Structural, dynamics and site selectivity investigations of platinum anticancer drug-nucleosome interaction

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**Structural, Dynamics and Site Selectivity
Investigations of Platinum Anticancer Drug-
Nucleosome Interaction**

WU BIN

SCHOOL OF BIOLOGICAL SCIENCES

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Abbreviations

AAS	atomic absorption spectrophotometer
ATM	ataxia-telangiectasia mutated
bp	base pair
CarPt	(SP-4-2)-diammine[1,1-cyclobutanedicarboxylato(2--)-O,O']platinum
CHD1	chromodomain helicase DNA binding protein 1
CisPt	cis-diammine-dichloroplatinum(II)
CIA	chloroform/iso-amyl alcohol
DACH	trans-1,2-diamminocyclohexane-dichloro-platinum (II)
DNA	deoxyribonucleic acid
DSB	double-strand breakage
DTT	1,4-Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoresis migration shift assay
Exo III	exonuclease III
FACT	facilitates chromatin transcription
GSH	glutathione
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
HAT	histone acetyltransferase
HDAC	histone deacetylase
HMG	high mobility group (protein)
HP1	heterochromatin-associated protein-1
HSQC	heteronuclear single quantum coherence
HU	histone unlike (protein)
IPTG	isopropyl-β-D-thiogalactopyranoside
MMTV	mouse mammary tumor virus
MPD	2-methyl-2,4-pentanediol
MRN	Mre11-Rad50-Nbs1
NCP	nucleosome core particle
NER	nucleotide excision repair
NFR	nucleosome free region
NMR	nuclear magnetic resonance
OxPt	oxalate (trans-1,2-diamminocyclohexane)platinum(II)
PAGE	poly-acrylamide gel electrophoresis
PCG	polycomb group complex
PEG	polyethylene glycol
PIC	pre-initiation complex
RNA	ribonucleic acid
SET	methylation catalyzing domain
SHL	Superhelix location
TamPt	triammino-monochloro-platinum (II)
TSS	transcription start site
XPA	xeroderma pigmentosum, complementation group A
XPE	xeroderma pigmentosum, complementation group E

Abstract

The nucleosome is an essential structural and functional feature of eukaryotic chromatin, which allows for hierarchical genomic regulation. Not only are histones heavily involved in genomic events, nucleosomal DNA is found to possess structural characteristics important for genomic function as well. The discovery of chromatin site-selective compounds could allow targeting of specific genes, which coincide with weak points of cancer cells.

The primary pharmacological effect of platinum-based chemotherapeutic agents is mediated through formation of DNA adducts, which interfere with genomic activities and ultimately trigger apoptosis. In spite of their widespread application in the treatment of specific cancers, current Pt drugs elicit severe toxicity and resistance effects. Thousands of Pt compounds have been synthesized and screened in an effort to discover improved anticancer agents, yet the drug design process likely suffers by not considering a defined nuclear target.

We investigated the reaction of cisplatin and oxaliplatin with a nucleosome core particle (NCP) and naked DNA. We discovered that histone octamer association is capable of differentially modulating Pt drug adduct formation at certain sites. Extensive DNA-Pt drug cross-link formation observed at the centre of the NCP suggests that this region of the nucleosome, known to be least accessible to protein factors, may provide a favorable target for the design of improved Pt anticancer agents. Moreover, crystallographic analysis revealed that DNA-Pt drug cross-links can be accommodated in the NCP without large conformational change, providing a starting point for structure-based design.

Mechanistic insight would also aid in the development of improved Pt drug therapies. We found that Pt adducts formed by reaction of cisplatin or oxaliplatin with the nucleosome core inhibit histone octamer-DNA sliding, but do not cause significant alteration of positioning. In fact, adduct formation reinforces positional preferences intrinsic to the DNA sequence. This may indicate that modulation of Pt drug site selectivity by histone octamer association may relate to nucleosome-specific properties of DNA. This sheds light on Pt drug-mediated inhibition of chromatin

remodeling *in vivo* and suggests that adducts can shield their own repair and interfere with genomic activities by directly altering nucleosome dynamics.

Based on our initial results, we explored alternative NCP constructs for structural and drug development studies. We discovered that alterations in DNA sequence can have a profound impact on how well NCP crystals tolerate platinum drug treatment. In combination with a repertoire of DNA footprinting data, high resolution structural analysis of NCP derivatized with compounds differing in leaving/carrier groups is providing unprecedented insight into platinum drug site selectivity and reaction mechanism. Moreover, we were able to obtain well diffracting crystals of an NCP composed of strong positioning TTAA elements flanking the nucleosome centre. A dramatic DNA-dependency of NCP packing in the crystal is observed, which may hold significance for internucleosomal interactions *in vivo*. Analysis of this novel structure is providing insight into the basis for sequence dependent nucleosome positioning.

CHAPTER I

DNA, NUCLEOSOME AND CHROMATIN

1.1 DNA Structure

In the last 56 years since the first paper describing the double helix structure of DNA, its molecular structure and biochemical significance have been determined. During the past five decades, we have also witnessed the establishment of the central dogma of molecular inheritance, the solving of the nucleosome structure, the completion of the Human Genome Project and the beginning of the even more laborious eukaryotic epigenetic studies. DNA is unambiguously proven to provide the genetic instructions for the development and functioning of all known cellular organisms and some of the viruses.

The structure and dynamics of DNA, even in the absence of exogenous factors is highly complex. Besides the three types of well known overall helical organizations, A, B and Z forms (Dickerson et al, 1982), the DNA double helix displays distinct conformational features and sequence-dependent structure. As illustrated in Figure 1.1, DNA presides over three rotational and three translational degrees of freedom for each of three basic conformational relationships. These include base-base, base pair and base pair step parameters. Among the total of 16 parameters, merely 5 base pair (bp) step parameters need be considered for understanding DNA bending and packaging in chromatin. Roll and tilt correspond to DNA bending, in which base unstacking occurs at the base edges or phosphodiester backbone edges, respectively, where tilt is energetically unfavorable relative to roll and thus plays a minor part. Moreover, positive roll, bending into the major groove is substantially more favored compared to negative roll, or bending into the narrower minor groove. Changes in the DNA bending parameters, roll and tilt, are furthermore coupled to alterations in the other three key bp step relationships, slide, twist and shift. Specifically, roll values are anticorrelated with those of twist and slide, while tilt is coupled to shift.

Figure 1.1 The nomenclature of the nucleic acid structure components. Adapted from (Dickerson, 1989)

Previous molecular dynamics demonstrated that each of the ten DNA bp steps adopted a sequence specific ensemble of conformations. (Lankas et al, 2003) Generally, the YR (pyrimidine-purine) type of bp step was found to be most flexible, especially the TA step, in terms of both twist and tilt values. However, similar study based on crystallographic data yielded different conclusions. The TA step was considered stiffer than GA and GC steps (Olson et al, 1998). To make the scenario more complicated, in the case of long sections of double helix, the exact DNA conformation is not a simple summation of all the structural parameters of the bp steps. Recently, a thorough crystallographic analysis demonstrated that the AT steps within an alternating AT context behave differently from those in an A-tract (Mack et al, 2001). In a similar way, these crystallographic data suggest that sequence and crystal packing have an effect on TA step structure and dynamics. Analysis of other crystal structures and molecular dynamics simulations also imply that conformations of dinucleotide steps are strongly context dependent. (Fujii et al, 2007; Packer et al, 2000)

Thus, contrary to popular view, oligonucleotides, especially B form DNA, are not just strings of genetic code, but polymers having distinct topologies similar to those of polypeptides. Additional *in vitro* experiments are needed to generate the database necessary for understanding the principles guiding DNA dynamics.

1.2 Nucleosome Structure and Positioning

Numerous transcription and architectural factors were discovered assisting in the readout and organization of the information encoded within the DNA sequence. Therefore, it is important to consider DNA structure in the presence of these factors, and in particular, in the context of chromatin, the protein-packaged state of DNA within the nucleus. In this manner, we can better understand the molecular basis of the sophisticated network governing DNA transactions.

Among all the components, the nucleosome emerges as a key regulator of the eukaryotic genome. As the basic functional unit of chromosomal structure, it consists of the nucleosome core particle (NCP) and linker DNA. Approximately 147 bp of DNA wrapped in roughly 1.7 turns around a histone protein octamer core (a tetramer of H3 and H4 and two dimers of H2A-H2B), compose each NCP as shown in Figure 1.2 (Davey et al, 2002; Richmond et al, 1984).

Figure 1.2 A frontal (left) and a side view (right) of an NCP (PDB entry 1KX5) showing H2A in blue, H2B in yellow, H3 in red, H4 in green and DNA strands in violet and cyan. The perspective at right is a tilted view looking into the dyad, demonstrating the two H2A/H2B dimers attached to the H3/H4 tetramer core, guiding the surrounding DNA wrapping into a left-handed superhelix.

The tight packing of DNA is achieved by 14 histone-DNA anchoring sites. At these interaction points, nucleosomal DNA is bent consecutively into the major and minor grooves (Fig. 1.3). DNA binding is mediated by direct hydrogen-bonds, hydrophobic interactions and salt bridge interactions with basic side chains, aliphatic residues, as well as main chain groups of the histones.

In most structures of the NCP, there are one or more regions in which the DNA is stretched and overtwisted relative to other sites (Fig. 1.3). Nevertheless, the overall average twist values for nucleosome core DNA is ~ 10.2 bp/turn, which represents overtwisting relative to the canonical naked DNA solution value of ~ 10.5 bp/turn. Depending on the degree of DNA stretching, the twist values have been observed to range from 10.1 to 10.3 bp/turn (Richmond & Davey, 2003). Histone octamer association may potentially induce the formation of up to four stretched locations in the nucleosome, which would result in further overtwisting (Luger et al, 1997; Widom, 2001). Earlier studies suggested that there are unusual DNA sites in the nucleosome, which display hypersensitivity to chemical or DNase I treatment and increased retroviral integration activity. These properties may coincide with DNA stretching within the nucleosome core. Occasionally, DNA that appears to be buried within nucleosomes could spontaneously detach from the histone periphery (Anderson et al, 2002; Thastrom et al, 1999).

Figure 1.3 DNA wrapping and stretching in the nucleosome core. (a) View of the NCP147 crystal structure down the DNA superhelix axis showing the major groove-inward (grey DNA bases) and minor groove-inward (white DNA bases) facing regions for approximately one half of the particle. Numbers correspond to double-helical turns from the nucleosome centre (0), which coincides with the central bp at the particle pseudo dyad axis where the major groove directly faces the histone octamer. Long magenta arrows indicate potential regions of DNA stretching around the 2-turn and 5-turn locations. Small black arrows correspond to base pairs ± 3 that are absent in NCP145. The phosphodiester backbone of the DNA strands appears as cyan and orange. Histone proteins are colored blue for H3, green for H4, gold for H2A, and red for H2B. (b) Comparison of DNA sequences and histone-DNA register in NCP crystal structures described in the main text. NCP145, NCP146, NCP147, NCP146b, NCPp147 and NCPp145 are particles with *Xenopus laevis* histones, whereas hNCP146 corresponds to a particle with the same DNA sequence as NCP146, but with histones from *Homo sapiens* (altered crystal packing interactions between particles in the crystal apparently underlie the differences in stretching between NCP146 and hNCP146). Minor groove-inward facing regions are highlighted in orange, and the base numbering scheme (b), relative to NCP147, corresponds to the 5' (–) to 3' (+) direction of either DNA strand in the duplex (only one strand is shown for each construct; SHL = superhelix location, turns from

center). Observed regions of stretching are underlined in magenta, and a gap in the DNA sequence represents the resulting shift in histone-bp register. Asterisks denote the extreme kinks into the major groove at the 1-turn location and into the minor groove at the 1.5-turn position associated with stretching in NCP145.

Previous bioinformatics studies of the nucleosome positioning in eukaryotic genomes reveals a non-random distribution of DNA sequence motifs. Fourier analysis of the spatial distributions of the 10 different dinucleotide steps shows a periodicity of 10.2 bp/turn for GC and AA base pairs, which displayed an opposite rotational phase angle with respect to one another (Prunell et al, 1979; Satchwell et al, 1986). It is recognized that nucleosome positioning is influenced by the DNA sequence. There is a general trend of A|T-rich sequences and G|C-rich sequences to position respectively on the nucleosome such that the minor groove and major groove are oriented to face inward. This comes about from the overall tendency of A|T-rich elements to prefer a narrow minor groove, opposite to that of G|C-rich sequences, which thus fits the overall histone-imposed DNA wrapping requirements (Bailey et al, 2000; Godde & Widom, 1992; Lowary & Widom, 1997; Prunell et al, 1979; Widom, 1992). However, the nucleosomal DNA positioning preferences suggested by bioinformatics and biochemical assays sometimes contradict results from structural or computational studies. For example, the TA step is found to favor large positive roll in crystal structures and simulations (Packer et al, 2000), while it is actually identified to be the most preferred element at minor groove inward locations, which should require negative roll angle values. Further studies of NCPs with newly designed DNA sequences would provide a clearer view of the sequence-governed nucleosome positioning.

The structural and dynamic constraints on the DNA arising from histone octamer association appear to be crucial for eukaryotic genomic regulation (Cosgrove et al, 2004; Luger et al, 1997; Suto et al, 2000; Tsunaka et al, 2005). The interactions between histone octamer core and the nucleosomal DNA are likely to be the result of co-evolution. The structural influence of nucleosome structure reshapes the overall appearance of the eukaryotic genome, and is also utilized by the genome to perform complicated functions. Furthermore, it acquired multi-dimensional regulatory elements by adopting increasingly versatile histones through a gradual evolution of the nucleosomal structure (Malik & Henikoff, 2003).

1.3 Evolution of Nucleosome and Chromatin Architecture

Early on in evolution, chromatin-like architecture was adopted mainly to physically compact DNA. Extended nucleic acids are highly space consuming for the organism and are too vulnerable to volatile chemicals and electrostatic collapse at the same time. Positively charged protein had to be recruited to compact the negatively charged DNA into more stable and organized entities. In certain viruses, several thousands of nucleotides wrap onto the positively charged inner surface of the virion head (Dokland et al, 1997). In prokaryotes, HU (histone unlike) proteins assumed a form of a positively charged 60 Å wide infinite ladder for DNA packaging (Guo & Adhya, 2007).

However, toroidal DNA compaction in prokaryotes does not have any individual functional unit. The DNA is non-differentially and smoothly packed into a 60 Å supercoil (Figure 1.4). The HU dimers are globular particles lacking any tail structures, incapable of holding the DNA tightly together. The weak interface interaction of HU dimers renders the HU ladder sensitive to ionic concentration and more responsive to overall transcription activities.

In some archaea, proteins more similar to eukaryotic histones emerged and were utilized to package the genome in a different way. Besides serving the compaction role, the histone fold structure is capable of performing many other regulatory functions. Formation of a compact core allows (1) regulated positioning of nucleosome; (2) peripheral modification of individual histone core and tails to achieve gene specific regulation; (3) flexible packaged nucleosome arrays, enabling even higher order structure and regulation. The histone-like proteins were suggested to be phylogenetically related to present generation histones both structurally and functionally (Malik & Henikoff, 2003). Figure 1.4 illustrates the gradual evolution of the histone-like fold structure from archaea to eukaryotes, as well as the HU ladder organization in prokaryotes. Nucleosome and chromatin structures seem to have evolved from primitive DNA packaging proteins. The discrete packaging of genomic DNA appeared first, followed by the basic tails of the histones growing longer to better neutralize DNA and to elaborate on more regulatory mechanisms (Slesarev et al, 1998). Later on, multiple specialized histones with distinct regulatory roles differentiated from the wild type histones to fulfill the increasingly more functional

requirements. Thus, it was not unexpected that the rise of a mature nucleosome and chromatin structure coincided with the emergence of eukaryotes from the archaic ancestors (Ouzounis & Kyrpides, 1996).

Figure 1.4 Structure of HU ladder, archaeal and *Xenopus* histone dimers. (a) The core ladder formed by HU dimers, wrapped by prokaryotic genomic DNA, adapted from (Guo & Adhya, 2007). (b–e) Crystal structures of various archaeal and eukaryotic histone dimers. These include (b) a homodimer of HMfA from *Methanothermobacter fervidus* (PDB entry 1B67), (c) the doublet histone from *Methanopyrus kandleri* (PDB entry 1F1E) and (d) H3–H4 and (e) H2A–H2B heterodimers from *Xenopus laevis* (PDB entry 1AO1). Note the presence of an extra N-terminal α -helix for H3 (grey). For clarity, the C-terminal α -helix extension in H2B is not shown. Adapted from (Malik & Henikoff, 2003).

The higher levels of chromatin hierarchical architecture allowed a sophisticated packaging of an enormous amount of genetic material in higher eukaryotes. Under normal circumstances, an array of nucleosomes compacts into a 30 nm fiber, which chromosome scaffold proteins and adhering proteins condense to form further compacted chromosome. Since each individual chromosome is found to be restricted to a distinct nuclear region, the entire nucleus can be considered as the highest hierarchical level (Figure 1.5). Finally, this multiple-level organization of genetic material could boost the capacity of the eukaryotic genome tremendously.

When considering the coding fraction of the genomes, the more complex organisms with more sophisticated cellular differentiation appear more 'empty' than the simpler organisms, as shown in figure 1.6. In the mammalian genome, the actual coding DNA fraction is merely 2-3%, and this percentage increases all the way up to over 80% in prokaryotes.

Figure 1.5 Organization hierarchy of eukaryotic chromosome, adapted from (Lodish et al, 2000)

However, the low proportion of coding DNA in the genome does not necessarily mean that the genome is storing less information. The non-coding DNA

along with histones and other nuclear proteins could organize the limited coding DNA into interactive signaling networks, which might be significantly more efficient than coding the individual functional pathways. Hence, the DNA distortions in eukaryotic genome induced by histones and other nuclear factors are deliberate and relevant, as well as functionally essential for performing proper genetic activities. For example, nucleosomes function as a physical barrier during transcription. Local chromatin structure has to decondense itself and deplete the nucleosome components at the promoter region to allow the productive binding of an RNA Polymerase II complex. The degree of the chromatin compaction/decompaction is regulated by the presence of nearby enhancer or repressor binding sites, or by the content of the histone modifications/variants of the given chromatin locus (Saunders et al, 2003).

Figure 1.6 Plot of the proportion of coding DNA in entire genome in various species. The diagonal lines plot points of equal proportional contribution to the total genome. As shown, the coding efficiency is decreasing (Lynch, 2006).

1.4 Nucleosome Modification and Dynamics

A. Histone variants

The histone octamer comprises the core of the nucleosome, with many histone variants present in eukaryotes, especially in higher eukaryotic organisms. Table 1.1 summarizes several of the most extensively studied histone variants. Different types of histone octamers might package the DNA differentially and have distinct functional consequences.

Some of the variants are only slightly altered from the canonical histones, such as H3.3 and H2A.X. For example, H3.3 is differently regulated and expressed outside of S phase and plays an important role during gametogenesis (Henikoff, 2009). Whilst in response to double-strand breaks, H2A.X preferentially accumulates at the disrupted chromatin region. This event, followed by a propagation of phosphorylation of both newly and originally deposited H2A.X, marks the ‘injury’ zone (Rogakou et al, 1998).

In contrast, CenH3 and macroH2A have variants quite dissimilar to their original versions. CenH3 has an N-terminal tail completely different from H3.3, likely specialized for packaging chromatin at eukaryotic centromeres and binding sites for many regulatory factors. As the result of the incorporation of CenH3, centromeric nucleosomes package the DNA in a right-handed superhelical pattern and make the centromere drastically distinct from other chromosome regions (Furuyama & Henikoff, 2009). MacroH2A comprises an unusual, vertebrate-specific H2A lineage, which is believed to induce a tighter compaction of the nucleosome array. This coincides with the fact that macroH2A is enriched in inactive X chromosome (Costanzi & Pehrson, 1998). The large C-terminal domain which has phosphoesterase activity might also interfere with the binding of other chromatin regulatory factors.

Finally, H2A.Z and H2ABbd have an overall structure similar to their canonical counterpart, but the dozens of alternative residues endow them with novel functions. H2A.Z is a notable histone variant, as it is highly conserved and critical for maintaining a transcription permissive ‘open’ state (Hartley & Madhani, 2009). The analysis of the H2A.Z nucleosome structure shows that the docking domain near the H2A.Z C-terminus, where normally the H2A/H2B dimer extensively interact with the

H3/H4 tetramer, is compromised, suggesting a relatively loose compaction of the nucleosome (Rogakou et al, 1998). On the other hand, the negative charge of the acidic patch which is involved in higher order chromatin fiber formation is enhanced in H2A.Z, thus compacted nucleosome array is stabilized by enrichment of H2A.Z under certain circumstances. In addition, specific H2A.Z modifications impeding its dominant role in transcriptional regulation were identified, illustrating how H2A.Z is subjected to multidimensional regulation. The most recently discovered H2A variant is H2A.Bbd. The distribution pattern of H2A.Bbd strongly correlates with transcriptionally active domains and seems to be mutually exclusive with that of macroH2A. A weakened charge of the acidic patch which blocks specific nucleosome-nucleosome stacking might provide a structural explanation for its activity.

Name	Difference	Function
H3.3	Minor sequence changes	Provides H3 source outside S phase
CenH3 (CENP-A in mammals and CID in <i>Drosophila melanogaster</i>)	Large alterations in amino acid sequence, longer loop1	Organizes the centromere; N-terminal tail functions as anchoring sites for many centromeric proteins
H2A.Z (H2Avd in <i>Drosophila melanogaster</i>)	Different 'docking' domain, at the interface with H3/H4 tetramer; extended acidic patch at the inter-nucleosome interaction site	Helps to define specific chromatin region (e.g. TATA-less transcription start site)
H2A.X	C-terminal extension that fits the consensus sequence: SQ(E/D) Φ , where Φ indicates a hydrophobic residue	Plays crucial role in chromatin compaction and DNA repair
macroH2A	200 residue long C-terminal domain	Defines the inactive X-chromosome; nucleosome possesses increased salt dependent stability
H2A.Bbd	Weaker acidic patch (DEE $\rightarrow$ DRL). 118 bp not 147 bp DNA protected during micrococcal nuclease digestion	Assists unfolding of 30-nm chromatin fiber; necessary for high level expression of specific genes

Table 1.1 Summary of selected histone variants. (Albert et al, 2007; Bao et al, 2004; Chodaparambil et al, 2007; Malik & Henikoff, 2003; Zhou et al, 2007).

