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The Effects of Histone H1 and Benzo[a] Pyrene on Chromatin Modifying Complexes.

Zeina Salim Al Natour

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Modifying Complexes

By

Zeina Salim Al Natour

A thesis
Submitted to

United Arab Emirates University
In partial fulfillment of the requirements
For the Degree of M.Sc. in Environmental Sciences

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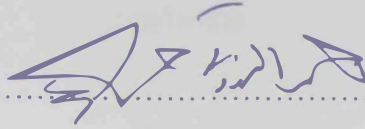
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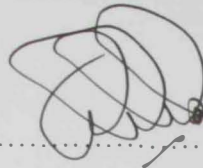For the Degree of M.Sc. in Environmental Sciences

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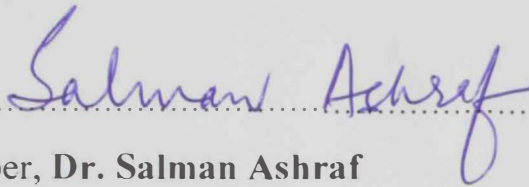
The Thesis of **Zeina Salim Al-Natour** for the Degree of Master of Science in Environmental is approved.

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Dedications

To my parents, my sister, and my brothers

Acknowledgment

I am deeply grateful to my supervisor, Dr. Ahmed Al-Marzouqi for his encouragement, patience and great support. I would also like to thank all the staff at the Faculty of Medicine and Heal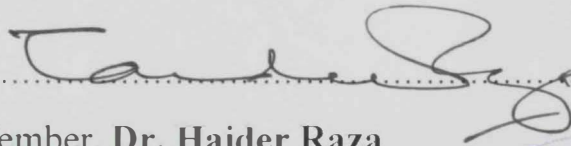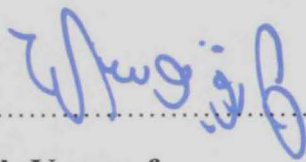th Sciences, especially the Biochemistry and Microbiology and Immunology Departments, for their kind advice, support and help during my work. I would like to thank Ms. Milena Mechkaraska for her valuable help in the beginning of my work. I am deeply grateful to my friends Salma Awad, Hussain El Hasasna, and Samah Othman for their great support and help. I would also like to thank Dr. Salman Ashraf, Dr. Haidar Raza and Dr. Mahmoud Talib Al Ali for kindly revising my thesis, which gave me a great chance to learn and finally special thanks to my family for their full care and concern.

Abstract

Packaging of DNA into the condensed structure of chromatin presents a barrier to many cellular processes that require DNA access such as transcription and replication. This problem is solved, in part, by the action of various complexes that modify the chromatin structure so that it becomes more accessible and, therefore, a more suitable platform for these processes. ATP-dependent chromatin remodeling complexes and histone acetyltransferases are examples of many chromatin modifying complexes that work to alleviate chromatin-mediated repression. Histone H1 is an important component of chromatin which serves in both stabilizing and folding of chromatin into a higher order structure. Many studies have demonstrated the importance of histone H1 in gene regulation. Furthermore, histone H1 has been suggested to affect the functions of modifying proteins. In this study, we have used pull-down assay to test the effects of histone H1 on the binding of two chromatin modifying complexes (SWI/SNF and SAGA) to *in vitro* assembled unmodified and accetylated nucleosomal arrays. Gel shift assay was also performed to check for the effect of histone H1 on the binding of these complexes to mononucleosomes. Furthermore, we have tested the effects of histone H1 on the remodeling activity of SWI/SNF using restriction enzyme accessibility assay. Our results show reduced binding of both SWI/SNF and SAGA complexes to both unmodified and acetylated nucleosomal array templates in the presence of histone H1. However, the histone H1-dependent inhibition of binding was specific to SWI/SNF when unmodified mononucleosomes were used. Furthermore, histone H1 was found to decrease the activity of the SWI/SNF complex.

Additionally, we investigated the effects of benzo[a]pyrene [B(a)P] on two cell lines, WRL-68 and HepG2 cells in an attempt to find whether chromatin modification is a

possible pathway in the carcinogenesis of this compound. Benzo[a]pyrene is a chemical carcinogen that belongs to the polycyclic aromatic hydrocarbons, and is known to induce DNA damage by forming DNA adducts. We tested both cells lines with different concentrations of benzo[a]pyrene and for different durations. Cell viability and apoptosis were checked by cell cytotoxicity assay and flow cytometery, respectively followed by studies on the expression patterns of three chromatin remodeling proteins (BRG1, Gcn5 and BAF155). Results show toxic effects of benzo[a]pyrene on both cells lines with increasing concentration and duration, while no changes in the expression of either BRG1, Gcn5, and BAF155 was observed. This suggests that the carcinogenicity of benzo[a]pyrene doesn't affect the expression pattern of at least these three proteins. However, our results don't exclude chromatin modification as a possible pathway in the carcinogenesis of this compound. The expression of other chromatin modifying proteins need to be checked as well as the effects of B[a]P on the activity of these proteins before we can completely rule out a link between B[a]P effects on cells viability and chromatin modifying.

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List of Abbreviations

ACF - ATP-utilizing chromatin assembly and remodeling factor

ATP - Adenosine Tri Phosphate

B[a]P-Benzo[a]Pyrene

BAFs - Brg1 associated factor

bp - base pair

BSA- bovine serum albumin

CBP- CREB binding protein

DMSO- Dimethyl sulfoxide

G5E4-5S- A DNA fragment which contains five consensus Gal4-binding sites upstream of an adenovirus 2 E4 minimal promoter flanked on both sides by five 5S rDNA nucleosome positioning sequences

HAT- Histone acetyltransferase

HDAC- Histone deacetylase

HepG2 -Human hepatocellular liver carcinoma cell line

ISWI - Imitation SWI

MNase- Micrococal nuclease

NURF- Nucleosome remodeling factor

PAGE – Polyacrylamide gel electrophoresis

PAHs- Polycyclic aromatic hydrocarbons

PMSF- Phenyl methyl Sulphonyl Fluoride

Rb- Retino blastoma

RSC- Remodel the structure of chromatin

SAGA- Spt-Ada- Gcn5 acetyaltransferase

SDS- Sodium Dodecyle sulfate

SIN- SWI- independent

SNF- Sucrose Non fermentor

Spt- suppressor of Ty

SWI- Switch

WRL-68 human liver embryonic cells

Chapter 1: Chromatin remodeling complexes

Introduction

The prokaryotic genome is mostly contained in a single large circular DNA molecule, with a minor fraction present in small plasmids. This genome is supercoiled and complexed with various proteins existing free in the cytosol, a structure that we refer to as the nucleoid. In eukaryotes the situation is quite different. The eukaryotic cell has enormous amount of DNA, for example the diploid DNA content of a human cell is about 8×10^9 base pairs corresponding to a total length of nearly 3 meters. All this DNA should be packed into a nucleus of about 10 μm in diameter. This enormous amount of DNA poses some serious challenges, 1) the effective compaction of this large genome so that it fits into the small area available in the nucleus, 2) the selective transcription since only 5%-10% of the whole genome is ever transcribed in a typical eukaryotic cell. Both compaction and the control of gene expression in eukaryotes are achieved by having the DNA complexed with a set of special proteins to form a protein/DNA complex called chromatin.

Proteins complexed with DNA fall into two major classes of histone and nonhistone chromosomal proteins. Histones are small in size and are very basic proteins rich in lysines and arginines. They constitute the basic building blocks of the chromatin structure. Histones include five types of proteins. They are H1, H2A, H2B, H3, H4, which are always found in equimolar quantities (Mathews and van Holde, 1996). The most conserved histones among living eukaryotes are H3 and H4, which differ very little, even between extremely diverse species. The H2A and H2B are less conserved than H3 and H4 but still exhibit substantial evolutionary stability, especially within their nonbasic portions. Linker histone H1 is quite

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distinct from other histones, being larger, more basic and by far the most tissue-specific and species specific histones. Vertebrates contain an additional linker histone, H5, which has a function very similar to H1. The high content of basic amino acids in histones makes them polycationic and leads to their interaction with the polyanionic phosphate backbone of DNA to produce uncharged nucleoproteins. All five histones are characterized by a central nonpolar domain, which forms a globular structure, and N-terminal and C-terminal regions that contain most of the basic amino acids. The basic N-terminal regions of H2A, H2B, H3 and H4 are the major sites of interaction with DNA.

The nucleosome

Histones interact with DNA to form a periodic "beads-on-a-string" structure, called a polynucleosome, in which the basic building block is the nucleosome. Each nucleosome is a disk-shaped structure of about 11 nm in diameter and 6 nm in height that consists of a DNA segment and an octameric histone cluster composed of two molecules of each of H2A, H2B, H3, and H4 histones. Each cluster consists of a tetramer of $(H3)_2$- $(H4)_2$ with a H2A-H2B dimer stacked on each face of the disk. The DNA is wrapped around the octamer as a negative toroidal superhelix with the central H3- H4 tetramer core interacting with the central 70-80 bp of the surrounding DNA. Approximately 146 bp of DNA is wrapped around the histone octamer complex. Histones are in contact with the minor groove of DNA and leave the major groove available for interaction with proteins that regulate gene expression and other DNA functions.

To form the beads-on-a-string structure nuleosomes are joined by "linker DNA" of about 20-90 bp long. This DNA is associated with H1 that locks the coiled DNA in place, producing

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a complex called the chromatosome. Formation of nucleosomes , known as the 10-nm fiber, achieves one level of compaction which is about tenfold reduction in the apparent length of DNA. Since the distribution of these nucleosomes is not random with respect to the DNA base sequence it has been suggested that DNA binding is sequence dependent. In fact it has been shown that nucleosomes tend to associate preferentially with certain DNA regions. DNA sequences such as long A tracts or G-C repeats are not usually associated with nucleosoems. In contrast, certain bent DNA regions, for instance, periodically phased A tracts, associate strongly with histones (Devlin, 2002).

The higher structure of chromatin

To achieve further compaction the 10-nm fiber is condensed into a solenoid arrangement involving six to seven chromatosomes per solenoid turn. The linker histone H1 has an important role in the higher level of compaction since histones H1 bind to one another cooperatively, bringing the neighboring nucleosomes together to form the 30 nm fiber. This condensation step compacts the DNA structure with a ratio that may be as high as two orders of magnitude. The 30 nm fiber comprises only selected regions of DNA that are characterized by the absence of binding of sequence-specific nonhistone DNA-binding proteins. The presence of DNA binding proteins and the effects on the formation of 30 nm fibers may depend on the transcriptional status of the regions of DNA involved. The 30 nm fiber can further condense and fold to form chromosomes, which are thicker chromatin fiber visible in metaphase. This is achieved by the formation of looped and condensed domains of the 30 nm fiber. These domains bind to a nuclear scaffold consisting of histone H1 and several non histone proteins such as Sc1 (a topoisomerase) and Sc2, which leads to the accumulation of

supercoils. These loops are further packed by being arranged into stacked helical coils, a structure we refer to as the chromatid (Devlin, 2002). Figure 1 shows the hierarchical folding of chromatin.

Metaphase chromosome — 1400 nm

Condensed scaffold-associated form — 700 nm

Chromosome scaffold

Extended scaffold-associated form — 300 nm

30-nm chromatin fiber of packed nucleosomes — 30 nm

"Beads-on-a-string form of chromatin — 11 nm

Short region of DNA double-helix — 2 nm
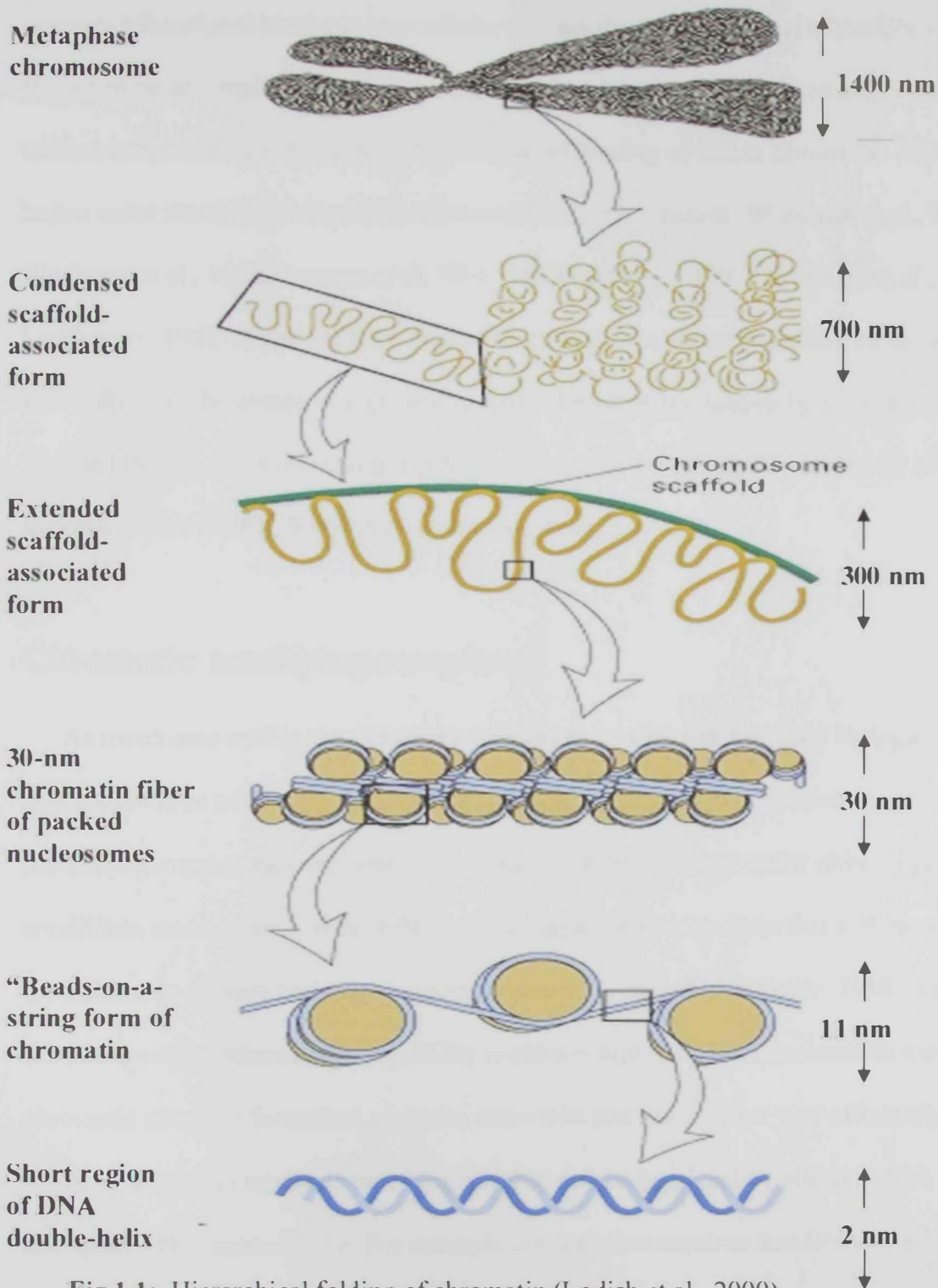
Fig 1.1: Hierarchical folding of chromatin (Lodish et.al., 2000).

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Chromatin involvement in gene regulation

While the packaging of cellular DNA into chromatin serves to compact the eukaryotic genome into a small nucleus, the physical structure and the compact nature of the chromatin presents a functional barrier to any cellular process that require access to the DNA such as transcription and replication (Hill et al., 2000). Biochemical studies have implicated both nucleosome core assembly and/or the subsequent binding of linker histone H1 which leads to higher order structure of chromatin in transcriptional repression (Workman et al., 1988; Workman et al., 1990; Croston et al, 1991; Workman et al., 1991; Laybourn et al., 1991; Lorch et al., 1992). Repression by nucleosomes seems to be carried either by nucleosomes on DNA blocking the access to sequence specific DNA binding factors by steric inhibition with histone DNA contacts occluding regulatory sites or by inhibiting the movement of RNA polymerase II (Wolffe, 2001; Paranjepe et al., 1994).

Chromatin modifying complexes

As mentioned earlier, the chromatin appears to be a barrier for many biological processes that use DNA as a substrate such as transcription, replication, recombination, and repair. Nucleosomes are remarkably stable to physical perturbation and under physiological conditions, nucleosomal arrays fold to stable higher order structures that self-associate within the nucleus to achieve concentrations in excess of 50 mg/ml (van Holde, 1988; Wolffe, 1998). Despite the compaction and accessibility problems that arise from nucleosome assembly and chromatin structure formation, complex metabolic processes occur very efficiently *in vivo*.

In principle, all reactions that involve DNA can be regulated by altering DNA packaging and hence DNA accessibility. For example transcription requires that DNA be accessible to

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sequence specific transcription factors and RNA polymerase and requires the melting and reformation of the double helix throughout the length of the transcript. Chromatin structure impedes all the steps required for transcription. So repression of transcription can be achieved by creating a stable, inaccessible chromatin structure while activation can be achieved by creating an accessible chromatin structure. Chromatin structure regulation is not a random process and proper regulation requires many factors that collaborate to ensure successful work (Narlikar and Fan., 2002). The contrasting requirement between storage and functional utility is met through the use of specialized molecular machines that reversibly disrupt and modify chromatin.

The term chromatin remodeling has been used to describe transitions in chromatin structure that can include physical alterations to the histones, histone post-translational modifications, DNA methylation, the non- histone protein content of the chromatin, and chromatin conformation through the action of special complexes (Flaus and Hughes., 2004). The most widely characterized chromatin-modifying complexes studied to date can be classified based on their modes of action into two major groups, as follows: (1) ATP-dependent complexes, which use the energy of ATP hydrolysis to locally disrupt or alter the association of histones with DNA and (2) histone modifying complexes such as histone acetyltransferases (HAT) and histone deacetylases (HDAC) complexes, which regulate genes expression by determining the level of acetylation of the amino termini tails of nucleosomal histones associated with them (Vignali, 2000).

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ATP-dependent chromatin remodeling complexes

All of the ATP-dependent remodeling complexes contain an ATPase subunit that belongs to the SNF2 superfamily of proteins. Based on this subunit, the complexes have been classified into two main groups: The SWI2/SNF2 group and the imitation SWI (ISWI) group. Mi-2, a third class of ATP-dependent complexes which contains a Snf2-like ATPase and also shows deacetylase activity has been recently described (Eisen et al., 1995).

The SWI2/SNF2 group. Complexes belonging to this group contain a highly conserved ATPase subunit, which belongs to the Swi2/Snf2 subfamily of proteins. This group includes the yeast SWI/SNF (ySWI/SNF), the yeast RSC, the *Drosophila* Brahma complex, and the human BRM (hBRM) and BRG1 (hBRG1) complexes. The ATPase subunit they contain are Swi2/Snf2, Sth1, Brm , hBRM and hBRG1, respectively. The homology of these proteins extends beyond the ATPase domain, as they all contain a bromodomain in the C-terminal region and two other conserved regions of unknown function called domains 1 and 2 (Laurent et al., 1993; Tamkun et al., 1992).

The ySWI/SNF complex was the first remodeling complex to be described and characterized. It contains 12 known subunits, including Swi2/Snf2. Several of the subunits were initially identified genetically as gene products involved in the regulation of either the *HO* endonuclease gene or the *SUC2* gene, which encodes for an invertase enzyme. *HO* is required for mating type switching, hence SWI, while SUC2 mutants are classified as sucrose non-fermenters, thus SNF (Imbalzano, 1996; Peterson, 1996). The SWI/SNF was subsequently shown to be involved in the transcriptional regulation of a wider subset of yeast genes (Holstege et al., 1998). About 5% of yeast genes have now been shown to be regulated by the SWI/SNF complex. Other studies have provided a connection between the functions of

SWI/SNF complex and chromatin. Several mutations that suppressed SWI/SNF phenotypes corresponded to genes encoding histones and other proteins (Kurger et al., 1995; Recht and Osley., 1999). The link between ySWI/SNF complex and chromatin was strengthened when the SWI/SNF complex was purified and found to alter the nucleosome structure in an ATP-dependent manner (Cote et al., 1994; Peterson and Herskowitz, 1992).

The yeast RSC complex contains many proteins that are homologues to the SWI/SNF subunits, they share at least two identical subunits. These two complexes also have similar biochemical activities, however, the yeast RCS complex is far more abundant than SWI/SNF in the yeast cell (thousands of molecules compared to 100-200 molecules of SWI/SNF). In addition, the 15 subunit, RCS complex contains several subunits that are essential for the viability of the cell, whereas, none of the SWI/SNF subunits is essential. These 15 RCS subunits include a homolog to the Swi2/Snf2 ATPase, called Sth1, and homologues of Snf5, Swi3, and Swi73 which are Sfh1, Rsc8/Swh3, and Rsc6, respectively (Cairns at al., 1996). In addition the yeast RCS complex contains two actin related proteins Arp7 and Arp9 that are identical to the actin related proteins in SWI/SNF complex, the Swp61 and Swp59, respectively (Cairns et al., 1998; Peterson et al., 1998).

Genetic screening for repressors of the transcriptional polycomb protein in *Drosophila melanogaster* lead to the identification of proteins which were homologues of SWI/SNF proteins (Tamkun et al., 1992) and were found to form part of a large multirprotein complex called Brahma (BRM) (Dinwall et al., 1995). The Brahma complex is composed of eight major proteins; including the ATPase subunit Brm. Proteins that copurify with Brm have been called BAPs for Brm Associated Proteins. Brm complex subunits Bap45/Snr1, Bap155/Miora, and Bap60 are conserved between yeast and humans (Papoulas., 1998; Dingwall et al., 1995). Moira/Swi3D is a homologue of yeast Swi3 and the human proteins Baf155 and is reportedly

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identical to Bap170, (Papulas et al., 1998; Corsby et al ., 1999).The similarities between the *Drosophila* Brm protein and its human homologue are extensive and are across many regions. The first region is rich in prolines and hydrophobic and aromatic amino acids. The second is the tryptophan-rich SANT domain. This domain might be involved in Moira's association with Brm. The third region of homology is a leucine zipper motif, which is thought to be involved in self- association ability of Moira (Crosby et all., 1999). Brm complex includes a protein that seems to be unique to higher eukaryotes, Bap111, which contains an HMG domain and is homologous to Baf57. Bap111 seems to be present in human complexes but not in the related yeast RSC and SWI/SNF complexes. Other components of the *Drosophila* Brm complex are Bap74 and Bap47 (Papulas et al., 1998).

Two SWI/SNF-like multisubunit complexes of approximately 2 MDa have also been purified from human cells (Wang et al., 1996; Wang et al., 1996b) Those complexes contained different DNA-dependent ATPase/ helicase subunits, BRG1 and hBRM, and are called hBRG1 and hBRM, respectively. The two subunits are 70% identical and show homology to the yeast Swi2/Snf2 across the entire gene (Eisen et al., 1995). The proteins that are associated with hBRG1 and hBRM in these complexes, called BAFs or BRG1 Associated factors, are also very similar. It has been suggested that there might be multiple complexes in different cell types, each containing a different subset of BAF proteins and either hBRG1 or hBRM as the catalytic subunit (Kown et al., 1994). Later studies have shown that hBRG1 and hBRM have been found in different cell lines from a wide range of tissues, and the complexes containing them might have slightly different subunit compositions (Wang et al., 1996).

The ISWI group. This group of ATP- dependent remodeling complexes contains the ISWI protein as the ATPase subunit. The most studied members of this group are ACF (ATP-utilizing Chromatin Assembly and remodeling Factor), NURF (Nucleosome Remodeling

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factor), CHRAC (Chromatin Accessibility Complex), which were purified from *Drosophila* extracts by using biochemical methods based on the their ability to disrupt and/or generate regularly spaced nucleosomal arrays (Ito et al., 1997;Tsukiyama and Wu, 1995; Vagra-Weisz et al., 1997). These complexes contain the nucleosome-dependent ATPase ISWI, which has significant homology with the Swi2/Snf2 proteins exclusively over the region of the ATPase domain (Elfring et al., 1994). ISWI complexes are smaller and have fewer subunits than their SWI/SNF counterparts. For example, the NURF complex has a molecular mass of 500 kDa and is composed of 4 subunits (Martinez-balbas et al., 1998), the CHRAC complex has a molecular mass of approximately 670 kDa and is composed of 5 subunits (Varga-weisz et al., 1997), while the smallest of all is the ACF complex with a molecular mass of 220 kDa and contains two subunits, one of which is the ISWI subunit (Ito et al., 1997).

