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Mechanistic Analysis of Chromatin Remodeling Enzymes: a Dissertation

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Mechanistic Analysis of Chromatin Remodeling Enzymes

Dissertation Presented

by

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Mechanistic Analysis of Chromatin Remodeling Enzymes

A Dissertation Presented

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DEDICATION

A mi familia

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ABSTRACT

The inherently repressive nature of chromatin presents a sizeable barrier for all nuclear processes in which access to DNA is required. Therefore, eukaryotic organisms ranging from yeast to humans rely on a battery of enzymes that disrupt the chromatin structure as a means of regulating DNA transactions.

These enzymes can be divided into two broad classes: those that covalently modify histone proteins, and those that actively disrupt nucleosomal structure using the free energy derived from ATP hydrolysis. The latter group, huge, multisubunit ATP-dependent chromatin remodeling factors, are emerging as a common theme in all nuclear processes in which access to DNA is essential. Although transcription is the process for which a requirement for chromatin remodeling is best documented, it is now becoming clear that other processes like replication, recombination and DNA repair rely on it as well.

A growing number of ATP-dependent remodeling machines has been uncovered in the last 10 years. Although they differ in their subunit composition, organism or tissue restriction, substrate specificity, and regulating/recruiting partners, it has become increasingly evident that all ATP-dependent chromatin remodeling factors share a similar underlying mechanism.

This mechanism is the subject of the studies presented in this thesis. Chromatin-remodeling factors seem to bind both the histone and DNA components of nucleosomes.

From a fixed position on nucleosomes, the remodeling factors appear to translocate on the DNA, generating torsional stress on the double helix. This activity has several consequences, including the distortion of the DNA structure on the surface of the histone octamer, the disruption of histone-DNA interactions, and the mobilization of the nucleosome core with respect to the DNA.

The work presented in this thesis, along with data reported by other groups, supports the hypothesis that yeast SWI/SNF chromatin remodeling complex and the recombinational repair factor, Rad54p, both employ similar mechanisms to regulate gene transcription, and facilitate homologous DNA pairing and recombination, respectively.

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LIST OF ABBREVIATIONS

ACF- ATP-utilizing and chromatin accessibility factor

ADP- adenosine diphosphate

APY- apyrase

ATP- adenosine triphosphate

ARP- actin related protein

ARS- autonomously replicating sequence

ATM- ataxia telangiectasia mutated

bp- base pairs

BRCA1/2- breast cancer gene 1/2

BSA- bovine serum albumin

CBP- calmodulin binding protein

CHD- chromatin helicase-DNA binding proteins

ChIP- chromatin immunoprecipitation

CHRAC- chromatin remodeling and accessibility complex

Ci- curie

cpm- counts per minute

DNA- deoxyribonucleic acid

DSB- double-strand break

DTT- dithiotreitol

EDTA- ethylene diaminethylether tetraacetic acid

EGTA- ethyleneglycol diaminoethylether tetraacetic acid

Endo VII- T4 endonuclease VII

et al.- et alibi (and others)

EM- electron microscopy

hSWI/SNF- human switch/sucrose non-fermentable complex

IgG- immunoglobin G

ISWI- imitation SWI

FPLC- fast performance liquid chromatography

GST- glutathione S-transferase

H1- linker histone H1

H2A-core histone H2A

H2B- core histone H2B

H3- core histone H3

H4- core histone H4

H5- linker histone H5

HDAC- histone deacetylase

HO- mating type switching endonuclease promoter

HR- homologous recombination

IR- ionizing radiation

Kb- kilobase pairs

KDa- kilodaltons

k_{cat}- catalysis rate constant

Lk- linking number

M- molecular weight marker or DNA ladder

MDa- megadalton

Mnase- micrococcal nuclease

NBS1- Nijmegen Breakage Syndrome protein 1

NHEJ- non-homologous end joining

NRD/ NURD - nucleosome remodeling and deacetylase complex

NURF- nucleosome remodeling factor

PAGE- polyacrylamide gel electrophoresis

PMSF- phenlymethylsulfonyl fluoride

Rad- Radiation sensitive

RNA- ribonucleic acid

RPA- Replication Protein A

rpm- revolutions per minute

RSC- remodels the structure of chromatin

SAGA- Spt-Ada-Gcn5-Acetyltransferase

SANT- Swi3, Ada2, N-CoR, TFIIIB"

s.c.- supercoiled

SDS- sodium dodecyl sulfate

SMART- simple modular architecture research tool

SWI/SNF- switch/sucrose non-fermentable complex

TAE- tris acetic EDTA

TAP- tandem affinity purification

TBP- TATA box binding protein

TFO- triplex-forming oligonucleotide

TLC- thin layer chromatography

Topo I- topoisomerase I

Tw- DNA twist

U- units

UV- ultraviolet radiation

WCE- whole-cell extract

Wr- DNA writhe

WT- wild type

YEPD- yeast extract, peptone, and dextrose media

CHAPTER I

INTRODUCTION

Introduction

Eukaryotic DNA is packaged and organized in the nucleus by histone and non-histone proteins, giving rise to chromatin. The fundamental unit of chromatin structure is the nucleosome, which consists of DNA wrapped around an octamer of the four core histones. The assembly of DNA into nucleosomes and derived higher order structures constitutes a highly restrictive environment for all DNA-mediated processes, like transcription, replication and repair (Wolffe, 1998). Consequently, it is not surprising that eukaryotic organisms possess a variety of evolutionarily conserved enzymes to modulate the architecture of chromatin, allowing the precise temporal and spatial regulation of genetic processes. These enzymes can be divided into two major classes: those that introduce covalent modifications on histone NH_2- or COOH-terminal "tail" domains and those that use the free energy derived from ATP hydrolysis to actively disrupt nucleosomal structure.

This chapter discusses the relationship among chromatin structure, chromatin modifying enzymes, and DNA-mediated processes, with emphasis on the roles of yeast SWI/SNF complex in transcriptional activation, and yeast Rad54p in homologous recombination and DNA repair.

Chromatin Structure

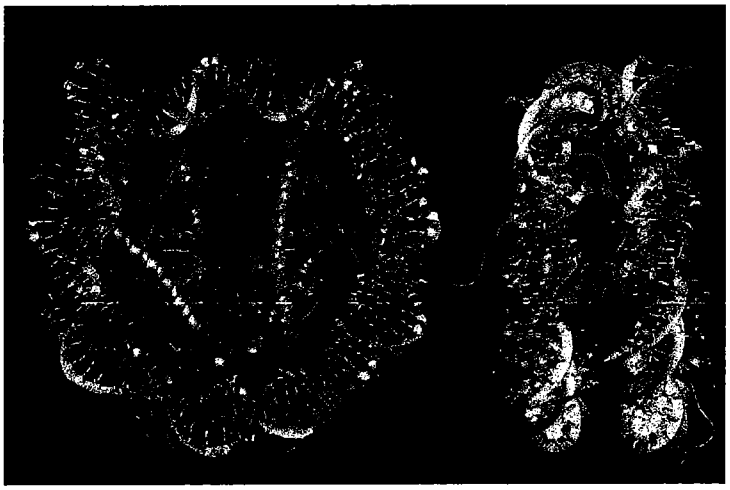


Figure I.1: Representation of the nucleosome structure
Nucleosome core particle: ribbon traces for the 147-bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (H3, blue; H4, green; H2A, yellow; H2B, red). The views are down the DNA superhelix axis for the left particle and perpendicular to it for the right particle. There are about 220 contacts between the phosphate backbone of DNA and the surface of the histone octamer. Adapted from Luger *et al.* (1997).

Chromatin has been historically seen as a static structure, with a primary role in packaging the eukaryotic genome within the boundaries of the cell nucleus. We now know that chromatin is an extremely dynamic, malleable structure that plays crucial roles not only in packaging, but also in the protection of eukaryotic DNA and the regulation of all DNA-mediated processes.

The basic building block of chromatin is the nucleosome core particle, consisting of 147 base pairs of DNA wrapped in a flat, left-handed superhelix ~1.75 times around a histone octamer (Figure I.1). The histone octamer consists of two copies of each of the four core histone proteins, H2A, H2B, H3 and H4. Each one of these highly basic proteins contains two separate functional domains: a signature "histone-fold" motif (sufficient for the histone-histone interactions, and histone-DNA contacts within the nucleosome), and NH_2-terminal and COOH-terminal "tail" domains that flank the central domain (Arents *et al.*, 1991). These tails have not yet been resolved in the crystal

structure of the nucleosome, but they seem to extend radially from the nucleosome core (Bohm and Crane-Robinson, 1984; Luger *et al.*, 1997).

Chromatin contains not only core histone proteins, but also linker histones and non-histone proteins associated with the DNA filament (Thomas and Furber, 1976). The DNA wrapping around the core particle, forming a linear array of nucleosomes ("beads-on-a-string", or 10nm fiber) accounts for only the first degree of compaction, or primary structure of chromatin. The second level of condensation is attained by short range internucleosomal interactions, and is stabilized by the association of regulatory proteins, such as linker histones H1 and H5 or non-histone proteins, resulting in a 3-dimensional arrangement known as the 30nm fiber (Woodcock and Dimitrov, 2001; Horn and Peterson, 2002a and 2002b). Tertiary structures are formed by self-association of these fibers into 100-400nm structures (chromonema filament), as visualized by electron microscopy in interphase cells or detected *in vitro* by biophysical methods (Fletcher and Hansen, 1996; Carruthers *et al.*, 1998). Recent *in vivo* studies indicate that transcription actually occurs on these enormous, 100-400 nm thick chromatin fibers (Memedula and Belmont, 2003).

The histone tails are not required for assembly of the octamer or the nucleosome, but they are essential for both intramolecular and intermolecular folding of nucleosomal arrays into higher order compacted structures *in vitro* (Fletcher and Hansen, 1996; Horn *et al.*, 2002).

Chromatin Modifying Enzymes

Chromatin fibers are the natural substrates for all DNA-mediated processes such as replication of the genome, gene transcription, sensing and repair of DNA damage, or generation of genetic diversity by recombination. These processes are commonly initiated and regulated by DNA-binding proteins that scan the chromatin fiber in search of their preferred recognition sequence (reviewed in Becker, 2002). Interaction of these factors with their target elements requires that the compact (and frequently repressive) chromatin organization be partially relaxed (Becker and Hörz, 2002). Therefore, eukaryotic organisms take advantage of a battery of enzymes to modulate the architecture of chromatin, allowing the precise regulation of genetic processes.

Two types of highly conserved chromatin remodeling enzymes have been implicated as regulators of the repressive nature of chromatin structure. The first group comprises those enzymes that covalently modify the nucleosomal histone proteins, such as histone acetyltransferases, deacetylases, methyltransferases, kinases, phosphatases, and ubiquitin ligases (reviewed in Geiman and Robertson, 2002; Zheng and Hayes, 2003). Combinations of post-translational marks on single histones, single nucleosomes and nucleosomal domains establish local and global patterns of chromatin modification. Overall, it is believed that histones are major carriers of epigenetic information, and that covalent modifications on the histone NH_2-terminal tails function as master on/off switches that determine whether a gene is active or inactive (reviewed in Turner, 2000 and 2002; Fischle *et al.*, 2003).

The functional consequences of these modifications, which are likely to have profound effects on recognition of the nucleosomal fiber by regulatory proteins and its higher-order folding, are currently being explored with great intensity by researchers in the fields of transcriptional initiation, elongation and termination, chromosomal architecture, cohesion and segregation, telomeric and centromeric structure and function, DNA damage sensing and repair, as well as others.

The second group of chromatin remodeling enzymes consists of multisubunit complexes that utilize the free energy derived from ATP hydrolysis to actively disrupt the interactions between histones and DNA that characterize canonical nucleosomes (reviewed in Sudarsanam and Winston, 2000; Peterson, 2002a and 2002b; Becker and Hörz, 2002). Nucleosomes are rather stable particles, in which DNA and histones are held together by 14 clusters of contacts, which involve over 200 hydrogen bonds and salt bridges. Thus, it is not surprising that nucleosome remodeling requires energy input. Alterations of the histone-DNA interactions by ATP-dependent chromatin remodeling enzymes seem to be subtle and transient, momentarily enhancing the accessibility of nucleosomal DNA to interacting proteins.

ATP-dependent chromatin remodeling is a fundamental principle involved in all major reactions in which chromatin is a substrate. Its role in activation and repression of transcription affects control of the cell cycle (Krebs *et al.*, 2000; Strobeck *et al.*, 2000; Zhang *et al.*, 2000; Muchardt and Yaniv, 2001), cell differentiation (de la Serna *et al.*, 2001a and 2001b; Roy *et al.*, 2002; Müller and Leutz, 2001; Zamoyska, 2003), and hence the development of multicellular organisms (Klochendler-Yeivin *et al.*, 2000; Bultman *et*

al., 2000; Guidi *et al.*, 2001; Reyes *et al.*, 2002). Accordingly, mutations in individual subunits of ATP-dependent chromatin remodeling complexes have been correlated with a number of human cancers (reviewed in Klochendler-Yeivin *et al.*, 2002). In addition, BRCA1, a tumor suppressor linked to breast cancer, is associated with a human SWI/SNF-related chromatin remodeling complex (Bochar *et al.*, 2000).

Genetic, biochemical, and genome expression analyses have demonstrated that histone modifying enzymes and ATP-dependent chromatin remodeling complexes do not necessarily act independently of one another (Pollard and Peterson, 1997; Holstege *et al.*, 1998; Cosma *et al.*, 1999; Krebs *et al.*, 1999; Sudarsanam *et al.*, 2000). Indeed, there is increasing evidence for a direct role for modified histones in the recruitment of ATP-dependent chromatin remodeling enzymes to their target sites (Hassan *et al.*, 2002). Alternatively, ATP-dependent chromatin remodeling is a prerequisite for recruitment of histone-modifying enzymes at certain promoters (reviewed in Fry and Peterson, 2001). Collectively, chromatin-modifying enzymes greatly influence chromatin structure. Therefore, investigating how these enzymes alter and regulate chromatin structure in such a dynamic fashion, allowing DNA transactions to occur properly and efficiently is a fundamental issue for understanding the mechanisms of DNA-mediated processes.

SWI2/SNF2 Family of ATPases

Each member of the ATP-dependent family of chromatin remodeling enzymes contains an ATPase subunit that is related to the SWI2/SNF2 subfamily of the DEAD/H superfamily of nucleic acid-stimulated ATPases (Eisen *et al.*, 1995). Members of the

SNF2-like family exhibit an impressive range of biological functions, including gene-specific transcriptional activation (SWI2/SNF2 subfamily), transcriptional repression (MOT1), destabilization of reconstituted nucleosomes (SWI2/SNF2 and SNF2L subfamilies), transcription-coupled repair (ERCC6 subfamily), nucleotide excision repair of non-transcribed regions of the genome (Rad16), recombination repair (Rad54 subfamily), and chromosome segregation (Lodestar). In spite of the presence of a conserved helicase-like motif in these proteins, helicase activity has not yet been detected in any SNF2-like family member (reviewed in Pazin and Kadonaga, 1997).

The members of the SWI2/SNF2 family of proteins share a nucleic acid-stimulated ATPase domain (see Figure I.2; Eisen *et al.*, 1995; Pollard and Peterson, 1998). This ATPase domain contains seven regions (I-VII), which are strongly conserved among family members, and in the case of Swi2/Snf2p, each region is crucial for SWI/SNF function *in vivo* (Gorbalenya *et al.*, 1989; Okabe *et al.*, 1992; Richmond and Peterson, 1996).

Seventeen members of the SWI2/SNF2 subfamily have been identified in *Saccharomyces cerevisiae*, and four of these ATPases have been purified as subunits of distinct chromatin remodeling complexes (ySWI/SNF, yRSC, ISW1 and ISW2). Additional ATP-dependent remodeling complexes that contain SWI2/SNF2 family members have been identified in *Drosophila* (dACF, dNURF, dCHRAC, Brahma), human (hSWI/SNF, hNURD, hRSF) and frog (xMi-2).

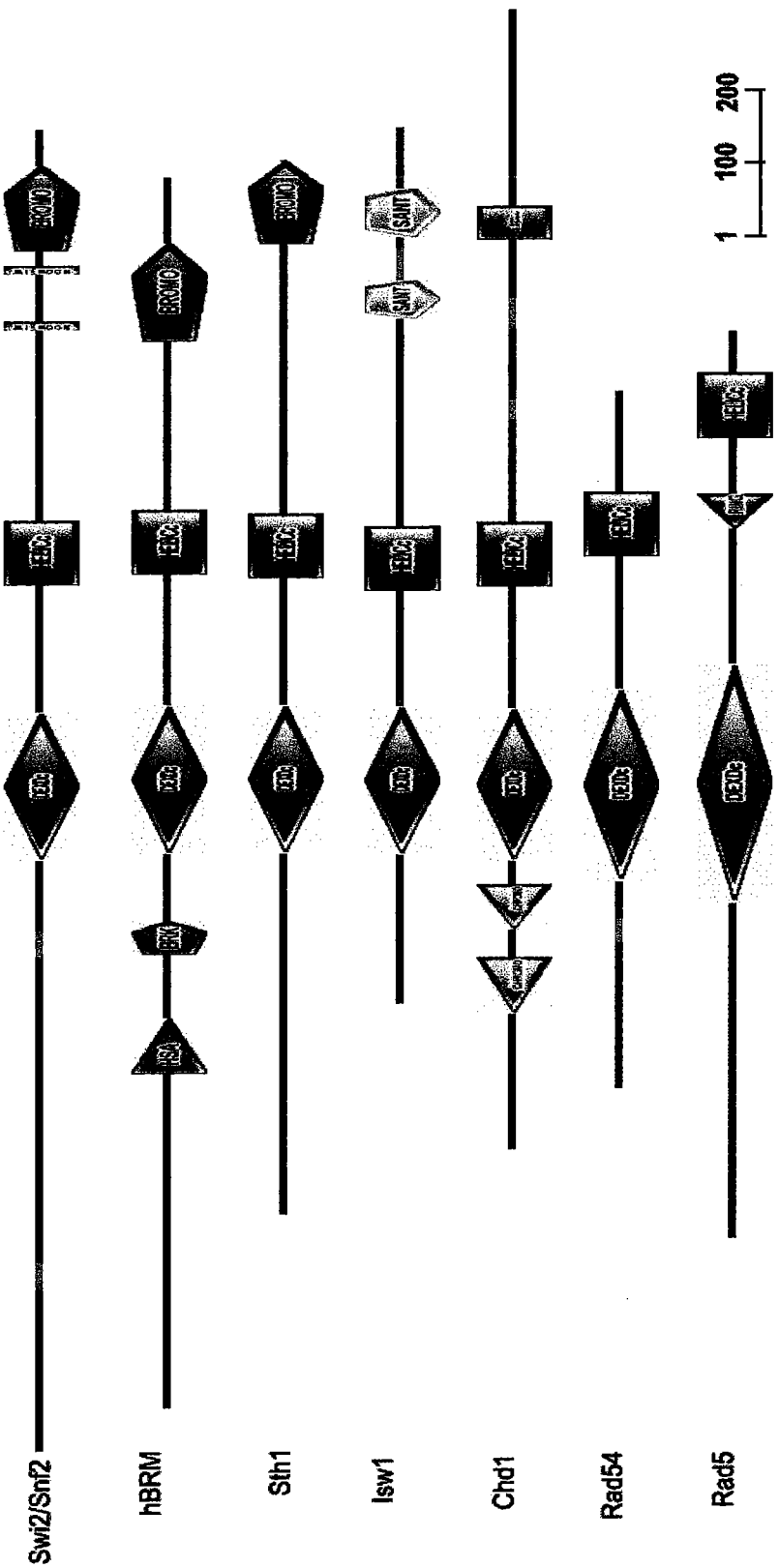

Swi2/Snf2
hBRM
Sth1
Isw1
Chd1
Rad54
Rad5
1
100
200

Figure 1.2: Schematic representation of a subset of members of the SWI2/SNF2 family of proteins.

The hallmark of ATP-dependent chromatin remodeling complexes is a Swi2/Snf2p homologue subunit. All homologues contain the strongly conserved SWI2/SNF2-like ATPase motif, composed of a DEXH box (orange), and a HELICc3 domain (red). The DEXH box contains the putative nucleotide binding and ATP hydrolysis sites. The enzymes in the SWI2/SNF2 family can be grouped into several subfamilies according to sequence features outside of their ATPase domain, like bromodomains (purple), SANT domains (green), chromodomains (light blue), or RING domains (mauve). This figure was generated using the Simple Modular Architecture Research Tool (SMART) (Schultz *et al*, 2000; http://smart.embl-heidelberg.de).

These complexes can be further sub-divided into three groups based on whether their ATPase subunit is similar to either yeast Swi2/Snf2p, *Drosophila* ISWI (Imitation SWI), or human Mi-2/CHD proteins. A number of studies have shown that at least some ATP-dependent remodeling enzymes maintain activity as individual polypeptides or minimal complexes, suggesting that the ATPase components are fundamental to the enzymatic reaction (Corona *et al.*, 1999; Phelan *et al.*, 1999 and 2000).

ATP-dependent chromatin remodeling complexes vary in the number of subunits and the types of assays used to monitor their activity, but apparently they all utilize the free energy derived from ATP hydrolysis to alter chromatin structure and enhance the association of proteins with nucleosomal DNA-binding sites (Boyer *et al.*, 2000). The mechanism by which this is accomplished has not yet been unequivocally established, and has been the subject of much speculation and controversy over the past 10 years.

ySWI/SNF Chromatin Remodeling Complex

The yeast SWI/SNF complex, considered the archetype of ATP-dependent chromatin remodeling enzymes, is required for many transcriptional activators to enhance

transcription in yeast (reviewed in Winston and Carlson, 1992; Peterson and Tamkun, 1995; Fry and Peterson, 2002). Originally, five SWI/SNF subunits were identified in two independent genetic screens. The SWI (mating type switching) mutant screen was designed to identify regulatory factors of the HO gene, which encodes an endonuclease required for mating type switching (Stern *et al.*, 1984). In an independent screen, factors involved in the regulation of the SUC2 gene, which encodes the enzyme invertase that is required for growth on non-fermentable carbon sources, were identified and called SNF (for sucrose non-fermenting) (Neigeborn *et al.*, 1987). A subset of mutants identified by these two screens, swi1, swi2, swi3, and snf2, snf5, snf6, respectively, share similar mutant phenotypes, such as slow growth, inefficient growth on non-fermentable carbon sources and gene-specific transcriptional defects (Abrams *et al.*, 1986; Estruch and Carlson, 1990; Laurent *et al.*, 1990; Happel *et al.*, 1991; Peterson and Herskowitz, 1992). A genetic suppressor screen of *swi/snf* mutants revealed that mutations affecting core histones and non-histone chromatin associated proteins could bypass the need for SWI/SNF complex. This implied that the SWI/SNF complex counteracts chromatin structure in order to facilitate gene activation (reviewed in Pollard and Peterson, 1998; Vignali *et al.*, 2000; Martens and Winston, 2003).

The cloning of *swi2* and *snf2* revealed that they were the same gene, and biochemical purification of Swi2/Snf2p led to the discovery that Swi2/Snf2p, Swi1p, Swi3p, Snf5p and Snf6p proteins are part of a large (~1.15 MDa) multi-subunit complex (Peterson and Herskowitz, 1992; Peterson *et al.*, 1994; Cairns *et al.*, 1994; Côté *et al.*, 1994). The purified complex contains six additional polypeptides: Arp7p, Arp9p,

Swp82p, Swp73p, Swp29p and Snf11p (Peterson *et al.*, 1998; Côté *et al.*, 1994; Cairns *et al.*, 1994 and 1996a;Treich *et al.*, 1995). Consistent with the genetics, numerous biochemical studies have shown that the SWI/SNF complex uses the free energy derived from ATP hydrolysis to disrupt nucleosome structure *in vivo* and *in vitro* (reviewed in Pazin and Kadonaga, 1997; Kornberg, 1999; Peterson, 2002a and 2002b).

With the exception of the Swi2/Snf2p ATPase, the biochemical functions of the individual SWI/SNF components remain unknown (reviewed in Vignali *et al.*, 2000). The importance of the ATPase motor (Swi2 polypeptide) for the complex function is underscored by the fact that a single residue change in the putative nucleotide binding loop (*swi2K798A*) completely eliminates SWI/SNF activity (Laurent *et al.*, 1993; Côté *et al.*, 1994; Richmond and Peterson, 1996).

SWI/SNF complex displays various biochemical activities, which are either ATP-dependent or independent. SWI/SNF-like complexes have the ability to bind naked and nucleosomal DNA with high affinity, in an ATP- and sequence-independent manner (Quinn *et al.*, 1996; Côté *et al.*, 1998; Moreira and Holmberg, 1999).

ATP-dependent functions of the SWI/SNF-like complexes include enhancement of gene-specific activator binding (Burns and Peterson, 1997), transcriptional repression of a subset of genes (Murphy *et al.*, 1999; Dimova *et al.*, 1999; Martens and Winston, 2002; reviewed in Narlikar *et al.*, 2002), stimulation of replication from certain ARS elements (Flanagan and Peterson, 1999), and viral integration (Iba, 2003; Wang, 2003).

In vitro biochemical studies have shown that human and yeast SWI/SNF complexes are able to disrupt DNase I digestion patterns of rotationally phased

nucleosomal DNA, drive the *trans*-displacement of histone octamers from a particular piece of DNA to competing DNA (Lorch *et al.*, 1999; Phelan *et al.*, 2000) and stimulate binding of a number of activators (TBP, GAL4 derivatives, NF-KB, and more) to nucleosomal binding sites in an ATP-dependent manner (Côté *et al.*, 1994; Kwon *et al.*, 1994; Imbalzano *et al.*, 1994).

Understanding the mechanism of SWI/SNF remodeling has been a major area of investigation. When the studies included in this thesis were initiated, the established view in the chromatin field was that SWI/SNF and other ATP-dependent chromatin remodeling complexes could alter the nucleosome structure in an ATP-dependent manner, as visualized by an increase in the accessibility of nucleosomal DNA to endonucleases or transcription factors, but the mechanism underlying these alterations had not been determined. Moreover, the exact nature of the "remodeled state" was not known and there were conflicting reports regarding the stability or reversibility of this state. The different outcomes of SWI/SNF remodeling activity *in vitro* reflected, at least in part, the particular substrate and condition used in each type of experiment (Martens and Winston, 2003). Specifically, studies performed using mononucleosomes as a substrate suggested that the alterations introduced by SWI/SNF were stable and did not require the continuous hydrolysis of ATP (Imbalzano *et al.,* 1996; Lorch *et al.*, 1998; Schnitzler *et al.*, 1998; Côté *et al.,* 1998; Guyon *et al.*, 1999). In contrast, studies carried out with arrays of nucleosomes showed that the "remodeled state" was highly reversible and absolutely dependent on the presence of SWI/SNF and ATP (Logie and Peterson, 1997 and 1999; Logie *et al.*, 1999).

Our data indicates that ySWI/SNF can slide histone octamers along the DNA in an ATP-dependent manner, both on mononucleosomes and nucleosomal array substrates, resulting in repositioned nucleosomes. Studies performed on ySWI/SNF and other ATP-dependent chromatin remodeling complexes support our findings (Hamiche *et al.*, 1999; Längst *et al.*, 1999; Längst and Becker, 2001a; Brehm *et al.*, 2000; Whitehouse *et al.*, 1999; Guschin *et al.*, 2000; Havas *et al.*, 2000; Gavin *et al.*, 2001; Guyon *et al.*, 2001; Aoyagi and Hayes, 2002; Kassabov *et al.*, 2003) and substantiate the current interpretation that chromatin remodeling enzymes specifically perturb the writhe or twist of DNA on the surface of the nucleosome core by introducing superhelical torsion on DNA, thus lessening the DNA-histone interactions and resulting in nucleosome repositioning. Still, it is not clear whether nucleosome movement is the primary outcome of ATP-dependent chromatin remodeling (reviewed in Becker and Hörz, 2002; Martens and Winston, 2003). In fact, a number of studies suggest that drastic nucleosome movements are not required for nucleosomal DNA perturbation or for enhancement of DNA accessibility to restriction enzymes by SWI/SNF (Bazett-Jones *et al.*, 1999; Gavin *et al.*, 2001).

DNA Repair by Homologous Recombination

Although transcription is the process for which a requirement for chromatin remodeling is best documented, replication, recombination, and DNA repair may also rely on it. In fact, members of the SWI/SNF2 family of proteins have been implicated in these processes. Particularly, Rad54p (see Figure I.2) is required for the homologous

recombination (HR) pathway used by eukaryotic cells to repair DNA double-strand breaks (DSBs).

DNA DSBs are generated when the two complementary strands of the DNA double helix are broken at sites that are sufficiently close to one another that base-pairing and chromatin structure are insufficient to keep the two DNA ends juxtaposed. As a consequence, the two DNA ends are likely to become physically dissociated from one another, making repair difficult to perform, and providing the opportunity for inappropriate recombination with other sites in the genome (reviewed in Jackson, 2002).

Chromosomal DSBs may arise through exposure of cells to harmful environmental agents such as ionizing radiation (IR) or cytotoxic chemicals (radio-mimetics, alkylating agents, etc). Alternatively, DSBs may be caused by endogenously-produced oxygen radicals, stalled replication forks, or as physiological intermediates during programmed cellular processes, such as meiosis or V(D)J recombination (Pâques and Haber, 1999; Hiom, 2001; Wood, 2001; Jackson, 2002). A DSB is a particularly dangerous type of DNA lesion. It can interrupt the coding sequence of a gene, disrupt the linkage between coding and regulatory sequences, alter chromosome organization, and perturb the systems that ensure correct DNA replication, chromosome packaging, and chromosome segregation (Cromie, 2001). Cell survival and maintenance of genome integrity are critically dependent on efficient repair of DSBs, since unrepaired or misrepaired DSBs may result in mutations, gene translocations, gross chromosomal rearrangements, or cellular lethality.