B. Histone modifications

Besides having variants, histones can undergo various modifications. Their long tails contain numerous potential modification sites, making the nucleosome highly versatile in terms of their capacity for being genetically regulated as shown in Figure 1.7. These modifications directly exert influence upon the DNA-histone interactions and higher order chromatin formation. Furthermore, they are also docking sites for many regulatory factors.

The N-terminal tails of histones, as well as more recently characterized defined positions of the globular domain, can carry post-translational modifications such as methylation, acetylation, phosphorylation, ubiquitination, sumoylation and ADP ribosylation (Kouzarides, 2007). Covalent modifications of histones are closely related to various cellular functions, many of these reversible modifications can crosstalk and govern dynamic activities of chromatin. Sometimes, they can be important indicators of certain biological conditions, such as the pluripotency of stem cells and the activity of specific genes (Dodge et al, 2004; Margueron et al, 2005; Shahbazian & Grunstein, 2007; Torres-Padilla et al, 2007).

Figure 1.7 Post-translational modifications of human nucleosomal histones. Adapted from (Bhaumik et al, 2007). The modifications include acetylation (ac), methylation (me), phosphorylation (ph) and ubiquitination (ub1). Most of the known histone modifications occur on the N-terminal tails, with some exceptions including ubiquitination of the C-terminal tails of H2A and H2B and acetylation and methylation of the globular domain of H3 at K56 and K79, respectively. Globular domains of each core histone are represented as colored ovals.

(1). methylation

Both arginine and lysine residues on histones could be multi- (mono-, bis-, tri-) methylated in higher eukaryotes. Histone methylation has several different roles in biological processes. The methylation of lysines 4, 9, 27, 36 and 79 of H3 and Lys20 of H4 functions as a docking site for many transcriptional coactivators and repressors. The states of methylation at specific sites are key indicators of gene activities (Shilatifard, 2006).

(2). acetylation

Acetylation is the modification wherein an acetyl group gets attached to a histone lysine, thereby neutralizing the positive charge. It is proposed that this modification could weaken the DNA-histone interaction and induce a more open chromatin state, which encourages the binding of many more transcriptional coactivators (Dodge et al, 2004).

(3). phosphorylation

Histones are often phosphorylated at specific sites during cell division (Bradbury, 1992). Phosphorylation of H2A (H2A.Z) is induced by double strand DNA breakage. Phosphorylation at histone H3S10 and H3S28 during mitosis is regulated by Aurora kinases (Nowak & Corces, 2004).

(4). ubiquitination

Ubiquitination has been reported to occur on H2A (Lys119 in higher eukaryotes), H2B (human Lys120; yeast Lys123) and H3. Histone ubiquitination is catalyzed by linking the carboxy-terminal glycine of ubiquitin and the ϵ -group of a lysine residue on histones together. The reversible ubiquitination of H2A and H2B is required at particular stages of gene activation and transcription (Zhang, 2003).

C. Nucleosome repositioning

DNA sequence plays a principle role in nucleosome positioning. Although the left-handed supercoiled structure is largely preserved in the nucleosome, DNA association to the histone core is far from being static. DNA on the nucleosome is able to slide along the histone surface to reposition itself translationally (Luger, 2003). Frequently, the dynamic repositioning behavior of DNA is catalyzed by a specific regulatory machine to give rise to a defined local chromatin structure. The positioning of nucleosomes is vital in transcription initiation events. In yeast, there is a nucleosome free region (NFR) before the transcription start site of many genes and a strongly positioned nucleosome right after the start site in spite of transcriptional activity, as shown in Figure 1.8. The figure demonstrates how nucleosome localization/density correlates to functional categorization of yeast genome. Notably, a nucleosome free region followed by a strongly positioned nucleosome indicates a potential 5' boundary of transcriptionally active gene.

Figure 1.8 Nucleosomal landscape of yeast genes, adapted from (Mavrich et al, 2008). The consensus distribution of nucleosomes (grey ovals) around all yeast genes is shown, aligned by the beginning and end of every gene. The resulting two plots were fused in the genetic region. The peaks and valleys represent similar positioning relative to the transcription start site (TSS), marked by the arrow under the green circle preceded by the 5' nucleosome-free region (NFR). Transitions observed in nucleosome composition illustrated by green-blue shading (green corresponds to high H2A.Z levels, acetylation, H3K4 methylation and phasing, whereas blue represents low levels). Transcriptional termination within the 3' NFR is indicated by the red circle. The TSS region is always free of nucleosomes regardless of the transcriptional state.

Although in higher eukaryotes there are more divergent transcription regulation modes, in a large proportion of genes, the binding of the pre-initiation complex reduces nucleosome occupation at the 5' region of the transcription start site whether transcription is successful or not, as demonstrated in Figure 1.9.

The dynamic nature of the nucleosome enables two distinct modes of regulation, the compactness of individual nucleosomes or the nucleosome array, and the translational positioning of the histone octamer on the DNA. These two features open up possibilities for more sophisticated signaling networks, which are both resistant and sensitive to direct environmental stimuli, and consequently underlie cell differentiation. Hence, nucleosome repositioning dynamics are essential to eukaryotic gene regulation. To a certain degree, nucleosome distribution provides an additional dimension to the way DNA encodes information.

Figure 1.9 Nucleosomal positioning in transcriptional initiation. PIC = pre-initiation complex. Y-axis indicates the number of positioned nucleosomes found at a certain distance away from the TSS (transcription start site) normalized by the total number of genes in each class. The TSS is shielded by a nucleosome in most of the inactive genes (without PIC). Unexpressed human promoters have higher TSS nucleosome occupancy. Adapted from (Ozsolak et al, 2007)

1.5 Multidimensional Regulation of the Eukaryotic Genome

To further understand how histone variants/modifications and nucleosome positioning regulate DNA transactions, and conversely how DNA sequence influences these dynamics, several other factors have to be taken into consideration. Certain categories of factors, such as ATP-dependent chromatin remodeling complexes and histone chaperones overlap, while each genetic regulation process involves multiple groups of factors. The combination of all these players determines the actual state of the genome, granting eukaryotic cells countless possibilities and a remarkable regulatory potential (Figure 1.10).

Figure 1.10 The multidimensional regulation network of molecular genetic processes.

A. Histone modification enzymes

(1). methyltransferases and demethylases

Nearly all histone methyltransferases characterized to date contain a domain named after *D. melanogaster* Su(var)3-9, Enhancer of zeste (E(z)) and Trithorax (trx) [SET]. This domain could catalyze methylation of specific lysines on H3 and H4. For example, H3K9 (Histone 3 lysine 9) is recognized and methylated by Suv39h1, Suv39h2, G9A and ESET (Sims & Reinberg, 2006). Similarly, Ezh2 catalyzes histone H3K27 methylation; while PR-Set7 (also known as Set8) and two other SET domain-containing proteins, Suv4-20h1 and Suv4-20h2, catalyze histone H4K20 methylation. Surprisingly, the enzymatic complexes which share the specificity for the same residue seem to have different gene specific activity profiles. Set1 (yeast), MLL (human) and Set2 appear to methylate H3K4 and H3K36 respectively (Shilatifard, 2006). The methylation of these two residues has been intensively investigated, since they are apparently the hallmark of actively transcribed genes, as illustrated by Figure 1.11. The large complex associating with Set1 named COMPASS (complex of proteins associated with Set1), defines localization and recruits transcription elongation factors (Saunders et al, 2006). In contrast, Dot1 complex which is responsible for mono, di- and tri-methylation of H3K79 does not have a SET domain and is unable to methylate free histones. The methylation of this residue is linked to telomere-associated gene silencing (Bolzer et al, 2005; Saunders et al, 2006; van Leeuwen et al, 2002).

The methylation of lysine residues is reversible and is mediated by demethylases. For instances, LSD1 demethylates mono- or dimethylated H3K4 or K9, depending on the proteins present in its complex. Members of the JMJC family display unique specificity and have demethylation activity for all three kinds of methylation (Shi, 2007).

Methylation of histone lysines does not directly induce transcription. The site specific methylation acts as docking site for many genomic regulatory proteins. Figure 1.12 demonstrates how the specific binding is achieved. The 'pro-transcription' H3K4 methylation is recognized by the double chromo domain of CHD1 protein. The 'gene-silencing' H3K9 methylation is targeted by transcriptional repressor HP1 chromo domain.

Figure 1.11 The typical histone modification pattern of an active gene in yeast, adapted from (Saunders et al, 2006). The presence of the histone modifications may correlate with genes that are undergoing active transcription elongation. High levels of H3 and H4 acetylation are typically detected at the promoter and 5' regions. On the other hand, H3-K36 trimethylation is present throughout the transcription unit, with highest levels at the 3' end. Trimethylated H3-K4 usually peaks at promoter-proximal regions, dimethylated H3-K4 predominantly localizes at the 5' end and the middle of the transcription unit and monomethylated H3-K4 tends to localize towards the 3' end of the transcription unit. The yeast Rad6–Bre1 complex and the mammalian UBCH6–RNF20/40 complex ubiquitylate histone H2B; monoubiquitylated H2B is associated with both the promoter and coding regions.

Figure 1.12 Illustrations of factors recognizing H3K4me3 and H3K9me3. Adapted from (Taverna et al, 2007). (a) Overall secondary structure of methylated lysine recognizing factors. (b, d) HP1 binds to H3K9me3. K9me3 is protruding into the recognition groove. (c, e) CHD1 specifically recognizes H3K4me3, in contrast, the entire peptide terminus is recognized by the binding pocket.

(2). acetylases and deacetylases

Acetylation is catalyzed by histone acetyltransferases (HATs) through the transfer of the acetyl moiety from acetyl-coenzyme A to the ϵ -amine of target lysine residues. This modification neutralizes the positive charge on lysine, which in turn creates a loose chromatin structure. Consequently, many transcriptional coactivators, such as Gcn5/PCAF, CBP/p300 and SRC-1, have been shown to possess intrinsic HAT activity.

Histone acetylation could be reversed by the action of histone deacetylases (HDACs). Thus, the interplay between HATs and HDACs determines the histone acetylation level. Many transcriptional corepressor complexes, such as mSin3a, NCoR/SMRT and NURD/Mi-2, contain subunits with HDAC activity. For example, Rpd3S was identified to associate with RNA polymerase II transcription elongation complex. However, the seemingly positive role in transcription actually provides a negative feedback control to prevent nonspecific transcription initiation (Keogh et al, 2005).

(3). kinases

Phosphorylation of H2A (H2A.X) histones carried out by phosphatidylinositol-3-OH kinases, such as Mec-1 in yeast, is induced by DNA damage and is important for DNA repair signaling events (Foster & Downs, 2005). Phosphorylation at H3S10 and H3S28 is catalyzed by Aurora kinases, which are highly conserved from yeast to human. H4S1 phosphorylation is related to chromatin compaction during gametogenesis in fly and mouse (Krishnamoorthy et al, 2006). Overall, histone kinases have important roles in cell cycle regulation. H3 phosphorylation is catalyzed by Ras-MAP kinase pathway (Nowak & Corces, 2004).

(4). ubiquitintransferases

Rad6-mediated H2B monoubiquitination is crucial in the regulation of transcription in a specific subset of RNA polymerase II-transcribed genes. The identification of Rad6 (HR6A/HR6B in human), as an E2-conjugating enzyme of H2B monoubiquitination, helps to explain the pro-transcriptional role of monoubiquitinated H2A/H2B dimer (Shilatifard, 2006), as illustrated in Figure 1.13. Inhibition of active transcription abolishes ubiquitination of H2B. For example, H2BK123 monoubiquitination is required to activate the transcription of SAGA-

Figure 1.13 Mechanistic model for the role of H2B monoubiquitination in transcription elongation in chromatin. It is speculative whether the H2A-H2B dimer is monoubiquitinated at H2BK120 and displaced by FACT, and it is possible that only one of the two H2B molecules undergoes monoubiquitination and that the unmodified dimer is displaced from the nucleosome. Adapted from (Pavri et al, 2006).

regulated genes such as GAL1, SUG2 and PHO5. The machinery responsible for H2BK123 monoubiquitination is highly conserved from yeast to human.

B. ATP-dependent remodeling complexes

The nucleosome localization of specific genomic regions is altered during genetic processes, especially transcription initiation. There are four main types of ATP-dependent nucleosome remodeling complexes: the ISWI (including ACF complex), SNF2 (including SWI/SNF complexes and RSC complexes in yeast), CHD and INO80/SWR families, as shown in Figure 1.14a (Lusser & Kadonaga, 2003). The families share the conserved ATPase domain but differ in their accessory protein components. ATP-dependent remodeling complexes are important in regulating promoter architecture and defining the state of active transcription (Whitehouse et al, 2007), while histone chaperones play a key function during transcription elongation. Deletion of any individual ATP-dependent remodeling complex was found to affect only a small proportion of genes, which indicates that these complexes have redundant roles in gene regulation. A 'loop recapture' mechanism has been proposed to explain the nucleosome repositioning activity of ACF (Strohner et al, 2005) and RSC complexes (Chaban et al, 2008), as shown in Figure 1.14b.

As mentioned above, CHD1 possessing the chromo domain specifically recognizes methylated H3K4, sensing the pro-transcription signature and inducing structural changes at the promoter region (Flanagan et al, 2005; Sims et al, 2005). While in the case of the SWI/SNF family, region specific recognition is achieved by the interaction between the C-terminal bromodomain and the acetylated histone tails. On the other hand, the INO80 complex is recruited by Ser129 phosphorylated H2A.X, as the result of DNA double strand breakage. This ATP-dependent remodeling complex is believed to facilitate DNA repair by loosening nucleosomes.

Figure 1.14 (a) Nucleosome remodeling and disassembly/reassembly enzymes. The four main families of ATP-dependent chromatin remodeling complexes are ISWI, SNF2, CHD, and INO80. The SNF2, CHD and INO80 are capable of both evicting H2A/B dimers and sliding the nucleosome along the DNA, while ISWI repositions the nucleosomes. Adapted from (Lusser & Kadonaga, 2003). **(b)** Structure-based model for SWI/SNF activity. Translocation domain pulls ~50 bp DNA away from the surface of nucleosome. The propagation of this loop causes the repositioning of nucleosome. Adapted from (Dechassa et al, 2008).

C. Histone chaperones

The complexes and factors which associate with histones and stimulate their transfer, without being part of the final product, are defined as histone chaperones (De Koning et al, 2007). They are classified into three major categories: (i) chaperones, including Asf-1, HIRA, Spt6, Rtt106, nucleolin, nucleoplasmin, Chz1 and Nap1, are capable of binding, transporting and transferring histones without involvement of other factors; (ii) multichaperone complexes that combine several histone chaperone subunits, like CAF-1 and FACT; and (iii) histone chaperone modules which increase binding affinity in large enzymatic complexes, including Arp4 in INO80 complexes, rsf-1 in RSF/SNF2 complexes, Arp7, Arp9 in SWI/SNF and RSC complexes (Peterson et al, 1998), acf-1 in ACF complexes (Ito et al, 1997) and hif1 in HAT1/HAT2 complexes (Ai & Parthun, 2004). Furthermore, functionally, histone chaperones have been shown to: (i) deposit histone components to their DNA/protein-complex targets as well as to evict them out of specific chromatin region; (ii) act as the reservoir during development and to buffer transient histone overload; and (iii) transport histone components in and out of nucleus (Harata et al, 1999).

In terms of binding selectivity, histone chaperones could be grouped into H3-H4 and H2A-H2B subclasses. Moreover, some of histone chaperones show selectivity towards several H3, H2A variants and linker histones. For instance, Chz1 exclusively transports H2A.Z-H2B dimers, while HIRA favors H3.3-H4 tetramers as binding partners (Loyola & Almouzni, 2007; Luk et al, 2007; Ray-Gallet et al, 2002). The interaction between chaperones and histones may involve an acidic patch, which neutralizes the positive charge on the histone surface (Muto et al, 2007). However, more specific and delicate interactions between the two have also been shown to be essential for the function of the chaperones. For example, recent high resolution structures of nucleoplasmin and Nap1 show that these chaperones have a conserved β -structure involved in histone binding. This kind of dynamic interaction modulates the affinity under various cellular conditions (different available binding partners). Figure 1.15 summarizes the complex relationships of the histone chaperones.

Figure 1.15 Histone chaperones modulate histone dynamics during transcription elongation. During transcription, histones are thought to be partially or totally evicted from the transcribed DNA. The evicted histones may then be recycled upon chromatin restoration after passage of the polymerase. Alternatively, newly synthesized histones might be incorporated at this step. This cycle of histone eviction and deposition is facilitated by the cooperation (red lines) of the candidate histone chaperones with histone modifiers and remodeling factors that are involved in transcription elongation. The binding of the histone modifiers and remodeling factors to their substrates (indicated in parentheses) may assist in targeting histone chaperones to sites of active transcription. (ac), Acetylation; (TF), transcription factor; (N-ter), N terminus. Adapted from (De Koning et al, 2007).

D. Chromatin localization and clustering

A classic example of nucleus organization illustrates the distinction between the decondensed and relatively active euchromatin and more condensed, generally inactive heterochromatin (Sexton et al, 2007). Recently, it has been discovered that

individual chromosomes occupy distinct positions in the nucleus, referred to as nuclear territories (Bolzer et al, 2005; Cremer et al, 2006), shown in Figure 1.16. The active genes are clustered into transcribing chromosomal segments, and are usually positioned towards the nuclear interior (Foster & Bridger, 2005).

Figure 1.16 (a) A model of nucleus territories: three hypothetical chromosome territories — green, blue and red — are shown (Lanctot et al, 2007). (b) Reconstituted fluorescence map of all chromosomes in human male fibroblast nuclei, adapted from (Bolzer et al, 2005).

The compartmentalization of the nucleus is not static, but dynamic, especially for the active gene-rich interior. The restructuring of nuclear organization was found to be highly relevant to genetic regulation. Recent developments in technology (large scale immunoprecipitation, fluorescence labeling and parallel sequencing methods) allow the identification of a spatial interacting network of genomic loci. Sometimes these co-localized gene clusters contain activated genes from different chromosomes, demonstrating coordinated gene expression (Takizawa et al, 2008).

The formation and maintenance of nuclear loci seems to be self-organized and spontaneous. Upon the initiation of transcription of a particular gene, chromatin undergoes remodeling as the result of the binding of RNA Polymerase II, which is the predominant physical force in restructuring of chromosomes. In fact, the influence of the RNA Polymerase II complex is more powerful than kinesins and myosins. The further recruitment of transcription factors to the gene, as the result of remodeled chromatin, establishes the formation of an active gene locus. As the transcription continues, other splicing and transcription elongation factors become involved. When

multiple genes within the loci begin to be actively transcribed, the concentrated transcription factors stabilize a transcription center (Misteli, 2007). Furthermore, histone modifications play a role in signal propagation. However, the structural details regarding the formation of individual loci are still not fully resolved.

E. Transcription regulation complexes

During transcription initiation, all kinds of transcription activators are accumulated at the promoter and enhancer regions, functioning as probes to evaluate the state of readiness. On the other hand, certain genetic elements (PHO, GAF, and other polycomb response elements) recruit repressors and transcription inhibitory complexes, like polycomb complexes (King & Kingston, 2001; Schwartz & Pirrotta, 2007).

There are three main types of polycomb group complexes (PCG), PRC1 (Shao et al, 1999), PRC2 and PhoRC. In different cell types, these complexes have various recognition profiles. In *D. melanogaster*, PCG are responsible for suppression of *hox* genes and maintenance of the transcription activity after every cell cycle, even if the initial repressing signals are no longer there. All three families of PCG complexes are responsible for the methylation of H3K27, which is normally associated with inactive genes (Francis et al, 2004; Muller et al, 2002), as illustrated in Figure 1.17.

Other transcription regulation complexes function in a less defined and more peculiar way. Generally they recognize and utilize pre-established epigenetic signals (CpG islands, transcription factor binding motifs or histone modifications), and amplify, propagate and translate the signals into meaningful molecular genetic language, depending on the context. The interplay of complexes with opposing functions determines the activity of specific genes or gene clusters.

Figure 1.17 Methylation of histone H3 by the PRE complexes. **(a)** The model depicts DNA-binding proteins, binding to the Polycomb response element (PRE), which is shown free of nucleosomes (grey spheres), possibly with the involvement of a chromatin remodeling complex, and cooperative recruitment the PRC1 and PRC2 complexes. **(b)** The PRE-bound complexes methylate flanking nucleosomes (indicated by red pin balls on the nucleosomes) on either side of the PRE. **(c)** The methylation domain is extended by looping of the PRE-bound complexes to contact nucleosomes over a broad region. The looping is mediated by interactions of the PC chromodomain and possibly other methyl-binding domains, such as PHD fingers, MBD, Tudor and SET domains in other PcG proteins, creating and maintaining a broad methylation domain. The PRE also constitutively binds Trithorax (TRX), which would be carried along in the looping contacts and could, in principle, also deposit the histone H3 methylation of lysine 4 (H3K4) mark (not shown). Adapted from (Schwartz & Pirrotta, 2007).

F. Non-histone chromatin proteins

Besides histones and enzymatic complexes, there are also several categories of proteins that participate in chromatin maintenance. Among them, actin and microtubule related filament proteins function as the physical support of the nucleus. There is also a group of ‘high mobility group’ (HMG) proteins, constituting three well studied subgroups, HMGA, HMGB and HMGN, which are ubiquitous and crucial for

chromatin function. Each has its own unique and conserved motif, exerting structural/architectural roles onto chromatin (Hock et al, 2007).