In yeast ISWI1 and ISWI2 are two ISWI-related proteins that have been recently identified based on their homology to the ATPase domain of *Drosophila* ISWI. They were found to be components of multisubunit complexes with diverse nucleosomal- remodeling and spacing abilities (Tsukiyama et al., 1999). In human an ISWI-containing complex was also purified based on its ability to facilitate transcription from chromatin templates (LeRoy et al., 1998).

The Mi-2 group. Complexes of this group posses both chromatin remodeling and deacetylase activities (Tong et al., 1998). In human the complex is termed hNURD and it contains HDAC1, HDAC2, the retinoblastoma protein (Rb) associated proteins, RbAp46 and 48, and the Swi2/Snf2 ATPase homologue, CHD4 also called Mi-2B. This complex has the ability to both deacetylase histones and remodel chromatin, presumably by means of HDAC and Mi-2/CHD subunits respectively (Xue et al., 1998). A Mi-2 complex related to hNURD has also been identified in Xenopus egg extracts (Wade et a., 1998). Xenopus Mi-2 complex have peptides that are homologus to mammalian NURD complex subunits. These peptides are

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thought to be involved in recruiting remodeling activities of Xenopus Mi-2 to methylated DNA. Human NURD was also found to contain two forms of MBD3, however, only the MBD2 , a homologue of MBD3, is capable of binding to both methylated DNA and the NURD complex. Thus, it is believed the NURD complex is tethered to methylated DNA via its interaction with MBD3 (Zhang et al., 1999). These contrasting activities of remodeling and deacetylation displayed by both human and Xenopus Mi-2 complexes suggest that remodeling activity of these complexes might be specifically directed to methylated regions of the genome, which in turn, can lead to repression either by compaction of the chromatin or by allowing the binding of repressor proteins. Other possibility is that Mi-2 complexes are recruited to specific genes by repressors (Kehle et al., 1998; Kim et al., 1999).

Comparing different chromatin remodeling complexes

As previously mentioned ATP-dependent chromatin remodeling complexes can be divided into three main classes based on the identity of their ATPase subunits which display homology through their ATPase domains, but contain different additional domains. This subunit binds to additional different subunits in different complexes. To date SWI/SNF family and ISWI-based family are the two best studied families of remodeling complexes. (Kingston and narlikar, 1999; langst and becker , 2001).

In general chromatin remodeling can be defined as a stable alteration in the nucleosomes and the chromatin structure (vignali et al., 2000). The exact mechanisms by which ATP-dependent remodeling complexes work are becoming more clear now. Recent studies have revealed some additional facts about the activities of chromatin remodeling complexes.

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Biochemical comparisons performed found similarities and differences among different families. Comparisons included the nature of substrates to which they bind and the outcome of their remodeling activities which could suggest variations in their mechanisms of action. To illuminate the similarities and differences in the mechanistic functions of chromatin remodeling proteins, it would be very helpful to discus how they differ in binding to substrates to remodel as well as their potential activities.

A. Binding of chromatin-remodeling complexes to DNA and nucleosomal substrates

A first step for remodeling requires that complexes recognize and bind to their substrates. Different complexes have been found to bind differently to DNA and nucleosomes. For example, while the NURF Complex was not found to form stable complex with nuleosomes or DNA *in vitro*, it may interact with nucleosomes in a manner dependent on the core histone tails. The ISWI subunit of NURF, CHRAC and ACF has been shown to binds to DNA at least transiently (Gorgel et al., 1997; Tsukiyama and Wu, 1995). The ability of recombinant ISWI to have some nucleosomal activity on its own indicates its ability to directly interact with nucleosome substrates (Tsukiyama and Wu, 1995; Corona el al., 1999; Hamiche et al., 1999). The SWI/SNF complex, however binds to DNA and nucleosomes with high affinity (Côté et al., 1998; Lorch et al., 1998; Quinn et al ., 1996), This binding to naked DNA was shown to be ATP- dependent in ySWI/SNF with a k_d in the nanomolar range (Quinn et al., 1996). Displacement of the complex by distamycin A or chromomycin A3 suggested that this binding occurs through minor grooves interactions (Côté et al., 1998., Quinn et al., 1996). The binding of the SWI/SNF to DNA nonspecifically in a length-dependent manner with a preference for

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four way junctions and cruciforms has also been demostrated. This could be achieved by the IN11/hSNF11 and BAF57 subunits of SWI/SNF which contain HMG box that could bind to DNA containing such structural features (Morozov et al .,1998; Wang et al , 1998).

The inability to easily disrupt ySWI/SNF binding to nucleosomes by the addition of distamycin A, excludes the possibility of SWI/SNF binding to nucleosomes through minor groove interaction (Côté et al., 1994). The SWI/SNF interaction with nucleosomes was found to be slightly higher than that for naked DNA, a possible reason would be the additional interactions of SWI/SNF with the core histones. In fact the ability to bypass SWI/SNF requirement by H2A/H2B dimer depletion either *in vivo* by core histone mutations or *in vitro* by adding chaperones suggests the interaction of SWI/SNF with the core histones (Côté el al., 1994; Hirschhorn el al., 1995). A more recent experiment demonstrated interaction of core histones with SWI/SNF. That was done by inducing site directed mutagenesis in the core histone tails which resulted in SWI/SNF independent (Sin) mutations (Recht and Osley, 1999). Those mutations targeted regions needed for H2A/H2B dimerization and dimer/tetramer association and also the H2B amino terminus , which is suggested to have inhibitory role that can be antagonized by SWI/SNF. The presence or absence of histones N- terminal tails in nucleosomes and its effect on binding and activation of ATP-dependent remodeling complexes would also suggest other biochemical differences among the different complexes.

The *Drosophila* ISWI-based complex, NURF, was not able to remodel nucleosomes lacking the histone N-terminal tails, while, these tailless nucleosomes were remodeled by yeast and the human SWI/SNF complexes (Langst and becker, 2001b). Another work has shown the requirement of H4 N terminal tail for the stimulation of the ATPase activity of the ISWI. These tails were suggested to play a role in coupling ATP hydrolysis to conformational changes in nucleosomes rather than nucleosome binding (Clapier el al., 2001). However, the

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case for SWI/SNF was different as the removal of the H4 N-terminal tail did not affect the rate of remodeling (Guyon et al., 1999; Logie et al., 1999), but rather it increased the affinity of SWI/SNF for the nucleosomal array (Logie et al., 1999).The human SWI/SNF was found to be able to remodel tail-less mononucleosomes as well as nucleosomal arrays suggesting that hSWI/SNF is activity was not dependent on core histone tails (Guyon et al., 1999).

B. Biochemical comparisons of ATP-dependent remodeling complexes

Understanding the mechanisms of action of chromatin remodeling requires biochemical as well genetic assays. Initial biochemical approaches were not very helpful since the biochemical assays used for the ISWI were different from those used for the SWI/SNF complex. For example, the ISWI complexes were tested for both their ability to disrupt or assemble spaced nucleosomal arrays and their ability to stimulate *in vitro* transcription (Ito et al., 1997; LeRoy et al., 1998; Tsukiyama et al., 1995; Varga-Weisz et al., 1997). On the other hand, SWI/SNF and RCS complexes were assayed for their ability to disrupt rotational phasing of DNA sequences on nucleosome core particles as well as for their ability to stimulate the binding of transcription factors to nucleosomes (Imbalzano et al., 1994; Côté et al., 1994; Kwon et al., 1994; Wang et al., 1996). Although NURF was found to disrupt the rotational phasing of DNA on nucleosomes cores but their mode of action was not similar to SWI/SNF complex (Tsukiyama and Wu, 1995).

Although different approaches were followed with different classes of remodeling proteins, some important data were revealed. Later (side-by-side) experiments allowed comparisons of the different families by using the same remodeling assays. For example, BRG1 (the human homolog of yeast SWI2/SNF2) and SNF2H (the human homolog of ISWI)

were tested for their ability to remodel nucleosomal arrays and only BRG1 was able to alter restriction enzyme and DNase accessibility on mononuleosomes. In addition, in another experiment BRG1 was able to introduce topological changes in a closed nucleosomal array (Aalfs et al., 2001). Octamer sliding assays showed that dMi-2 remodeling complex has caused the histone octamer to move toward central positions within a 248 bp DNA fragment, while the ISWI complex moved the histone octamer towards the ends of DNA. In addition Mi-2 could remodel nucleosomes that lacked the N- terminal tails of histones H4, H3, and H2A which was not possible with ISWI (Brehm et al., 2000). DNA extrusion assay is another assay that suggested mechanistic difference among different complexes. This assay detects the ability to promote the formation of cruciform DNA structures from inverted repeats of DNA. Both BRG1 and ySWI/SNF could extrude cruciform DNA from both naked DNA and chromatin templates, while ISWI and Mi-2 Complexes could perform this function only on chromatin templates, which confirms the importance of nucleosomal substrates in stimulating ATPase activities of ISWI and Mi-2 (Langst and becker, 2001).

Mechanisms of chromatin remodeling

The previous comparison of remodeling complexes suggests mechanistic difference among some of them in their modes of actions. In general remodeling can be considered a consequence of nucleosome conformation disruption which can itself represent remodeling or this disruption can lead to a subsequent movement of nucleosomes. Remodeling mechanisms can be classified into: I). remodeling by mobilizing and repositioning of nucleosomes. II). Remodeling by causing conformational changes to nucleosomes.

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I. Remodeling by mobilizing and repositioning of nucleosomes.

It is clear that the histone octamer blocks the access of factors to nucleosomal DNA and a way to expose these blocked sites would be to reposition the whole octamer or mobilize it. There are two known nuleosome rearrangement models: sliding of histone octamers along DNA *in cis* or displacement of histones *in trans*.

A. Sliding of histone octamers *in cis* (Spooling, Twist defect diffusion, and Bulge diffusion)

Sliding, in general, means sliding the DNA with respect to the histone octamer. It involves identical amounts of movement of the entry and exit points of the DNA in the same direction, which leads to an octamer which is translationaly repositioned and so DNA that is free of histones, see figure 1.2 (Meersseman et al ., 1992). Using assays that distinguish between translational position of mononucleosomes assembled on ~ 240-350 bp of DNA by NURF, CHARC,and ISWI has demonstrated sliding of the histone octamer (Hamiche et al., 1999; langst et al., 1999). In addition the existence of an array of randomly positioned nucleosomes provided further evidence (Langst and Becker, 2001b). The ySWI/SNF has also been shown to slide the histone octamer to remodel chromatin (Whitehouse et al., 1999). Other study have found nucleosome sliding induced by CHRAC or NURF appears to occur without displacement of the histones octamer from DNA but rather is suggested to be done in small steps and not large leaps (Hamishe et al., 1999; Längst et al, 1999). Further, restriction enzyme accessibility in the linker regions of nucleosomal arrays was blocked indicating repositioning nucleosomes over these regions by sliding (Jaskelioff et al., 2000). Nucleosome mobility by

sliding can be explained by three models: Spooling, twist diffusion, and Bulge diffusion (Figure 1.2).

The "spooling model" can explain the repositioning of nucleosomes (Pazin and Kadonaga, 1997). This idea developed by experiments that monitored the transcription of RNA polymerases through nucleosomes (Bednar et al., 1999). In this scenario, the movement of the enzyme on DNA leads to the peeling off the histone octamer surface of larger DNA segments. The patch of free histone is then available to capture a different DNA segment.

The "twisting model" suggests that a remodeling complex as ISWI alters the topology of DNA and, thereby, changes histone-DNA interactions. It was found that the generation of superhelical torsion represents a primary biomechanical activity shared by all Snf2p-related ATPase-containing proteins. The generation of superhelical torque provides a potent means by which ATP-dependent chromatin remodeling complexes can manipulate chromatin structure (Havas et al., 2000; Varga-Weisz and Becker, 1998). Thermal energy could alter the twist of DNA (van Holde and Yager, 1985), effectively disrupting a set of DNA-histone interactions at the site of entry into the nucleosome and replacing them by analogous interactions involving the neighboring base-pair. Since small distortions of the helix geometry can be accommodated in the nucleosome (Luger and Richmond, 1998), it is possible that the locally altered twist is propagated over the surface of the nucleosome. Once the helix distortion emerges on the other side of the nucleosome, the DNA will have been displaced by one base pair relative to the octamer surface.

The "bulge diffusion" is a model that suggests the unpeeling of DNA from the histone octamer at the entry and exit of the nucleosome and the subsequent rebinding of more distal sequences to the same histone contact points which would cause the nulceosomal particles to harbor excess DNA, looping out in a bulge. The migration of the bugle around the

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nucleosomal superhelix would result in the nucleosome stepping in the direction where the bulge has initiated(Flaus and Owen-Hughes, 2003; Kulic and Scheissel, 2003). Many observations of nucleosomal sliding *in vivo* and *in vitro* are consistent with movements in this bulge diffusion model (Flaus and Owen-Hughes, 2003b; Fazzio and Tsukiyama, 2003).

B. Histones displacement *in trans*

In this model, the histone octamers are transferred to other DNA regions. In general, although many remodeling complexes showed nucleosome sliding, it was not the only mechanism in which nucleosomes were repositioned or remodeled. For example, sliding alone couldn't explain how DNA is made accessible in regions of tightly packed nucleosomes. Remodeling was seen in mononucleosmes with no flanking DNA into which histone octamer can slide (Kingston and Narlikar, 1999). Moreover restriction sites which were closer to the center of DNA were exposed faster and more when compared to DNA ends, an event that can't be explained by the simple sliding of histones (Narlikar et al., 2001). Furthermore, site-specific cross linking of DNA to the histone octamer didn't prevent remodeling by hWI/SNF (Lee etal., 1999). Remodeling mechanisms other than sliding as histone displacement in trans, and nucleosomal conformational change, looping can explain many remodeling activities which can't follow the easy way of sliding of nucleosomes.

Both the RCS and SWI/SNF complexes have been found to displace histone octamer *in trans* (Lorch et al., 1999; Owen-Hughes et al., 1996). The binding of transcription factors was found to further destabilize histone-DNA interactions and facilitate trans displacement (Owen-Hughes and Workman, 1996). The RSC complex was found to catalyze the transfer of a histone octamer from a nucleosome core particle to naked DNA. The newly formed octamer-DNA complex is identical with a nucleosome in all respects. The reaction requires ATP and

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involves an activated RSC-nucleosome intermediate. The mechanism may entail formation of a duplex displacement loop on the nucleosome, facilitating the entry of exogeneous DNA and the release of the endogenous molecule (Lorch et al., 1999). Neighboring nucleosomes were found to form a barrier for sliding and causes SWI/SNF to switch to trans displacement (Owen-Hughes et al., 1996).

The same result was concluded in another work in which SWI/SNF complex was found to prefer to slide histone octamers in the presence of barrier that prevent sliding (Whitehouse et al., 1999). Unlike SWI/SNF, complex CHARC and NURF complexes has not been shown to have any trans displacement activity (Längst et al., 1999; Hamiche et al., 1999). The ability of SWI/SNF to perform displacement of the octamer could be explained by the strong binding to DNA compared to the ISWI containing complexes. The additional binding of transcription factors to nucleosomes may contribute to this destabilizing of histone-DNA interactions within the disrupted nucleosome (Owen-Hughes et al., 1996; Owen-Hughes and workman, 1996).
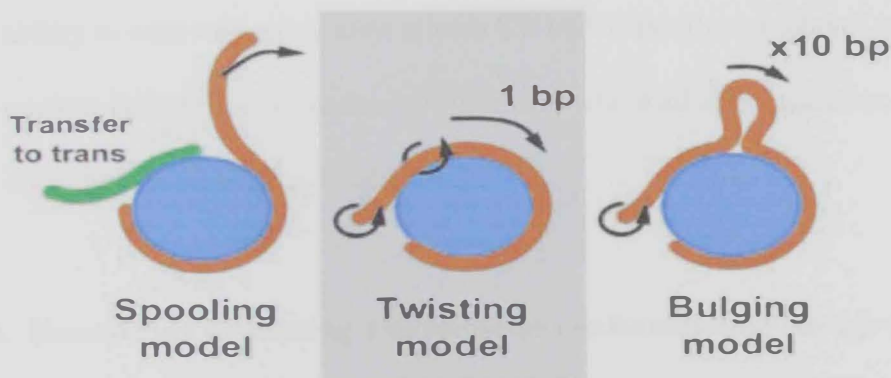
Fig 1.2: The three distinct models (spooling, twisting and bulging) for nucleosome mobility (Längst and Becker, 2001).

II. Remodeling without mobilizing of nucleosomes

These mechanisms present ways in which nucleosomes are locally disrupted and DNA-histone interactions are weakened to expose occluded DNA without the need of repositioning of nucleosomes. However the mobilizing of nuclosomes can be a subsequent step after nucloesomal conformational disruption.

A. Creating DNA loops on the surface of nucleosomes

SWI/SNF was found to create DNA loops on the surface of nucleosomal arrays. Loop formation on nucleosome surface causes the loss of DNA content on nucleosomes allowing the unwrapping of part of the nucleosomal DNA (David et al 1999). When BRG1 and SNF2h were compared for their abilities to make nucleosomal sites accessible on a mononucleosome and on the central nucleosome of a trinucleosome, it was found that BRG1 opens centrally located sites more than an order of magnitude better than SNF2h. This capability of BRG1 probably due to the ability to create DNA loops on the surface of a nucleosome, even when that nucleosome is constrained by adjacent nucleosomes. This ability to unravel central sites allows SWI/SNF family complexes to facilitate binding of nuclear factors in chromatin environments where adjacent nucleosomes might otherwise constrain mobility (Fan et al., 2003).

B. Remodeling by causing a nucleosome conformational change (or nucleosomal disruption)

Altering the conformation of nucleosome is an alternative model that would describe remodeling without the mobilizing of nucleosomes. The features of SWI/SNF- or RSC-

disrupted nucleosomes conformation are as follows: i) the loss of rotational phasing of the DNA on the surface of the histone octamer, although the DNA remains at least partly associated with the histone octamer surface, ii) increased accessibility of nucleosomal DNA to transcription factors and restriction enzymes, iii) reduction of the total amount of DNA associated with the histone octamer, iv) the formation of dinucleosome-like species perhaps by the interaction of loosened DNA with histone octamer from other nucleosomes, v) reduction of the stability of nucleosomes at elevated ionic strengths, vi) the relocation of H2A N-terminal tail from a position approxiamatly 40 bp on either side of the nucleosome dyad to a location near the nucleosome dyad (reviewed in Vignali et al., 2000). Previous data have not shown helicase-like activity by SWI/SNF complex despite sequence homology between Swi2/Snf2 ATPase domain and that of helicase (Côté et al., 1998). Moreover, loss of H2A/H2B dimer was not demonstrated since cross linking of histones didn't inhibit remodeling by chromatin remodeling complexes (Côté et al., 1998; Bazett-jones et al., 1999). However, more recently the involvement of loss or exchange of H2A/H2B dimer in an octamer by some of these complexes have been demonstrated as will be discussed later.

Histone dimer removal or replacement by chromatin remodeling complexes

Loss of histones dimer was suggested after observing that depletion of H2A and H2B levels enabled the SUC2 gene to be induced in the absence of the SWI/SNF complex. It was also found that the depletion of H2A and H2B caused a transition in chromatin structure at the SUC2 promotor that mimics SWI/SNF acitity *in vivo* (Hirschhorn et al., 1992). Unraveling of DNA from the surface of nucleosomes can explain how dimer exchange occurs. However, other factors can do the dimer removal or exchange such as the interaction of the Snf5 subunit with H2B (Bruno et al., 2003) and the generation of negatively superhelical torsion during

remodeling activity (Brooks and Jackson, 1994). In contrast to SWI/SNF complex ISWI

complexes were not found to have features as altering the conformation of nucleosomes, this

was observed by the inability of those complexes to form stable complex between with

nucleosomes nor a persistently altered nucleosome conformation has been observed. In fact

most mechanistic studies on the ISWI-containing NURF and CHRAC complexes suggest a

gradual movement of nucleosomes along DNA in *cis* as a consequence of their action

(Imbalzano et al., 1994; Hamiche et al., 1999). Many events take place as a consequence of

dimer loss from a nucleosome. The removal of the dimers may act as a first step in the

complete removal of histone octamers from DNA (Mozziconacci and Victor, 1994). The loss

of dimers can destabilize the organization of the remaining histones, since the direct contact

between histone dimers is thought to increase the stability of their association (Luger et al.,

1997). Recently SWI/SNF was found to be involved in the removal of nucleosomes from the

yeast PHO5 promoter *in vivo* (Boeger et al., 2003; Reinke and Hörz, 2003) and cooperation

between the *Drosophila* SWI/SNF complex and the histone H3/H4 chaperone ASFl was also

supports dimer/tetramer exchange and displacement (Moshkin el al., 2002). The potential

consequences of dimer removal could be (i) removal of the remaining dimers or tetramers, (ii)

allowing a closer approach of nucleosomes, (iii) the increasing of DNA accessibility, (iv)

enabling conformational changes, (v) affecting the higher order structure (Flaus and Owen-

Hughes, 2004).

The H2A/H2B dimer could also be exchanged with different H2A/H2B forms resulting in

a nucleosome having an altered histone composition.The Yeast Swr1 protein has been shown

to direct replacement of H2A-type dimers with Htzl p-Htbl p in an ATP- dependent manner

(Mizuguchi el al, 2004). This action of incorporating type Htzp1 protein may act to prevent

the spread of heterochromatin into normally euchromatin regions (Meneghini et al., 2003).

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The swapping of histone composition allows the generation of non-canonical nucleosomes which would allow the interaction of transcription factors with nucleosomal DNA. This prevents the formation of well ordered heterochromatin fibers or might alter the number of supercoils constrained by the chromatin. Dimer exchange potential consequences are: (i) controling of chromatin higher structure, (ii) alter the propensity for subsequent remodeling or polymerase passage events, (iii) reprogram signals implicit in nucleosome modification state and composition (Flaus, A and Owen-Hughes, 2004).

Chromatin modifying complexes

ATP-dependent chromatin remodeling is not the sole mechanism by which chromatin structure is altered. The chromatin could also be covalently modified which doesn't require the use of energy. The covalent modifications include acetylation, phosphorylation, ubiquitination, ADP-ribosylation and methylation (Bradbury, 1992). Acetylation of histones is the most understood of all and will be discussed since other modifications are beyond the scope of the thesis.

A. Chromatin modification by histones acetylation

Histone acetylation was found to play a major role in eukaryotic transcription regulation. Histone acetyltransferases or HATs work by transferring an acetyle group from acetyl-coenzyme A(acetyle-CoA) to the ε-amino group of some lysine side chains within a histone's basic N- terminal tail region (Loidl, 1994). Extending out, from the globular domains and harboring a positive charge these N-terminal regions of histones are believed to bind with the negatively charged DNA to form the nucleosomes of histones (Fletchur and Hansen, 1995;

Lurgur et al., 1997). These histone tails also mediate interactions between nucleosomes. Lysine acetylation which neutralizes part of a tail region's positive charge is believed to weaken histone/DNA (Hong et al., 1993; Kelly and Kuroda, 1995), or nucleosome/nucleosome interactions (Fletcher and Hansen, 1996; Lurger and Richmond, 1998) and/or signal a conformational change (Norton et al., 1989). This will destabilize the nucleosome structure or arrangement and gives other nuclear factors, such as transcription factors more access to the genetic material. This is supported by the fact that acetylated chromatin is associated with transcription activation (Hebbes et al., 1988; Turner, 1993). The acetylation of histones is a reversible process which was found to be associated with activation, deacetylation tends to correlate with transcriptional repression. The regulation of such opposing processes ensure appropriate level of transcription of various genes (Kuo and Allis, 1998).