Because of the threats posed by DSBs, eukaryotic cells have evolved complex and highly conserved systems to rapidly and efficiently detect these lesions, signal their presence and bring about their repair. There are two main pathways for DNA DSB repair— homologous recombination and non-homologous end joining (NHEJ). These two pathways are essentially distinct from one another and function in complementary ways to effect DSB repair. They are intimately coupled to DNA damage surveillance and checkpoint functions, and constitute the effector arm of DNA damage responses (van Gent *et al.*, 2001; Jackson, 2002).

NHEJ involves the ligation of the two free DNA ends at regions of little or no homology. The disadvantage of this approach is that it can lead to loss of information from the DNA due to resection of the broken ends. The alternative to using an end-joining strategy is to repair the broken DNA molecule using another DNA duplex of identical or near-identical sequence by homologous recombination. Both NHEJ and HR are highly conserved throughout eukaryotic evolution but their relative importance differs from one organism to another (Haber, 2000a). Simple eukaryotes such as the yeasts *S. cerevisiae* or *S. pombe* rely mainly on HR to repair radiation-induced DNA DSBs. In contrast, in mammals the NHEJ pathway predominates in many stages of the cell cycle — particularly in G_0 and G_1 — although HR is also of importance, particularly during S and G_2 phases (Johnson and Jasin, 2000).

During homologous recombination, the damaged chromosome enters into synapsis with an undamaged DNA molecule with which it shares extensive sequence homology (e.g., sister chromatid), and retrieves genetic information from it, resulting in

the accurate reversal of the damage. In organisms ranging from yeast to human, HR is mediated by the Rad52 (radiation sensitive) epistasis group of proteins. This group consists of Rad50p, Rad51p, Rad52p, Rad54p, and Mre11p, along with Rad55p, Rad57p, Rad59p, and Xrs2p in yeast, and Rad51B-D, XRCC2, XRCC3, and NBS1 in humans. Mutations in these proteins result in pleiotropic defects in DNA damage repair, including extreme sensitivity to IR and other DSB-inducing agents.

The events of HR are complex and, based on studies under various biological circumstances and in different organisms, there are various models for precisely how they take place. Figure I.3 shows an outline of a general model. DSBs are first processed into long single-stranded DNA regions by specialized exonucleases. The efficiency of this reaction *in vivo* relies upon, and probably involves, a complex containing Rad50p, Mre11p and Xrs2p (NBS1 in humans). The resulting 3' single-stranded overhangs are bound by Rad51p, a homologue of the prokaryotic recombinase RecA, in a process that is influenced by a range of other proteins, including replication protein A (RPA) (Baumann and West, 1997), Rad52p (Van Dyck, 1999), Rad54p (Van Komen *et al.*, 2002; Mazin *et al.*, 2003; Wolner *et al.*, 2003), BRCA1 and BRCA2 (reviewed in Jasin, 2002). Rad51p polymerizes onto these single-stranded tails to form a nucleoprotein filament that interacts with an undamaged duplex DNA molecule as it searches for a sufficiently extended region of sequence homology. When the search has been successfully completed, Rad51p catalyzes the central reaction in HR, a strand-exchange event in which the damaged molecule invades the other DNA duplex, displacing one strand as a D-loop (see Figure I.3). This reaction is influenced by other factors, primarily Rad54p

(Swagemakers *et al.*, 1998; Petukhova *et al.*, 1998 and 1999; Van Komen *et al.*, 2000; Dronkert *et al.*, 2000). The strand exchange reaction generates a joint molecule between the homologous damaged and undamaged DNA molecules.

The 3' terminus of the damaged DNA molecule is then extended by a DNA polymerase (δ or ε) that copies the missing information from the undamaged partner, and the ends are ligated by DNA ligase I. Finally, after migration, the crossed DNA strands (Holliday junctions) are resolved by cleavage and ligation to yield two intact duplex DNAs (reviewed in Kanaar *et al.*, 1998; Flores-Rozas and Kolodner, 2000). In contrast with NHEJ, HR is generally accurate and non-mutagenic, although there are exceptions (for instance, when direct repeats flank the two DNA ends).

This general model for homologous recombination has been challenged by the finding that the donor sequence remains unaltered in most cases, whereas heteroduplex is formed on the recipient (originally damaged) molecule. This led to the consideration of models involving synthesis-dependent strand annealing (SDSA), in which essentially all of the newly synthesized DNA is found in the recipient locus (reviewed in Haber, 2000b).

Rad51p encodes a recombinase capable of carrying out *in vitro* strand-exchange reactions, in a fashion similar to bacterial RecA. Rad54p, a DNA-dependent ATPase related to the SWI2/SNF2 family, has been shown to physically interact with Rad51p

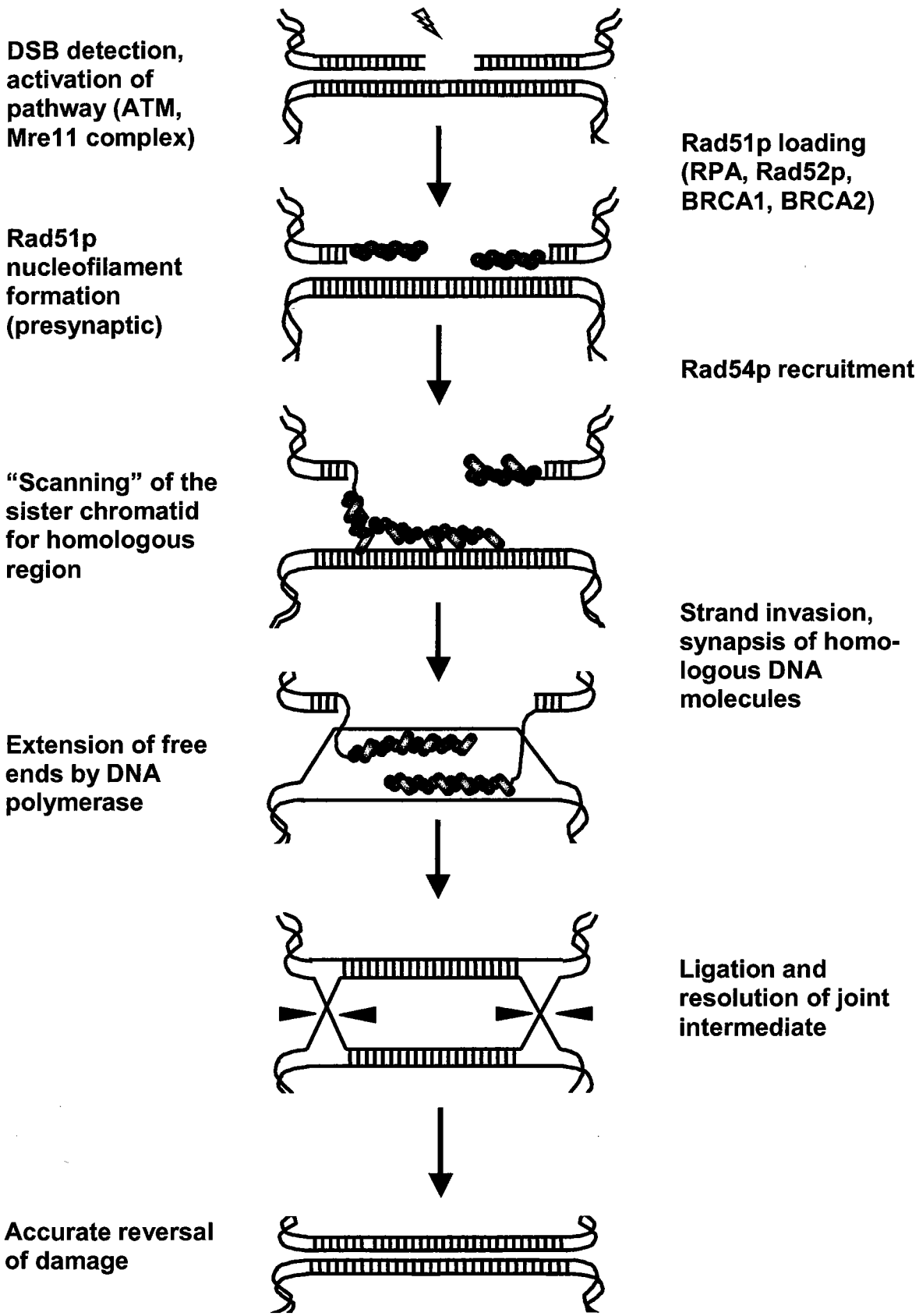

DSB detection, activation of pathway (ATM, Mre11 complex)
Rad51p loading (RPA, Rad52p, BRCA1, BRCA2)
Rad51p nucleofilament formation (presynaptic)
Rad54p recruitment
"Scanning" of the sister chromatid for homologous region
Strand invasion, synapsis of homologous DNA molecules
Extension of free ends by DNA polymerase
Ligation and resolution of joint intermediate
Accurate reversal of damage

Figure I.3: Schematic representation of DSB repair through HR

The differently colored double-stranded DNAs represent homologous sequences (sister chromatids). Double-strand break formation triggers cell-cycle checkpoints, and the nucleolytical processing of the 5' ends at both sides of the break. Rad51p polymerizes onto the single-stranded overhangs with the aid of multiple factors. (RPA, Rad52p, Rad55/57p, Rad54p, and in mammals BRCA1, BRCA2, and Rad51 orthologues). The Rad51p nucleoprotein filament searches for the homologous duplex DNA. When a considerable stretch of homology is found, DNA strand exchange generates a joint molecule between the two DNA molecules. DNA synthesis fills in the break in the damaged strands, and resolution of the joint intermediate results in the faithful reconstitution of the genetic information lost at the site of damage.

(Jiang *et al.*, 1996; Clever *et al.*, 1997), and to significantly increase the efficiency of the *in vitro* strand-exchange reaction (Petukhova *et al.*, 1998). Its relevance in the HR repair pathway is underlined by the fact that mutations in Rad54 (Matsuda *et al.*, 1999) and a Rad54 homologue, Rad54B (Hiramoto *et al.*, 1999), have been found in human primary lymphoma and colon cancers. Moreover, Rad54 (Gonzalez *et al.*, 1999) and Rad54B (Rasio *et al.*, 1997) have been correlated with breast carcinomas in a loss of heterozygosity analysis.

Among the Rad52 group of proteins required for recombination, Rad54p is the only one for which there does not appear to be a structural or functional homologue in prokaryotes, suggesting a potential role in overcoming the inherent repressive nature of chromatin (Petukhova *et al.*, 1998). As other SWI2/SNF2 family members, yeast and human Rad54 carry highly conserved ATPase and helicase domains. They hydrolyze ATP in the presence of DNA, but they possess no helicase activity (Petukhova *et al.*, 1998; Swagemakers *et al.*, 1998). The ATPase activity of Rad54p is much stronger than the ssDNA-dependent ATPase activity of Rad51p (k_{cat}>1,000/min and k_{cat}<1/min, respectively) (Pâques and Haber, 1999). Rad54p is related to other DNA repair proteins,

Rad5p (implicated in post-replicative repair), Rad16p (implicated in UV repair), and Rad26p (implicated in transcription-coupled repair, Citterio *et al.,* 2000), all of them members of the SWI2/SNF2 family (see Figure I.2). This led to the idea that these proteins "open up" chromatin for repair and recombination (Peterson, 1996), an idea supported by a genetic study that suggested that Rad51p, Rad54p, Rad55p and Rad57p are required for HO-induced gene conversions only when the donor sequence is inaccessible, embedded in chromatin, but not when it is placed on a plasmid (Sugawara *et al.*, 1995).

Rad54p is required for efficient formation of heteroduplex DNA, a key intermediate in recombination processes, but the details of the molecular mechanism through which Rad54p stimulates this reaction are poorly understood. Rad54p induces a change in DNA topology (Petukhova *et al.*, 1999; Van Komen *et al.*, 2000) that could be required for formation of the joint DNA intermediate. A scanning force microscopy study of the hRad54-DNA complex suggests that Rad54 can introduce both negative and positive supercoils on DNA, reminiscent of protein translocation along the DNA (Ristic *et al.*, 2001).

Synopsis

ATP-dependent chromatin remodeling enzymes are emerging as a common theme in the fields of transcriptional regulation, replication and DNA repair. In general, chromatin remodeling complexes alter nucleosomal structure to ensure the proper

regulation of all DNA-mediated processes. The main question we intended to tackle is how the free energy derived from ATP hydrolysis is translated into a biomechanical force that disrupts the contacts between nucleosomal histones and DNA. Dissecting the mechanism of ATP-dependent chromatin remodeling will help understand how these complexes contribute to normal execution of diverse nuclear processes and the way in which their deficiency can play a part in human disease.

The research described in this thesis investigates the mechanism of chromatin remodeling used by two *Saccharomyces cerevisiae* factors: the SWI/SNF ATP-dependent chromatin remodeling complex, and Rad54p, a member of the Rad52 epistasis group required for homology-based DNA repair. *In vivo,* SWI/SNF modulates the accessibility of regulatory sequences on specific gene promoters; Rad54p assists Rad51p in the search for homology and/or strand invasion steps of the homologous recombination pathway. Here we present data supporting the hypothesis that both SWI/SNF and Rad54p translocate along nucleosomal DNA, generating superhelical stress and altering histone-DNA interactions.

Both these factors are highly conserved in evolution from yeast to man. Examination and dissection of the mechanism used by these enzymes will provide a better understanding of their role in the regulation of DNA-mediated processes. Moreover, this study should be applicable to similar factors in higher eukaryotic systems.

Preface to Chapter II

In Chapter II, we provide evidence suggesting that SWI/SNF induces the mobilization of histone octamers along the DNA. Our data reconciles previous contradicting reports regarding the stability of the SWI/SNF-generated "remodeled state".

This work embodies the joint effort of Colin Logie, Ph.D., who contributed the data represented in Figures II.1 and II.2; Igor Gavin, Ph.D., who contributed the data represented in Figures II.6 and II.7; and myself (Figures II.3, II.4 and II.5).

The data presented in this Chapter was published in the journal **Molecular and Cellular Biology**, Volume 20, No.9 (May 2000).

CHAPTER II

SWI/SNF-MEDIATED NUCLEOSOME REMODELING: ROLE OF HISTONE OCTAMER MOBILITY IN THE PERSISTENCE OF THE REMODELED STATE

CHAPTER II

SWI/SNF-Mediated Nucleosome Remodeling: Role of Histone Octamer Mobility in the Persistence of the Remodeled State

INTRODUCTION

Eukaryotic chromatin has seen a rebirth of intense study over the past few years. Foremost among the biochemical reactions impinging on chromatin structure is ATP-dependent chromatin remodeling which leads to an enhanced accessibility of nucleosomal DNA (reviewed in Kingston and Narlikar, 1999; Kornberg and Lorch, 1999; Peterson, 2002a). This reaction plays a key role in the regulation of transcription by RNA polymerase II, and it has been proposed to be a prerequisite for a variety of other cellular processes that require access to the chromatin template (reviewed in Varga-Weisz and Becker, 1998; Müller and Leutz, 2001; Muchardt and Yaniv, 2001). In addition to ATP dependent nucleosome remodeling, multi-subunit complexes that can acetylate (Owen-Hughes *et al., 1*996; Grant *et al.*, 1997; Saleh *et al.,* 1997; Pollard and Peterson, 1998) or methylate (Chen *et al.*, 1999) histone and nonhistone proteins have the potential to directly modify chromatin structure and function.

A host of ATP dependent chromatin remodeling complexes has been identified via either biochemical fractionation of cell extracts, yeast genetics, or through genome

database mining (Pennings *et al.*, 1991; Kwon *et al.*, 1994; Tsukiyama *et al.*, 1995 and 1999; Cairns *et al.*, 1996b; Ito *et al.*, 1997; Varga-Weisz *et al.*, 1997; LeRoy *et al.*, 1998; Armstrong *et al.*, 1998; Tong *et al.*, 1998; Wade *et al.*, 1998; Xue *et al.*, 1998; Zhang *et al.*, 1998). A hallmark of these multi-subunit complexes is that they contain a member of the SWI2/SNF2 subfamily of DNA-stimulated ATPases. Seventeen members of the SWI2/SNF2 family have been identified in the yeast genome (Eisen *et al.*, 1995; Pollard and Peterson, 1998), and to date, four of these ATPases have been purified as subunits of distinct chromatin remodeling complexes [SWI/SNF, (Côté *et al.*, 1994); RSC, (Cairns *et al.*, 1996b); ISW1 and ISW2, (Tsukiyama *et al.*, 1999)]. Additional ATP-dependent remodeling complexes have been identified in *Drosophila* [ACF (Ito *et al.*, 1997), CHRAC (Varga-Weisz *et al.*, 1997), NURF (Biggar and Crabtree, 1999), brm (Papoulas *et al.*, 1998)], human [hSWI/SNF (Kwon *et al.*, 1994), NURD (Tong *et al.*, 1998; Zhang *et al.*, 1998; Xue *et al.*, 1998), RSF (LeRoy *et al.*, 1998)], and frog [Mi-2 (Wade *et al.*, 1998)]. Each of these complexes appears to catalyze a similar reaction in which the energy of ATP hydrolysis is used to weaken histone-DNA interactions which leads to an increase in nucleosomal DNA accessibility. In the case of the yeast SWI/SNF, *Drosophila* brm, and human SWI/SNF complexes, this reaction is required for transcriptional regulation of target genes *in vivo* (reviewed in Kingston and Narlikar, 1999).

Many of the in vitro studies that have focused on the mechanism of ATP-dependent remodeling have utilized mononucleosome substrates. In these cases, ATP-dependent remodeling is often scored as a disruption of the DNase I digestion pattern of

rotationally phased nucleosomal DNA or an enhancement of transcription factor binding to nucleosomal sites. For yeast SWI/SNF, RSC, and human SWI/SNF complexes, the remodeled state of mononucleosomes is stable after removal of ATP or SWI/SNF (Imbalzano *et al.*, 1996; Lorch *et al.*, 1998; Côté *et al.*, 1998; Schnitzler *et al.*, 1998; Guyon *et al.*, 1999). Furthermore, this persistent disruption of mononucleosome structure is accompanied by formation of a novel, stably remodeled species that resembles a dinucleosome and retains a full complement of histones and DNA (Schnitzler *et al.*, 1998; Lorch *et al.*, 1999; reviewed in Travers, 1999). This is probably the result of the ability of hSWI/SNF and RSC to transfer the histone octamer onto acceptor DNA under certain reaction conditions (Lorch *et al.*, 1999; Phelan *et al.*, 2000).

In contrast to studies with mononucleosomes, ATP-dependent remodeling of nucleosomal array substrates by yeast SWI/SNF and RSC leads to the formation of an unstable remodeled state that requires continuous ATP hydrolysis (Logie and Peterson, 1997 and 1999; Logie *et al.*, 1999). In these studies remodeling was monitored by quantifying the enhanced kinetics of restriction enzyme digestion of a unique *SalI/HincII* site located within the central nucleosome of a positioned nucleosomal array. Addition of a remodeling enzyme leads to a 30-40 fold increase in digestion kinetics, but removal of ATP results in almost immediate reversal of the nucleosomal restriction site to the occluded state.

The apparent differences in stability of the remodeled state between mononucleosome and nucleosomal array substrates might be due to the different assays used to detect remodeling events; alternatively, remodeling of mononucleosome

substrates may yield novel, stable products that do not occur on nucleosomal arrays. Here we have tested these two possibilities by directly comparing the lability of remodeled mononucleosomes and nucleosomal arrays using a quantitative restriction enzyme coupled remodeling assay. Using this assay we confirm that the remodeling of mononucleosome substrates by yeast SWI/SNF leads to a persistent accessibility of DNA that was previously occluded by a nucleosome, whereas the remodeled state of nucleosomal arrays is labile and requires continuous ATP hydrolysis. We then show that the stable, accessible state of mononucleosomes correlates with the SWI/SNF-dependent movement of the histone octamer to the DNA ends. Likewise, SWI/SNF action leads to a persistent change in nucleosome positioning on the array substrate, but in this case the resulting random nucleosome positions cause a general and persistent occlusion of DNA sites.

MATERIALS AND METHODS

Plasmid constructions

A partial *Eco*RI digestion was carried out on pCL7b (Logie and Peterson, 1999) to release a DNA fragment encompassing 5 head to tail repeats of the 208 bp *L. variegatus* 5S rDNA nucleosome positioning element. This *Eco*RI fragment was then cloned into the unique *Eco*RI site of pCL6 (Logie and Peterson, 1999), to yield pCL113, where the last repeat bears the unique *SalI/Hinc*II site and is flanked by a unique *Pst*I site distal to the *Not*I site of pBS- SKII (+). To generate pCL114 (which contains a total of seven 5S repeats), a single, blunt ended, *Eco*RI 208 bp *L. variegatus* 5S rDNA nucleosome positioning element was sub-cloned into the filled-in *Xba*I site of pCL113 to introduce a wild type 208 bp rDNA repeat between the *Not*I and the modified *SalI/Hinc*II site bearing repeat of pCL113. pCL115 contains a single 5S repeat and was generated by fill-in of the *Sal*I site located in the polylinker of pCL6 so as to leave a unique *SalI/Hinc*II site at the predicted dyad axis of the single 5S repeat.

Reagent preparation and nucleosome reconstitutions

SWI/SNF and histone octamers were purified as described (Logie *et al.*, 1999). Apyrase was purchased from Sigma (A-6410) and was diluted to a concentration of 1U/µl as described (Imbalzano *et al.*, 1996). Nucleosomal array DNA templates (*Not*I–*Eco*RV fragments derived from pCL7b (Logie and Peterson, 1999), pCL113 or pCL114) were labeled by the Klenow polymerase fill-in reaction using [α-^{32}P] dCTP

(6000μCi/mmol, Amersham). Linear nucleosomal arrays were reconstituted at a ratio of 1.0-1.3 octamers per 5S DNA repeat, and samples were characterized by *Eco*RI analysis as previously described (Logie *et al.*, 1999). Reconstituted nucleosomal arrays were loaded on top of a 10%-40% linear glycerol gradient and centrifuged in a SW-28 rotor (Beckman) at 33,000*g* for 16 hours for sedimentation studies. 0.4 ml fractions were collected from the top of the gradient and counted by scintillation.

Circular minichromosomes were reconstituted as the linear arrays, but in this case 2 μg supercoiled pCL115 and 2 μg purified chicken histone octamers were assembled in 100 μl reactions (a histone:DNA ratio of ~1 octamer per 150 bp).

Mono- and di-nucleosomes were obtained by digestion of the appropriate labeled nucleosomal arrays with *Pst*I. The *Pst*I digestion reactions contained 3 μg of the corresponding labeled nucleosomal array ($1.5x10^6$ cpm), 100U *Pst*I (NEB), 10mM NaCl, 5mM Tris-HCl, 1mM $MgCl_2$, 0.1mM DTT, in a final volume of 200 μl. After 2 hours at 37°C, the reactions were loaded on top of a 15 ml 10%-30% linear glycerol gradient containing 1% BSA, 0.2% PMSF, 0.1% Tween 20, 1mM DTT, 10mM Tris-HCl (pH: 8.0), 125mM NaCl. The gradients were centrifuged for 17 hours at 28,000 rpm in a SW 28 rotor. The gradients were fractionated into 500 μl fractions, the position of the labelled DNA was determined by scintillation counting, and 1/30th of the radioactive fractions (~$1x10^4$ cpm) was analyzed on 4% native polyacrylamide gels. Mononucleosomes (216 bp) were detected in fractions 9-12; dinucleosomes (427 bp) in fractions 14-19. Mononucleosomes reconstituted on the 427 bp DNA fragment were detected in fractions 9-12 of the "dinucleosome" gradient. For reconstitution of the 154 bp 5S

mononucleosome, an *Nru*I-*Bam*HI fragment (154 bp) from pCL113 was used in a fast salt dilution reconstitution protocol (Ito *et al.*, 1997). After the reconstitution procedure, mononucleosomes were purified through a 5ml 5%-30% linear glycerol gradient, and 150 µl fractions were collected, counted, and analyzed on a 4% native polyacrylamide gel.

Reaction conditions

For the coupled SWI/SNF reactions, reconstituted arrays, mono-, or di-nucleosomes (0.3 - 2nM final DNA concentration) were mixed with 3nM SWI/SNF complex and 10 units of restriction enzyme in a buffer containing final concentrations of 125mM NaCl, 5mM $MgCl_2$, 1mM DTT, 10mM Tris-HCl (pH: 7.9), 100 µg/ml BSA and 3% glycerol. Where indicated, ATP was added to a final concentration of 1mM. Where indicated, 0.5U of apyrase was added per 50 µl of reaction mixture. The reactions were incubated at 37°C. Under these conditions, removal of ATP by apyrase was complete in <2 minutes. At the indicated time points an aliquot of the reaction was vigorously mixed for 10 s with 25 µl TE and 50 µl of a 1:1 solution of phenol/chloroform. After this extraction, samples were treated with 1 mg/ml proteinase K for 1 hour at 37°C. The purified DNA fragments were resolved either by non-denaturing agarose gel electrophoresis in the presence of ethidium bromide or on 4% native polyacrylamide gels. For SWI/SNF remodeling reactions containing the circular minichromosomes, DNA topoisomers were resolved on 20 cm 1.75% agarose gels in 40 mM Tris (pH: 8.0), 30 mM $NaPO_4$, 1 mM EDTA at 40 V for 2 days (Guyon *et al.*, 1999), followed by Southern blotting and probing with pCL115 sequence. The fraction of topoisomers, cut and uncut

DNA were obtained by PhosphoImager analysis using the ImageQuant software (Amersham Biosciences). To resolve the nucleosomal species with different translational positions, the reactions received a 100-fold molar excess of unlabeled chicken oligonucleosomes to compete for binding of SWI/SNF to the mononucleosome substrate prior to loading to a 4% polyacrylamide native gel, run at room temperature for 16 hours at 40V.

Micrococcal nuclease digestion.

Reconstituted arrays at a concentration of 0.8 nM were digested with either 1, 2 or 4 units/ml micrococcal nuclease (Sigma) in 50mM NaCl, 10mM Tris-HCl, 2.5mM $MgCl_2$, 0.25mM $CaCl_2$, 1mM DTT, 0.1mg/ml BSA at 37°C for 10 min. [^{32}P]-labeled DNA was digested under the same conditions at a concentration of 10 pM. For "nucleosome protection" experiments 3 nM arrays was digested with 500U/ml Mnase. Purified DNA was analyzed either on a 1% agarose gel or a 4% acrylamide gel (acrylamide to bisacrylamide ratio of 30:0.8).

RESULTS

SWI/SNF-dependent enhancement of nucleosomal array DNA accessibility requires the continuous presence of ATP.

In order to quantify the accessibility of nucleosomal DNA in the context of nucleosomal arrays, we have developed a biochemical assay where nucleosome remodeling activity is coupled to restriction enzyme activity such that remodeling is revealed as an enhancement of restriction enzyme cleavage rates (Logie and Peterson, 1999). In our previous studies the central nucleosome of an 11-mer nucleosomal array contained a unique *Sal*I/*Hinc*II site located at the predicted dyad axis of symmetry (Logie and Peterson, 1997 and 1999; Logie *et al.*, 1999; Polach and Widom, 1995; Yudkovky *et al.*, 1999). Restriction enzyme kinetics are bi-phasic in this system; the first phase is rapid and reflects the fraction of restriction sites that are not occluded by a nucleosome (40-15% of the total population, depending on the ratio of histone octamer to DNA used for reconstitution; see also Logie and Peterson, 1997 and 1999; Logie *et al.*, 1999). The second phase is very slow and reflects a dynamic equilibrium between the occluded and "open" nucleosomal DNA states (Figure II.1B; Logie and Peterson, 1999; Polach and Widom, 1995). Addition of SWI/SNF and ATP stimulates the second phase of *Sal*I/*Hinc*II digestion 20- to 30-fold, but, in contrast to the persistent remodeling of mononucleosome substrates, the enhancement of nucleosomal array digestion by *Sal*I or

*Hinc*II requires continuous ATP hydrolysis (Logie and Peterson, 1999; Logie *et al.*, 1999).