HMGAs bind preferentially to AT-rich DNA, and induce conformational changes. HMGA-loaded DNA subsequently serves as a platform for the binding of additional components (Fedele et al, 2001). Recently, HMGA2 was found to have a direct DNA repair role, exhibiting dRP/AP site cleavage activity (Summer et al, 2009). Overall, HMGAs are implicated in stem cell development and differentiation. HMGBs are involved in both intracellular and extracellular signaling pathways. They share a motif of an L-shape structure which recognizes and sharply kinks the DNA. It was also shown that HMGBs specifically target lesions caused by CisPt, residing on the exact site of cross-linking (Ohndorf et al, 1999). They are also important components of the DNA repair machinery, responsible for recognition of abnormal DNA structures. HMGNs have a positively charged domain in the middle, named the nucleosome binding domain (NBD) and another acidic motif in the C-terminal region, characterized as the chromatin regulatory domain. HMGNs bind specifically to nucleosomes and are critical in general for cellular differentiation, by modulating both local and higher order structure changes. The level of HMG protein concentration varies according to cell cycle stage and cell type (Hock et al, 2007).

G. DNA repair complexes

The eukaryotic genome is constantly exposed to environmental hazards, which lead to DNA damage. Double-strand breakage (DSB), as a result of ionizing radiation, is the most undesirable scenario, which is difficult to repair and is the main cause for oncogenic genetic recombination. Thus, eukaryotic cells adopt a highly complicated system to efficiently detect, isolate and repair DSB or even annihilate the cell, when necessary.

For example, the Mre11-Rad50-Nbs1 (MRN) complex acts as the sensor of DSB (Lee & Paull, 2005). It binds to long stretches of single stranded DNA, which is a characteristic feature of DSB. Bound MRN greatly increases the affinity of interaction between ataxia-telangiectasia mutated (ATM) kinase and DNA. Then ATM activates several downstream factors, including p53, Chk2, Brca1, and H2A.X, which trigger cell-cycle arrest, leading to DNA repair or apoptosis (Pandita & Richardson, 2009). The role of phosphorylated H2A.X as a marker of injured

chromatin regions has been discussed previously. Some unclassified histone chaperones and ATP-dependent remodeling complexes are involved in the stages which follow DSB detection, leading to the removal of histones and release of the DNA ends.

H. The role of RNAi in genome maintenance

Heterochromatin is defined by the presence or the absence of specific molecular markers, mainly the post-translational modifications of histones. For example, methylation of H3K9, H3K27 and H4K20 strongly correlates with reduced gene activity, and is considered to be a marker of heterochromatin. In typical heterochromatic regions, such as centromeres, H3K9 methylation recruits HP1 protein and other suppressor complexes. In turn, HP1 recruits cohesin to promote sister-chromatid cohesion at the centromere. At the same time, pro-transcriptional features such as hyperacetylation are not usually observed in the centromeric region (Jenuwein & Allis, 2001).

Recently, this classic view of silenced centromeres has been challenged by new findings. Transcription of many heterochromatic DNA repeats in many cell types has been reported (Volpe et al, 2002). The RNA products of these repeats appear to be substrates for the RNAi-dependent regulation pathways (Fukagawa et al, 2004). They participate in the initial assembly of the heterochromatin structures, and recruit the H3K9 histone methyltransferases. Once the histone modification pattern is established, the RNAi-related complexes are no longer necessary for centromere formation (Kagansky et al, 2009). Besides the direct inhibitory effects of the RNAi related gene silencing, the RNAi complexes in heterochromatin recruit a different group of RNA polyadenylation complex, the TRAMP. This complex tags the transcribed RNA differently, so that the RNA is transported to exosomes for degradation, instead of being translated (Wyers et al, 2005).

RNAi-related gene suppression also contributes to other signaling pathways. RNAs associated with piwi members of the Argonaute family, called piRNAs have been recently identified, which are shown to be involved in transposon silencing in the germline (Lau et al, 2006). Thus, noncoding RNAs have versatile roles in genomic regulation. They are not only involved in the feedback degradation, but also actively

participate in up-stream regulation, influencing the histone modification pattern and serving as platforms for site directed regulation complexes (Buhler & Moazed, 2007).

1.6 Development of Drugs Targeting Chromatin

When considering the complexity of the eukaryotic genome, it becomes apparent that therapeutic agents targeting naked DNA alone may be generally insufficient and ineffective. A newer generation of drug design should consider chromatin structure, which is the predominant form of DNA existing in eukaryotic cells. As the basic but crucial functional unit of the genome, a selectively targeted nucleosome is not easily repaired or bypassed and has more significant impact on genomic regulation.

Consequently, site specific targeting of nucleosomal DNA should be possible if the structural characteristics of the nucleosomes were thoroughly elucidated. The highly constrained conformation of nucleosomal DNA sometimes applies additional structural restrictions for intercalating agents or other bulky groove binding agents. However, alternative DNA conformations induced by histone-DNA interactions or the unique biochemical environment in chromatin structure enables new strategies of site specific targeting (Davey & Davey, 2008). For example, highly sequence-specific minor groove-binding polyamides associate with DNA sites inside the nucleosome in a defined way, not observed in the case of naked DNA binding (Edayathumangalam et al, 2004).

Although some of the drugs targeting the DNA are already amongst the most effective anti-cancer treatments currently available, a better understanding of DNA structurally and functionally within the context of the nucleosome would provide invaluable insights in the battle against different diseases.

CHAPTER II

PLATINUM ANTI-CANCER THERAPEUTICS

2.1 Platinum Drug History

Platinum derivatives (mainly cisplatin [CisPt], carboplatin [CarPt] and oxaliplatin [OxPt]; Figure 2.1) alone or combined with other anti-cancer therapeutics, have been proven remarkably effective in treatment against testicular cancer, and prolonging the lifespan of colon and ovarian cancer patients (Rabik & Dolan, 2007). With increasingly more clinical applications world-wide, these chemicals have gone 'platinum' in the times of basic struggle against cancer. However, these drugs are still far from ideal. The intolerable side effects and fast developing drug-resistance quickly render platinum derivatives obsolete during treatment (Wu et al, 2004).

Marketed platinum anticancer drugs

Figure 2.1 Chemical structures of platinum anti-cancer therapeutics, adapted from (Jung & Lippard, 2007).

The antiproliferative activity of CisPt was accidentally discovered in 1965 (Rosenberg et al, 1965). As further functional investigation of CisPt proceeded, the extraordinary anticancer activity drew attention into searching for new platinum derivatives. CarPt was introduced in 1982 with the hope of easing the toxicity of CisPt, followed by more soluble OxPt first synthesized in the 1980s (Kidani et al, 1980). Unexpectedly, clinical trials of OxPt revealed a distinct pharmacological profile from that of CisPt; some CisPt resistant cell lines responded to OxPt treatment (Wong & Giandomenico, 1999). This discovery stimulated another wave of search for

Figure 2.2 History of platinum anticancer therapeutics, from (Kelland, 2007).

better platinum derivatives (Fuertes et al, 2003; Jung & Lippard, 2007; Ziegler et al, 2000).

2.2 Activation and Reaction Mechanism

Although platinum agents bind to a variety of biological macromolecules, including proteins, which are also responsible for undesirable side effects of the treatment, DNA appears to be the major pharmacological target. The N7 atom of purines is electrophilically attacked by the centrally localized Pt (II) ion, and if there are other purines in the vicinity, a cross-linking of two purines by platinum drugs can take place. The interaction between platinum drugs and DNA is a multistage process.

Hydration and pre-association of Pt complexes with halogeno leaving groups has been demonstrated by many [^1H , ^{15}N] HSQC NMR studies to take place prior to reaction (Davies et al, 2000). Additionally, the presence of oligonucleotides in solution slows down the CisPt aquation rate by 30-50%, which suggests that pre-association between CisPt and DNA and aquation can occur simultaneously. This was further supported by the fact that the monoaquated species (not fully aquated population) of CisPt accounts for the majority of the platination to DNA (Hambley, 2001).

Monofunctional adduct formation takes place after the initial step. The reaction rate has been extrapolated by means of NMR experiments (Davies et al, 2000; P. J. Sadler, 1996). The binding to the 3' G of a GpG pair is approximately three-fold faster than the binding to the 5' G, probably due to platinum agents favoring the structural parameters of the 3' G.

Closure to a bifunctional adduct is the final step in platination. Mainly 1,2 GG, 1,2 AG and some minor fraction of 1,3 GNG and interstrand cross-links have been identified in the genomic DNA of CisPt-treated cells *in vivo* and *in vitro* (Akaboshi et al, 1994; den Hartog et al, 1983; Lippard & Hoeschele, 1979). In the *in vitro* studies, the closure from a 3' monofunctional adduct is approximately an order of magnitude faster than closure from a 5' adduct, irrespective of the local DNA sequence, possibly due to the chirality of the DNA double helix (Davies et al, 1998). Several simulation studies also confirmed this observation (Mantri et al, 2007).

Figure 2.3a and 2.3b show the crystal structures of CisPt- and OxPt-cross-linked GG. The base-to-base dihedral angle is ~40 degrees for CisPt and ~25 degrees for OxPt in these adducted dodecamers (Brabec et al, 1992; den Hartog et al, 1983). It is widely accepted that the platination induced distorted conformation (e.g. kinked and partially unwound GG step) perturbs normal cellular function and eventually results in apoptosis (Jamieson & Lippard, 1999; Sherman et al, 1985; Takahara et al, 1995).

2.3 Mechanism of Action *in Vivo*

The induced conformational distortions observed in the X-ray crystal structures of platinum-DNA complexes do not straightforwardly explain all the pharmacological activities of these agents. Although CisPt and OxPt adductions result in similar kinking and unwinding of the DNA, their efficacies are quite distinct. OxPt displays a less severe clinical toxicity and a retained activity against CisPt-resistant cell lines (Kelland & McKeage, 1994). The differential responses to the agents by other biological elements might provide the explanation to these observations. Firstly, the platinum scavengers in serum and cytosol neutralize a significant proportion of platinum agents. Sulfur-rich protein factors, including glutathione (GSH), metallothioneins, and anti-oxidants, are usually responsible for platinum drug resistance (Rabik & Dolan, 2007). Secondly, several ion channels and receptors are involved in the cellular uptake of platinum agents. Some types of platinum agents are better recognized by 'transporters' on the surface of specific cell populations, making them sensitive to platinum drugs (Muggia, 2009).

On the other hand, some common ions have been identified as having inhibitory effects on certain platinum agents. For instance, high chloride concentration has an adverse effect on hydrolysis of the majority of platinum drugs, especially CisPt. This is one of the mechanisms making this type of drug more environmentally specific, considering the 100 mM to 3 mM drop in chloride concentration from serum to cytosol.

Furthermore, different cell types at distinct stages of the cell cycle have drastically different cellular environments, and the concentrations of numerous cellular factors responsible for maintaining the integrity of the genome may vary greatly. For example, suppressed cellular levels of ERCC1/XPF (involved in NER,

nucleotide excision repair) makes cells sensitized to CisPt treatment even in those cell lines, which developed CisPt resistance (Martin et al, 2008). Moreover, CisPt and OxPt were found to generate slightly different types of adducts as well as to prefer different regions of genomic DNA, which could yield distinct physiological responses and allow for differential recognition by cellular components (Woynarowski et al, 1998).

Lastly, the variations in chemical structure among platinum drugs give rise to unique biochemical properties, activating specific types of biological response. Several biological models were proposed to explain the pharmacological activity of platinum-based drugs. However, it is still not fully understood how the platinum lesions generated by these drugs stimulate downstream apoptosis pathways (Jung & Lippard, 2003; Zamble et al, 1998).

As shown in Figure 2.3c, CisPt adducts are capable of selectively arresting the RNA polymerase II complex. Cross-linked bases will create a significant energy barrier for translocation between the +2 and +1 transcription sites. Furthermore, cross-linking related supercoiling stress causes strong resistance to topoisomerase activity during transcription. In the end, inhibited RNA polymerase II triggers catastrophic downstream signals, resulting in cell cycle arrest and ultimately in apoptosis (Damsma et al, 2007). In contrast, CisPt lesions readily escape the check points of DNA replication (Alt et al, 2007).

Besides blocking transcription, CisPt-dinucleotide cross-linking was discovered to be selectively recognized by several groups of non-histone nuclear proteins (Zlatanova et al, 1998). Most of these are chromatin accessory proteins such as HMGB1, linker histones or DNA repair complex components, like XPA and XPE. It is possible that these factors bind and shield the CisPt adducts, preventing lesions from being repaired (He et al, 2000). In Figure 2.3d, HMGB1 protein is bound at the CisPt 1,2GG cross-linked site. Because these factors are essential for chromosomal dynamic regulation, their depletion to platinum lesion sites leads to genomic disorder, and ultimately apoptosis.

The current theories explain chemical and biological consequences of platinum drug treatment to a certain extent. However, most studies used only naked DNA as the substrate. Furthermore, temporarily stalled RNA polymerase II is not degraded and could be fully restored after chemically removing the platinum adducts (Jung & Lippard, 2006). Moreover, HMGB1 knock out mice were demonstrated

viable and showed no significant enhanced or reduced sensitivity to CisPt treatment (Wei et al, 2003). Thus, there is still a need to explain the differential anti-cancer pharmacological effects as well as drug resistance mechanisms of the various platinum derivatives.

Figure 2.3 Crystal structures of (a) 1,2 CisPt and (b) OxPt cross-links at GG dinucleotide sites in oligonucleotide DNA. The C1 atom of the sugar-phosphate backbone is colored black. Polar hydrogen atoms associated with the amine groups of platinum (magenta) are shown in white (Spingler et al, 2001; Takahara et al, 1996; Takahara et al, 1995). (c) RNA polymerase II complex arrested at a CisPt adduct (Damsma et al, 2007). (d) HMGB1 protein specifically bound to a 1,2 GG CisPt-cross-linked oligonucleotide. HMGB1 is orange-colored (Ohndorf et al, 1999).

2.4 Recent Strategies for Drug Development

Current efforts in improving platinum drugs are focusing on the pharmacological aspects by reducing toxicity as well as cellular drug resistance. Tissue- and cellular-specific design is exploiting the potential of currently available agents. Some of the novel approaches include radioactivation of Pt (IV) (+4 charged) compounds for tissue specific targeting. This strategy combines radioactive chemotherapy to convert the inactive Pt (IV) compounds into the active form of Pt (II) drugs by radiation in defined target tissues. In this way, the side effects in other parts of the body are reduced (Hall et al, 2007). Similarly, smart delivery tags are being used in platinum drug based therapy. The precursor drugs chemicals are linked to nanocarbon tubes or other carrier groups, which are selectively endocytosized by specific cell types, and later released intra-cellularly (Mukhopadhyay et al, 2008).

Multinuclear platinum anti-tumor compounds have been synthesized to test the potential for linking multiple Pt centers with aliphatic linkers, and more recently with bifunctional thiourea, spermine, spermidine or modified tetraamine linkers (Wong & Giandomenico, 1999). One of the trinuclear chemicals, BBR3464 (Figure 2.4), exhibits a complete lack of cross-resistance to CisPt resistant cell lines in recent preclinical trials and shows significantly more potency against osteosarcoma cell lines *in vitro*. This increased activity was suggested to be due to the elevated cellular Pt uptake and extent of DNA cross-linking (Perego et al, 1999).

Cocktailing with other drugs has been adopted as a way to counter the cellular resistance to platinum derivatives. For example, combination with glutathione inhibitors significantly increases the cellular response to CisPt. The increase of GSH levels is the common cellular defense mechanism used by cells tackling metallo-toxins. The decrease of GSH results in a much higher proportion of platinum adducts ending up on DNA. Some platinum agents display greater synergistic efficacy when combined with other inhibitors of nucleotide excision repair machinery (Kelland, 2007).

Figure 2.4 Chemical structure of BBR3464 (cationic part), adapted from (Perego et al, 1999).

2.5 Thesis Scope and Aims

The identification of relevant biological targets comprises an urgent task in rational drug development. Gaining more insight into the structural aspects of platinum drug binding to genomic DNA should allow for development of more effective anti-cancer drugs. Discovery of new chemicals mimicking the apoptotic properties of platinum agents, while avoiding the side effects and cell resistance, is the ultimate direction for the next generation ‘platinum’ therapies. As discussed previously, a proper chromatin platform is needed for all genomic activities. By not considering the state of the eukaryotic genome (nucleosomal structure), we are missing essential clues for constructing the bigger picture.

Figure 2.5 *In vivo* restriction enzyme analysis of chromatin and transiently transfected MMTV (mouse mammary tumor virus) templates in cells treated with cisplatin, transplatin, or Dex as indicated, adapted from (Mymryk et al, 1995). Analysis was performed by using Taq polymerase primer extension with a radioactive-labeled single-stranded primer specific for either the stable or transiently introduced MMTV promoter. The amplitude of the signal represents the amount of the fragment, which is an indicator of whether the Sst I digestion site is exposed or still protected by nucleosome structure. The Sst I site is located at the dyad of a well positioned nucleosome in a stable MMTV template, if the activator Dex is not applied.

As shown in Figure 2.5, a paper by Mymryk and co-workers sheds light on the therapeutic effects of platinum drugs. In the experiment they performed, cells with both, stable viral template (2.5A) and transient template (2.5B), were treated with CisPt for 16 hours. As a result, transcription factors could no longer displace histones and free nucleosome-bound DNA, while the non-therapeutic analog transplatin did not have this effect. The inhibition was only profound in cells with stable viral template and not a transient one. This was an indication that platinum anticancer derivatives interfered with normal nucleosome and chromatin function. Unfortunately, at that time, the authors of this study did not realize it and concluded the difference they had observed was due to HMGB1-like regulators. As part of this thesis work, drug-nucleosome DNA interactions and the intrinsic sequence dependent nucleosomal DNA dynamics were studied by employing highly homogeneous reconstituted nucleosomes. We also looked at the biochemical consequences of DNA adductions in the nucleosome using quantitative repositioning assays, which demonstrated that even a single DNA lesion could influence nucleosome dynamics considerably.

With newly gained knowledge about eukaryotic genomic processes, the critical role of nucleosome structure mediating the platinum-induced cellular toxicity becomes more apparent. Thus, we focused on elucidating the details of the structural influence of the DNA sequence and nucleosome scaffold on the site selectivity of platinum anticancer drugs, as well as biochemical consequences of platinum-DNA adducts within the nucleosome.

Furthermore, we asked how the distribution of platinum adducts is affected by the context of the nucleosome, as compared to naked DNA. We employed several techniques, such as atomic absorption spectrophotometry (AAS) and exonuclease III digestion assay to generate the adduction maps. At the same time, the crystallographic investigation of platinum-soaked NCP crystals provided invaluable structural information. Finally, a series of NCPs with *de novo* designed DNA constructs were employed as a means to unveil the extent of sequence dependence on nucleosome positioning. Addressing these questions will hopefully provide an initial platform for identification of appropriate targets within chromatin and set the stage for finding improved, more specific medicinal agents.

CHAPTER III

MATERIALS AND METHODS

3.1 Histone Octamer Preparation

X. laevis histone genes (with optimized codons) were cloned into pET3a or pET3d plasmids (gifts from Prof. Timothy J. Richmond ETH, Zurich). Below are the complete amino acid sequences of the four core histones overexpressed to assemble and purify histone octamer (HO), according to published protocols (Davey et al, 2002; Luger et al, 1999).

H2A

SGRGKQGGKTRAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAP
VYLAADVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAVRNDEELNKLLG
RVTIAQGGVLPNIQSVLLPKKTESSKSKSK

H2B

AKSAPAPKKGSKKAVTKTQKKDGKKRRKTRKESYAIYVYKVLKQVHPD
TGISSKAMSIMNSFVNDVFERIAGEASRLAHYNKRSTITSREIQTAVRLLLP
GELAKHAVSEGTKAVTKYTSK

H3

ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVAL
REIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAY
LVALFEDTNLCAIHAKRVTIMPKDIQLARRIRGERA

H4

SGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLI
YEETRGLVKVFLENVIRDAVTYTEHAKRKTVTAMDVVYALKRQGRTLY
GFGG

Annotation 3.1 Amino acid sequences of core histone proteins used in this study.

A. Cell transformation

An aliquot of 50 µl BL21DE3-PLysS cells (Promega, Madison, WI, USA) were transformed with 2 µg of plasmid (pET3a, ampicillin) encoding the individual histone. The transformation mix was then incubated on ice for 20 minutes, heat

shocked at 42 °C for 45 seconds, then 500 µl of LB was added to the transformation mixture, which was incubated in the shaker for 40 minutes at 37 °C, before plating.

TYE agar plates containing appropriate antibiotics (final concentration, chloramphenicol 25 µg/ml + ampicillin 100 µg/ml) were used for plating. [BL21 (DE3) pLys S cells are inherently resistant to chloramphenicol (camp) and the transformed plasmid (eg: pET 3d-H3) provides ampicillin (amp) resistance.] For every 100 ml of agar medium, allowed to cool to 50 °C, 100 µl of each of the antibiotic stocks (1000 fold) were added.

B. Protein overexpression

Ingredients of 2xTY medium:	1Liter	7.5L	8L
Bacto Tryptone	16 g	120 g	128 g
Yeast Extract	10 g	75 g	80 g
Sodium chloride	5 g	37.5 g	40 g
Media was mixed and dissolved in MQ-water and transferred to 2-L culture flasks with a maximum volume of 500 ml per flask.			

Protocol 3.1 The ingredients of the protein expression medium.

Four to five individual colonies were inoculated into 12-ml inoculation tubes with 2 ml of LB with ampicillin (Amp, 100 µg/ml) and chlorampicillin (Camp, 25 µg/ml) separately. After a 4 hour incubation, these tubes were combined and added into a 500 ml flask with 100 ml fresh 2 fold TY medium (ingredients described in Protocol 3.1) supplemented with Amp and Camp. The 100 ml culture was allowed to grow for 2-3 hours and then distributed into another fifteen 2-L flasks with 500 ml fresh medium each with Amp and Camp. This final stage of culture medium was induced with IPTG (at a level of 0.4mM) and incubated at 37 °C for ~3 hours, to reach an OD₆₀₀ of 0.6.