B. Histone acetyltransferases (HATs)

In general histone acetyltransferases or (HATs) can either be located in the nucleus or in the cytoplasm. Although some might present in many complexes and locations and don't fit this classification. The nuclear HATs acetylate nucleosomal histones within the chromatin in the nucleus and are linked to transcription (Bronwell and Allis,1996 ; Garcea and Alberts, 1980). While cytoplasmic HATs acetylate newly synthesized free histones in the cytoplasm, which are transported into the nucleus where they are deacetyled again and incorporated into the chromatin (Allis el al., 1985; Ruiz- Carrillo et al., 1975). HATs are very diverse proteins and they belong to different protein families including Gcn5-related N-acetyle transferase superfamily (GNAT), the MYST family, the p300/CPB family, nuclear receptor coactivators,

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TBP- Associted factor TAF$_{II}$250, and TFIIC (sturner and Berger, 2000). Since this thesis is only concerned about SAGA histone acetyltransferase complex which contains a HAT from the GNAT superfamily, we will summarize the available information about the SAGA complex.

The Gcn5 HAT

Gcn5 belongs to the GNAT superfamily. The proteins of this family were grouped together based on similarity in several regions and acetylation-related motifs, and they include eukaryotic and prokaryotic acetyle transferases with different specificity (Neuwald and Landsman, 1997). Four sequence motifs A,B,C and D with not fully understood functions define this family with motif A being the most conserved region (Dutnall et al. 1998; Wolf el al, 1998). GCN5 was first recognized in the ciliate *Tetrahymena thermophila* and was found to be a transcription related HAT (Bronwel el al., 1996). This 55-KDa polypeptide was able to accelytate free histones (Bronwell and Allis, 1996). Homologs of Gcn5 have been found later on in many organisms including *Saccharomyces cerevisiae* (Georgakopoulos and Thireos, 1992), humans (Candau et al., 1996), mouse (Xu et al., 1998) *Schizosaccharomyces pombe, Drosophila melanogaster* (Smith et al., 1998), *Arabidopsis thalania*, and *Toxoplasma gondii* (Hettman and Soldati., 1999). Such wide spread homology in organisms suggests the highly conserved role throughout eukaryotes. Yeast Gcn5 contains a C-terminal bromodomain, an Ada2 interaction domain and a HAT domain which was found to be required by adaptor-mediated transcriptional activation *in vivo* (Candau et al., 1996). Results of alanine scan mutagenesis perfomed in the Gcn5 HAT domain have demonstrated the correlation of Gcn5 HAT function with cell growth, *in vivo* transcription, and histone acetylation at the Gcn5-

dependent *HIS3* promotor in vivo (Kuo et al., 1998). Further mutation studies have also revealed the effect of HAT activity of Gcn5 in chromatin remodeling at the *PHO5* promotor *in vivo* (Gregory et al., 1998). Other investigations have found that recombinant Gcn5 acetylates histone H3 strongly and H4 weakly *in vitro* in a free histone mixture. Analysis of these reactions products revealed primary sites of acetylation were lysine 14 in histone H3 and lysines 8 and 16 in histone H4 (Kuo et al., 1996). This acetylation activity was not found in nucleosomal histones (Grant et al., 1997, Kuo et al., 1996; Scott et al., 2000), except under special conditions and at high enzyme concentrations (Tse et al., 1998).

In the humans the Gcn5 subclass of acetyltrasnferase is represented by two closely related proteins, GCN5 and p300/CREB – binding protein associated factor (PCAF). These proteins share remarkable degree of homology throughout their sequence, but they contain an aproximatley 400-residue amino terminal region which is not present in the yeast GCN5 (Xu et al., 1998). *In vivo* and *in vitro* studies on human GCN5 revealed its role as a transcriptional adaptor analogous to those of yeast Gcn5 (Candau et al., 1996). In addition, the human GCN5 was shown to have HAT activity in *vitro* (Yang et al., 1996) and its HAT domain was able to substitute for that of yeast in vivo indicating evolutionary conservation (Wang et al., 1997). The bromodomain of GCN5, similar to other bromodomains has been shown to bind acetylated lysines in histone N-terminal tails (Jeanmougin et al., 1997; Winston, 1992). *In vitro* binding studies have shown direct interactions between yeast GCN5 bromodomain with H3 and H4 N- terminals tails (Ornaghi et al., 1999) indicating its role in contributing to substrate interaction and tethering to chromatin sites (Brownell and Allis, 1996). Using GST (Glutathione S- transferase) pull down assay, Hassan et al. (2007) demonstrated the specificity of binding of some bromodomains including the GCN5 bromodomain to differentially acetylated H3 and H4 peptides as well as HAT- acetylated histones.

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The SAGA histone acetyltransferase complex (SAGA)

The inability of Gcn5 to acetylate nucleosomes which are the physiological substrates for HATs was explained by the requirement for other factors. Identification of native yeast complexes that were capable of acetylating nucleosomal substrates showed the importance of other subunits in their function (Grant et al., 1997). At least four distinct complexes have been identified from the yeast *S. cerevisiae* extracts. These complexes include the SAGA, ADA, NuA4 and NuA3 histone acetyletransferases which mainly acetylate. Below is a brief description of the yeast SAGA and the Human SAGA complexes.

The yeast SAGA is a large 1.8 MDa in size complex was. This complex brings together four different groups of transcription related proteins: Transcription adaptors (Ada proteins), subset of the Spt proteins, and a subset of Tafs (Grant et al., 1998) and the Tra1 protein (Gant et al., 1998; Saleh et al., 1998). The yeast SAGA complex also contains the transcriptional regulator Sin4 (Yu et al., 2000). Using various biolchemical and genetic screening studies, these subunits were found to have various roles, which range from retaining complex integrity to, effect on growth (Grant et al., 1997, Roberts and Winston., 1997, Sterner et al., 1999) activator interactions, nucleosome acetylation to TBP interaction. *In vitro* the SAGA complex was found to be able to stimulate transcription using various chromatin-templates (kundu et al., 1999; Utley et al., 1998; Wallberg et al., 1999). A similar role of transcription stimulation was also found *in vivo* at a subset of genes (Dudley et al., 1999). The SAGA complex was found to give Gcn5 the ability to acetylate nucleosomes and affect the histone substrate specificity of Gcn5, conferring a primary specificity for histone H3 and to a lesser extent for H2B (Grant et al., 1997.) More over the Gcn5 within the SAGA complex expanded its lysine

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specificity on histone H3 indicating the effects of other subunits on GCN5 specificity (Grant et al., 1999). A human GCN5-containing complex has been also identified and purified from HeLa nuclear extracts (Ogryzko et al., 1998) with similar subunits to the yeast SAGA complex (Yu et al., 1998, McMahon et al., 1998) and similar fuctions (McMahon et al., 1998, Vassilev et al., 1998).

Cooperation between ATP- dependent remodeling complexes and HATs

Several studies have indicated cooperation between ATP-dependent chromatin remodeling complexes and HAT complexes to achieve gene regulation. Gcn5/ SAGA and chromatin remodeling complex SWI/SNF were found to display genetic interactions (Pollard and Peterson., 1997, Roberts and Winston., 1997) and together play a role in regulation of some genes (Bigger et al., 1999; Gregory et al., 1999; Sudarsanam et al., 1999).

For example, single mutations in the yeast SAGA subunits or in the ySWI/SNF subunits didn't lead to severe growth defects while simultaneous mutations in both complexes were lethal, suggesting genetic interaction between components of these two complexes. Another evidence was with chromatin immunoprecipitation (ChIP) where it was found that both BRG1 and p300/CBP are present on estrogen receptor-responsive promoters following estrogen treatment of MCF7 cells (DiRenzo et al. 2000; Shang et al. 2000).

Cooperation between the different chromatin modifying complexes can happen in two different ways. One way is through physical interactions between the ATP-dependent remodelers and chromatin modifiers which could increase their affinities for the chromatin template. This potenial interaction could also affect the activities of each complex. The second way is through genetic interaction between various complexes. Genetic studies and genome-

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wide microarray analysis indicates that *ISW2* and *SIN3-RPD3* function synergistically to regulate gene expression (Fazzio et al., 2001; Goldmark et al., 2000). In addition, an *in vitro* transcription system, p300 and P/CAF enhanced NURF-mediated transcriptional activation from a chromatin template (Mizuguchi et al., 2001).

Another possibility is that the alteration of the chromatin template by one complex could make it a better substrate for the other complex. The yeast SWI/SNF complex has been shown to be stabilized on the chromatin template following acetylation in vivo using an altered *PHO5* promoter in yeast. This study suggested that the Gcn5-containing SAGA complex might serve two functions which are acetyling of nucleosomes as well as the stabilization of SWI/SNF complex to the newly acetylated nucleosomes (Syntichaki et al., 2000). This was also shown using an *in vitro* system, the SWI/SNF was shown to preferentially bind and be retained on acetylated templates. This data shows that acetylation stabilizes SWI/SNF association (Hassan et al., 2001). The order in which these complexes would work together might depends on the nature of promoters, thus at different promoters, a different order of recruitment is envisioned. For example, the recruitment of ATP-dependent chromatin remodelers was found to precedes that of HAT complexes on the yeast *HO* promoter (Cosma et al., 1999; Krebs et al., 1999). While the reverse order of complex recruitment has been observed on the IFN-β promoter and for retinoic acid-induced promoters (Agalioti et al., 2000; Dilworth et al., 2000).

The overall objectives of this proposal are to 1) investigate the effects of the linker histone H1 on the binding of SWI/SNF and SAGA to nucleosomal templates as well as to study its effect on the function of SWI/SNF and 2) to investigate the effects of benzo[a]pyrene on cell viability and apoptosis of HepG2 and WRL-68 cell lines and to test the effects of benzo[a]pyrene on the expression of some proteins that are subunits of chromatin remodeling Complexes.

Chapter 2: The effect of histone H1 on chromatin remodeling complexes

Introduction

The previously mentioned organization of DNA into a "beads on a string" structure is not the end of DNA compaction. For efficient compaction this 10-nm fiber is further condensed into a solenoid arrangement involving six to seven nucleosomes per solenoid turn. This is often referred to as the 30 nm fiber. Most of nucleosomes in eukaryotic chromatin are closely associated with histones of the lysine-rich class such as histones H1, H1°, H5, etc. These are often referred to as linker histones (LHs) because they are bound, at least in part, to the linker DNA between nucleosomes. Linker histones' role is believed to create and/or maintain the 30 nm fiber structure of the chromatin fiber. (Zlatanova and Holde, 1996; Holde and Zlatanova, 1996; Ramakrishnan, 1997; Widom, 1998). Since linker histone H1 is the main focus of this study and it will be discussed in more details.

The linker histone H1 is important since H1 proteins, in addition to binding to linker DNA, also bind to one another in a cooperative manner bringing the neighboring nucleosomes together to form the 30-nm fiber. This condensation step provides a compaction ratio that is as high as two orders of magnitude. The 30- nm fiber is found to form only over selected regions of DNA that are characterized by the absence of binding with other sequence- specific non-histone DNA-binding proteins. The presence of DNA-binding proteins and the effects on the formation of 30-nm fibers may depend on the transcriptional status of the regions of DNA (Devlin, 2002). Histone H1 has also been found to have preferential binding to four-way helical juction DNA resembeling the histone H1 binding site in the linker region between

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nucleosomes (Vagra- Weisz st al., 1993). While the linker histones facilitate the formation of higher order structure of chromatin, their presence was not found to be as essential as core histones for chromatin and chromosome assembly. However linker histones play an important role in stabilizing the chromatin structure (Dasso et al., 1994; Shen et al., 1995). Linker histones have three structural domains that include a central globular domain flanked by N- and C- terminal tails (Ramakrishnan et al., 1993). The N- and C-terminal tails of the linker histones bind to the DNA within the nucleosome core and in the linker DNA between the nucleosome cores. These tails harboring basic residues serve to neutralize polyanionic backbone of the DNA which serves in folding the chromatin into higher order structure (Allan et al. 1981; Allan et al 1986; Howe et al., 1998; Carruthers and Hansen, 2000).

Linker histone H1 involvement in gene regulation

As mentioned previously the packaging of cellular DNA into chromatin serves to compact the large genome into a small volume but at the same time the compact nature of the chromatin presents a functional barrier to many cellular process that require access to the DNA (van Holde, 1988). Both *in vivo* and *in vitro* experiments have provided proof of the involvement of histone H1 in gene regulation. The nonrandom distribution of histone H1 on the genome ensures its major role in gene regulation, for example it was abundant in inactive chromatin, and it was suggested to cross link adjacent nucleosomes in inactive regions. Moreover it was found that histone H1 binds differently in expressed regions (Weintraub, 1984). Other experiments showed that histone H1 was less abundant in active genes sequences (kamakaka and Thomas, 1990; Bresnick et al., 1991; Necheva et al., 1989). A later study found that histone H1 was absent in CpG-rich islands which are a major characteristic of

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house keeping genes (Tazi and Bird, 1990). Histone H1 was also found to be abundant in nucloesomes rich in 5- methylcytosine (Ball et al., 1983). Histone H1 has been shown to affect transcription in various ways. In a study Shimamura et al. (1989) found that transcription from the *Xenopus* 5S rRNA gene was repressed by adding histone H1 to minichromosomes that were assembled *in vitro*. This inhibition was reversed when the histone H1 was removed. Another *in vitro* study demonstrated that the addition of histone H1 to the transcriptionally active *Xenopus laevis* chromatin results in the dominant and selective repression of oocyte 5S rRNA genes and satellite 1 DNA, indicating the role of H1 in gene regulation (Wolffe, 1989). Other studies have shown the repression of oocyte-specific 5S rRNA genes by H1 in *vivo* (Kandolf, 1994; Bouvet et al., 1994).

Histone H1 was found to participate in repression of the genome in ground state and that sequence – specific transcription factors induce selective genes by combination of true activation and release of basal repression that is imposed, in part, by H1(Croston et al., 1991). Histone H1 was also shown to bind to and cause increased transcription inhibition on methylated templates compared to unmethylated templates.This higher inhibition of transcription on methylated DNA was at the transcription initiation stage and had no effect on transcription elongation (Levine et al., 1993). Although in one of the studies histone H1 was not found to cause trascriptional repression in reconstituted preblastoderm *Drosophila* chromatin and repression was found only to be confered by nucleosomal structure (Sandaltzopoulos et al., 1994), other studies provided evidence for H1-mediated transcriptional repression (Neil et al; 1995). In this study H1 deposited on nucleosomal arrays was shown to inhibit both transcription initiation and elongation by T7 RNA prolymerase. Inhibition of trasncription was shown to result from premature termination of transcripts. This indicates that histone H1 binding to chromatin might form structures which repress

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transcription. Histone H1 was also found to reduce the amount of RNA synthesis from *in vitro* reconstituted templates to 1-4 percent of that observed with chromatin containing only nucleosome core histones (Laybourn and Kadonaga, 1995). Another evidence was also found when repeat-induced point mutations in histone H1 gene in *Neurispora crassa* caused depression in pyruvate decarboxylase gene, which has a key role in the respiratory-fermentative pathway (Folco et al., 2003). Some studies have shown histone H1 can cause trascription reperession by repressing transcription factor USF binding to stable nucleosomal templates and that this repression was not simply due to steric occlusion (Juan et al., 1995). Instead it was caused by H1 binding leading to reduced transient dynamic exposure of the DNA from the histone octamer surface as was suggested by Polach and Widom, (1995). In another study H1-mediated repression of factor binding was shown to be dependent on the core histone amino-terminal tails. It was suggested that there would be less stable interaction of histone H1 with the core particle in the absence of the amino termini (Juan et al. 1994).

On the other hand, histone H1 was also found to be required for activation of certain genes ,which makes its role much more difficult to understand (Shen and Gorovsky, 1996). Another study has shown parallel results in which histone H1 encoding gene deletion in yeast didn't result in increased gene expression but rather in a modest reduction (Hellaur et al, 2001). Moreover another *in vitro* study has revealed the fact that H1 enhances transcription initiation from a MMTV promoter on which nucleosomes were assembled (Koop et al., 2003). Such findings suggests that histone H1 does not have major effect on global transcription but can be either a positive or negative gene- specific regulator of transcription *in vivo*. Whether H1 role was repression or compaction both facts show its contribution to gene regulation.

The effects of histone H1 on chromatin remodeling

Histone H1's role in stablizing and promoting the formation of the higher order sturcture of chromatin structure causes problems to many processes that require open chromatin sturcutre. Since ATP-dependent remodeling complexes such as SWI/SNF work mainly to make chromatin an open structure for many of the nuclear processes such as replication and trascription, it would be very likely that histone H1 could affect its activity due to its binding to the chromatin structure. Chromatin modification by acetylation is another process that might be affected by the binding of H1. In this study, we will focus on the effects that the binding of H1 to nucleosomal arrays would have on the binding and fuction of these two groups of chramtion-modifying complexes.

Very few studies have shown the effects of histone H1 on the binding or activity of SWI/SNF. One study showed partial inhibition of SWI/SNF activity on nucleosomal templates with H1, which was due to the structure formed by the addition of H1 to nucleosomes and was not due to the presence of histone H1 alone (Hill et al., 2000). Another study supporting the same has shown that abolished activity of hSWI/SNF, xMi-2 and XAcf complexes was not mainly due to histone-induced folding of the array and that phosphorylation of the histone H1 by Cdc2/cyclin B kinase could rescue remodeling of SWI/SNF (Horn et al., 2002). In another study, histone H1 was found to affect the nature and the position of hSWI/SNF products which provides evidence for histone H1 effect on SWI/SNF products (Ramachandran et al., 2003).

Since acetylation of chromatin is a process of chromatin modification and it was found to be a solution to overcome the repressive nature of chromatin by accessing their targets in the highly compacted chromatin, it is logical to test whether involvement of histone H1 can

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affect their binding as well as activities. Histone H4 acetylation during nuleosomal assembly has been sugested to regulate the binding of histone H1 to regulate higher structure of chromatin (Perry and Annunziato, 1991; Perry and Annunziato, 1989). Other studies have tested the incorporation of H1 in hyperacetylated chromain and has demonstrated that histone acetylation alters the capacity of histone H1 to condense chromatin (Ridsdale et al., 1990) and that its presence affects the ability of transcription factors to interact with DNA (Juan et al., 1994; Schultz et al, 1996). While all these studies presents the effect of acetylation on histone H1 incorporation, other studies show the reverse effect, that is the possible effect of histone H1 incorporation on the acetylation process. H1 and H5 were found to specifically inhibit the acetylation of mono- and oligonucleosomes carried by p300/CPB and that inhibition was found to be due to steric hindrance of H3 with the tails of linker histones and was not due to condensation of chromatin (Herreraa et al., 2000). Such inhitory effect of H1 was also examined in an *in vivo* study testing the acetyaltion activity of nuclear extracts prepared from normal cells on the chromatin templates prepared from normal and H1-overexpressing cells, this study has proven reduced activity on templates with excess H1 and such an inhibiton was suggested to result from changes in chromatin stucture which might modulate the level or the rate of core histone acetylation *in vivo* (Gunjan et al., 2001). In a more recent study it was shown that H1 repressed p300 acetyltrasferase activity at the Human T- Cell Luekemia Virus type 1 promotor (Konesky et al., 2006).

Previous studies were only able to present the many effects that histone H1 might have on chromatin remodeling but others were with more specific findings about how H1 can affect the remodeling process. Pennings et al. (1994) found that adding histone H1 to nucleosomes suppressed a short range mobility of nucleosome cores that generally occurs at low ionic strength conditions. This can point to its effect in any acitvity that requires nucleosomes

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mobility such as ATP- dependent chramatin remodeling by SWI/SNF. Another study has demosntrated that HMG-D and histone H1 could cause local changes in the accessiblity of nucleosomal DNA upon binding to linker DNA (Ragab and Travers., 2003).

Regulating chromatin remodeling can be one of the ways that histone H1 works to regulate transcription or other processes. Not much information is available on how histone H1 interferes with chromatin remodeling complexes but still these information sugests an inhibitory effect of histone H1 on their binding and their activity. Based on studies which demonstrates histone H1 involvement in regulation, we wanted to test the effects of histone H1 on the binding of SWI/SNF and SAGA complexes to *in vitro* assembeled nucleosomes templates. In this study we will use nucleosomal arrays as well as mononculeosomes. Nucleosomal arrays provide a good model for compacted chromatin while, mononucleosomes exclude such possiblity and would shed light on whether repression of both complexes has a common mechanism and function. On the other hand, since less binding reveals less remodeled templates and so less products, the other goal of this study is to test possible inhibitory effect of H1 on ATP- dependent remodeling activity of the SWI/SNF complex which might suggest an effect on any subsequent process of chromatin remodeling.

Experimental procedure

Purification of the SWI/SNF and SAGA chromatin modifying complexes

The SWI/SNF and the SAGA complexes were purified from the yeast strain Saccharomyces cerevisiae using Tandem Affinity Purification (TAP) method (Rigaut et al., 1999; lee at al., 2004; Hassan el al., 2006). The Snf6 and Spt7 subunits of the SWI/SNF and SAGA complexes are tap-tagged as described by . These tagged strains were grown in six liters of Yeast Peptone Dextrose (YPD) media until an optical density (OD600) of 3.0 was reached. Cells where then harvested and yeast whole cell extract was obtained by bead beating using glass beads in extraction buffer that contained 40 mM HEPES-KOH pH 7.5, 350 mM NaCl, 0.1 % Tween-20, 1μg/ml pepstatin A, 2 μg/ml leupeptin, 0.5 mM DTT, 1 mM PMSF. Whole cell extract was then cleared by centrifuging at 13000 rpm for 30 min. The supernatant was then ultracentrifuged and the resulting supernatant which contains the cellular proteins was bound to 0.5 ml of IgG affinity resin (Amersham, Sweden) which had been pre-washed three times with the extraction buffer. Binding was carried out by rotating over night at 4 ^{0}C. IgG resin was then washed once with 20 ml of extraction buffer and then with 10 ml of TEV cleavage buffer (10 mM Tris, p H 8, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 10% Glycerol, 1 mM PMSF, 2 μg/ml leupeptin, 1 μg/ml Pepstatin A, 1 mM DTT). The IgG resin was then re-suspended in 1 ml of TEV cleavage buffer and cleaved overnight with TEV protease on a rotator. The cleaved protein was then eluted and resin was washed with 3 mL of calmodulin binding buffer (10mM Tris p H 8 , 1 mM MgAc, 1 mM imidazole, 2 mM CaCl$_2$, 2 mM PMSF, 2 μg/ml leupeptin, 1 μg/ml Pepstatin A, 0.5 mM DTT). The elute was then supplemented with 3 μl of 1 M CaCl$_2$ and incubated with 0.5 ml of washed calmodulin resin (Amersham, Sweden) overnight. The calmodulin resin was then washed and the bound

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proteins eluted with calmodulin elution buffer (150 mM NaCl, 10mM Tris pH 8, 1 mM MgAc, 1 mM imidazole, 2 mM EGTA, 0.1 NP- 40, 10% glecerol, 1 mM PMSF, 2 µglml leupeptin, 1 µg/ml Pepstatin, 0.5 mM DTT). Elution was in 5 fractions of 200 µl after which the elution was concentrated by a concentrator from Millipore. This was followed by elustions with calmodulin elution buffer that contained 0.5 M and 1 M NaCl. Protein quality was checked both SDS-PAGE electrophoresis followed by silver stain and Western blot using Anti –Tap antibodies. Figure 2.1.A shows the silver stain of purified SWI/SNF and SAGA complexes. Figure 2.1.B shows the Western blot of the same purified protein complexes.

The Biotinylated G5E4-5S nucleosomal array template

G5E4-5S template was prepared by using pG5E4T plasmid (Figure 2.2.A) which contains five consensus Gal4-binding sites upstream of the adenovirus 2 E4 minimal promoter flanked on both sides by five 5S rDNA nucleosome positioning sequences (Figure 2.2.B) (Ikeda et al., 1999). 80 µg of pG5E4T plasmid was digested by 5 units of Asp718 restriction enzyme (Roche) in 1X Boehringer Manheim B (10 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM MgCl, 1 mM Beta-mercaptoethanol at 37 °C for 3 hours. Complete digestion was verified on 1% agarose gel (Figure 2.2.C).The digestion product was then biotinylated using Klenow enzyme (Gibco) 1X Boehringer Manheim buffer B in the presences of 0.1 mM of dNTPs – dATP plus Biotin-14 dATP (Invitrogen, USA) at a final concentration of 0.4 mM. The labeling reaction was carried on at 30 °C for 30 minutes. The biotinylated product was purified using PCR purification kit from QIAquick PCR purification kit (Qiagen, Hilden, Germany). This was followed by a second digestion using 10 units and 6 units of restriction enzymes Cla I and Eae I (New England BioLabs, USA) respectively. Digestion was carried out at 37 °C for

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3 hours. The digestion produces the biotinylated 2.5 kb G5E4-5S fragment of interest and other fragments of smaller sizes. To separate the 2.5 kb fragment, the DNA was run on 0.8 % agarose gel at a voltage of 5 V/ cm (Figure 2.2D) excised and purified by Qiagen Gel Extraction kit. G5E4 DNA concentration was measured by UV spectrophotometry.