To investigate whether these kinetics of SWI/SNF remodeling are unique to the central nucleosome of an array, we constructed new DNA templates where the nucleosome positioning sequence marked by the *Sal*I/*Hinc*II site was located within the last or second to last position of the array (Figure 1A). These new DNA templates were then reconstituted into nucleosomal arrays and the kinetics of nucleosomal array remodeling by SWI/SNF was compared with the rates of remodeling of our original array template (Figure II.1B). Addition of SWI/SNF to remodeling reactions resulted in a dramatic enhancement of

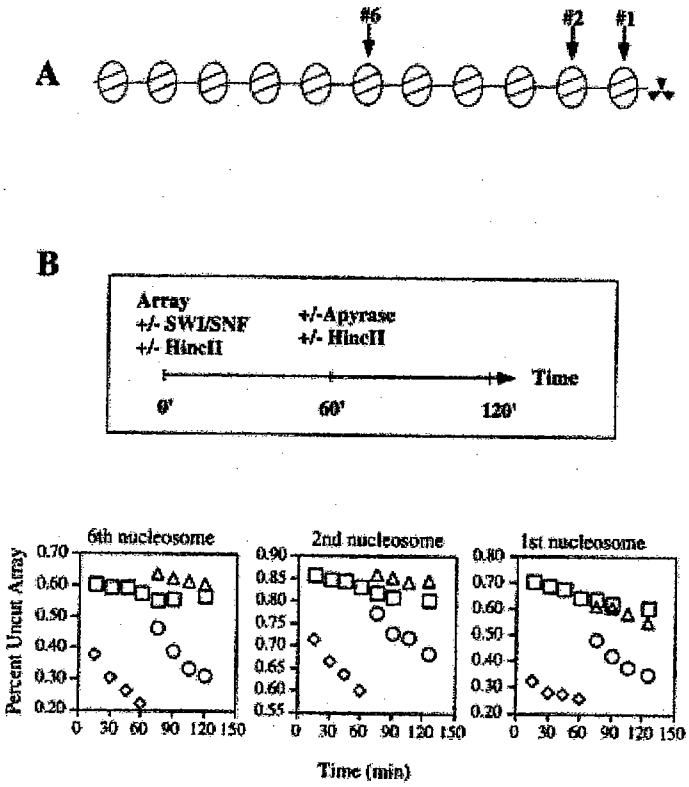


Figure II. 1: Remodeled nucleosomes do not accumulate in the context of linear nucleosomal arrays

(A) Schematic representation of the nucleosomal DNA templates used for the coupled restriction enzyme-SWI-SNF remodeling assay. Each template is composed of head-to-tail repeats of a 5S rDNA nucleosome positioning sequence from *L. variegatus*. The first, second, or sixth nucleosome is tagged by a unique *Sal*I/*Hinc*II restriction site.

(B) The nucleosomal arrays were incubated with *Hinc*II (□), SWI-SNF (○, △), or both (◇). After 1 hour, *Hinc*II (○) or *Hinc*II and apyrase (△) were added to the reaction to test for accumulation of remodeled substrate or for ATP dependence, respectively. Cleavage rates were quantified as described in Materials and Methods. Similar results were obtained in three separate experiments.

restriction enzyme activity for all three nucleosomal array substrates (Figure II.1B; diamonds). Pre-incubation of the arrays with SWI/SNF for 1 hour prior to restriction enzyme addition also resulted in enhanced cleavage rates (Figure II.1B; circles). Pre-incubation of the arrays with SWI/SNF for 1 hour, followed by co-addition of restriction enzyme and apyrase (to enzymatically remove ATP) resulted in cleavage kinetics that were identical to reactions where SWI/SNF was omitted from the reaction (Figure II.1B; triangles). These results indicate that remodeled, accessible nucleosomes do not accumulate during the preincubation with SWI/SNF and ATP regardless of their positions within the array.

Nucleosome remodeling is not persistent on closed circular nucleosomal arrays.

In contrast to our studies with linear nucleosomal arrays, Kingston and colleagues have reported that SWI/SNF-dependent remodeling of closed circular nucleosomal arrays, as assayed by a decrease in the number of constrained negative supercoils, does not require continuous ATP hydrolysis (i.e., a persistent change in minichromosome structure; Guyon *et al.*, 1999; Imbalzano *et al.*, 1996; Schnitzler *et al.*, 1998). In order to test whether the topology of the nucleosomal array influences the stability of the remodeled state, as assayed by restriction enzyme digestion, we reconstituted an average of seven nucleosomes onto a ~3kb plasmid DNA template that contains a single 5S nucleosome positioning sequence harboring a unique *Sal*I/*Hinc*II site (Figure II.2). This array was then subjected to a remodeling/reversal experiment identical to those displayed

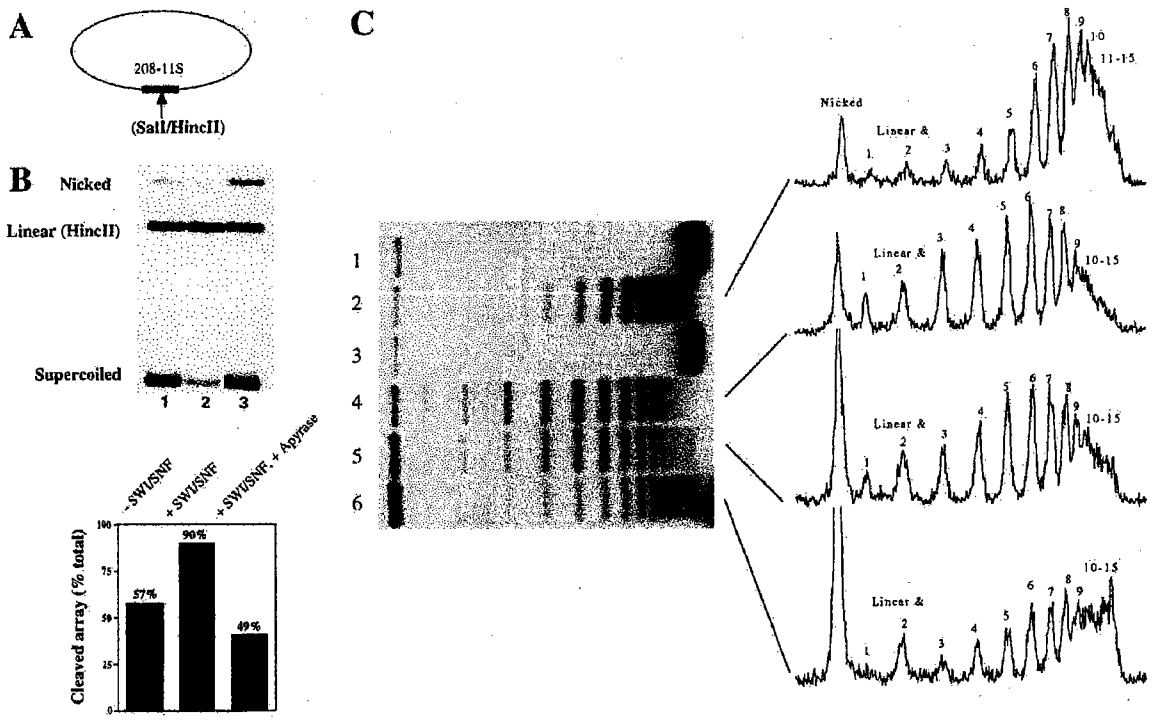


Figure II.2: Remodeled nucleosomes do not accumulate on closed circular nucleosomal arrays

(A) Nucleosomal arrays were reconstituted on negatively supercoiled closed circular plasmids bearing a unique *SalI*/*Hinc*II site on a single 5S nucleosome positioning sequence.

(B) The closed circular arrays were subjected to *Hinc*II digestion for 30 min followed by a 30-min incubation with (lane 2) or without (lane 1) SWI-SNF. Alternatively, the arrays were incubated for 30 min with SWI-SNF, followed by removal of ATP with apyrase and digestion with *Hinc*II for 30 min (lane 3).

(C) A supercoiling assay was performed on the closed circular nucleosomal arrays (lane 1) in order to detect remodeling as changes in plasmid linking number. Addition of calf thymus topoisomerase I resulted in the appearance of approximately 15 bands after deproteination and agarose gel electrophoresis (lane 2). Incubation of the arrays with SWI-SNF and ATP for 30 min had no effect on topology (lane 3). Addition of SWI-SNF, ATP, and topoisomerase I resulted in a redistribution of the topoisomers (lane 4). Removal of ATP by addition of apyrase after 30 min of incubation in the presence of SWI-SNF plus topoisomerase did not affect the distribution of topoisomers (lane 5). Incubation of the arrays with SWI-SNF and ATP for 30 min followed by apyrase and then topoisomerase resulted in a topoisomer distribution similar to that in lane 2 (lane 6). Quantification of the autoradiograph indicates that for lanes 2 and 6, the predominant band corresponds to a linking number of 8; for lanes 4 and 5, the predominant band corresponds to a linking number of 6. Note that apyrase treatment of the circular array results in an increase in the nicked plasmid form (B, lane 3; C, lanes 5 and 6). Apyrase-induced plasmid nicking does not affect *HincII* activity or the relative distribution of topoisomers.

in Figure II.1. In the absence of SWI-SNF, we found that 57% of the plasmid was linearized during the 30-min *Hinc*II digestion, which reflects the fraction of *Hinc*II sites not occluded by histone octamers (Fig II.2B, lane 1). Addition of SWI/SNF and ATP resulted in 90% of the plasmid being cleaved (Fig II.2B, lane 2).

However, pre-incubation of the circular nucleosomal array with SWI/SNF for 30 minutes, followed by removal of ATP with apyrase and subsequent exposure to *Hinc*II, resulted in only 49% cleavage (Fig II.2B, lane 3). Thus, as we observed for linear nucleosomal arrays, the SWI/SNF-dependent stimulation of *Hinc*II cleavage of circular nucleosomal arrays requires continuous ATP hydrolysis.

Previous studies with circular nucleosomal array substrates detected a persistent decrease in plasmid linking number due to a combined incubation of the arrays with SWI/SNF, ATP, and topoisomerase I (Imbalzano *et al.*, 1996; Schnitzler *et al.*, 1998; Guyon *et al.*, 1999). One possibility is that SWI/SNF-dependent changes in the topology of minichromosomes are persistent, whereas the enhanced accessibility of nucleosomal DNA within a single nucleosome requires continuous ATP hydrolysis. Alternatively, inclusion of topoisomerase I during the remodeling reaction may trap the remodeled state irreversibly (e.g. by inducing nucleosome loss). To investigate this latter possibility, we monitored the changes in minichromosome topology under conditions where we could temporally separate topoisomerase I and SWI/SNF activities. Figure II.2C shows that addition of calf thymus topoisomerase I to our reconstituted minichromosomes resulted in the appearance of ~15 discernible topoisomers after deproteination and agarose gel electrophoresis (Figure II.2C). Quantification of the intensity of the topoisomers

indicated that an average of 7 nucleosomes had been reconstituted onto this 3.2 kb plasmid (peak topoisomer has a linking number of 8; about 50% saturation). This degree of saturation correlates well with the percentage of nucleosomal *Hinc*II sites (Figure II.2B). Incubation of the minichromosome with topoisomerase I, SWI/SNF, and ATP resulted in a redistribution of the topoisomers corresponding to a loss of about two constrained supercoils per plasmid (Figure II.2C, lane 4; the peak topoisomer has a linking number of 6). When apyrase was added after the incubation with SWI/SNF and topoisomerase I, the pattern of topoisomers was not greatly altered, indicating that the effect of SWI/SNF action was persistent in this assay as previously observed (Figure II.2C, lane 5; Imbalzano *et al.*, 1996; Schnitzler *et al.*, 1998; Guyon *et al.*, 1999). However, if the minichromosomes were incubated for 30 minutes with SWI/SNF and ATP, and topoisomerase I was added *after* treating the SWI/SNF reaction with apyrase, then the topoisomer distribution becomes similar to the control reactions that lacked SWI/SNF (peak topoisomer has a linking number of 8; Figure II.2C, lane 6). In fact there is an increase in the proportion of topoisomers that appear to contain 11-15 constrained supercoils, which may reflect Topo I-induced DNA knotting of stable SWI/SNF- nicked array DNA complexes (Fig II.2C, lane 6; Dean *et al.*, 1985). Importantly, apyrase does not inhibit topoisomerase I activity (data not shown). We conclude that only the topoisomerase I-relaxed and remodeled state is stable after removal of ATP. In contrast, in the absence of topoisomerase I, SWI/SNF-induced changes in minichromosome topology rapidly collapse (reverse) after removal of ATP. These results are consistent

with the kinetics of *Hinc*II digestion, and they indicate that the topology of a nucleosomal array does not influence the stability of the remodeled state.

Persistent remodeling of isolated di- and mono-nucleosomes.

In contrast to nucleosomal arrays, several studies have reported persistent alterations in mononucleosome structure due to SWI/SNF action (Imbalzano *et al.*, 1996; Schnitzler *et al.*, 1998; Lorch *et al.*, 1999; Guyon *et al.*, 1999). Moreover, novel, "stably remodeled species" that resemble di-nucleosomes were formed from mononucleosome substrates (Schnitzler *et al.*, 1998; Lorch *et al.*, 1999). In light of the above results we wished to study remodeling on mono- and di-nucleosomes using the coupled remodeling-restriction enzyme assay to test whether it could also detect a stable remodeled species. To obtain pure di- and mono-nucleosomes, we engineered nucleosomal array DNA templates where a *Pst*I site flanks the ultimate or the penultimate (*Sal*I/*Hinc*II tagged) nucleosome positioning sequence (Figure II.3A and data not shown). Nucleosomal arrays were reconstituted on these templates, digested with *Pst*I, and the products of the restriction reaction were fractionated on glycerol gradients to isolate homogeneous 216 bp mono- or 427 bp di-nucleosome particles.

Isolated mono- or di-nucleosomes were used as substrates in restriction enzyme digests in the presence or absence of SWI/SNF. In the absence of SWI/SNF complex, 24% of the mononucleosomes were rapidly cleaved by *Sal*I, indicating that this population contains accessible *Sal*I sites (Figure II.3A).

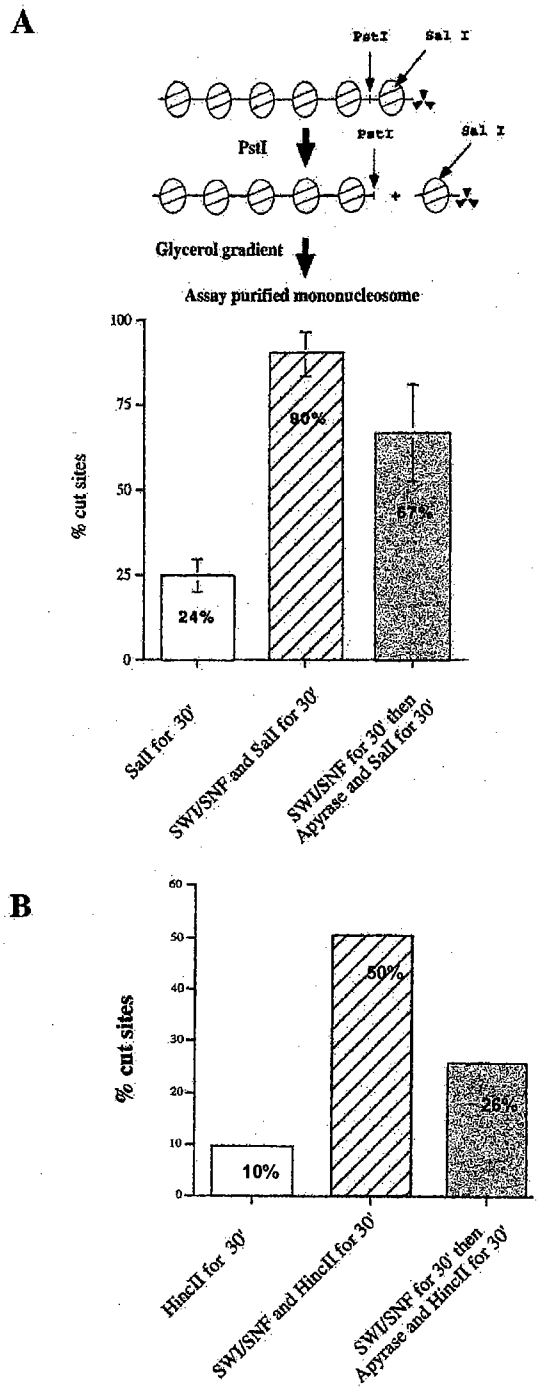


Figure II.3: SWI-SNF remodeling of isolated mononucleosomes leads to persistent DNA accessibility

(A) Purified mononucleosomes, obtained following *Pst*I digestion of a 6-mer nucleosomal array, were treated with SWI-SNF, *Sal*I, and/or apyrase as indicated.

(B) Mononucleosomes assembled on a 154-bp DNA fragment were used as substrates in the same assays as panel A. Similar results were obtained in two additional, independent experiments.

The remaining 76% of the mononucleosomes were cleaved with the slow digestion kinetics diagnostic of nucleosomal *Sal*I sites (data not shown). In contrast, when SWI/SNF and ATP were included in the reaction, 90% of the mononucleosomes were cleaved by *Sal*I during a 30 minute incubation. No stimulation of mononucleosome digestion was observed if ATP was omitted from the reactions (data not shown). In contrast, pre-incubation of the mononucleosomes with SWI/SNF and ATP, followed by co-addition of apyrase and *Sal*I, resulted in 67% cleavage of the mononucleosomes (Figure II.3A).

Identical results were obtained when, after the preincubation step, the binding of SWI/SNF to the mononuclesome substrate was competed for by addition of a 20 fold excess of chicken erythrocyte oligonucleosomes (data not shown). Similar results were also obtained with the di-nucleosome substrates (data not shown). Thus, in contrast to our results with nucleosomal array substrates (Figure II.1 and II.2), the majority of the SWI/SNF-dependent enhancement of mono- or di-nucleosome accessibility, as assayed by *Sal*I digestion, does not require continuous ATP hydrolysis.

Previous studies have used mononucleosome substrates assembled onto short DNA fragments (e.g. 154 bp; Côté *et al.*, 1994; Imbalzano *et al.*, 1996), and thus we wished to confirm that our restriction enzyme assay would also be able to detect persistent remodeling of these types of mononucleosome substrates. To address this issue, we reconstituted 154 bp mononucleosomes using the reconstitution method described by Imbalzano *et al.* (1994; see Materials and Methods; Figure II.3B). In this case only 10% of the *Hinc*II sites were cleaved after 30 minutes of digestion in the

absence of SWI/SNF. Incubation of these 154 bp mononucleosomes with SWI/SNF and ATP led to 50% cleavage after the 30 minute incubation. And finally, if the mononucleosomes were preincubated with SWI/SNF and ATP, and then apyrase and *Hinc*II were added, 26% of the sites were cleaved (Figure II.3B). Although the remodeled state of the 154 bp mononucleosomes was clearly less stable than the 216 bp mononucleosomes, much of the stimulation of *Hinc*II digestion due to SWI/SNF action was stable in the absence of continuous ATP hydrolysis.

SWI/SNF remodeling of mononucleosomes results in a protection of the ends of the DNA.

Our data indicates that SWI/SNF-dependent remodeling of mononucleosomes leads to a more stable, accessible reaction product than does SWI/SNF remodeling of nucleosomal arrays. One possibility we considered is that SWI/SNF action might lead to translational movement of histone octamers. A prediction of this hypothesis is that the stable, increased accessibility of the *Hinc*II site should be accompanied by an increased protection of DNA elsewhere on the template. To test this idea, we digested a 216 bp mononucleosome with two additional restriction enzymes, *Bam*HI and *Nco*I, whose sites are located 9 and 23 bp, respectively, from the ends of the DNA template (Figure II.4A).

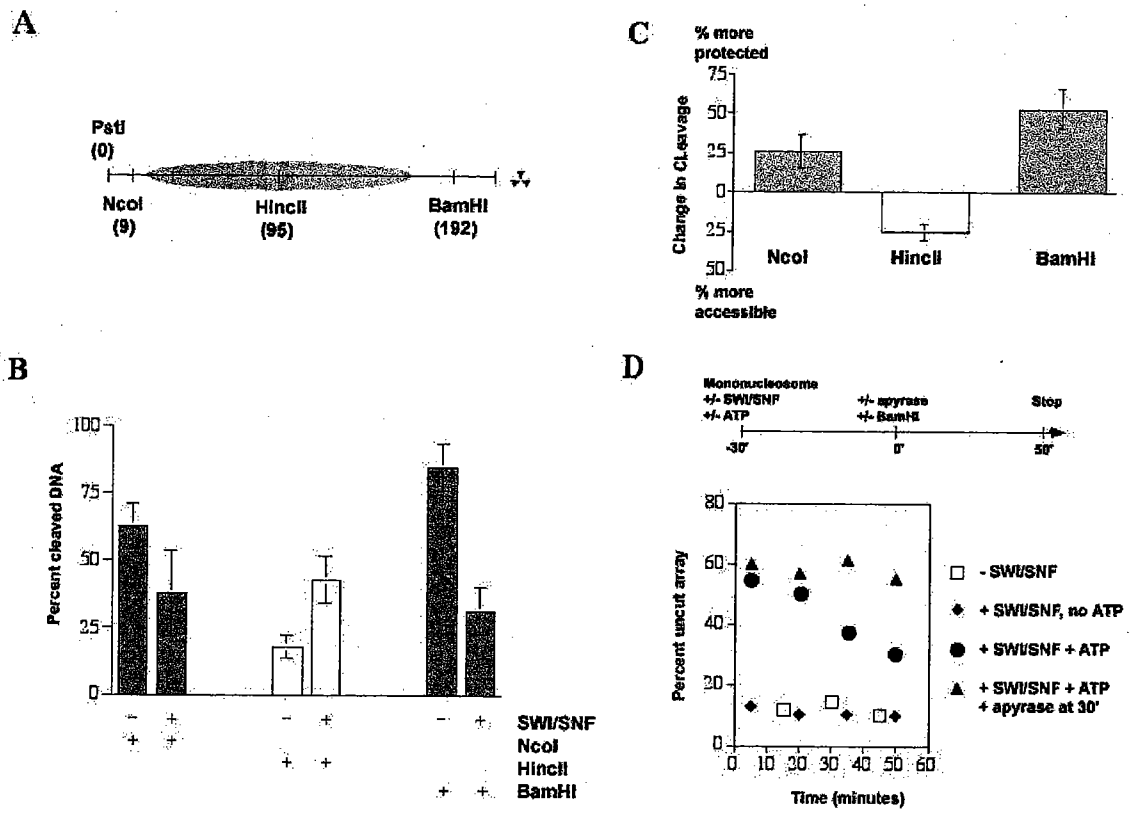


FIGURE II.4: SWI/SNF action leads to increased protection of DNA ends

(A) Schematic representation of the 216 bp mononucleosome substrate.

(B) The ends of the DNA fragment are more protected after a 30 minute incubation with SWI/SNF. The graph represents the percentage of cleaved nucleosomal DNA after restriction enzyme digestion without SWI/SNF (-), or after a 30 minute incubation with SWI/SNF, followed by removal of ATP with apyrase (+). Error bars represent the standard deviation from at least 3 experiments.

(C) The graph represents the difference in percent nucleosomal DNA cleaved by the restriction enzyme in the absence (-) and presence (+) of SWI/SNF (see panel B).

(D) Time course of *Bam*HI DNA cleavage. Mononucleosomes were pre-incubated for 30 minutes in the absence of SWI/SNF (□), in the presence of SWI/SNF without ATP (□), or in the presence of SWI/SNF and ATP(●,▲). Reactions containing SWI/SNF and ATP were then incubated with (▲) or without (●) apyrase, and BamHI was added to all reactions and the amount of cleavage was determined throughout a 50 minute timecourse.

In the absence of SWI/SNF, we found that 65% to 90% of the *Nco*I and *Bam*HI sites were freely accessible, consistent with the known preferred position of the 5S nucleosome (Figure II.4B). Upon incubation of the mononucleosomes with SWI/SNF for 30 minutes, followed by addition of apyrase, we observed an increased protection of the *Nco*I and *Bam*HI sites (25 and 50% increase in proportion of occluded sites, respectively, Figure II.4C). This contrasts with the *Hinc*II sites, of which 25% were rendered accessible by SWI/SNF action and subsequent removal of ATP (Figure II.3, Figure II. 4B). Similar results were observed if the binding of SWI/SNF to the mononucleosome substrate was competed for by addition of a 20-fold excess of chicken erythrocyte oligonucleosomes (data not shown). These experiments strongly imply that SWI/SNF remodeling can result in altered translational positioning of histone octamers on DNA. Furthermore, the dramatic increase in protected restriction sites near the ends of the DNA fragment indicate that DNA ends may act as sinks where SWI/SNF remodeled nucleosomes preferentially accumulate.

To further investigate the SWI/SNF-dependent protection of mononucleosomal DNA, we determined the kinetics of *Bam*HI cleavage in the presence or absence of SWI/SNF and in the presence or absence of continued ATP hydrolysis. In the absence of SWI/SNF, 72% of the *Bam*HI sites were rapidly cleaved, and only 18% of the *Bam*HI sites were cleaved at a rate diagnostic of nucleosomal DNA (Figure II.4D). Thus, the majority of histone octamers do not appear to be positioned over the *Bam*HI site. After a 30 minute incubation with SWI/SNF and ATP, 60% of the mononucleosomes were more resistant to subsequent *Bam*HI cleavage, reflecting the possible movement of the histone

octamer (time zero; Figure II.4D). If apyrase was added after the 30 minute preincubation to remove ATP, mononucleosomes were digested by *Bam*HI at the slow rate that is diagnostic of nucleosomal DNA (Figure II.4D, triangles). In contrast, if ATP hydrolysis was allowed to continue, SWI/SNF was able to remodel the newly occluded *Bam*HI sites as assayed by the increased kinetics of *Bam*HI digestion (Figure II.4D, closed circles). These results support our view that SWI/SNF action can lead to movement of histone octamers to the DNA ends, and furthermore that these "remodeled nucleosomes" represent cannonical nucleosomes that can inhibit the accessibility of DNA as well as serve as new substrates for SWI/SNF remodeling.

SWI/SNF action alters the translational positioning of mononucleosomes

In order to verify that the persistent aspect of SWI/SNF-mediated mononucleosome remodeling is due to a repositioning of histone octamers, we employed a nucleosome mobility assay where populations of mononucleosomes with heterogeneous translational positions are resolved by virtue of their different electrophoretic properties on native polyacrylamide gels. First, we used the strategy outlined in Figure II.3B to isolate mononucleosomes reconstituted onto a 427 bp DNA fragment that contains two 5S nucleosome positioning sequences. This substrate is nearly identical to the 416 bp mononucleosome described by Meersseman *et al.* (1992), and, as this group previously observed, these purified mononucleosomes resolve into eight electrophoretically distinct species on 4% native acrylamide gels (Figure II.5, lane 1). Two of the species make up

~60% of the total population (Figure II.5, lane 1) and the fastest migrating species represent octamers located close to the DNA ends (Meersseman *et al.*, 1992). In the absence of ATP, exposure to SWI/SNF had no effect on the distribution of species (Figure II.5, lane 2). In contrast, addition of SWI/SNF and ATP resulted in a quantitative switch of > 90% of the total population into the faster migrating species (Figure II.5, lane 3). Incubation of these mononucleosomes with SWI/SNF and ATP did not increase the amount of "free" DNA (Figure II.5, compare lanes 2 and 3), and these "remodeled" mononucleosomes still co-sedimented in glycerol gradients with mononucleosomes that had not been remodeled (data not shown). Furthermore, prolonged incubation of the mononucleosomes (up to 2 hours) resulted in the same pattern (data not shown), suggesting that the reaction rapidly reaches an equilibrium where the distribution of nucleosome translational positions is strongly biased towards histone octamers occupying end positions.

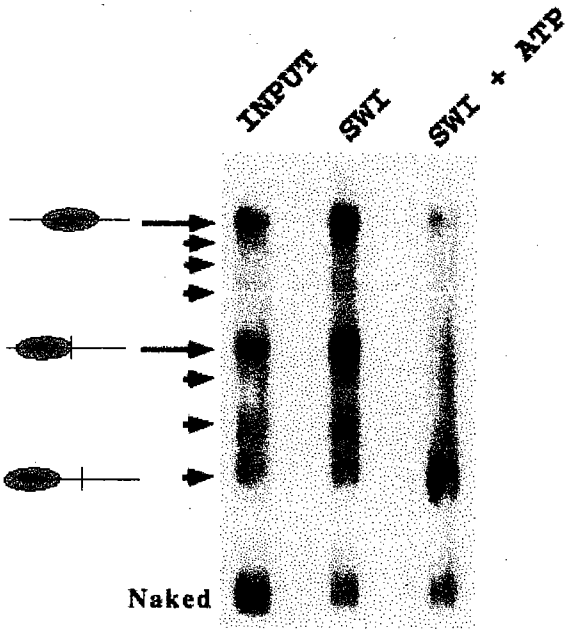


Figure II.5: SWI/SNF action alters the translational position of mononucleosomes

Dimers of the nucleosome positioning sequence (416 bp) bearing single histone cores resolved in 8 distinct electrophoretic species on 4% native acrylamide gels (lane 1). Each one represents a different translational position of the octamer on the DNA fragment (Meersseman *et. al.*, 1992). Upon exposure to SWI/SNF and ATP (lane 3) but not SWI/SNF alone (lane 2), the distribution pattern of these species is dramatically altered (see text for discussion).

SWI/SNF action mobilizes histone octamers within positioned nucleosomal arrays

The observed difference in the stability of the remodeled state for mononucleosome and nucleosomal array substrates might be due to an inhibition of nucleosome mobility within an array context. In this case, the remodeled state may represent a novel, accessible nucleosomal structure whose maintenance requires continuous ATP hydrolysis. On the other hand, the remodeling of nucleosomal arrays might also reflect the ATP-dependent movement of histone octamers. In this case, continuous ATP hydrolysis might also be needed to maintain nucleosome positions that create an accessible *Sal*I/*Hinc*II site. To test this latter possibility, we first monitored the accessibility of the unique *Bam*HI and *Nco*I restriction sites located adjacent to the *Sal*I/*Hinc*II-marked central nucleosome (Figure II.6A). In the absence of SWI/SNF, 65% of the *Nco*I sites and 90% of the *Bam*HI sites were accessible to restriction enzyme (Figure II.6B), which is consistent with the major translational frame of the 5S nucleosome and is similar to what we observed for the 216 bp 5S mononucleosome (Figure II.4). However, in contrast to our results with the mononucleosome substrates, addition of SWI/SNF and ATP led to a further increase in the accessibility of *Nco*I and *Bam*HI sites (Figure II.6B). If the nucleosomal arrays were pre-incubated with SWI/SNF and ATP, and then apyrase was added with the restriction enyzme, an increased occlusion of the *Nco*I and *Bam*HI sites resulted (25% change for *Nco*I and 15% change for *Bam*HI; Figure II.6B). These results suggest that the enhanced accessibility of the *Hinc*II, *Bam*HI, and *Nco*I sites requires continuous ATP hydrolysis, and furthermore, as we observed for

mononucleosome substrates, these results suggest that SWI/SNF may stably alter nucleosome positioning within the array.

