotides	Sangon Biotech (Shanghai, China)
pUC 19 Vector, pBR322 Vector	New England Biolabs (Singapore)
1 kb DNA ladder	New England Biolabs (Singapore)
T4 DNA ligase	New England Biolabs (Singapore)
ATP	New England Biolabs (Singapore)
Proteinase k	Thermo Scientific (Singapore)
Topoisomerase I (E. coli)	New England Biolabs (Singapore)
Nt. BspQI nicking endonuclease	New England Biolabs (Singapore)
Nucleosome Assembly Kit	New England Biolabs (Singapore)
Histone H1 ⁰ (human, recombinant)	New England Biolabs (Singapore)
QIAquick PCR Purification Kit	Qiagen (Singapore)
Agarose	Invitrogen
Ethidium bromide	Bio-Rad Laboratories (Singapore)
TAE, Tris	1st Base (Singapore)
Eppendorf tubes, Pipette tips	Greiner (Singapore)
Gel documentation system	Syngene (Cambridge, UK)

Most all the chemicals used in this research were listed above otherwise were obtained from Sigma-Aldrich with analytical grade or molecular biology grade.

3.2.2 Nucleosome assembly

Nucleosomes were prepared from the Epimark Nucleosome Assembly Kit (New England Biolabs #E5350S) following the dilution assembly protocol for 50 pmol DNA substrate. A 20 μ l mixture containing 5 μ g DNA, 2 M NaCl, 5 μ M Histone H2A/H2B Dimer and 2.5 μ M Histone H3.1/H4 Tetramer (dosages of core histones should be adjusted according to the required molar ratio of nucleosome to DNA) was incubated at room temperature (25 $^{\circ}$ C) for 30 minutes. Subsequently, 7 μ l dilution buffer (10 mM Tris, pH 8.0) was added into the mixture to bring the sodium concentration to 1.48 M. After incubation at room temperature (25 $^{\circ}$ C) for 30 minutes, the mixture was diluted by adding 13 μ l dilution buffer (10 mM Tris, pH 8.0) to bring the sodium concentration to 1.0 M. Afterwards, the mixture was incubated at room temperature (25 $^{\circ}$ C) for additional 30 minutes and further diluted by adding 27 μ l dilution buffer (10 mM Tris, pH 8.0) to bring the sodium concentration to 0.6 M. Finally, the mixture was incubated at room temperature (25 $^{\circ}$ C) for 30 minutes again and diluted by adding 93 μ l dilution buffer (10 mM Tris, pH 8.0) to bring the final concentration of NaCl to 0.25 M. The obtained mixture was further incubated at room temperature (25 $^{\circ}$ C) for 30 minutes and stored at 4 $^{\circ}$ C.

3.2.3 Histone H1-DNA binding assay

A 20 μ l aqueous solution containing 5 μ g DNA, 50 mM NaCl (concentration of NaCl should be adjusted according to experimental requirements) and 2.5 μ M recombinant human histone H1⁰ (New England Biolabs #M2501S,

dosage of histone H1 should be adjusted according to the required molar ratio of histone H1 to DNA) was incubated at 37 °C for 30 minutes.

3.2.4 Chromatosome assembly

Nucleosomes were prepared from the Epimark Nucleosome Assembly Kit (New England Biolabs #E5350S) following the dilution assembly protocol as described before. A 20 µl solution containing nucleosomal arrays (nucleosome concentration of 2.5 µM), 250 mM NaCl and 2.5 µM recombinant human histone H1⁰ (New England Biolabs #M2501S, dosage of histone H1 should be adjusted according to the required molar ratio of histone H1 to DNA) was incubated at 37 °C for 30 minutes.

3.2.5 Preparation of open-circular DNA

A 30 µl solution containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 µg of supercoiled pUC19 (New England Biolabs #N3041S), pBR322 (New England Biolabs #N3033S) or pAdEasy-1 (gift from Addgene #16400) vector DNA and 2 U of Nt.BspQI nicking endonuclease (New England Biolabs #R0644S) was incubated at 50 °C for 1 hour. The obtained open-circular DNA product was then purified by QIAquick PCR Purification Kit (Qiagen), and further examined by agarose gel electrophoresis.

3.2.6 Ligation of nicked circular DNA

A 30 µl solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol, 0.5 pmol of nicked circular DNA or DNA-histone complex and 50 U of T4 DNA ligase (New England Biolabs

#M0202S) was incubated at 16 °C for 1 hour. The obtained open-circular DNA product was then purified by QIAquick PCR Purification Kit (Qiagen), and further examined by agarose gel electrophoresis.

3.2.7 Removal of proteins from DNA samples

A mixture containing 5 µg of DNA-histone complex and 5 U of recombinant Proteinase K (Thermo Scientific #EO0491) was incubated at 37 °C for 10 minutes. The obtained solution was further purified by QIAquick PCR Purification Kit.

3.2.8 Agarose gel electrophoresis

The agarose gel slab was prepared by heating an 1.2 % (w/v) agarose suspension in the absence or presence of 1.5 µg/ml chloroquine (concentration of chloroquine should be adjusted according to experimental requirements) in a boiling water bath to melt the agarose, and allowing the gel to solidify at room temperature for 1 hour. A 20 µl of DNA sample was mixed with gel loading buffer (New England Biolabs #B7021S) and loaded into the well of agarose gel. Then, the gel was run at 2 volts/cm in 1 × TAE buffer for 12 hours. After gel electrophoresis, the gels were stained with 1.5 nM of ethidium bromide in 1 × TAE buffer for 30 minutes, and analyzed using G:BOX iChemi gel documentation system (Syngene).

3.3 Results and Discussion

3.3.1 Gel electrophoretic analysis of DNA topological properties

Gel electrophoresis is a routinely used biochemical technique for separating biomolecules. As discussed in Section 1.3, the separation of electrophoresis is achieved by moving negatively charged DNA molecules from cathode to the positively charged anode in a porous gel. For our purpose of DNA topological study, agarose gel electrophoresis is the most effective technique to separate the DNA molecules in different topological states. Agarose which is a polysaccharide polymer material, contains relatively large pores. In comparison with the polyacrylamide gel, agarose gel provide better resolution for large DNA fragments (100 bp to 25 kbp) separation. The migration rate of these biomolecules through the gel mainly based on their size, charge and shape. Accordingly, four topologically-different forms of double-stranded DNA were analyzed by agarose gel electrophoresis as shown in Figure 3-1. The structures of DNA in these bands are linear (L form, lane 2), open circular (OC form, lane 3), relaxed circular (RC form, lane 4) and supercoiled circular DNA (SC form, lane 5, respectively. Therefore, it can be seen that the DNA molecules of the same size but different conformation migrate differently in the agarose gel. The order of increasing migration distance of these DNA molecules is as follows:

$$\text{CC form} \approx \text{RC form} < \text{L form} < \text{SC form}$$

Supercoiled circular DNA molecules migrate faster than other forms of DNA due to their compact structure, whereas relaxed and open circular DNAs migrate with the slowest mobility.

Figure 3-1. Agarose gel electrophoretic analysis of four topological forms of double-stranded DNA. Lane 1: 1 kb DNA marker; Lane 2: EcoRI (restriction endonuclease) digestion products of pBR322 DNA (linear form); Lane 3: Nt. BspQI (nicking endonuclease) digestion products of pBR322 DNA (open-circular form); Lane 4: E.coli topoisomerase I digestion products of pBR322 DNA (relaxed circular form with a thermal distribution of topoisomers); Lane 5: negatively supercoiled pBR322 DNA (supercoiled form).

As discussed in Section 1.1.3, the DNA topoisomers are DNA molecules with the same formula but different topologies. Typically, supercoiled DNA topoisomers contain different numbers of superhelical turns and differ in their overall shape under certain conditions. Mathematically, the writhing number (the number of times the duplex axis crosses itself) is used to describe the degree of DNA supercoiling. As illustrated in Figure 3-2, the highly supercoiled DNA molecules with large absolute values of writhing number are more compact than the less supercoiled ones, and are expected to migrate faster in agarose gel.

Figure 3-2. Schematic illustration of negatively supercoiled double-stranded circular DNA with different superhelical turns (writhe numbers).

Separation of DNA topoisomers were tested in our following electrophoretic studies. *E. coli* topoisomerase I, as one of the type I DNA topoisomerases, catalyzes the relaxation of negative supercoiled DNA and produces the less supercoiled DNA molecules. In our experiments, pUC19 plasmid DNA which has 2,686 base pairs in length was treated with increasing amount of *E. coli* topoisomerase I, followed by standard agarose gel electrophoresis. As shown in Lane 2 of Figure 3-3, the plasmid DNA extracted from bacteria remains highly negatively supercoiled and migrates faster in the agarose gel. After treatment with topoisomerase, DNA supercoils are partially released, while the obtained DNA topoisomers moves slower as shown in Lane 3 and Lane 4. Quantitative analysis of the thermal distribution of circular DNA topoisomers is crucial for determining the topological changes occurred in the DNA molecules. It can be found that the distribution of less supercoiled DNA topoisomers can be resolved by agarose gel into several discrete bands (as seen in Lane 4). However, the topoisomers with plenty of superhelical turns can hardly be separated in the standard gel (as seen in

Lane 2). Instead, all the highly supercoiled DNA topoisomers co-migrate in a single band, which is due to the insufficient resolution of the standard agarose gel.

Figure 3-3. Electrophoretic separation of negatively supercoiled pUC19 topoisomers on standard agarose gel. Lane 1: 1 kb DNA ladder; Lane 2: 500 ng of negatively supercoiled pUC19 DNA; Lane 3: partially relaxation of negatively supercoiled pUC19 by 0.3 U of E.coli topoisomerase I at 37 °C for 1 h; Lane 4: partially relaxation of negatively supercoiled pUC19 by 1 U of E.coli topoisomerase I at 37 °C for 1 h. Electrophoretic separation was carried out in 1.2 % w/v agarose gel in 1 × TAE buffer at 25 °C for 12 h at 2 volts/cm.

In order to solve this problem, DNA-intercalating agents were used for analyzing the DNA topoisomers harboring high degrees of negative supercoiling. As reported, chloroquine is one of the commonly used DNA intercalators that can efficiently intercalate between the DNA base pairs and unwind the DNA double helix¹⁵⁻¹⁶. On the basis of the topological rules which given in Section 1.1.3, the linking number which is always equivalent to the sum of twist and writhe remains unchanged in covalently closed circular DNA. Therefore, unwinding of DNA double helix which reduces the helical twist will result in an increase of writhe

accordingly. As a consequence, intercalation of chloroquine into double-stranded DNA will generate additional positive supercoils (or relieve negative supercoils) in circular DNA molecules. Theoretically, with addition of chloroquine in agarose gel and electrophoresis running buffer, the overall distribution of DNA topoisomers will shift from highly negatively supercoiled toward more relaxed forms. By using this method, the circular DNA with a broad range of supercoiling degrees can be effectively analyzed by agarose gel electrophoresis.

Accordingly, examinations of the negatively supercoiled DNA with different superhelical turns (same as the samples used in Figure 3-3) were conducted in the presence of 1 $\mu\text{g/ml}$ or 2 $\mu\text{g/ml}$ chloroquine respectively, results of which are shown in Figure 3-4. As expected, the highly supercoiled topoisomers in this experiment were partially relaxed, and can be successfully separated as discrete bands in the gel (as seen in Figure 3-4A Lane 3-4 and Figure 3-4B Lane 2-4). These experimental results suggest that in order to achieve high resolution separation of DNA topoisomers, appropriate concentration of DNA intercalators is required according to their degree of supercoiling.

Figure 3-4. Electrophoretic separation of supercoiled pUC19 topoisomers on

chloroquine-containing agarose gel. Lane 1: 1 kb DNA ladder; Lane 2: 500 ng of negatively supercoiled pUC19 DNA; Lane 3: partially relaxation of negatively supercoiled pUC19 by 0.3 U of E.coli topoisomerase I at 37 °C for 1 h; Lane 4: partially relaxation of negatively supercoiled pUC19 by 1 U of E.coli topoisomerase I at 37 °C for 1 h. Electrophoretic separation was carried out in 1.2 % w/v agarose gel with (A) 1.0 µg/ml, or (B) 2.0 µg/ml chloroquine at 25 °C for 12 h at 2 volts/cm.

In addition, since either positively or negatively supercoiled DNA migrates faster than relaxed form DNA in the gel, it is a challenge to distinguish different types of supercoiled DNA molecules of the same superhelical density. To overcome this issue, two-dimensional agarose gel electrophoresis has been developed for analyzing complex mixtures of DNA topoisomerases^{12, 17-20}. On the other hand, with the aid of DNA intercalators, one-dimensional electrophoresis can also be used for discrimination of positive or negative supercoiled topoisomers in our studies. It has been shown in our previous experiments that the highly negatively supercoiled DNA can be partially relaxed by chloroquine. One step forward, if we keep on increasing chloroquine concentration, its unwinding effect will then convert the negatively supercoiled DNA to the positively supercoiled form (as seen in Figure 3-5B Lane 4). At this point, by carefully controlling the concentration of DNA intercalators, negatively and positively supercoiled topoisomers will be respectively converted into slightly and highly positively supercoiled species. Afterwards, by comparing their migration rates with that of relaxed circular form DNA in the same intercalator-containing gel, we could accordingly identify the sign and degree of these DNA supercoiling. The detailed

information as well as the experimental observations was discussed in the following sections.