C. Cell harvesting

Cells from a 7.5 liter culture were spun down at 7000 rpm for 7 minutes (Beckman JA10 rotor) and resuspended in 100 ml wash buffer (50 mM Tris, pH 7.5,

100 mM NaCl with 1 mM beta-mercaptoethanol) and flash frozen in liquid nitrogen. After transferring into a 250-ml centrifuge bottle, the cell pellet was stored at -20 °C. In the next step, frozen cells were thawed at 37 °C until the suspension became homogenous and viscous. The 100 ml of cell mass was sonicated until complete cell lysis (sonication for 10–60 minutes until no severe viscosity observed; 30% amplitude, with a pulse of 1 sec and rest of 1 sec). The 100 ml cell lysate was kept chilled on ice during the sonication process. Samples were then centrifuged at 18,000rpm in PPCO tubes (JA25.50 rotor) for 15 min at 4 °C. The supernatant fraction was saved on ice until the presence of histone inclusion bodies in the pellet was confirmed.

After discarding the supernatant, the collected cell pellet was dissolved in 150 ml of wash buffer containing 1% Triton X-100. If the pellet appeared “spongy”, the partially processed sample was to be sonicated and spun down again, as described previously. Sample was centrifuged at 18,000rpm for 15 min at 4 °C. After a few washes, the pellet, which was majoritively histone inclusion body, was resuspended by Triton X-100-free wash buffer. The last fraction of insoluble protein pellet was transferred using a sterile spatula into a 50-ml Falcon tube and stored at -20 °C. Crude histones could be stored at this stage for longer duration.

D. Protein purification

The histone inclusion body was dissolved in a buffer (7M guanidinium hydrochloride, 20 mM Na-Acetate, pH 5.2, 10 mM DTT) and purified by passing through a S-200 (GE Healthcare, Uppsala, Sweden) gel filtration column under (7 M urea, 1 M NaCl, 20mM Na-Acetate, pH 5.2, 5 mM beta-mercaptoethanol, 1 mM EDTA) denaturing conditions. 1 ml DMSO was added to the histone inclusion body first to soften the pellet. A relatively pure histone fraction eluted after 120 ml when a 26/60 sephacryl S-200 column was used. The elution was then dialyzed against water to get rid of the excessive urea/salt in the solution. The desalted histone solution was then lyophilized and prepared for long-term storage and further purification.

Further purification was achieved using a Mono-S/Resource-S column (GE Healthcare, Uppsala, Sweden), under denaturing conditions (7 M urea, NaCl gradient from 300 mM to 1 M, 20 mM Na-Acetate, pH 5.2). Histones were dialyzed

(Spectra/Por dialysis membrane, 3 kDa cut-off) against water and lyophilized again (Figure 3.1).

Figure 3.1 SDS-PAGE analysis of purified individual histones. Lanes: 1, histone octamer marker; 2, H2A; 3, H2B; 4, H3; 5, H3-C110A; 6, H4

E. Octamer refolding

Individually purified and lyophilized histones were dissolved in 7M guanidinium hydrochloride solution (also containing 10 mM Tris pH7.5, 10 mM DTT) and mixed in a specific stoichiometry. This mixture was gently dialyzed (Spectra/Por dialysis membrane, 6 - 8 kDa cut-off) against 2 M NaCl solution (Tris pH 7.5, 10 mM, 1 mM Na-EDTA) to refold into native histone octamer. Reducing agent (5 mM 2-mercaptoethanol or 2 mM DTT) was added to prevent disulfide bond formation. Three changes of dialyzing buffer with minimum dilution factor of 30 were necessary, while ensuring that the first and the second dialyses were longer than 5 hours and the last step longer than 3 hours. The extinction coefficient values given by previous literature appeared to be empirically incorrect and hence, based on denatured extinction coefficients, we had to mix the individual histones with a molar ratio of $H2A:H2B:H3:H4 = 1:1:1.8:1.8$, instead of equimolar, to get better yields during histone octamer refolding (Table 3.1).

Histone	Native extinction coefficient (OD276/Mole)	Denatured extinction coefficient (OD276/Mole)
H2A (13960Dalton)	4050	4350
H2B (13493Dalton)	6700	7250
H3 (15273Dalton)	4040	4640
H4 (11236Dalton)	5400	5800
H3 C110A	4040	4640

Table 3.1 Theoretical extinction coefficients of individual histones

F. Octamer purification

Refolded histone octamer was centrifuged to get rid of precipitated histone aggregates. Soluble histone octamer was purified by passing through a S-200 size exclusion gel filtration column under 2 M NaCl condition (10 mM Tris pH 7.5, 1 mM Na-EDTA, 10 mM beta-mercaptoethanol). The octamer fraction, H3H4 tetramer fraction and H2A/H2B dimer fraction eluted out at 120 ml, 145 ml and 160 ml, respectively, as shown in Figure 3.2. Purified fractions were combined and concentrated up to at least 10 mg/ml using Milipore concentrators (cutoff 10 kD). An equal volume of glycerol was then added to the sample, and the octamer (in 50% glycerol) maintained good quality for several weeks at -20 °C (Dyer et al, 2004). The histone octamer concentration could be estimated by the OD276 readings; roughly 0.45 unit represents 1 mg/ml protein concentration.

Figure 3.2 FPLC S-200 gel filtration purification of histone octamer. The main peak represents the histone octamer fraction, for which a sample is shown in the SDS PAGE gel inset.

3.2 DNA Preparation

DNA with palindromic sequences is extremely useful in reconstituted NCP studies. By taking advantage of the symmetry properties of the NCP, palindromic DNA can assist in reconstituting a more homogenous NCP population, and can significantly facilitate crystal packing order.

As summarized in the previous Figure 1.3, six palindromic NCPs were used in our study. In this section, we discuss the DNA component of the NCP. The first three DNAs: 145, 146 and 147 were derived from the same alpha satellite DNA sequence. Compared to DNA147, DNA145 lacks two base pairs at ± 3 base sites. DNA146 has an additional T at +2 base site, in contrast to DNA145.

The palindromic DNA sequences were divided into two halves to avoid complication in amplification due to their palindromic nature. This makes the expression and purification of these DNA fragments less straightforward. The last two sequences, DNAp147 and DNAp145, were specifically designed based on previous human alpha-satellite derived sequences (DNA145, 146, 147). The dyad region (from SHL -2 to SHL 2) sequence was modified (Figure 1.3).

As illustrated in Figure 3.3 and Figure 3.4, the two halves of palindromic DNA were cloned into a carrier vector, separated by EcoRV restriction sites. After inoculation and amplification using a prokaryotic system (HB101 cells) in TB medium (12 g Bacto tryptone and 24 g Yeast extract per liter, no salt) with additional 17 mM KH_2PO_4 , 36 mM K_2HPO_4 and 4 ml 50% glycerol, the cells were alkaline lysed after harvesting. Every 15 g of cell mass was resuspended in 50 ml alkaline lysis buffer I (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0), and lysed by addition of 100 ml alkaline lysis buffer II (200 mM NaOH, 1% SDS). Very vigorous shaking was required for complete cell lysis at this step. The lysis was then quenched by addition of 175 ml lysis buffer III (4 M K-acetate, 2 M acetic acid). After spinning at 10'000 g for 20 minutes at 4 °C, cell extracts (supernatant fraction) were isopropanol precipitated (addition of 0.52 volume of isopropanol, room temperature incubation for 20 minutes and spinning at 16'000 g for 30 minutes). The pellet was then air-dried and dissolved by a buffer containing 10 mM Tris, pH 8.0 and 50 mM EDTA. Finally, the solubilized DNA fraction was submitted to overnight RNase treatment (RNase A 10 $\mu\text{l/ml}$) to eliminate RNA contaminants.

Figure 3.3 Schematic representation of DNA147 preparation procedure.

Figure 3.4 Overall strategy of making reconstituted nucleosomes. Individual histones are to be refolded into histone octamer and halves of palindromic DNA are to be ligated. In the end, by performing the salt gradient dialysis, the homogeneous reconstituted nucleosomes are prepared.

The next day, this crude plasmid solution was phenol extracted twice to get rid of lipid and small hydrophobic contaminants and CIA (24 parts Chloroform to 1 part isoamyl alcohol) extracted once to remove remaining phenol (all hydrophobic extractions were performed using only TEFLON tubes). Then, this semi-purified plasmid DNA was selectively precipitated (0.5 M NaCl and 10% PEG 6000) to get rid of smaller RNA remnants. After resuspension using a solution of 10 mM Tris 8.0 with 0.1 mM EDTA, another one or two rounds of CIA extraction were followed immediately to remove PEG contaminants. Ethanol precipitation was performed after the last extraction to prevent inactivation of the enzymes, caused by traces of chloroform. Purified plasmid was resuspended using TE0.1 (10 mM Tris pH8.0 buffer with 0.1 mM EDTA), and the yield was estimated by a spectrophotometer reading at 260 nm.

EcoRV digestion was done to release the 84mer DNA fragments (for reaction conditions, refer to Table 3.2). NaCl to a final concentration of 0.5 M was added prior to PEG into the system, with the aim of neutralizing the ionic repulsion between the DNA. The separation of insert and the empty vector plasmid was achieved by a precise 9.6% PEG 6000 precipitation, and followed by 30 minute on-ice incubation and centrifugation at 27'000 g for 20 minutes at 4 °C. The 84mer DNA containing supernatant was kept. This fraction was CIA extracted twice and then ethanol precipitated. Upon resuspension in TE0.1, 84mer DNA was ready for the next step.

Dephosphorylation of these fragments was performed by CIAP (calf intestine alkaline phosphatase, see Table 3.2) to ensure specificity of the subsequent ligation step. After thorough extraction using phenol chloroform 1:1 mixture to arrest the dephosphorylation reaction, the 84mer was purified by ethanol precipitation. A small scale (<50 ul) test ligation of this dephosphorylated material was required to ensure the dephosphorylation efficacy.

Confirmed fully dephosphorylated 84mer was cleaved by HinfI enzyme to release the 'protection fragment' of 9bp with an overhang of 3 bases. The remaining 72 bp with overhang was the half-piece of the final 147 bp palindromic product. The smaller 'protection fragment' was removed by passing the entire HinfI digested sample through a Mono-Q column. A two-step increase in the NaCl gradient (20 mM Tris 7.5, 0.1 mM EDTA) separated the two fragments well (Figure 3.5). The 9bp with 3 base overhang eluted at the 550 mM NaCl point, while the 72 bp with 3 base overhang started to elute at the 620 mM NaCl point.

Figure 3.5 FPLC and DNA PAGE result of 72 bp fragment purification. The pink line in the chromatography graph indicates the injection time point; the brown line represents the ionic strength; the green line shows the percentage of the buffer ‘B’ component; the blue line is the OD254 reading. In the PAGE picture, from left to right, the 4 lanes are (1) 100 bp ladder as marker. (2) *HinfI* digested 84mer before purification. (3) 9 bp ‘protection’ fragment. (4) 72 bp fragment.

The ligation reaction using T4 DNA ligase was performed to put the 72+3 bp pieces together. This step was challenging, since it was difficult to push the ligation reaction yield to 100%. However, by optimizing conditions, we were able to push the final yield to over 97%. Table 3.2 summarizes the enzymatic reaction conditions involved in DNA preparation. A last round of MonoQ purification was performed to separate the product and the unligated half fragments. The 72 bp with 3 base overhang eluted at the 620-635 mM NaCl point, closely followed by the 147 bp end product, as shown in Figure 3.6.

Reaction	Buffer and DNA Concentration	Remarks
Restriction Enzyme Digestion (EcoRV, EcoRI, <i>HinfI</i> , <i>AvaII</i>)	Standard reaction buffer 2 mg/ml DNA substrate optimum for large scale digestion	shaking at 220 rpm for 16 hours at 37 °C
Dephosphorylation (CIAP)	Standard reaction buffer 2 mg/ml DNA and 60 U/ml enzyme	shaking at 220 rpm for 16 hours at 37 °C
Ligation (T4 ligase)	100 mM Tris (pH = 7.6), 20 mM MgCl ₂ , 5 mM dTT, 2 mM rATP, 2 mg/ml DNA, 1000 U/ml ligase	Incubation for 1 day at room temperature, followed by 16 hours at 4 °C

Table 3.2 Summary of reaction conditions during DNA preparation.

Figure 3.6 Purification of 147 bp fragment by FPLC and DNA PAGE. The injection time point in the chromatography graph indicated by a pink line; the ionic strength represented by the brown line; the percentage of the buffer ‘B’ component illustrated in green; the OD254 reading shown in blue. Analysis by PAGE, from left to right, (1) 100 bp ladder as marker. (2) Ligated samples before purification. (3) The content of 72 bp peak. (4) The content of 147 bp peak.

Not all our palindromic DNAs were prepared by employing EcoRV and HinfI enzymes. Due to sequence compatibility, alternative restriction enzymes were used to process other DNA constructs. Table 3.3 summarizes information on the usage of restriction enzyme in all the DNA preparations.

DNA construct (number of semi-repeats within each plasmid)	1 st restriction enzyme to release the repeat	Enzyme concentration	2 nd restriction enzyme to expose ligation site	Enzyme concentration
DNA147 (24)	EcoRV	300 U/mg	HinfI	300 U/mg
DNA146 (24)	EcoRV	300 U/mg	EcoRI	400 U/mg
DNA145 (24)	EcoRV	300 U/mg	HinfI	300 U/mg
DNA146b (16)	EcoRV	250 U/mg	EcoRI	400 U/mg
DNAp147 (16)	EcoRV	250 U/mg	AvaII	2500 U/mg
DNAp145 (16)	EcoRV	250 U/mg	AvaII	2500 U/mg

Table 3.3 Restriction enzymes used during DNA preparation. Digestion conditions were as recommended in the standard protocol.

3.3 NCP Reconstitution

Purified homogeneous histone octamer and DNA fragment were added into a reconstitution solution (10 mM DTT, 2 M KCl) in equimolar ratio according to calculation (Protocol 3.2). The equilibrated reconstitution solution was then transferred into dialysis tubing and subjected to NaCl gradient dialysis against a salt free NCP dialysis buffer (TCS buffer: 20 mM Tris, pH 7.5, 1 mM EDTA, with additional KCl in units of Mole/L; Figure 3.7).

Initially, it was difficult to pinpoint the optimal ratio, due to the error in concentration measurement of histone octamer mother stocks. Thus, a few test buttons were set up to calibrate the DNA/octamer ratio, before large scale experiments were conducted. For both test and large-scale reconstitutions, the initial mix contained ~ 5.5 μ M of DNA and ~5 μ M octamer.

X μ l	DNA stock
Y μ l	octamer stock
4 μ l	DTT
100-(Y/4) μ l	4 M KCl
96 - X - Y*3/4 μ l	water

Protocol 3.2 Calculations for a 200 μ l NCP reconstitution. Note that the sequence of adding the ingredients should follow from the bottom up.

Figure 3.7 Simplified cartoon showing the set up of gradient dialysis (the darker the color of the buffer the higher the salt concentration).

Extra caution was taken when the KCl concentration dropped to the 0.45 – 0.25 M range (corresponding to a 20–27 hour time point, Figure 3.8), during which white precipitant might appear. This reversible ‘pellet’ would ultimately disappear at the TCS 0 stage. The reconstituted NCP was further purified by magnesium chloride precipitation, if necessary. Excess DNA was effectively removed by precipitating the NCP using 10 mM MgCl_2 and discarding the supernatant. This divalent driven precipitation was reversible and had no negative effect on NCP quality, as shown in Figure 3.9. The precipitated NCP was resuspended in buffer containing 10 mM EDTA. This method was developed based on a similar protocol which was used to purify nucleosome array.

Figure 3.8 KCl concentration plot during a typical 2 day machine-pump reconstitution. The machine pumping speed setting was 18 hours 1/20 beaker volume per hour, followed by 8 hours 1/15 beaker volume per hour, and 15 hours 1/10 beaker volume per hour. If the procedure started at 6 pm on day one, then at about 9 am day three the pumping stage would be over, and the [KCl] would be lower than 70 mM. Direct change of the beaker buffer to TCS 0 for another 3 hours would complete the reconstitution process. *Masterflex* machine and size 13 tubing was used.

Figure 3.9 Mg precipitation-based purification of reconstituted NCP shown on a 5% DNA PAGE. **Lane:** 1. Reconstituted NCP sample before purification; 2. Resuspended pellet; 3. The supernatant fraction after Mg precipitation; 4. NCP standard with bands corresponding to both centered and off-centered forms.

3.4 Gel Electrophoresis

Both native and denaturing polyacrylamide gels were used to verify specific chemical and structural properties of DNA. Native DNA polyacrylamide gel electrophoresis was utilized throughout our projects to identify the size and purity of DNA/NCP populations. Urea denaturing gels were used mostly for chemical DNA sequencing, which revealed the chemical modifications of DNA. SDS PAGE enabled the analysis of individual protein components and cross-linked species. Agarose gels were used for visualizing large DNA fragments and plasmid DNA (summarized in Table 3.4).

In native PAGE, about 3-5 μ l DNA/NCP ($\sim 5 \mu$ M) samples containing 10% sucrose were loaded into each well. Gels were stained with ethidium bromide (EtBr, 1 μ g/ml) solution for 5 min and rinsed a few times with distilled water to remove nonspecifically bound EtBr. DNA was visualized using the *GeneSnap* gel-doc system. Finally the program *Genetools* was used to analyze the images.

Denaturing DNA PAGE required a much larger set up (21 X 50 cm), compared to the 10 X 15 cm plates used in native DNA PAGE. After 1 hour of pre-running at 1700 V, samples were loaded. The gel was kept hot at 55 $^{\circ}$ C during the 'actual' run. After taking off the gel from the plate the gel was briefly fixed (by 5 % methanol, 5 % acetate) and subjected to vacuum drying. The dried gel was placed in

the cassette facing a Bio-Rad K film. The K film, which absorbed radioactive signals was developed using phosphorimager (Bio-Rad).

For SDS PAGE, 2-mercaptoethanol was occasionally required in the loading buffer to suppress disulfide bond formation in the protein samples. These gels were stained with Coomassie Blue staining solution (0.025 % Coomassie Blue in 40 % methanol, 7 % acetate) for 20 minutes, and destained with water overnight.

Main Ingredient	Mono : Bis	Buffer	Running Condition	Application
5-6 % acrylamide	40 : 1	0.25 X TBE	110 Volt, 70 min	NCP, DNA longer than 30 bp
10-12 % acrylamide	40 : 1	1 X TBE	150 Volt, 30-40 min	DNA longer than 10 bp
24 % acrylamide	40 : 1	1 X TBE	170 Volt, 150 min, 4 °C	5 bp < DNA < 30 bp
0.1 % SDS, 10 -18 % acrylamide for resolving, 5 % for stacking	60 : 1 for resolving, 20 : 1 for stacking	300 mM Tris pH8.8 for resolving, 125 mM Tris pH6.8 for stacking	30 mA per plate running, 30 – 60 min	Proteins, crosslinked complex having protein components
0.5 – 2 % agarose	N. A.	1 X TAE	100 Volt, 30 – 60 min	Large DNA fragment, plasmid
10 % acrylamide, 7 M urea	29 : 1, premix	0.5 X TBE	55 – 65 Watt, 110 min	Maxam-Gilbert Sequencing

Table 3.4 Electrophoresis protocols. 1 X TBE contains 89 mM Tris, 89 mM Boric acid and 1 mM EDTA. 1 X TAE has 40 mM Tris, 20 mM acetate and 1 mM EDTA. 1 X SDS running buffer has 25mM Tris, 200 mM Glycine and 1 g per liter SDS. All PAGE gels have 0.1 % APS (ammonium persulfate) and 0.1 % TEMED (N, N, N', N'-tetramethylethylenediamine) as a common ingredient. The same buffer was used as the running buffer for all the gels, except for SDS PAGE (1 X SDS running buffer).

3.5 Platination

For Exo III digestion assays, 20 μM DNA or NCP in a buffer of 20 mM K-Cacodylate (pH 6.0) was incubated with 100 μM cisPt or 200 μM oxPt at room temperature for 48 hours. NCP samples (5 μM) in a buffer of 20 mM K-Cacodylate (pH 6.0) were treated with different Pt:NCP stoichiometry for 48 hr at 4 $^{\circ}\text{C}$ and subjected to heat-shifting to assess propensity for repositioning. Similarly, DNA and NCP samples (5 μM) treated for 24 hr or 48 hr were used in AAS experiments.

3.6 Nucleosomal DNA Extraction

The interaction between histones and DNA is mainly electrostatic in nature. High salt concentrations disrupt the NCP and cause release of the DNA. Incubating NCP samples under a series of buffers with a range of salt concentrations (50 mM – 2 M) was used to assess the stability of the NCP structure (with histone variants/modifications or an alternative DNA sequence). On the other hand, incubating NCP samples under high salt (e.g. >1.5 M NaCl) and high temperature (50-60 $^{\circ}\text{C}$) conditions for 1 hour denatured the majority of the material, allowing separation of histones from the DNA. Further phenol/chloroform extraction and ethanol precipitation was used to purify nucleosomal DNA in this respect.

3.7 Atomic Absorption Spectroscopy

The graphite furnace mode of a Hitachi Z-2000 Polarized Zeeman Atomic Absorption Spectrophotometer was used to measure the precise Pt concentration in designated samples. A default program designed for Pt element detection was adapted (40 seconds 80-110 $^{\circ}\text{C}$ drying, 20 seconds 800 $^{\circ}\text{C}$ ashing, 10 seconds 2700 $^{\circ}\text{C}$ atomizing). Each sample was diluted to such an extent so that it had ~ 0.01 nM (20 μl X 0.5 μM) Pt element when manually loaded into the reaction chamber. For completely unknown samples, trial runs were performed, since the concentration measurement was accurate only to ~ 0.01 nM. Pt drug stock was pre-calibrated with $\text{PtCl}_4(\text{NH}_3)_2$ standards based on AAS reading, while the working stock of Pt drugs was used to prepare the standard curve. Finally, the Zeeman polarized absorbance

was compared to the plotted standard to calculate the Pt concentration of the unknown diluted samples. As shown in Figure 3.10, the standard curve was linear. The AAS graphite furnace mode was ideal for platinum concentration determination.

Figure 3.10 The working interface of Z-2000 AAS graphite furnace mode.

3.8 Thermally-Induced Nucleosome Repositioning

A. Repositioning PAGE

In this assay, nucleosomes at a less energetically favored position slide along the DNA and reside at another more preferred position, after moderate heat treatment (37 to 60 °C) for a duration ranging from 15 minutes up to 60 hours. The positioning of the NCP was probed by PAGE, based on the different migration rates of these two conformational isomers (Figure 3.11).