To investigate the accessibility of DNA adjacent to every nucleosome within the array, we analyzed the accessibility of the *Eco*RI sites that are located between each of the eleven 5S DNA repeats (Figure II.6A). In the absence of SWI/SNF, *Eco*RI digestion of the nucleosomal array yields primarily the 208 bp 5S DNA limit product, as well as some di- and tri-nucleosome-size partial digestion products (Figure II.6C). This result is consistent with the majority of histone octamers occupying positions between *Eco*RI sites. When arrays are incubated with SWI/SNF and ATP, a small increase in *Eco*RI cleavage is observed which is best visualized by a decrease in the amount of di- and tri-nucleosome sized DNA fragments (Figure II.6C). However, when arrays are pre-incubated with SWI/SNF and ATP for 30 minutes, followed by addition of apyrase and *Eco*RI, accessibility of the *Eco*RI sites is dramatically reduced as visualized by a decrease in the amount of 208 bp DNA product and a large increase in the amount of partial digestion products (Figure II.6C). This SWI/SNF- and ATP-dependent decrease in DNA accessibility throughout the array is similar to the changes in *Nco*I and *Bam*HI accessibility at the central nucleosome, and is also consistent with the hypothesis that SWI/SNF might alter nucleosome positioning within the array.

To further investigate persistent changes in nucleosome positioning within the array due to SWI/SNF action, we performed partial micrococcal nuclease (Mnase) digestions. Mnase cleavages occur only within the linker regions between nucleosomes, and thus in the absence of SWI/SNF, Mnase digestion reveals a repeating pattern of

cleavages and protections indicative of a positioned array of eleven nucleosomes (Figure II.7A). Addition of SWI/SNF and ATP disrupts the positioned nucleosomal array, yielding digestion products that are nearly identical to those of the unassembled 5S DNA template (Figure II.7A; note that Mnase cleavage of the 5S array DNA yields a repeating pattern of cleavages and protections that is the inverse of the nucleosomal pattern). Furthermore, this disruption of the positioned 5S array is persistent and does not require continuous ATP hydrolysis (Figure II.7A, +APY lanes). This effect of SWI/SNF remodeling represents changes in translational positioning of nucleosomes rather

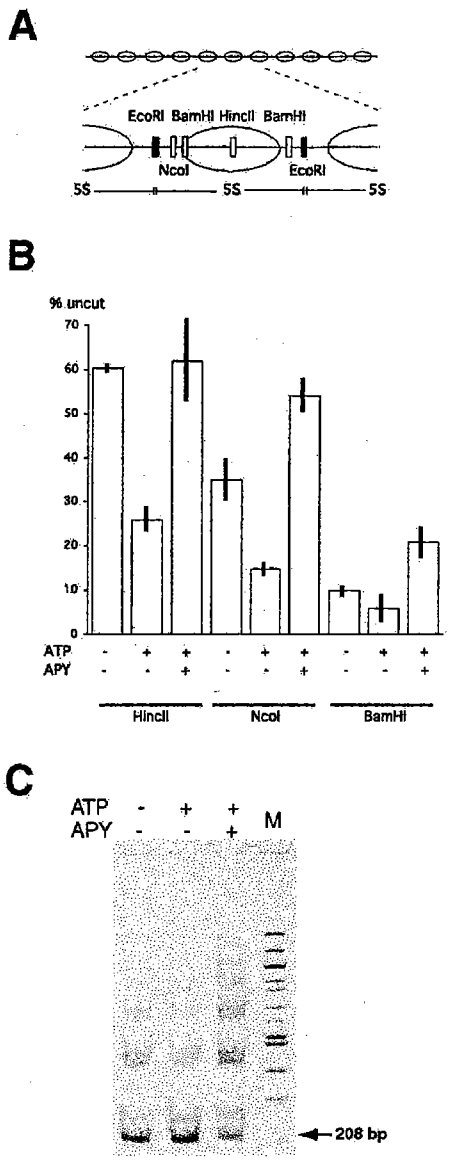


Figure II.6: SWI/SNF-dependent remodeling changes nucleosome positions within reconstituted arrays.
(A) Schematic representation of the template showing positions of restriction sites unique to the central nucleosome (open bars). *Eco*RI sites (filled bars) are in the linker region between every 5S repeat.
(B) Change in the accessibility of restriction endonuclease sites at the nucleosome dyad (*Hinc*II) and linker region (*Nco*I and *Bam*HI) following SWI/SNF reaction. The bars represent percentage of uncut arrays after restriction endonuclease cleavage in the presence of ATP and/or SWI/SNF. Where indicated, apyrase was added previous to the restriction enzyme. Error bars represent the standard deviation from at least three experiments.
(C) SWI/SNF reaction results in a decrease in accessibility of linker regions throughout nucleosomal arrays. The *Eco*RI digestion of reconstituted nucleosomal arrays was carried out as in B. The limit digestion product (208 bp) is marked with an arrow. The 232 bp product represents the central nucleosome. Larger products represent partial digestion products. M - 100 bp DNA ladder (NEB).

than nucleosome loss, as the remodeled arrays still co-sediment with control arrays on glycerol gradients (Figure II.7B), and they still contain nucleosomes that protect ~150 bp of DNA after extensive digestion with Mnase (Figure II.7C). Thus, these results indicate that SWI/SNF remodeling of 5S arrays is associated with a dramatic randomization of nucleosome translational positions. In the presence of SWI/SNF and ATP this randomized state is dynamic as it is associated with an enhanced restriction

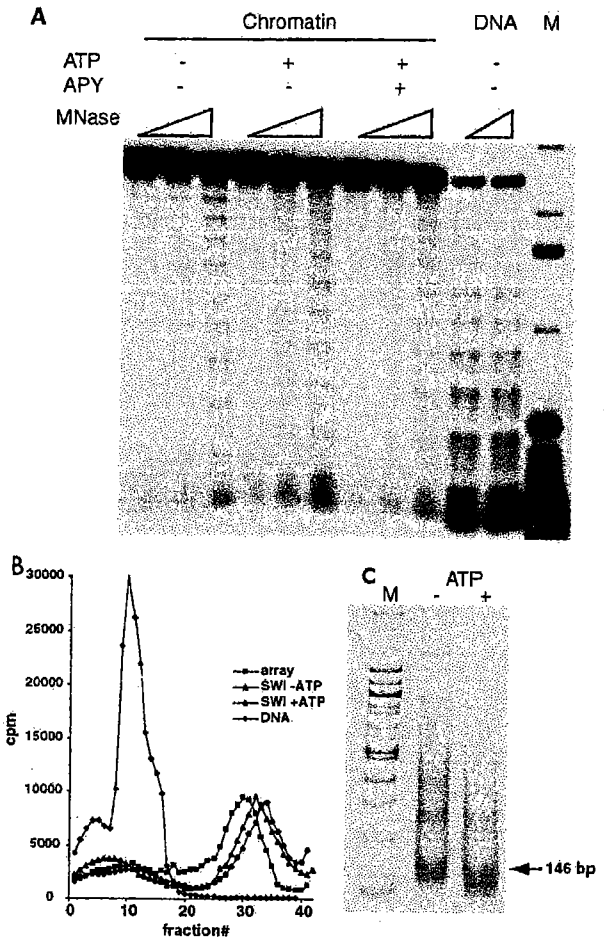


Figure II.7: Persistent randomization of nucleosome positions within reconstituted arrays as a result of SWI/SNF-dependent remodeling

(A) Micrococcal nuclease digestion of reconstituted 11-mer arrays. Reconstituted nucleosomal arrays were digested with increasing amounts of micrococcal nuclease in the presence of SWI/SNF +/- ATP before or after addition of apyrase. Note that 5S array DNA also exhibits a repeating pattern of Mnase cleavages that is the inverse pattern of nucleosomal arrays. M – 1Kb DNA ladder (Gibco BRL)

(B) SWI/SNF-dependent remodeling does not change the sedimentation properties of nucleosomal arrays. Sedimentation profiles of naked DNA and reconstituted nucleosomal arrays either prior to or after SWI/SNF-dependent remodeling.

(C) Nucleosomes within remodeled arrays protect 146 bp of DNA. Nucleosome arrays were incubated in the presence of SWI/SNF with or without ATP, the reaction was stopped with apyrase and remodeled arrays were digested with a high concentration of micrococcal nuclease. 146 bp nucleosomal DNA is marked with an arrow. M – 100 bp DNA ladder.

enzyme accessibility throughout the array. In contrast, the subsequent inactivation of SWI/SNF remodeling activity leads to a randomized array where many restriction sites are persistently occluded.

DISCUSSION

In the present study we have carried out experiments aimed at reconciling two seemingly contradictory experimental observations concerning the persistence of the remodeled nucleosome state induced by yeast SWI/SNF and related ATP-dependent remodeling complexes (Imbalzano *et al.,* 1996; Côté *et al.*, 1998; Schnitzler *et al.,* 1998; Guyon *et al.*, 1999; Logie and Peterson, 1999; Lorch *et al.*, 1999). First, when the remodeling activity of SWI/SNF and RSC was analyzed on mononucleosome substrates, the remodeled, accessible state (detected by restriction enzyme cleavage, transcription factor binding, or DNase I digestion patterns) appeared to persist for over 30 minutes upon removal of ATP or SWI/SNF from the reaction (Imbalzano *et al.,* 1996; Côté *et al.*, 1998; Schnitzler *et al.,* 1998; Guyon *et al.*, 1999; Lorch *et al.*, 1999). However, when SWI/SNF, RSC, NURF, CHRAC, or Mi-2 activity was analyzed on nucleosomal array substrates (detected by restriction enzyme digestion), remodeling was found to be reversible unless multiple dimers of a transcription factor were bound to the previously occluded DNA (Logie and Peterson, 1997 and 1999; Papoulas *et al.*, 1998). The experiments presented here suggest that the "stable", accessible state that is detected on mononucleosomes is due to a SWI/SNF-induced translational movement of the histone octamer which results in the accumulation of histone octamers closer to DNA ends. Furthermore, the "reversibility" of nucleosomal array remodeling is also a misnomer since it too reflects stable changes in nucleosome positions that in this case re-establishes occlusion of DNA sites after SWI/SNF inactivation. Our data are consistent with a recent

study on yeast SWI/SNF by Whitehouse *et al.* (1999), and with two studies that showed that chromatin remodeling complexes that contain the ISWI ATPase can induce nucleosome mobility (Hamiche *et al.,* 1999; Längst *et al.,* 1999).

Nucleosome mobilization: mononucleosomes vs arrays

We find that ATP-dependent remodeling of mononucleosomes and nucleosomal arrays leads to changes in nucleosome positions. In the case of a 216 bp mononucleosome substrate, octamer movements appear to be in excess of 70 bp given the relative positions of the *Hinc*II, *Bam*HI, and *Nco*I sites (Figure II.4C). In this case, SWI/SNF action leads to a nucleosome-free *Hinc*II site (which is initially located at the nucleosomal dyad of a centrally-positioned nucleosome), and newly generated nucleosomal occlusion of the DNA ends. This switch in restriction enzyme accessibility is established and maintained (for at least 30 minutes) even in the presence of continued ATP hydrolysis, and it is also stable after removal of ATP. In contrast, whereas SWI/SNF action on nucleosomal arrays also leads to dramatic changes in nucleosomal positions, the continued presence of SWI/SNF and ATP leads to increased accessibility of all restriction enzyme sites tested. Thus, on nucleosomal arrays, SWI/SNF remodeling appears to establish a dynamic state of continual nucleosome mobilization that generates accessible DNA sites throughout the array. When ATP or SWI/SNF is removed from the reaction, these random nucleosome positions are "fixed" and DNA sites are in general occluded within the population. Thus, remodeling of mononucleosomes appears to score

movement of histone octamers to the DNA ends, whereas remodeling of nucleosomal arrays scores a dynamic state of octamer mobilization.

Given that persistent disruption of mononucleosomal DNA seems to correlate with the translational movement of the histone octamer, it seems surprising that SWI/SNF action can lead to a stable disruption of mononucleosomes that are reconstituted on very short DNA fragments (e.g. 154 bp). On these substrates the *Hinc*II site is located ~75 bp from the nucleosomal edge, and thus a movement of the octamer to the end of the DNA fragment will still leave the *Hinc*II site buried within the nucleosome. One possibility is that SWI/SNF remodeling of 154 bp mononucleosomes yields novel reaction products that are not generated on nucleosomal arrays or on mononucleosomes with longer stretches of linker DNA. Alternatively, we favor a model in which SWI/SNF induces movement of the histone octamer off the end of the DNA fragment, leading to a histone octamer with less than 147 bp of DNA wrapped onto its surface. This type of reaction may not be favored, and, consistent with this view, remodeling of the 154 bp mononucleosomes does lead to less of the stable remodeled product after SWI/SNF inactivation (Figure II.3B). Previous studies have shown that reconstitution of histone octamers onto a 145 bp DNA fragment can lead to the preferential assembly of only 128 bp of DNA (Ramsay *et al.,* 1984). Furthermore, visualization of SWI/SNF remodeling by electron microscopy indicates that nearly 20 bp of DNA is lost from a remodeled nucleosome (Bazett-Jones *et al.,* 1999). If SWI/SNF generates remodeled mononucleosomes that contain a significant number of unoccupied DNA binding sites, then these particles may show a propensity to self-associate via histone-DNA

interactions. This model may provide a simple explanation for the previously described novel reaction product that behaves biochemically as a di-nucleosome (Schnitzler *et al.*, 1998; Lorch *et al.*, 1999).

Is ATP-dependent remodeling equivalent to histone octamer mobilization?

One possibility is that SWI/SNF and related enzymes use the energy of ATP hydrolysis to directly move histone octamers, perhaps by "screwing" DNA over the octamer surface as suggested by Varga-Weisz and Becker (Varga-Weisz *et al.*, 1997). Alternatively, SWI/SNF-like enzymes might use the energy of ATP hydrolysis to generate a high energy intermediate where DNA-histone contacts have been disrupted, but where the translational frame of the histone octamer on DNA has not yet been altered. This "activated" state may also constrain fewer negative supercoils due to the weakened histone-DNA interactions (see below). A similar "activated" intermediate consisting of weakened histone-DNA contacts has been proposed for remodeling catalyzed by ISWI-containing complexes (Ito *et al.*, 1997; Alexiadis *et al.*, 1998) and human SWI/SNF (Narlikar *et al.*, 2001). This high energy state might then decay into stable changes in nucleosome positions. In this model, the preferred stable outcome for mononucleosomes appears to be nucleosomes at the DNA ends, but for nucleosomal arrays, where DNA ends do not flank each nucleosome, remodeling leads to a more random positioning of nucleosomes. Consistent with this "high energy intermediate model", Gavin *et al.* (2001) reported that yeast SWI/SNF enhances the accessibility of a restriction site on small,

circular nucleosomal arrays in which histone octamer movement is highly constrained, suggesting that the generation of "accessible nucleosomes" by SWI/SNF does not depend solely on the repositioning of histone octamers.

Furthermore, ATP-dependent remodeling by human SWI/SNF is still proficient on "immobilized" substrates which contain histone octamers crosslinked to nucleosomal DNA (Lee *et al.,* 1999; Aoyagi *et al.*, 2002). Thus, remodeling, as assayed by disruption of the DNase I digestion pattern of a rotationally positioned nucleosome, can occur even in the absence of dramatic octamer movement. Importantly, accumulation of the SWI/SNF-remodeled state is impaired on a crosslinked mononucleosomal particle, in agreement with our hypothesis that nucleosome movement is responsible for the persistent alterations observed on SWI/SNF-treated mononucleosomes (Aoyagi *et al.*, 2002).

Stability of the remodeled state as a function of chromatin topology

Kingston and colleagues have used a minichromosome topology assay to monitor the persistence of nucleosomal array remodeling by human SWI/SNF complex (Imbalzano *et al.,* 1996; Schnitzler *et al.,* 1998; Guyon *et al.,* 1999). In this remodeling assay, nucleosomes are reconstituted onto a closed circular DNA template in the presence of topoisomerase I, and these substrates are then incubated with SWI/SNF, ATP, and topoisomerase I. In these reactions, ATP dependent remodeling by human SWI/SNF results in the loss of a large number of constrained supercoils, and these topological changes persist for several hours after inactivation or removal of the remodeling enzyme.

Based on these results, it was proposed that the stability of remodeled minichromosomes (as detected by these changes in topology) reflects a novel remodeled nucleosome species. In contrast, we hypothesized that the persistent nature of the remodeled product detected in the topology assay might be due to the combined incubation of minichromosomes with SWI/SNF and a topoisomerase, rather than a property inherent to the SWI/SNF remodeling reaction. To test this possibility we designed a remodeling experiment where the activities of SWI/SNF and topoisomerase I were temporally uncoupled (Figure II.2). Whereas co-incubation of SWI/SNF and topoisomerase I led to a change in minichromosome topology that persisted in the absence of ATP or SWI/SNF, incubation of the minichromosome with SWI/SNF and ATP, followed by removal of ATP and subsequent addition of topoisomerase I, eliminated the stable change in topology. Thus, persistent changes in minichromosome topology requires the combined action of SWI/SNF and topoisomerase I. Although the nature of this persistent change is not clear, it is possible that topoisomerase I traps a transient change in DNA topology that is induced during the remodeling reaction. In the absence of topoisomerase I, this change in topology would collapse, due either to the artificially high level of supercoiling on the minichromosomes or to an inherent reversibility of the remodeling reaction. Alternatively, the combined action of SWI/SNF and a topoisomerase might lead to more efficient eviction of histone octamers from circular chromatin.

Stability of the remodeled state in vivo

Two studies have recently addressed the issue of continuous versus transient requirement of the yeast SWI/SNF complex *in vivo* (Biggar and Crabtree, 1999; Sudarsanam *et al.,* 1999). In both cases, inactivation of SWI/SNF led to the rapid loss of gene expression, indicating that SWI/SNF is continuously required to maintain activated levels of gene expression. Previously it had been proposed that this continuous requirement for SWI/SNF observed in vivo might reflect the activity of other remodeling complexes (such as RSC) that might use the energy of ATP hydrolysis to re-establish a repressive chromatin structure (Travers, 1999). The data presented here, however, imply that there is no *a priori* need to invoke a balance between positively and negatively acting ATP dependent remodeling complexes since termination of the nucleosome remodeling reaction leads to reappearance of *bona fide,* DNA-occluding nucleosomes.

Our data also provides a simple model to explain how SWI/SNF can be required for transcriptional repression of some genes in vivo (Holstege *et al.,* 1998). As we observe here, SWI/SNF might in some instances use the energy of ATP hydrolysis to alter the positions of nucleosomes surrounding cis-acting regulatory sites in vivo. In some cases, these movements might enhance DNA accessibility (either by continuous action of SWI/SNF or by placing DNA sites between nucleosomes), but in other instances SWI/SNF action might lead to the occlusion of DNA sites that are required for gene expression. In fact, SWI/SNF action might first act to promote accessibility of DNA to

key transcription factors, but inactivation or loss of SWI/SNF from the target gene would help re-establish a repressed state by "freezing" inhibitory nucleosome positions.

Preface to Chapter III

In Chapter III, we provide evidence indicating that yeast Rad54p is an ATP-dependent chromatin remodeling enzyme. Furthermore, our data suggests that both Rad54p and SWI/SNF exploit a DNA translocation mechanism to alter nucleosomal structure.

The work presented here is part of an ongoing collaboration with Dr. Patrick Sung, who supplied the purified yeast Rad51, Rad54 and RPA proteins. Jocelyn Krebs, Ph.D., made the initial observation that Rad54p enhances the accessibility of nucleosomal DNA. Stephen Van Komen, Ph.D., from Dr. Sung's laboratory, contributed the data represented in Figure III.1.

The data presented in this Chapter was published in the **Journal of Biological Chemistry**, Volume 278, No.11 (March 2003).

CHAPTER III

Rad54p IS A CHROMATIN REMODELING ENZYME REQUIRED FOR HETERODUPLEX DNA JOINT FORMATION WITH CHROMATIN

CHAPTER III

Rad54p is a Chromatin Remodeling Enzyme Required for Heteroduplex DNA Joint Formation with Chromatin

INTRODUCTION

Chromosomal DNA double-strand breaks (DSBs) arise through exposure of cells to harmful environmental agents such as ionizing radiation or mutagenic chemicals (radiomimetics, alkylating agents, etc). DSBs can also be caused by endogenously-produced oxygen radicals, by errors in DNA replication, or as obligatory intermediates during programmed cellular processes, such as meiosis or V(D)J recombination (Pâques and Haber, 1999; Hiom, 2001; Wood *et al.,* 2001). Cell survival and maintenance of genome integrity are dependent on efficient repair of DSBs, as unrepaired or misrepaired DSBs may lead to mutations, gene translocations, gross chromosomal rearrangements, or cellular lethality.

Several pathways for repairing DSBs have evolved and are highly conserved throughout eukaryotes. Homologous recombination (HR) is a major pathway of DSB repair in all eukaryotes and has a distinct advantage over other mechanisms in that it is mostly error-free. In organisms ranging from yeast to human, HR is mediated by members of the *RAD52* epistasis group (*RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*,

RAD57, *RAD59*, *MRE11*, and *XRS2*). Accordingly, mutations in any one of these genes result in sensitivity to ionizing radiation and other DSB-inducing agents (Pâques and Haber, 1999). The importance of the HR pathway in maintaining genome integrity is underscored by the fact that mutations in each one of its critical factors have been correlated with chromosomal instability-related ailments, including ataxia telangiectasia-like disease, Nijmegen breakage syndrome, Li Fraumeni syndrome, as well as various forms of cancer (Khanna and Jackson, 2001).

In vivo and *in vitro* studies have suggested the following sequence of molecular events that lead to the recombinational repair of a DSB. First, the 5' ends of DNA that flank the break are resected by an exonuclease to create ssDNA tails (Melo and Toczyski, 2002). Next, Rad51p polymerizes onto these DNA tails to form a nucleoprotein filament that has the capability of searching for a homologous duplex DNA molecule. After DNA homology has been located, the Rad51-ssDNA nucleoprotein filament catalyzes the formation of a heteroduplex DNA joint with the homologue. The process of DNA homology search and DNA joint molecule formation is called "homologous DNA pairing and strand exchange". Subsequent steps entail DNA synthesis to replace the missing information, followed by resolution of DNA intermediates to yield two intact duplex DNA molecules (Kanaar *et al,*. 1998).

The homologous DNA pairing activity of Rad51p is enhanced by Rad54p (Petukhova *et al.,* 1998). Rad54p is a member of the SWI2/SNF2 protein family (Eisen *et al.,* 1995) that has DNA stimulated ATPase activity and physically interacts with Rad51p (Jiang *et al.,* 1996; Clever *et al.,* 1997). Because of its relatedness to the SWI2/SNF2

family of ATPases, Rad54p may have chromatin remodeling activities in addition to its established role in facilitating Rad51p-mediated homologous pairing reactions. In this study we show that Rad51p and Rad54p mediate robust D-loop formation with a chromatin donor, whereas the bacterial recombinase, RecA, can only function with naked DNA. Furthermore, we find that the ATPase activity of Rad54p is essential for D-loop formation on chromatin and that Rad54p can use the free energy from ATP hydrolysis to enhance the accessibility of nucleosomal DNA. Experiments are also presented to suggest that chromatin remodeling by Rad54p and yeast SWI/SNF involves DNA translocation.

MATERIALS AND METHODS

DNA constructions

All DNA manipulations were carried out using standard methods (Sambrook *et al.,* 1989). Oligonucleotides were obtained from Operon Technologies (Alameda, California). Plasmid pXG540 and T4 EndonucleaseVII used in the cruciform extrusion experiments were a kind gift of Dr. T. Owen-Hughes.

The oligonucleotide used for triplex formation was TFO (triplex forming oligonucleotide) (5'-TTCTTTTCTTTCTTCTTTCTTT-3'). To generate pMJ5, the annealed oligonucleotides TFOB5 (5'-TCGAGAAGAAAAGAAAGAAGAAAGAAAC-3') and TFOB3 (5'-TCGAGT TTCTTTCTTCTTTCTTTTCTTC-3') where ligated to the product of a XhoI digestion carried out on pCL7c (Logie and Peterson, 1997). This yielded a pBluescript SKII (-) plasmid containing 5 head to tail repeats of the 208 bp *L. variegatus* 5S rDNA nucleosome positioning element flanked by a TFO binding site.

The DNA template (208-11) used to reconstitute nucleosomal arrays for the ATPase, remodeling, and Mnase assays consists a NotI--HindIII fragment derived from pCL7b (Logie and Peterson, 1997), containing 11 head-to-tail repeats of a 5S rRNA gene from *L. variegatus*, each one possessing a nucleosome positioning sequence. The sixth nucleosome occludes a unique SalI restriction site.

For the DNA length dependence assays, single-stranded oligonucleotides (random N-mers ranging from 10 to 100 nucleotides in length) were PAGE-purified to ensure length homogeneity (Integrated DNA Technologies, Inc., Coralville, IA).

Reagent Preparation

Recombinant yeast Rad51p, Rad54p, rad54 K341A, and rad54 K341R were overexpressed in yeast and purified as previously described (Petukhova *et al.,* 1998). SWI/SNF purification was as described (Logie and Peterson, 1999). Histone octamers were purified from chicken erythrocytes as described by Hansen *et al.* (1989). Octamer concentrations were determined by measurements of A_{230} (Stein, 1979). Nucleosomal array DNA templates (pXG540, pMJ5, or 208-11) were labeled by the Klenow polymerase fill-in reaction using [α-^{32}P] dCTP (3000μCi/mmol, Amersham (Piscataway, New Jersey)). Nucleosomal arrays were reconstituted by salt dialysis as previously described (Logie and Peterson, 1999), and the nucleosome saturation was determined to be 60-80% by digestion analysis.

D-loop reactions

Oligonucleotide D1 (90-mer) used in the D-loop experiments has the sequence: 5'- AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGC TTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT-3', being complementary to positions 1932 to 2022 of pBluescript SK(-) replicative form I DNA. Oligonucleotide D1 was 5' end-labeled with [γ-^{32}P] dATP and polynucleotide kinase, as described (Petukhova *et al.,* 1998). Buffer R (35mM Tris-HCl, pH: 7.4, 2.0mM ATP, 2.5mM $MgCl_2$, 30mM KCl, 1mM DTT, and an ATP regenerating system consisting of 20mM creatine phosphate and 30μg/ml creatine kinase) was used for the reactions; all the incubation steps were carried out at 30°C. Rad51 (0.8μM) and Rad54 (120nM) were

incubated with radiolabeled Oligonucleotide D1 (2.4μM nucleotides) for 5 minutes to assemble the presynaptic filament, which was then mixed with naked pBluescript replicative form I DNA (38μM base pairs) or the same DNA assembled into chromatin (38μM base pairs). Chromatin assembly was monitored by following topological changes as well as measuring the degree of occlusion of a unique *Eco*RI restriction site close to the D1 sequence. Substrates were estimated to be ~80% saturated with nucleosomes. The reactions containing RecA protein (0.8μM) were assembled in the same manner, except that they were supplemented with an additional 12.5mM $MgCl_2$ at the time of incorporation of the duplex substrates. At the indicated times, 4μl portions of the reactions were withdrawn and mixed with an equal volume of 1% SDS containing 1mg/ml proteinase K. After incubation at 37°C for 5 min, the deproteinized samples were run in 1% agarose gels in TAE buffer (40mM Tris-HCl, pH: 7.4, 0.5mM EDTA) at 4°C. The gels were dried and the radiolabeled DNA species were visualized and quantified by PhosphorImager analysis (Personal Molecular Imager FX, Bio-Rad).

ATPase Assay

Recombinant yeast Rad54p (5nM), Rad51p (5nM) or purified yeast SWI/SNF (10nM) was incubated at 30°C with 50μM single-stranded oligonucleotides (random N-mers ranging from 10 to 100 nucleotides in length), in the presence of 100μM ATP, 2.5μCi [γ-^{32}P] dATP (6000μCi/mmol, Amersham), 2.5% glycerol, 0.1% Tween 20, 20mM Tris-HCl, pH: 8.0, 200μM DTT, 5mM $MgCl_2$, 100μg/ml BSA. Samples were taken after 2, 5, 15 and 30 minutes and resolved by TLC. The proportion of liberated ^{32}P-

pyrophosphate was determined using the Molecular Dynamics PhosphorImager and ImageQuant Software. ATPase assays were independently repeated 3 times which yielded very similar results. The average values from the 3 experiments were plotted in Figure III.4.