Reconstitution of nucleosomal arrays and chromatosomes

The quality of hyperacetylated or unacetylated core histones purified from Hela cells as well as linker histone H1 (purchased from Sigma) was checked after running on 15% SDS-PAGE followed by silver staining (Figure 2.1.C). Reconstitution was done as described previously. Briefly, 0.6 μg of the G5E4 array fragment was incubated with 0.6 μg of each of hyperacetylated or unacetylated core histones to achieve a 1.2:1 molar ratio of core histones to nucleosomal sites on DNA. The reconstitution reaction contained 2 M NaCl in a final volume of 10 μl. The reaction was first incubated for 15 min at 37 °C, then it was serially diluted by adding 3.3, 6.7, 5, 3.6, 4.7, 6.7, 10, 30, and 20 μl of buffer A (50 mM HEPES (pH 7.5), 1 mM EDTA, 5 mM DTT, 0.5 mM phenylmethylesulfonyl fluoride(PMSF)) with 15-minutes incubation at 30 °C for each dilution step. The reaction was brought to a final NaCl concentration of 0.1 mM by adding 100 μl of buffer B (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% Nonidet P-40, 5 mM DTT, 0.5 mM PMSF, 20% glycerol and 100μg/ml of BSA) and incubated for 15 minutes at 30 °C. To make nucleosomal arrays in the presence of H1, it was added in the step at which the NaCl concentration was 0.6 mM. The molar ratio of H1 to nucleosomes was approximately 1:1. Reconstitutions quality was checked by running them on a 1% agarose gel in 1x TBE buffer at 5 v/1 cm and products were stained with Ethidium bromide (Figure 2.3.A).

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Micrococal Nuclease digestion to check the reconstitutions

Approximately 500 ng of each of naked DNA and nucleosomal array template were digested separately with 2 units of MNase enzyme in a reaction buffer that contained 50 mM KCl and 3 mM of caclium chloride. Digestion was carried on at room temperature for 2 minutes, after which it was stopped by adding EGTA to a final concentration of 10 mM. Products were then extracted with phenol/chloroform and ethanol precipitated, and then resolved on a 2 % agarose gel that was stained with ethidium bromide (Figure 2.3.B).

Immobilizing nucleosomal arrays on magnetic beads

The G5E4 and nucleosomal templates with and without H1 were bound to streptavidin paramagnetic beads (Promega) (Hassan et al., 2002; Carrozza et al., 2003). First, 30 μl of magnetic beads were washed twice with a buffer that contained (10 mM HEPES, 5 % glycerol, 50 mM KCl, 0.25 mg/ml BSA and 2 mM $MgCl_2$). Separation of beads was done by using a magnetic separator. The templates were then added to these washed magnetic particles in a binding buffer containing (10 mM HEPES, 5 % Glycerol, 300 mM KCl, 0.25 mg/ml BSA and 2 mM $MgCl_2$) and incubated for 3 hours at 30 °C with mixing. The supernatant was saved and the beads were washed twice resuspended in 30 μl of the buffer. Binding efficiency was checked by both running reconstitutions before binding to magnetic particles and the supernatant after binding on a 0.8% agarose gel.

Biotinylated pull-down assay

After the nucleosomal arrays are reconstituted with and without H1 and bound to magnetic particles either of the purified SWI/SNF or SAGA complexes were added to it in a pull down assay. Briefly, 5 µl of magnetic particles alone or bound with different DNA templates (nucleosomal arrays with or without H1) was incubated with either SWI/SNF or SAGA in a reaction volume of 20µl in a binding buffer that contained (10 mM HEPES, 5 % glycerol, 50 mM KCl, 0.25 mg/ml BSA and 2 mM $MgCl_2$). After 2 hours incubation at 30^oC with occasional mixing, the beads were separated from the supernatant by a magnetic separator. The beads were then washed twice with binding buffer and re-suspended in the binding buffer. Both the supernatant and beads were run on a 10% SDS-PAGE at 150V for 2 hours. The protein was then transferred onto nitrocellulose membrane, blocked in 5% skimmed milk, 0.05% Tween-20 containing phosphate buffered saline (PBST) for one hour. The blots were then washed twice with PBST and then incubated with anti-TAP primary antibody which is an antibody against the calmodulin epitope (Openbiosystems) with a dilution of 1:1000. The blot were then washed three times with PBST and incubated for one hour at room temperatures with an anti-rabbit secondary antibody conjugated with horse radish proxidase (HRP). The blot was again washed three times with PBST and the signal was detected on blots by SuperSignal chemiluminescence kit (Pierce, Biotechnology, Rockfor, IL, USA) and visualized by atutoradiography.

Generation of a radio- labeled mononucleosomal template

Polymerase chain reaction (PCR) was used to generate a 183 base pair DNA fragment from a plasmid that contains the Gal4 and USF binding sites, called pGUB (Juan et al., 1994; Prochasson et al., 2003). The primers sequences used for the PCR are 5': 5'- GAT CCT CTA GAC GGA GGA CA -3', 3' : 5'- GAT CCC TCG ATT CCA TGG-3'. First, the 5' primer was radio-labeled with ^{32}P by using T4 polynucleotide kinase enzyme (T4 PNK) in 1 X NEBuffer T4 PNK reaction buffer that contains (70 mM Tris-HCl (pH 7.6), 10 mM MgCl, 5 mM Dithiothreitol). In this reaction 0.1 μM of the 5'primer, 10 units of T4 PNK and (25 μci) of ^{32} P-ATP (Institute of Isotopes Co., Ltd, Budapest) in a 10 μl reaction volume was incubated for one hour at 37°C followed by incubation for 10 minutes at 70 °C to stop the reaction . After labeling the primer with ^{32}P, a PCR was set to generate the GUB fragment. The PCR reaction volume was 100 μl and had 0.2 mM deoxynucleotides (dNTPs), 0.1 μM of the 3' primer and all the kinase reaction (10μl) that contains the labeled 5'primer and Taq polymerase and the polymerase buffer. Using denaturing, annealing and extension temperatures of 94 °C, 57 °C and 72 °C respectively for one minute in each of these steps. The PCR reaction was done for 30 cycles using PCR machine (Thermocycler). The PCR product was then separated on a 2 % agarose gel and purified by Qiagen Gel Extraction kit. The radiolabeled fragement was eluted in 20 μl TE buffer and radioactivity was counted.

Reconstitution of the GUB mononuclesomes with and without H1

The ^{32}P labeled GUB DNA fragment was used to reconstitute mononucleosomes with and without the presence of histone H1. Reconstitution was done by mixing 2 μl of GUB template

in a reaction buffer that contained 1 M NaCl and the template was titrated with different amounts of short oligonucleosomes. A step wise dilution of the reaction was done by adding buffer A that contained 50 mM Hepes (pH 7.5), 1 mM EDTA, 5 mM DTT, 0.5 mM phenylmethylesulfonyl fluoride (PMSF) to achieve a serial dilution in the salt concentration with the reaction, followed by the addition of Buffer B (10 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM beta mercaptoethanol). The final NaCl concentration in the reconstitutions was 0.1 M. After that the products were separated on a 8 % polyacrylamide gel using a 0.5 X TBE buffer, exposed to X-ray film and developed to check the signal intensity. Based on signal intensity the optimum amount of oligonucleosomes used for the reconstitutions was determined. The same reaction was repeated this time but the template was titrated with increasing amounts o histone H1. Histone H1 was added on the second step of salt dilution when salt concentration was 0.6 M the incorporation of H1 to form chromatosome particles was then checked by running products on a 8 % poly acrylamide native gel as before (Figure 2.6.A).

Gel mobility retardation assay

Naked DNA and mononuleosomes were titrated with different amounts of both SWI/SNF and SAGA complexes to find out the amount needed to cause a band shift. The Reaction was carried in a 20 μl reaction buffer containing (10 mM HEPES, 5 % glycerol, 50 mM KCl, 0.25 mg/ml BSA and 2 mM $MgCl_2$) , The reaction was then incubated at $37^{\circ}C$ for 1 hour and resolved on a 4% polyacrylamide gel and visualized as before. After determining the amount of SWI/SNF and SAGA needed for the gel shift, the same reaction was repeated with nucleosomes in the presence or absence of H1 (Figure 2.6.B and C).

Restriction enzyme accessibility assay

SWI/SNF complex was incubated with nuleosomes ±H1 in the presence or absence of

ATP to allow remodeling (Prochasson et al., 2003; Hassan et al., 2006). This reaction was

similar in conditions to the gel shift explained above except for the addition of ATP. Then 1

µL of Sal1 restriction enzyme was added and allowed to digest DNA for 30 minutes at 30 °C.

The enzyme activity was stopped by adding 22 µl of stopping mixture that contained (20 mM

Tris, 50 mM EDTA, 2 % SDS,0.2 mg/ml Protinase K, 1 mg/ml glycogen) and incubating the

reaction for one hour at 70 °C. The products of digestion were then ethanol precipitated and

boiled in 5 µl of 95% formamide dye prior to resolving on a 6% denaturing. Results were

visualized by using X-rays films (Figure 2.7).

Results

Purification of chromatin modifying protein complexes and the G5E4 nucleosomal

array template. Following the purification of the TAP-tagged yeast SWI/SNF and SAGA

complexes by tandem affinity purification method, both protein complexes eluted at three

different salt concentrations (0.150, 0.5 and 1M) were resolved on 10 % SDS- PAGE gel

and were visualized by silver staining (Figure 2.1.B). This figure demonstrates the purity of

protein complexes eluted since different subunits can be seen in the gel. Different amounts of

the same proteins complexes were analyzed by Western blot as seen in Figure 2.1.A. In

addition, the purity of core histones, acetylated core histones, and histone H1 was also tested

by running them on a 15% - SDS PAGE followed by sliver staining (Figure 2.1.C).

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Nucleosomal arrays were assembled on the G5E4-5S fragment containing tandem repeats of the sea urchin 5S rDNA nucleosome positioning sequences. This template for nucleosomal assembly was generated by enzymatic digestions of the p2085S-G5E4 (Figure 2.2.A). The efficiency of digestion was tested by checking DNA after each digestion step on a 1% native agarose gel. Figure 2.2.C shows the uncut plasmid in lane1, while complete linearization of the plasmid after digestion with restriction enzyme *Asp718* is shown in lane 2, lane 3 shows subsequent digestion by the restriction enzymes *Cla I* and *Eae I* to generate the 2.5 kb DNA template. Figure 2.2.D shows total DNA loaded after complete digestion and
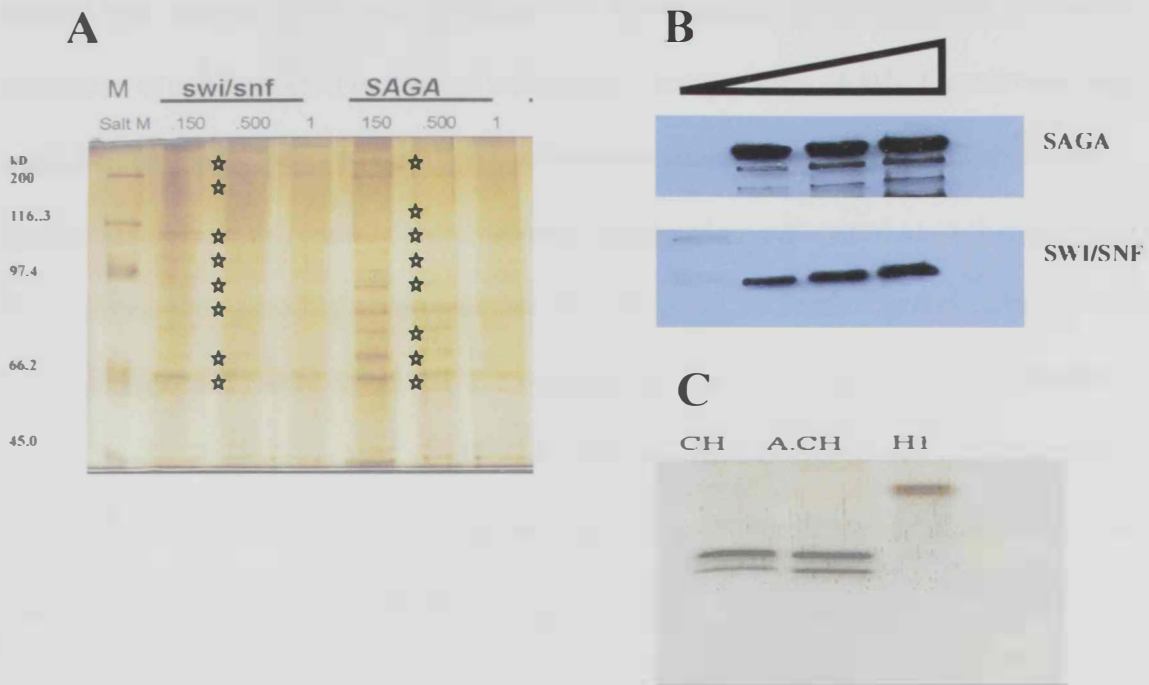
Fig 2.1: Purified SWI/SNF, SAGA, and histones. (A) TAP tagged SWI/SNF and SAGA complexes were purified by the Tandem Affinity Purification method. Complexes were eluted from calmodulin affinity resin at different salt concentrations, 0.15 M, 0.50 M and 1 M NaCl. The purified complexes were run on a 10% SDS- PAGE gel and silver stained. (B) Western bolt for different amounts of the purified SWI/SNF and SAGA protein complexes were run on 10% SDS-PAGE (C) Acetylated, unmodified core histones, and H1 were run on a 15% SDS-PAGE gel, followed by silver staining.

separation of fragments on a 0.8% agarose gel. The 2.5 kb fragment was excised and purified by QIAquick gel extraction kit from Qiagen. DNA concentration was measured by using UV Spectrophotometry and was found to be 0.2 μg/μl.

After the preparation of the 5S-G5E4 template, it was used to assemble nucleosomal arrays by salt dilution. The template was titrated with core histones till a ratio of 1:1 was achieved. Successful nucleosome assembly was detected by slower migration of the assembled nucleosmes on a 0.8% agarose gel.(Figure 2.3.A). Lane 2 of the figure shows the 2.5kb naked DNA, lane 3 shows the nucleosomal array. Lanes 4 and 5 show the same nucleosomal arrays titrated with two concetrations of histone H1. The increasing amounts of H1 led to slower migration of nucleosomes on the gel indicating, incorporation of H1. The slowest migrating band in lane 5 was found to match H1 to nuclosomes molar ratio of 1:1. This figure demonstrates how different reconstitutions migrate when compared to each other.   To further demonstrate the assembly of nucleosmes, a micrococal nuclease digestion assay (MNase) was perfomed on both naked DNA and the nucleosomal array. In Figure 2.3.B, digestion for 2 minutes with MNase revealed a ladder like pattern when nucleosomes were digested, indicating protection of DNA sites by the nucloesomes (lane 2) while digestion of naked DNA produced complete digested products (lane 1).
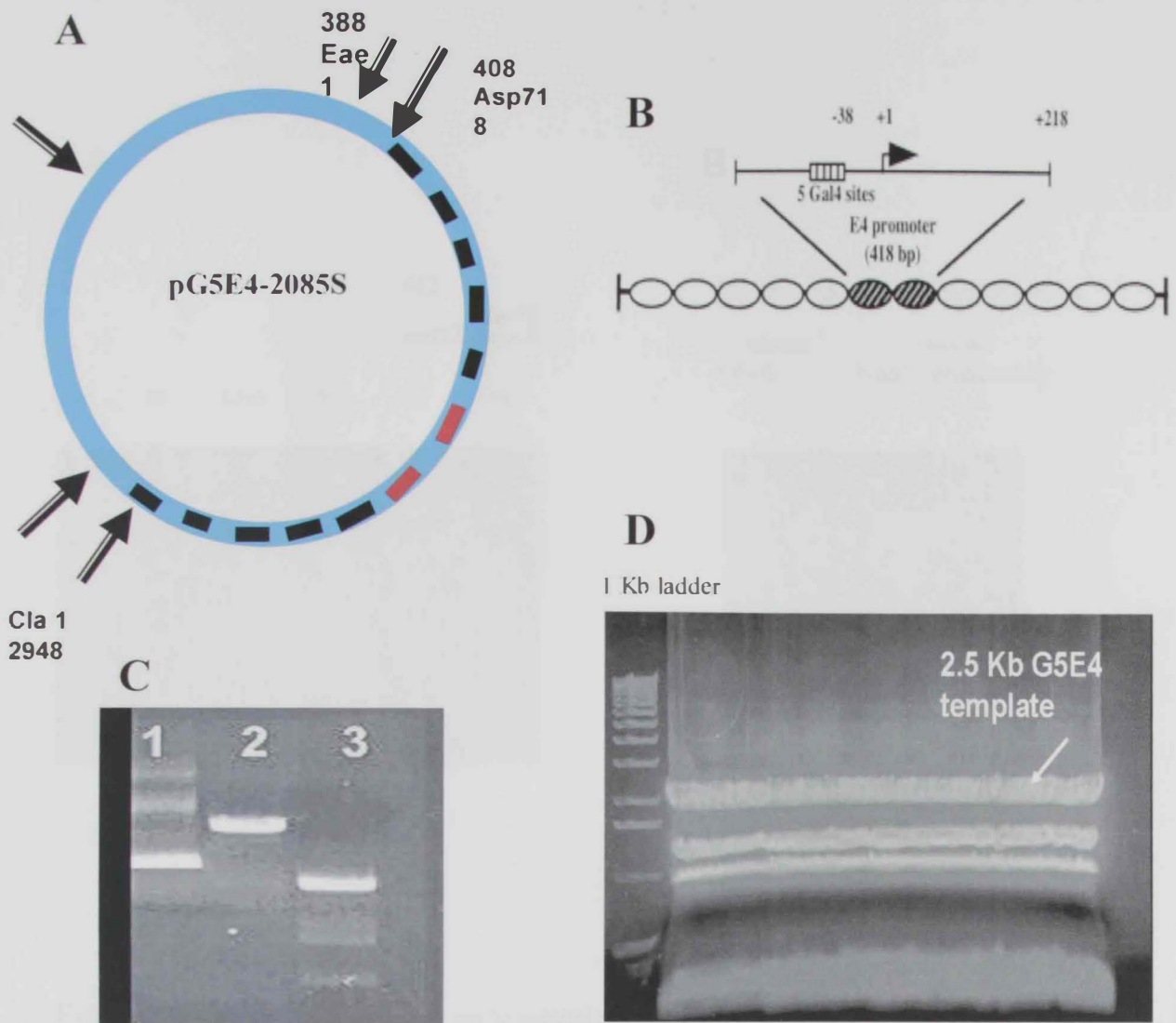
Fig 2.2: Generation of a 2.5 kb DNA fragment containing 5S-rDNA nucleosome positioning sequences. (A) A map of the pG5E4 with the position of the restriction sites that were used.(B) A diagram of the 2.50 bp G5E4 fragment, It has a 5Gal4 binding sites upstream of the adenovirus minimal E4 promoter (Utley et al, 1998).(C) An agarose gel showing 1 μg of each of uncut pG5E4 in lane 1, pG5E4 cut with Asp718 in lane 2 and cut with *Eae I* and *Cla I* in lane 3. (D) An agarose gel showing the 2.5 kb G5E4 template that was cut from a 1% agarose gel for purification.

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A

H1

M   DNA   Nu   Nu   Nu

B

Mnase digestion of nucleosomes

2 minutes   2 minutes
DNA   Nuclesomal array
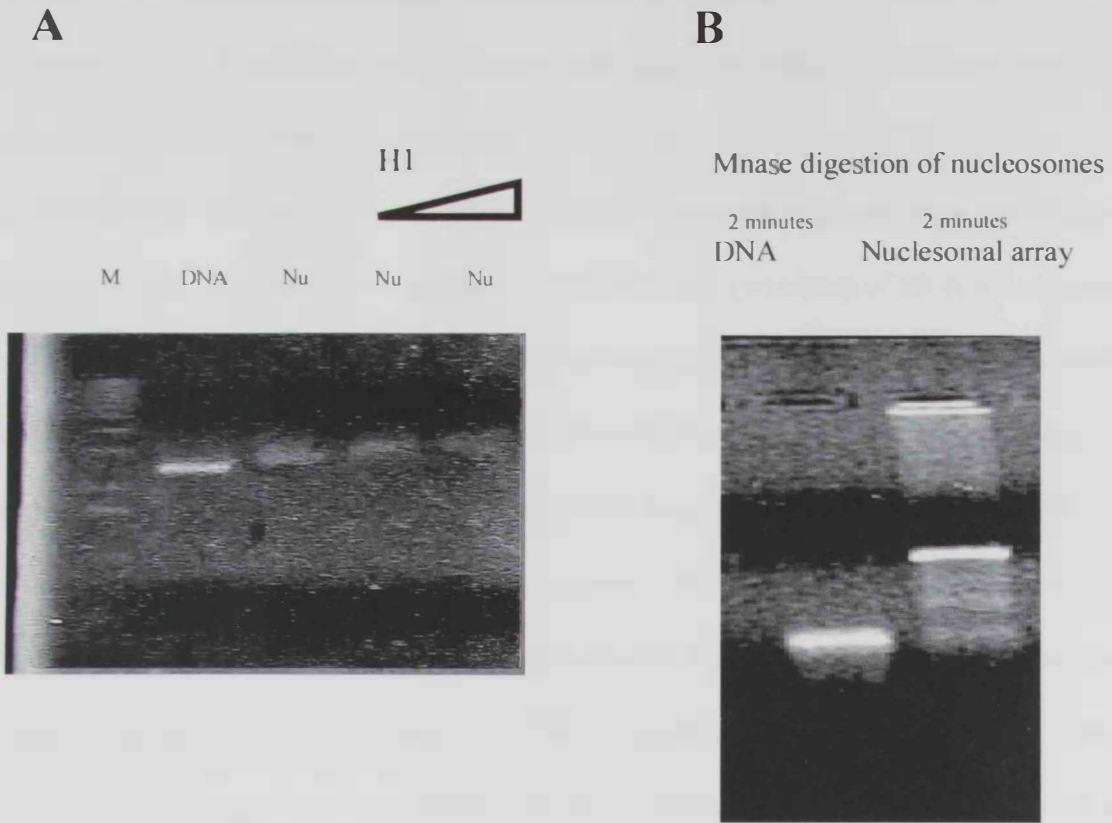
Fig 2.3: Preparation of the G5E4 nucleosomal array.(A) An agarose gel showing the reconstitution of G5E4 template into nucleosomal array and chromatosomal array, lane 1 is the 1 kb DNA ladder, lane 2 shows the migration of the 2.5 kb DNA template alone and lane 3 reconstituted with core histones and lanes 4 and 5 shows nucleosomes with increasing amounts of H1 (B) MNase digestion of the reconstituted G5E4 template, lane 2 shows the laddering of the array after a brief digestion with MNase, while, this laddering is not observed with the same amount of MNase digestion for the naked DNA shown in lane 1.