Figure 3.11 Electrophoretic migration of naked and nucleosomal DNA (a) 1. 100bp DNA marker; 2. DNA146b; 3. DNA 147; 4. NCP146b with two positions; 5. NCP147 with two positions; 6. NCP146b fully heat shifted to centered form (50 °C for 1hour); 7. NCP147 fully heat shifted to centered form (37 °C for 20 minutes). Note that DNA146b is rigid and kinked, and displays a retarded mobility on gel. (b) View of the NCP with the particle pseudo two-fold axis running vertically through the middle (DNA, colored; histone proteins, grey). The centered (bottom) versus off-centered (top) forms of NCP differ in positioning by a single turn of the double helix.

B. Sample preparation

NCP material resting at off-centered positioning site of the DNA was a starting point to monitor the repositioning dynamics. Thus, a 4 °C reconstitution was generally employed to generate NCP majoratively trapped at the less favored position. After the reconstitution of ‘mispositioned’ NCP, samples could be stored for up to 3 days at 4 °C.

C. Heat treatment and evaluation

Before performing the heat treatment, ‘mispositioned’ NCP aliquots of 2-3 μl were placed at the bottom of 600 μl tubes. During the heat treatment, these tubes were submerged completely under water to prevent sample evaporation. Tubes were taken

out and left on ice immediately after the heat treatment, before placing them at 4 °C. DNA PAGE was used to evaluate the samples, according to protocols in Section 3.4.

Densitometric analyses were carried out using the program *Gene Tools* and *Quantity One*. The *Gene Tools* program can automatically recognize the lanes and banding pattern once the image is loaded. Then the total intensity inside each banding box was taken as a useful indicator for assigning band intensity. Background subtraction was performed by manually designating another background box. Most types of images were compatible with this program, including TIF, BMP and raw image format.

Figure 3.12 The working interface of *Gene Tools*.

In *Quantity One* program, after defining the band, a wide range of parameters were defined to interpret the data in multiple ways. Additionally, the intensity of each individual lane or multiple lanes could be chosen to plot, tracking the profile of given samples. Usually, peak intensity was used to represent the intensity. In this case, additional background correlation was also required.

Overall, since the DNA PAGE stained with EtBr had less background features, it was easier to make use of EtBr stained gels to estimate the proportion of bands in different lanes. However, because the free DNA was over represented in EtBr staining,

Coomassie Blue stained DNA PAGE showed more accurate proportions of band intensity within each lane.

Figure 3.13 Band detection in *Quantity One*.

Figure 3.14 Multiple density definitions in *Quantity One*

Quantity One provided more band analysis options, which made it less compatible with other image formats, unfortunately. Users have to manually define the lanes and the bands, in order to control all the tunable sensitivity and size parameters.

3.9 Endonuclease Digestion Assay

Endonuclease digestion can differentiate ‘platinated’ sites that block enzyme activity from lesion-free sites. Naked DNA samples were desalted and purified by one additional round of ethanol precipitation. Nucleosomal DNA was extracted according to the extraction protocol. Then these samples were subjected to specific restriction enzyme digestion reactions, as stated below:

DNA (2.5uM)	5µl
10X reaction buffer	5µl
restriction enzyme (10unit/ul)	2.5µl
water	37.5 µl

Protocol 3.3 Diagnostic restriction enzyme digestion conditions.

Incubation conditions varied according to properties of the enzymes in use. For complete digestion, sometimes over night incubation was required. After digestion, the reaction mix was directly loaded onto the DNA PAGE apparatus to examine the digestion products. The figures in the results section demonstrate fully how we conducted this assay to examine the differences between nucleosomal and naked DNA platination distributions.

3.10 Exonuclease III Footprinting

Exonuclease III (Exo III) footprinting is an assay based on the phenomenon that the exonuclease enzyme stops and dissociates at certain DNA regions with abnormal conformation or permanent adducts. Exo III is an enzyme that non-processively cleaves the double-stranded DNA from 3’ to 5’ direction. At 37 °C, a minimum DNA fragment of 12bp is required for the Exo III in order to bind. This

exonuclease also exhibits other activity including 3'-repair diesterase, AP DNA-repair endonuclease and 3'-phosphomonoesterase. Exo III has a 2-fold symmetric and 4-layered $\alpha\beta$ fold structure. Similar to DNase I, the exonuclease performs a hydrolytic reaction. The interaction of the enzyme and the substrate DNA involves a long section (55 Å) of DNA.

Figure 3.15 Cartoon showing how different Exo III fragments were generated. X represented adduction site. Platinated DNA blocked the Exo III and resulted in DNA fragments with varying lengths. By comparing to a modified Maxam-Gilbert purine marker (3' dephosphorylated, otherwise marker bands run ~one half-base faster on gel), one is able to determine the sites of platination.

A. Sample preparation

NCP and naked DNA (20 μM) samples (both platinated and controls) were diluted to 5 μM . The NCP samples were extracted as described above. Addition of glycogen helped to push up the yield of ethanol precipitation in case of a lower concentration DNA. Naked DNA samples were treated in the same way to provide more relevant controls.

Extracted DNA samples were then resuspended in TE0.1 (10mM Tris pH7.5, 0.1 mM EDTA) and further adjusted to 2 μM in concentration. T4 PNK was used to radioactively label the 5' end (Protocol 3.5). 5'-labeled DNA samples were then

purified using dyeEx2.0 (Qiagen) spin columns spun at 800 g for 3 minutes. Labeled DNA samples were then subjected to Exo III digestion.

Figure 3.16 Sequencing gel results of Exo III digested naked DNA.

DNA (2 μ M)	15 μ l
10 X PNK buffer	3 μ l
PNK (10 units/ μ l)	1 μ l
γ^{32} P-ATP (10 μ Ci/ μ l)	1 μ l

1 hour incubation at 37 °C

Protocol 3.4 T4 PNK 5' radioactive labeling reaction

DNA (1.5 μ M, ~135 μ g/ml)	5 μ l
10*NEB buffer 1	5 μ l
Exo III (100 units/ μ l)	2.5 μ l
MilliQ water	37.5 μ l

50-65 minutes at 37 °C or room temperature

Protocol 3.5 Exo III digestion

In order to stop the reaction, 25 μ l 3X digestion stop buffer (0.9 M Sodium acetate, 0.09 M EDTA) was added to the reaction system, immediately followed by addition of 190 μ l pre-chilled 100% ethanol. After a 10 minute incubation on ice, DNA fragments were spun down (12k rpm, 3 minutes). The invisible pellet was further rinsed with 70% cold ethanol. Precipitated DNA could be either directly dissolved in

1X loading dye for evaluating the digested but un-deplatinated samples, or in 1 M thiourea buffer in order to deplatinate the DNA fragments. The radioactivity signal was diminished to approximately one half after Exo III digestion.

Figure 3.17 Overnight thiourea treatment removes platinum adducts.

DNA fragments	~ 300 ng
Aqueous thiourea 1M	200 µl

Overnight incubation at 58 °C

Protocol 3.6 Deplatination

At the end of the deplatination reaction, 30 µl of 3 M NaAc was added to the reaction system, followed by 750 µl of cold ethanol. After 10 minute incubation on ice, the samples were spun at 12000 rpm, 3 minutes in order to precipitate the DNA fragments. Additional 70% ethanol rinse was optional at this point. Samples were then dissolved in 1X denaturing DNA gel loading dye, heated for 3 minutes at 95 °C, chilled and spun down.

B. Preparation of G+A marker

A Maxam-Gilbert G+A reaction marker was used to help identify the digested fragments. Unlike the usual Maxam-Gilbert protocol, an extra 3'-dephosphorylation of the marker was performed to ensure coherent migration with the exonuclease digested and deplatinated fragments. Naked DNA, with exactly the same sequence as the platinated samples, was 5' labeled with ³²P as described above. Later on, this labeled DNA was treated with formic acid to modify all the purine sites (Protocol 3.7).

Formic acid reaction		Formic acid stop buffer
DNA (100 µg/ml)	10 µl	0.3 M NaAc
Formic acid 80%	25 µl	0.1 M EDTA
		RNA (25 µg/ml tRNA/RNA salt)

The length of incubation was 6-8 min depending on the region of interest within segment at room temperature.

Protocol 3.7 Formic acid fragmentation

Two hundred µl of formic acid stop buffer was added into the system to quench the reaction, followed by immediate addition of 750 µl cold ethanol. After a 10 minute incubation on ice, DNA was spun down and resuspended following the

protocol, as described above. An optional 70% ethanol rinse was performed to get rid of the salt contaminants.

Two hundred μl of 10% (v/v) piperidine was added into the tube to dissolve the DNA pellet. The same tube was incubated for 45 minute at 95 °C to induce the fragmentation of the modified DNA. The reaction was again stopped by double ethanol precipitation.

A 1X loading dye was used to solublize the normal Maxam-Gilbert DNA purine markers. However, the DNA marker fragments obtained still contain 3'phosphate groups, which do not co-migrate with the Exo III-generated deplatinated samples, thus the DNA marker samples were resuspended in TE0.1 and subjected to 3'dephosphorylation (Protocol 3.9).

Figure 3.18 Comparison of Maxam-Gilbert purine markers before and after 3'dephosphorylation by 10% denaturing PAGE.

As usual, this last reaction was stopped by ethanol precipitation, and 1X denaturing DNA loading dye was used to dissolve the final DNA marker (heated, chilled and spun down).

DNA (~50 µg/ml)	10 µl
10X PNK Buffer	5 µl
PNK (10 units/µl)	2.5 µl
MilliQ water	32.5 µl

16 hour (complete) at 37 °C

Protocol 3.8 3' Dephosphorylation

3.11 Crystallization of NCP

NCP crystallization was achieved exploiting the salting-in phenomenon at medium Mn^{2+} concentrations (30-40mM). Crystals of reconstituted NCPs (with different DNA sequences) were grown at 18 °C by the hanging drop vapor diffusion method over wells containing reservoir buffer (35-43 mM MnCl_2 , 23-30 mM KCl, 20 mM K-Cacodylate pH6.0). A volume of 5 µl precipitation solution (140-170 mM MnCl_2 , 90-120 mM KCl, 20 mM K-Cacodylate) was mixed with an equal volume of NCP solution (8 mg/ml). Crystals with a size of approximately 0.2 X 0.3 X 1.0 mm appeared after about 2 weeks as the hanging drop swelled. Mature crystals were stabilized and dehydrated gradually in stabilizing buffer (with additional 24% MPD and 2% trehalose, compared to reservoir buffer) before further buffer exchange and diffraction analysis.

Two methods of preparing 'platinated' NCP crystals were tested; soaking platinum derivatives solution into pre-formed NCP crystals and direct crystallization of adducted NCP samples. Specific stabilization buffer with additional ligands was added into the pre-harvested crystals to perform designated soaking experiments. For extended soaking experiment, at least 600 µl buffer was used for individual soaking, in order to minimize damage of the crystals resulting from evaporation. 600 µl lockable microtubes (Axygen, CA, USA) filled with harvesting/soaking buffer were used for transporting crystals. Exchanges with fresh NCP crystal storage buffer removed the excess platinum chemicals in the soaking solution and stopped the reactions. Pre-treated NCP samples were crystallized under similar conditions as the

native NCP samples. Unreacted platinum agents were removed before setting up crystallizations.

Figure 3.19 Image of NCP147 crystals.

3.12 X-ray Diffraction, Data Processing and Structure Solution

NCP crystals were mounted as described before (Davey et al, 2002). The diffraction intensities of the datasets reported here were collected on a charge-coupled device detector or a PILATUS detector on the PXI beamline at Swiss Light Source, Villigen, Switzerland. Integration, scaling and merging of the intensities were carried out using programs MOSFLM and SCALA from the CCP4 suite.

In order to process the PILATUS data, specific command lines had to be used to exclude ‘blind’ regions on the image plate, as shown in Protocol 3.3.

```
DETECTOR PILATUS
DIVERGENCE    0.001    0.001
DISPERSION    0.00020
GAIN 0.87
ADCOFFSET    0
NULLPIX      -1
```

```

LIMITS EXCLUDE      0.0 338.8 434.6 340.2
LIMITS EXCLUDE      0.0 253.8 434.6 255.3
LIMITS EXCLUDE      0.0 168.8 434.6 170.2
LIMITS EXCLUDE      0.0  83.9 434.6  85.2
LIMITS EXCLUDE 398.2   0.0 401.3 423.6
LIMITS EXCLUDE 361.7   0.0 364.8 423.6
LIMITS EXCLUDE 325.2   0.0 328.3 423.6
LIMITS EXCLUDE 288.8   0.0 291.9 423.6
LIMITS EXCLUDE 252.3   0.0 255.4 423.6
LIMITS EXCLUDE 215.9   0.0 219.0 423.6
LIMITS EXCLUDE 179.4   0.0 182.5 423.6
LIMITS EXCLUDE 142.9   0.0 145.9 423.6
LIMITS EXCLUDE 106.5   0.0 109.6 423.6
LIMITS EXCLUDE  70.0   0.0  73.1 423.6
LIMITS EXCLUDE  33.5   0.0  36.6 423.6

```

Protocol 3.9 MOSFLM command lines prior to load the PILATUS images.

After starting Mosflm 7.0.1 (only this version works for PILATUS data), these command lines were pasted to the Mosflm prompt, followed by pressing enter. The wavelength and beam center information could be incorporated into these initial command lines manually, if necessary. This was followed by spot identification and auto indexing (note that you also have to manually key in the oscillation range for the first image used for spot identification). Cell unit refinement based on two sections of images was performed, in which at least three images were included. After saving the refined space group and unit cell information, this information was pasted to the executive command lines, for example 'comm_cis145_19h_1mM'. Lastly, this command file was executed by typing in @comm_cis145_19h_1mM in Mosflm prompt line (full content listed in Protocol 3.4).

```

#
DETECTOR PILATUS
WAVELENGTH 1.07030
DIVERGENCE 0.001 0.001
DISPERSION 0.00020
BEAM 215.37 212.04
GAIN 0.87
ADCOFFSET 0
NULLPIX -1
DISTANCE 519.230
DISTORTION YSCALE 1.0005 TILT 45 TWIST -15
MATRIX 145_cis19_1m_1_00_091.mat
!This matrix was obtained from postrefinement
!using 2 segments starting with images 20 50
SYMMETRY 19
MOSAIC 0.43
TEMPLATE 145_cis19_1m_1_00###.cbf

```

```

RASTER      15    13    11      3      3
SEPARATION  1.0  1.0
OVERLOAD CUTOFF 3538100
LIMITS EXCLUDE      0.0 338.8 434.6 340.2
LIMITS EXCLUDE      0.0 253.8 434.6 255.3
LIMITS EXCLUDE      0.0 168.8 434.6 170.2
LIMITS EXCLUDE      0.0  83.9 434.6   85.2
LIMITS EXCLUDE 398.2      0.0 401.3 423.6
LIMITS EXCLUDE 361.7      0.0 364.8 423.6
LIMITS EXCLUDE 325.2      0.0 328.3 423.6
LIMITS EXCLUDE 288.8      0.0 291.9 423.6
LIMITS EXCLUDE 252.3      0.0 255.4 423.6
LIMITS EXCLUDE 215.9      0.0 219.0 423.6
LIMITS EXCLUDE 179.4      0.0 182.5 423.6
LIMITS EXCLUDE 142.9      0.0 145.9 423.6
LIMITS EXCLUDE 106.5      0.0 109.6 423.6
LIMITS EXCLUDE  70.0      0.0   73.1 423.6
LIMITS EXCLUDE  33.5      0.0   36.6 423.6
PROCESS 1 TO 360 START 0 ANGLE 0.5
POSTREF OFF
SPOTALL
HKLOUT Cis145_19h_1m_360.mtz
GO
RUN
END
eof
#

```

Protocol 3.10 The content of the command file, 'comm_cis145_19h_1mM'.

More options could be added to the command file with the aim to improve the integration, for example, adjusting the box size by changing the RASTER values, or replacing the POSTREF OFF command by PROFILE NOOPTIMISE command to enable partial optimization, or restricting the resolution by giving RESOLUTION values.

NCP structures were solved by molecular replacement with the program Amore from CCP4 package (Collaborative, 1994), using NCP147 (1kx5) as a starting model. Automated refinement was carried out using REFMAC5, and manual model building was executed with Coot (Emsley & Cowtan, 2004). In order to reduce noisiness of anomalous difference electron density maps, 0.1-0.3 Å was trimmed from the highest resolution data for map calculations. Initial phase determination was obtained through molecular replacement procedures. In model refinement, for example in optimizing the DNA conformation in Pal147, regional omit maps and site mutagenesis functions of COOT were used. Pymol and COOT were utilized to generate figures for further analysis and presentation.

3.13 Oligonucleotide Alkylation and Purification

A. Introduction

In order to obtain a high resolution structure of ENA (N-epoxymethyl-1,8-naphthalimide) bound to DNA oligos, we had to first derivitize and purify oligos.

Figure 3.20 Chemical structure of ENA

As both an intercalator and an alkylating agent, ENA is a hydrophobic chemical with very poor water solubility. Hence, DMSO was used in the preparation of a high concentration ENA:DNA (1 mM, 1:1) reaction system to prevent ENA precipitation. DNA oligos in solution were pre-treated by the addition of about 10-20% DMSO in TE0.1. Then, 10 mM ENA stock solubilized in 100% DMSO was added. Finally, the addition of a 5-10% DMSO TE0.1 solution was required to dilute the DMSO concentration down to no higher than 20% (DMSO concentrations above 20% were inhibitory to the reaction). Reactions took place at room temperature over ~48 hours. Beyond this time, the reactivity of ENA appeared to be negligible due to inactivation by hydrolysis.

B. Mass spectrometric analysis

Samples of oligonucleotides were diluted (final concentration > 50 μ M) in water. Five to 10 μ l of the diluted sample was sufficient for preparing each measurement spot for MALDI-TOF mass-spectroscopy. In order to get sharp peaks, the 3-HPA matrix was used during sample immobilization.

C. HPLC purification

ENA adducted DNA oligos were subjected to 3-5 minutes of centrifugation at 14.5 krpm in order to eliminate the insoluble unreacted ENA. Then samples were either auto or manually injected into the HPLC column (Jupiter 300, C18). Different programs with varying lengths were used to purify ENA-oligo complexes (Table 3.5).

Time	6 b	8 b	10 b	12 b
0 min	2 % B	2 % B	2 % B	2 % B
2 min	2 % B	8 % B	8 % B	8 % B
25 min	92 % B	70 % B	60 % B	50 % B
30 min	92 % B	92 % B	92 % B	92 % B
35 min	2 % B	2 % B	2 % B	2 % B
36 min	End	End	End	End

Table 3.5 HPLC protocols used for ENA-oligo purification. Buffer A constituted 100 mM triethylamine acetate (TEAA, pH 7.5, made by titrating TEA with acetic acid). 0.05 % TFA in acetonitrile constituted buffer B (with an optional 100 mM TEAA). The column was heated by the Shimadzu HPLC system up to 48 °C throughout the procedure.

RESULTS AND DISCUSSION

CHAPTER IV

DERIVATIZATION OF DNA

4.1 Reactivity of Platinum Drugs

Understanding how platinum anticancer drugs differentially recognize naked *versus* nucleosomal DNA is the first step toward deciphering platinum drug site selectivity. The overall platination reactivity of different Pt compounds was determined by AAS. For each measurement, 5-20 pg of Pt were required. The number of average adducts per DNA/NCP was generated by triplicate AAS readings. The intra-batch (same substrate and drug, multiple readings) standard deviation of the AAS experiments were about 7%, and the inter-batch (different batches of raw materials and drugs) standard deviation was found out to be on average 11%. The variations came from fluctuations in the lamp intensities of nanodrop as well as AAS (affecting DNA concentration measurement and Pt concentration reading, respectively), errors in pipetting and sample qualities.

A full scale analysis based on AAS results was done to investigate the differences of CisPt and OxPt reactivity towards several NCP and DNA samples, at different stoichiometries. DNA extracted from platinated NCP was less adducted compared to directly platinated naked DNA. In Figure 4.1, apparently from the raw number of average hits per DNA, the difference was not pronounced (extracted DNA from adducted NCP was merely 15-20% less platinated). This difference could be explained by two factors: first, some of the Pt drugs were scavenged by the histone components of the NCP; second, the presence of histone elements influenced the DNA conformation so that certain potential adduction sites were hindered from reacting with Pt agents. The first hypothesis was supported by the fact that almost 40% OxPt in platinated NCP were found on the histone components, in the case of CisPt, this figure was ~10-15%, depending on the drug ratio.

Figure 4.1 Plot of platinum drug adducts per DNA/NCP molecule (Y-axis) at different stoichiometries. Experiment was conducted at room temperature.

Previously, there were arguable reports regarding the reactivity of OxPt compared to CisPt. Some *in vivo* studies showed that the difference in reactivity between the two drugs was not significant, approximately 2-fold (Ta et al, 2006). However, other groups reported that the difference obtained from room temperature *in vitro* systems was roughly 3- to 5-fold. Interestingly, our 4 °C adduction experiments yielded a 19-fold difference in terms of drug reactivity. None of the above observations was incorrect, since OxPt activation was found to be temperature dependent (Figure 4.2), while CisPt reactivity was less sensitive to temperature variations. Overall reactivity of CisPt was reduced by only 20-30% from room temperature as compared to 4 °C. The reduction was more than 60-70% for OxPt.

Figure 4.2 Plot of number of platinum drug adducts per DNA/NCP molecule (Y-axis) at different temperatures.

Thus, other than the nucleosomal DNA, methione and histidine residues in histones could be potential sites for platination as well. In particular, the reversible proteins adducts probably serve as *in vivo* platinum reservoirs (Reedijk, 1999). Nevertheless, the degree of overall preference of DNA over histones was demonstrated by our AAS result.

4.2 Distribution of Platinum Adducts on DNA

Information on the overall reactivity of Pt agents towards different substrates does not provide details of platinum–nucleosome site selectivity. A base-resolution Pt adducts distribution on naked and nucleosomal DNA can provide much more insight. This method can reveal enhanced platination sites inside the nucleosome, which are a result of their unique nucleosomal structural environment.