All of the above mentioned electrophoretic studies were carried out using pUC19 as DNA substrate. This widely used plasmid is a relative small circular DNA which has 2686 base pairs. Under physiological conditions, the DNA double helix repeat as 10.4 base pairs per turn in solution. Accordingly, pUC19 plasmid DNA isolated from bacterial cells is expected to have about 15 negative superhelical turns. Another commonly used plasmid, pBR322 vectors, were used in our following investigations as a larger substrate. pBR322 is a double-stranded circular DNA which has 4361 base pairs in length. Theoretically, each pBR322 DNA molecule contains approximately 25 negative superhelical turns. As a comparison, parallel experiments have been conducted to examine the topological properties of this circular DNA. Similarly, the highly negatively supercoiled topoisomers migrate as a single band in agarose gel without DNA intercalators as shown in Figure 3-6A (lane 2). With the addition of chloroquine, approximately fifteen individual topoisomer bands were resolved in the gel (as seen in Figure 3-6B), which indicates a broad distribution of pBR322 topoisomers. This may due to the larger circular DNA own better torsional flexibility than smaller ones. Plasmids that much larger than pBR322 have been also tested in our studies. However, experimental separation of such plasmids appeared as smeared DNA bands rather than a set of discrete bands in a 20-cm-long 1% agarose slab gel (data not shown here). In this case, pUC19 and pBR322 plasmids were used as the DNA substrates in our subsequent investigations on the DNA topological properties in the interaction with histone proteins.

Figure 3-5. Electrophoretic separation of negatively supercoiled pBR322 topoisomers on agarose gel in the absence or presence of chloroquine. (A) Electrophoresis of 500 ng negatively supercoiled pBR322 on 1.2 % w/v agarose gel (2 volts/cm, 12 h, 25 °C). (B) Electrophoresis of 500 ng negatively supercoiled pBR322 on 1.2 % w/v agarose gel with 1.5 µg/ml chloroquine in gel and running buffer (2 volts/cm, 12 h, 25 °C).

In order to obtain quantitative information from our electrophoretic results, additional experiments were carried out accordingly. Negatively supercoiled plasmid DNA was completely relaxed by DNA topoisomerase, followed by agarose gel electrophoresis in the presence of 0.5 µg/ml chloroquine. Syngene imaging and analysis systems have been used to determine individual band intensity from digital images of gel lanes. Electrophoretic images were captured first using the G:BOX imaging system with GeneSnap software. Densitometric analysis of grayscale intensity was then performed on these obtained images using GeneTools program. Fluorescence intensities of bands in each lane was applied to

the individual linegraph as shown on the right of the gel image in Figure 3-6, which represents the mass of nucleotides in a certain area of gel. In this approach, the thermal distribution of DNA topoisomers can be thoroughly characterized for our further statistical analysis.

Figure 3-6. Quantitative gel electrophoretic analysis of the equilibrium distribution of circular DNA topoisomers by Syngene gel imaging system using GeneTools software. Left: the gel electrophoretic image captured using G:BOX imaging system (Syngene, UK); Right: Quantification of band intensities using GeneTools software. Ten bands (band 1-10 as indicated) were resolved on this agarose gel.

As described by Depew and Wang²¹ as well as Pulleyblank *et al.*²² in 1975, the thermal equilibrium of DNA molecules gives a Boltzmann distribution of states in solution as illustrated in the following equation,

$$N_i/N_t = A \exp(-E_i/RT) \quad (3.1)$$

in which A is a normalization factor, while R and T stand for the gas constant and the absolute temperature respectively. The percentage of molecules in a given state (N_i/N_t) is related to its state energy (E_i).

In our studies, the relative concentrations of various topoisomers are calculated further plotted on a linear scale as shown in Figure 3-7. This graph represents the integrated peak areas of the individual bands on the gel shown in Figure 3-6. The resulting distribution was then mathematically fitted by a Gaussian curve using Origin software:

$$f(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-\mu)^2}{2\sigma^2}} \quad (3.2)$$

According to the exact fit equation that resulted from the fitting process, the Gaussian center (expected value μ_0) of this distribution of topoisomers is 5.2 as indicated in Figure 3-7. To further investigate the topoisomer distributions of other DNA samples, DNA molecules with certain supercoiling degrees were analyzed under same condition. Subsequently, the relative intensity of each DNA band was calculated and then fitted by Gaussian curve. By comparing the μ_0 and μ_1 which obtained in the new Gaussian equation, the shift in topoisomer distribution ($\Delta\mu = \mu_1 - \mu_0$) can be quantified. The absolute value of $\Delta\mu$, in this case, indicates the average superhelical turns of the set of DNA topoisomers.

Figure 3-7. Determination of the Gaussian center of DNA topoisomers. The best-fit Gaussian curve was obtained from the relative intensities of the gel bands shown in Figure 3-6.

In summary, we have systematically characterized the topological properties of plasmid DNA using one-dimensional agarose gel electrophoresis. Furthermore, the Gaussian-center method has been developed and tested here in our studies for effectively determining the superhelical turns of the covalently closed circular DNA. This method will be further used in our following investigations of histone binding effects on circular DNA.

3.3.2 Determination of DNA supercoiling generated by the formation of nucleosome core particle

In eukaryotic cells, DNA wraps in 1.67 left-handed turns around a histone octamer core, forming the nucleosome core particle. The left hand toroidal rotation, topologically, is equivalent to one negative superhelical turn within a DNA topological domain. The difference between the toroidal turns which obtained by X-ray analysis²³⁻²⁵ and the observed degree of supercoiling²⁻⁵ is the so-called linking number paradox. It has been demonstrated by Richmond *et al.*²⁶ that this topological puzzle may be attributed to the overtwisting of DNA double helix that wrapped around histone octamer surface.

Basically, in our following studies, we are focusing on the DNA supercoiling that generated by the formation of nucleosome cores under physiological conditions. As illustrated in Figure 3-8, one nucleosome core particle that forms on the completely relaxed circular DNA induces one negative supercoil in the toroidal nucleosomal DNA. Hence, the net supercoiling of this covalently closed structure will be maintained as zero by the generation of a positive superhelical turn in the linker DNA. It can thus be imagined that a chromatin-like circular DNA structure containing a number of nucleosome cores will result in the

same number of positive supercoils in its linker regions between the nucleosomes. It was therefore our initial expectation that the histone-associated topological variation of circular DNA could be determined by examining these positive supercoils in the linker DNA. However, it has been carefully verified by Clark and colleagues that the conformation of nucleosomes formed on positively supercoiled DNA is greatly changed compared with the classical nucleosomes that formed on negatively supercoiled or relaxed circular DNA²⁷. That is to say, if the relaxed circular DNA is used as starting material in our studies, the nucleosomes formed afterwards will be in an altered conformation, which will result in unreasonable and unanalyzable experimental data.

Figure 3-8. Schematic illustration of the topological changes occurring in relaxed circular DNA by the formation of nucleosome core particles.

In order to overcome this challenge, both the open-circular and the negatively supercoiled DNA were selected as substrates for examining their binding effects with histone octamers in our following studies. Two distinct experimental routes were accordingly designed and shown in Figure 3-9 and Figure 3-10. As illustrated in Figure 3-9, the open-circular DNA without superhelical tension (Structure 2 in Figure 3-9) was first prepared by breaking one of the two strands of plasmid DNA (catalyzed by DNA nicking endonuclease

Nt.BspQI). Next, the stepwise nucleosome assembly reaction was performed by mixing the nicked circular DNA substrate and the four core histones in certain molar ratio in high levels of salt (~2 M NaCl), followed by a slow dilution to low salt concentration to permit nucleosome formation. The detailed nucleosome assembly procedures was thoroughly provided in Section 3.2.2. At this point, all of the torsional tension within the linker DNA should be released spontaneously owing to the presence of the single-stranded nick (as seen in Structure 3 in Figure 3-9). These nucleosome-containing nicked plasmids were subsequently resealed enzymatically to give back to the covalently closed structure (Structure 4 in Figure 3-9). Theoretically, the superhelical turns that remained in this circular structure were entirely contributed by the nucleosome cores. In order to remove the histone proteins, these nucleosome-DNA complexes were treated by proteinase K at 50 °C for 10 min and further purified using QIAquick PCR Purification Kit. Consequently, these obtained protein-free DNA molecules (Structure 5 in Figure 3-9) were topologically equivalent with the ones associated with histones (Structure 4 in Figure 3-9). Finally, by comparing the topoisomer distribution of these finally obtained circular DNA (Structure 5 in Figure 3-9) and that of completely relaxed circular DNA (Structure 6 in Figure 3-9) prepared by nick-ligation of pBR322, we could therefore characterize the effects of nucleosome formation on the DNA topological properties.

Figure 3-9. Schematic illustration of our designed experimental route for determining circular DNA supercoiling changes generated by the formation of nucleosome core particles (Route 1).

Different from the experimental route (Route 1) discussed above, another experimental route in which negatively supercoiled DNA was used as substrate for nucleosome assembly was designed and further tested in our studies. It has been known that the plasmids isolated from bacteria are highly supercoiled with an superhelical density of -0.06. In this case, the pBR322 (4,361 base pairs in length) that was used in our experiments contains approximately 25 negative superhelical turns. Theoretically, these inherent negative supercoils could help nucleosomal DNA maintain their favorable geometry and avoid conformational alteration that may be induced by positive supercoils²⁷.

As illustrated in Figure 3-10, nucleosomal arrays were reconstituted by stepwise dilution method (described in Section 3.2.2) using the negatively supercoiled circular DNA (Structure 1 in Figure 3-10) and core histones. DNA nicking endonuclease Nt.BspQI was then used to generate single-strand breaks on these nucleosome-containing DNA molecules. At this point, the rest of superhelical tension in the linker DNA could be relieved after the equilibrium reached in a short period of time (Structure 3 in Figure 3-10). Subsequently, excess

amount of T4 DNA ligase was added into the mixture to reseal the nick-site, providing the covalently closed nucleosomal DNA (Structure 4 in Figure 3-10). Similarly, removal of proteins by Proteinase K is required before analysis by gel electrophoresis. The proteinase digestion product was subsequently purified using QIAquick PCR Purification Kit and then examined by agarose gel electrophoresis.

Figure 3-10. Schematic illustration of our designed experimental route for determining circular DNA supercoiling changes generated by the formation of nucleosome core particles (Route 2).

Topological properties of these DNA products were then determined by agarose gel electrophoresis in the presence of 1.2 $\mu\text{g/ml}$ chloroquine. As a comparison, the distribution of relaxed pBR322 DNA topoisomers (Structure 6 in Figure 3-9 and Figure 3-10) was shown in Lane 2 of Figure 3-11 and further analyzed by GeneTools program. The obtained distribution graph was plotted in Figure 3-12A. Expected value (μ_0) of the best-fit Gaussian curve is found to be 11.0.

The supercoiled DNA molecules obtained by Route 1 (Structure 5 in Figure 3-9) were analyzed using the same method as shown in Lane 3 of Figure 3-11. Accordingly, the Gaussian distribution of these supercoiled topoisomers was plotted using Origin fitting program and given as an equation in Figure 3-12B.

Gaussian center (μ_1) of this distribution is found to be 9.7, so that the changes of DNA supercoiling can be calculated as: $\Delta\mu_1 = \mu_1 - \mu_0 = -1.3$. As Structure 6 is originally relaxed form circular DNA whose original writhing number is zero, and became positively supercoiled in chloroquine gel. Structure 5 whose visible superhelical turns on chloroquine gel is less than that of Structure 6 indicates its negatively supercoiled form with an average superhelical turns of -1.3. Based on the fact that formation of each nucleosome generate one negative supercoil^{2-5, 28}, our current experimental results suggest that on average there are around 1.3 nucleosomes formed on each of pBR322 plasmids DNA under such conditions.

Similarly, the thermal distribution of supercoiled DNA products obtained by Route 2 (Structure 5 in Figure 3-10) were shown in Lane 4 of Figure 3-11. Quantification of the gel bands was carried out and the Gaussian fitting of these topoisomers was plotted in Figure 3-12C. The mean value (μ_2) of this obtained Gaussian curve is found to be 9.2, indicating the average superhelical turns in these DNA molecules are -1.8 ($\Delta\mu_2 = \mu_2 - \mu_0$).

Figure 3-11. Electrophoretic analysis of DNA supercoiling changes generated by the formation of nucleosome core particles via Route 1 and Route 2. Lane 1: 1 kb DNA ladder; Lane 2: 300 ng of completely relaxed pBR322 DNA (ligation of nicked circular DNA by Nt.BspQI and T4 ligase); Lane 3-4: ligation and proteinase digestion products of circular nucleosomal arrays constructed from 500 ng of pBR322 DNA and 50 nM histone octamer via Route 1 (Lane 3) or Route 2 (Lane 4). Electrophoretic separation was carried out in 1.2 % w/v agarose gel with 1.2 µg/ml chloroquine at 25 °C for 12 h at 2 volts/cm.

Figure 3-12. Quantitative analysis and determination of the supercoiling levels of DNA topoisomer distributions produced via Route 1 or Route 2. The best-fit Gaussian curve was obtained from the relative intensities of the gel bands shown in Figure 3-11, Lane 2 (A), Lane 3 (B) and Lane 4 (C) (the unligated products have been excluded from our analysis).

It can be observed that nucleosomal arrays are successfully constructed on circular plasmid DNA using both experimental routes. However, the obtained topoisomer distributions vary based on different methods used. Less nucleosomes are formed on open-circular DNA compared to that formed on highly-negative supercoiled DNA, which may be due to the preferential binding affinity of core histones to negatively supercoiled form of DNA as described by Patterson *et al.*²⁹.

On the other hand, formation of nucleosomes on supercoiled DNA results in a wide distribution as can be seen in Lane 4 of Figure 3-11 and Figure 3-12C, which make it difficult to be analyzed and quantified. As a consequence, Route 1 were primarily used in most cases during our following investigations.

Furthermore, additional nucleosome concentration-dependent experiments have been conducted to systematically investigate the effect of nucleosome formation on circular DNA topological properties. As shown in Figure 3-13A, completely relaxed pBR322 DNA was run in Lane 2 as topological standard with zero superhelical turn. A series of nucleosome assembly reactions were carried out via Route 1 varying the concentration of nucleosome core histones. With increasing nucleosome concentration, it can be observed that the distribution of bands gradually shifted up on the gel. Accordingly, statistical analysis of these gel bands was performed using GeneTools and Origin software, the results of which were combined in Figure 3-13B. Obviously, all of these plasmids associated with nucleosomes were shown to be negatively supercoiled, and most importantly, their superhelical densities are increasing at a rate proportional to the molar ratio of nucleosome cores to DNA.