Cruciform Formation Assay

Cruciform formation assays were performed as previously described (Havas, *et al.*, 2000). Briefly, 8ng of AvaI-linearized pXG540 (either naked, N, or nucleosomal, C) were incubated with various concentrations of Rad54p, Rad51p or rad54 K341A and 0.15mg/ml EndoVII (except where noted), in the presence of 10mM Hepes, pH: 7.9, 50mM NaCl, 3mM $MgCl_2$, 5% glycerol, 0.1mM DTT, 1mM ATP (except where noted), 3mM phosphoenolpyruvate, and 20U/ml pyruvate kinase for 30 min at 30°C. The products were resolved in 1.2% agarose gels and visualized Sybr Gold staining (Molecular Probes, Eugene, OR), followed by analysis with ImaQuant software.

Chromatin Remodeling Reaction

For the coupled SWI/SNF- or Rad54p-*Sal*I reactions, reconstituted 208-11 nucleosomal arrays (~1 nM final concentration) were pre-incubated at 37°C for 20 min with 2.5U/µl *Sal*I in a buffer containing final concentrations of 50mM NaCl, 5mM $MgCl_2$, 1mM ATP, 3mM phosphoenolpyruvate, 10U/ml pyruvate kinase, 1mM DTT, 10mM Tris-HCl, pH: 8.0, 100µg/ml BSA and 3% glycerol. Nucleosomal arrays were approximately 80% saturated with nucleosomes. Buffer, 2nM SWI/SNF complex, or

various concentrations of recombinant Rad51p and Rad54p were added and samples were taken at the indicated time points, vigorously mixed for 10s with 25µl TE and 50µl 1:1 solution of phenol/chloroform. The purified DNA fragments were resolved by electrophoresis in 1.2% agarose gels in the presence of 50µg/ml ethidium bromide. The gels were then dried on 3MM Whatman paper. The fraction of cut and uncut DNA was determined by Phosphorimager analysis using a Molecular Dynamics PhosphorImager and ImageQuant software. Experiments were repeated independently at least 3 times, which yielded very similar results.

Micrococcal Nuclease Digestion

15nM reconstituted 208-11 nucleosomal arrays were incubated at 37°C with 2nM SWI/SNF, 100nM Rad54p, or buffer, in the presence of 2mM ATP, 5mM NaCl, 2.5mM Tris-HCl, pH: 8.0, 0.25mM $MgCl_2$, 0.3mM $CaCl_2$, 3mM phosphoenolpyruvate, 10U/ml pyruvate kinase, 1mM DTT, 10µg/ml BSA, 0.5% glycerol. After 20 min, 0.0005U of micrococcal nuclease (Worthington Biochemical Corporation, Lakewood, NJ) was added to the reaction and aliquots were taken at the indicated time points, then treated for 20 minutes with 2µg/µl proteinase K and extracted twice with a 1:1 solution of phenol:chloroform. The resulting digestion products were resolved by electrophoresis in 2% agarose gels, run at 2.5V/cm for 12 hours. The gels were fixed, dried, and analyzed using a Molecular Dynamics PhosphorImager and ImageQuant Software.

Triple-Helix Displacement Assay

Triple-helix formation was performed as described (Firman and Szczelkun, 2000). Briefly, equimolar concentrations (100nM) of *Ssp*I-linearized pMJ5 and ^{32}P-labeled TFO were mixed in buffer MM (25mM MES, pH: 5.5, 10mM $MgCl_2$) at 57°C for 15 minutes and left to cool to room temperature overnight. The resulting triplex was either used directly or reconstituted into nucleosomal arrays. To introduce nicks into the DNA, pMJ5 was exposed to various concentrations of DNAseI (Promega, Madison, WI) for 2 minutes at 37°C, the reactions were stopped with 5mM EDTA, vigorously mixed for 10 seconds with a 1:1 solution of phenol/chloroform, ethanol-precipitated, and resuspended in water. The degree of nicking introduced by DNAseI treatment was assessed by electrophoretic analysis of native and heat-denatured samples (in the presence of 15% formamide) on denaturing 1.3% agarose gels, followed by Sybr Gold Stain (Molecular Probes, Eugene, OR).

The triplex-containing substrates (5nM) were incubated at 30°C with 5nM recombinant Rad54 protein or SWI/SNF complex, in a buffer containing 35mM Tris-HCl, pH: 7.2, 3mM $MgCl_2$, 100µg/ml BSA, 50mM KCl, 1mM DTT, 3mM phosphocreatine, 28µg/ml creatine phosphokinase, and where noted, 3mM ATP. Samples were taken at the indicated time points, the reactions were quenched with GSMB buffer (15% (w/v) glucose, 3% (w/v) SDS, 250mM MOPS, pH: 5.5, 0.4mg/ml bromophenol blue), and analyzed in 1.2% agarose gels (40mM Tris-acetate, 5mM sodium acetate, 1mM $MgCl_2$, pH: 5.5) at 10V/cm for 1.5 hours at 4°C. Gels were fixed in 5% acetic acid, 50%

methanol for 1 hour and dried. The proportion of bound and free TFO was determined using a Molecular Dynamics PhosphorImager and ImageQuant Software.

RESULTS

Rad51p and Rad54p promote DNA pairing with a chromatin donor

Repair of a DSB by homologous recombination begins with the invasion of a double stranded, homologous donor by a Rad51-ssDNA nucleoprotein filament, also referred to as the pre-synaptic filament. This strand invasion reaction is typically monitored *in vitro* by following the Rad51p-dependent formation of a D-loop between a radiolabeled oligonucleotide and a homologous double-stranded DNA donor (Figure III.1A). In this case, efficient D-loop formation also requires the ATPase activity of Rad54p. *In vivo*, however, the search for homology and strand invasion involves a homologous donor that is assembled into chromatin. Given that the Rad54p ATPase shows sequence relatedness to known chromatin remodeling enzymes, it was of considerable interest to examine the ability of Rad54p to promote Rad51p-dependent D-loop formation with a nucleosomal donor.

Figure III.1 shows the results of D-loop assays that use either a circular, naked DNA donor or this same circular DNA assembled into nucleosomes. Consistent with previous studies, the combination of yeast Rad51p and Rad54p led to rapid and highly efficient D-loop formation on the naked DNA donor (Figure III.1B). A similar level of D-loop formation was also obtained when the bacterial recombinase RecA was used in these assays with naked DNA (Figure III.1C). Surprisingly, assembly of the circular donor into

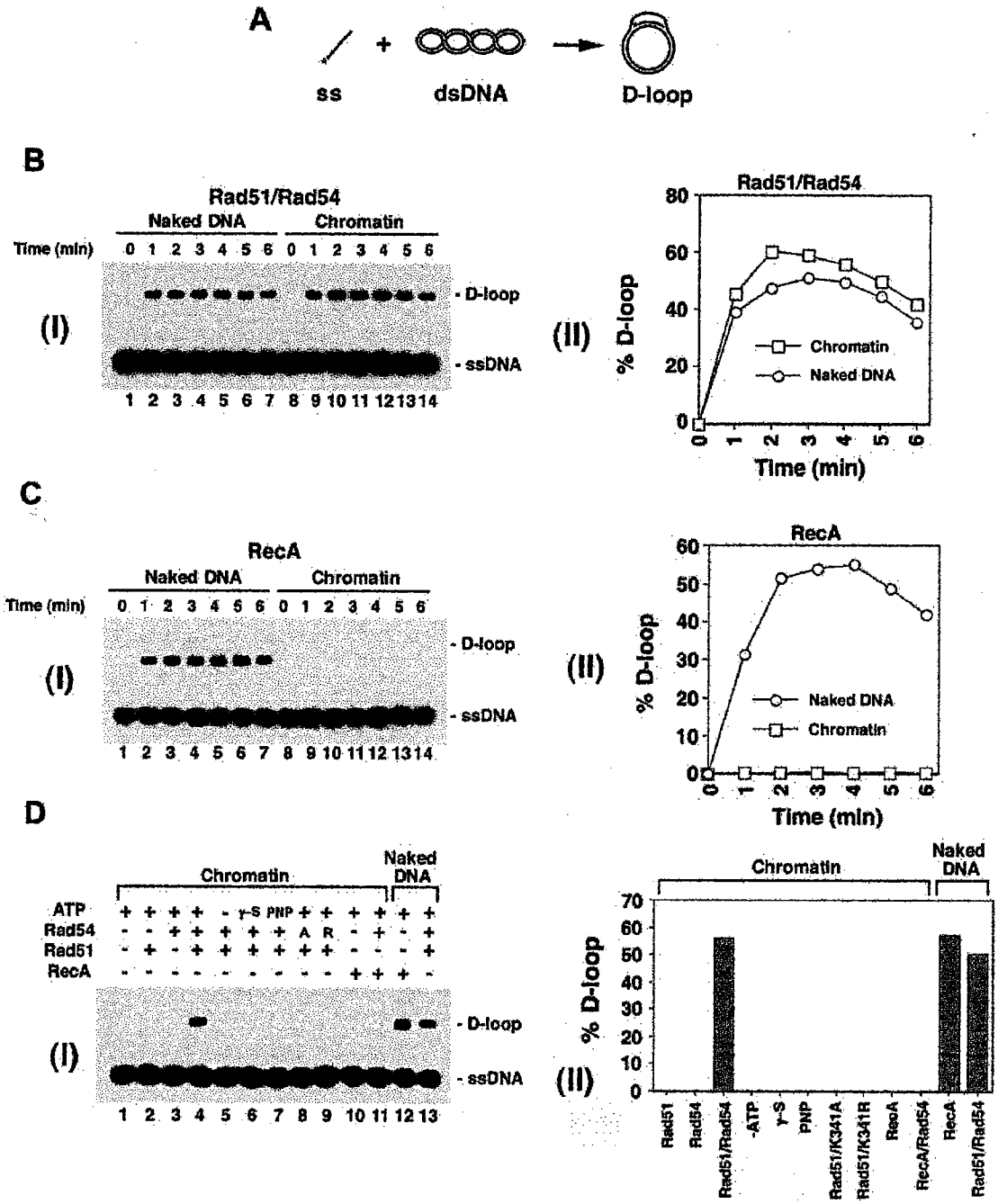


Figure III.1: Rad51p and Rad54p promote efficient DNA strand invasion with chromatin.

Figure III.1: Rad51p and Rad54p promote efficient DNA strand invasion with chromatin.

(A) Schematic of the D-loop reaction. A radiolabeled oligonucleotide (ss) pairs with a homologous duplex target (dsDNA) to yield a D-loop, which, after separation from the free oligonucleotide on an agarose gel, is visualized and quantified by PhosphorImager analysis of the dried gel.

(B) Panel I shows D-loop reactions mediated by Rad51p and Rad54p with the naked homologous duplex (Naked DNA) and the homologous duplex assembled into chromatin (Chromatin). The results from the experiments in panel I are graphed in panel II. The % D-loop refers to the proportion of the homologous duplex converted into D-loop.

(C) Panel I shows D-loop reactions mediated by RecA with the naked homologous duplex (Naked DNA) and the homologous duplex assembled into chromatin (Chromatin). The results from the experiments in panel I are graphed in panel II.

(D) Panel I shows D-loop reactions in which Rad51p and RecA were used either alone or in conjunction with Rad54p or rad54 mutant variants with the naked homologous duplex (Naked DNA) and the homologous duplex assembled into chromatin (Chromatin), as indicated. ATP was omitted from the reaction in lane 5, and ATP-γ-S (γS) and AMP-PNP (PNP) replaced ATP in lanes 7 and 8, respectively. The reactions in lanes 8 and 9 contained ATP, but Rad54p was replaced with the ATPase defective variants rad54 K341A (A) and rad54 K341R (R), respectively. The results from the experiments in panel I are summarized in the bar graph in panel II.

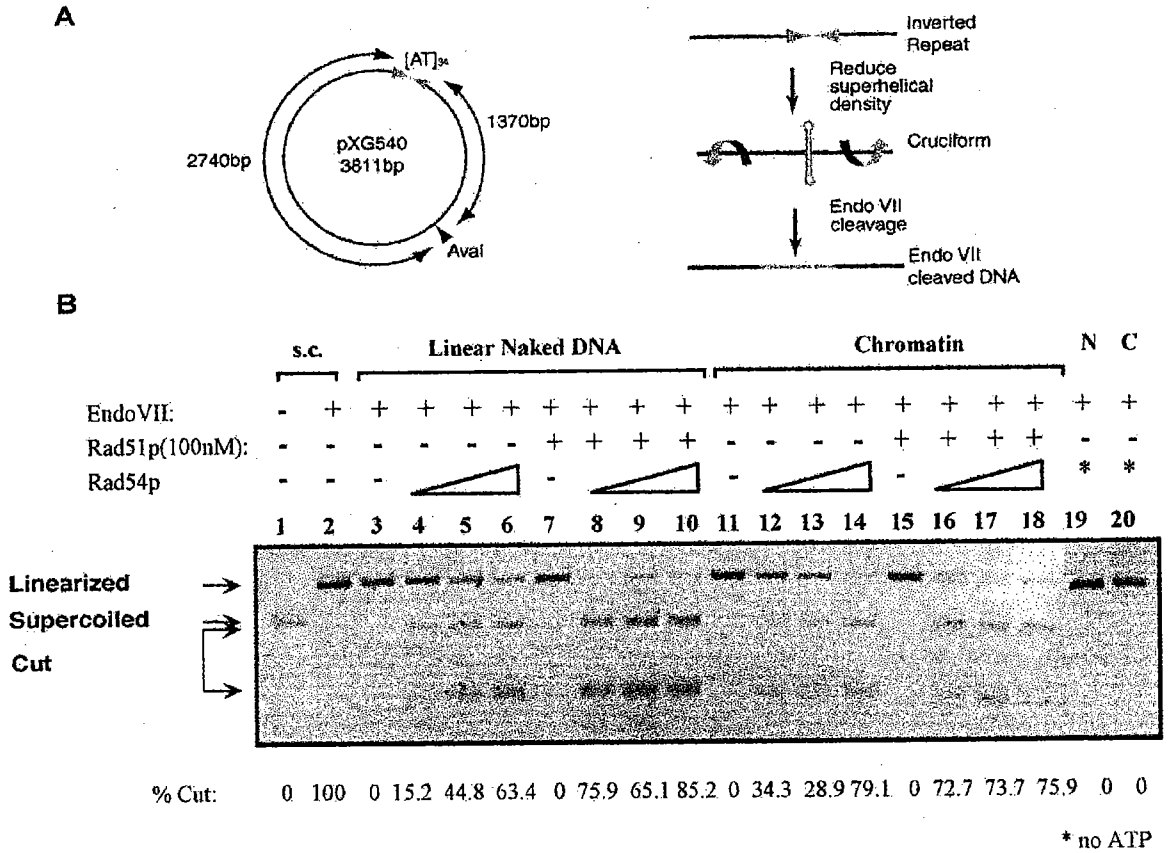
chromatin had no effect on the efficiency of D-loop formation by Rad51p and Rad54p (Figure III.1B). D-loop formation on the chromatin donor required ATP hydrolysis by Rad54p, since non-hydrolyzable ATP analogs (ATP-γ-S and AMP-PNP) were unable to substitute for ATP (Figure III.1C, panel I, lanes 6 and 7), and two ATPase-defective mutant variants of Rad54p, rad54 K341A and rad54 K341R (Petukhova *et al.,* 1999), were inactive (Figure III.1D). In contrast to reactions that contained Rad51p/Rad54p, the activity of RecA was completely eliminated when the donor was assembled into nucleosomes (Figure III.1C). Furthermore, addition of Rad54p to the RecA reaction did not rescue D-loop formation on chromatin (Figure III.1D, Panel I, lane 11). Thus, the

eukaryotic recombination proteins have the unique capability of performing the DNA strand invasion reaction with a chromatin donor.

Rad54 generates unconstrained superhelical torsion in nucleosomal DNA

A number of chromatin remodeling complexes that contain SWI2/SNF2-related ATPases have been shown to alter chromatin structure by generating superhelical torsion in DNA and nucleosomal arrays (Havas *et al.,* 2000). Indeed, the ability to introduce superhelical stress may represent a primary biomechanical activity of all SWI2/SNF2-like ATP-dependent DNA motors, and this activity is likely to be crucial for catalyzing alterations in chromatin structure. Previous studies have shown that Rad54p can also generate both negative and positive supercoiled domains in dsDNA, and it has been suggested that this activity reflects the tracking of Rad54p along DNA (Van Komen *et al.,* 2000; Ristic *et al.,* 2001).

We investigated whether Rad54p is able to introduce superhelical torsion on nucleosomal substrates, using a cruciform extrusion test that has been used for examining other chromatin remodeling enzymes (Figure III.2A). In this assay, superhelical torsion leads to extrusion of a cruciform that is then recognized and cleaved by bacteriophage T4 endonuclease VII that has high specificity for this DNA structure (Havas *et al.,* 2000). Consistent with previous studies (Van Komen *et al.,* 2000), Rad54p action generates torsional stress on a linear, dsDNA substrate (N) which leads to cruciform extrusion



(Figure III.2B; lanes 4-6). Importantly, Rad54p was able to generate torsional stress on the nucleosomal substrate (C) with comparable efficiency (lanes 12-14).

Figure III.2: Rad54 generates superhelical torsion on nucleosomal DNA

(A) Schematic illustration of the cruciform extrusion assay: A linearized plasmid (pXG540) containing an inverted repeat sequence is incubated with T4 Endonuclease VII, a highly selective junction resolving enzyme, and Rad54p, in the presence of ATP. Rad54p increases the local unconstrained superhelical density, resulting in the extrusion of a cruciform structure, which is recognized and cut by Endo VII. Adapted from Havas *et al.* (2000).

(B) Results of a typical cruciform formation assay. Supercoiled (lanes 1, 2), AvaI-linearized pXG540 DNA (lanes 3-10, 19), or nucleosomal pXG540 (lanes 11-18, 20) was incubated with 12.5, 25 or 50nM Rad54p as indicated, in the presence or absence of 100nM Rad51p. EndoVII was omitted in lane 1. ATP was omitted in lanes 19 and 20. The numbers below each lane (% cut) represent the percentage of pXG540 molecules cleaved by EndoVII. s.c., supercoiled substrate; C, chromatin substrate; N, naked linear DNA substrate.

The addition of 100nM Rad51p greatly stimulated the ability of Rad54p to promote the formation of cruciform structures on both naked and nucleosomal substrates (compare lanes 4 and 8, and 12 and 16, respectively). Note that Rad51p fails to support cruciform formation by itself (lanes 7 and 15). As expected, the generation of torsional stress required ATP (lanes 19 and 20). Furthermore, the ATP hydrolysis mutant variant rad54 K341A was inactive in these assays (data not shown). These data indicate that Rad54p, like other SWI2/SNF2 family members, utilizes the free energy from ATP hydrolysis to alter DNA topology, and that nucleosomal arrays constitute excellent substrates for this activity.

Rad54p can disrupt a DNA triple-helix

How Rad54p introduces topological stress in nucleosomal DNA is unclear. Previously, we suggested that superhelical torsion might result from translocation of Rad54p along the DNA double helix (Van Komen *et al.,* 2000). Recently, chromatin remodeling by the yeast RSC complex (which contains the SWI2/SNF2-related ATPase, Sth1p) has been shown to involve ATP-dependent DNA translocation (Saha *et al.,* 2002). In order to further evaluate the ability of Rad54p to translocate on DNA, we used a DNA triple helix displacement assay that was originally developed to follow the translocation of a type I restriction endonuclease along DNA (Firman and Szczelkun, 2000). The substrate used (see Figure III.3A) consists of a radioactively labeled oligonucleotide (TFO*) bound via Hoogsteen hydrogen bonds to the major groove of a 2.5 kb linear

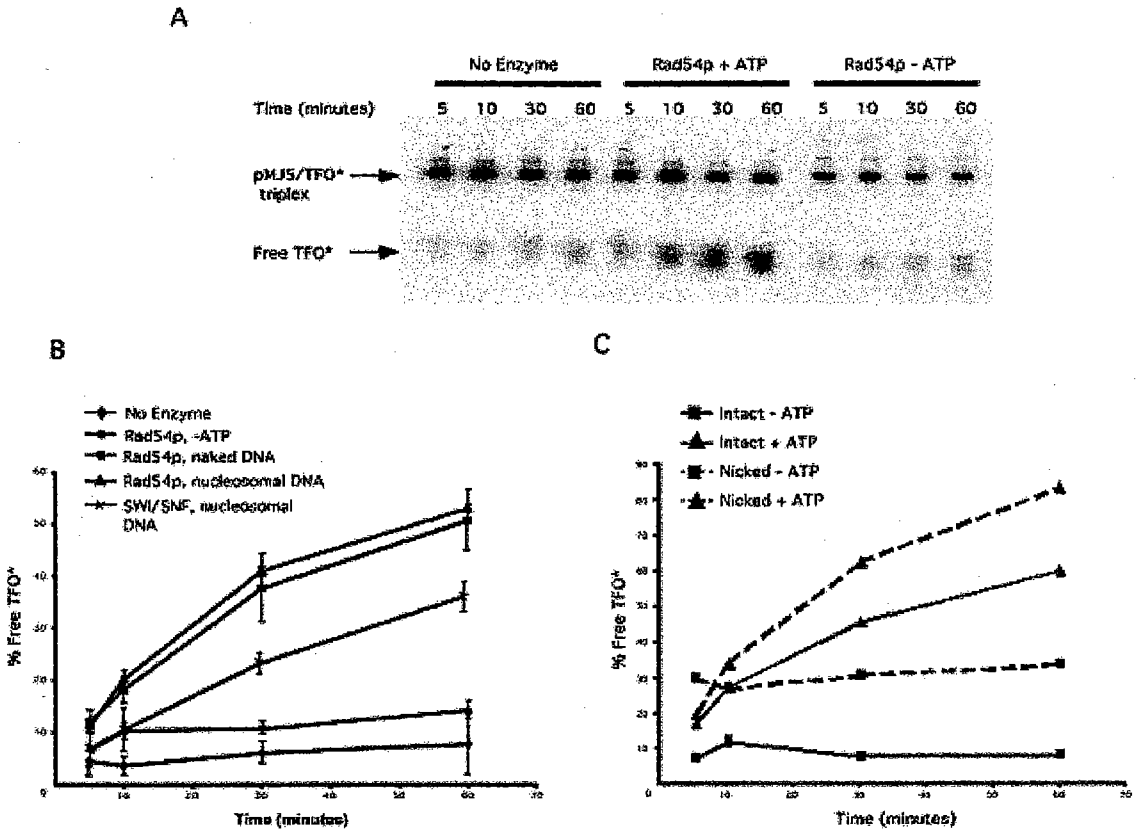


Figure III.3: Rad54p action displaces a pre-formed triplex

(A) Typical results obtained with naked triplex-containing substrate. The upper band corresponds to the duplex-bound TFO*; the lower band corresponds to free TFO*. Reactions contained 5nM triplex substrate and 5nM Rad54p.

(B) The percentage of free TFO* in four or more experiments were averaged and plotted as a function of time. Note that triplex displacement from the nucleosomal template (▲) occurs at equal efficiency to that of naked DNA (■). SWI/SNF (✕) also displaces the triplex in an ATP-dependent manner.

(C) Rad54p action efficiently dissociates a pre-formed triplex from nicked substrates (■, broken line), in an ATP-dependent manner (▲, broken line). Note that the triplex is inherently less stable on nicked DNA (■, broken line) than on intact DNA substrates (■, solid line), but nevertheless, the ATP-dependent action of Rad54p on this substrates has a strong effect on the rate of TFO dissociation (▲, broken line).

dsDNA. Translocation of a protein along the DNA displaces the triplex, which can be detected as dissociation of the radioactive TFO* from the DNA triplex. Figure III.3B shows typical levels of triplex displacement in the absence or presence of Rad54p. Rad54p was able to efficiently displace a pre-formed triplex from both naked (squares) and nucleosomal (triangles) substrates, in an ATP-dependent manner. Also shown are the results for ySWI/SNF on a nucleosomal substrate (**x**). Similar results were obtained when the TFO*-bound substrate contained single-strand nicks (Figure III.3C), strongly suggesting that the displacement of the TFO* reflects translocation of Rad54p and that it is not due simply to the generation of torsional stress. Thus, yeast RSC (Saha *et al.,* 2002), ySWI/SNF, and Rad54p all share the ability to use the free energy from ATP hydrolysis to disrupt triplex DNA. *Drosophila* ISWI has been recently reported to have a similar activity (Whitehouse *et al.*, 2003), suggesting that all chromatin remodeling factors share the ability to displace a pre-formed triplex.

Rad54p has ATPase kinetics diagnostic of a DNA translocating enzyme

The "inch-worm" model for DNA translocation, originally envisioned (Yarranton and Gefter, 1979) for DNA helicases, and later modified by Velankar *et al.* (1999), proposes that the translocating enzyme progresses along the contour of the DNA in steps of a single base, and each step requires the hydrolysis of one ATP molecule. This model predicts that the rate of ATP hydrolysis of a unidirectional DNA translocating enzyme will depend on the length of the DNA (Dillingham *et al.,* 2000).

To investigate whether Rad54p has ATPase properties characteristic of a unidirectional DNA translocating enzyme, the rate of ATP hydrolysis was measured in the presence of saturating amounts of single-stranded oligonucleotides ranging from 10 to 100 nucleotides in length (Figure III.4). For comparison, we also monitored the ATPase activity of yeast SWI/SNF (triangles). In the case of Rad54p, oligonucleotides shorter than 40 bases failed to stimulate the ATPase activity of Rad54p (diamonds), while oligonucleotides between 40 and 70 bases led to a stimulation of ATPase activity that was proportional to DNA length. For oligonucleotides longer than 70 bases, the ATPase activity no longer increased with the DNA length. When Rad51p was added to these reactions (squares), shorter oligonucleotides became more effective in promoting ATP hydrolysis, and the overall activity was enhanced. Likewise, the ATPase activity of ySWI/SNF (triangles) was also proportional to the DNA length, with a plateau reached at 60 bases.

These results are fully consistent with both Rad54p and ySWI/SNF coupling ATP hydrolysis to unidirectional translocation, in which the rate of DNA binding (or more likely, the transition to translocation) is slower than the rate of DNA translocation (Saha *et al.,* 2002). The rate-limiting step could consist in the conformational change of Rad54p (or Swi2p) caused by binding of ATP, which would promote translocation along the DNA. In this case, no ATP hydrolysis is observed with very short substrates, presumably because a minimum DNA length is required for Rad54p or ySWI/SNF to bind and to translocate before reaching an end and releasing the DNA. When the substrate is ~30-40 nucleotides in length, Rad54p and ySWI/SNF readily bind the substrate and more

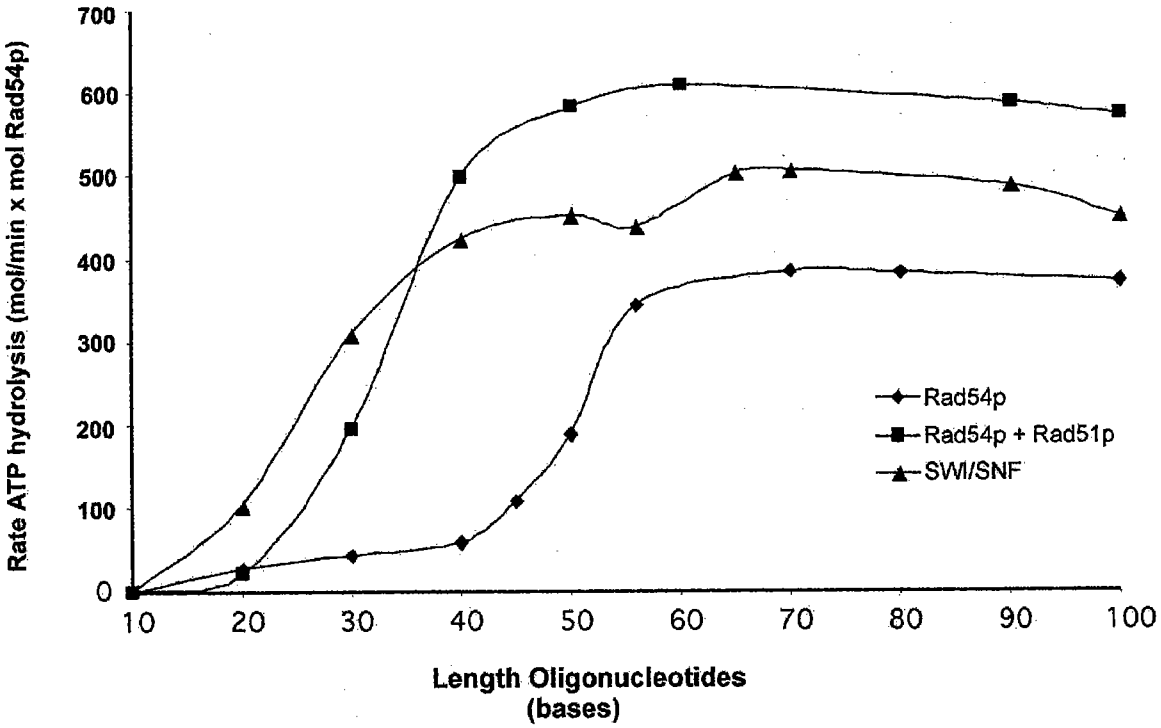


Figure III.4: Rad54 has ATPase kinetics typical of a unidirectional DNA translocating enzyme

The rate of ATP hydrolysis by 5nM Rad54p (diamonds), 5nM Rad54p + 5nM Rad51p (squares), or 10nM SWI/SNF (triangles) was measured in the presence of 50μM ssDNA (n-mers) oligonucleotides of different lengths. The average values from 3 independent experiments are plotted. Rates were determined from experiments with at least four timepoints.

extended translocation events take place. The rate of ATP hydrolysis is fairly constant with DNA substrates longer than 60-70 nucleotides, reflecting the possibility that Rad54p and ySWI/SNF have little processivity, and thus they release their substrate after ~60-70 bases regardless of the total length of the DNA molecule. Although the tri-phasic kinetics of ATPase activity are consistent with a DNA translocation mechanism, it remains a possibility that the longer single stranded oligonucleotides exhibit more extended

secondary structures which are either more proficient at binding Rad54p (or SWI/SNF) or stimulating its ATPase activity.