There were several assays we could choose from to map the adduction sites. Chemical/thermal fragmentation-, polymerase- and nuclease assays were among the possible options. However, since platinum adducts do not introduce a formal positive charge into the base ring system, chemical/thermal fragmentation assays, such as those used to map alklylation events, are not functional. Furthermore, the DNA sequences we used were palindromic, rendering the reannealing procedures in polymerase assays unsuitable. Nuclease based assays were the most appropriate tools to study the base-resolution of platinum adduct distribution on DNA in solution.

Figure 4.3 Illustration of endonuclease digestion assay. (a) Restriction site map of DNA147. (b) On adducted nucleosomal DNA, a HinfI restriction enzyme recognition site was protected by Pt crosslinking. (c) The same site on naked DNA was less adducted, thus the restriction site was more exposed and susceptible for digestion. Red square indicate the adduction sites. The gels adjacent to the illustrations show the samples before (left lanes) and after (right lanes) digestion.

A. Endonuclease assay

Restriction enzyme digestion was used previously to differentiate the adduction products of different DNA sequences (Cohen et al, 2002). This assay yielded rather useful results about differences in the platination profiles of distinct DNA species (Figures 4.3 and 4.4). DNA147 contains a centrally positioned *Hinf*I site, two adjacent *Alu* sites and another two *Pst*I sites at the terminal regions (Figure 4.3). As shown in Figure 4.4, platinated nucleosomal DNA had stronger protection at the *Hinf*I site and weaker at *Alu* sites. The enzymatic digestion was roughly the same at the *Pst*I sites. There were notable minor differences with regard to relative protection against restriction digestions between *Cis*Pt and *Ox*Pt.

Figure 4.4 Restriction enzyme digestion results of different ‘platinated’ samples (a) 2-hour long *Hinf*I digestions of 4 samples. From left to right: control unadducted Nuc DNA147, 5:1 *Cis*Pt adducted naked DNA, 5:1 *Cis*Pt adducted Nuc DNA, 10:1 *Cis*Pt adducted Nuc DNA. (b,c) Overnight digestion of (b) 5:1 *Cis*Pt adducted DNA samples and (c) 50:1 *Ox*Pt adducted DNA samples: 1. control undigested Nuc DNA; 2. digested control DNA; 3. adducted naked DNA; 4. digested platinated naked DNA; 5. adducted Nuc DNA before digestion; 6. platinated Nuc DNA after digestion.

It appears that conclusions should be drawn cautiously when interpreting the results of this assay. The restriction digestion could only indirectly reflect the structural differences in DNA. For example, some of the Alu digestion results were found contradictory to other observations. Platination at locations nearby the restriction sites could affect the efficiency and the fidelity of the endonuclease, making the analysis more complicated. Moreover, this assay was limited to a few suitable restriction digestion sites present in the DNA constructs. Additionally, either over-digestion revealing no differences in site protection, or partial digestions showing 'false' differential protection confused the interpretation. Thus, endonuclease digestion assay provided some insightful information in this case, but it was not appropriate for systematic examination of the distribution of DNA lesions. A more comprehensive method had to be employed to determine the distribution of platinum adducts on DNA.

B. Exonuclease assay

To assess the differences in adduct formation between nucleosomal and naked DNA upon Pt treatment, the Exo III assay was a more suited technique. The scheme of the exonuclease assay is summarized in Figure 3.16. In order to make a systematic comparison, samples with equal radioactive counts were loaded onto the gel.

Exo III is not active on a single-stranded DNA substrate, but it should digest duplex DNA having minimally about 20 bp, depending on sequence. Thus, it may be possible that the enzyme fails to digest even long fragments that are largely single-stranded and have only small complementary sections. This might occur if extensive digestion occurs roughly equivalently on both ends of a duplex DNA. However, as shown in Figure 4.5, as long as the digestion duration and enzyme concentration were sufficient, Exo III was capable of digesting all DNA substrates down to the last 10-15 bp.

Figure 4.5 Real and artifactual residual DNA fragments after Exo III digest. Artifactual OxPt adduct sites (red parentheses) showed the same on-gel mobility in sample 5 and 6. Blue arrows point to the examples of authentic OxPt adducts.

(1). DNA sequence specific platination in nucleosome

An Exo III digestion assay of CisPt- and OxPt-adducted naked and nucleosomal DNA was performed first. The fragmentation patterns of the different samples were marked and compared (Figure 4.6). We found that certain regions of nucleosomal DNA were relatively more adducted compared to the naked form. The results suggested that different drugs had slightly varying targeting preferences, which might account for their differential pharmacological properties. In the absence of any other cellular factors, nucleosomal DNA was found to display differential susceptibility to platinum chemicals (Wu et al, 2008).

Moreover, more in-depth Exo III digestion analysis of samples with several different DNA sequences and Pt analogs provided more details. These highly related sequences were used to demonstrate the structural and functional consequences of relative nucleosomal positioning, while minimizing the influence of local DNA

sequence changes. At the same time, a less related DNA146b was used, with the aim to illustrate the structural commonalities of nucleosomal DNA with different sequence contents.

The number of the agents used in the study was increased to 5, to also include (SP-4-2)-diammine[1,1-cyclobutanedicarboxylato(2--)-O,O']platinum (II) (CarPt), trans-1,2-diamminocyclohexane-dichloro-platinum (II) (DACH-Pt) and triammino-monochloro-platinum (II) (TamPt). CarPt and TamPt are analogs of CisPt, whereas DACH-Pt is an analog of OxPt, with different pharmacological effects. CarPt was demonstrated to have very mild toxicity, but on the other hand, it required a very high dosage for the desired effect. As the precursor of OxPt, DACH-Pt (provided by our collaborators) is potent in terms of cellular toxicity, but unfortunately suffers from extremely low water solubility. TamPt (provided by our collaborators) has theoretically very low therapeutic effects, because of only a single free reactive site present in its structure. Consequently, crosslinking is unlikely to occur in this case, making TamPt a useful chemical for negative control purposes. The chemical structures of these analogs are shown in Figure 4.7.

Figure 4.6 Exonuclease footprinting analysis revealing drug-specific differences in platination site-selectivity between naked and nucleosomal DNA. NCP147 and naked DNA147 were treated with CisPt or OxPt (color-coded), followed by end-labeling of the purified DNA and Exo III digestion. Red arrow denotes central bases. **(a,b,c)** Denaturing PAGE of exonuclease-treated DNA samples shows digest termination sites resulting from encounter of platinum adducts. **(a)** Overall footprint and resolved sections corresponding to the **(b)** central and **(c)** 3' regions are shown. **(d)** DNA sequence for 79 of 147 bp. Regions where the DNA minor groove faces inward, towards the histone octamer, are colored orange. Exonuclease stop sites are depicted as arrowheads adjacent to the terminal 3' nucleotide, pointing towards the apparent platinum adducts. Filled symbols indicate relatively strong and open symbols moderate termination points.

Figure 4.7 Chemical structures of platinum chemicals used in Exo III assay sequencing gel analysis. (a) CisPt, pattern can be found in Figure 4.8 (b) CarPt, Figure 4.12 (c) TamPt, Figure 4.10 (d) OxPt, Figure 4.9 (e) DACH-Pt, Figure 4.13.

Platination of DNA or NCP was achieved by directly adding platinum compounds to the samples. Chloride ion and acetate were avoided when preparing the reaction solutions, while cacodylate, phosphate and sulfate solutions were shown to be compatible with platination activity. The incubation was done under different temperatures (4–37 °C), avoiding exposure to a strong light source. The reaction was stopped by ethanol precipitation (DNA samples) or magnesium precipitation (NCP samples), allowing removal of unreacted platinum agent. Some of the Pt drugs displayed a very low water solubility, such as DACH-Pt (< 200 μM at room temperature), whereas CisPt showed a temperature dependent solubility (< 3 mM at 4 °C, while > 5 mM at 20 °C).

Based on the new data, we were able to assign the adduction sites down to a single base pair. Almost all the drug treated samples, with an exception of TamPt, naked and nucleosomal DNA, shared similar distributions of platinum adducts. The

overall similarity between naked and nucleosomal DNA samples indicated that the adduction preferences of the platinum chemicals were largely determined by the intrinsic DNA sequence. In certain regions of nucleosomal DNA, specific enhancements in terms of the percentage in the total adductions were observed, for example, in the dyad region (base -3) and SHL -1.5 site (base -16) of NCP147 (areas colored in pink in Figure 4.8 & 4.9). At the sites of differential selectivity, histone elements apparently played a more significant role. The extensive presence of positively charged histone tails and side chains make the DNA near the dyad region least accessible. The DNA at SHL -1.5/1.5 sites has extensive interactions with histones as well.

Not surprisingly, TamPt treated samples had much fewer fragments after digestion compared to crosslinking platinum chemicals (shown in Figure 4.10), even in the presence of a high Pt:DNA ratio (confirmed by AAS). Indeed, under normal degrees of enzymatic digestion, only a few exonuclease resistant sites were detected. An overall three-fold weaker digestion condition had to be employed to detect sites of mono-adduction.

Figure 4.8 Sequencing gel of radioactive-labeled CisPt adducted DNA samples digested by Exo III. The red arrow indicates the approximate dyad location. From left to right: (1) Maxam-Gilbert purine marker of DNA145; (2) CisPt 5:1 naked DNA145; (3) CisPt 5:1 nucleosomal DNA145; (4) Maxam-Gilbert purine marker of DNA146a; (5) CisPt 5:1 naked DNA146a; (6) CisPt 5:1 nucleosomal DNA146a; (7) Maxam-Gilbert purine marker of DNA147; (8) CisPt 5:1 naked DNA147; (9) CisPt 5:1 nucleosomal DNA147; (10) Maxam-Gilbert purine marker of DNA146b; (11) CisPt 5:1 naked DNA146b; (12) CisPt 5:1 nucleosomal DNA146b. Regions showing clear site selectivity differences are highlighted in pink. Stop site pairs on opposing strands corresponding to likely GC-interstrand crosslinks are highlighted in blue, green and yellow.

Figure 4.9 Sequencing gel of radioactive-labeled OxPt adducted DNA samples digested by Exo III. The red arrow indicates the approximate dyad location. From left to right: (1) Maxam-Gilbert purine marker of DNA145; (2) OxPt 20:1 naked DNA145; (3) OxPt 20:1 nucleosomal DNA145; (4) Maxam-Gilbert purine marker of DNA146a; (5) OxPt 20:1 naked DNA146a; (6) OxPt 20:1 nucleosomal DNA146a; (7) Maxam-Gilbert purine marker of DNA147; (8) OxPt 20:1 naked DNA147; (9) OxPt 20:1 nucleosomal DNA147; (10) Maxam-Gilbert purine marker of DNA146b; (11) OxPt 20:1 naked DNA146b; (12) OxPt 20:1 nucleosomal DNA146b. Regions showing clear site selectivity differences are highlighted in pink. Stop site pairs on opposing strands corresponding to likely GC-interstrand crosslinks are highlighted in blue, green and yellow.

Figure 4.10 Sequencing gel of radioactive-labeled TamPt adducted DNA samples digested by Exo III. The red arrow indicates the approximate dyad location. From left to right: (1) purine marker of DNA145; (2) TamPt 5:1 naked DNA145 digested by Exo III (half enzyme concentration and two-thirds incubation duration relative to the standard condition*); (3) TamPt 5:1 adducted nucleosomal DNA145 digested by Exo III*; (4) purine marker of DNA146b; (5) TamPt 5:1 naked DNA146b digested by Exo III*; (6) TamPt 5:1 nucleosomal DNA146b digested by Exo III*; (7) purine marker of DNA145; (8) TamPt 5:1 naked DNA145 digested according to standard condition; (9) TamPt 5:1 nucleosomal DNA145 digested according to standard condition; (10) purine marker of DNA146b; (11) TamPt 5:1 naked DNA146b digested according to standard condition; (12) TamPt 5:1 nucleosomal DNA146b digested according to standard condition.

(2). the chemical nature of the Exo III stop sites

Since monoadducts did not inhibit the Exo III digestion significantly, this indicates that the majority of the bands in sequencing gels represented crosslinks at those sites. Most of the intrastrand crosslinks resulted in one or two bands in series at the first or second purine of a crosslinked purine-purine bp step. Another study which used Exo III to verify the presence of a synthetically designed intrastrand CisPt crosslink demonstrated this phenomenon as well (Ober & Lippard, 2008). However, several bands that appeared in the vicinity of GC elements were unlikely to be intrastrand crosslinked sites. Further investigations suggested these outliers corresponded to interstrand crosslinks.

In the denaturing gels of Figure 4.11, a fraction of the undigested platinated DNA samples were found to migrate much slower than a typical single stranded species (145-147 nucleotides). To verify the identity of these bands, palindromic DNA145 was heat-denatured and flash-cooled to convert it into a hairpin conformation. Using adducted hairpin (Figure 4.11b, lane 2) as a reference point, we confirmed that the bands with retarded on-gel mobility (highlighted in colors) were in fact interstrand crosslinked DNA, as opposed to potentially conformationally constrained intrastrand crosslinked DNA.

Some of the upper bands in the DNA146b and DNAp147 samples appeared to be much weaker than those of DNA145 (Figure 4.11, bands highlighted in blue). As such, they likely represent interstrand crosslinking at SHL 0.5 and SHL 1.5 sites, since DNA146b and DNAp147 lack GC elements at these locations. This assignment was also consistent with the conclusion of a previous study on mitomycin C-adducted short DNA oligomers, where the least mobile band corresponded to the most centrally crosslinked isomers (Millard et al, 1991). Following the same logic, one of the upper bands can be assigned as the SHL 2.5 GC-interstrand crosslinked species, which is indeed a common motif among the three different sequences (colored in green). The relatively faster migrating bands (in yellow) appeared to be the two conformational isomers (cis and trans) of a SHL 5.5 interstrand crosslinked population.

Figure 4.11 Undigested radioactive-labeled DNA samples analyzed on denaturing sequencing gels revealing interstrand crosslinking. The slower migrating species represent interstrand crosslinking at different locations. (a) 1 and 10 are unadducted control DNA145; 2 and 3 are CisPt 10:1 naked and nucleosomal DNA145, respectively; 4 and 5 are OxPt 20:1 naked and nucleosomal DNA145, respectively; 6 and 7 are CarPt 200:1 naked and nucleosomal DNA145, respectively; 8 and 9 are TamPt 10:1 naked and nucleosomal DNA145, respectively. (b) 1 is control DNA 145; 2 is hairpin DNA145 crosslinked by CisPt 10:1 ratio, thus showing only 'internal interstrand' cross-linked species, and anything larger than these bands should be an actual interstrand crosslinked DNA; 3 and 4 are CisPt 10:1 naked and nucleosomal DNA145, respectively; 5-12 contain alternating naked and nucleosomal samples of CisPt 10:1, OxPt 20:1, CarPt 200:1 and TamPt 10:1, respectively, of the DNA146b construct; 13 is control DNA146b. (c) 1 and 2 CisPt 10:1 naked versus nucleosomal DNA145; 3 is control DNA145; 4-11 contain alternating naked and nucleosomal samples of CisPt 5:1, OxPt 10:1, CarPt 200:1 and DACH-PT 10:1, respectively of the DNAP147 construct. The interstrand-crosslinked species at SHL 1.5, SHL 2.5 and SHL 5.5 regions are highlighted by blue, green and yellow highlighted areas, respectively. Apparently, conformational isomers of SHL 5.5 crosslinked population resulted in two populations with different on-gel mobility.

(3). carrier group dominates the site selectivity of platinum drugs

Notably, the Exo III digested CarPt- and DACH-Pt-treated samples resemble to a high degree the patterns of those treated with CisPt and OxPt, respectively. Not surprisingly, the structures of the corresponding platinum derivatives reveal a commonality of the chemical nature in the carrier groups (Figure 4.7). The electrostatic and Van der Waals interactions between the carrier group elements and the surrounding bases (sometimes even deoxyribose groups) inevitably influence the sequence preference in drug binding. Similar conclusions were reached by comprehensive molecular modeling analysis of the interactions between the carrier groups and the adjacent DNA features (Jamieson & Lippard, 1999; Pilch et al, 2000).

Additionally, a few unexpected favored adduction sites were identified, such as the dyad GGAA(T)T motif of NCP147. The central GA step of this DNA section was not considered to be a favourable platination site in previous studies. Computational modeling suggested that the chemical elements determining priority of the 3' purine mono-adduction played similar roles to discourage formation of a GA crosslink (Mantri et al, 2007). However, the primary site preference of platinum chemicals could be affected by the complex nucleosomal environment in the immediate vicinity. Positively charged histone side chains nearby and altered DNA configuration could potentially explain the differences observed with naked DNA studies. With more detailed structural information on the elements governing selectivity, we hope to be able to design new drugs with predictable site selectivity in the context of chromatin.

Figure 4.12 Sequencing gel of radioactive-labeled CarPt adducted DNA samples digested by Exo III. The red arrow indicates the approximate dyad location. From left to right: (1) purine marker of DNA145; (2) CarPt 200:1 naked DNA145; (3) CarPt 200:1 nucleosomal DNA145; (4) purine marker of DNA146; (5) CarPt 200:1 naked DNA146; (6) CarPt 200:1 nucleosomal DNA146; (7) purine marker of DNA147; (8) CarPt 200:1 naked DNA147; (9) CarPt 200:1 nucleosomal DNA147; (10) purine marker of DNA146b; (11) CarPt 200:1 naked DNA146b; (12) CarPt 200:1 nucleosomal DNA146b. Regions showing clear site selectivity differences are highlighted in pink. Stop site pairs on opposing strands corresponding to likely GC-interstrand crosslinks are highlighted in blue, green and yellow.

Figure 4.13 Sequencing gel of radioactive-labeled DACH-PT adducted DNA samples digested by Exo III. The red arrow indicates the approximate dyad location. From left to right: (1) purine marker of DNA145; (2) DACH-PT 5:1 naked DNA145; (3) DACH-PT 5:1 nucleosomal DNA145; (4) purine marker of DNA146; (5) DACH-PT 5:1 naked DNA146; (6) DACH-PT 5:1 nucleosomal DNA146; (7) purine marker of DNA147; (8) DACH-PT 5:1 naked DNA147; (9) DACH-PT 5:1 nucleosomal DNA147; (10) purine marker of DNA146b; (11) DACH-PT 5:1 naked DNA146b; (12) DACH-PT 5:1 nucleosomal DNA146b. Regions showing clear site selectivity differences are highlighted in pink. Stop site pairs on opposing strands corresponding to likely GC-interstrand crosslinks are highlighted in blue, green and yellow.

4.3 Structures of Platinated NCPs

Exo III digestion assay revealed the platination site distribution on nucleosomal DNA. However, this assay only produced a limited map without structural details of the lesions. Thus, X-ray crystallographic investigation was conducted to provide critical insight to understand the site selectivity of platinum derivatives.

Initial X-ray structural analysis was performed based on several datasets collected in 2007 (Table 4.1). Since the presence of Mn^{2+} ions in the crystallization stabilizing buffer was necessary to preserve the crystal diffraction quality, we kept the Mn concentration at 10 mM. Thus, a cross comparison of signals at both the Mn edge (1.893 Å) and Pt edge (1.072 Å) was done to unambiguously confirm the identities of the metal atoms (see Table 4.2). Anomalous X-ray scattering by platinum atoms was utilized as the key reference to assign platination sites. As shown in Figure 4.14, the relative anomalous signal of Mn is ~ 6.7 -fold lower than Pt at the Pt edge, whereas it is only ~ 2.4 -fold lower at the Mn edge.

In the next phases of data collection in 2008 and 2009, 11 new datasets (Table 4.3) were collected, utilizing NCP145 and NCP146b crystals, which demonstrated much better tolerance towards Pt treatment compared to NCP147. Furthermore, only datasets at the Pt edge were collected, since previous experiments demonstrated that comparison of the F_o - F_c difference and the anomalous maps was sufficient to confirm the identity of the atoms. Generally, confirmed Pt atom sites display a much higher anomalous compared to difference peak.

Figure 4.14 The anomalous scattering coefficient plot of Pt and Mn.

Figure 4.15 X-ray diffraction image of NCP146b

A. Distribution of platination sites in crystals

In the NCP147 datasets, several DNA and histones Pt adducts were clearly identifiable. Consistent with previous biochemical studies, most of the sites on DNA were at 1,2 GG and AG steps. Anomalous peaks were situated between the N7 atoms of two purine bases, strongly indicating a crosslinking between the two bases. Furthermore, an unexpected 1,2 GA crosslink near the dyad (b -2) was identified in the CisPt, as well as OxPt treatments, in agreement with the Exo III digestion assay result. In addition, a 1,3 GTG (b +62) crosslink was apparent in CisPt treated NCP. The free DNA terminal adenine was almost fully platinated by OxPt, likely due to the steric freedom of the terminal base (Figure 4.16a, b, c).

Data Collection ^b		untreated ^a	oxPt-1d	oxPt-2d	cisPt-1d	cisPt-2d	cisPt-3d
Space group		P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions							
<i>a</i> (Å)		106.1	106.1	106.3	106.1	106.3	106.9
<i>b</i> (Å)		109.7	110.0	109.7	109.4	109.4	109.3
<i>c</i> (Å)		181.5	182.6	181.8	181.0	180.6	180.1
Resolution (Å)		94-3.40	110-3.41	110-3.45	109-3.41	109-3.45	109-3.97
<i>R</i> _{merge}		(3.58-3.40) ^c	(3.59-3.41)	(3.64-3.45)	(3.59-3.41)	(3.64-3.45)	(4.18-3.97)
<i>I</i> / <i>σI</i>		8.1 (15.4)	8.5 (49.0)	7.1 (48.5)	8.0 (49.5)	7.6 (48.5)	7.3 (49.8)
Completeness (%)		23.1 (13.1)	18.4 (3.6)	20.0 (3.9)	18.8 (3.1)	20.7 (4.3)	18.0 (3.2)
Redundancy		99.3 (100)	99.3 (100)	98.9 (100)	99.7 (100)	99.1 (100)	99.4 (100)
		7.1 (7.3)	7.3 (7.3)	7.3 (7.5)	7.3 (7.3)	7.3 (7.5)	7.2 (7.4)
Refinement							
Resolution (Å)		94-3.40	94-3.41	94-3.45	94-3.41	94-3.45	93-3.97
No. reflections		28,960	28,969	27,702	28,698	27,518	18,309
<i>R</i> _{work} / <i>R</i> _{free}		25.0 / 29.5	33.7 / 39.9	33.9 / 43.5	32.0 / 37.7	33.0 / 40.2	33.2 / 43.2
No. atoms							
Protein		6269	6269	6269	6269	6269	6269
DNA		6021	6021	6021	6021	6021	6021
Ion		1	1	1	1	1	1
B-factors							
Protein		23	111	146	114	140	201
DNA		66	175	238	196	232	301
Ion		6	84	80	59	84	113
R.m.s. deviations							
Bond lengths (Å)		0.008	0.008	0.008	0.009	0.008	0.008
Bond angles (°)		1.34	1.35	1.28	1.36	1.25	1.19

^a Abbreviations for data sets: native NCP (untreated) and one-, two-, or three-day cisPt or oxPt treatments (-1d, -2d, -3d)
^b Single crystal data; X-ray wavelength=1.072 Å
^c Values for highest-resolution shell are shown in parentheses.