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Histone H1 represses the binding of both SWI/SNF and SAGA complexes to nucleosomal arrays. To study whether histone H 1 affects the binding of SWI/SNF and SAGA complexes to nucleosomal arrays, a pulldown assay was done. SWI/ SNF and SAGA binding to nucleosomal arrays reconstituted with or without H1 was tested in separate experiments. Each complex was incubated with magnetic beads with different templates and then magnetic beads were separated and resolved on a 8 % SDS- PAGE and was immunoblotted followed using anti-TAP antibody incubation. Beads alone were included as a control for specific binding to magnetic beads and the supernatant of all reactions were included to ensure that less protein binding to templates on beads was not due to unequal protein loading input. Figures 2.4.A and B show the binding of SWI/SNF and SAGA respectively to nucleosomal array templates. The nonspecific binding of SWI/SNF complexes to beads is shown in lane 1 of Figure 2.4.A. Lane 2 shows the binding of SWI/SNF complex to the naked DNA and lane 3 shows similar binding to nucleosomal arrays, while, in lane 4 there is very clear reduction in binding of SWI/SNF complex to nucleosomal arrays in the presence of H1. Lanes 5-8 show the supernatants of the four binding reactions, they show the amount of SWI/SNF complex that was left unbound in lanes 1-4 respectively. Same inhibitory effect of H1 upon the binding of SAGA complex to nucleosomal arrays is observed in Figure 2.4.B. Nonspecific binding of SAGA to magnetic beads alone in lane 1, lane 2 shows binding of naked DNA and lane 3 shows similar binding to nucleosomal arrays, while, the binding in the presence if H1 was diminished in lane 4. Lanes 5-8 show the supernatant of the four binding reactions, they show the amount of SAGA complex that was left unbound in lanes 1-4 respectively. All these results show inhibitory effect of histone H1 on the binding of both the SWI/SNF and SAGA complexes to nucleosomal arrays.

Fig 2.4. Autoradiograms of western blot showing that histone H1 inhibits the binding of SWI/SNF and SAGA complexes to nucleosomal arrays **(A)** The binding of SWI/SNF to nucleosomal array templates in the presence or absence of H1. Lane 1 shows the binding of SWI/SNF complex to magnetic beads as a control. Lanes 2 and 3 show the binding of SWI/SNF complex to DNA and nucleosomes respectively. Lane 4 shows the binding to nucleosome +H1. Lanes (1-8) show the presence of unbound SWI/SNF in supernatant in the same order. (B) The binding of SAGA to nucleosomal array templates of with and without H1. Lane 1 shows the binding of SAGA complex to magnetic beads as a control. Lanes 2 and 3 show the binding of SAGA complex to DNA and nucleosomes respectively. Lane 4 shows the binding to nucleosome +H1. Lanes (1-8) show the presence of unbound SAGA in supernatant in the same order.

Histone H1 represses the binding of SWI/SNF and SAGA complexes to acetylated nucleosomal arrays. To examine the effect of histone H1 on the binding of SWI/SNF and SAGA on acetylated nucleosomal arrays, arrays were reconstituted as previously described using hyperacetylated core histones rather than unmodified ones (Figure 2.5.A). Fig 2.5.B and C show the results of the biotinlyated pull-down experiments in which increased binding of SWI/SNF as well as SAGA complexes to hyperacetylated nucleosomal array was observed (lane 2 in both figures) when compared to unmodified templates (lane 1 in both figures), demonstrating better binding of SWI/SNF to acetylated nucleosomal arrays when compared to unmodified templates which is due to the presence of bromodomain that recognizes acetylated histones. Moreover the binding of both of these complexes of both was reduced when hyperacetylated nucleosomes in the presence of H1 was used to reconstitute the template (Figure 2.5.B and C lanes 3 and 4). This suggests that even with acetylated nucleosomes, histone H1 can repress the binding of SWI/SNF and SAGA complexes to nucleosome. In other words, while acetylation can increase the binding of these complexes to nucelosomes, the repression by histone H1 is still significant. The acetylation and histone H1 binding seems to have almost equal but opposing effects in the binding of SWI/SNF and SAGA complexes to nucleosomal arrays.
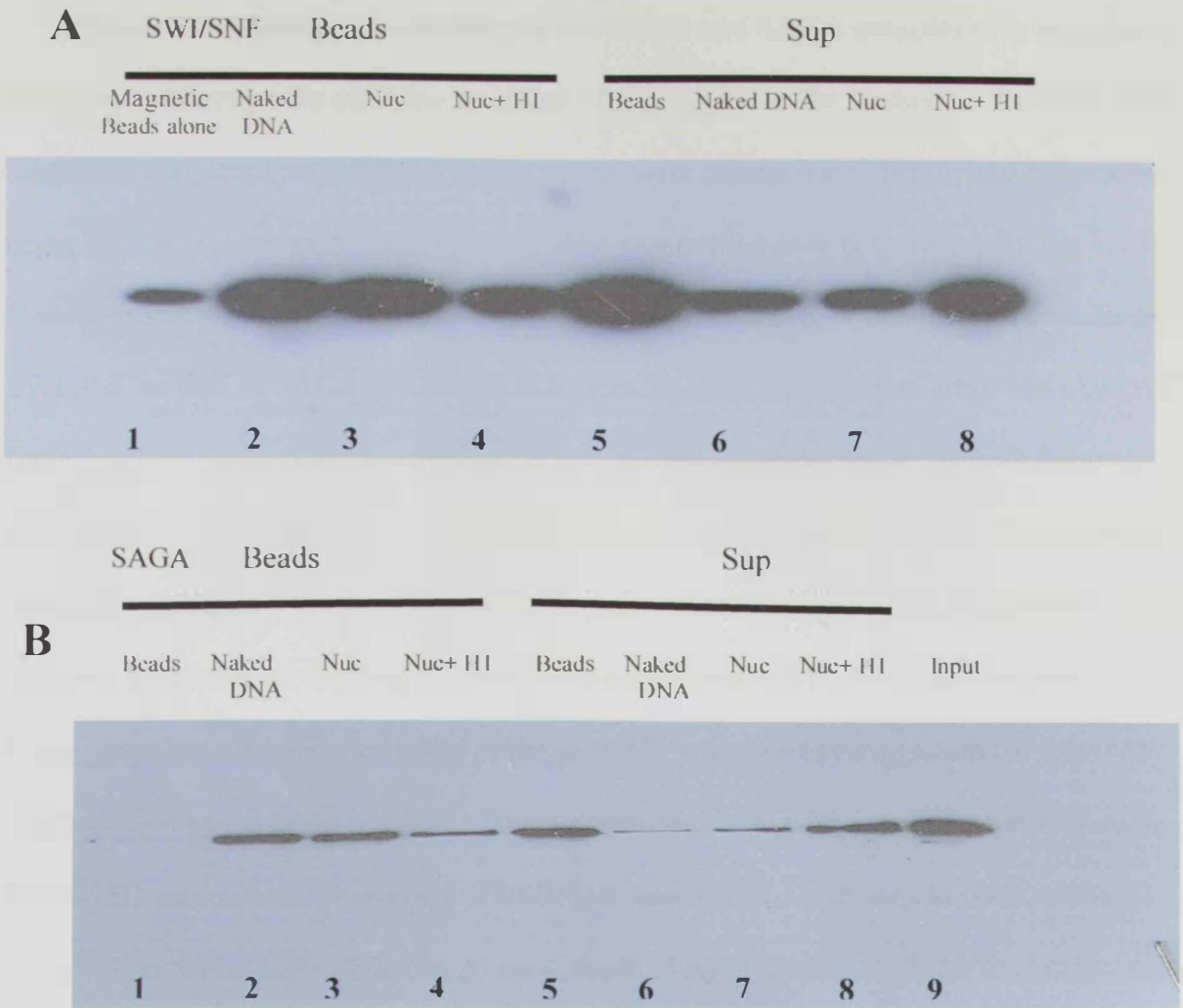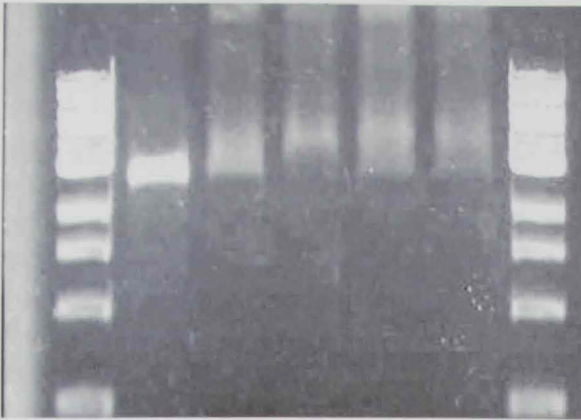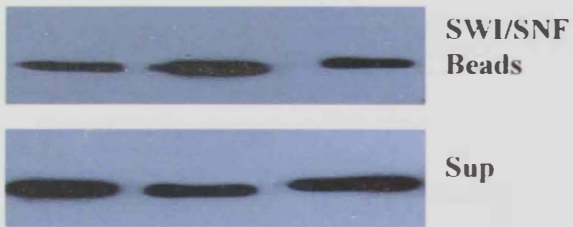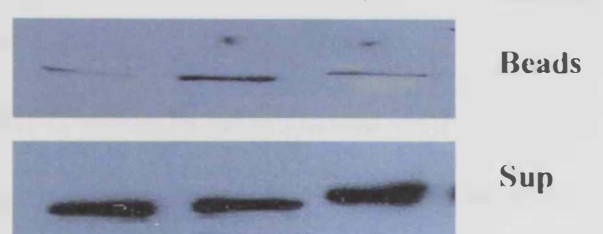
Fig 2.5: Autoradiograms of western blot showing that histone H1 inhibits the binding of SWI/SNF and SAGA to acetylated nucleosomal arrays (A) An agarose gel showing the reconstitution of the 2.5 kb G5E4 template with hyperacetylated histones in the presence and absence of histone H1. (B) Western blot showing the binding of the SWI/SNF complex to nucleosomal array reconstituted with hypracetylated histones in the presence and absence of H1. (C) Binding of the SAGA complex to nucleosomal array reconstituted with hypracetylated histones in the presence and absence of H1.

Histone H1 inhibits the binding of SWI/ SNF but not SAGA to monoculeosomes.
A gel mobility retardation assay performed on to find out the effect of histone H1 on the
binding of SWI/SNF and SAGA complexes to mononucleosme template. This was done by
generating 183 bp DNA fragment from the pGUB by PCR. Amplification was preceded by
end labeling using ^{32}P- ATP followed by PCR. The template was then reconstituted into
mononucleosomes, this was followed by another reconstitution to form mononucleosomes
with histone H1. Reconstitution products were resolved on a 6% acrylamide native gel in
which the success of reconstitution was recognized by a band shift caused by slower migration
(Figure 2.6.A lanes 2 and 3) compared to the DNA alone (lane 4) The reconstituted templates
in the presence or absence of histone H1 were incubated with increasing amounts of SWI/SNF
(Figure 2.6.B) or SAGA (Figure 2.6.C) in separate reactions and the products of these
reactions were resolved on 4% native gel. The results show partial binding of SWI/SNF to
naked DNA (Figure 2.6B, compare lanes 1 and 2). The binding of SWI/SNF to
mononucleosomes in the absence or presence of H1 is shown in lanes 3-6 and 7-10
respectively. Lanes 4-6 show the aggregation of mononucleosomes upon SWI/SNF addition,
while lanes 8-10 show a decrease in the intensity of the shifted band (corresponding to
SWI/SNF binding) when the nucleosomes are reconstituted in the presence of histone H1. In
other words histone H1 inhibits the binding of SWI/SNF to mononucleosomes indicating an
inhibitory effect of H1 on SWI/SNF binding. Figure 2.6.C shows the effect of H1 on the
SAGA binding to mononucleosomes. In contrast to the SWI/SNF data, histone H1 didn't
seem to affect the binding of SAGA to mononucleosomes (compare lanes 5-7 and 2-4).

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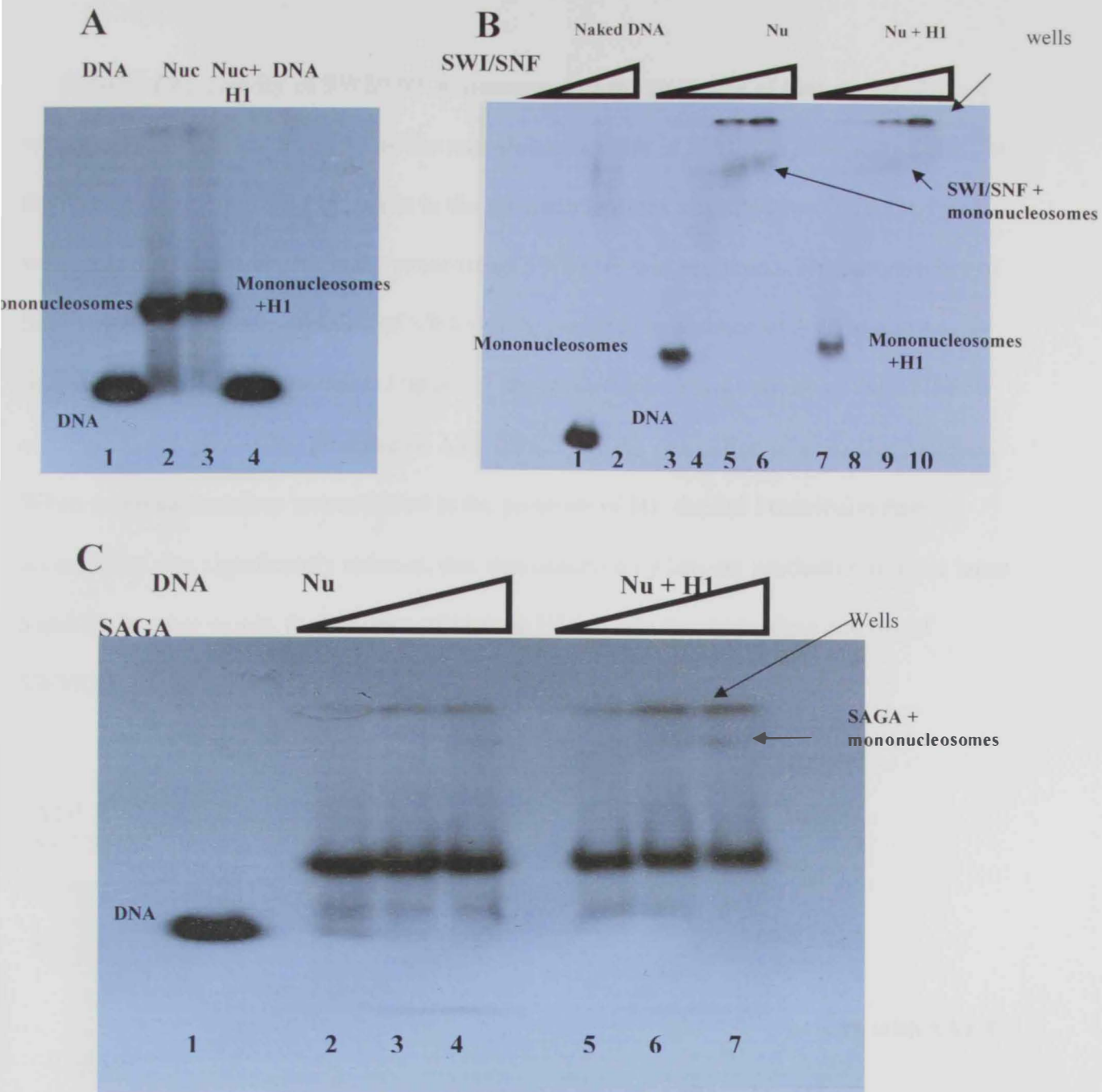
Fig 2.6: Autoradiograms showing that histone H1 inhibits the binding of SWI/SNF, but not SAGA to mononucleosomes.(A) Autoradiogram showing the reconstitution of the 183 bp GUB template into mononucleosomes with and without H1.(B) Gel mobility retardation of the binding of SWI/SNF complex to mononucleosomes reconstituted in the presence or absence of histone H1.(C)

Gel mobility retardation of the binding of SAGA complex to mononucleosomes reconstituted in the presence or absence of histone H1.

Remodeling activity of SWI/SNF is decreased in the presence of histone H1. To test whether histone H1 has an effect on the remodeling activity of SWI/SNF, the accessibility of the restriction enzyme Sal I to its site in the mononucleosmes assembled on GUB fragment with and without histone H1 in the presence of SWI/SNF was measured. The accessibility of Sal I was tested after the addition of SWI/SNF in presence or absence of ATP to allow remodeling of mononucleosomes. Figure 2.7 shows the remodeling activity of the SWI/SNF on mononuclesomes in the presence of ATP (lane 5) by the generation of a smaller fragment. When mononucleosomes reconstituted in the presence of H1, the Sal I restriction enzyme accessibility was significantly reduced, this was observed by less cut products (compare lanes 5 and 8). In other words, the presence of histone H1 inhibits the remodeling activity of SWI/SNF on mononucleosomal templates.
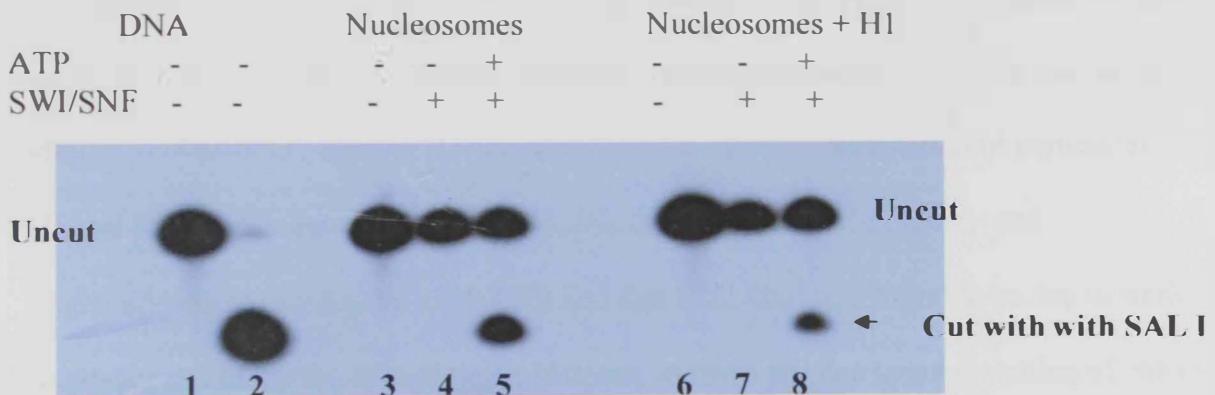
Fig 2.7 Autoradiogram showing that remodeling activity of SWI/SNF is reduced in the presence of histone H1. Chromatin remodeling was followed by the restriction accessibility of the enzyme *Sal I to* mononucleosmal templates. Products were resolved on a 6% denaturing gel.

Discussion

Histone H1 plays a major role in organizing chromatin into higher order structure. Many studies have suggested histone H1 to be a potent inhibitor of transcription (Shinamura et al. 1989; Croston et al., 1991; Laybourn and Kadonaga). It is also well established that the SWI/SNF remodeling complex functions mainly to make chromatin more accessible for the transcriptional machinery (Narlikar and Fan., 2002). Having a common role in gene regulation raised questions on whether histone H1 could affect the binding of SWI/SNF to nculeosomes and its activity. Very few studies have tested how histone H1 affects the SWI/SNF function. One study showed inhibition of SWI/SNF activity on nucleosomal templates with H1, which was found to be confered by the structure formed by the addition H1 to nucleosomes rather than the presence of histone H1 alone (Hill et al., 2000). Another study has supported the same idea but such an inhibtion was not found due to histone-induced folding of the array (Horn et al., 2002). Further studies have shown that histone H1 affects the nature and the position of hSWI/SNF products (Ramachandran et al., 2003). Beside these effects on SWI/SNF, histone H1 was also found to regulate acetylation of chromatin. Histone H1 and H5 were found to specifically inhibit the acetylation of of mono- and oligonucleosomes carried by p300/CPB and that inhibition was found to be due to steric hindrance of H3 by the tails of linker histones and was not due to condensation of chromatin. (Herreraa et al., 2000). Such inhitory effect of histone H1 was also tested in which *in vivo* and was suggested to result from changes in chromatin stucture which might modulate the level or rate of core histone acetylation (Gunjan et al., 2001). In addition histone H1 was found to repress p300 acetyltransferase activity at the human T- cell luekemia virus type 1

promotor (Konesky et al., 2006). All these data demonstrate the repession effects of histone H1 on the trascription from nucleosomal templates (Juan et al. 1994; Juan et al., 1995) and suggests a possible effect on the binding of SWI/SNF and HATs to nucleosomal arrays. Utilizing a purified system to test the binding of SWI/SNF and SAGA to nucleosomal templates with or without H1 we found that histone H1 repressed the binding of both SWI/SNF and SAGA complexes to *in vitro* assembled nucleosomal arrays. This reduced binding is suggested to be either due to chromatin higher order structure promoted by the addition of H1 (Devlin, 2002) by steric occlusion or masking of the sites by histone H1 sites where both complexes might prefer to bind. Such a suggestion is supported by data that showed the preferential binding of histone H1 to four-way helical junction DNA, which is similar to the histone H1 binding site in the nucleosome structure (Patrick et al., 1993). The binding to the four-way helical junction DNA was also found to be a property of SWI/SNF complex as well (Zlatanova and Van holde, 1989). It is possible that histone H1 occupies the binding site where SWI/SNF might prefer to bind. Since SAGA has not been shown to bind to four-way helical junction DNA, the inhibition of SAGA binding might be due to chromatin condensation rather than competition for binding sites as is the case with SWI/SNF. To test whether this histone H1 repression is observed on modified chromatin templates as well, the effect of histone H1 on SWI/SNF and SAGA binding to nuclesomal arrays was repeated using hyperacetylted nucleosomal arrays. Results revealed that same level of inhibition was still seen even when templates were hyperacetylated. To find out whether chromatin higher order structure was behind the repression, mononucleosomes assembled on a ^{32}P- 183 bp labeled DNA fragment reconstituted with or without H1 were used. In a gel shift assay, there was a clear inhibition on SWI/SNF binding to the monucleosmes with H1 while, there was no effect on the binding of SAGA to mononucleosomes suggesting a different mode of repression of

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histone H1 on the binding of SWI/SNF and SAGA to nucleosomal arrays. It is likely that the

inhibition of SAGA binding by histone H1 is due to chromatin condensation rather than steric

occlusion of the binding site. Less binding implies less remodeling activity on nucleosomes

and thus a remodeling assay on mononculeosomes reconstituted in the presence or absence of

H1, SWI/SNF remodeling activity was found to be reduced significantly in the presence of

H1. Such inhibitory effect on remodeling activity might be explained by less SWI/SNF protein

available for remodeling due to less binding, even though we can't exclude the inhibitory

effect of histone H1 on the remodeling function of SWI/SNF itself.

Chapter 3: The effect of benzo[a]pyrene on chromatin remodeling complexes

Introduction

As I have discussed in previous chapters, it is well established that chromatin is an important regulator of transcription and that genes which are packaged into chromatin are transcriptionaly inactive. This compacted structure of chromatin can be remodeled to a more accessible structure to enable transcription activation (Workman and Kingston, 1998). Factors that mediate chromatin transitions are (i) histone acetyltransferases (HATs); (ii) histone deacetylases (HDACs); and adenosine triphospate ATP-dependent chromatin remodeling complexes (Kingston et al., 1996). Alteration in either recruitment or the function of any of these factors can lead to misregulation of transcription; which can affect key regulatory genes leading to improper proliferation and differentiation of cells, events that can lead to cancer. Many studies have linked ATP- dependent remodeling complexes and HATs to cancer which has made chromatin-modifying complexes an important pathway in cancer development. Since chromatin is the substrate on which chromatin remodeling complexes and modifying complexes work, this can raise a question on whether DNA damage can affect their work. Such damage in DNA accounts for the mutagenicity and carcinogenicity of many chemical carcinogens such as the well known polycyclic aromatic hydrocarbons (PAHs). Some studies have provided primary data on how these compounds can modulate gene expression of many chromatin related proteins. Such data encouraged the search for the involvement of chromatin remodeling in the carcinogenicity of those compounds. In this chapter, we study the effects of benzo [a] pyrene (B[a]P) which is a frequently used model compound for PAHs on two cells

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lines, HepG2 (Human hepatocellular liver carcinoma cell line) and WRL-68 (human liver embryonic cells) and investigate whether protein expression of particular subunits of modifying complexes is altered upon treatment with benzo[a]pyrene. In addition, we were interested in understanding how a possible alteration in gene expression can be related to effects seen as apoptosis or increased cell proliferation. First, we will discus the roles of ATP-dependent chromatin remodeling complexes and HATs in cancer followed by a brief description of PAHs and how they induce mutagenicity. Finally, the results of our studies on the effect of PAHs on chromatin remodeling will be presented.