Rad54 is an ATP-dependent chromatin remodeling enzyme

Saha and colleagues (2002) proposed that short range translocation events may be the key feature of chromatin remodeling enzymes, leading to a "pumping" of DNA across the surface of the histone octamer which then results in enhanced DNA accessibility and nucleosome movements. To investigate whether Rad54p might also enhance the accessibility of nucleosomal DNA, we used an assay where nucleosome remodeling activity is coupled to restriction enzyme activity such that remodeling is revealed as an enhancement of restriction enzyme cleavage rates (Logie and Peterson, 1997). This assay uses a nucleosomal array substrate in which the central nucleosome of an 11-mer array contains a unique *Sal*I site located at the predicted dyad axis of symmetry (see Figure III.5A). In the absence of a remodeling enzyme, the rate of *Sal*I cleavage is very slow (Figure III.5A, solid diamonds), whereas addition of a remodeling enzyme, such as yeast SWI/SNF, leads to enhanced digestion (Figure III.5A; solid squares).

When Rad54p was added to the remodeling reactions, *Sal*I digestion was also dramatically enhanced (solid circles, triangles), although a higher concentration of this protein (50nM) was required to achieve a rate of digestion comparable to that of reactions that contained yeast SWI/SNF (2nM, squares). However, when Rad51p (50nM) and Rad54p (50nM) were both present in the reaction, much higher levels of remodeling were

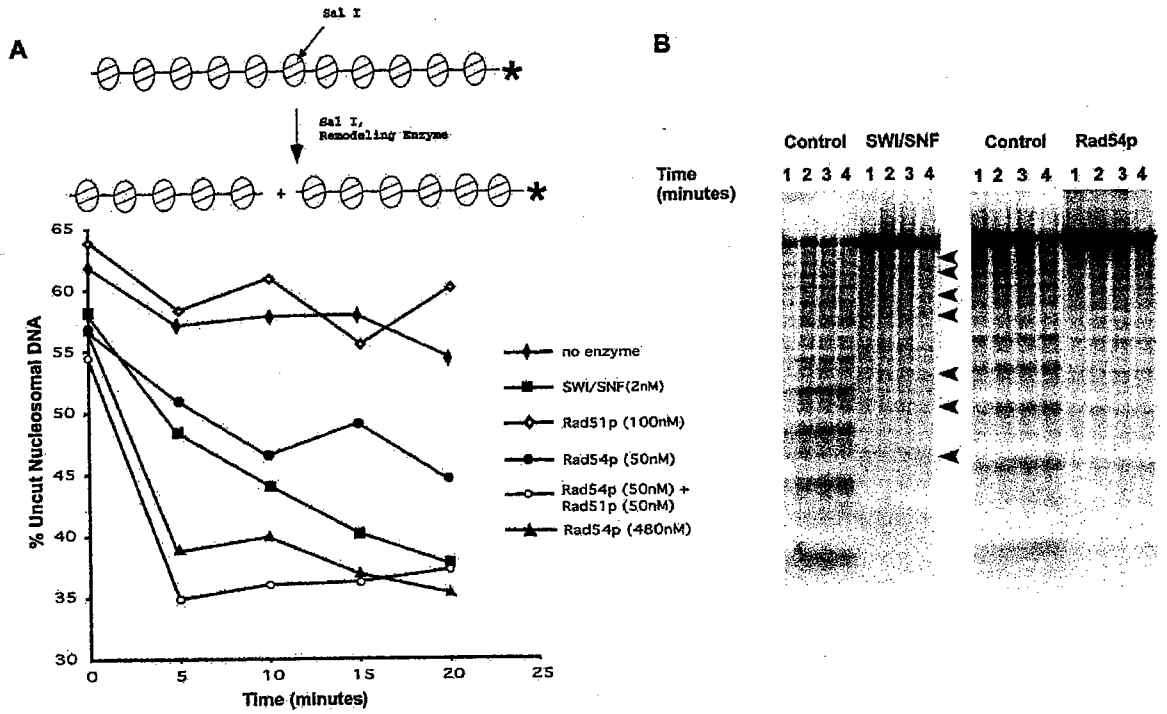


Figure III.5: Rad54 is an ATP-dependent chromatin remodeling enzyme

(A) Various concentrations of recombinant Rad54p were tested for chromatin remodeling activity in a coupled remodeling-restriction enzyme cleavage assay. The nucleosomal substrate was incubated with 50nM (closed circles) or 480nM Rad54p (triangles), 100nM Rad51p (open diamonds), 50nM Rad54p + 50nM Rad51p (open circles), 2nM ySWI/SNF (squares), or buffer (closed diamonds).

(B) 208-11 reconstituted nucleosomal arrays were incubated at 37°C with 2nM SWI/SNF, 100nM Rad54p, or buffer (control lanes). Aliquots were treated with Mnase at the indicated time points. The arrowheads in the first panel indicate the alternate banding pattern as a result of SWI/SNF-induced nucleosome movement.

attained (open circles). Note that Rad51p has no intrinsic chromatin remodeling activity (open diamonds). The stimulation of the Rad54p chromatin remodeling activity by Rad51p is congruent with previous studies showing that Rad51p enhances the rate of ATP hydrolysis and DNA supercoiling by Rad54p (Mazin *et al.*, 2000; Van Komen *et al.*, 2000). Thus, the above data indicate that Rad54p is sufficient for chromatin remodeling

activity, but that the combination of Rad51p and Rad54p constitutes a more potent remodeling machine.

Rad54p does not induce significant nucleosome mobilization

A number of chromatin remodeling complexes that contain a SWI2/SNF2-related ATPase (ySWI/SNF, dCHRAC, dNURF and xMi-2) can use the energy of ATP hydrolysis to move nucleosomes in cis (see Chapter II; Hamiche *et al.,* 1999; Längst *et al.,* 1999; Whitehouse *et al.,* 1999; Guschin *et al.,* 2000; reviewed in Becker and Hörz, 2002; Flaus and Owen-Hughes, 2003). In order to investigate whether Rad54p can also catalyze nucleosome mobilization, ^{32}P-end-labeled nucleosomal arrays were incubated with buffer, ySWI/SNF, or Rad54p, and nucleosome positions were mapped by micrococcal nuclease (Mnase) digestion (Figure III.5B). Mnase only cleaves DNA between nucleosomes, which leads to a periodic ladder of digestion products indicative of a positioned 11-mer nucleosomal array (Figure III.5B). Consistent with our previous studies, incubation with ySWI/SNF (2 nM) and ATP leads to a complete disruption of the Mnase digestion pattern, indicative of nucleosome sliding (Figure III.5B, left panel; also see Chapter II). In contrast, addition of Rad54p (100 nM) and ATP had very little effect on the cleavage periodicity (Figure III.5B, right panel). Likewise, addition of both Rad51p and Rad54p (100 nM each) to these assays did not change the Mnase digestion profile (data not shown). Importantly, these experiments used concentrations of

ySWI/SNF and Rad54p that yielded similar levels of chromatin remodeling in the restriction enzyme accessibility assay (Figure III.5A).

Furthermore, Rad54p does not induce histone octamer mobilization in a defined, mononucleosomal system (data not shown). Thus, although Rad54p can enhance the accessibility of nucleosomal DNA to restriction enzymes, this activity does not appear to reflect large scale re-arrangement of nucleosome positions.

DISCUSSION

In eukaryotes, chromatin presents an accessibility dilemma for all DNA-mediated processes, including gene transcription and DNA repair. Whereas much progress has been made on identifying the enzymes that remodel chromatin structure to facilitate transcription, less is known of how the DNA repair machinery gains access to damaged DNA within chromatin (reviewed in Green and Almouzni, 2002). In particular, it has not been clear how the recombinational repair machinery can locate regions of DNA homology when those DNA donor sequences are assembled into chromatin. Here we have shown that the yeast recombination proteins, Rad51p and Rad54p, are sufficient to promote heteroduplex DNA joint formation with chromatin. In contrast, the bacterial recombinase RecA is completely inactive with a chromatin donor. The unique capacity of the eukaryotic machinery to contend with chromatin likely reflects the chromatin remodeling activity of Rad54p in which the free energy from ATP hydrolysis enhances the accessibility of nucleosomal DNA. Strand invasion with chromatin may also require a specific interaction between Rad51p and Rad54p since the chromatin remodeling activity of Rad54p does not facilitate RecA-dependent D-loop formation with chromatin (Figure III.1D). Recently, Alexiadis and Kadonaga (2002) have reported that the *Drosophila* Rad51 and Rad54 proteins can also facilitate strand invasion with chromatin.

How does Rad54p remodel chromatin structure?

Several studies have shown that SWI/SNF-like chromatin remodeling enzymes can perform two separable reactions: (1) they can use the free energy from ATP hydrolysis to enhance the accessibility of nucleosomal DNA and (2) they can use this free energy to mobilize nucleosomes in *cis* (reviewed in Peterson, 2000). Recent work from Cairns and colleagues have suggested that both of these activities may be due to ATP-dependent "pumping" of DNA into the nucleosome (Saha *et al.,* 2002). In this model, small amounts of DNA translocation might lead to transient exposure of small "loops" of DNA on the surface of the histone octamer, whereas larger quantities of DNA "pumped" into the nucleosome would lead to changes in nucleosome positions. Our data supports this model, as we find that both yeast SWI/SNF and Rad54p, like yeast RSC (Saha *et al.,* 2002) and *Drosophila* ISWI (Whitehouse *et al.*, 2003), can disrupt a DNA triplex in an ATP-dependent reaction, presumably by translocation of DNA along the surface of the enzyme or by translocation of the enzyme along the DNA. Furthermore, the ATPase activities of ySWI/SNF and Rad54p are sensitive to DNA length which is diagnostic of DNA translocating enzymes (Dillingham e*t al.*, 2000).

Although ySWI/SNF and Rad54p can both enhance the accessibility of nucleosomal DNA, only ySWI/SNF appears to be proficient at significantly altering nucleosome positioning as measured by a disruption in the pattern of Mnase digestion of the remodeled nucleosomal substrate.

Our results suggest that the precise mechanism of chromatin remodeling by Rad54p may be distinct from that of ySWI/SNF. For instance, Rad54p may only be able to pump small amounts of DNA across the histone octamer surface. Alternatively, Rad54p may translocate along DNA, rather than pumping DNA into the nucleosome (see Chapter V and Figure V.2). In this model, Rad54p may "pull" the Rad51-ssDNA nucleoprotein filament along the chromatin fiber, leading to changes in nucleosomal DNA topology and DNA accessibility. Such a DNA tracking mechanism might play a key role in facilitating both the search for homology as well as the strand invasion step.

Multiple roles for Rad54p during homologous recombination

Our results suggest that Rad54p is an extremely versatile recombination protein that plays key roles in several steps of homologous recombination. Recently, Wolner and colleagues (2003) reported that Rad54p is required for optimal recruitment of Rad51p to a double strand break *in vivo*, and likewise Rad54p can promote formation of the presynaptic filament *in vitro* by helping Rad51p contend with the inhibitory effects of the ssDNA-binding protein RPA. Several studies over the past few years have also shown that the ATPase activity of Rad54p plays key roles subsequent to formation of the presynaptic filament. For instance, Rad54p is required for the Rad51p-nucleoprotein filament to form a heteroduplex joint DNA molecule, even when the homologous donor is naked DNA (Figure III.1A; see also Petukhova *et al.,* 1998; Petukhova *et al.,* 1999; Solinger *et al.,* 2001). In this case, it has been proposed that Rad54p might use the free

energy from ATP hydrolysis to translocate along DNA which facilitates the homology search process. This DNA translocation model is fully consistent with our findings that Rad54p can displace a DNA triplex and that the ATPase activity of Rad54p is proportional to DNA length. Rad54p also stimulates heteroduplex DNA extension of established joint molecules (Solinger and Heyer, 2001). Finally, we have shown that Rad54p is required for Rad51p-dependent heteroduplex joint molecule formation with a chromatin donor. In this case, our results suggest that the ATPase activity of Rad54p is used to translocate the enzyme along the nucleosomal fiber, generating superhelical torsion which leads to enhanced nucleosomal DNA accessibility. It seems likely that this chromatin remodeling activity of Rad54p might also facilitate additional steps following heteroduplex joint formation.

Preface to Chapter IV

In Chapter IV we provide evidence indicating that yeast Rad54p physically interacts with histone H3 and H4 NH_2-termini. The significance of these interactions *in vivo* is discussed.

The work presented here is part of an ongoing collaboration with Dr. Patrick Sung, who supplied the purified yeast Rad51, Rad54 and RPA proteins. Dong Hyun Roh, and Stephen Van Komen, Ph.D., from Dr. Sung's laboratory, contributed the data represented in Figure IV.4 (D.H.R.) and IV.6 (S.V.K.).

CHAPTER IV

Rad54p INTERACTION WITH NUCLEOSOMAL HISTONE NH_2-TERMINAL DOMAINS

CHAPTER IV

Rad54p Interaction with Nucleosomal Histone NH_2-Terminal Domains

INTRODUCTION

The genome of eukaryotes is packaged into chromatin. The basic repeating unit of chromatin is the nucleosome, a stable complex of the four core histone proteins and DNA. The DNA wraps ~1.75 times around the core histone octamer, adopting a condensed conformation that is further compacted by inter-nucleosomal interactions. Chromatin structure has a strong influence on the regulation of all nuclear processes in which access to DNA is required, such as transcription, replication and DNA repair. A number of proteins are able to modulate the inherently restrictive chromatin environment and allow these processes to take place.

The two major classes of enzymes capable of altering chromatin structure are (a) histone modifying enzymes, that covalently modify histone proteins by phosphorylation, methylation, acetylation, or ubiquitylation (reviewed in Fischle *et al.*, 2003); and (b) ATP-dependent chromatin remodeling complexes, that utilize the free energy derived from ATP hydrolysis to actively disrupt the histone-DNA interactions (reviewed in Becker and Hörz, 2002).

ATP-dependent chromatin remodeling complexes contain an ATPase subunit related to the SWI2/SNF2 subfamily of DNA-stimulated ATPases. The activity of these complexes (or in some cases, the ATPase subunit itself) leads to changes in the rotational path of nucleosomal DNA on the surface of the histone octamer, enhanced accessibility of nucleosomal DNA to transcription factors and restriction enzymes, movement of histone octamers in *cis* and *trans*, formation of dinucleosome-like particles (reviewed in Havas *et al.*, 2001) and changes in the topology of circular (Kwon *et al.*, 1994; Chapter II) and linear chromatin (Havas *et al.*, 2000; Chapter III). The ability to generate superhelical stress on DNA may provide the biomechanical force necessary for the disruption of histone-DNA contacts and the creation of short regions of DNA that are transiently dissociated from the octamer surface (Varga-Weisz and Becker, 1998; Havas *et al.*, 2000; Flaus and Owen-Hughes, 2001; Gavin *et al.*, 2001; Aoyagi *et al.*, 2002).

We have previously shown that Rad54p, a member of the SWI2/SNF2 family that is required for DNA repair and homologous recombination in organisms ranging from yeast to human, is an ATP-dependent chromatin remodeling enzyme (Chapter III, figure III.5A). Rad54p works in conjunction with Rad51p, a eukaryotic homologue of the RecA recombinase, to drive the pairing and recombination of homologous DNA molecules on chromatin (Chapter III, figure III.1). Like other members of the SWI2/SNF2 family, Rad54p is able to translocate and generate torsional stress on chromatin (Chapter III, figures III.2 and III.3; Van Komen *et al.*, 2000; Ristic *et al.*, 2001).

In contrast with other chromatin remodeling enzymes, Rad54p does not seem to cause detectable nucleosome mobilization (Chapter III, figure III.5B and data not shown).

This could be an indication that the mechanism used by Rad54p to remodel chromatin is different than the one used by SWI/SNF and other chromatin remodeling enzymes (see Chapter V, and figure V.2). Alternatively, Rad54p might require other factors to achieve its maximal remodeling activity, or to enhance its processivity.

During the past few years, several models have been proposed to explain the mechanism of ATP-dependent chromatin remodeling. Three attractive models, which are not necessarily mutually exclusive, involve DNA twisting, DNA translocation or structural alteration of the octamer (reviewed in Flaus and Owen-Hughes, 2003). The combination of elements from these mechanisms gives rise to a prevalent model, which posits that ATP-dependent chromatin remodeling factors remain in a fixed position, bound to both the histone octamer and the DNA on the nucleosome. ATP hydrolysis initiates translocation, causing translational movements of the DNA, accompanied by DNA twist. The DNA being "pushed" or "pulled" from the chromatin remodeling enzyme towards the first histone-DNA contact becomes under- or over-twisted, initiating a DNA wave across the surface of the nucleosome (Saha *et al.*, 2002). This wave transiently disrupts histone-DNA interactions, generating a window of opportunity for DNA-binding proteins (transcription factors, endonucleases) to bind their preferred sequence. An additional outcome of this model is the repositioning of the histone octamer along the DNA (1 bp "movement" per 34° twist of the DNA double helix around its axis), resulting in the direct alteration of the accessibility of underlying DNA sequences (reviewed in Flaus and Owen-Hughes, 2003).

In order to test whether the chromatin remodeling model depicted above correlates with the mechanism used by Rad54p, we investigated the possibility that Rad54p directly interacts with both DNA and the histone octamer. Here we report that Rad54p specifically binds the proximal NH_2-terminal region of histones H3 and H4. These results correlate with *in vivo* experiments showing that both histone H3 and H4 are modified in response to DNA damage, and that these modifications are critical for downstream DNA repair events (Qin and Parthun, 2002; Bird *et al.*, 2002).

MATERIALS AND METHODS

DNA constructions

All DNA manipulations were carried out using standard methods (Sambrook *et al.,* 1989). The DNA template (208-11) used to reconstitute nucleosomal arrays for the ATPase and remodeling assays consists of a *Not*I–*Hind*III fragment derived from pCL7b (Logie and Peterson, 1997), containing 11 head-to-tail repeats of a 5S rRNA gene from *L. variegatus*, each one possessing a nucleosome positioning sequence. The sixth nucleosome occludes a unique *Sal*I restriction site.

Reagent Preparation

Recombinant yeast Rad51p and Rad54p were overexpressed in yeast and purified as previously described (Petukhova *et al.,* 1998). SWI/SNF purification was as described (Smith *et al.,* 2002). Recombinant full-length or globular domain (tail-less) versions of the four histone proteins were expressed in *E. coli*, and purified according to Luger *et al.* (1999). Octamers were prepared from purified histones (Luger *et al.*, 1999) and reconstituted into chromatin on 208-11 or pBluescript DNA by salt dialysis as previously described The nucleosome saturation level was determined by *Eco*RI digestion analysis (see figure IV.3B; Logie and Peterson, 1999).

Histone binding assays

GST and GST-histone fusions were expressed in *E. coli* BL21 (DE3) as described (Smith and Johnson, 1988). 25µg of GST, GST-H3 (1-46), GST-H4 (1-34), GST-H4 (1-16), GST-H2A (1-35), GST-H3 (1-25) or GST-H3 (21-46), GST fusion histone NH_2-termini proteins were incubated with 6µl of Glutathione Sepharose-4B beads (Amersham Pharmacia) in 30µl of PK1 buffer (20mM Tris-HCl, pH: 7.2, 75mM KCl, 0.5% Triton X-100) containing 5µg bovine serum albumin (BSA) in a final volume of 30µl on ice for 30 minutes with agitation. The bead-bound GST-histone NH_2-tails were washed with 100µl of PK1 buffer, then resuspended and incubated in a final volume of 30µl of PK1 buffer containing 5µg BSA and 5µg of Rad54p or Rad51p on ice for 30 minutes with agitation. After centrifugation, the supernatant (S) was removed and the beads were washed three times with 30µl of PK2 buffer (20mM Tris-HCl, pH: 7.2, 150mM KCl, 1% Triton X-100). After removing the third wash fraction (W), the beads were resuspended in 55µl of SDS sample buffer to yield the eluate (E). SDS sample buffer (25µl) was also added to the supernatant (S) and wash fraction (W). 10µl aliquots of the samples were separated in a 12 % SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue.

D-loop reactions

Oligonucleotide D1 (90-mer) used in the D-loop experiments has the sequence: 5'- AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGC TTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT-3', being complemen-

tary to positions 1932 to 2022 of pBluescript SK(-) replicative form I DNA. Oligonucleotide D1 was 5' end-labeled with [γ-^{32}P] dATP and polynucleotide kinase, as described (Petukhova *et al.,* 1998). Buffer R (35mM Tris-HCl, pH: 7.4, 2.0mM ATP, 2.5mM $MgCl_2$, 30mM KCl, 1mM DTT, and an ATP regenerating system consisting of 20mM creatine phosphate and 30µg/ml creatine kinase) was used for the reactions; all the incubation steps were carried out at 30°C. Rad51p (0.8µM) and Rad54p (120nM) were incubated with radiolabeled Oligonucleotide D1 (2.4µM nucleotides) for 5 minutes to assemble the presynaptic filament, which was then mixed with naked pBluescript replicative form I DNA (38µM base pairs) or the same DNA assembled into chromatin (38µM base pairs). Chromatin assembly was monitored by following topological changes as well as measuring the degree of occlusion of a unique *Eco*RI restriction site close to the D1 sequence. Substrates were estimated to be ~80% saturated with nucleosomes. 4µl portions of the reactions were withdrawn after 5 minutes, and mixed with an equal volume of 1% SDS containing 1mg/ml proteinase K. After incubation at 37°C for 5 minutes, the deproteinized samples were run in 1% agarose gels in TAE buffer (40mM Tris-HCl, pH: 7.4, 0.5mM EDTA) at 4°C. The gels were dried and the radiolabeled DNA species were visualized and quantified by PhosphorImager analysis (Personal Molecular Imager FX, Bio-Rad).

ATPase Assay

Recombinant yeast Rad54p (1nM) was incubated at 30°C or 37°C with 5nM of either naked 208-11 dsDNA or reconstituted nucleosomal arrays, along with 1mg/ml

BSA, 15nM RPA, 9ng/µl free histones, or buffer, in the presence of 100µM ATP, 2.5µCi [γ-^{32}P] dATP (6000µCi/mmol, Amersham), 2.5% glycerol, 0.1% Tween 20, 20mM Tris-HCl, pH: 8.0, 200µM DTT, 5mM $MgCl_2$, 100µg/ml BSA. Samples were taken after 2, 5, 15 and 30 min and resolved by TLC. The proportion of liberated ^{32}P-pyrophosphate was determined using the Molecular Dynamics PhosphorImager and ImageQuant Software. ATPase assays were independently repeated 3 or more times which yielded very similar results.

Chromatin Remodeling Reaction

For the coupled Rad54p-*Sal*I reactions, reconstituted 208-11 nucleosomal arrays (~1 nM final concentration) were pre-incubated at 37°C for 20 min with 2.5U/µl *Sal*I in a buffer containing final concentrations of 50mM NaCl, 5mM $MgCl_2$, 1mM ATP, 3mM phosphoenolpyruvate, 10U/ml pyruvate kinase, 1mM DTT, 10mM Tris-HCl, pH: 8.0, 100µg/ml BSA and 3% glycerol. Nucleosomal arrays were approximately 60% saturated with nucleosomes. Buffer or 100nM of recombinant Rad51p and Rad54p were added and samples were taken at the indicated time points, vigorously mixed for 10s with 25µl TE and 50µl 1:1 solution of phenol/chloroform. The purified DNA fragments were resolved by electrophoresis in 1.2% agarose gels in the presence of 50µg/ml ethidium bromide. The gels were then dried on 3MM Whatman paper. The fraction of cut and uncut DNA was determined by PhosphorImager analysis using a Molecular Dynamics PhosphorImager and ImageQuant software (Amersham Biosciences).

RESULTS

Nucleosomal DNA protects Rad54p from thermal denaturation

The ATPase activity of Rad54p is required for many of its biological functions *in vivo* and for enhancing Rad51p-mediated homologous DNA pairing reactions *in vitro*, both on naked DNA (Petukhova *et al.,* 1999) and on chromatin (see Chapter III). We have recently shown that this ATPase activity is also required for translocation of Rad54p, and consequently, for introducing superhelical stress on DNA. It is noteworthy that all of Rad54p's *in vitro* biochemical activities are efficiently carried out on either naked DNA or chromatin substrates. We were therefore interested in determining whether chromatin influences the ATPase activity of Rad54p.

As shown in Figure IV.1, both naked DNA (closed squares) and chromatin (closed triangles) stimulate the ATPase activity of Rad54p at 30°C, with naked DNA being somewhat more effective. At the low protein concentrations at which these assays were performed (1nM), purified Rad54p is extremely temperature-labile and is rapidly inactivated at 37°C (Figure IV.1; Van Komen *et al.,* 2002). Thus, as expected, the ATPase activity of Rad54p was not detectable in the presence of naked DNA when the reactions were performed at 37°C (Figure IV.1, open squares). Importantly, when the reaction was carried out in the presence of chromatin (open triangles), the rate of ATP hydrolysis at 37°C was even greater than the rate obtained in reactions conducted at 30°C.

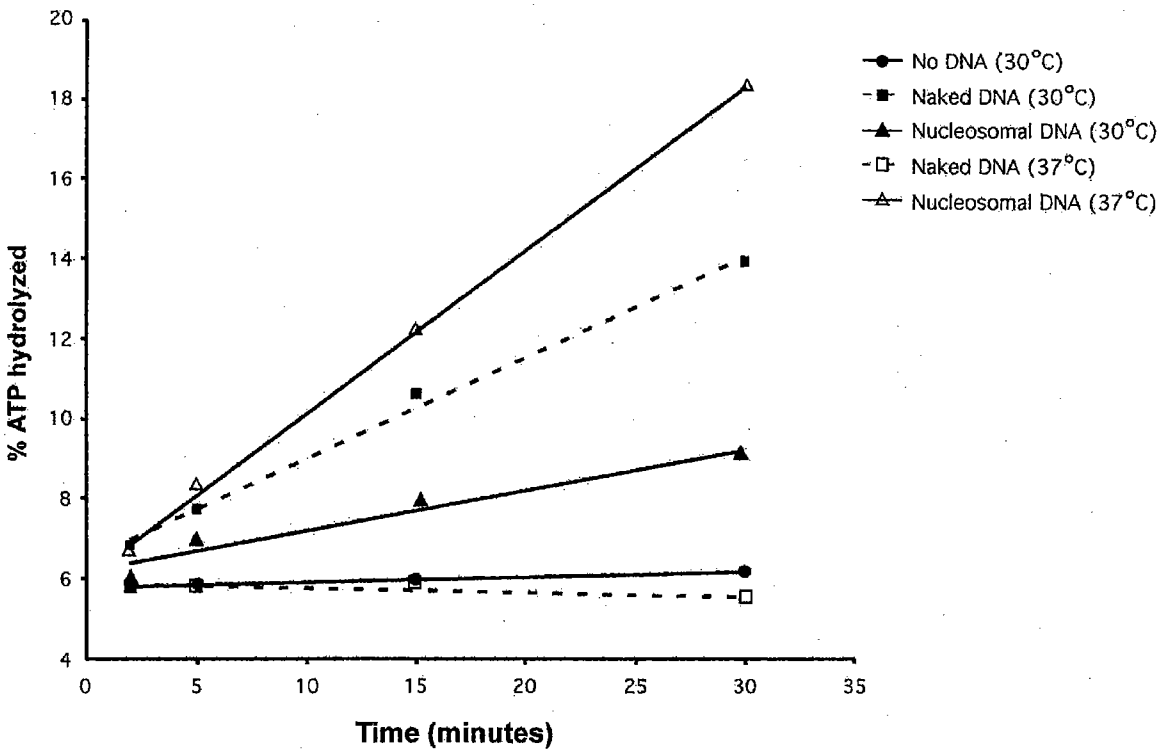


Figure IV.1: Nucleosomal DNA protects Rad54p from thermal inactivation

ATPase assays. 1nM Rad54p was incubated at 30°C with no DNA (circles), 5nM naked (closed squares) or nucleosomal dsDNA (closed triangles), or at 37°C with 5nM naked (open squares) or nucleosomal dsDNA (open triangles). Samples were taken after 2, 5, 15, and 30 minutes. The rate of ATP hydrolysis is proportional to the slope of the respective trend line.

This result suggests that Rad54p physically interacts with one or more components of the histone octamer, and that this interaction not only stimulates the rate of ATP hydrolysis, but also stabilizes Rad54p.