As is known, each nucleosome core particle consists of about 146 base pairs of DNA. In this case, even the concentration of histone octamer reach 120 nM (molar ratio of nucleosome cores to DNA bases is $\sim 1:400$ bp) in our studies, the nucleosomes are still far from saturated occupation of the entire DNA molecule. However, as can be seen in the Lane 3-5 of Figure 3-13A, with the increasing concentration of nucleosome core proteins, the yields of re-ligated products decreased distinctly. This may be due to the physically blocking of nick site by nucleosome particles, and thus hinders the approach of nicking enzymes in correct

geometry. As a consequence, for accurate determination of DNA superhelical turns induced by histone-DNA interaction, the concentration of histone octamer should be kept in a reasonable range (~60 nM) in the following investigations.

Figure 3-13. Electrophoretic analysis of DNA supercoiling changes generated by the formation of nucleosome core particles with increasing concentration of histone octamer. (A) Lane 1: 1 kb DNA ladder; Lane 2: 300 ng of completely relaxed pBR322 DNA (ligation of nicked circular DNA by Nt.BspQI and T4 ligase); Lane 3-5: ligation and proteinase digestion products of circular nucleosomal arrays constructed from 500 ng open-circular pBR322 DNA and 60 nM (Lane 3), 90 nM (Lane 4), or 120 nM (Lane 5) histone octamer. Electrophoretic separation was carried out in 1.2 % w/v agarose gel with 1.2 µg/ml chloroquine at 25 °C for 12 h at 2 volts/cm. (B) Correlations between the level of

DNA supercoiling generated by the formation of nucleosome core particles and the concentration of histone octamer.

The conformation of nucleosome core structure are known to be influenced by various environmental factors. Polyamines are a group of ubiquitous small basic molecules that mostly present in eukaryotic cells, and play significant roles in the formation of nucleosome core particles. Recent *in-vitro* investigations suggest that these aliphatic polycations may help stabilize chromatin structures and participate in DNA aggregation process³⁰⁻³⁵. In our following studies, the topological variation of circular chromosomal DNA induced by spermidine (one of the polyamine molecules with three amino groups) has been systematically examined by electrophoretic mobility shift assays. Nucleosomes were constituted on the open-circular plasmid DNA by stepwise salt dilution procedure (as previously described in Figure 3-9, Route 1). Ligation of these circular DNA were performed in the absence or presence of various concentrations of spermidine. The obtained covalently-closed DNA were treated by proteinase and purification kit before loading to the chloroquine-containing agarose gel, results of which were shown in Figure 3-14A. As described in the figure legend, the thermal distribution of relaxed DNA was given in Lane 2 as DNA topoisomer standard. It has been shown in Figure 3-14A Lane 3 to Lane 7 that, in the absence or presence of increasing concentration of spermidine, the degree of DNA supercoiling increases firstly reaching its peak at the spermidine concentration of 5 mM but dramatically decrease when the concentration keep rising to 10 mM. Similar observations were found when putrescine or spermine was used instead of spermidine in our previous studies. Quantitative analysis of these topoisomer distributions in Lane 3 to Lane 7

was provided in Figure 3-14B (detailed information described in the previous sections).

Figure 3-14. Electrophoretic analysis of DNA supercoiling changes generated by the formation of nucleosome core particles with increasing concentration of spermidine. (A) Lane 1: 1 kb DNA ladder; Lane 2: 300 ng of completely relaxed pBR322 DNA (ligation of nicked circular DNA by Nt.BspQI and T4 ligase); Lane 3-7: ligation and proteinase digestion products of circular nucleosomal arrays constructed from open-circular pBR322 DNA and 100 nM histone octamer in the absence (Lane 3) or presence of 1 mM (Lane 4), 2 mM (Lane 5), 5 mM (Lane 6), or 10 mM (Lane 7) spermidine. Electrophoretic separation was carried out in 1.2 % w/v agarose gel with 1.2 μ g/ml chloroquine at 25 $^{\circ}$ C for 12 h at 2 volts/cm. (B)

Correlations between the level of DNA supercoiling generated by the formation of nucleosome core particles and the concentration of spermidine.

These remarkable findings encourage us to re-examine and further investigate the role of polyamines on the alteration of nucleosome topological structures. It can be conjectured that the physical interactions between the positively charged groups on these polyamines and the negatively charged DNA backbone may have an effect on the local geometry of nucleosome-wrapped DNA, and further result in an supercoiling change on the entire nucleosomal arrays. Our experimental results suggest that the nucleosomes formed on circular DNA can be stabilized or undergo some kind of conformational change in the presence of certain concentration of spermidine, whereas high levels of spermidine lead to the dissociation of nucleosome core particles. The reduction of negative supercoiling at high concentration of polyamines may be attributed to an unwinding of the left-handed turns of DNA through ionic interactions. As a consequence, our following studies will focus on the variations of DNA supercoiling and their associations with nucleosome cores and linker histones.

3.3.3 Determination of DNA supercoiling generated by the action of histone H1

It has been discussed in our previous sections that the formation of nucleosome cores generates additional negative supercoils into circular DNA molecules and thus greatly affects their topological properties. The degrees of supercoiling have been analyzed and statistically determined in our studies by means of agarose gel electrophoresis. Furthermore, we have investigated the

influences of positively charged polyamines on nucleosome conformation using our systems. All these works provide us detailed observations and insights into the correlation between DNA topological properties and the formation of nucleosome core particles.

Besides the nucleosome core histones, linker histones that present in almost all eukaryotes also play essential roles in the formation of chromatin filament structures. Histone H1 is one of the commonly studied linker histones, and was believed to bind the nucleosomal DNA at its entry and exit sites, resulting in an entire chromosome particle³⁶⁻³⁹. Other than this cooperative binding model of linker histone on chromosome, the lysine-rich terminal segments of histone H1 were proved to bind strongly to naked DNA in its minor groove⁴⁰⁻⁴³. In addition, Ivanchenko *et al.* reported in 1997 their findings that histone H1 has a preference for binding the DNA with higher superhelicity⁴⁴. However, the physiological significance of these two types of binding in chromatin packaging remain elusive nowadays. It is our speculation that both these two binding actions of linker histone may introduce local superhelical tension into DNA molecules. In addition, accumulation of these supercoils may further change the topological properties of entire circular DNA. In order to investigate the linker histone's binding effects on the changes in nucleosome-free DNA supercoiling, a series of experiments have been methodically designed and carefully conducted in our following studies. The proposed model of non-specific interactions between histone H1 and circular DNA has been shown in Figure 3-15, in which the linker histones exhibit similar actions as nucleosome cores, introducing global writhes into the covalently closed DNA molecule.

Figure 3-15. Schematic illustration of the topological changes occurring in relaxed circular DNA by non-specific binding of linker histone H1.

Our experimental routes were accordingly designed and illustrated in Figure 3-16. One of the commonly used plasmids, pBR322, served as DNA substrates in our following studies. In Route 1 (Figure 3-16A), the negatively supercoiled pBR322 (Structure 1 in Figure 3-16A), which contains 4361 base pairs in length, was first treated by DNA nicking endonuclease Nt. BspQI. This enzyme hydrolyzes one strand of duplex DNA at a specific site and relieves the superhelical tension within the supercoiled DNA. The obtained open-circular DNA (Structure 2 in Figure 3-16A) was then incubated with recombinant human histone H1⁰ in the presence of NaCl at 37 °C for a certain period of time. After that, the nick site on each histone H1-DNA complexes (Structure 3 in Figure 3-16A) was sealed by T4 DNA ligase with the help of ATP. As a result, the supercoils generated by the binding action of linker histone H1 was constrained in the covalently closed circular DNA (Structure 4 in Figure 3-16A). In order to quantitatively determine the levels of DNA supercoiling, these DNA-histone complexes were then treated by proteinase K at 50 °C, followed by purification using QIAquick Purification Kit (Structure 5 in Figure 3-16A).

Different from Route 1, negatively supercoiled plasmid DNA was used directly as starting material in Route 2 (Figure 3-16B). Topoisomerase-mediated DNA relaxation method has been widely used to study DNA unwinding effect of intercalators⁴⁵⁻⁴⁸. It was our expectation that similar methods could be utilized to examine the binding effect of histones. However, inhibitory effect of histones on DNA topoisomerase I was observed and reported by Richter and colleagues⁴⁹. As such, a nick-ligation reaction performed by DNA nicking endonuclease and DNA ligase was then proposed in our studies instead of topoisomerase-catalyzed relaxation reaction. Although it has been demonstrated histone H1 may also cause the inhibition of T4 DNA ligase⁵⁰, the unligated DNA product can be separated distinctly by gel electrophoresis, without affecting the results of our analysis.

In the next experimental route, the mixtures of untreated pBR322 (Structure 1 in Figure 3-16B) and certain amount of human histone H1⁰ were incubated at the same conditions. After histone H1-DNA complexes have formed (Structure 2 in Figure 3-16B), the nicking endonuclease Nt. BspQI was used to induce a single-stranded break, and as a result, release the negative supercoils in each histone H1-bound DNA molecules (Structure 3 in Figure 3-16B). Enzymatic ligation reaction was then carried out using excess amount of T4 DNA ligase (Structure 4 in Figure 3-16B). Proteinase K has been subsequently used to remove the histone proteins, giving a closed circular DNA (Structure 5 in Figure 3-16B).

Figure 3-16. Schematic illustration of our designed experimental route for determining circular DNA supercoiling changes generated by the binding of linker histone H1. (A) Binding of linker histone H1 to open-circular DNA (Route 1). (B) Binding of linker histone H1 to negatively supercoiled DNA (Route 2).

Gel electrophoresis was used in our following studies for quantitative analysis of the supercoiling levels of these circular DNA. As shown in Figure 3-17, the DNA topoisomers can be separated in chloroquine-containing agarose gel. Relaxed circular pBR322 DNA, as topoisomer standard, was loaded in Lane 2, while the supercoiled DNA obtained via Route 1 and Route 2 were run in Lane 3 and Lane 4, respectively. It can be seen obviously that the distribution of topoisomers in Lane 3 shift towards the top of the gel compared to the relaxed topoisomers in Lane 2, which suggests that additional negative supercoils were generated indeed into the circular DNA through the non-specific binding of linker histone H1. However, the broad distribution of topoisomers shown in Lane 4 was

further proved to be negatively supercoiled DNA (data not shown). This unexpected result may be due to the binding of histone H1 to the nicking endonuclease recognition site hinders the enzyme from approaching its substrates and catalyzing its reaction. In addition, this could explain the relative low yield of T4 ligase-catalyzed ligation reaction in Route 1 (as seen in Lane 3 of Figure 3-17).

Figure 3-17. Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 via Route 1 and Route 2. Lane 1: 1 kb DNA ladder; Lane 2: 300 ng of completely relaxed pBR322 DNA (ligation of nicked circular DNA by Nt.BspQI and T4 ligase); Lane 3-4: ligation and proteinase digestion products of circular nucleosomal arrays constructed from 500 ng of pBR322 DNA and 2 μ M histone H1⁰ (the molar ratio of DNA to histone is 1:120) via Route 1 (Lane 3) or Route 2 (Lane 4). Electrophoretic separation was carried out in 1.2 % w/v agarose gel with 1.2 μ g/ml chloroquine at 25 °C for 12 h at 2 volts/cm.

To further investigate the correlation between the superhelical turns generated by linker histone binding and the amount of linker histone H1, concentration-dependent experiments were carried out via Route 1 (described in Figure 3-16) accordingly. Various concentrations of human recombinant histone H1⁰ were incubated with 500 ng of nicked pBR322 DNA in the presence of 50 mM NaCl. After 30 minutes, these circular DNA were re-ligated followed by proteinase digestion and gel electrophoretic analysis. As shown in Figure 3-18A, the topoisomer distributions gradually shift from their relaxed states to their negative supercoiled forms with the increasing concentration of histone H1. The best-fit Gaussian curves of these distribution provide statistical proofs for our experimental outcomes. The relationship between the level of DNA supercoiling and the concentration of histone H1 has been plotted in Figure 3-28B. When histone H1 concentration comes to 2 μ M, the corresponding molar ratio of DNA to histone H1 is 1:120. In this case, the binding of each histone H1 molecule to naked DNA generates about -0.02 superhelical turns into the entire circular DNA. This coarse value, although highly dependent on a number of environmental factors, could still provide us a rough estimate on the DNA supercoiling effects of linker histones.

Figure 3-18. Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 to naked pBR322 DNA with increasing concentration of histone H1. (A) Lane 1: 1 kb DNA ladder; Lane 2: 300 ng of completely relaxed pBR322 DNA (ligation of nicked circular DNA by Nt.BspQI and T4 ligase); Lane 3-5: ligation and proteinase digestion products of DNA-histone H1 complexes from 500 ng open-circular pBR322 DNA and 0.5 μM (Lane 3), 1 μM (Lane 4), or 2 μM (Lane 5) histone H1⁰. Electrophoretic separation was carried out in 0.8 % w/v agarose gel with 1.0 $\mu\text{g/ml}$ chloroquine at 25 $^{\circ}\text{C}$ for 12 h at 2 volts/cm. (B) Correlations between the level of DNA supercoiling generated on naked pBR322 DNA and the concentration of histone H1.

In order to further confirm our findings, similar experiments were conducted using pUC19 as DNA substrate. Different from pBR322, the pUC19 molecule is a smaller size DNA circle with 2,686 base pairs in length, which could in theory help narrow the thermal distribution of DNA topoisomers. Accordingly, formation of histone H1-DNA complex was investigated here under the same conditions. The binding effects of linker histone H1 on the supercoiling of pUC19 DNA were examined in our following electrophoretic studies (Figure 3-19A). In comparison with the result of pBR322 experiments, the discrete bands that observed in each lane were much less as expected. Similarly, the correlation between the mean value of each Gaussian curve and the concentration of histone H1 was plotted and shown in Figure 3-19B. Consequently, the number of supercoils generated per histone H1 molecule can be calculated as -0.02 (-1.79 superhelical turns/72 histone H1 molecules), which is the same as the number obtained in our pBR322 experiments. These topological binding effects of histone H1 have been taken into consideration in our further investigations on the interactions between linker histones and nucleosome cores.