The role of ATP-dependent chromatin-remodeling complexes in cancer

Many studies have implicated ATP-dependent-remodeling complexes in the regulation of cellular growth and proliferation. For example, hBRG1 and Sfh1 protein have been shown to play a role in cells cycle progression (Cao et l., 1997; Khavari et al., 1993), while BRM knockout mice show increased cell proliferation (Reyes et al., 1998), Human ATP-dependent remodeling complexes have also been identified as co-regulators of genes involved in cellular transformation (Muchardt and Yaniv, 1999). It is believed that this sort of regulation is mediated indirectly by the ability of ATP-dependent remodeling complexes to remodel promoters of genes involved in cell cycle control. Other studies demonstrate different possible ways of cell cycle control as physical interactions between subunits of theses complexes and other proteins, or the phosphorylation status of subunits of these protein complexes. For example, it was shown that hBRG1 and hBRM subunits of human SWI/SNF complex physically interact with retinoblastoma protein forming a complex inducing cell cycle arrest

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(Dunaief et al., 1994; Strober et al., 1996). Another study shows that hBRG1 co-immunoprecipitates with cyclin E, which in turn, associates with the cycle dependent kinase Cdk2 to control the G1/S checkpoint of the cell cycle. Moreover in the same study other SWISNF subunits were found to coimmunoprecipitate suggesting that cyclin E/Cdk2 might interact with the SWI/SNF as a whole. Cyclin E/Cdk2 complex was also found to phosphorylate both hBRG-1 and BAF155 subunits which were proposed to regulate the activity of hSWI/SNF (Shanahan et al., 1999). Phosphorylation of subunits in ATP-dependent chromatin remodeling complexes was also suggested to regulate the complex function during the cells cycle. For example, the phosphorylational status of Sfh1p, a component of yeast RSC, was found to oscillate during cell cycle (Cao et al., 1997). Another study have found that hBRM and hBRG1 in SWI/SNF were phosphorylated during mitosis and were excluded from condensed chromatin. Moreover, hBRM was partially degraded in mitotic cells which is believed to lead to inactivation of SWI/SNF during cell division (Muchardt et al., 1996). The phosphorylation of another subunit of SWI/SNF, hswi3 which followed that of Brg1 has also been demonstrated. It was found that the phosphorylated complex with the lost ability to disrupt nucleosomes recovered its activity as cells exited mitosis and became dephosphorylated (Sif et al., 1998). Another study shows phosphoinositol pathway mediated targeting of hSWI/SNF complex to chromatin upon lymphocyte activation (Zhao et al., 1998).

The above mentioned studies provide evidence on how ATP- remodeling complexes are involved in the control of the cell cycle progression. It is also well known that cancer might develop when cell cycle is disrupted. Many studies have demonstrated a relation between ATP-dependent remodeling complexes and cancer. For example rhabdoid tumors, which is an aggressive cancer of the brain and soft tissue, was found to consistently express a mutated hSNF5-INI1, a component of human SWI/SMF complex. In addition, hSNF5-INI1 was found

to display the properties of a tumor-suppressor gene (Versteege et al., 1998; Sevenet et al., 1999). Some studies have shown that mutations in the ATPase subunit of SWI/SNF, Brg1, are also associated with multiple types of tumors (Wong et al., 2000). BRCA1 which is a tumor suppressor gene was also found associated with a SWI/SNF-related complex. It has been shown that BRCA1 can directly interact with the BRG1 subunit of the SWI/SNF complex (Bochar et al., 2000). Moreover, p53-mediated stimulation of transcription by BRCA1 was completely abrogated by either a dominant-negative mutant of BRG1 or the cancer-causing deletion in exon 11 of BRCA1 (Bochar et al., 2000). In another study, c-Myc was found to interact directly with the hSNF5-INI1 component of SWI/SNF and that its transactivation requires an intact and active SWI/SNF complex (Cheng et al., 1999).

The role of histone acetyltransferase (HAT) complexes in cancer

Studies of oncogenic fusion proteins have revealed evidences of the role of HAT complexes to cancer. It was found that a significant number of chromosomal translocations, that are often associated with leukemia, had a significant number of genes encoding HATs (Rowley, 1998). One of the oncogenic fussion proteins which cause acute leukemia in humans is formed by the fussion of the human mixed lineage leukemia/trithorax protein (MLL) and CREB binding protein (CBP), resulting in the MLL-CBP fussion protein. MLL is a DNA-binding transcriptional regulator that interacts with chromatin remodeling complexes, and is important for maintaining the expression of HOX genes, which play an important role in development and hematopoieses (Yu et al., 1998). CBP is a large protein that contains a HAT domain, a bromodomain, and several other domains that bind a wide variety of gene-specific activators (Shikama et el., 1997). Many models have been proposed to explain transcriptional

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misregulation caused by this type of oncogenic fusion. One suggests recruitment of CBP to MLL target sites, resulting in their acetylation and activation (Yu et al., 1998). An alternative model suggests that this fusion protein is recruited to targets not normally bound by MLL. Other models suggest MLL to alter the acetylation activity of CBP or alter interactions of CBP with other proteins, resulting in misregulation of the target. Finally, another possibility is that MLL-CBP is sequestered at MLL- binding sites and thus there wouldn't be enough CBP for other targets (Cairins, 2001). Fusion proteins were also found to form between two HAT proteins such as CBP and other HAT related proteins as Monocytic leukemia zinc finger (MOZ) and MOZ-related factor (MORF) (Borrow et al., 1996; Panagopoulos et al., 2001). One explanation for misregulation by MOZ-CBP and MORF-CBP complexes is the presumably increased acetylation at CBP targets. Also other model is the recruitment of CBP-MOZ and MORF to undefined MOZ and MORF targets (Cairins, 2001). Such fusion was also found to occur between MOZ and transcriptial intermediary factor 2 (TIF2) (Sterner and Berger, 2000). All of these data illustrate that misregulation of HAT targeting and activation could lead to cancer.

Polycyclic aromatic hydrocarbons as cancer causing agents

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants with high carcinogenic potencies that have been linked to the etiology of human cancers through their presence in cigarette smoke and environmental pollutants (Colon et al., 1999). Since they are ubiquitous compounds (present in several places at the same time) they have received much attention and numerous studies reflect that importance. PAHs are a large group of organic compounds with two or more fused aromatic rings (Fig.3.1) They have relatively low

solubility in water, but are highly lipophilic. PAHs are formed mainly as a result of pyrolytic

processes, especially the incomplete combustion of organic materials during industrial and

other human activities, such as processing of coal and crude oil, combustion of natural gas (for

heating) , combustion of refuse, vehicle traffic, cooking and tobacco smoking, as well as

natural processing such as carbonization. Several hundreds of different PAHs, in addition a

number of heterocyclic aromatic compounds as well as nitro-PAHs can be generated by

incomplete combustion (Colon et al., 1999).

Naphthalene   Acenaphthene   Anthracene

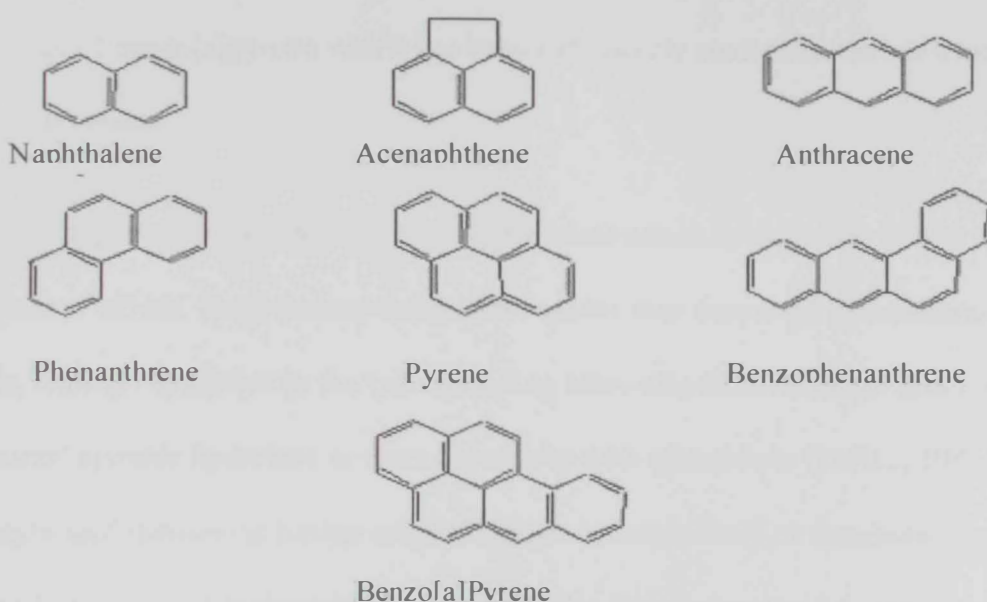Phenanthrene   Pyrene   Benzophenanthrene

Benzo[a]Pyrene

Fig 3.1 Poly cyclic aromatic hydrocarbon PAHs

Metabolism of PAHs

The lipophilic nature of PAHs enables them to readily penetrate cellular membranes and

remain in the body indefinitely. Metabolism of PAHs in the body renders them more water

soluble and more excretable. Metabolism of PAHs occurs in all tissues and it involves several

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possible pathways involving various enzyme activities. A tissue's metabolic route is determined by the activities and the affinities of the enzymes in that tissue. The enzyme system primarily responsible for PAH metabolism is the microsomal mixed function oxidase system (MFO) which converts the non-polar PAHs into polar hydroxy and epoxy derivatives (Hall et al., 1989). Epoxides are the major intermediates in the oxidative metabolism of aromatic double bonds. The epoxides are reactive and ezymatically metabolized to other compounds such as dihydrodiols and phenols (Glatt and Oesch, 1987). The structural similarity of PAHs contributes to the similarities that exist in their metabolism, and the metabolism of benzo[a]pyrene which has been extensively studied is used as a model for PAHs metabolism.

Benzo[a]pyrene is metabolized initially by microsomal cytochrome P- 450 systems to several arene oxides. Once formed these arene oxides may rearrange spontaneously to phenols, undergo hydration to the corresponding trans-dihydrodiols in a reaction catalyzed by microsomal epoxide hydrolase or react covalently with glutathione (IARC., 1983). 6-Hydroxybenzo[a]pyrene is further oxidized either spontaneously or metabolically to 1, 6-, 3, 6- or 6,12- quinones. 3-hydroxybenzo pyrene is also metabolized to 3,6- quinone, and the 9-hydroxy- benzo[a]pyrene is further oxidized to k- region 4,5 oxide which is hydrated to the corresponding 4, 5 dihydrodiol. The phenols, quinones and dihydrodiols can all be conjugated to glucuronides and sulfate esters, the quinone also form glutathione conjugates (IARC., 1983; Agrawal et al., 1991). In addition to being conjugated, the dihydrodiols undergo further oxidative metabolism. The cytochrome P-450 system metabolizes benzo[a]pyrene- 4,5-dihydrodiol to a number of uncharacterized metabolites, while 9, 10 dihydrodiol is metabolized predominantly to its 1- and/or 3 phenol derivative with only minor quantities of

9,10- diol -7, 8- epoxide being formed. In contrast to the 9,10-diol, benzopyrene -7, 8- diol is metabolized to a 7,8 – dihydrodiol -9,10 epoxide and a phenol – diol which is a minor pathway. The diol epoxides can be conjugated with glutathione either spontaneously or by glutathione-S-transferase catalyzed reaction. They may also hydrolyze spontaneously to tetrols (Hall et al., 1987). Fig 3.2 summarizes the metabolism of benzo[a]pyrene.
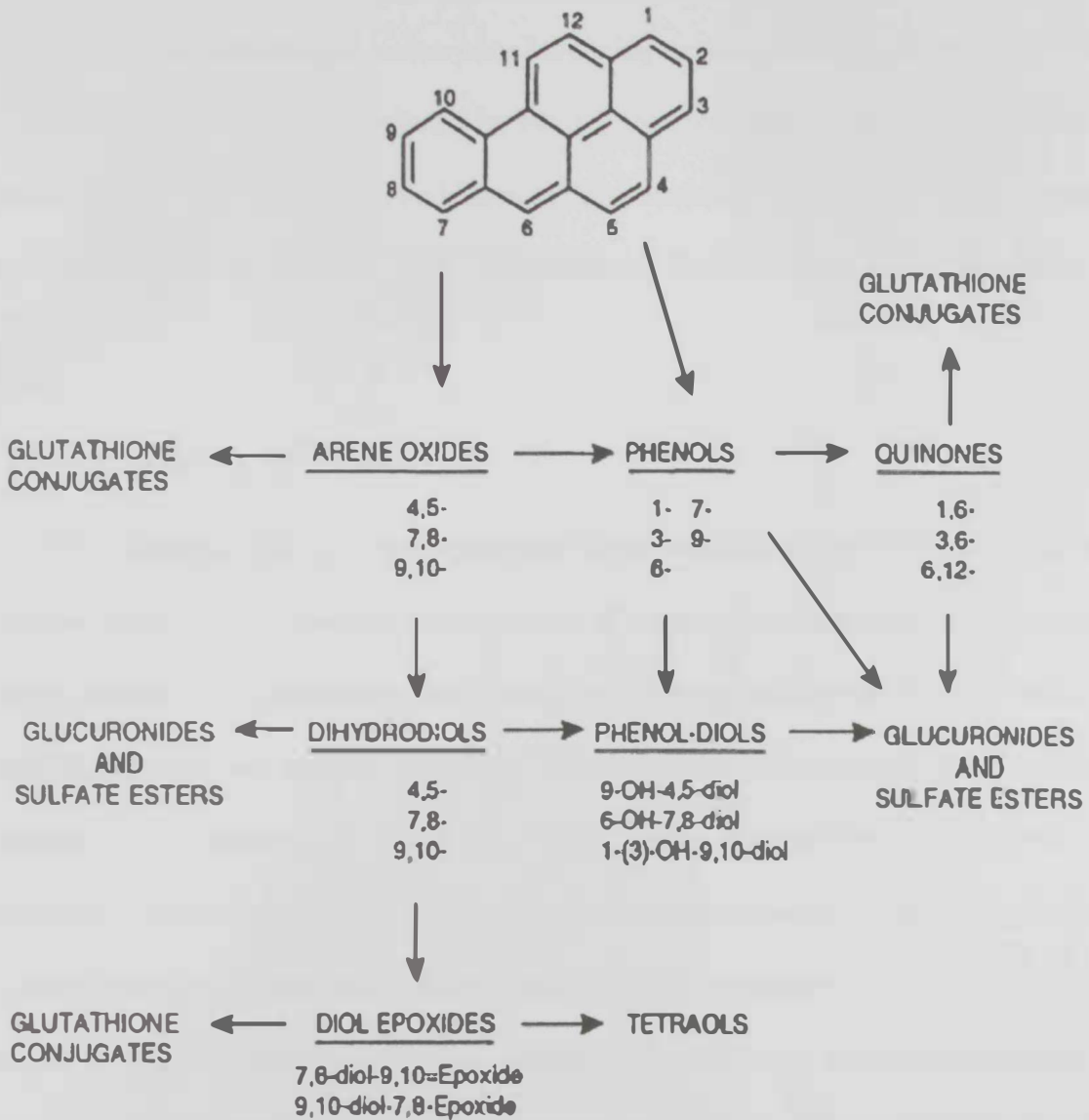

Fig 3.2: Metabolic scheme for Benzo[a]pyrene.

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Enzyme systems that metabolize PAHs are widely distributed in the cells and tissues of humans and animals. The highest metabolizing capacity is present in the liver, followed by lung, intestinal mucosa, skin and kidneys, but metabolism may take place also in nasal tissues, mammary gland, spleen, brain, hair follicles , erythrocytes, platelets, leukocytes, placenta and uterus. Animal and human fetal tissues have the capacity to metabolize PAHs, but at a low rate compared to the adult tissues (Anderson et al., 1989). PAHs stimulate their own metabolism by inducing microsomal cytochrome p-450 monooxygenase and epoxide hydrolases. The induction of isoenzymes belonging to the cytochrome P-450 1A subfamily (CYP1A1 and CYP1A2) is mediated by binding to a cytosolic receptor protein, the Ah receptor (Nebert et al., 1993). In addition to the previous system, other enzyme systems have been suggested to be involved in the metabolism of PAHs (Philipson and Ioannides, 1989).

Mutagenicity of PAHs

The mutagenic effects of benzo[a]pyrene is well established and it is frequently used as a positive control to demonstrate the sensitivity of various test systems to detect the genotoxic action of unknown compounds. Moreover, it serves as a model compound for PAHs. PAHs exert their mutagenic and carcinogenic activity through biotransformation to chemically reactive intermediates which bind covalently to cellular macromolecules.The level of binding of PAH to DNA correlates with the relative carcinogenic potency of the PAH (Brookes and Lawly, 1989). Extensive and systemic studies on the tumerigenicity of individual PAH metabolites in animals have led to the conclusion that vicinal or so called bay-region diol epoxides are the ultimate mutagenic and carcinogenic species of some PAHs, although not the only one (Graslund and Jernstorm, 1994). Two pathways are involved in mutagenicity i) the

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formation of dihydrodiols epoxides that covalently bind to exocyclic amino groups of purines in DNA to form stable adducts. ii) the formation of radical cations that bind to N7 and C8 of guanine to form unstable adducts.

i) Dihydrodiol Epoxides and the formation of stable DNA adducts

Bezo[a]pyrene utilized as a model helps our understanding of how dihydrodiol epoxides are formed, in the case of benzo[a]pyrene, it is called 7, 8-diol -9,10 epoxide. The formation of this metabolite requires three enzymatic reactions: initial epoxidation to yield the 7,8-epoxide, hydrolysis of this epoxide to yield the (-)- trans- 7, 8 diol and finally a second epoxidation of the diol to produce benzo[a]P -7,8 diol- 9,10 – epoxide, (B[a]PDE) (Yang et al., 1977) (Figure.3.3).

(+)-(7R,8S)-epoxide

(−)(7R,8R)-7,8-dihydrodiol

(+)(7R,8R,9S,10R)-7,8-dihydro-diol-9,10-epoxide
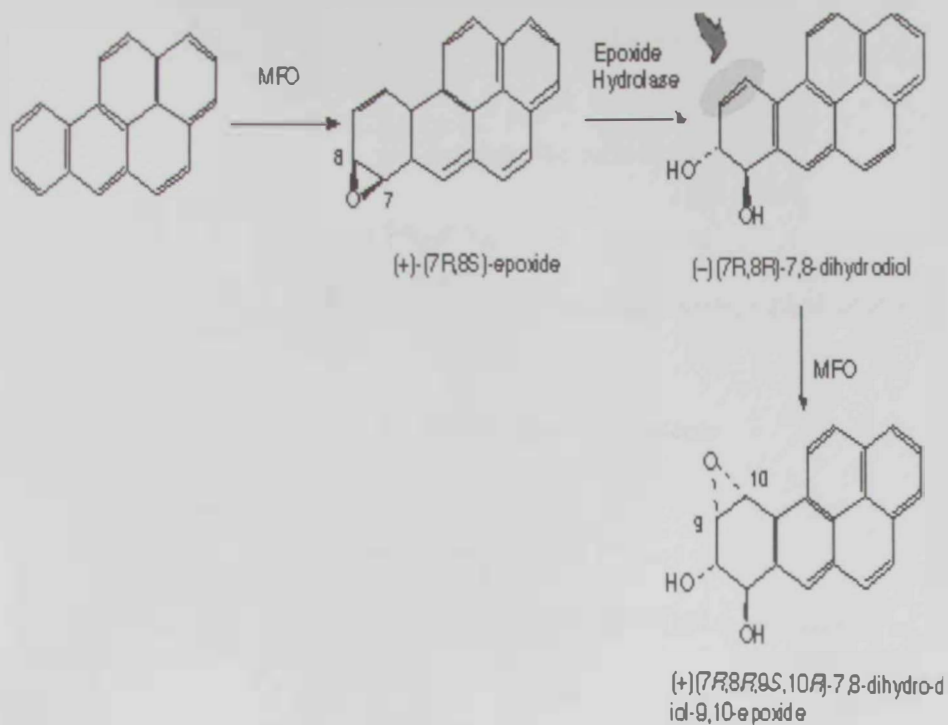
Fig 3.3 : Formation of 7,8 – diol- 9,10 epoxide.

Base adduct: benzo[a]pyrene

 benzo[a]pyrene

⇓ metabolic activation

 benzo[a]pyrene diol-epoxide (BPDE)

⇓ DNA incorporation

 BPDE - DNA adduct
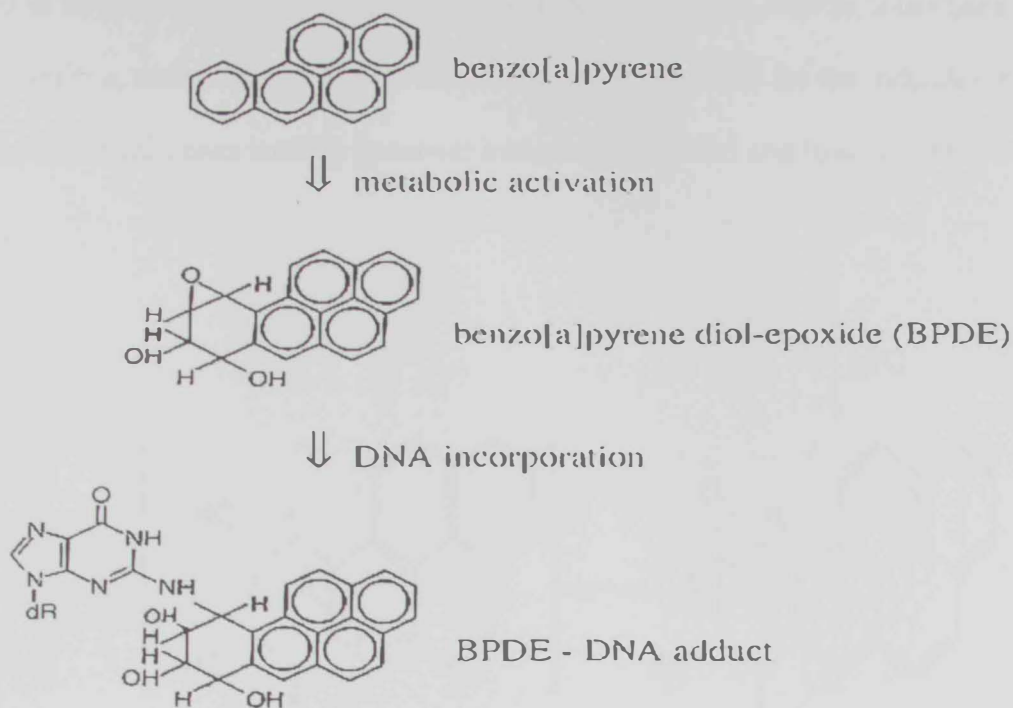
Fig 3.4. Benzo[a]pyrene diol-epoxide adduct

B[a]PDE form binds to the DNA by forming stable adducts on the exocylic amino group of deoxyguanosine (dG) (Figure 3.4) (Meehan and Calvin, 1979).

ii) Radical cations and the unstable adducts

This is the alternative activation pathway in which radical cations are generated by one electron oxidation of diol epoxides. These diol epoxides are easily converted by epoxide ring opening into electrophilic carbonium ions (Figure 3.5), which are alkylating agents that covalntently bind to nucleophilic sites in the DNA bases and in proteins (Brookes and Lawly, 1964). Due to the binding of these intermediates, mainly at N7- or C8- positions of purine bases, the resulting adducts are unstable and generate apurinic (AP) sites in the DNA by

spontaneous depurination (Cavalieri and Rogan, 1995). AP sites have also been reported to be produced in DNA by reaction with dihydrodiol epoxides (Li et al., 1995). It has been proposed that these lesions, rather than stable DNA adducts, are responsible for the induction of mutations in critical genes leading to cancer initiation (Cavalieri and Rogan, 1995).