One obvious concern is that the stabilization of Rad54p by chromatin might be non-specific. To address this possibility, we monitored the ATPase activity of Rad54p at 30°C or 37°C, in the presence of naked DNA and a non-relevant protein (BSA), or DNA-binding proteins (RPA or free histones). As shown in Figure IV.2, none of these factors

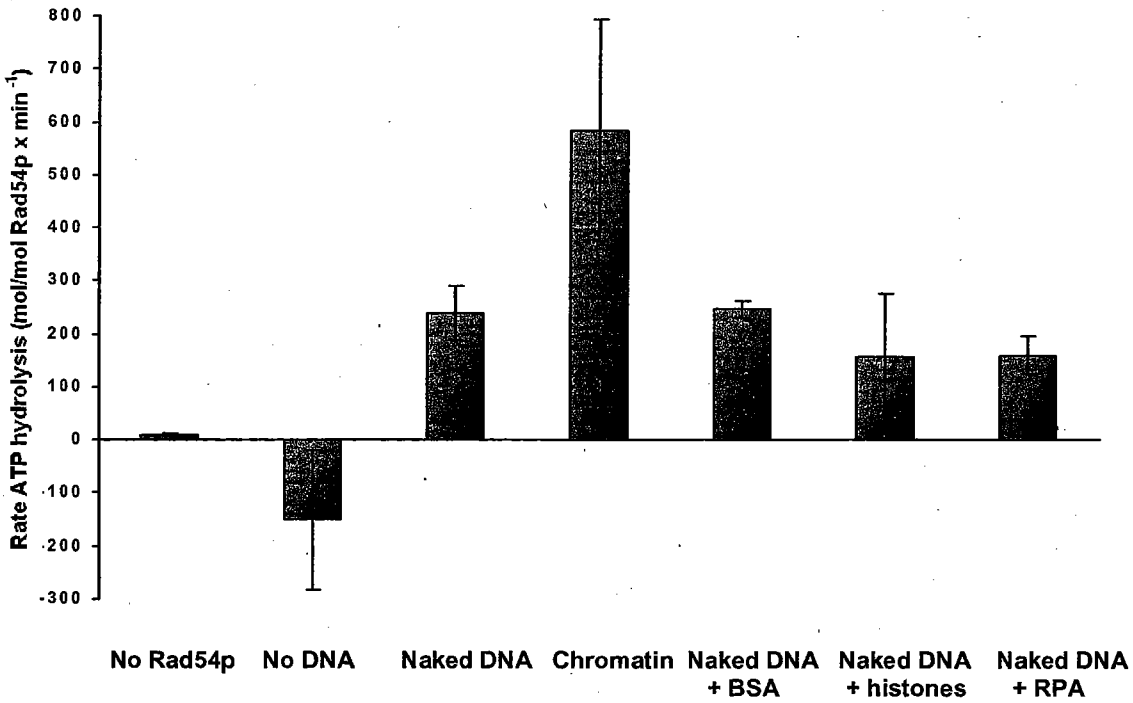


Figure IV.2: Rate of ATP hydrolysis at 37°C in the presence of different co-factors
All reactions contain the same molar ratio of DNA (either naked or nucleosomal) to Rad54p, as well as the same molar ratio of protein (either BSA, RPA, nucleosomal or free histones) to Rad54p, except where noted. The columns represent the average of at least 3 independent experiments.

stabilizes Rad54p, and the DNA-stimulated ATPase activity at 37°C is low, comparable to the rate detected in the presence of naked DNA alone. Figure IV.2 also shows that in the absence of Rad54p, there is no measurable ATPase activity associated with the nucleosomal arrays ("No Rad54p" sample). These results indicate that nucleosomal DNA is uniquely able to protect Rad54p from thermal inactivation, suggesting that Rad54p may physically interact with one or more components of nucleosomes.

Histone NH_2-terminal tails are required for protection of Rad54p from thermal denaturation

The fact that Rad54p is stabilized by nucleosomal DNA might reflect a physiologically relevant functional interaction between DNA repair proteins and chromatin components. Specific lysine residues on histone H3 NH_2-terminal tail and their acetylation by Hat1p are required for efficient DNA DSB repair *in vivo* (Qin and Parthun, 2002). Similarly, acetylation of histone H4 at its NH_2-terminal domain by Esa1p is required for DNA repair (Bird *et al.,* 2002). One simple explanation for the stabilizing effect that nucleosomal DNA has on Rad54p posits that Rad54p might directly interact with nucleosomes. We therefore decided to test whether Rad54p could be protected from thermal denaturation by "tail-less" chromatin reconstituted with the globular domains of the four core histones (i.e., deletion constructs of the core histone proteins that lack the NH_2-terminal domains).

The globular domains of the core histones form a stable octamer (Figure IV.3A) that can be reconstituted into chromatin by a standard salt-dialysis protocol (Figure IV.3B). At 30°C, tail-less chromatin stimulates Rad54p's ATPase activity to a level comparable to that of WT chromatin (i.e., chromatin reconstituted with full-length histone proteins). Nevertheless, when the reaction is performed at 37°C, the rate of ATP hydrolysis in the presence of tail-less chromatin drops to levels comparable to those obtained in the presence of naked DNA (Figure IV.3C). These results suggest that tail-less chromatin is unable to protect Rad54p from thermal inactivation, and that the NH_2-

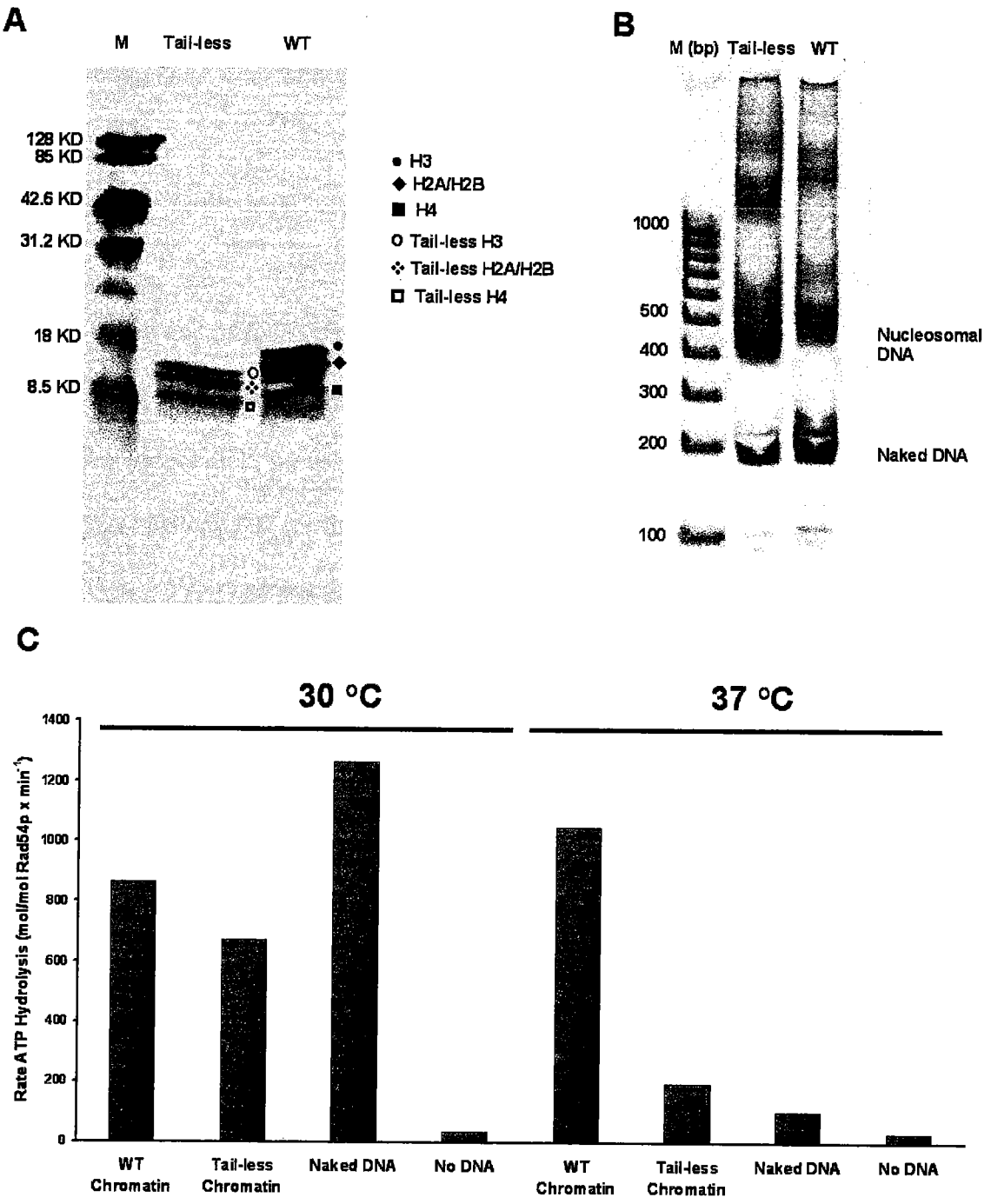


Figure IV.3: Histone NH_2-terminal domains are involved in protecting Rad54p from thermal denaturation

A. SDS-PAGE of tail-less and WT histone octamers purified through a size-exclusion column.

B. Native PAGE of *Eco*RI-digested nucleosomal arrays reconstituted with tail-less or WT histone octamers.

C. Rates of ATP hydrolysis by Rad54p at 30°C and 37°C, in the presence of different co-factors.

terminal domains of the histone proteins play a crucial role in the stabilizing effect that nucleosomal DNA exerts on Rad54p.

Rad54p specifically interacts with histone H3 and H4 NH_2-terminal tails

Given that histone NH_2-terminal tails are required to protect Rad54p from thermal denaturation, we hypothesized that Rad54p directly interacts with these moieties. In order to test this proposition, we asked whether Rad54p could bind to GST-fusion proteins that harbor yeast histone NH_2-termini.

Figure IV.4A shows that Rad54p interacts with GST-H4 (lane 3, residues 1-34 of H4; lane 6, residues 1-15), and GST-H3 (lane 9, residues 1-46). In contrast, Rad54p does not interact with GST-H2A (lane 12) or the GST control (lane 15). Binding to GST-H2B was not determined. The interaction with histone H3 is specific to the proximal half of the NH_2-terminal tail, containing amino acid residues 1-25 (Figure IV.4B, lane 3), since a GST construct encompassing the distal half of the H3 NH_2-terminal tail (residues 21-46) fails to pull down Rad54p (lane 6). This is particularly interesting, since these two H3 peptides possess a similar number of positively charged amino acids (Figure IV.4D), indicating that the binding is strictly sequence-dependent and not due to non-specific electrostatic interactions. Similar results were observed for histone H4 NH_2-terminal tail (Figure IV.4A, lane 6, and data not shown).

Rad51p fails to associate with any of the GST-histone tail fusions (shown for GST - H3 (1-25) in Figure IV.4B, lanes 7 - 9). The ability of Rad54p to bind histone tail

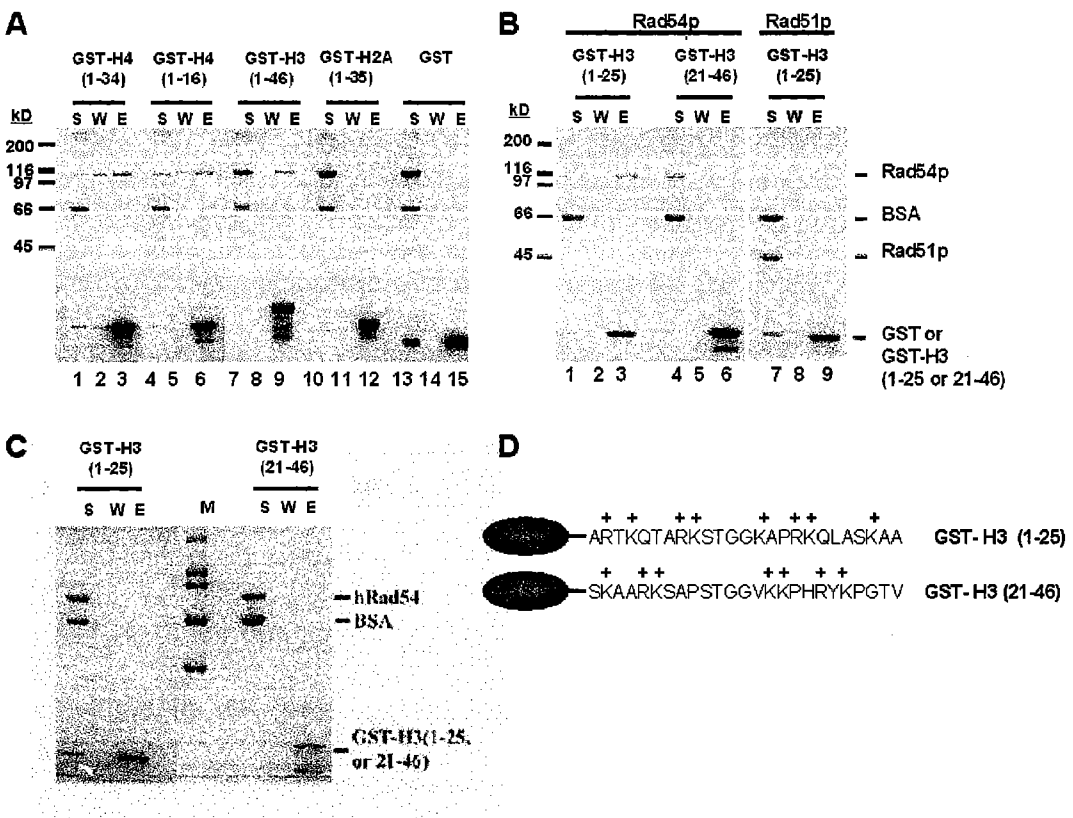


Figure IV.4: Rad54p interacts with histone H3 and H4 NH_2-terminal tails

A. A mixture of Rad54p and BSA was incubated with glutathione Sepharose beads containing either GST or GST fused to the tail portion of histones H2A, H3, or H4. **S**, supernatant; **W**, washed beads; **E**, SDS eluate. Note that Rad54p, but not BSA, binds to GST-H3 and GST-H4. In contrast, Rad54p does not bind GST-H2A.

B. A mixture of Rad54p and BSA was incubated with glutathione Sepharose beads containing GST fused to two different portions (residues 1-25 and residues 21-46) of the histone H3 NH_2-terminal tail. Note that Rad54p interacts with the proximal portion (residues 1-25) of the H3 tail, but no binding to the distal portion (residues 21-46) could be detected. As expected, neither Rad51p nor BSA bind the H3 tail.

C. Human Rad54 fails to bind either portion of histone H3 NH_2-terminal tail.

D. The amino acid sequences of the two H3 tail portions are shown. Positively charged residues are marked by a "+" symbol.

constructs reveals species-specific interactions, since human Rad54 fails to associate with the GST-yeast histone fusion proteins (shown for GST-H3 in Figure IV.4C).

Thus, the results indicate that Rad54p specifically interacts with the proximal portion of histone H3 and H4 NH_2-terminal domains. The direct contact with histone tails emanating from the nucleosome core could account for the ability of nucleosomal DNA to protect Rad54p from thermal denaturation (Figure IV.1).

Chromatin remodeling by Rad54p is independent of histone NH_2-terminal tails

NH_2-terminal domains have been shown to be required for multiple rounds of catalytic chromatin remodeling by the yeast SWI/SNF and RSC complexes (Logie *et al.*, 1999). Moreover, the chromatin remodeling and ATPase activities of ISWI-containing complexes have been found to require nucleosomal histones for maximal stimulation; specifically, the *Drosophila* NURF complex requires residues 16 to 19 of histone H4 (Clapier *et al.*, 2001 and 2002; Hamiche *et al.*, 2001).

In light of these facts, we decided to test whether the histone NH_2-terminal domains are necessary for Rad54p *in vitro* chromatin remodeling activity. To this effect, we performed chromatin remodeling-*Sal*I digestion coupled assays on nucleosomal substrates reconstituted with either full-length recombinant histones (WT chromatin), full-length H2A and H2B combined with the globular domains of H3 and H4 (H3, H4 tail-less chromatin), full-length H3 and H4 combined with the globular domains of H2A and H2B (H2A, H2B tail-less chromatin), or the globular domains of all four core

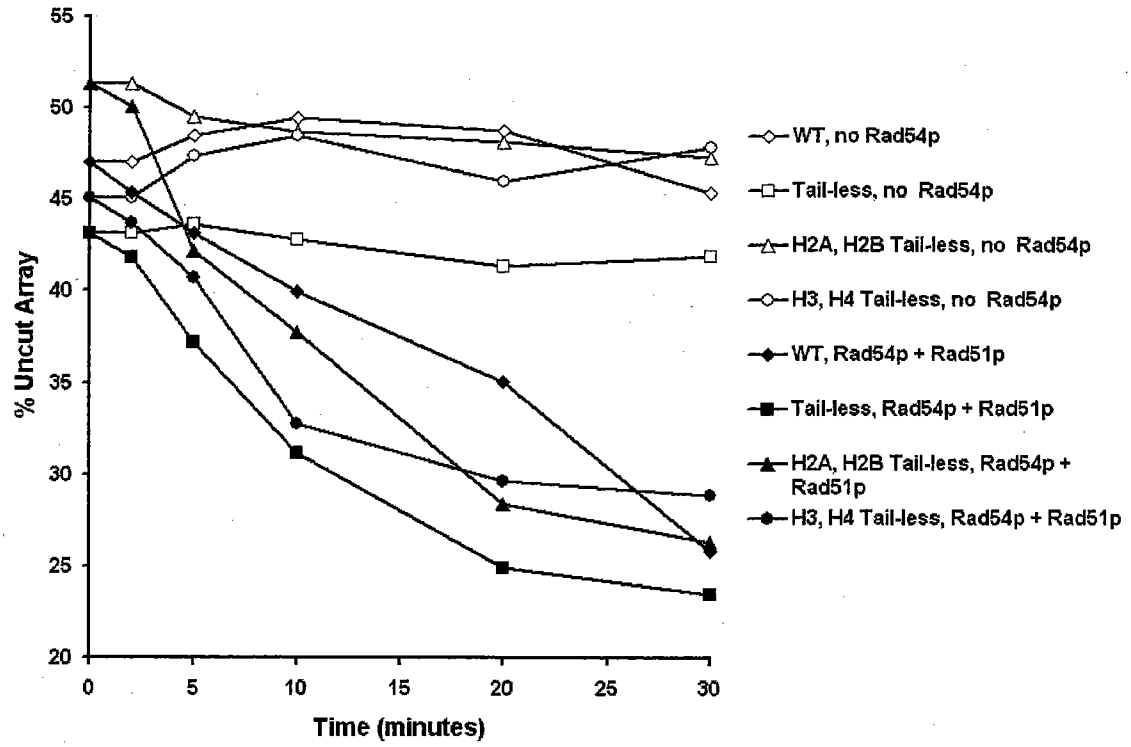


Figure IV.5: Histone NH_2-terminal tails are not essential for Rad54p chromatin remodeling
The different nucleosomal substrates were subjected to *Sal*I digestion for 20 minutes, followed by addition of buffer (open symbols) or 100nM Rad54p + 100nM Rad51p (closed symbols) at time 0. The shown values are the average of 3 independent experiments.

histones (tail-less chromatin). Figure IV.5 shows that in the absence of Rad54p (open symbols), the rate of *Sal*I cleavage is very slow for all nucleosomal species. The addition of Rad54p along with Rad51p leads to dramatically enhanced *Sal*I digestion kinetics (closed symbols), regardless of the nature of the nucleosomal substrate.

The ability of Rad54p to enhance the accessibility of nucleosomal sequences is thus independent of its interaction with histone NH_2-terminal domains. This result is not surprising, since previous studies with yeast SWI/SNF and RSC showed that under similar assay conditions, these complexes efficiently remodel tail-less chromatin (Logie

et al., 1999). Interestingly, both SWI/SNF and RSC require intact histone termini for catalytic chromatin remodeling (i.e., multiple rounds of remodeling). Apparently, the core histone NH_2-terminal tails influence a step of the reaction that occurs after nucleosome remodeling but prior to intermolecular transfer of the remodeling complex to new nucleosomal arrays.

D-loop formation by Rad51p and Rad54p is independent of histone NH_2-terminal tails

To further characterize the functional interactions between Rad54p and nucleosomal histones, we investigated the ability of Rad54p to facilitate the formation of D-loops by Rad51p on nucleosomal substrates that contained full-length histones (WT chromatin), full-length H2A and H2B combined with the globular domains of H3 and H4 (H3, H4 tail-less chromatin), full-length H3 and H4 combined with the globular domains of H2A and H2B (H2A, H2B tail-less chromatin), or the globular domains of all four core histones (tail-less chromatin).

Figure IV.6 depicts the results from a representative single-time point experiment. Rad51p, in the presence of Rad54p, proficiently catalyzes the pairing of a supercoiled, double-stranded DNA molecule ("donor") and a homologous, single-stranded oligonucleotide that is radioactively labeled.

The faster migrating species in Figure IV.6 represents the free, unincorporated oligonucleotide. The higher band represents the heteroduplex product of the Rad51p

reaction. The efficiency of this reaction is similar for naked dsDNA substrates (lane 2), WT chromatin substrates containing full-length versions of all four core histones (lane 3), tail-less chromatin substrates containing the globular domains of all four core histones (lane 4), H3, H4 tail-less chromatin substrates (lane 5), or H2A, H2B tail-less chromatin substrates (lane 6). The slower migrating band that appears in the last three lanes (marked by an asterisk) corresponds to nicked circular DNA, accidentally generated during substrate manipulations.

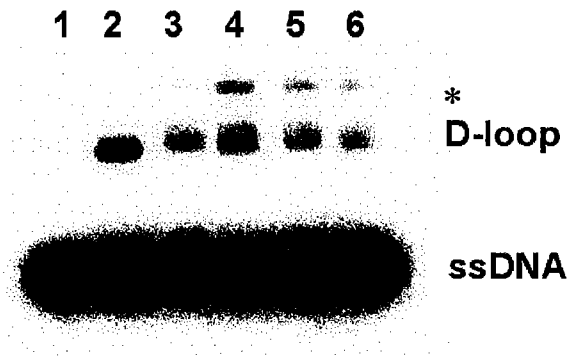


Figure IV.6: D-loop formation on tail-less chromatin substrates

Rad51p/Rad54p drive the recombination of a single-stranded oligonucleotide to a homologous site on a duplex DNA molecule. Lane 1, no duplex donor; lane 2, naked DNA donor; lane 3, WT chromatin substrate; lane 4, tail-less chromatin substrate; lane 5, H3, H4 tail-less substrate; lane 6, H2A, H2B tail-less substrate.

These results indicate that Rad54p does not require histone NH_2-terminal tails to facilitate the Rad51p-driven strand invasion and D-loop formation reaction on chromatin substrates. This is in agreement with our finding that Rad54p efficiently remodels the structure of nucleosomes that lack some or all of the core histone NH_2-terminal domains (Figure IV.5).

DISCUSSION

Rad54p physically interacts with histone NH_2-termini

Here we report that Rad54p specifically interacts with the NH_2-terminal domain of histones H3 and H4. This result suggests that, as proposed for other chromatin remodeling enzymes (Côté *et al.*, 1998; Hassan *et al.*, 2002; Saha *et al.*, 2002), Rad54p contacts both the DNA and the nucleosome core. This interaction was made evident in two different ways: on one hand, purified yeast Rad54p directly binds GST-fusion proteins harboring the NH_2-terminal portion of core histones H3 and H4, but not H2A. The interactions seem to be specific, since related proteins (yRad51, hRad54) fail to bind any of the yeast histone NH_2-terminal tail constructs. Furthermore, Rad54p preferentially binds the proximal segment of the NH_2-terminal domain of histones H3 and H4, encompassing residues 1-25 and 1-16, respectively (Figure IV.4).

The observation that chromatin, but not naked DNA, protects Rad54p from thermal denaturation provides an additional piece of evidence for a physical interaction between Rad54p and histone NH_2-termini (Figure IV.1). The protective effect is attributable specifically to nucleosomal DNA containing intact histone proteins (Figures IV.1 and IV.3). Other nucleoprotein complexes, like RPA-DNA, fail to stabilize Rad54p. The presence of non-relevant proteins, like BSA, is ineffective as well. Furthermore, the incubation of Rad54p with unassembled (free) histones and DNA is not sufficient to keep Rad54p from being thermally inactivated (Figure IV.2).

These results suggest that Rad54p is sensitive to structural elements of the nucleosomal complex, and its interacting surface specifically recognizes both the conformation of the DNA, and the presence of intact histone NH_2-termini. Current efforts are aimed at establishing the regions of Rad54p that are responsible for the contacts with the different nucleosomal components.

Histone NH_2-termini are not essential for Rad54p *in vitro* function

Given that Rad54p interacts with histone H3 and H4 NH_2-terminal tails, we wanted to investigate if these interactions are required for Rad54p activity. We therefore tested whether Rad54p could utilize chromatin substrates that lack histone NH_2-termini in two functional *in vitro* assays. Interestingly, Rad54p does not seem to discriminate between chromatin substrates that contain tail-less histones from those that contain full-length histone proteins in either assay (Figures IV.5 and IV.6). The ability of Rad54p to enhance the accessibility of a nucleosomal restriction site, as well as facilitate the formation of heteroduplex DNA joint molecules on chromatin are absolutely independent of the presence of histone NH_2-terminal tails.

These results do not necessarily mean that the interactions between Rad54p and core histones are not required for the function of Rad54p. In fact, yeast SWI/SNF complex can remodel tail-less chromatin under the same conditions in which Rad54p was tested (Logie *et al.*, 1999, and data not shown). Nevertheless, histone NH_2-terminal domains have been shown to be required for multiple rounds of catalytic chromatin

remodeling by both the yeast SWI/SNF and RSC complexes. Furthermore, both SWI/SNF and RSC complexes exhibit higher apparent affinity for tail-less arrays, presumably due to a slower rate of dissociation (Logie *et al.,* 1999).

Therefore, it is possible that under different assay conditions, Rad54p would show a preference for either type of substrate. We are currently developing more suitable *in vitro* functional tests to investigate the role of histone NH_2-termini in Rad54p activity, substrate binding kinetics, etc.

As mentioned above, specific histone modifications are required for *in vivo* DNA DSB repair. The inability to recapitulate the requirement for histone NH_2-termini for DNA repair *in vitro* (see Figure IV.6) might be indicative of the fact that histone post-translational modifications are required for a different step in the homologous recombination pathway, such as damage signaling, or recruitment of other repair factors that act upstream of Rad51p/Rad54p (reviewed in Iizuka and Smith, 2003). Alternatively, the presence of post-translational modifications on histone NH_2-terminal tails might modulate the affinity of Rad54p for its substrate. Therefore, in the absence of these modifications, Rad54p exhibits a seemingly comparable affinity for WT and tail-less chromatin. A third alternative is that histone NH_2-terminal tails might be involved in stabilizing the heteroduplex intermediate after the strand invasion step of homologous recombination, hence the requirement for histone NH_2-termini cannot be detected in our assays.

CHAPTER V

PERSPECTIVES

Chapter V

Perspectives

SWI/SNF chromatin remodeling: mononucleosomes *versus* nucleosomal arrays

SWI/SNF is an ATP-dependent chromatin remodeling complex that disrupts DNA-histone interactions. Several studies of SWI/SNF activity on mononucleosome substrates have suggested that remodeling leads to altered, accessible nucleosomes, which persist in the absence of continuous ATP hydrolysis. In contrast, we have reported that SWI/SNF-dependent remodeling of nucleosomal arrays is rapidly reversed after removal of ATP. One possibility is that these contrasting results are due to the different assays employed; alternatively, the liability of the SWI/SNF-remodeled state might be different on mononucleosomes versus nucleosomal arrays. To investigate these possibilities, we used a coupled SWI/SNF remodeling-restriction enzyme assay to directly compare the remodeling of mononucleosome and nucleosomal array substrates.

We found that SWI/SNF action causes a mobilization of histone octamers for both the mononucleosome and nucleosomal array substrates, and these changes in nucleosome positioning persist in the absence of continued ATP hydrolysis or SWI/SNF binding. In the case of mononucleosomes the histone octamers accumulate at the DNA ends even in the presence of continued ATP hydrolysis, which results in accessibility of DNA sequences elsewhere on the DNA fragment.

On nucleosomal arrays, the ATP-dependent action of SWI/SNF leads to a dynamic state where nucleosome positions may be continually randomized and all DNA sites throughout the array have increased accessibility. The random positioning of nucleosomes persists after removal of ATP, but inactivation of SWI/SNF is accompanied by an increased occlusion of many restriction enzyme sites. Thus, SWI/SNF action generates persistent changes in nucleosome positioning for both types of substrates. The results indicate, however, that the remodeling of mononucleosomes or nucleosomal arrays does not lead to an accumulation of "altered" nucleosomes that maintain an accessible state in the absence of continuous ATP hydrolysis.

Is ATP-dependent chromatin remodeling equivalent to nucleosome mobilization?

Short-range nucleosome movements in *cis* are a particularly attractive principle by which the packaging of DNA is maintained, yet rendered "transparent" through stochastic exposure of individual DNA segments in the more accessible linker regions between nucleosomes (reviewed in Becker, 2002). Indeed, all classes of ATP-dependent chromatin remodeling enzymes are able to induce nucleosome "sliding", arguing that this reaction is fundamental to the remodeling process (Längst *et al.*, 1999; Hamiche *et al.*, 1999; Whitehouse *et al.*, 1999; Brehm *et al.*, 2000; Guschin *et al.*, 2000; Eberharter *et al.*, 2001; Lorch *et al.*, 2001)

Nevertheless, ATP-dependent remodeling by hSWI/SNF is still proficient on nucleosomes that have been "immobilized" by a single chemical crosslink between the

histone octamer and DNA (Lee *et al.*, 1999; Aoyagi *et al.*, 2002). Furthermore, ySWI/SNF can enhance restriction enzyme accessibility of small circular nucleosomal substrates in which drastic nucleosome movements are sterically hindered (Gavin *et al.*, 2001). Therefore, it seems unlikely that ATP-dependent chromatin remodeling consists solely in nucleosome mobilization. As a matter of fact, the reaction catalyzed by SWI/SNF and other chromatin remodeling complexes seems to comprise two elements: the dynamic, reversible perturbation of histone-DNA interactions, and the translational movement of nucleosomes with respect to the DNA.