Figure 3-19. Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 to naked pUC19 DNA with increasing concentration of histone H1. (A) Lane 1: 1 kb DNA ladder; Lane 2: 300 ng of completely relaxed pUC19 DNA (ligation of nicked circular DNA by Nt.BspQI and T4 ligase); Lane 3-5: ligation and proteinase digestion products of DNA-histone H1 complexes from 500 ng open-circular pUC19 DNA and 0.5 μM (Lane 3), 1 μM (Lane 4), or 2 μM (Lane 5) histone H1⁰. Electrophoretic separation was carried out in 1.2 % w/v agarose gel with 1.2 $\mu\text{g/ml}$ chloroquine at 25 $^{\circ}\text{C}$ for 12 h at 2 volts/cm. (B) Correlations between the level of DNA supercoiling generated on naked pUC19 DNA and the concentration of histone H1.

As reported by Natour *et al.* in 2007, the stabilities of the linker histone-DNA complexes are greatly affected by the ionic strength in solution⁵¹. It is therefore our expectation that the salt-dependent binding of linker histone to DNA may have impacts on the superhelicity of circular DNA as well. Accordingly, experiments have been conducted here to study the correlation between the NaCl concentration and the histone H1-induced superhelical turns that constrained in the circular DNA. In this experiment, the same nick-ligation route were used as discussed previously. After the enzymatic removal of proteins, the histone-free plasmids was analyzed by gel electrophoresis in the end, result of which was shown in Figure 3-20A. The fully relaxed pBR322 was prepared and run in the second lane as topoisomer standard, while lanes 3-8 show the histone H1 binding products in the presence of 0 mM, 10 mM, 25 mM, 50 mM, 100 mM or 250 mM NaCl, respectively. Furthermore, the relation between salt concentration and the degree of DNA supercoiling was then obtained and plotted in Figure 3-20B through the quantification and statistical analysis of the electrophoretic result. It has been verified in our previous studies that NaCl in itself has little influence on DNA supercoiling. Thus, according to our experimental outcomes, these salt-dependent differential effects on DNA superhelicity were most likely caused by the salt-mediated formation or dissociation of the DNA-histone H1 complex. In this case, the results shown in Figure 3-20 suggest that histone H1 binds tightly to naked DNA at NaCl concentrations between 50 mM and 100 mM. Reduction in histone H1 binding affinity was observed when salt concentration is either too high or too low.

Figure 3-20. Electrophoretic analysis of the effect of salt on the level of DNA supercoiling generated by linker histone H1. Lane 1: 1 kb DNA ladder; Lane 2: 300 ng of completely relaxed pBR322 DNA (ligation of nicked circular DNA by Nt.BspQI and T4 ligase); Lane 3: ligation of histone H1-nicked DNA complexes by T4 DNA ligase in the absence of NaCl (concentration of histone H1 is 0.5 μ M); Lane 4-8: ligation of histone H1-nicked DNA complexes by T4 DNA ligase in the presence of 10 mM (Lane 4), 25 mM (Lane 5), 50 mM (Lane 6), 100 mM (Lane 7), or 250 mM (Lane 8) of NaCl (concentration of histone H1 is 0.5 μ M). Electrophoretic separation was carried out in 0.8 % w/v agarose gel with 1.0 μ g/ml chloroquine at 25 $^{\circ}$ C for 12 h at 2 volts/cm. (B) Correlations between the level of DNA supercoiling generated by linker histone H1 and the concentration of NaCl.

In conclusion, our electrophoretic studies on the interactions of histone H1 and naked DNA revealed that circular DNA exhibits a detectable linking number change under the action of histone H1. On the basis of our quantitative results, we have made a rough estimate that the linker histone H1 binds to circular DNA varying its superhelicity at about -0.02 turns per molecule. Furthermore, the effect of salt on the formation of DNA-histone H1 complexes has been carefully examined. It is therefore our hope that our studies could provide a better understanding about the topological actions of linker histones. Going one step further, we will discuss in the next section on the role of linker histone during chromatosome formation and its topological effect on chromosomal DNA.

3.3.4 Examination of DNA topological changes in the closed circular nucleosomal arrays by linker histone H1

Linker histones are highly abundant chromatin proteins that present in all multicellular eukaryotes and play essential roles in their chromatin condensation and compaction⁵²⁻⁵⁷. Binding of linker histones direct the compaction of nucleosomal arrays into a folded 30 nm fibre which is known as the second level of chromosomal DNA organization⁵⁸⁻⁶⁰. The solenoidal model was proposed by Finch and Klug in 1976, where the linker DNA between adjacent nucleosomes is bent by the action of linker histone and coil around a central cavity to form the 30-nm chromatin fibre⁶¹. Recent investigations of chromatosome crystal structures provide us valuable information on the linker histone binding mechanism and further elucidate their roles in the process of 30-nm-fibre formation⁶²⁻⁶⁵. It has been recognized in 1980's that linker histones bind to the dyad of the nucleosome core particle and protect ~168 bp of DNA from micrococcal nuclease digestion⁶⁶⁻⁶⁷,

which suggests the linker histones associate exactly with nucleosomal DNA at its entry and exit site of the nucleosome. Additionally, it has been demonstrated that a linker histone-dependent stem-like motif is formed at the entry-exit site, and reduce the linker DNA entry-exit angle of consecutive nucleosomes⁶⁸. Although the model of nucleosome arrangement still remains controversial to this day, all the information collected from previous studies could help us develop our understanding of the structural and functional roles of linker histones in nucleosomal DNA packaging.

It is our speculation that the linker histone upon binding to the nucleosome core particle, may generate localized torsional stress into linker DNA, which as a consequence facilitates the formation of the supercoiled 30-nm chromatin fibre. In order to verify such hypothesis, experiments have been accordingly designed and conducted in our following studies. Circular plasmid DNA was used as substrate in our experiments for (i) construction of nucleosomal arrays, and (ii) constraining and quantifying the DNA superhelical turns generated by the binding of linker histones.

As illustrated in Figure 3-21, nucleosomal arrays are first constructed on the open-circular DNA (one single strand break on each DNA molecule) following the nucleosome assembly protocol as described in Section 3.2.2. Then, linker histones bind to the nucleosome cores, forming the chromatosomes on circular plasmid. After reaching equilibrium, the single-stranded DNA breaks are ligated by T4 DNA ligase to give covalently closed circular DNA molecules. A parallel study, at the same time, should be conducted without the addition of linker histone proteins. In the end, both the experimental products (Structure 8 in Figure 3-21)

and the control groups (Structure 5 in Figure 3-21) have to be treated by proteinase K before electrophoretic analysis.

Figure 3-21. Schematic illustration of our designed experimental route for determining the changes in chromosomal DNA supercoiling induced by linker histone H1 binding to nucleosomes.

Accordingly, electrophoretic experiments were conducted to verify our proposed topological effect of linker histones as shown in Figure 3-22. Complete relaxation of pBR322 DNA was performed by mixing the supercoiled DNA and the nicking endonuclease (Nt. BspQI) followed by ligation reaction using T4 DNA ligase. The obtained circular DNA was run in the chloroquine-containing (1.2 $\mu\text{g/ml}$) agarose gel as topoisomer standard (Lane 2 in Figure 3-22A). The final products of control group without incubation with linker histone H1 (Structure 5 in Figure 3-21) was obtained via the experimental routes described before and given in Lane 3. Due to the inhibitory effect of linker histones on DNA ligase, some of unligated DNA still remain in the final mixture of products and can be seen in the

gel as the slowest migrating band (similar to that reported by Sivolob *et al.*⁶⁹). These open-circular DNA bands have been excluded from our following quantitative analysis of topoisomer distribution. According to our previous studies as discussed in Section 3.3.2 and Section 3.3.3, the concentrations of NaCl and spermidine in the reaction mixtures were 50 mM and 5 mM respectively. The changes in DNA supercoiling induced by the binding of linker histone H1 to pre-formed nucleosomal arrays were shown in Lane 3-5 of Figure 3-22A. It can be observed that increasing concentration of histone H1 gives a shift of topoisomer bands towards its relaxed form. Since either histone octamer or linker histone alone is known to generate local positive supercoils into the linker region of covalently closed circular DNA (as discussed in Section 3.3.2 and Section 3.3.3).

Our findings here indicate that the binding of linker histone H1 to nucleosome core particles introduces additional negative superhelical stress into the linker DNA, which is subsequently removed by the nicking-ligation process and results in an observation of reduction in negative superhelical turns on gel. This binding effect of histone H1 on DNA supercoiling which is the exact opposite of that produced by nucleosome cores was quantitatively analyzed using the method described in our previous sections. As shown in Figure 3-22B, the average number of superhelical turns of the DNA topoisomers in Lane 3 can be calculated as: $\Delta\mu_1 = 4.1 (\mu_1) - 6.8 (\mu_0) = -2.7$, which suggests that around 2.7 nucleosome core particles were formed on each plasmid DNA molecule. After the binding action of linker histone H1 (the molar ratio of histone octamer to histone H1 was 1:1 in Lane 4 of Figure 3-22A), additional 0.9 ($5.0 (\mu_2) - 4.1 (\mu_1) = 0.9$) negative supercoils were removed from these chromatosome-containing structures. As a result, it can be accordingly obtained that the binding of each histone H1

molecule to the nucleosome core particle may generate roughly -0.3 ($0.9/-2.7 \approx -0.3$) superhelical turns into the linker region of covalently closed circular DNA. This particular effect of linker histones on chromosomal DNA topological properties was first uncovered here and will be further investigated in our following studies.

A

B

Figure 3-22. Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 to the nucleosomal arrays constructed on pBR322 DNA. (A) Lane 1: 1 kb DNA ladder; Lane 2: 500 ng of completely relaxed

pBR322 DNA (ligation of nicked circular DNA by Nt.BspQI and T4 ligase); Lane 3: ligation and proteinase digestion products of circular nucleosomal arrays constructed from 500 ng open-circular pBR322 DNA and 60 nM histone octamer. Lane 4-5: ligation and proteinase digestion products of circular nucleosomal arrays associated with 60 nM (Lane 4, histone octamer/histone H1 molar ratio of 1:1) or 120 nM (Lane 5, histone octamer/histone H1 molar ratio of 1:2) linker histone H1. Electrophoretic separation was carried out in 1.2 % w/v agarose gel with 1.2 µg/ml chloroquine at 25 °C for 12 h at 2 volts/cm. (B) Quantitative analysis and determination of the supercoiling levels of DNA topoisomer distributions in Lane 3-5 of Figure 3-22A (the unligated products have been excluded from our analysis).

In order to further confirm the linker histone binding effect on chromosomal DNA, additional experiments were conducted using a smaller plasmid (pUC19) as DNA substrate. Experimental procedures and the reaction factors involved in these trials were the same as the ones described before. Gel electrophoretic results as well as the quantitative analysis are given in Figure 3-23. Similarly, a reduction in negative superhelicity was observed as the concentration of linker histone H1 increases. Analysis of these topoisomer distributions show that the number of nucleosomes formed on each plasmid is around 1.3 ($\Delta\mu_1 = 3.4 (\mu_1) - 4.7 (\mu_0) = -1.3$), while the additional DNA supercoils generated by the binding of histone H1 (the molar ratio of histone octamer to histone H1 was 1:1 in Lane 4 of Figure 3-23A) is 0.3 ($3.7 (\mu_2) - 3.4 (\mu_1) = 0.3$). In this case, the cooperative binding of each histone H1 molecule to the nucleosome core particle

gives -0.2 ($0.3/-1.3 \approx -0.2$) superhelical turns into the circular arrays of nucleosomes, which is close to the number obtained in our pBR322-based study.

Figure 3-23. Electrophoretic analysis of DNA supercoiling changes generated by the binding of linker histone H1 to the nucleosomal arrays constructed on pUC19 DNA. (A) Lane 1: 1 kb DNA ladder; Lane 2: 500 ng of completely relaxed pUC19 DNA (ligation of nicked circular DNA by Nt.BspQI and T4 ligase); Lane 3: ligation and proteinase digestion products of circular nucleosomal arrays constructed from 500 ng open-circular pUC19 DNA and 60 nM histone octamer.

Lane 4-5: ligation and proteinase digestion products of circular nucleosomal arrays associated with 60 nM (Lane 4, histone octamer/histone H1 molar ratio of 1:1) or 120 nM (Lane 5, histone octamer/histone H1 molar ratio of 1:2) linker histone H1. Electrophoretic separation was carried out in 1.2 % w/v agarose gel with 1.2 µg/ml chloroquine at 25 °C for 12 h at 2 volts/cm. (B) Quantitative analysis and determination of the supercoiling levels of DNA topoisomer distributions in Lane 3-5 of Figure 3-22A (the unligated products have been excluded from our analysis).

Taken together, we demonstrated for the first time that the cooperative binding of linker histone H1 to the nucleosome cores introduces additional negative supercoiling into the linker DNA between chromatosomes formed on covalently closed circular DNA. In addition, these supercoils accumulated in DNA molecules were technically identified in our gel electrophoresis experiments, while the number of superhelical turns generated per chromatosome formation has been measured approximately as -0.2~-0.3 by our quantitative analysis of topoisomer distributions. Most importantly, this surprising action of linker histone H1 has the reverse topological effect compared to that produced by histone octamer or histone H1 alone. It is therefore our hope that our current findings could help facilitate our understanding of linker histone's role and function in the process of nucleosome condensation.