10-carbocation (+)(7R,8R,9S,10R)-7,8-dihydro-d
iol-9,10-epoxide
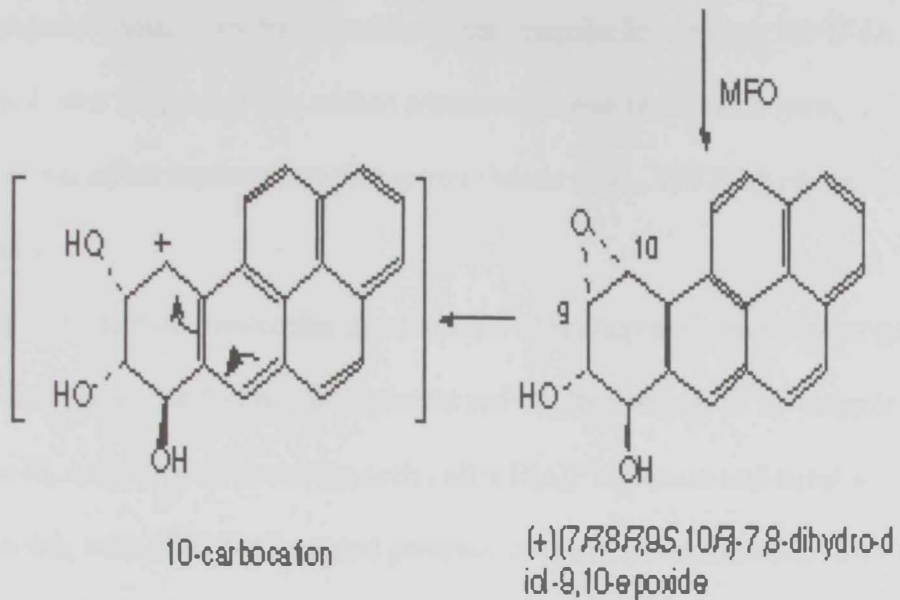
Fig 3.5: The formation of carbonium ions

A Link between the carcinogenesis of PAHs and chromatin remodeling.

As mentioned above, metabolic activation of PAHs results in the formation of reactive intermediates that can damage DNA by covalently binding (Melendez-Colon et al., 1999), and DNA adducts by PAHs has been shown to form *in vitro* and *in vivo* (Nesnow et al., 1998; Ross et al., 1995). Some studies have shown that the presence of nucleosomes suppresses BPDE

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induced damage levels within the central area of nucleosmes by up to 60% (Cavalieri and Rogan, 1995; Melendez-Colon et al., 1999). On the other hand, another study found that BPDE adducts in *X. borealis* 5S rRNA gene enhance nucleosome formation in a damage-dependent manner. It was suggested that adduct formation in nucleosome could potentially fix DNA in a conformation favorable for nucleosome formation. An alternative explanation was that a flexible hinge point induced by BPDE adducts may require less energy for DNA to wrap around the histones. It was suggested that within a transcriptional region of a gene, a more stable nucleosome could affect nucleosome disruption (Mann et al., 1997) by chromatin modifying complexes.

Some studies tried to explore molecular mechanisms of mutagenesis and carcinogenesis induced by B[a]P. One group used a two-dimentional gel electrophoresis to investigate protein expression levels in FL cells (human amnion cells) after B[a]P exposure and results revealed alteration of 47 proteins, with SWI/SNF related proteins, and other bormodomain-containing proteins among the identified proteins (Gao et al, 2003). The same group tested the effect of B[a]P on the expression of proteins in human amnion epithelial cells and expression changes were again detected in a number of transcriptional regulators including SNF2L1 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1). This protein, which is mainly involved in the chromatin remodeling and transcription regulation was found to be down-regulated greatly after B[a]P exposure (Gao et al, 2004). In anther study, the treatment of HepG2 cells with B[a]P was also shown to result in modulation of gene expression with slight reduction in genes expressing histone 1 (H2a1), histone 1 (H3d) and histone deacetylase 1 (HDAC1) (Staal et al., 2006). Yet another study has shown time and concentration dependent changes in gene expression induced by B[a]P in MCF-7 and HepG2 cells lines (Hockley et al., 2006). The over all response consisted of up-regulation of tumor

suppressor genes and down-regulation of oncogenes promoting cell cycle arrest and apoptosis. Anti-apoptotic signals were also affected. In this study, genes involved in nuclear assembly like histone 1(H2bg), histone 1 (H4b), histone 1 (H2bj), histone 1 (H3d) and histone 1 (H4c) were down regulated in MCF- 7 cells while down-regulated and then up-regulated or vice versa in HepG2 cells. All the above data showing modulation of expression of some genes involved in chromatin assembly or even chromatin remodeling suggest the possible involvement of chromatin remodeling in the carcinogencity of B[a]P. Additional studies have shown the involvement of chromatin remodeling in apoptosis, which could be used to explain a possible pathway that cells go through after B[a]P treatment. For example, it was shown that the over expression of INI1 protein, a chromatin remodeling factor associated with SWI/SNF, in Ini1-deficient cells caused induced G1 arrest and apoptosis (Keisuke et al., 2002). Similarly, a more recent study have shown that forced expression of Brg1 in mesenchymal cells by adenoviral trasndcution induced growth arrest and apoptosis (Napolitano et al., 2007). In this study we will investigate the effects of B[a]P on the expression of BRG-1, BAF55 and GCN5 which are chromatin-modifying related proteins.

Experimental procedure

Chemicals and reagents:

B[a]P (purity 100%, CAS no. 50-32-8) was purchased from AccuStandard®, Inc. (New Haven, CT), and was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich Chemie GmbH steinheim, Germany) to prepare a 50 mM stock solution and stored at -20 °C protected from light. Protease inhibitors (PMSF, leupeptin, aprotenin and pepstain A) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Anti- Brg1, anti-Gcn5, and anti-Baf115 antibodies were all obtained from Santa Cruz Biotechnology (CA, USA). The anti-actin antibody was purchased from Sigma (St. Louis, MO, USA).

Cell culture

WRL-68 and HepG2 Cell lines (ATCC, USA) were cultured in Roswell Park Memorial Institute media (RPMI 1640 + Glutamax™; GIBCO-BRL) supplemented with 10% fetal calf serum (GIBCO-BRL) and 100 units/ml penicillin- streptomycin (GIBCO-BRL). Cells were maintained at 37 °C in 5% CO_2 incubator. After reaching confluency, cells were subcultured into 6-, 12-, and 96- wells culture plates for subsequent treatment. Treatment was done by replacing old media with freshly prepared media containing various concentrations of B[a]P (0.1 μM, 1 μM, 10 μM, and 100 μM). DMSO concentration in all media as well as control media was maintained at a concentration of 0.2 %.

Cell cytotoxicity assay (CCK-8 assay)

Cytotoxicty in WRL-68 and HepG2 cells, after treatment by B[a]P, was evaluated using the CCK- cell counting kit (Dojindo Laboratories). Cells were plated in three 96-well plates at a density of 5000 cells/well/100 μl of RPMI 1640 plus 10 % FCS and 100 units/ml penicillin- streptomycin at 37 °C. Next day old media was replaced with freshly prepared media with B[a]P at concetrations of 0.1 μM, 1 μM, 10 μM and 100 μM for 1, 3 and 6 days. Controls contained media with 0.2% DMSO only. Toxicity was evaluated after each treatment according to manufacturers instructions, by adding 10 μL of CCK-8 solution to each well. Reduction time was allowed to proceed for 1-3 hours. Absorbance at 450 nm was measured with a micoplate reader. Data were calculated as a percent of absorbance compared to DMSO control group.

Detection of Cell Viability and Cell Death (Flow Cytometry)

Detection of cell viability and cell death by flow cytometry was performed using Annexin V-FITC and propidium iodide stains (BD Biosciences, USA). Cells were seeded in two 12-wells culture plates with approprate media, and on the next day, old media was replaced with media containing different concetrations of B[a]P. Treatments lasted only for three and six days, after which the cells were washed with phosphate-buffered saline. Briefly, Cells were resuspended in 100 μl of Annexin V binding buffer (BD Pharmingen). Annexin V-FITC (4 μl) and Propidium Iodide (PI) (4 μl) were added to each treatment in the dark. Cells were incubated at room temperature for 30 minutes and kept in the dark. Cell suspensions

were raised to a final volume of 500 µl with Annexin V binding buffer. Flow cytometry was performed using a Becton-Dickenson FACScan flow cytometer and Cell Quest software.
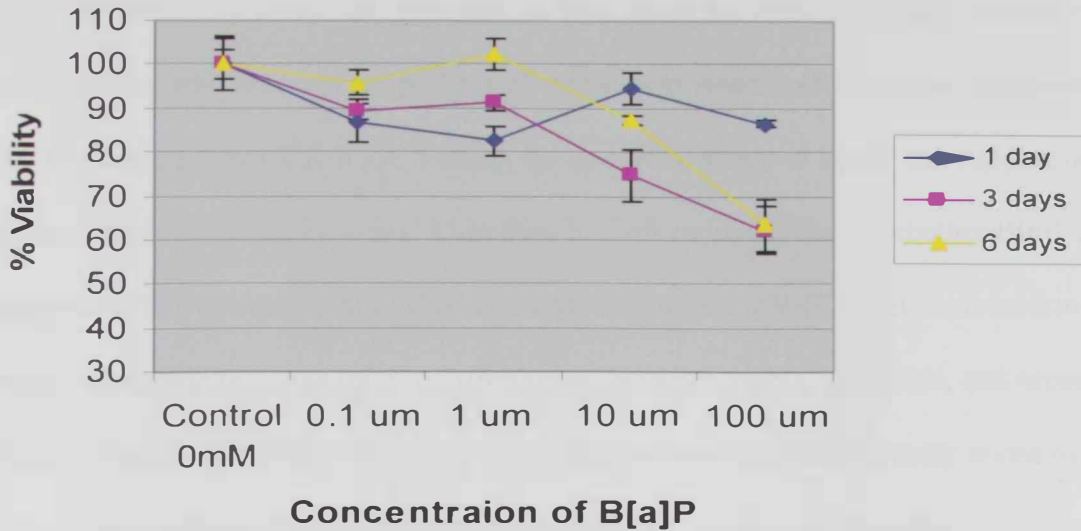
SDS-PAGE and Western blot analysis

Both WRL-68 and HepG2 cells were exposed to the same concetrations of B[a]P mentioned above with DMSO concetration maintained of 0.2 % in all wells. Controls contained only 0.2 % DMSO. Cell were trpsinized, washed and collected after 1, 3 and 6 hours exposure and then lysed in 100 µl of lysis buffer (100 mM Hepes, pH 7.5, 10 % sucrose, 10 mM DTT, 0.1% CHAPS, 150 mM NaCl, protease inhibitors (1 mM PMSF and 1 µg/ml each of leupeptin, aprotenin and pepstain A). Cells were lysed by 6 consecutive cycles of freezing and thawing, and spun at 14000 rpm for 30 minutes at 4 °C. Supernatent was seprated from pellet and stored at -80 °C. Concetration of protein in cellular extracts was estimated by a an assay based on the Bradford colormetric reaction (BioRad, USA). For Western bloting, 30 µg of cellular protein was electrophoretically separeted on a 10% SDS-PAGE. Following electrophoresis gels, were transferred onto nitrocellulose membranes (Millipore, USA). Following the transfer, the membranes were blocked by incubation in 5% nonfat dry milk in phospate buffered saline with 0.05% Tween-20 (PBST) for 2 hours at room temperature. Then the, membranes were incubated at 4 °C overnight with the following antibodies diluted in PBST: anti-Brg1 (1:1000), anti-155 (1:1000), anti-Gcn5 (1:1000), and anti-actin (1:1000). Blots then were washed with PBST and were incubated with the appropriate horseradish peroxidase-conjugated secondary antinodies (1:1000 dilution) for 2 hours at room temperature. After washing with PBST, binding of antibodies was detected on blots by SuperSignal chemiluminescence kit (Pierce, Biotechnology, Rockfor, IL, USA) visualized by

atutoradiography. Equal, loading of protein was confirmed using mouse monoclonal secondary antibody to detect actin.

Results

Cytotoxic effect of B[a]P on WRL-68 and HepG2 cells. Cell counting kit-8 (CCK-8) was used to test the cytotoxic effect of B[a]P on WRL-68 and HepG2 cells. The kit allows sensitive colorimeteric determination of the number of viable cells. It is based on the reduction of WST-8 [2-(2-methoxy-4-nitrphenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] by dehydrogenase in viable cells which produces a water-soluble formazan dye. The amount of the yellow formazan dye generated in cells is directly proportional to the number of living cells. Both dose and time dependent effects of B[a]P on the viability of both HepG2 and WRL-68 cells were tested. Both cells lines were treated with various concentrations of B[a]P, treatment was also done for different duration of one, three or six days. HepG2 Cells treated with B[a]P for three and six days showed a clear dose-dependent reduction in viability, where increasing concentration of B[a]P resulted in reduced viability (Figure 3.6.A). At highest B[a]P concentration (100 μM), viability was reduced down to 63%. On the other hand, viability of HepG2 cells after one day of treatment was slightly reduced even at the highest concentrations. WRL-68 cells (Fig 3.6.B) treated for one or six days shows only a slight-dose dependent change in viability while viability of WRL-68 cells treated with B[a]P for three days was reduced to 59%, at the highest B[a]P concentration. In general, these data indicate that B[a]P was more toxic at high concentrations and that toxicity was more apparent with longer treatments, but the pattern of toxicity was also cell type dependent, possibly because of metabolic differences in the two different cells lines.
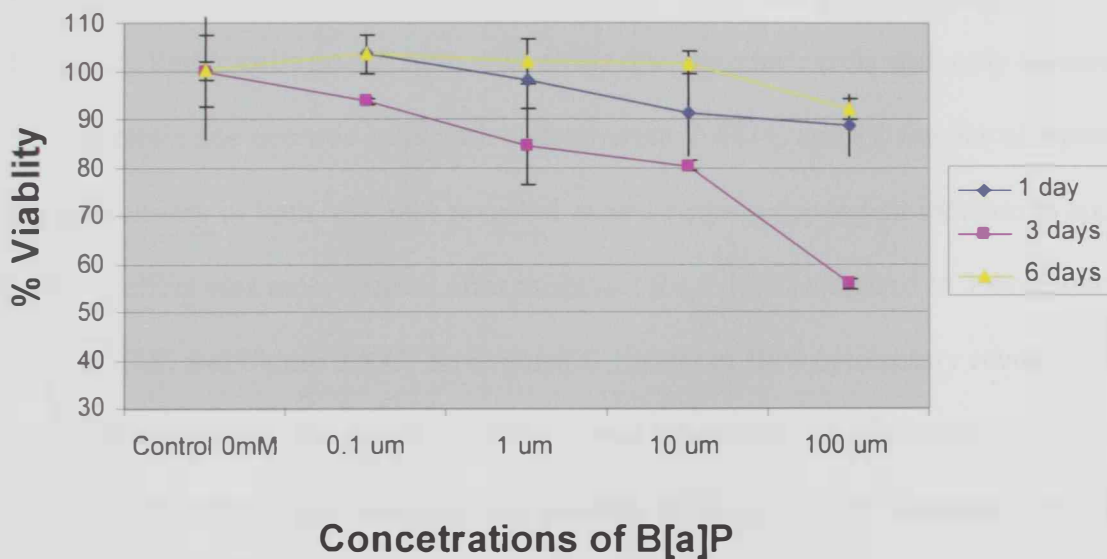
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Fig 3.6 : Cytotoxic effects of B[a]P on two cell lines. (A) Viability of HepG2 cells after treatment with different concentration of B[a]P for 1, 3 and 6 days.(B) Viability of WRL-68 after treatment with different concentrations of B[a]P for 1, 3 and 6 days.

Apoptotic effect of B[a]P on HepG2 and WRL-68 cells

Cytotoxicity assay only provides an idea about the effects of B[a]P on cell viablity but cannot differentiat between apoptotic and viable cells since both have dehydrognease activity which is detected by CCK-8 kit. Testing the apoptotic effect of B[a]P can support data obtained from cytotoxicity assay. Moreover it gives more details on whether B[a]P induces apoptosis. Flow cytometry after dual-staining with annexin V-FITC in combination with propidium iodide (PI) was used to differentiate between viable, apoptotic, and secondary necrotic HepG2 and WRL-68 cells. Loss of plasma membrane asymmetry is one of the earliest features of apoptosis. This feature can be detected by binding of Annexin V to the membrane phospholipids phosotidyle serine (PS) that is translocated from the inner to the outer leaflet of the plasma membrane of apoptocic cells. Since PS translocation also occurs during necrosis, PI is used with Annexin V. PI can penetrate plasma membrane when membrane integrity is breached. Viable cells do not stain with either dye, apoptotic cells with only annexin V-FITC, late apoptotic and necrotic cells with both annexin V-FITC and PI. Graphical representation of flow cytometry of both cell lines revealed a concetration-dependent increase in apoptotic cells and that effect was more evident after treatment for 6 days compared to 3 days treatment (see Figure 3.7.C and Figure 3.8.C) So in general results of flow cytometery revealed that 6 days treament was more toxic than 3 days treatement which was not similar to results obtained from cytotoxicity assay. These results can be possibly because of B[a]P interfering with the metabolic rate in the cells and after long durations giving that false result of increased viablity. Flow cytometery results for both HepG2 and WRL-68 cell lines look similar, however the variation in toxicity results in CCK-8 can be expalined by possible metabolic difference in

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both cell lines.
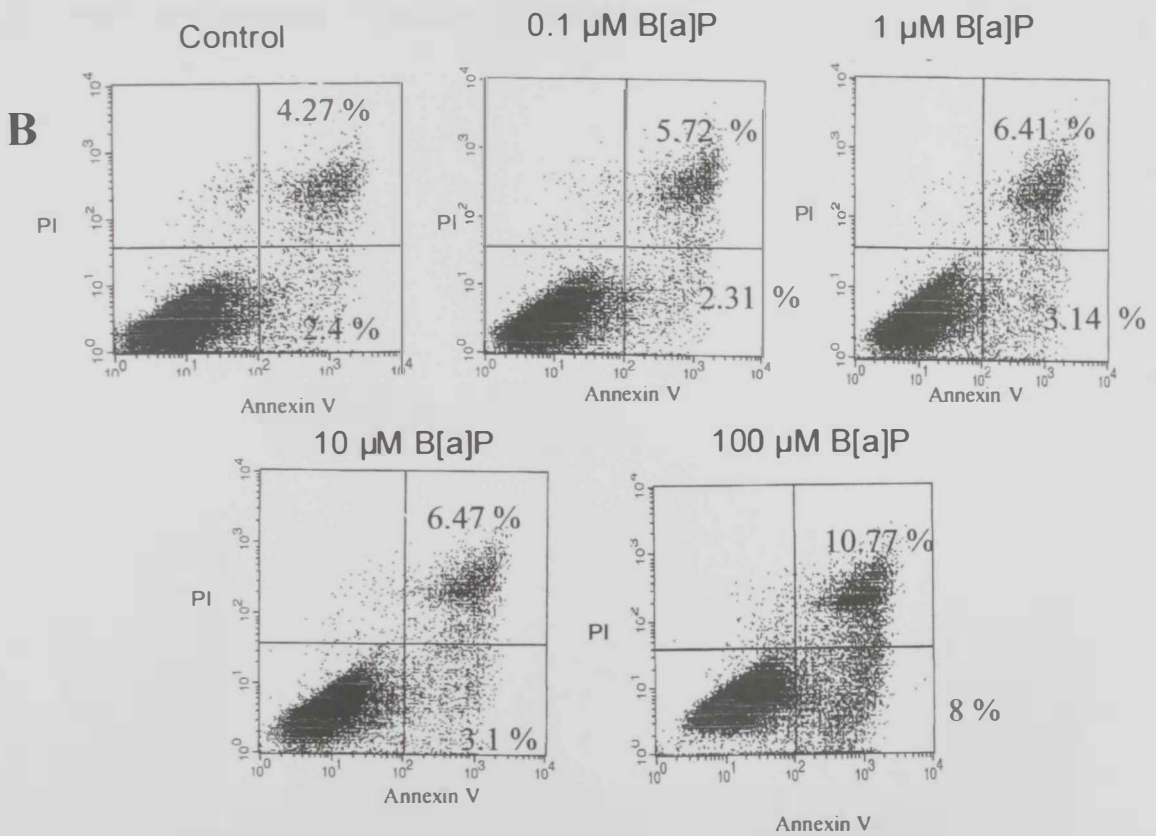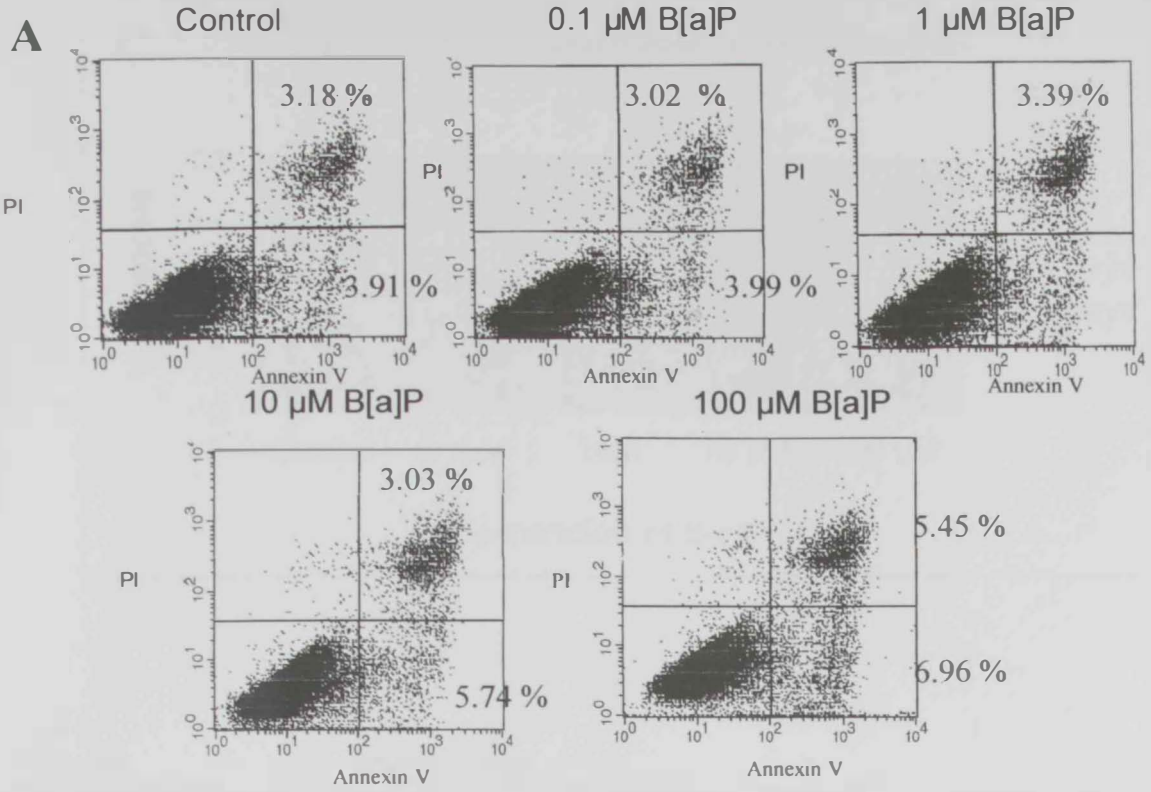
Fig 3.7: Flow cytometric analysis of HepG2 cells after treatment with B[a]P. (A) Treatment with B[a]P for 3 days. (B) Treatment with B[a]P for 6 days. (C) Graphical representation of the flow cytometry data for 3 and 6 days treatment of HepG2 cells from A and B.