Table 4.1 X-ray diffraction data collection and refinement statistics.

Site	untreated ^a $\Delta Pt/\Delta Mn/id.$ ^b	oxPt-1d $\Delta Pt/\Delta Mn/id./oc.$ ^c	oxPt-2d $\Delta Pt/\Delta Mn/id./oc.$	cisPt-1d $\Delta Pt/\Delta Mn/id./oc.$	cisPt-2d $\Delta Pt/\Delta Mn/id./oc.$	cisPt-3d $\Delta Pt/\Delta Mn/id./oc.$
-73	<u>A</u>	11.9/4.1/Pt/0.73	7.9/4.6/Pt/0.79			
-73'		13.7/6.6/Pt/0.83	9.2/5.6/Pt/0.76			
64/65	TGGA	4.7/ /Pt/<0.1	4.7/ /Pt/0.10			
64/65'		4.3/ /Pt/0.21	(3.7)/ /Pt/0.35			
62/63	GTG					4.2/ /Pt/**
61/62	AGGT	5.2/19.7/Mn	/4.5/Mn	/4.8/Mn		
61/62'		4.4/16.8/Mn		/4.1/Mn		
60/61	CAGG			4.9/ /Pt/0.24		
-57/-56	CAGA	/5.6/Mn	(3.6)/ /Pt/0.18	5.4/4.3/Pt/0.21		
48/49	TGGT	6.2/16.0/Mn	/4.2/Mn			
48/49'		5.2/15.7/Mn	/4.2/Mn	/5.7/Mn		
-35/-34	TGGA	/10.8/Mn	/4.5/Mn	/4.4/Mn		
-35/-34'		/10.8/Mn	/4.2/Mn	/4.7/Mn		
27/28	AGCA ^d	4.1/16.5/Mn	/4.0/Mn	/4.0/Mn		
24/25	TGGA	/4.5/Mn	4.5/ /Pt/0.21			
24/25'		/4.3/Mn	4.0/ /Pt/0.21			
-3/-2	TGGA	/9.0/Mn	4.1/ /Pt/0.16	(3.7)/ /Pt/0.18		
-3/-2'		/12.4/Mn	5.4/ /Pt/<0.1			
-2/-1	GGA ^A / _T			4.9/ /Pt/0.36	4.6/ /Pt/0.20	
-2/-1'				4.5/4.1/Pt/0.34	4.1/ /Pt/0.26	
H4-M84						
H4'-M84				4.2/ /Pt/0.11	5.0/ /Pt/0.15	4.1/ /Pt/0.11
H3-M120				7.1/ /Pt/0.10	4.6/4.5/Pt/0.19	8.7/4.4/Pt/0.23
H3'-M120				6.5/ /Pt/0.20	9.6/4.9/Pt/0.21	10.6/4.4/Pt/0.19
H2B-V45 ^d						
H3'-D77 ^d						
		9.3/32.7/Mn	6.8/11.6/Mn	8.3/14.3/Mn	7.1/16.4/Mn	4.5/12.5/Mn

^a Abbreviations for data sets: native NCP (untreated) and one-, two-, or three-day cisPt or oxPt treatments (-1d, -2d, -3d)
^b Bijvoet difference Fourier peak heights (in sigma) were calculated using phases from the refined models (Suppl. Table 1 & 2). Values listed are 4σ or greater, with the exception of reference sites common to related compounds, where peaks above 3.5σ are shown in parentheses.
^c Occupancy value estimates (oc., %) for platinum atoms incorporated into test models were refined with B-factors remaining within 10% of those for the DNA/protein ligands. Values can be exaggerated by partial occupancy of Mn²⁺ at sites on the DNA common to both metal ions. Due to uncertainty in conformation, occupancies could not be calculated for the GTG cross-link site (**).
^d Mn²⁺ ions involved in interparticle contacts. An apostrophe distinguishes between the two pseudosymmetry-related, halves of the NCP.

Table 4.2 Anomalous difference peaks at platinum and manganese X-ray absorption edges.

Treatment conditions												
Data Collection												
Space group	Untreated	CisPt	TamPt	OxPt	DACH-Pt	Untreated	CisPt	TamPt	OxPt1	OxPt2	OxPt3	DACH-Pt
Resolution (Å)	NCP145 control	NCP145 1 mM 19 hours	NCP145 2 mM 55 hours	NCP145 0.6 mM 57 hours	NCP145 0.1 mM 46 hours	NCP146b control	NCP146b 1 mM 19 hours	NCP146b 2 mM 55 hours	NCP146b 0.6 mM 14 hours	NCP146b 0.6 mM 25 hours	NCP146b 0.6 mM 57 hours	NCP146b 0.1 mM 71 hours
	P212121	P212121	P212121	P212121	P212121	P212121	P212121	P212121	P212121	P212121	P212121	P212121
Rmerge	91.29 - 2.50 (2.64 - 2.50)	51.03 - 2.85 (3.00 - 2.85)	58.42 - 2.65 (2.79 - 2.65)	61.2 - 2.95 (3.11 - 2.95)	48.85 - 2.5 (2.64 - 2.5)	92.85 - 2.55 (2.69 - 2.55)	52.34 - 2.5 (2.64 - 2.5)	58.52 - 2.7 (2.85 - 2.7)	105.41 - 3.0 (3.16 - 3.0)	54.72 - 2.5 (2.64 - 2.5)	57.45 - 2.6 (2.74 - 2.6)	46.32 - 2.3 (2.42 - 2.3)
I / σI	5.9 (40.6)	9.0 (47.1)	4.8 (43.7)	5.7 (44.0)	7.0 (44.6)	7.9 (43.0)	5.9 (36.4)	7.5 (47.0)	6.9 (25.4)	4.6 (34.8)	6.1 (31.8)	5.5 (46.9)
Completeness (%)	21.6 (3.1)	20.1 (3.1)	26.0 (2.1)	30.1 (2.0)	22.8 (2.8)	17.5 (2.6)	21.4 (2.4)	19.8 (1.8)	23.3 (7.8)	29.0 (2.0)	18.4 (1.8)	22.0 (2.2)
Redundancy	99.6 (97.3)	99.9 (100.0)	88.4 (55.6)	92.8 (66.6)	96.2 (80.0)	91.6 (60.4)	99.6 (97.9)	96.4 (81.7)	89.6 (55.2)	89.1 (58.8)	93.5 (69.8)	98.3 (89.0)
	6.9 (4.8)	5.9 (6.1)	5.3 (3.5)	5.3 (3.9)	5.9 (5.6)	6.5 (4.5)	5.4 (3.4)	4.9 (2.7)	7.3 (7.2)	5.0 (2.7)	4.5 (2.4)	5.7 (4.6)
Refinement												
Resolution (Å)	91.29 - 2.50	51.03 - 2.85	58.42 - 2.65	61.2 - 2.95	48.85 - 2.5	92.85 - 2.55	52.34 - 2.5	58.5 - 2.7	92.85 - 3.0	54.72 - 2.5	57.45 - 2.6	46.32 - 2.3
No. reflections	72054	49540	53670	41252	70061	60540	70475	53327	36217	61625	57610	89364
Rwork/Rfree	23.6/27.0	22.1/28.0	26.0/27.6	27.3/29.9	24.2/27.8	23.4/26.2	22.5/26.7	24.9/30.2	23.9/28.3	25.4/27.7	26.0/28.1	24.5/28.5
No.atoms	12202	12018	12042	12042	12018	12413	12018	12012	12012	12012	12012	11996
B-factors	64.07	99.075	95.354	123.608	75.854	70.538	86.107	85.593	73.704	78.945	90.86	79.204
R. m. s. deviations												
Bond lengths (Å)	0.009	0.015	0.01	0.011	0.018	0.009	0.02	0.01	0.009	0.01	0.01	0.021
Bond angles (°)	1.42	2.013	1.48	1.332	2.156	1.432	2.261	1.447	1.416	1.469	1.433	2.188

Table 4.3 Statistics summary of platinated NCP145 and NCP146b datasets (the values in parentheses are from the highest resolution shell).

Figure 4.16 X-ray crystallographic analysis revealing DNA (CisPt, 1/5-7; OxPt, 1-4/8/9) and histone (Met120, Met84) platinum adduct positions in the NCP147. The central bp at the NCP pseudo two-fold symmetry axis is denoted by a red arrow. An apostrophe indicates the opposing NCP half. Anomalous difference maps, contoured

at 4σ (a), 4.1σ (b), or 3.4σ (c) and superimposed on the refined models for 48-hour OxPt (a,b) and CisPt (c) treatments, show platinum atom locations (black mesh). (a) View of a thin slice of the NCP. DNA strands are colored orange/cyan and histone proteins blue (H3), green (H4), yellow (H2A), and red (H2B) (a,e). (b) An OxPt-AG adduct. (c) CisPt adducts at GA and methionine in the NCP centre. (d) DNA sequence and adduct sites for 79 of 147 bp. Bases with major versus minor groove facing inward towards the histone octamer are colored, respectively, black (d,e) and orange (d) or white (e). (e) Summary of CisPt and OxPt adduct sites, with approximately one NCP half shown. Methionine sites are circled, whereby large arrows show corresponding locations in the opposing NCP half. (f) Model of an OxPt adduct at the NCP centre. An OxPt-GG adduct from the oligonucleotide crystal structure was modified to an OxPt-GA adduct and superimposed onto the OxPt1 site adjacent to the central bp in the high resolution NCP crystal structure. The platinum (magenta) and diaminocyclohexane atoms are shown in space-filling representation to emphasize potential interactions with histone elements, such as H3 lysine 115 and 115' (black arrows).

On the histones, the methionine sulfur was found to be a platination hotspot. H3-M120 was preferably attacked by CisPt, while H4-M84 was more heavily adducted by OxPt (Figure 4.16a).

The more recent data at much higher resolution ($2.5\text{\AA}$ - $3.1\text{\AA}$) confirmed the earlier findings, and further highlighted more than 50 potential adduction sites for different drugs. The higher resolution diffraction was apparently a result of the two 145/146 bp NCP crystal types being more able to accommodate platination-induced distortion to the DNA. In Figure 4.17, when comparing different derivatives, the TamPt adducts were the least site specific, and co-localized to the sites preferred by other agents, except for the methionines near the dyad. On the other hand, bifunctional platinum drugs (CisPt and OxPt) with different carrier groups share mainly the histone methionines as targets. In agreement with the Exo III assay, different Pt chemicals favored distinct DNA motifs. Due to limitations of experimental data, the exact factors determining the differences in Pt drug site selectivity were not completely obvious. Steric access to the purine-N7 atom, based on DNA conformation and site-specific occupancies of Pt, appears to be an important factor (work in progress). In a few cases, we could see a few sites that were differentially platinated by the OxPt and DACH-Pt treatment, which is not obvious from the Exo III assay analysis. As we discussed in the previous section, mono-adducts were bypassed by the digestion assay frequently, while they gave strong signals in crystallographic investigation. The differential mono-adduct distribution

map provided an indication of how the mono-hydrated leaving group intermediate influences the site preference of Pt drugs.

Figure 4.17 Anomalous density of TamPt (pale blue), CisPt (red) and OxPt (green) at 3.7σ for derivitized NCP146b crystals. Except for the dyad methionines (black arrows), most of the CisPt and OxPt peaks overlap with those of TamPt.

B. Interstrand crosslinking in nucleosome

Apart from the intra-strand adducts, a few possible inter-strand crosslinked regions were also identified. As shown in Figure 4.18, in the SHL 0.5 region, all the CisPt, OxPt and DACH-Pt anomalous signals were concentrated in the middle of two guanines at a GC base pair. This bp step displays a positive slide, which brings the two guanine N7 atoms in closer proximity. Such positive slide configurations are specifically promoted by minor groove bending at the minor groove-inward locations

in the nucleosome. This phenomenon was largely unnoticed in previous studies, because the biochemical assays which had been used, such as hydroxyl fragmentation, extensive DNase I digestion or polymerase stop assay could not differentiate interstrand from the intrastrand crosslink configurations.

Figure 4.18 Anomalous densities superimposed at a possible interstrand crosslink site (I chain -5, I chain -4 of NCP145). Density of the 2Fo-Fc map of NCP145-DACH-Pt dataset, contoured at 1.5σ is colored in pale violet. The CisPt anomalous density contoured at 3.2σ is shown in dark blue. Density of the anomalous map of -DACH-Pt treated sample contoured at 3.1σ is in yellow. Density map of anomalous signal at 3.2σ of the OxPt treated sample is in pink. The model shown was refined based on the NCP145-DACH-Pt dataset.

The SHL 0.5 GC site was also detected by the Exo III assay as a possible interstrand crosslink, although its nature could not be confirmed. The crystallographic data unambiguously demonstrated that it is an interstrand crosslink in the nucleosome. However, this crosslink does not appear to be nucleosome specific, since the Exo III assays showed that platinated naked DNA yields a comparable pattern (Figure 4.11). Nevertheless, in rigid and constrained surroundings, such as in a nucleosome crystal, platinum interstrand crosslinks are still permitted to form.

C. Step-wise formation of 1,2-GG-intrastrand crosslinking

Many of the identified Pt adducts, especially in the briefly soaked crystals were apparently mono-adducts. From the ‘time-lapse’ OxPt soaking analysis, we ascertained a few progressive Pt crosslinking events. For example, as seen in Figure 4.19, in the 12-hour time point dataset, OxPt first platinated the I(-53) Guanine. Then as the incubation continued, there was a new anomalous signal which appeared in the middle of the I(-53/-52) GG bp step. This signal was further strengthened in the longest soak. For the first time, the stepwise crosslinking hypothesis of the Pt adduction was directly demonstrated by a series of X-ray structures. The expected large conformational change due to the transition from mono-adduct into a crosslink at the GG site was suppressed by the highly compact nucleosomal DNA bp stacking. The same observation was reported in a previous NMR study, where the 5’ end purine was found to be preferably platinated at the initial stage. However, the half-life of the mono-adducted species in our study is longer than what was previously estimated (P. J. Sadler, 1996), probably due to the presence of a more structurally constrained environment in NCP, which restricted the molecular dynamics.

Figure 4.19 Overlaid density maps showing a time-lapse 1,2 OxPt crosslink formation at I(-52), I(-53) site of NCP146b. The 14-hour (red), 25-hour (blue) and 57-hour (green) OxPt anomalous signals contoured at 3.2σ . The Pt crosslink signal in between the GG bases develops over time. Only the 14-hour soaking model is shown, since mixed conformational states made the models of the longer soakings highly distorted.

4.4 ENA Derivatization of Oligo DNA

In order to screen for well diffracting crystals, mono-nucleotides and single stranded oligonucleotides with a length of 6, 8, 10 and 12 base pairs were used to generate duplex DNA or nucleotide that is site specifically alkylated by ENA. Overall, maximally around 5% to 60% of the substrate guanine sites were adducted by ENA, independent of the initial drug to DNA ratio in the reaction system. The reactivity (when ENA was still in excess) and completeness of derivatization seemed to be DNA sequence- and length-dependent. More flexible and longer sequences appeared to be better substrates for ENAlation.

When analyzing dodecamer Oligo3, we noted that unadducted single strand eluted out from the column at 17 minutes (peak A), according to the 12 b program, while the adducted peak appeared 3 minutes later (peak B). Other constructs behaved slightly differently (Figure 4.20). The collected peaks were sent for MALDI-TOF examination, which showed unambiguously clear separation of the native and unadducted oligo DNA (Figure 4.21 and 4.22).

Figure 4.20 Plot of multiple wavelength light absorbance of HPLC elutions, plot of Buffer B gradient during ENAlated oligo3 (12 bp) purification and enlarged view of peak A and peak B. The OD 220 values (magnified 1.8 times) are represented by the pink curve; the OD 260 values are represented by the blue curve; the OD 280 values (magnified 1.8 times) are represented by the brown curve. The first peak is the flow through fraction, where the absorbance only at 220 nm appears to correspond to unreacted ENA.

Figure 4.21 Mass spectrometry result of un-adducted dGMP (upper) and adducted dGMP-ENA (below). 1 is Guanine; 2 is dGMP; 3 is unreacted ENA; 4 is Guanine adducted by ENA; 5 is dGMP-ENA. The presence of guanine may be due to the impurity of the dGMP stock or depurination during laser excitation.

Figure 4.22 Mass spectrometry result demonstrated separation of ENA adducted DNA oligo.

After purification, the double stranded ENA-oligo was evaluated by 24% DNA PAGE (Gel protocol refers to Table 3.4). ENAlated DNA oligo hybridized into the double stranded state under gel running conditions (4 °C), and showed retarded mobility compared to unmodified double-stranded oligo, as demonstrated in Figure 4.23. Thus, high purity ENA-oligo DNA was generated in this regard for use in further applications— in particular crystallization trials.

Figure 4.23 24% DNA-PAGE result of ENAlated DNA (oligo) samples. (a) ENAlated oligo3 before HPLC purification. (b) Peak B, collected, reannealed, precipitated and resuspended. (c) Peak C, collected, reannealed, precipitated and resuspended.

4.5 New Insights on an Old Topic

Platinum drugs as a group of established anti-cancer therapeutics have been on the market for more than four decades. However, the chemical and biological properties of these drugs have been only extensively explored at the chemical and the clinical levels. Modest effort was made to investigate the anti-cancer rationale at the biomacromolecular-chromatin level. In our studies, platinum drugs were found to be quite invasive, nucleosomal DNA was only slightly protected by the nucleosome structure during platination. The experimental results also suggest that these chemicals are capable of discriminating between differences in sequence content and rotational settings of the nucleosomal DNA. The carrier group plays a more

significant role in differential site selectivity, while the leaving group is more related to the initial hydration rate and has limited influence on the site selectivity.

Nucleosomes, as the fundamental functional units in the eukaryotic genome, are relevant as drug targets. Since they possess the structural information which is crucial for genomic processes, it would be impossible for the cancer cells to bypass nucleosomal signatures while preserving regulatory functionality. For example, the absence of a strong positioning nucleosome located right after transcription initiation sites would cause the binding of the RNA Polymerase II complex to the promoter to be severely hampered. In Chapter 2, we described mounting evidence pointing to the fact that platinated nucleosomes could be a vital link in the drug induced apoptotic signaling pathways. Furthermore, as relatively enclosed entities, nucleosomes are also less accessible to the repair machinery. The adducts within the nucleosome boundary could be more lethal due to less efficient detection and repair (Davey & Davey, 2008).

On the other hand, there are more structural features associated with nucleosomal DNA, making it a better target compared to the naked DNA. The characteristic DNA conformation induced by the histone-DNA contacts or the histone elements themselves can be selectively targeted. The dyad region of NCP147 is more heavily adducted compared to naked DNA, probably due to the cooperativity from the local histone elements, while the SHL 1.5 site platination of NCP145 is hindered, likely due to a wider GG bp distance induced by nucleosome-specific stretching.

All the derived structure-function information could ultimately contribute towards rational design of a more gene- or even single nucleosome-specific drug by either improving current Pt drugs or developing completely new genres of small molecules. For example, we could calculate the optimum distance needed between the reactive groups of a multi-functional alkylating agent for targeting different structural features; for instance to create more favorable interstrand crosslinking utilizing site selectivity rules. In another approach, crosslinking of histone components to the nearby nucleosomal DNA could also be a valid choice for locking the nucleosome into a lethal, fixed position.

CHAPTER V

KINETICS OF NUCLEOSOME REPOSITIONING

5.1 NCP Repositioning Fits First Order Reaction Kinetics

Heat-induced repositioning of the nucleosome has been observed *in vitro*. Although the detailed mechanism of this phenomenon is still not fully understood, it is generally agreed that it shares mechanistic commonalities with ATP-dependent nucleosomal repositioning *in vivo* (Becker, 2002). However, since nucleosome repositioning is a rapid, transient and reversible process, probing the mechanistic constants of this activity presents a challenge in experimental design. Using the protocol described in Chapter 3 and homogeneously reconstituted NCP, we were able to collect repositioning information with accuracy and precision. We fitted the empirical repositioning process to a first order reaction kinetic model.

Partially off-centered NCP146b samples prepared at 4 °C were subjected to heat-shifting at three different temperatures. As shown in Figure 5.1, the off-centered population gradually diminished as the heat-treatment continued. The higher the temperature, the faster the repositioning process proceeded. By plotting the natural logarithmic (ln) value of the off-centered NCP portion versus time, we got a roughly linear relationship, as illustrated in Figure 5.2, which fulfills the characteristics of first order reaction kinetics. Thus, independent trials were performed to obtain the figure of the energy barrier. ΔG was found to fluctuate ± 0.2 kcal/mol in figure 5.1, 5.2 & 5.3, from three tests, while this value is ± 0.3 kcal/mol in figure 5.4, from two tests.