3.4 Conclusion

This chapter discusses our electrophoretic studies on the topological changes in circular nucleosomal DNA. We described our separation of DNA topoisomers on the basis of chloroquine-containing agarose gel, and our

characterization of DNA superhelical turns by fitting the distribution of topoisomer intensities. Quantitative analyses of DNA-histone interactions and their topological effects were performed in covalently closed circular DNA molecules in order to constrain any supercoils generated or released. Firstly, nucleosomal arrays were constructed on certain plasmid DNA, and a series of experimental routes were accordingly designed to determine the number of nucleosome core particles formed. In addition, effects of salts and polyamines on the formation of nucleosomes were subsequently examined.

On the other hand, we investigated the interactions between linker histone H1 and naked circular DNA molecules and found that the binding of histone H1 constrained some detectable negative DNA supercoils in a salt-dependent manner. Furthermore, we reported a rough estimate of the number of superhelical turns stored by each histone H1 molecule in our quantitative analysis.

Additionally, our subsequent studies on the formation of chromatosomes suggest that the cooperative binding of linker histone on the nucleosome core particle generates additional negative supercoils into the linker region of DNA between chromatosomes. As illustrated in the model shown in Figure 3-24, it is our expectation that this linker histone-induced DNA supercoiling provides the torsional stress for chromatin higher-order compaction in eukaryotic cells.

Figure 3-24. Illustration of our proposed model of linker histone-induced nucleosome condensation. Binding of linker histone H1 generates additional negative supercoils into the linker DNA between chromatosomes.

In the following chapter, we will focus on the linker DNA's path on histone octamer for the purpose of disclosing the structural origin of linker histone-induced DNA supercoiling.

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Chapter 4

FRET-based Studies of Linker DNA's Path on Cylindrical Histone Octamer

4.1 Background Information

In the previous chapters, we have verified the existence of negative superhelical tension constrained in the compacted chromosome array structures using AFM (as discussed in Chapter 2). In addition, we have quantitatively determined the variation of DNA supercoils in the nucleosome-containing circular DNA induced by the binding of linker histones using agarose gel electrophoresis (as discussed in Chapter 3). It is therefore our belief that the interactions between linker histones and the nucleosome core particles may generate additional negative supercoils into the linker DNA, and thus facilitate the assembly of chromatin higher-order structure. In order to figure out the structural origin of these supercoils, an in-depth investigation on the linker DNA's orientation has been conducted and discussed in this chapter.

Histone proteins in the eukaryotic cells can be divided into two groups based on their positions relative to nucleosomal structures: core histones (H2A, H2B, H3 and H4) and linker histones (*e.g.* H1, H5)¹⁻⁵. Two copies of each of the four core histone proteins are capable of forming octameric complexes *in vitro* and *in vivo* that resemble “cylinders” in their overall shapes⁶⁻⁸. In the absence of histone H1, 146 base-paired segment of DNA wraps itself around the cylindrical

surfaces of histone octamers for ~1.65-1.67 turns, in which the two types of biomacromolecules are in close contact through electrostatic and van der Waals' interactions⁹⁻¹⁰. When histone H1 is present, on the other hand, binding between this linker histone and nucleosome core particles takes place. This binding facilitates the further wrapping of DNA to histone octamers for extra 0.35 turn, which add up the wrapping of DNA segments around the cylindrical surfaces of histone octamers to 2 full turns^{2, 11} (1.65 turns + 0.35 turn = 2 turns). The interactions between histone octamers and their bound DNA segments were studied extensively in the past⁹⁻¹⁰. However, the orientation of linker DNA's paths on the cylindrical surface of histone octamers in the additional 0.35 turn has not yet been well understood¹². In the following sections, we reported our studies on the orientations of linker DNA's paths on cylindrical histone octamers on the basis of fluorescence resonance energy transfer.

As introduced in Chapter 1, fluorescence resonance energy transfer (FRET) has been developed as a powerful method for measuring the distance between fluorophores on the nm scale¹³⁻¹⁶. Experimentally, FRET efficiency can be determined from the emission spectrum when exciting at a wavelength where only acceptor absorbs. The FRET efficiency (E) can be described as:

$$E = 1 - \frac{I_{DA}}{I_D} \quad (4.1)$$

where I_{DA} and I_D are the total donor fluorescence intensities in presence and absence of acceptor molecule, respectively. Due to the dipole-dipole coupling mechanism, the FRET efficiency is inversely proportional to the sixth power of the donor-to-acceptor distance as expressed by the following equation:

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} \quad (4.2)$$

where R is the distance between donor and acceptor fluorophores, and R_0 is the characteristic distance (Förster distance) at which the energy transfer efficiency is 50 %. The correlation between energy transfer efficiency and the distance (R) is shown in Figure 4-1.

Figure 4-1. FRET-based distance determination on the nanometre scale (adapted from "A Practical Guide to Single Molecule FRET", by Rahul Roy *et al.*, 2013¹³). FRET Efficiency as a function of inter-dye distance (R) for a $R_0 = 5$ nm.

As a consequence, such FRET-based distance measurement has been used in our following investigations to quantify the conformational variation of the DNA by association with histone proteins. It is therefore our hope that our studies on the orientation of linker DNA can help us better understand the role of linker histones in the process of higher-order DNA compaction in eukaryotic chromosomes.

4.2 Materials and Methods

4.2.1 Oligodeoxyribonucleotides, enzymes, chemicals and equipments

Product(s)	Manufacturer
Oligodeoxyribonucleotides	Sangon Biotech (Shanghai, China)
Fluorophore-labeled oligodeoxyribonucleotides	Bio-Synthesis (Lewisville, US)
100 bp DNA ladder	Thermo Scientific (Singapore)
T4 DNA ligase	New England Biolabs (Singapore)
Nucleosome Assembly Kit	New England Biolabs (Singapore)
Histone H1 ⁰ (human, recombinant)	New England Biolabs (Singapore)
QIAquick PCR Purification Kit	Qiagen (Singapore)
QIAquick Gel Extraction Kit	Qiagen (Singapore)
Acrylamide, bis-acrylamide	Sigma-Aldrich (Singapore)
TEMED, Ammonium persulfate	Bio-Rad Laboratories (Singapore)
Ethidium bromide	Bio-Rad Laboratories (Singapore)
TBE, Tris	1st Base (Singapore)
Eppendorf tubes, Pipette tips	Greiner (Singapore)
Atomic force microscopy	Veeco, (Santa Barbara, US)
AFM probes	Bruker (Camarillo, US)
Gel documentation system	Syngene (Cambridge, UK)
Cary eclipse fluorescence spectrophotometer	Varian (Mulgrave, Australia)

Most all the chemicals used in this research were listed above otherwise were obtained from Sigma-Aldrich with analytical grade or molecular biology grade.

4.2.2 Nucleosome assembly

Nucleosomes were prepared from the Epimark Nucleosome Assembly Kit (New England Biolabs #E5350S) following the dilution assembly protocol for 50 pmol DNA substrate. A 20 μ l mixture containing 5 μ g DNA, 2 M NaCl, 5 μ M Histone H2A/H2B Dimer and 2.5 μ M Histone H3.1/H4 Tetramer (dosages of core histones should be adjusted according to the required molar ratio of nucleosome to DNA) was incubated at room temperature (25 $^{\circ}$ C) for 30 minutes. Subsequently, 7 μ l dilution buffer (10 mM Tris, pH 8.0) was added into the mixture to bring the sodium concentration to 1.48 M. After incubation at room temperature (25 $^{\circ}$ C) for 30 minutes, the mixture was diluted by adding 13 μ l dilution buffer (10 mM Tris, pH 8.0) to bring the sodium concentration to 1.0 M. Afterwards, the mixture was incubated at room temperature (25 $^{\circ}$ C) for additional 30 minutes and further diluted by adding 27 μ l dilution buffer (10 mM Tris, pH 8.0) to bring the sodium concentration to 0.6 M. Finally, the mixture was incubated at room temperature (25 $^{\circ}$ C) for 30 minutes again and diluted by adding 93 μ l dilution buffer (10 mM Tris, pH 8.0) to bring the final concentration of NaCl to 0.25 M. The obtained mixture was further incubated at room temperature (25 $^{\circ}$ C) for 30 minutes and stored at 4 $^{\circ}$ C.

4.2.3 Histone H1-DNA binding assay

A 20 μ l aqueous solution containing 5 μ g DNA, 50 mM NaCl (concentration of NaCl should be adjusted according to experimental requirements)

and 2.5 μ M recombinant human histone H1⁰ (New England Biolabs #M2501S, dosage of histone H1 should be adjusted according to the required molar ratio of histone H1 to DNA) was incubated at 37 °C for 30 minutes.

4.2.4 Chromatosome assembly

Nucleosomes were prepared from the Epimark Nucleosome Assembly Kit (New England Biolabs #E5350S) following the dilution assembly protocol as described before. A 20 μ l solution containing nucleosomal arrays (nucleosome concentration of 2.5 μ M), 200 mM NaCl and 2.5 μ M recombinant human histone H1⁰ (New England Biolabs #M2501S, dosage of histone H1 should be adjusted according to the required molar ratio of histone H1 to DNA) was incubated at 37 °C for 30 minutes.

4.2.5 Annealing of oligonucleotides

Oligonucleotides that were purchased from Sigma-Aldrich or Bio-Synthesis were received in dry form. Respective amount of deionized water was added to each oligonucleotide to prepare a stock solution of 100 μ M. A 30 μ l solution containing 10 μ M of each oligonucleotide, 10 mM Tris-HCl (pH 8.0) and 50 mM NaCl was prepared and incubated at 95 °C for 3 minutes and was allowed to cool to 25 °C over a period of 45 minutes. The obtained duplex DNA was stored at 4 °C.

4.2.6 Ligation of duplex DNA fragments

A 30 μ l solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol, 10 μ M of each DNA fragment and

50 U of T4 DNA ligase (New England Biolabs #M0202S) was incubated at 16 °C for 1 hour. The obtained long duplex DNA was then purified by QIAquick PCR Purification Kit (Qiagen), and further examined by polyacrylamide gel electrophoresis.

4.2.7 Agarose gel electrophoresis

The agarose gel slab was prepared by heating an 1.2 % (w/v) agarose suspension in the absence or presence of 1.5 µg/ml chloroquine (concentration of chloroquine should be adjusted according to experimental requirements) in a boiling water bath to melt the agarose, and allowing the gel to solidify at room temperature for 1 hour. A 20 µl of DNA sample was mixed with gel loading buffer (New England Biolabs #B7021S) and loaded into the well of agarose gel. Then, the gel was run at 2 volts/cm in 0.5 × TBE buffer for 1 hour. After gel electrophoresis, the gels were stained with 1.5 nM of ethidium bromide in 0.5 × TBE buffer for 30 minutes, and analyzed using G:BOX iChemi gel documentation system (Syngene).

4.2.8 Polyacrylamide gel electrophoresis

A mixture of 142.5 g acrylamide (Sigma-Aldrich) and 7.5 g bis-acrylamide (N,N'-Methylenebisacrylamide, Sigma-Aldrich) was dissolved in 1 L of 1 × TBE buffer to give a 15 % (w/v) non-denaturing gel stock solution. The non-denaturing polyacrylamide gel was prepared by mixing 50 ml stock solution, 500 µl of freshly prepared 10 % (w/v) ammonium persulphate solution and 50 µl TEMED and allowing the gel to solidify between the plates assembled with spacers at room temperature for 30 minutes. A 20 µl of DNA sample was mixed with gel loading

buffer (New England Biolabs #B7021S) and loaded into the well of polyacrylamide gel. Then, the gel was run at 10 volts/cm in $1 \times$ TBE buffer for 5 hours. After gel electrophoresis, the gels were stained with 1.5 nM of ethidium bromide in $1 \times$ TBE buffer for 30 minutes, and analyzed using G:BOX iChemi gel documentation system (Syngene).

4.2.9 AFM imaging of DNA and DNA-histone complexes

AFM images were obtained in Tapping ModeTM on a MultimodeTM AFM (Veeco) in connection with a Nanoscope VTM controller. Antimony (n) doped Si cantilevers with nominal spring constants between 20 and 80 N/m were selected. Scan frequency was 2.0 Hz per line and the modulation amplitude was in a nanometre range. All DNA samples are deposited on the APS-modified mica and visualized in air at room temperature. The AFM images were further analyzed by NanoScope Analysis Version 1.50 program.

4.2.10 Fluorescence measurements

Fluorophore-labeled oligonucleotides were purchased from Bio-synthesis (Lewisville, TX). Fluorescence resonance energy transfer (FRET) experiments were performed using Cy3 as the donor, and rCy5 as the acceptor, with an excitation wavelength of 500 nm, and emission wavelength of ~560 nm and ~660 nm for Cy3 and Cy5, respectively. The DNA-containing sample was diluted to 100 μ l and transferred to a 1 cm path length quartz cuvette. Fluorescence measurements were carried out on a Cary Eclipse Fluorescence Spectrometer (Varian) at 25 °C with excitation at 500 nm or 600 nm. Emission spectra were

recorded for every nanometre from 530 to 830 nm. Excitation and emission slits were set at 10 nm.

4.3 Results and Discussion

4.3.1 Construction of DNA-based FRET systems for determination of nanoscale distances

As previously discussed, fluorescence resonance energy transfer occurs between two appropriately positioned fluorophores with overlapping emission and excitation spectra. Over the past decades, numerous organic molecules have been used as FRET-pairs in studies of molecular structures and dynamics¹⁷⁻²⁰. Among the fluorescent dyes, cyanine dyes Cy3 and Cy5 are frequently used in biological systems²¹⁻²⁵ due to their high photostabilities and quantum yields, and will be therefore utilized in our investigations of nucleosomal DNA structural and topological properties. Normally, the fluorophore with high quantum yield is required to maximize the FRET signal and minimize the noise background. Furthermore, the Cy3-Cy5 pair has a relatively large Förster radius (R_0) of 54 Å²⁶⁻²⁷, which is suitable for measuring long distances in our following studies on long-range molecular interactions. In addition, as illustrated in Figure 4-2, both these two cyanine dyes can be post-synthetically attached to the oligonucleotide at specific locations via a flexible linker, allowing FRET to be used to measure DNA conformational changes.