A

Control

0.1 μM B[a]P

1 μM B[a]P

4.09 %

4.52 %

4.42 %

3.42 %

3.08 %

3.47 %

10 μM B[a]P

100 μM B[a]P

4.58 %

6.65 %

4.13 %

5.83 %

B

Control

0.1 μM B[a]P

1 μM B[a]P

4.56 %

4.36 %

6.21 %

2.58 %

3.01 %

2.53 %

10 μM B[a]P

100 μM B[a]P

5.82 %

1.63 %

3.3 %

65 %

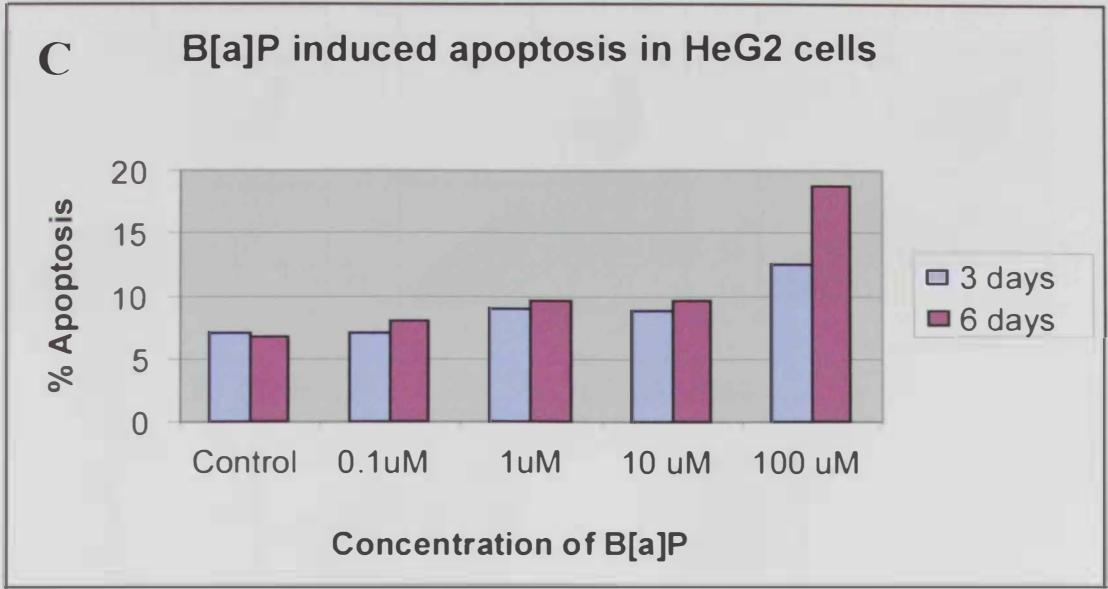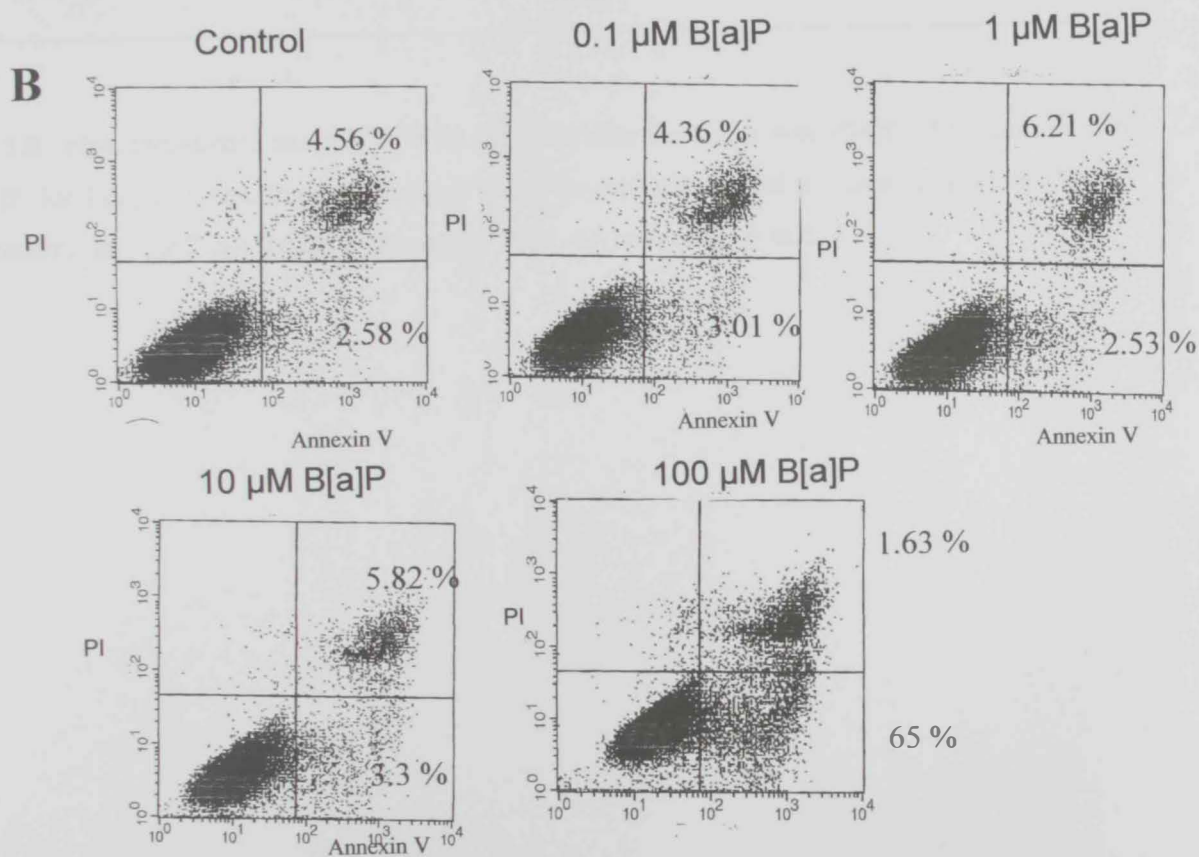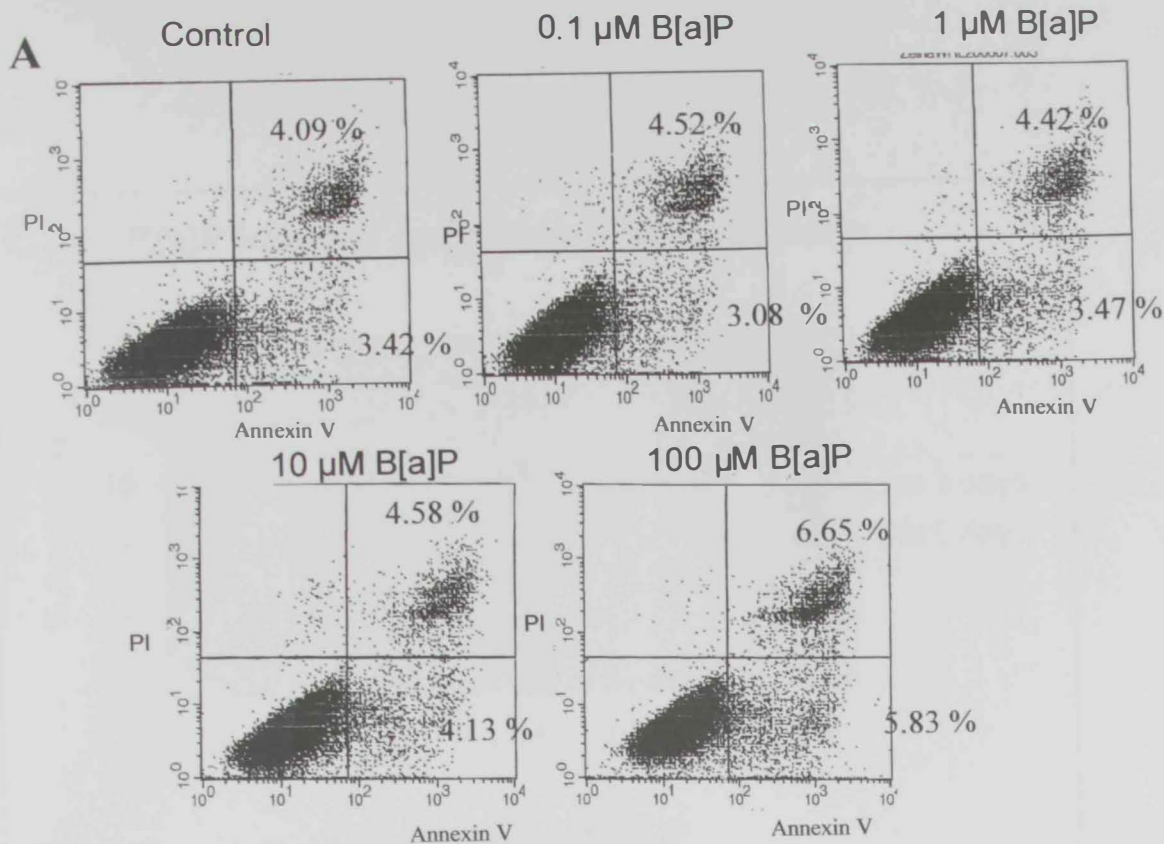**C**   **B[a]P induced apoptosis in WRL-68 cells**
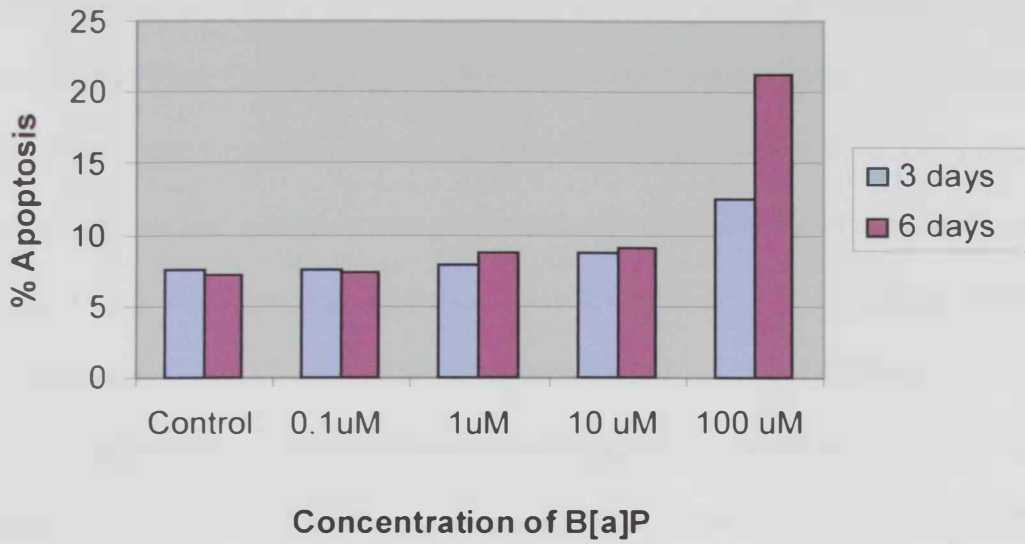
Fig 3.8: Flow cytometric analysis of WRL-68 cells after treatment with B[a]P. (A) treatment with B[a]P for 3 days. (B) treatment with B[a]P for 6 days (C) Graphical representation of the flow cytometry data for 3 and 6 days treatment of WRL-68 cells from A and B.

The expression of BRG-1, GCN5 and BAF155 in HepG2 and WRL-68 cells after treatment with B[a]P.

After observing apoptosis in both cell lines upon treatment with higher concentration of B[a]P, we wanted to test if this apoptosis was accompanied with a change in the expression BRG-1, BAF155 or Gcn5. Cells were harvested after treatment with different concentrations of B[a]P (0.1 µM, 1 µM, 10 µM and 100 µM) for 1, 3 and 6 days. Total cell protein was run on SDS-PAGE gels and detection was performed with antibodies against BRG-1 and GCN5 (Figure 3.9.A). The expression of the components of chromatin-remodeling proteins in HepG2 cells after treatment for 3 and 6 days were measured. It is clear that there is no significant change in expression even with treatment with higher concentration of B[a]P at which apoptosis was detected. In addition, we measured the expression of BRG-1, BAF155 and Gcn5 after treatment of HepG2 and WRL cells at different times points (1, 3, an 6 days) with two concentrations of B[a]P and observed no significant time-dependent change in protein expression (Figure 3.10.A and B). The lanes of actin at the bottom of Figure 3.10.A and B serves as a loading control.

A

Treatment for 3 days with B[a]P

Con   0.1 uM   1 uM   10 uM   100 uM
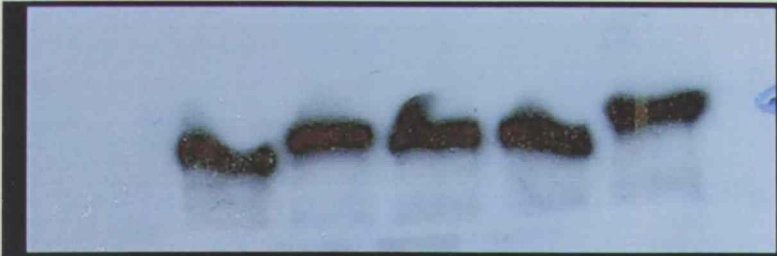
Brg1

Gcn5

B

Treatment for 6 days with B[a]P

Con   0.1 uM   1 uM   10 uM   100 uM
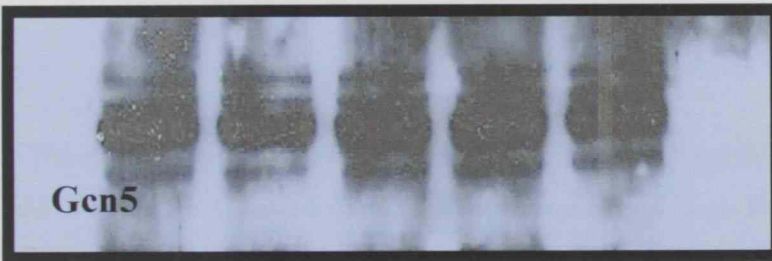
Brg1

Gcn5

Fig 3.9: The expression of both Brg1 and GCN5 after treatment of HepG2 cells with different concentrations of B[a]P for 3 and 6 days (A) treatment for 3 days. (B) Treatment for 6 days.
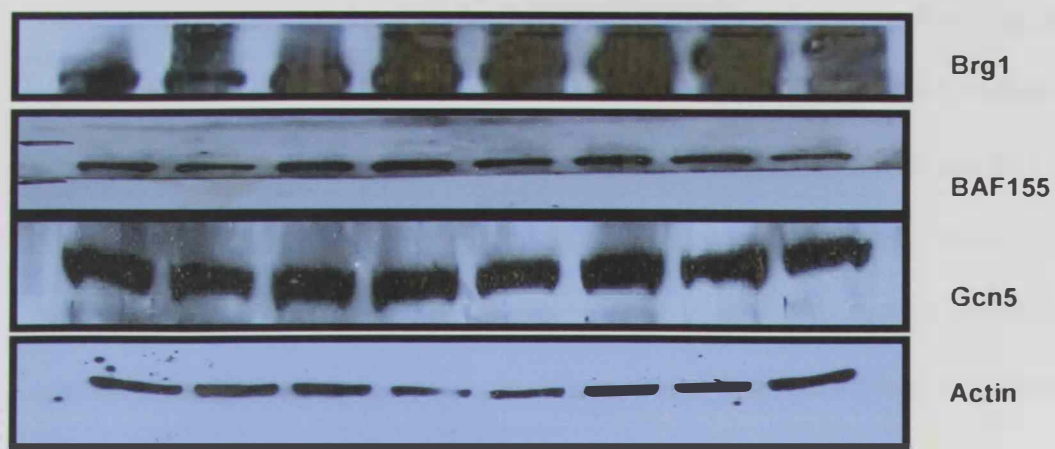
Fig 3.10 : The expression of Brg1, BAF155 and Gcn5 in HepG2 and WRL-68 cells treated with 10 μM and 100 μM of B[a]P for 1,3 and 6 days. (A) The expression of Brg1, BAF155 and Gcn5 in HepG2 cells (B) The expression of Brg1, BAF155 and Gcn5 in WRL-68 cells

Discussion

Carcinogenicity and mutagenicity of B[a]P is mostly explained by the covalent binding of B[a]P metabolites to DNA and the formation of DNA adducts. Such adducts might alter and introduce point mutations in a gene, an event that can lead to cancer development if genes that were affected are related to cell cycle control. Some studies have shown that chromatin remodeling might play a major role in inducing cancer or apoptosis. Most of these studies have analyzed the expression of chromatin remodeling complexes or tested the effects of their loss on cells. For example, one study demonstrated that BRG-1 loss renders cells resistant to retinoblastoma mediated cell cycle arrest an event that might lead to cancer (Strobeck et al., 2000). Another study has linked increased expression of BRG1 to the development and progression of gastric carcinoma (Sentani et al., 2001). In this study gastric carcinomas showed increased BRG1 expression in tumor tissue with gastric carcinomas with lymph node metastasis expressing BRG-1 at higher levels. Another evidence for the implication of BRG-1 in cancer development was demonstrated when the expression level of both BRG-1 and BRM was anaylyzed in primary lung adenocarcinoma and lung squamous cell carcinoma. The results showed that 10% of tumors showed concomitant loss in BRG1 and BRM expression (David et al., 2003). The role of BRG-1 in cancer was also shown when it was found that BRG-1 with ablated function in T lumphocytes resulted in profound abnormalities in mice demonstrating the role BRG-1 plays in regulating thymocyte cell proliferation and survival (Gebuhr et al., 2003). In addition, the deletion of BRG-1 locus AT 19p13 as a predictive marker for the prognosis of the patients that have oral carcinomas, such a conclusion was inspired from analyzing clinicopathological data for patients with oral squamous cell carcinoma (Gunduz et al., 2006). Finally, microarray data revealed that aberrant expression of

BRG-1 is associated with tumor development and increased invasiveness in prostate cancer (Sun et al., 2006).

BAF155 is one of the SWI/SNF subunits that was shown to stimulate the remodeling activity of BRG-1. BAF155 is one among the four subunits that are needed for efficient chromatin remodeling (Phelan et al., 1999). It was found that targeted deletion of BAF155 in mouse is embryonicaly lethal. Similar to BRG-1, Gcn5, a histone acetyltransferase was also proved to play an important role in cell cycle progression through transcription regulation of various cell cycle related genes. For example, Gcn5 deficiency in DT40 mutants resulted in influencing apoptotis- related genes and G1/S phase transition related genes (Kituchi et al., 2005). Similarly, deletion of Gcn5 gene was found to result in embryonic lethality in mice indicating its role in embryogenesis (Phan et al., 2005). In this study, we were interested in whether the cytotoxicity of observed in cells leading to their apoptosis after B[a]P treatment is due to changes in expression of subunits of chromatin-modifying proteins such as Brg1, Baf155 and Gcn5.

Here, we show high toxicity of B[a]P on both HepG2 and WRL-68 cells using CCK-8 cytotoxicity. These results were confirmed by flow cytometry, however, time dependent changes were not observed in the assay. In cytotoxicity assay, cells were more viable with longer duration of incubation while less viable with shorter treatment durations. This was not found in flow cytometry analysis in which longer durations of treatment were more toxic to cells. A possible explanation for this might be that B[a]P interferes with dehydrogenase activity especially after longer durations showing increased viability. Western blot analysis showed neither a time- or dose-dependent change in the expression of three subunits within chromatin-remodeling or histone acetyltransferase complexes. This demonstrates that reduced cell viability observed or increased incidence of apoptosis was not due to changes in the

expression of these proteins. These results, however, don't exclude a possible role of chromatin remodeling in the carcinogenesis of B[a]P. Measuring the expression of other subunits of chromatin-modifying proteins could shed more light into the possible involvement of remodeling as a possible pathway in B[a]P carcinogenesis. In addition to that change in expression, the activation and deactivation of subunits of chromatin-modifying proteins such as those were demonstrated in some studies could help greatly in pathway identification (Muchardt et al., 1996; Sif et al., 1998). Furthermore, chromatin immunoprecipitation (ChIP) can help in detecting any change in recruitment of remodeling complexes to genes involved in cell cycle regulation, as this can be one of the ways in which chromatin remodeling activity can be increased at certain genes.

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وفترات مختلفة عن طريق تقنيتا ال Cell cytotoxicity assay و ال Flow cytometry واللتان تكشفان عن

وضع الخلايا و تعرضها للموت المبرمج للخلية. بعد ذلك قمنا بالكشف عن إنتاج بروتينات لها دور في تعديل بنية

الكروماتين وهي ال BRG1 ،Gcn5 و BAF155. تبين النتائج أن للبنزو (أ) بايرين تأثير سمي على الخلايا وذلك

بزيادة التركيز والمدة بينما لم يرافق هذه التأثيرات أي تغير في إنتاج هذه البروتينات. تلك النتائج تبين أن الآلية التي

يعمل بها هذا المركب لا تعدل من إنتاج هذه البروتينا ت الثلاث على الأقل، ولكن هذا لا يستبعد أن يكون لتعديل بنية

الكروماتين دور في آلية التسبب بالسرطان. للحصول على صورة أكبر يجب الكشف عن إنتاج بروتينا ت أكثر ذات

علاقة بتعديل بنية الكروماتين كما يجب الكشف عن تأثير البنزو(أ) بايرين على فعالية هذه المركبات وذالك للوصول

إلى إمكانية وجود أي علاقة بين تعديل بنية الكروماتين والتأثير لت الملاحظة على هذه الخلايا.

الخلاصة

يمثل الكروماتين، وهو الشكل الذي يخزن به الحمض النووي في الخلية ، عائقا أمام العديد من العمليات الخلوية التي تتطلب وصول سهل للحمض النووي، كعملية نسخ المادة الوراثية وتعبير الجينات. تحل هذه المشكلة جزئيا عن طريق مركبات بروتينية تعمل على تعديل بنية الكروماتين أو تحويره بحيث يصبح بيئة أسهل لهذه العمليات. يوجد نوعان من المركبات المحورة للكروماتين، نوع يستهلك جزيء الطاقة ال ATP للقيام بعمله بينما الآخر يعدل الكروماتين عن طريق إضافة جزيء الأستيل للهستونات. يلعب اللهستون H1 دور مهم في ثبات و تكثيف الكروماتين، وقد أثبتت العديد من الدراسات أهمية الهستون H1 في تعديل التعبير الجيني كما يوجد دراسات تبين تأثير الهستون H1 على البروتينات المحورة والمعدلة لبنية الكروماتين. في هذه الداسة تم اختيار تأثير الهستون H1 على ارتباط نوعان من المركبات المحورة للكرماتين وهي مركبات ال SWI/SNF و ال SAGA على قوالب كروماتينية مصنعة مخبريا متعددة النيوكليوسومات بحيث كانت معدلة بإضافة مجموعة الأستيل أو غير معدلة، وقد استخدمت تقنية ال Pull down assay لدراسة هذا التأثير. كما تم استخدام قوالب كروماتينية غير معدلة أحادية النيوكليوسوم لدراسة نفس التأثير ولكن باستخدام تقنية ال Gel shift assay. بالإضافة لذلك، تم دراسة تأثير الهستون H1 على وظيفة مركب ال SWI/SNF باستخدام تقنية ال Restriction enzyme accessibility assay. تظهر النتائج أن الهستون H1 يثبط ارتباط كل من المركبين SWI/SNF و الSAGA على القوالب الكروماتينية العديدة النيوكليوسومات المعدلة والغير معدلة, بينما التثبيط كان فقط ملاحظا عند الSWI/SNF عند استخدام قوالب كروماتينية أحادية النيوكليوسوم. كما تبين النتائج أن للهستون H1 دور في تثبيط فعالية مركب ال SWI/SNF في تعديل بنية الكروماتين.

في القسم الثاني من هذه الدراسة تم إخبار تأثير مركب البنزو (أ) بايرين على نوعين من الخلايا ال HepG2 و ال WRL -68 وذالك لدراسة إحتمالية وجود علاقة بين تعديل بنية الكروماتين والآلية التي يسبب بها هذا المركب السرطان. ينتمي مركب البنزو(أ) بايرين لمجموعة الهيدروكربونات الأرومية متعددة الحلقات والمعروف عنها تدميرها لمادة الحمض النووي عن طريق الإرتباط به.اختبرنا التأثير على هذه الخلايا بعد تعريضها لتركيزات

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دراسة تأثير الهستون H1 والبنزو (أ) بايرين على البروتينات المحورة للكروماتين

رسالة مقدمة من /

زينة سليم الناطور

مقدمة إلى/
جامعة الامارات العربية المتحدة
استكمالا لمتطلبات الحصول على درجة الماجستير في علوم البيئة

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