In Figure 5.2, the slopes representing the first order reaction rate constant $-k$ were calculated, according to first order reaction formula (1).

$$\ln[\text{NCP}^{\text{off-centered}}] = -kt + \ln[\text{NCP}^{\text{off-centered}}_0] \quad (1)$$

Then since this reaction is spontaneous and no external forces are involved, the reaction rate constant should fit the Arrhenius equation (2).

$$k = A * e^{(-E/RT)} \quad (2)$$

Figure 5.1 Partially off-centered NCP146b repositioned by heating at three different temperatures. Double-stained gels (EtBr stained, upper , Coomassie Blue stained, lower). The number above each pair of gels indicates the duration of the heat-shift for individual sample. The numbers to the right of the gels are the incubation temperature in Kelvin degrees, used in the thermodynamic calculation.

By plotting the $\ln(k)$ value against $1/\text{temperature}$, we obtain ' $-E/R$ ' as the slope, according to calculations in (3).

$$\ln(k_1/k_2) = (-E/RT_1 + E/RT_2) \rightarrow \ln(k_1) - \ln(k_2) = (-E/R) * (1/T_1 - 1/T_2) \quad (3)$$

In Figure 5.3, the $\ln(k)$ values versus $1/\text{temperature}$ were found to indeed fulfill a linear relationship. Based on this plot, we deduced that $-E/R = 15130$. Thus it was straightforward to deduce that E_{146b} was $\sim 15120 * 8.314 = 125.7 \text{ kJ/mol}$ or $\sim 30.0 \text{ kcal/mol}$. The linearity of the Arrhenius plot indicates that the initial phase of heat-induced nucleosome repositioning follows a first order reaction mechanism.

Figure 5.2 The plot of natural log (ln) values of the off-centered NCP portion versus heat-shift duration. The 318 K shifted samples are represented in blue, 323K in yellow and 328K in pink.

Figure 5.3 A plot of ln(k) values from Figure 5.2 versus 1/temperature.

5.2 Rigidity Impedes Nucleosome Dynamics

As illustrated in Figure 5.4, NCP147 appeared to be more mobile than NCP146b with respect to heat-induced repositioning. Based on the Arrhenius Equation, formula (4) was derived from (2a)/(2b):

$k_1 = A \cdot e^{(-E_1/RT_1)}$ (2a) & $k_2 = A \cdot e^{(-E_2/RT_2)}$ (2b)

$k_1/k_2 = e^{(-E_1/RT_1 + E_2/RT_2)}$ (4)

Figure 5.4 Heat-shifting of the off-centered NCP147 over time. A double-stained gel (EtBr stained, upper, Coomassie Blue stained, lower). Plot of the (ln) values of the off-centered portion versus time.

We calculated the repositioning energy barrier for NCP with DNA147, by using formula (4). Based on the values $k_1 = 0.0116$ per minute, $k_2 = 0.1568$ per minute, $T_1 = 318$ K, $T_2 = 310$ K and $E_1 = E_{146b} = 125.7$ kJ/mol, E_{147} was calculated to be ~ 116.0 KJ/mol or ~ 27.7 Kcal/mol. Thus, NCP146b has an energy barrier 2.3 kcal/mol higher than NCP147. Considering the fact that both of the DNA sequences were derived from human α -satellite DNA, and had similar G|C content, it was intriguing that one of them was much more resistant to heat-shifting. By examining the intrinsic flexibility of these two DNA constructs, it was apparent from the slower mobility of DNA146b on native DNA-PAGE gels (Figure 3.11) that this sequence is more rigid and kinked than DNA147.

An obvious interpretation of the above phenomenon can come from considering the dynamic repositioning process. In order to propagate a 10 bp segment of DNA from one end to the other, it requires the nucleosomal DNA to be bent and kinked, to form stressed or twisted intermediates, and to transmit a bulged loop to the opposing terminus (Schiessel et al, 2001; Widom, 1999). Reduced flexibility or increased rigidity would certainly make such processes less energetically favorable. It had been shown that minor groove-binding polyamines are capable of suppressing repositioning processes (Suto et al, 2003), which agrees with our results.

5.3 Platinum Adducts Inhibit NCP Repositioning

Pt anti-cancer therapeutics are well known for their ability to crosslink DNA and induce a local kinked structure. Figure 5.5 shows that CisPt- and OxPt-adducted DNA displays discrete slower migrating species on the gel.

Figure 5.5 Platination resulted in retarded mobility of naked DNA on native PAGE gels. (a) DNA147 extracted from adducted NCP samples from left to right: control DNA; 1.4, 3.2 and 6.4 CisPt adducts per DNA; OxPt to DNA adduct ratio 0.5, 1.1 and 2.3. (b) DNA146b extracted from adducted NCP samples from left to right: control DNA; 1.1, 2.5 and 4.5 CisPt adducts per DNA; OxPt to DNA adduct ratio 0.5, 1.3 and 2.4. Adduct:DNA stoichiometry was determined by AAS.

Figure 5.6 Native PAGE analysis of prolonged heat shifted platinum drug-treated NCPs. Samples were treated with (a) 5:1 CisPt:NCP or (b) 50:1 OxPt:NCP stoichiometry and subjected to thermal incubation at 37 °C (NCP147) or 50 °C (NCP146b) for up to 6 hr (--, before heat treatment). The first time point for NCP146b is 30 min, as opposed to 10 min for NCP147, due to the lower intrinsic mobility of the former. Gels were stained both with EtBr (upper) and Coomassie Blue (lower). (c) Band densitometry analysis of EtBr stained gels in (a) and (b) showing positioning (% off-centered NCP after heat treatment) as a function of incubation time. Note that the upward smearing of centered NCP bands resulting from core particle disassociation upon extended thermal incubation gives rise to an overestimation of the off-centered NCP fraction. Figure 5.5 showed the on-gel migration of the DNA extracted from the samples used in this Figure.

As demonstrated previously (Figure 3.12), DNA with intrinsic curvature generally migrates slower on DNA PAGE gels compared to relatively linear DNA of the same length (Diekmann, 1987; Diekmann & Porschke, 1987). A DNA construct with periodic AAAA motifs, which had an overall accumulated curvature of ~ 180 degrees, would migrate about two thirds the distance as compared to non-curved DNA (Diekmann, 1987). DNA147 had a major adducted population running about 5% slower than native DNA, while platinated DNA146b had two slower migrating species running roughly 10% and 20% slower. This indicated that these DNA species were kinked and locally rigidified after Pt derivatization. A population of platinated DNA147 might have an ~ 30 degree bending, while platinated DNA146b might have two major species with additional ~ 60 degree and ~ 120 degree bending, respectively. In Figure 5.6, CisPt- and OxPt-crosslinked DNA was shown to influence the nucleosome repositioning, even when there was on average less than one adduct per nucleosome. These results demonstrated that extra kinking caused by platination translates to an even higher energy barrier for NCP repositioning.

5.4 TA Motifs in Minor Groove Region Inhibit NCP Repositioning

Restricted DNA flexibility was found to correlate with a higher repositioning energy barrier. Surprisingly, another 147 bp construct containing flexible TA motifs was shown to have an elevated repositioning energy barrier as well. Four TTAAA/TTTAA motifs were incorporated into the DNA147 sequence, creating a new sequence named DNAp147 (sequence shown in Figure 1.3). Figure 5.7 illustrates that the NCPp147 was resistant to heat-shifting at 310 K, in contrast to the original NCP147. The energy barrier derived from the plot indicated that this modified sequence has an energy barrier of ~ 30.7 kcal/mol, higher than that of the NCP147. Both NMR studies and molecular dynamics simulations suggested that TA elements exhibited rather high flexibility in solution (Lankas et al, 2003; Mack et al, 2001). However, after introducing these motifs into the nucleosome, the entire nucleosomal DNA behaved in a more rigid fashion, raising the energy barrier for repositioning. As discussed in Chapter 1, these TA related motifs were found to be statistically key

NCP-positioning factors. It seems that DNA flexibility alone is not the only determinant for NCP positioning and sliding.

Figure 5.7 Repositioning behavior of off-centred NCPp147 **(a)** Ethidium-stained gel of 310 K heat-shifted NCPp147 samples. The seven samples correspond to 0, 5, 10, 15, 20, 30 and 60 minute incubations. **(b)** Plot of $\ln(\text{off-centered})$ values versus incubation time.

5.5 DNA Sequence Dependent Nucleosome Repositioning

The conformational transition from the off-centered to centered forms is a specific case of nucleosome repositioning. However, the conclusions based on the observations about how the structural features of nucleosomal DNA influence the repositioning process still hold their significance, since the majority of the intra-nucleosome histone-DNA interactions remain the same in the *in vivo* nucleosomal state. Furthermore, the short arm/overhang of the off-centered NCP resembles the terminal site exposure of nucleosomal DNA, which is in common with intact nucleosomes having linker DNA (Anderson et al, 2002).

Previously, a competitive reconstitution assay was used to estimate the nucleosome association energy (Thastrom et al, 1999). In that assay, after a quick reconstitution process, the ratio of nucleosomal to naked DNA was estimated. The more DNA incorporated into the nucleosome, the higher the nucleosome affinity was assumed to be. Knowing that nucleosome repositioning might take days to reach equilibrium, the accuracy of this assay was compromised by not considering this process. In contrast to competitive reconstitution assays, with our repositioning experiments we can derive absolute values in terms of the nucleosome-association-related energy.

The energy barrier of nucleosome repositioning is composed of two main elements: the energy state of the initial conformation and the structural penalty for the intermediate state (Figure 5.8). A more stable initial state or a decreased flexibility will lead to an elevated repositioning energy barrier. ATP-driven nucleosome repositioning machines apparently utilize mechanisms analogous to these two principles to achieve efficient and site specific control of nucleosome sliding *in vivo*. One of the two main types of ATP-dependent chromatin remodeling complexes functions by evicting histones, and as a result of destabilizing the nucleosome, elevates the energy state of the initial configuration. The other remodeling complexes act as DNA benders, which create or stabilize bulging DNA loops, and in the end lower the energy state of repositioning intermediates. Our observations might provide insight linking the changes of nucleosomal DNA flexibility and conformation to alterations in nucleosome repositioning behavior, which is a relevant and crucial process in genetic regulation.

In summary, the results of our repositioning assay provided mechanistic insights into the sequence dependent nucleosome sliding. There are two modes by which nucleosomal DNA with specific sequences could induce strong resistance during repositioning. Namely, DNA with certain 8-10 bp motifs, which interact with histone octamer core in a specific way, might lead to a much more stabilized NCP at the initial state. At the same time, DNA with localized rigid motifs, usually spanning 2-5 bp, contribute to additional resistance affecting nucleosomal dynamics. Pt drugs, which tend to crosslink neighboring purines, alter the properties of those localized motifs (2-5 bp), rigidifying the DNA and inhibiting the nucleosome sliding.

Figure 5.8 Schematic illustration of factors proposed to underlie repositioning energy barriers under four different circumstances. In the case of comparing NCP146b and platinated NCP147, the elevated energy barrier of NCP146b is due to reduced DNA flexibility resulting in an intermediate state with a higher energy. In the case of NCPp147 with TA motifs, a more stable initial conformation also makes the repositioning less favorable.

CHAPTER VI

STRUCTURE OF NOVEL PALINDROMIC NCPp147

6.1 Overview of NCPp147 Structure with modified dyad region

NCPp147 (for DNA sequence, refer to Figure 1.3) was assembled, purified and crystallized. The structure revealed a new mode of inter-nucleosome interaction, and demonstrated a structural rationale behind sequence directed nucleosome positioning. Table 6.1 lists the crystallographic statistics of NCPp147. The most distinct feature of these new crystals is that they display the $P2_1$ space group (Figure 6.1), instead of the $P2_12_12_1$ symmetry found in all previous NCP crystals. Nevertheless, this new structure shows the same general conformational characteristics seen in other NCP constructs.

	NCPp147
Space group	$P2_1$
Cell dimensions	
a (Å)	106.747
b (Å)	178.509
c (Å)	110.412
unique angle β (degree)	102.78
Resolution (Å)	60.00 - 2.95 (3.11 - 2.95)
Rmerge	6.9 (47.1)
I / σ I	12.4 (1.5)
Completeness (%)	89.7 (55.6)
Redundancy	3.7 (3.2)
Refinement	
Resolution (Å)	60.00 - 2.95
No. reflections	74471
Rwork/Rfree	22.9/30.0
No. atoms	24335
B-factors	97.265
R. m. s. deviations	
Bond lengths (Å)	0.008
Bond angles (o)	1.345

Table 6.1 Statistics summary of the NCPp147 dataset (highest resolution shell is shown in parentheses).

Figure 6.1 The back, side and top views of P21 crystal packing for NCPp147. Pairs of NCPs in the same asymmetric unit are portrayed in the identical color.

A. The structure of TTAAA/TTTAA elements

In the NCPp147 crystal, the built-in TTAAA/TTTAA regions adopted alternative unique configurations, in response to a more stringent minor groove width restriction imposed by the SHL 1.5 local histone contacts. As shown in Figures 6.2 and 6.3, the width of the minor groove surrounding the SHL 1.5 TTAAA site is much narrower than the original NCP147. Figure 6.3 illustrates the experimentally determined electron density map around the SHL 1.5 region. The density corresponds well with the simulated contoured surface of the refined model. The histone residues (H3 and H4 histone fold) near the narrow minor groove are also shown. The histone elements confine the configuration of the DNA double helix at the anchoring site (sugar clamp). These extensive histone-DNA interactions force the local minor groove width to be extremely narrow.

A|T-rich motifs are known to have an intrinsically narrowed minor groove, which could better accommodate the spatial constraints enforced by the histone contacts at this anchoring point. On the other hand, in the original NCP147 structure, the wide minor groove-preferring G|C-rich sequence can not fit into such a conformation easily, leading to larger negative roll values for groove compression, which would require greater distortion energy.

The differences in energetic penalty associated with reconstituting DNA with different sequences into the nucleosome have a direct relationship to the overall

stability of the nucleosome. Thus, a TTAA section right at the SHL 1.5 site in the off-centered NCPp147 contributes to a more stable conformational state, endowing the NCP with a lower initial energy state. Any sequence alterations at this site can have a great impact on the overall stability and positioning activity of the nucleosome (Fernandez & Anderson, 2007; Thastrom et al, 2004). In the SHL 0.5 region, the built-in TA element was found to take on an alternate conformation relative to SHL 1.5, which is apparently induced by the unique local histone environment. The statistical frequencies of different dinucleotide step types were analyzed to establish that the TA step is clearly the strongest nucleosome positioning sequence element (Thastrom et al, 1999). The narrow minor groove and central flexibility features associated with TTAA motifs were previously suspected to be the reason, as discussed in Chapter 1. Our findings suggest a more definitive and precise structural explanation, and provide support for the existence of strong nucleosome positioning consensus sequences in the genome, such as those selected from *in vitro* experiment.

Figure 6.2 The (I-18)TTTAAA(I-13) region of NCPp147 (red) is superimposed with the same region of NCP147 (blue). The minor groove spanning the TTAA section is much narrower in NCPp147, while the contact points with histone elements remain nearly identical in both structures.

Figure 6.3 2Fo-Fc electron density map contoured at 1.3σ (left) and the DNA (space filling) and histone elements (sticks) cartoon (right) demonstrating the narrow minor groove at the SHL 1.5 site. H3 residues are shown in blue, and H4 in green.

B. An alternative inter-nucleosome interaction mode

In contrast to the classic nucleosome partial stacking, in which the H4 tail rests on another nucleosome's H2A/H2B acidic patch (Figure 6.4), the new structure demonstrates a stronger, more compact stacking (Figure 6.5). In this new configuration, there are more extensive histone-histone interactions as well as histone-DNA interactions between the NCPs within the same asymmetric unit. An H2A-H2B dimer-dimer, plus H3-H4 tetramer-tetramer pairing replace the typical H4-(H2A-H2B) dimer interaction, yielding a much larger contact surface. A similar mode of NCP stacking was seen before in yeast NCP crystals (White et al, 2001). However, due to the presence of a more 'compatible' H2B C-terminal tail in higher eukaryotes, this new *Xenopus* NCP structure illustrates a way to efficiently pack NCPs.

Considering there are multiple ways for eukaryotic nucleosomes to compact (Chodaparambil et al, 2007), and the crystallization of NCPp147 is a spontaneous aggregation process under physiological ionic strength with minimum energy, this structure might resemble an *in vivo* nucleosome interaction mode. The identification of such nucleosome-nucleosome stacking may enhance our understanding of

chromatin dynamics, and help to structurally interpret the effects of some histone modifications. Moreover, we find that a very modest change in nucleosomal DNA sequence can lead to a major difference in the NCP compaction configuration, demonstrating a possible critical role of DNA sequence in the mediation of chromatin organization. This finding might shed light on how nucleosome arrays or even higher order structures are influenced by DNA sequence.

Figure 6.4 The classical NCP-NCP interaction revealed in many previous NCP crystal structures (pdb code 1kx5). (a) The top view (left) and (b) side view (right) of this type of interaction demonstrate an H4 N-terminal tail-H2A-H2B acidic patch interaction mode. (c) The enlarged view of the interaction, the H4 N-terminal tail is shown as a grey flexible coil, while the residues surrounding the tail belong to the H2A-H2B dimer of a neighboring nucleosome.

Figure 6.5 The NCPp147 nucleosome-nucleosome interaction. **(a)** A frontal view of how two nucleosomes stack on each other, in cartoon display. **(b, c)** The back view and the side view, respectively, of the interface (cartoon display). Only one half of each nucleosome is shown for clarity; H2A, pink, H2B, yellow, H3, green, H4, cyan. From the back view **(b)** dimers interact with each other, while the H4 tail of the upper nucleosome has peripheral contacts with the lower tetramer surface.

CHAPTER VII

CONCLUSION AND FUTURE DIRECTIONS

In the past decade, there has been an explosion of information about the histone code (histone modification and related enzymatic complexes, reviewed in Chapter I). Hence, many chromatin researchers invested most of their efforts into unraveling the relationship between histones and nucleosome/chromatin dynamics. Although these studies have provided wealth of information, the biochemical and structural properties of DNA, which are the essential elements of eukaryotic genome, were mostly overlooked.

Our studies demonstrate that the DNA component of the nucleosome is functionally relevant as well. Firstly, the sequence of the nucleosomal DNA can influence the nucleosome-chromatin dynamics. Secondly, the different DNA sequences are selectively attacked by various platinum derivatives. Finally, as a result of site specific platination, the structurally modified DNA motifs exhibit differential effects on nucleosome-chromatin dynamics. In the previous studies, the association of minor groove binding polyamides has been observed to suppress histone octamer-DNA sliding in the nucleosomes (Suto et al., 2003). In addition, substitution of rigid DNA sequence elements into the nucleosome core, such as long poly(A) tracts, also resulted in a decrease in nucleosome mobility (Bao et al., 2006). Our findings on the effect of platinum adducts suggests that DNA cross-linking underlies the reduction in nucleosomes mobility. Furthermore, distinctions in nucleosomal site preference between different agents may be linked to differences in their pharmacological profiles.

In agreement with our conclusions, a recent review (Todd and Lippard 2009) suggested that the less mobile chromatin segment, in terms of nucleosome sliding, would definitely impede the progress of transcription. The retarded nucleosome mobility at the promoter region would also prohibit the initiation complexes from binding to the transcriptional activator binding sites (Kumar and Wigge, 2010). Thus, any abnormal nucleosome mobility and positioning could directly influence genomic progress, mainly due to their crucial roles in transcription.

Exploration of more means to achieve site specific DNA targeting in nucleosome by small molecules is the future direction of our research. The pharmaceutical utility of platinum drugs suffers from their severe side effects. The biochemical assays developed for this platinum project could be utilized to streamline screening of other potential nucleosomal DNA binding chemicals. If we could further demonstrate that the inhibited nucleosomal repositioning is the underlining apoptotic trigger, we could concentrate our efforts on development of other nucleosome-dynamics inhibitors, while bypassing the use of toxic heavy metals. Some follow-up projects in our group involve a design of organic and inorganic agents. The structural investigation of an organic chemical, ENA (mentioned in Section 3.13), suggests that it could intercalate into a nucleosome-specific stretching site, which might interfere with topoisomerase and DNA repair machinery, resulting in an accumulation of supercoiling stress in cells.

A series of *in vitro* transcription and chromatin regulatory assays are expected to be performed in the future in order to pinpoint the detailed events converting the nucleosome repositioning stress into a cascade of apoptotic signals. First of all, the inhibition of nucleosome dynamics could be reproduced, using nucleosome arrays or SV40 minichromosomes as the platforms, in the presence of regulatory complexes (purified homogeneous ATP-dependent chromatin remodeling complexes, histone chaperones or other regulatory factors). As a result, we could understand a lot of valuable underlying principles of chromatin regulation and the roles of individual regulatory factors. For instance, we could determine whether Pt lesions might affect the group of ATP-dependent chromatin remodeling complexes used to bend the DNA more severely than those which exchange the histones.

The comprehensive structural analysis of the NCPp147 and other constructs revealed the basis of DNA sequence directed nucleosome positioning and sliding, and suggested it might be the result of the co-evolution of DNA sequence and histone structure. We discovered a conserved motif hidden within the nucleosome, which illuminates the fundamentals of chromatin organization. This finding further emphasizes the critical role that DNA structure plays in genetic regulation.

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PUBLICATIONS RELATED TO THIS STUDY

1. **Bin Wu**, Peter Dröge and Curt A. Davey. Site selectivity of platinum anticancer therapeutics. *Nature Chemical Biology*. 2008 Feb;4(2):110-2. Profiled in "Research highlight." *Nature*. 2008 Jan 10;451:111.
2. **Bin Wu**, Curt A. Davey. Platinum drug adduct formation in the nucleosome core alters nucleosome mobility but not positioning. *Chemistry & Biology*. 2008 Oct 20;15(10):1023-8.
3. Gabriela E. Davey*, **Bin Wu***, Yuancai Dong, Uttam Surana and Curt A. Davey DNA stretching in the nucleosome facilitates alkylation by an intercalating antitumour agent. *Nucleic Acids Research*. 2010 in press.
4. **B. Wu***, K. Mohideen*, D. Vasudevan & C.A. Davey. 2010. Structural Insight into the Sequence Dependence of Nucleosome Positioning. *Structure (Cell Press)*. **in press**
5. **B. Wu** & C.A. Davey. Using Soft X-Rays for a Detailed Picture of Divalent Metal Binding in the Nucleosome. *Journal of Molecular Biology* **accepted**