Figure 4-2. Schematic illustration of fluorescence resonance energy transfer (FRET) occurring between Cy3 and Cy5 fluorescent moieties covalently linked to the phosphate groups on DNA.

DNA oligonucleotides, because of their well-defined three-dimensional structures, were widely used in biological systems to collect accurate information in FRET-based studies^{19, 24, 28-29}. However, recent studies have reported that several nucleotides, especially guanosine, exhibits strong quenching effect towards the attached fluorophore³⁰⁻³². In this case, the distance between the fluorophores may not be reliably determined with the consideration of intra-molecular quenching effect by the nearby nucleotides. In order to overcome such problem, on one hand, flexible spacers were introduced between DNA and the fluorophore to mitigate unwanted interference, while on the other hand, a group of DNA duplexes were constructed and used as the distance standards in our studies.

Our design of the duplex DNA FRET standards are shown in Figure 4-3. The Cy3 and Cy5 fluorescent dyes are incorporated (i) at the 5'- or 3'-terminal of the oligonucleotide respectively (as shown in Figure 4-3A), or (ii) internally into the sugar-phosphate backbone (as shown in Figure 4-3B). The detailed nucleotide sequences of these oligonucleotides are listed in Table 4-1. All these oligonucleotides were carefully checked for potential hairpin, homo-dimer and

hetero-dimer formation with OligoAnalyzer (provided by Integrated DNA Technologies, Inc.; available at: <https://sg.idtdna.com/calc/analyzer>).

In the first group of standard duplex DNA in which fluorophores located at both DNA termini, the high GC contents of these oligonucleotides (an extreme GC content of 100% in Oligo S1) are necessary for maintaining their structural stabilities. The donor and acceptor dyes are separated by a certain number of nucleic acid base pairs (8-22 base pairs). After these duplexes formed, the distances between the dyes are estimated to be approximately 2.6 (Oligo S1) to 7.0 (Oligo S8) nanometers (estimation of the distances obtained from RasMol program). On the other hand, the other group of DNA standards were subsequently designed in which the dyes were incorporated between the hydroxyl group on one base and the phosphate group on the adjacent base via phosphoroamidite chemistry instead of post synthesis. These two sets of fluorophore-modified DNA duplexes were used as FRET standards and tested in our following studies.

Figure 4-3. Illustration of our newly designed duplex DNA standards with Cy3

and Cy5 fluorophores labeled (A) terminally, or (B) internally.

Table 4-1. Sequence of oligonucleotides used for the construction of our newly designed duplex DNA standards.

Name of duplex DNA	Name of oligonucleotide	Sequence of oligonucleotide (5' to 3')
DNA S1	Oligo S1A	[Cy3]GGGCGGCC[Cy5]
	Oligo S1B	GGCCGCCC
DNA S2	Oligo S2A	[Cy3]CGGGCGGCCA[Cy5]
	Oligo S2B	TGGCCGCCCCG
DNA S3	Oligo S3A	[Cy3]ACGGGCGGCCAC[Cy5]
	Oligo S3B	GTGGCCGCCCCGT
DNA S4	Oligo S4A	[Cy3]GACGGGCGGCCACT[Cy5]
	Oligo S4B	AGTGGCCGCCCCGTC
DNA S5	Oligo S5A	[Cy3]CGACGGGCGGCCACTC[Cy5]
	Oligo S5B	GAGTGGCCGCCCCGTCG
DNA S6	Oligo S6A	[Cy3]GCGACGGGCGGCCACTCA[Cy5]
	Oligo S6B	TGAGTGGCCGCCCCGTCGC
DNA S7	Oligo S7A	[Cy3]AGCGACGGGCGGCCACTCAG[Cy5]
	Oligo S7B	CTGAGTGGCCGCCCCGTCGCT
DNA S8	Oligo S8A	[Cy3]CAGCGACGGGCGGCCACTCAGT[Cy5]
	Oligo S8B	ACTGAGTGGCCGCCCCGTCGCTG

DNA S9	Oligo S9A	GACAGGATCCCTATA[Cy3]CGGAATTCT GAACAGAAGCTTCAGG
	Oligo S9B	CCTGAAGCTTCTGTT[Cy5]CAGAATTCCG TATAGGGATCCTGTC
DNA S10	Oligo S10A	GACAGGATCCCTATA[Cy3]CGCGGAATT CTATTGAACAGAAGCTTCAGG
	Oligo S10B	CCTGAAGCTTCTGTT[Cy5]CAATAGAATT CCGCGTATAGGGATCCTGTC

Cy3 and Cy5 fluorophores were indicated as [Cy3] and [Cy5] respectively.

Formation of these duplex DNA was achieved by annealing complementary single stranded DNA oligonucleotides (experimental details provided in Section 4.2.5). In addition, these obtained duplex structures were verified by restriction enzyme digestion and polyacrylamide gel electrophoresis. The fluorescence properties of these double-stranded DNA molecules were examined using a Cary Eclipse Fluorescence Spectrophotometer operating in the fluorescence mode. Firstly, the excitation and emission spectra of oligonucleotides modified with Cy3 or Cy5 alone were obtained respectively and shown in Figure 4-4. The maximum (peak) wavelengths are lie within the range of previously reported values. In our subsequent FRET measurements, the wavelength of exciting light was set to 500 nm, with the aim of (i) maximizing the emission intensity, and (ii) simultaneously reducing the leakage of excitation light into the emission channel.

Figure 4-4. Fluorescence absorption (blue) and emission (red) spectra of (A) the Cy3-labeled oligonucleotide (Oligo S9A) and (B) the Cy5-labeled oligonucleotide (Oligo S9B). Fluorescence examinations were carried out at 3 μ M oligonucleotide concentration.

Fluorescence studies on our Cy3/Cy5-labeled duplex DNA were subsequently performed in aqueous solution in the presence of 50 mM NaCl and 10 mM Tris-HCl (pH 8.0) at 25 $^{\circ}$ C. The emission spectra were obtained using the same fluorescence spectrophotometer with excitation wavelength of 500 nm. As shown in Figure 4-5, with reducing the length of DNA between Cy3 and Cy5 fluorophores, significant decrease in donor emission accompanied by an increase of acceptor emission is observed, which indicates the occurrence of FRET in these duplex DNA standards.

Figure 4-5. Fluorescence spectra of our newly designed duplex DNA standards with Cy3 and Cy5 fluorophores labeled (A) terminally, or (B) internally excited at the wavelength of 500 nm.

According to these obtained emission spectra, experimentally-determined distances (R_e) between the donor and acceptor fluorophores can be calculated on the basis of formula 4.1 and formula 4.2 as described in the Section 4.1. On the other hand, computational-determined distances (R_c) were estimated by measuring

the distance between two phosphate groups in the crystal structure of a double-stranded B-form DNA fragment (obtained from the RCSB, PDB ID 3BSE³³) using the program RasMol. For comparison, all these distances were collected and listed in Table 4-2.

Table 4-2. Comparison of the experimentally-determined distances (R_e) between fluorophores and the computational-determined distances (R_c) between the corresponding fluorophore-linked phosphate groups.

DNA	Experimentally-determined distance R_e (nm)	Computational-determined distance R_c (nm)*	Nucleotide bases between fluorophores (bp)
DNA S1	3.96	2.64	8
DNA S2	4.29	2.97	10
DNA S3	4.71	3.90	12
DNA S4	5.02	4.73	14
DNA S5	5.30	5.17	16
DNA S6	5.71	5.88	18
DNA S7	6.05	6.31	20
DNA S8	6.68	6.98	22
DNA S9	4.55	3.69	10
DNA S10	5.27	5.16	15

* Computational-determined distances were estimated by measuring the distance between two phosphate groups in the crystal structure of a double-stranded B-form DNA fragment using the program RasMol.

On the whole there is reasonable accord between our experimental results (R_e) and the computational values (R_c) within the reliable region of 2.7-8.1 nm (with limits of $0.5 R_0$ and $1.5 R_0$), while the slight inconsistency when the donor-acceptor distance exceeds the R_0 value by 50 percent, from our understanding, may be due to (i) the three-carbon linker arms to which the fluorophores are attached, (ii) fluorescence quenching effects by adjacent guanosine nucleotides, and (iii) the relative orientation of the dipoles. In this case, the standard curve showing the correlation between the experimentally-determined distance and the computational-determined distance were plotted in Figure 4-6. It is therefore our belief that more reliable distance values can be obtained by interpolation of the resulting R_e values in this standard curve in our subsequent FRET-based distance measurements.

Figure 4-6. The standard curve showing the correlation between experimentally-determined distances (R_e) and computational-determined distances (R_c).

On the basis of the high FRET efficiencies of Cy3-Cy5 fluorophore pairs, we have designed and prepared a series of duplex DNA molecules with the fluorophore dyes modified either terminally or internally. Quantitative analysis of the fluorescence properties of these DNA-based systems has been carefully conducted. In addition, we investigated the correlation between the obtained

experimental distance (R_e) as well as the computational distance (R_c), and constructed the standard curve to which future assays can be compared. In our following studies, the molecular interactions between DNA and histone proteins will be systematically examined by using our DNA-based FRET systems.

4.3.2 Fluorescence studies of histone H1-induced inter-molecular DNA aggregation

As previously discussed, fluorescence resonance energy transfer has been used as a valuable technique for studying molecular level interactions due to its high sensitivity to small changes in distance. Typically, measurable FRET occurs only when donor and acceptor molecules are in close proximity. This property can be therefore utilized in our studies to investigate DNA conformational changes and characterize DNA-histone interactions. We described in Chapter 2 our AFM visualization of histone H1-induced DNA aggregation under physiological conditions. And also in Chapter 3, we demonstrated that histone H1 can binds to naked DNA and generate supercoils into covalently closed circular DNA molecules. Here in this chapter, intermolecular interactions between DNA and linker histone H1 will be further examined using our FRET-based systems. In addition, effects of environmental factors on the formation of DNA-histone H1 disordered complexes will be systematically studied.

Over the past decades, several studies have found that histone H1, at sufficiently high concentrations, binds tightly to naked DNA and forms specific structures with DNA molecules³⁴⁻³⁶. It is our speculation that linker histone H1 may facilitate the intermolecular aggregation of double stranded DNA fragments in a non-specific manner as illustrated in Figure 4-7. Therefore in our following

FRET-based studies, the donor and acceptor cyanine dyes were incorporated internally into a 206 bp-long DNA duplex spaced 166 bases apart (~55 nm apart along a DNA duplex). In this case, the FRET signal will only be detected when the two fluorophores are brought into close proximity by the action of histone H1.

Figure 4-7. Illustration of our proposed model of the histone H1-induced intermolecular aggregation of fluorophore-labeled DNA fragments.

As shown in Figure 4-8, the 206-bp-long double-stranded DNA fragment was constructed by a ligation reaction of five pieces of short DNA duplexes with different cohesive ends. This reaction was performed by the T4 DNA ligase at 16 °C for 2 hours. Each piece of short duplex DNA was prepared by annealing of two partially complementary synthetic oligonucleotides at the same molar concentration (detailed nucleotide sequences given in Table 4-3). Annealing reaction was carried out by mixing the oligonucleotides in annealing buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA) and decreasing the temperature from 95 °C to 25 °C with an increment of 0.1 K/s. The obtained 206 bp DNA fragment was purified using QIAquick PCR Purification Kit, and then examined on a non-denaturing polyacrylamide gel.

Figure 4-8. Illustration of our newly designed 206 base pairs fluorophore-labeled DNA duplex prepared from ten pieces of single-stranded oligonucleotides.

Table 4-3. Sequence of oligonucleotides used for the construction of our newly designed duplex DNA standards.

Name of oligonucleotide	Sequence of oligonucleotide (5' to 3')
Oligo 206A	CTTATGTGATGGACCCTATA[Cy3]CGCGGCCGCCCTGG
Oligo 206B	AGAATCCCGGTGCCGAGCCGCTCAATTGGTCGTAGACAGCT
Oligo 206C	CTAGCACCGCTTAAACGCACGTACGCGCTGTCCCCCGCGTTT
Oligo 206D	TAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTG
Oligo 206E	TCAGATATATACATCCTGGCATGTATTGAACAGCGACCTTGCCGGTGC
Oligo 206F	GCACCGGCAAGGTCGCTGTT[Cy5]CAATACATGCCAGGATG
Oligo 206G	TATATATCTGACACGTGCCTGGAGACTAGGGAGTAATC
Oligo 206H	CCCTTGGCGGTTAAAACGCGGGGGACAGCGCGTACGTGCGT
Oligo 206I	TTAAGCGGTGCTAGAGCTGTCTACGACCAATTGAGCGGCCTC

Cy3 and Cy5 fluorophores were indicated as [Cy3] and [Cy5] respectively.

As indicated in Table 4-3, Cy3 and Cy5 modification are incorporated into the Oligo 206A and Oligo 206F respectively via phosphoroamidite chemistry. After gel extraction and purification, the obtained 206 base pairs donor-acceptor labelled DNA substrate was applied in our following FRET studies. In order to verify the structural integrity of such DNA substrate, fluorescence measurements of each cyanine dye were first carried out with the excitation wavelength set at 500 nm (excitation wavelength of Cy3) and 600 nm (excitation wavelength of Cy5), respectively. As shown in Figure 4-9, fluorescence intensities of Cy3 and Cy5 are approximately proportional to their quantum yields (Cy3 quantum yield: 0.15, Cy5 quantum yield: 0.28), which indicates the molar ratio of donor to acceptor is around 1:1 as expected.

Figure 4-9. Fluorescence examination of our newly designed 206 base pairs fluorophore-labeled DNA duplex excited at 500 nm (blue), or 600 nm (red).

The fluorophore-labeled duplex DNA was then incubated with increasing concentrations of recombinant human histone H1⁰ at 25 °C in the presence of 50 mM NaCl. As shown in Figure 4-10, a decrease in donor emission accompanied by an increase in acceptor emission was observed with increasing amount of linker

histone H1 (a 1:2, 1:4 or 1:8 molar ratio of DNA to histone H1). According to the formulas 4.1 and 4.2 for FRET efficiency and the standard curve obtained in our last section, the average distance between these donor and acceptor dyes can be estimated to be 4.7 nm (DNA/histone H1 molar ratio of 1:8), which suggests that the fluorophores can be brought much closer upon binding of the linker histone H1. Each histone H1 molecule, however, has been proved to interact with only 10-20 base pairs of duplex DNA³⁷⁻³⁸. In this case, in spite of having a ~100 amino acid C-terminal domain, histone H1 may not bind and fold such a 206-bp-long DNA in a head-to-tail fashion to generate intramolecular FRET signal. Therefore, it is our expectation that these increases in the overall FRET efficiency should be attributed to the energy transfer caused by the intermolecular complexes of histone H1 proteins with DNA molecules.

Figure 4-10. FRET studies on the interaction between duplex DNA and linker histone H1. The mixtures of 0.1 μM of 206 base pairs fluorophore-labeled DNA duplex and 50 mM NaCl were incubated in the absence (blue), or presence of 0.2 μM (green), 0.4 μM (red), or 0.8 μM (purple) histone H1 at 37 $^{\circ}\text{C}$ for 2 hours.

In order to further confirm the formation of DNA intermolecular aggregates, the 206-bp duplex DNA was incubated with different amount of histone H1

followed by agarose gel electrophoresis. As shown in Figure 4-11, increasing the histone H1/DNA molar ratio up to 1:8 results in a smearing of the bands, which indicates the formation of protein-DNA disordered complexes at high stoichiometry of linker histone H1. This observation is consistent with similar results reported by other researchers³⁹.

Figure 4-11. Conformation of the histone H1-induced DNA duplex aggregation. Lane 1: 100 bp DNA ladder; Lane 2: 1.5 pmol of 206 base pairs fluorophore-labeled DNA duplex; Lane 3-7: DNA-histone H1 aggregates formed after incubation of 1.5 pmol DNA duplex and 3 pmol (Lane 3), 4.5 pmol (Lane 4), 6 pmol (Lane 5), 9 pmol (Lane 6), or 12 pmol (Lane 7) of linker histone H1 in the presence of 50 mM NaCl. Electrophoresis was performed in 1.5 % w/v agarose gel in $0.5 \times$ TBE buffer at 25 °C for 1 h at 2 volts/cm.

Histone H1 has been reported to bind naked DNA in a strongly salt-dependent manner^{38, 40-41}. Thus, our subsequent FRET-based study focused on the effects of NaCl concentration on the formation of histone H1-DNA aggregates. A mixture containing the 206 base pair DNA duplex and histone H1 in a molar ratio of 1:4 was incubated in the absence and presence of 25 mM, 50 mM, 100 mM, 250 mM and 500 mM NaCl, respectively, for 30 minutes at 37 °C. Fluorescence emission spectra were measured at excitation wavelength of 500 nm with Cary Eclipse Fluorescence Spectrophotometer and shown in Figure 4-12A. In addition,

the obtained FRET efficiency values (E) were summarized and plotted in Figure 4-12B. It can be observed that increasing the concentration of NaCl from up to 100 mM produces an increase in FRET between the fluorophores. These results suggest that the histone H1-DNA aggregates formed and maintained their stability at physiological salt levels (~50-100 mM NaCl). However, beyond this concentration there is a dramatic decrease in FRET efficiency, which indicates the dissociation of aggregates in a high ionic strength solution. These experimental outcomes provide us a better understanding of the salt effect on the histone H1 induced intermolecular association of duplex DNA fragments.

Figure 4-12. FRET studies on the effect of salt concentrations on the formation of histone H1-DNA aggregates. (A) The mixtures of 0.2 μ M DNA duplex and 0.8 μ M histone H1 were incubated in the absence (red), or presence of 25 mM (green), 50 mM (purple), 100 mM (tan), 250 mM (light blue), or 500 mM (orange) NaCl at

37 °C for 2 hours. The control group without addition of linker histone H1 was plotted in blue line. (B) Correlation between FRET efficiency and the concentration of NaCl.

In this section, we have designed and constructed a 206 base pairs duplex DNA substrate double-labeled with Cy3 and Cy5 fluorophores, which was subsequently used in our studies of histone H1 and DNA interactions. At higher excesses of histone H1, DNA molecules were found to undergo intermolecular aggregation into disordered histone-DNA complexes. The cyanine dyes located in close proximity in such complexes, which results in the occurrence of FRET between donor and acceptor fluorophores. In addition, we have examined the effects of ionic strength on the formation of histone H1-DNA aggregates. In our following studies, FRET-based DNA substrates will be used to investigate the path of linker DNA on cylindrical histone octamer with or without the binding of linker histone H1. Based on the findings discussed in this section, the concentration of histone proteins as well as other environmental factors, will be carefully controlled so as to avoid the intermolecular interactions between linker histones and DNA molecules.

4.3.3 FRET-based determination of the path of linker DNA under physiological conditions

In eukaryotic cells, nucleosome is the basic unit of DNA packaging, which consists of 146-147 base pairs of DNA wrapped around a core of eight histone proteins in 1.67 left-handed superhelical turns^{1, 10, 42-43}. These nucleosome core particles are connected by varying lengths (10-80 bp) of linker DNA, generating

the "bead-on-string" appearance of nucleosomal arrays. In addition, the linker histone binds and locks approximately 15-30 base pairs of linker DNA on the nucleosome core surface, resulting in two full turns of DNA^{2, 11, 44-49}. However, due to the lack of crystal structure of chromatosomes, the path of linker DNA on the cylindrical surface of histone octamer in the additional 0.35 turn still remains unclear¹². It is therefore our hope that the following FRET-based *in vitro* studies could help improve our understanding of the path of linker DNA and its roles in chromatin organization.

In order to investigate the path of the DNA entering and leaving the nucleosome core, several fluorophore-labeled DNA duplexes were accordingly designed and prepared based on the procedures described before (as shown in Figure 4-13 and Table 4-4). As reported by Widom et. al, nucleosomes are preferentially positioned over the "601 nucleosome binding element" at single nucleotide resolution⁵⁰, the nucleotide sequence of which is given in Figure 4-13A. This 601 element sequence was inserted into our newly-designed DNA duplexes with 9 to 14 flanking base pairs for precise placement of the nucleosome core. As illustrated in Figure 4-13B, the Cy3 fluorophore is labeled at one end of this duplex DNA, while the Cy5 is placed at the other end. During nucleosome assembly, the 146 base-paired high-affinity binding element is expected to be wrapped around the surface of histone octamer for 1.65 negative superhelical turns, leaving the 9 to 14 base pairs free linker DNA at both exit and entry sites of the nucleosome. In this case, the solenoidal wrapping of DNA on the nucleosome core particle can bring the DNA ends close together as shown in Figure 4-13C. The spatial distance between the fluorophores labeled at both ends of DNA can be therefore calculated based on the measurement of FRET efficiency.

Figure 4-13. Illustration of our FRET-based determination of linker DNA's path on cylindrical histone octamer. (A) Nucleotide sequence of the 601 nucleosome binding element reported by Lowary and Widom⁵⁰. (B) Our newly designed fluorophore-labeled 601-element-containing DNA duplexes. (C) Illustration of the fluorophore-labeled DNA duplex wrapped 1.65 turns around the histone octamer, leaving two linker DNA strands at the nucleosome entry/exit site.

Table 4-4. Sequence of oligonucleotides used for the construction of our fluorophore-labeled 601-element-containing DNA duplexes.

Name of oligonucleotide	Sequence of oligonucleotide (5' to 3')
Oligo 164A	[Cy3]GAAAATTCCCTGGAGAATCCCGGTGCCGAGGCC GCTCAATTGGTCG
Oligo 164C	AGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGAT ATATACATCCTGGCAATTTTG[Cy5]
Oligo 164D	CAAAATTGCCAGGATGTATATATCTGACACGTGCCTG GAGACTAGG
Oligo 164F	TAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACC GGGATTCTCCAGGGAATTTTC
Oligo 166A	[Cy3]CGAAAATTCCCTGGAGAATCCCGGTGCCGAGGC CGCTCAATTGGTCG
Oligo 166C	AGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGAT ATATACATCCTGGCAATTTTGC[Cy5]
Oligo 166D	GCAAAATTGCCAGGATGTATATATCTGACACGTGCCT GGAGACTAGG
Oligo 166F	TAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACC GGGATTCTCCAGGGAATTTTCG
Oligo 168A	[Cy3]GCGAAAATTCCCTGGAGAATCCCGGTGCCGAGG CCGCTCAATTGGTCG
Oligo 168C	AGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGAT ATATACATCCTGGCAATTTTGCT[Cy5]
Oligo 168D	AGCAAAATTGCCAGGATGTATATATCTGACACGTGCC TGGAGACTAGG
Oligo 168F	TAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACC GGGATTCTCCAGGGAATTTTCGC
Oligo 170A	[Cy3]GGCGAAAATTCCCTGGAGAATCCCGGTGCCGAG GCCGCTCAATTGGTCG
Oligo 170C	AGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGAT ATATACATCCTGGCAATTTTGCTG[Cy5]
Oligo 170D	CAGCAAAATTGCCAGGATGTATATATCTGACACGTGC CTGGAGACTAGG
Oligo 170F	TAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACC GGGATTCTCCAGGGAATTTTCGCC
Oligo 172A	[Cy3]TGGCGAAAATTCCCTGGAGAATCCCGGTGCCGA GGCCGCTCAATTGGTCG

Oligo 172C	AGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGAT ATATACATCCTGGCAATTTTGCTGG[Cy5]
Oligo 172D	CCAGCAAAATTGCCAGGATGTATATATCTGACACGTG CCTGGAGACTAGG
Oligo 172F	TAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACC GGGATTCTCCAGGGAATTTTCGCCA
Oligo 174A	[Cy3]GTGGCGAAAATTCCCTGGAGAATCCCGGTGCCG AGGCCGCTCAATTGGTCG
Oligo 174C	AGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGAT ATATACATCCTGGCAATTTTGCTGGC[Cy5]
Oligo 174D	GCCAGCAAAATTGCCAGGATGTATATATCTGACACGT GCCTGGAGACTAGG
Oligo 174F	TAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACC GGGATTCTCCAGGGAATTTTCGCCAC
Oligo B	TAGACAGCTCTAGCACCGCTTAAACGCACGTACGCGC TGTCCCCCGCGTTTAAACCGCCA
Oligo E	GAGTAATCCCCTTGCGCGTTAAAACGCGGGGGACAGC GCGTACGTGCGTTTAAAGCGGTGC

Cy3 and Cy5 fluorophores were indicated as [Cy3] and [Cy5] respectively.

A series of 601-element-containing DNA duplexes carrying different lengths of flanking region were accordingly prepared by annealing and ligating of six pieces of oligonucleotides. Detailed experimental procedures were described in Section 4.2.5 and Section 4.2.6. As shown in Figure 4-14A, the major band in Lane 5 (indicated by the arrow) of the expected size (~166 bp) is considered to be our desired duplex DNA product, whereas other bands are unligated small-size DNA fragments. This product band was subsequently excised while the DNA was extracted from the gel slices by QIAEX II Gel Extraction Kit and re-examined in Lane 6.

Figure 4-14. Conformation of our newly prepared fluorophore-labeled 601-element-containing DNA duplex. (A) Electrophoretic examination of the 166 base pairs DNA duplex constructed from three short DNA fragments. Lane 1: 100 bp DNA ladder; Lane 2-4: short DNA fragments with cohesive ends prepared by annealing of Oligo 166A and 166F (Lane 2), Oligo B and E (Lane 3) or Oligo 166C and 166D (Lane 4); Lane 5: ligation of three short DNA fragments by T4 DNA ligase; Lane 6: polyacrylamide gel extraction products of the DNA in the major band of Lane 5. Electrophoresis was performed in 15 % w/v non-denaturing polyacrylamide gel in $1 \times$ TBE buffer at 25°C for 5 h at 10 volts/cm. (B) Fluorescence examination of the 166 base pairs fluorophore-labeled DNA duplex excited at 500 nm (blue), or 600 nm (red).

After gel isolation, the desired DNA duplexes were collected and incubated with excess amount of histone core proteins in the presence of 2 M NaCl at 25°C for 30 min. To generate nucleosomes, the salt concentration was stepwisely diluted to the final 50 mM with dilution buffer (10 mM Tris-HCl pH 8.0). Fluorescence

measurements of these single nucleosome core particles were then carried out on a Cary Eclipse Fluorescence Spectrophotometer at room temperature. As shown in Figure 4-15, in the presence of core histones, DNA wrapped around the histone octamer, bringing the two fluorophores into close proximity to yield FRET signals at the wavelength of ~660 nm. However, there is no detectable FRET signal in the control group in which the DNA duplexes were incubated in the absence of histone octamer (shown in Figure 4-14B). In addition, the highest energy transfer efficiency (63.6 %) was observed when the 166 base pairs duplex DNA was used as substrate in which the linker DNA on both the entry and exit sides are approximately 10 bp long. In this case, according to Equation 4.1 and 4.2 given in Section 4.1, the spatial distance between these two fluorophores can be calculated as 4.32 nm. On the basis of our understanding, the slight differences in the distance between fluorophores in these nucleosomal DNA with different lengths of linker region may be due to the combined action of (i) the different directions of the linker DNA's paths leaving the nucleosome core surface, (ii) the orientation of the fluorophore within the DNA double helix, and (iii) the fluorescent-quenching effect of the nearby amino acids on core histones.

Figure 4-15. FRET studies on the distance between the ends of linker DNA in the histone H1-deficient mononucleosome particle. (A) Fluorescence spectra of 0.4 μ M 164 bp (blue), 166 bp (red), 168 bp (green), 170 bp (purple), 172 bp (light blue), or 174 bp (orange) fluorophore-labeled duplex DNA wrapped around excess amount of histone octamer in the presence of 250 mM NaCl. (B) Correlation between FRET efficiency and the length of duplex DNA.

In order to study the effect of temperature on the linker DNA's path, a series of experiments were conducted using the 166 base pairs fluorophore-labeled DNA duplex as substrate. FRET measurements were performed after 2 hours incubation of the nucleosomes under the corresponding temperatures. It can be seen in Figure 4-16 that the overall fluorescence intensity decreases with increase in temperature, which is consistent with the experimental observations reported previously and was demonstrated to be due to the increased molecular collisions

between fluorophores and other molecules⁵¹⁻⁵⁴. However, the ratio of donor (the peak at ~566 nm) and acceptor (the peak at ~666 nm) fluorescence intensities remains approximately constant with increasing temperature. This constant FRET efficiency indicates that the variation in temperature may not lead to a significant change in the spatial path of linker DNA. As a consequence, our subsequent nucleosome assembly experiments will be performed at 37 °C in order to mimic the environment and conditions in living cells.

Figure 4-16. FRET studies on the effect of temperature on the conformation of nucleosomal DNA. Nucleosomes were constructed from 166 base pairs fluorophore-labeled DNA duplex and excess amount of core histones. Fluorescence measurements were performed in the solution containing 0.3 μ M nucleosomes and 250 mM NaCl at 10 °C (blue), 20 °C (red), 30 °C (green), or 40 °C (purple).

The concentration of salt is considered one of the most crucial factors that determine and regulate chromatin compaction *in vivo*. Here in this study, FRET has been used to examine the stability of nucleosome core particle and the conformation of nucleosomal DNA in the presence of NaCl. As shown in Figure 4-17, no significant differences were observed with increasing the concentration of

NaCl, which suggests that the nucleosomal DNA and the linker DNA flanking the nucleosome core may maintain their overall conformation over a wide range of salt concentrations.

Figure 4-17. FRET studies on the effect of salt concentrations on the conformation of nucleosomal DNA. Nucleosomes were constructed from 166 base pairs fluorophore-labeled DNA duplex and excess amount of core histones. Fluorescence measurements were performed in the solution containing 0.3 μ M nucleosomes and 25 mM (blue), 50 mM (red), 100 mM (green), or 250 mM (purple) NaCl at 37 $^{\circ}$ C.

Polyamines are highly abundant in eukaryotic cells and essential for cell growth and differentiation⁵⁵⁻⁶⁰. Among them, spermidine which contain three amino groups have known to play significant roles in the formation of nucleosome and also in the regulation of chromatin higher order structures⁶⁰⁻⁶¹. It is our expectation that the interactions between these positively charged organic molecules and the negatively charged DNA backbones may alter the linker DNA's conformation, and further result in detectable variations in FRET efficiencies of our systems.

The single nucleosome particle was prepared accordingly in which the 166 base pairs Cy3/Cy5-labeled DNA wraps around a histone octamer core and leaves the ~10 bp free entering and exiting linker DNA segments. Fluorescence measurements for these nucleosome particles were performed in the presence of various concentrations of spermidine. As shown in Figure 4-18, spermidine profoundly affects the conformation of linker DNA and remarkably increases the distance between the fluorophores labeled on the ends of DNA duplex. According to Equation 4.1 and 4.2, the approximate distance between the ends of linker DNA at the entry site and the exit site can be calculated as 4.72 nm in the absence of spermidine but 8.38 nm in the presence of 9 mM spermidine. The concentration of polyamines in eukaryotic cells are reported to be around 1-20 mM in previous studies⁶²⁻⁶⁴. Therefore, our experimental results may suggest that the presence of certain concentration of cellular polyamines is essential for proper organization and accurately positioning of nucleosomal DNA *in vivo*.

Figure 4-18. FRET studies on the effect of spermidine on the conformation of nucleosomal DNA. Nucleosomes were constructed from 166 base pairs fluorophore-labeled DNA duplex and excess amount of core histones. Fluorescence measurements were performed in the solution containing 0.2 μ M

nucleosomes in the absence (blue) or presence of 4 mM (red), 5 mM (green), 6 mM (purple), 7 mM (aqua), 8 mM (orange), or 9 mM (light blue) spermidine at 37 °C.

In this section, a series of fluorophore-labeled DNA duplexes have been designed to characterize the path of linker DNA extending beyond the surface of the nucleosome core particle. With varying the lengths of linker DNA, the spatial distances between two DNA termini were systematically determined based on the measurements of their FRET efficiencies. The ends of linker DNA reach their closest point when the nucleosomal DNA is flanked by two ~10 bp long linker regions, where the donor and acceptor cyanine dyes are located 166 base pair apart. In addition, we have investigated the conformational changes of linker DNA under different physical and chemical environments in our FRET-based studies. All the information gathered throughout these experiments provided us with important insights into the path of entering and exiting linker DNA, and is of great importance for our further studies on the roles of linker histone in chromatin compaction.

4.3.4 FRET-based investigation of the topological roles of histone H1 in nucleosomal DNA condensation

Eukaryotic chromosomal DNA undergoes several levels of compaction to form the chromosome. Recent evidence revealed that the interactions between linker DNA and linker histone play essential roles in such DNA condensation process⁶⁵⁻⁶⁹. Linker DNA is the double-stranded DNA connecting nucleosome cores in eukaryotic chromatin, which varies from 10 to 80 base pairs in length.

Linker histone, on one hand, associates with the negatively charged linker DNA through electrostatic interactions, and at the same time, binds to nucleosomal DNA near the dyad axis of nucleosome core particle^{2, 47, 70-73}. However, due to the difficulties in obtaining crystal structure of the chromatosome (nucleosome core particle in complex with linker histone), the association mode of the linker histone with nucleosomal DNA and the fundamental roles of linker histones in nucleosome condensation still remain controversial and require additional clarification¹².

In this work we focused on the alteration of the linker DNA's path by the binding of histone H1 to the nucleosome core particle. Previous DNA footprinting studies suggest that the globular domain of linker histone is located over the nucleosomal dyad at the entry/exit region of DNA, and locks additional ~10 bp linker DNA on each side to the surface of cylindrical nucleosome core^{11, 47, 72, 74-76}. As illustrated in Figure 4-19, it is our speculation that the precise positioning of linker histone H1 may alter the orientation of the ~10 bp linker DNA's path, and thus produces a right-handed solenoidal wrap of DNA segment around the cylindrical histone octamer. Topologically, these positive supercoils constrained inside the chromatosome will consequently result in compensatory negative supercoiling of DNA in the linker regions of nucleosomal arrays, and further facilitate nucleosomal DNA condensation.

Figure 4-19. Our proposed model of histone H1-induced alteration in the linker DNA path and the positive supercoils constrained within the chromatosome.

In order to verify our speculation, the linker DNA's orientation was carefully examined in our following studies using the fluorophore-labeled nucleosomal systems. Similar to the procedures described in the previous sections, the cyanine-modified duplex DNA was prepared by annealing and ligating equimolar amounts of six oligonucleotide fragments. After gel isolation, the obtained 166 base pairs DNA duplex was used as the substrate for nucleosome assembly reaction in excess of core histones. Once the nucleosome core particle formed, the donor and acceptor fluorophores were brought into close proximity, thus resulting in the appearance of detectable FRET signals. Subsequently, additional FRET measurements were performed on the histone H1-bound chromatosome particles in order to quantitatively determine the conformational alteration of linker DNA. As shown in Figure 3-20A, nucleosome core particles were incubated with increasing concentration of histone H1⁰ under physiological conditions, followed by fluorescence examination.

Figure 4-20. FRET studies on the distance between the ends of linker DNA in the mononucleosome and the monochromatosome particles. (A) Fluorescence spectra of 0.2 μM fluorophore-labeled duplex DNA wrapped around excess amount of histone octamer and incubated in buffer solution (250 mM NaCl, 5 mM spermidine, pH 8.0) for 30 min at 37 $^{\circ}\text{C}$ in the absence (blue) or presence of 0.2 μM (red), or 0.4 μM (green) histone H1. (B) Effect of histone H1 on the path of linker DNA.

A dose-response effect was observed in which increasing amount of histone H1 correlated with remarkably increased FRET signal. These experimental results suggest that the entering and exiting linker DNA segments are brought much closer with the help of histone H1 as shown in Figure 4-21. In addition, the spatial distances between two fluorophores located at the termini of linker DNA

segments can be calculated according to their FRET efficiencies. When histone H1 is presented in sufficient amount, this distance is approximately 4.41 nm which is much shorter than the distance obtained in the histone H1-deficient nucleosome (5.10 nm) as given in Figure 20B. Furthermore, compared to the previously reported nucleosomal DNA superhelical pitch of 2.59 nm⁹, the distance between the ends of linker DNA in chromatosome is even shorter than twice the superhelical pitch. This demonstrates that the paths of the linker DNA segments locked on cylindrical histone octamer may not maintain their original left-handed orientations; instead tend to bend and change their directions of motions towards each other, giving a right-handed solenoidal wrap of DNA on nucleosome surface.

Figure 4-21. Our FRET-based spatial distance measurements between the entering and exiting linker DNA segments in the histone H1-deficient mononucleosome and the histone H1-bound monochromatosome particles.

As demonstrated in our previous studies, histone H1 alone, at relatively high concentrations, can bind to naked DNA and lead to the formation of disordered histone-DNA aggregates under particular conditions. It is therefore necessary to exclude the possibility of FRET signal emanating from the inter-nucleosomal interaction. As such, additional AFM experiments were carried out here to confirm the inexistence of the unspecific histone-DNA aggregates in the presence of histone H1. Nucleosome core particles were accordingly prepared and

incubated with or without histone H1 under the same conditions as that of our former studies. These obtained samples were fixed onto mica surface and visualized using a Multimode NanoScope V system operated in tapping mode in air. As seen from Figure 4-22, no obvious aggregates were found either in the absence or presence of linker histone H1 in our experiments, which suggests that the FRET signals observed in our previous investigations are indeed due to the conformational change of two linker DNA segments.

Figure 4-22. AFM conformation of the inexistence of the unspecific histone-DNA inter-nucleosomal aggregates.

In addition, some of the recent studies reported that the formation of 30-nm chromatin fibre can be affected by the ionic strength of the surrounding environment⁷⁷⁻⁷⁸. In order to verify whether physiological concentration of NaCl has an impact on the path of the linker DNA entering and exiting the nucleosome, additional experiments were carried out using our FRET-based monochromatosome system. Monochromatosome particles were prepared using the 166 base pairs fluorophore-labeled DNA as substrate according to the procedure described before, and subsequently incubated in the buffer solutions containing different concentrations (from 25 mM to 250 mM) of NaCl for 30 min at 37 °C. According to the results obtained in our FRET studies (shown in Figure

4-23), NaCl do not show any significant effect on the path of linker DNA in the histone H1-bound monochromatosome particle.

Figure 4-23. FRET studies on the effect of salt concentrations on the path of linker DNA in the histone H1-bound monochromatosome particle. Chromatosomes were constructed from 0.2 μ M fluorophore-labeled duplex DNA wrapped around excess amount of histone octamer and incubated with 0.4 μ M histone H1 in buffer solutions containing 5 mM spermidine and 25 mM (blue), 50 mM (red), 100 mM (green), or 250 mM (purple) NaCl for 30 min at 37 °C.

Combining all together, we get the conclusion that linker histone H1 may act as a lock for the entering and exiting linker DNA, holding these two ~10 bp linker DNA segments oriented towards each other and arranged in a right-handed helical conformation on the surface of cylindrical histone octamer. Due to this topological action of histone H1, positive DNA supercoils are expected to be constrained within the chromatosome structure. In order to compensate these locally constrained supercoils, theoretically, an overall DNA negative supercoiling will be generated into the linker region between chromatosomes, and may be responsible for chromatin condensation in eukaryotic cells

4.4 Conclusion

In this chapter we have discussed the design and development of our DNA-based FRET systems for studying the interactions between DNA and histone proteins. A series of duplex DNA fragments double-labeled with Cy3 and Cy5 fluorophores were accordingly constructed, and their FRET efficiencies were systematically examined. In the presence of an excess quantity of linker histone H1 and particular concentration of NaCl, double-stranded DNA molecules were found to interact with each other and form intermolecular aggregates in solution, which is proved by the occurrence of energy transfer between donor and acceptor fluorophores in such disordered DNA-histone complexes.

Unlike the nonspecific interactions of histone H1 with DNA, the core histones assemble on duplex DNA to form nucleosome core particles. In this case, the spatial distance between the ends of two linker DNA segments extending beyond the surface of histone octamer was determined by the measurement of FRET efficiency. It has been demonstrated in our studies that the ends of linker DNA reach their closest point when the nucleosomal DNA is flanked by two 10 bp long linker regions. Furthermore, effects of several environmental factors on the linker DNA conformation were carefully studied.

In addition to the nucleosome core particles, monochromatosomes were constructed in our investigations by adding linker histone H1 to the pre-formed nucleosome cores. Our FRET results showed that upon the binding of histone H1, two linker DNA segments orient themselves towards each other, which is opposite to the directions of nucleosomal DNA paths in the original 1.67 turns. Topologically, this right-handed solenoidal wrap of linker DNA on the cylindrical

histone octamer will generate local positive supercoiling within the chromatosome particle, and will be theoretically compensated with negative supercoils in chromatin fibres. It is therefore our belief that this linker histone-induced DNA supercoiling is likely to serve as the triggering event for eukaryotic DNA condensation.

4.5 References

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List of Publications

1. Zhang, H.; Guo, J. J.; Li, D. W.; Ng, M. T. T.; Lee, J. Y.; Lv, B.; Ng, C. W.; Lee, S.; Shao, F. W.; Li, T. H., Confirmation of quinolone-induced formation of gyrase-DNA conjugates using AFM. *Bioorg Med Chem Lett* **2013**, 23 (16), 4622-4626.
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