In effect, SWI/SNF and other chromatin remodeling complexes seem to generate high-energy intermediates in which the nucleosomal DNA is accessible. The "decay" of these intermediate conformations could lead to the repositioning of histone octamers (Narlikar *et al.*, 2001). In other words, histone octamer movement might be a consequence of the remodeling reaction and not an obligatory feature. Our results correlate with this hypothesis, which predicts that the ATP-dependent remodeling of mononucleosomal particles would lead to persistently disrupted structures (histone octamers "trapped" on DNA ends), whereas remodeling of nucleosomal arrays would generate an accessible structure while the reaction is taking place, and revert to the original, occluded structure, after the reaction is terminated.

ATP-dependent chromatin remodeling involves a DNA translocation mechanism

The finding of Owen-Hughes and colleagues that a number of members of the SWI2/SNF2 family of ATP-dependent chromatin remodeling factors are able to generate superhelical torsion on linear DNA provided support to an exciting new model (Havas *et al.*, 2000). According to this model, chromatin remodeling involves rotating the DNA, causing it to under- or over-wind and/or bulge on the octamer surface (reviewed in Havas *et al.*, 2001; Kassabov *et al.*, 2003). The repeated generation of such topological alterations and their diffusion along the DNA double helix could result in the disruption of histone-DNA interactions and the sliding of the histone octamers. Gavin *et al.* (2001) showed that in fact, these changes in DNA topology are required for ySWI/SNF chromatin remodeling.

The topology of a double-stranded DNA molecule is described by its linking number (Lk), which describes the number of times that the two strands of the DNA double helix wind around each other. Lk is made up of two geometrical parameters, twist (Tw) and writhe (Wr) (Cozzarelli *et al.,* 1990). Tw and Wr give information about the shape of the DNA. The local winding of the two strands of the double helix is described by Tw, whereas Wr describes the number of times that the axis of the double helix winds around itself. The relationship between these three parameters is expressed by the equation Lk= Tw + Wr. To mechanistically interpret the change in topology induced by ATP-dependent chromatin remodeling enzymes, it is necessary to determine whether the twist or the writhe is being modified (Ristic *et al.,* 2001).

Protein-constrained ΔTw and ΔWr might result from different DNA-binding modes of the protein. For example, proteins induce a ΔWr by wrapping the DNA around their surface; a ΔTw can be induced by stretching the helix in a protein-stabilized filament. Alternatively, the ΔLk generated by chromatin remodeling factors could be due to the introduction of unconstrained supercoils on the DNA, either by directly twisting the double helix along its long axis, or by a protein translocation mechanism.

The former mechanism (twisting) has been greatly debated in the past few years. According to this model, chromatin remodeling enzymes would "screw" the DNA along the surface of the histone octamer, thus advancing the nucleosome core along the DNA one base pair at a time (Whitehouse *et al.*, 2000). The DNA would maintain its writhe, and the majority of histone-DNA interactions would be maintained at any point in time. However attractive, this model seems to be flawed. hSWI/SNF was shown to efficiently slide nucleosomes on substrates containing branched and nicked DNA structures, which sterically hinder rotation of the DNA on the octamer surface and inhibit retention of torsional stress within the helix (Aoyagi and Hayes., 2002). Likewise, ISWI was shown to catalize nucleosome sliding on nicked substrates (Längst and Becker, 2001b). Moreover, electronic microscopy imaging (Bazett-Jones *et al.,* 1999) and high-resolution mapping of histone-DNA contacts before and after ySWI/SNF remodeling reaction support a model in which segments of DNA are transiently peeled off the surface of the histone octamer (Kassabov *et al.,* 2003)

The alternative mechanism, translocation along the DNA, could explain the topological changes that result from ATP-dependent chromatin remodeling, and the

generation of numerous, unstable intermediates of the histone octamer-DNA complex. The "pulling" or "pushing" of the DNA to or from the nucleosome by translocation would cause it to under- or over-wind and/or transiently bulge from the surface of the histone octamer, freeing it from the inhibitory histone interactions and thus momentarily enhancing the accessibility for DNA binding proteins like transcription factors or endonucleases (reviewed in Martens and Winston, 2003). Recently, two groups reported that the yeast RSC and *Drosophila* ISWI chromatin remodeling complexes translocate along the DNA, thereby destabilizing histone-DNA contacts and enhancing the accessibility of nucleosomal DNA (Saha *et al.*, 2002; Whitehouse *et al.*, 2003).

Both SWI/SNF and Rad54p (a member of the SWI2/SNF2 family of ATPases involved in DNA DSB repair) have been shown to introduce changes in the topology of DNA (SWI/SNF: Havas *et al.,* 2000; Gavin *et al.*, 2001; Rad54p: Tan *et al.*, 1999; Petukhova *et al.*, 1999; Van Komen *et al.,* 2000; Ristic *et al.,* 2001). Here we present evidence supporting a translocating mechanism for both these factors. The translocation of these enzymes along the DNA would account for the ATP-dependent disruption of nucleosome structure, nucleosome repositioning and enhanced transcription factor binding/endonuclease accessibility observed *in vitro* (Whitehouse *et al.*, 1999; Havas *et al.*, 2000; Gavin *et al.*, 2001; Kassabov *et al.*, 2003; reviewed in Pazin and Kadonaga, 1997; Flaus and Owen-Hughes, 2003).

The workings of ATP-dependent chromatin remodeling

Owen-Hughes and colleagues proposed that ATP-dependent chromatin remodeling complexes might be tethered to nucleosomes by direct interaction with histone NH_2-terminal domains, and from this position, apply torsional strain on the DNA and cause it to over- or under-wind and/or bulge on the octamer surface (Flaus and Owen-Hughes, 2001).

The combination of this model and the hypothesis of DNA translocation generates a new picture, in which chromatin remodeling factors would initiate the remodeling reaction by destabilizing histone-DNA interactions at the entry/exit point by translocating along the DNA (Saha *et al.*, 2002). Considering that the remodeling factor remains in a fixed position, bound to both the histone octamer and the DNA, the ATP-dependent translocation would cause translational movement of the DNA accompanied by DNA twist. The DNA emerging from the remodeling factor would be topologically constrained by the remodeling enzyme itself and the first histone-DNA contact (Becker and Hörz, 2002). This small DNA domain would become under- or over-twisted (>10.6 bp/turn) due to the threading of the DNA through the remodeling factor. The combination of translation and twist would provide the energy required for breaking the first histone-DNA contact. In addition, the DNA is likely to become distorted by a writhe component as well as twist, due to the interconvertible nature of these geometrical parameters (Figure V.1). This action, repeated over time, could have the effect of pushing the DNA off the surface of the nucleosome, and generate a DNA wave consisting of a small

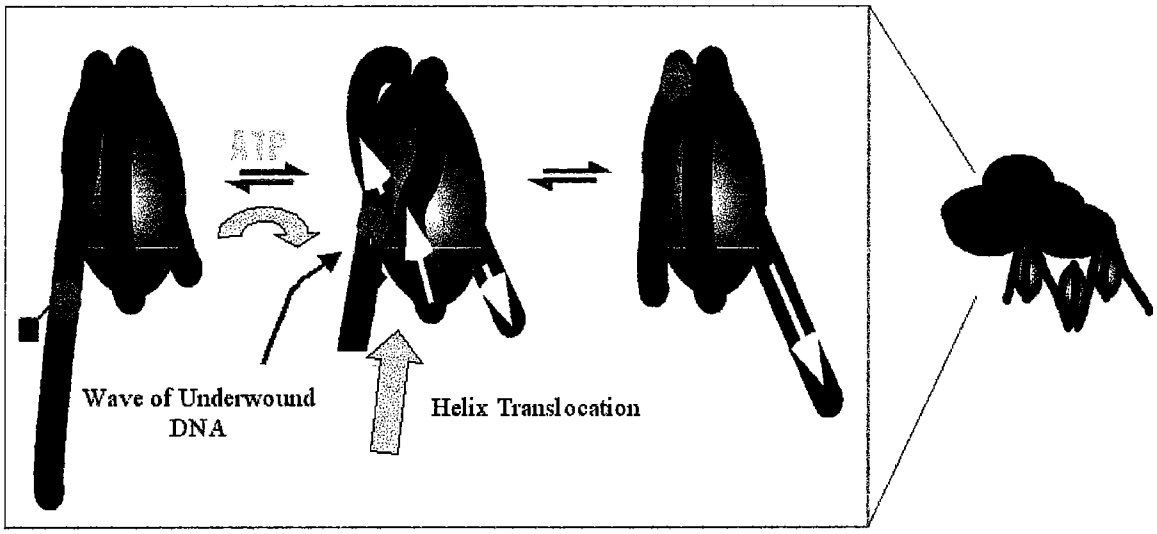


Figure V.1: A model for chromatin remodeling by translocation
In this model, the energy of ATP hydrolysis is used to drive the translocation of DNA across the surface of the histone octamer, resulting in distortion of the DNA structure. The distorted DNA may separate from the nucleosome surface, forming a bulge that is transmitted along the DNA, culminating in the repositioning of the histone octamer. (Adapted from Smith and Peterson, 2002)

segment of DNA that cannot lie in its preferred position on the nucleosome because of either its writhe or twist properties (Saha *et al.*, 2002).

The DNA wave or "bulge" could re-associate at a different site on the histone octamer surface and/or be transmitted along the length of the DNA by twist or writhe diffusion. When the DNA wave transiently uncovers the binding site for a transcription factor or endonuclease, it could be "caught" by the binding factor, providing a mechanistic basis for the transient enhancement in nucleosomal DNA accessibility observed in chromatin remodeling reactions.

Our data, along with that of other groups, suggests that the force of translocation might be the most important component of the DNA wave. Translocation can provide

sufficient mechanical force to break histone-DNA contacts, even in the case of nicked substrates, in which the torsional stress is liberated by the free rotation of one of the DNA strands. Moreover, the translational displacement of DNA could create intermediate nucleosomal structures with high potential energy (stored in the broken histone contact(s) and in DNA topology) that would be consistent with many of the properties of "remodeled chromatin".

Finally, if a 1bp DNA "bulge" were to migrate over the 147bp steps equivalent to the length of nucleosomal DNA, this would achieve an apparent nucleosome mobilization of 1bp (Flaus and Owen-Hughes, 2003). Each individual step in this wave propagation is reversible (the bulge can travel stochastically backward and forward along the DNA) and must transit through all the histone-DNA contact points. Therefore, the ability of a chromatin remodeling enzyme to induce nucleosome mobilization will be clearly dependent on the processivity and directionality of the translocation event, as well as the size of the bulge generated.

Chromatin remodeling during homologous recombination

In Chapters III and IV, we report that Rad54p, a conserved eukaryotic protein required for DNA repair by homologous recombination, presents many of the biochemical properties of an ATP-dependent chromatin remodeling enzyme. Rad54p enhances the accessibility of nucleosomal DNA, introduces torsional stress and displaces a pre-formed triplex from linear DNA, both naked and nucleosomal. Furthermore,

Rad54p efficiently displaces a triplex from nicked substrates, suggestive of a mechanism in which the translational effect is more relevant than the torsional strain introduced on the DNA double helix. In addition, Rad54p has ATPase kinetics diagnostic of a DNA translocation mechanism. Finally, Rad54p interacts with histone NH_2-termini, consistent with the general chromatin remodeling mechanistic model presented above.

Importantly, we have been able to recapitulate homologous DNA pairing and heteroduplex molecule formation on nucleosomal substrates, using purified yeast Rad51 and Rad54 proteins. These reactions are central to *in vivo* homologous recombination pathways, like the recombinational DNA DSB repair pathway. Our results suggest that one of the roles of Rad54p *in vivo* consists in facilitating the Rad51p-driven DNA recombination reaction by altering the chromatin structure at the homologous donor site. This hypothesis is backed by a genetic study that indicated that a subgroup of the Rad52 family of proteins, involved in DNA repair by homologous recombination (Rad51, Rad54, Rad55, Rad57), was only required when the homologous donor site was embedded in chromatin structure, but not when it was placed on a plasmid (Sugawara *et al.*, 1995). Furthermore, Rad54p is the only member of the Rad52 group of proteins for which there is no structural or functional homologue in prokaryotes (Petukhova *et al.*, 1999).

However, it is possible that Rad54p utilizes a chromatin remodeling mechanism that differs from the one used by other factors, like SWI/SNF. Even though Rad54p (alone or in conjunction with Rad51p) and SWI/SNF present similar activities in most of the biochemical and functional assays presented in this thesis, we have not been able to

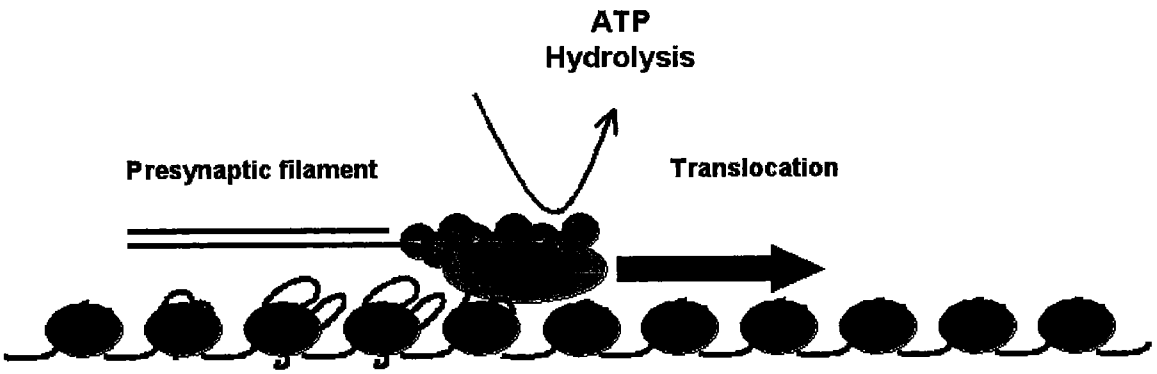


Figure V.2: A model for Rad54p as a DNA tracking chromatin remodeling enzyme
One possibility is that Rad54p tracks along the donor site, dragging the Rad51p nucleofilament along, and facilitating the homology search and/or strand invasion steps of HR by "loosening" the chromatin structure.

detect nucleosome mobilization as a result of Rad54p action on either nucleosomal arrays (Chapter IV) or mononucleosomal substrates (data not shown).

This disparity in the activities of Rad54p and SWI/SNF might stem from the fact that Rad54p requires other factors to reach its maximal activation, or processivity. The extent of nucleosome sliding depends on the size of the DNA "bulge" created by the remodeling enzyme, and its kinetics of diffusion on the surface of the nucleosome. Perhaps Rad54p is assisted *in vivo* by an unknown factor that stabilizes the interaction of Rad54p with the nucleosomal substrate, or it enhances its ability to pump larger DNA bulges across the nucleosome.

Alternatively, it is possible that Rad54p interacts with its substrate differently than SWI/SNF, and instead of inducing the translocation of DNA, Rad54p tracks along the DNA (Figure V.2). In this model, Rad54p might "pull" the Rad51-ssDNA nucleoprotein filament along the chromatin fiber, leading to changes in nucleosomal DNA topology and

DNA accessibility. Such a DNA tracking mechanism might play a key role in facilitating both the search for homology as well as the strand invasion step.

Although speculative, this model is consistent with our results, and with other reports indicating that Rad54p translocates along DNA (Van Komen *et al.*, 2000; Ristic *et al*, 2001). Furthermore, this model predicts that Rad54p would alter local histone-DNA interactions, without drastically affecting nucleosome positioning. Despite great progress seen in the fields of DNA repair and recombination in the past few years, many questions are still unanswered, and much more work will be needed to fully understand the intricacies of the process of recombinational DNA repair *in vivo*.

APPENDIX I

ISOLATION OF *Saccharomyces cerevisiae* Rad54p BY

TANDEM AFFINITY PURIFICATION

APPENDIX I

Isolation of Rad54p by Tandem Affinity Purification

INTRODUCTION

The repair of DNA DSB breaks in *Saccharomyces cerevisiae* and higher eukaryotes requires the members of the RAD52 epistasis group, including Rad51p, Rad52p, and Rad54p. The fact that these proteins interact in yeast two-hybrid assays led to the suggestion that repair of DSBs in yeast may be performed by a multiprotein complex, termed a recombinosome (Milne and Weaver, 1993; Hays *et al.* , 1995; Jiang*et al.*, 1996; Clever *et al.*, 1997).

In order to test the hypothesis that the recombinational repair protein Rad54 resides in a stable complex in the nucleus, we fused a Tandem Affinity Purification (TAP) tag to the C-terminus of the yeast Rad54 protein. This tag consists of Calmodulin Binding Protein (CBP), followed by a specific TEV cleavage site and three copies of the protein A coding sequence.

The standard purification protocol involves two successive affinity purification steps. In the first step, IgG-sepharose beads are used to bind the protein A fusion polypeptide present in whole cell extracts. The fusion protein is then cleaved off the beads with TEV protease, and this material is bound to calmodulin-agarose beads (Tasto

et al., 2001). The purified polypeptides are eluted from the beads by addition of EGTA. The purified polypeptides are then resolved by SDS-PAGE, and visualized by Western Blot analysis, or if required, silver staining and mass spectrometry analysis.

MATERIALS AND METHODS

Design of yeast strains

CY924 (HMLα, MAT**a**, HMR**a**, Δho, ade1, leu2, lys5, trp1::hisG, ura3-52, ade3::Gal10:HO), obtained from J. Haber, was transformed with a PCR product encompassing the TAP tag, flanked by sequences homologous to the 3 end of the endogenous RAD54 gene, and the immediately downstream region (60bp of homology on each side of the TAP tag). This DNA fragment was recombined into the endogenous RAD54 locus, yielding a 3 -tagged version of the endogenous gene.

CY915 (Δho,Δhml::ADE1, MATα, Δhmr::ADE1, ade1-110, leu2, 3-112, lys5, trp1::hisG, ura3-52, ade3::Gal10:HO, rad54::LEU2) was obtained from J. Haber, and used as a negative control.

Purification of TAP-Rad54p

6-liter cultures were grown in YP medium (1% yeast extract, 2% Bacto-Peptone, BD) containing 2% glucose until the OD_{600} reached ~1.0. Cells were treated with 0.05% MMS for 5 hours, to a final OD_{600} of ~1.5-2.0. The cells were harvested by centrifugation, washed once, and resuspended with extraction (E) buffer (20mM Hepes, pH: 7.4, 150mM NaCl, 10% glycerol, protease inhibitors). The cells were lysed in a 50-ml bead beater with zirconium beads (Biospec Products Inc.), with 6 pulses of 20 seconds each, separated by 2 minutes on ice. After lysis, the lysate was cleared in a Ti-45 rotor

(Beckman) at 40,000rpm for 45 minutes at 4°C. The supernatant was labeled whole-cell extract (WCE).

♦*Standard TAP purification*

The WCE was incubated for 2 hours at 4°C with 600µl of IgG-agarose (Sigma), followed by several washes with E buffer. The bound material was incubated O.N. at 4°C with 300U of TEV protease (Invitrogen) in the presence of 1mM DTT. The eluate was collected, and incubated for 2 hours at 4°C with 600µl calmodulin affinity resin (Stratagene) in the presence of 2.5mM $CaCl_2$. The beads were then washed several times with E buffer + $CaCl_2$, and the bound proteins were eluted with E buffer containing 10mM EGTA.

♦*Modified TAP purification*

The WCE was incubated for 2 hours at 4°C with 600µl calmodulin affinity resin (Stratagene) in the presence of 2.5mM $CaCl_2$. The beads were then washed several times with E buffer + $CaCl_2$, and the bound proteins were eluted with E buffer containing 10mM EGTA. The eluate was then incubated for 2 hours at 4°C with 600µl of IgG-agarose (Sigma), followed by several washes with E buffer. The bound material was eluted with 0.5M acetic acid.

Western Blots

The purification fractions were resolved in 8% PAGE, transferred to nitrocellulose membrane (Protran, Schleicher and Schuell), and probed with commercially available α-Rad51 or α-Rad52 antibodies (Santa Cruz Antibodies), or with

affinity-purified α-Rad54 antibodies (Petukhova *et al.,* 1998). Alternatively, the TAP-Rad54 protein was visualized with α-IgG secondary antibodies that recognize the protein A component of the tag.

RESULTS

In order to isolate endogenous Rad54p and potential interacting partners, we tagged the RAD54 gene with a tandem affinity purification (TAP) sequence. Three independent clones were tested for their ability to protect cells from a DNA damaging agent, methyl methanesulfonate (MMS). As shown in Figure A.1,none of the three clones interfered with Rad54p activity *in vivo*. Interestingly, clones 2 and 3 are more resistant to MMS than the wild-type (untagged) strain. The reason behind this phenomenon might be that Rad54p is stabilized by the TAP tag.

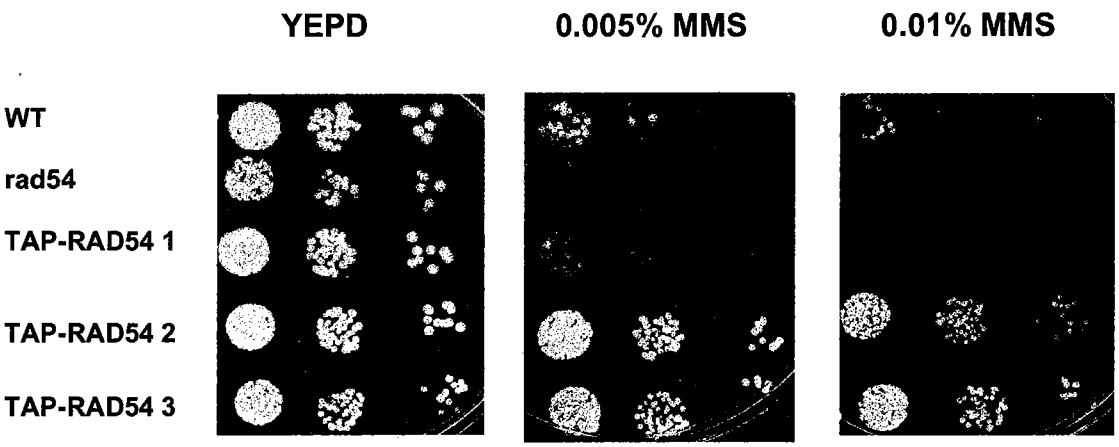


Figure A.1: The TAP tag does not interfere with Rad54p *in vivo* function.
Consecutive dilutions of strains harboring a WT RAD54 allele, a rad54 null-mutant allele, and three independent clones harboring tagged TAP-RAD54 alleles were spotted on YEPD media, or YEPD containing different concentrations of MMS.

Since the treatment of *Saccharomyces cerevisiae* with methyl methanesulfonate (MMS) increases Rad54p expression levels ~10-fold (Cole *et al.*, 1987; Clever *et al.*, 1999), we performed the purification process from both untreated and MMS-treated cell cultures, not only to obtain higher yields, but also to determine whether Rad54p resides in a pre-formed, stable complex, or in a DNA damage-induced complex.

The standard TAP purification method (see Materials and Methods section) produced extremely low yields. For clones 1 and 2, this was due to the low efficiency of the TEV cleavage step. For clone 3, it was a consequence of the low efficiency of the elution step from the calmodulin resin. These difficulties probably stem from errors in the PCR product used for tagging the endogenous gene.

Consequently, we modified the protocol, by reversing the order of the purification steps (see Materials and Methods section). Furthermore, we eluted the material from the last affinity binding step (IgG beads) with an acid treatment, instead of proteolytically cleaving the TAP-fusion protein. This modified protocol worked well for clones 1 and 2, although the yields were never extensive.

In order to determine whether Rad54p resides in a native complex, we probed a Western blot including samples from several purification steps with antibodies that recognize Rad54p, Rad51p or Rad52p. Figure A.2 shows that all of the detectable Rad51p flows through the first purification step, the calmodulin affinity column. About 50% of the Rad52p present in the WCE flows through the calmodulin binding step. The other 50% binds the column, but in a non-specific fashion, since it remains bound after

EGTA treatment (lane 3), and it is undetectable in the eluate. Rad54p, on the other hand, is efficiently retained by the calmodulin affinity column (no Rad54p detectable in the flow-through fraction, lane1). The elution step is not complete, with ~50% of the detectable protein coming off the column after EGTA treatment (lane 4). These results indicate that these three factors do not co-purify, suggesting that, at least under the conditions used, they are not part of a complex.

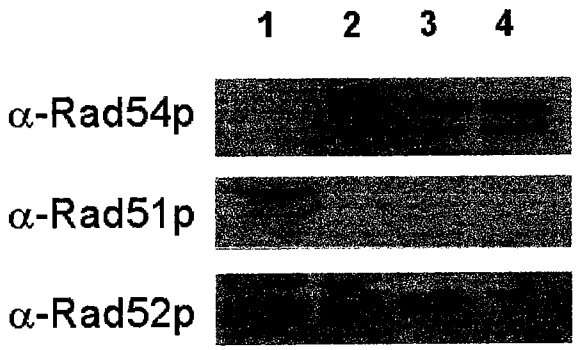


Figure A.2: DNA repair proteins Rad54, Rad51 and Rad52 are not part of a stable complex

Lane 1, material that does not bind to the calmodulin affinity column (flow-through); lane 2, material bound to the calmodulin affinity column; lane 3, material that does not elute from the calmodulin affinity column after EGTA treatment; lane 4, material that elutes from the calmodulin affinity column.

DISCUSSION

In spite of previous reports suggesting the existence of a native recombinosome complex, our results indicate that, at least under the conditions used, yeast Rad51p, Rad52p, and Rad54p do not reside in a stable complex. This is still true in cells that have been challenged with high concentrations of a DNA alkylating agent, MMS, which induces the formation of multiple double-strand breaks on the DNA.

It is possible that a complex exists, but the interactions between its components are weak and do not tolerate the extraction conditions. It is also possible that a complex forms only at a given time during the cell cycle, and is not detectable in asynchronously growing cultures. Alternatively, it is a possibility that the MMS-treatment induces the expression of Rad54p much more than that of Rad51p, Rad52p. Therefore, the majority of the tagged Rad54 polypeptide might be free because its interacting partners have been titrated out. Another possibility is that there is a recombinosome and that Rad54p is not part of it.

However, a study reported by Essers and colleagues (2002) supported our results. This group monitored the diffusion rates of Rad51p, Rad52p, and Rad54p in the yeast nucleus as a result of DNA damage, and found that they are extremely dissimilar. The repair proteins accumulate into foci at sites of DNA damage induction. These foci are dynamic structures of which Rad51p is a stably associated core component, whereas Rad52p and Rad54p rapidly and reversibly interact with the structure. This result suggests that the majority of the repair proteins are not part of a multi-protein complex,

consistent with our finding that Rda51p, Rad52p and Rad54p proteins do not co-elute in our purification scheme. Even though these factors functionally and physically interact *in vivo*, it now seems evident that they do so in a dynamic fashion, without formation of a recombinosome .

APPENDIX II

PUBLICATIONS

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SWI-SNF-Mediated Nucleosome Remodeling: Role of Histone Octamer Mobility in the Persistence of the Remodeled State

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SWI-SNF is an ATP-dependent chromatin remodeling complex that disrupts DNA-histone interactions. Several studies of SWI-SNF activity on mononucleosome substrates have suggested that remodeling leads to novel, accessible nucleosomes which persist in the absence of continuous ATP hydrolysis. In contrast, we have reported that SWI-SNF-dependent remodeling of nucleosomal arrays is rapidly reversed after removal of ATP. One possibility is that these contrasting results are due to the different assays used; alternatively, the lability of the SWI-SNF-remodeled state might be different on mononucleosomes versus nucleosomal arrays. To investigate these possibilities, we use a coupled SWI-SNF remodeling–restriction enzyme assay to directly compare the remodeling of mononucleosome and nucleosomal array substrates. We find that SWI-SNF action causes a mobilization of histone octamers for both the mononucleosome and nucleosomal array substrates, and these changes in nucleosome positioning persist in the absence of continued ATP hydrolysis or SWI-SNF binding. In the case of mononucleosomes, the histone octamers accumulate at the DNA ends even in the presence of continued ATP hydrolysis. On nucleosomal arrays, SWI-SNF and ATP lead to a more dynamic state where nucleosomes appear to be constantly redistributed and restriction enzyme sites throughout the array have increased accessibility. This random positioning of nucleosomes within the array persists after removal of ATP, but inactivation of SWI-SNF is accompanied by an increased occlusion of many restriction enzyme sites. Our results also indicate that remodeling of mononucleosomes or nucleosomal arrays does not lead to an accumulation of novel nucleosomes that maintain an accessible state in the absence of continuous ATP hydrolysis.

Eukaryotic chromatin has seen a rebirth of intense study over the past few years. Foremost among the biochemical reactions impinging on chromatin structure is ATP-dependent chromatin remodeling, which leads to an enhanced accessibility of nucleosomal DNA (for recent reviews, see references 18, 19, and 48). This reaction plays a key role in the regulation of transcription by RNA polymerase II, and it has been proposed to be a prerequisite for a variety of other cellular processes that require access to the chromatin template (for reviews, see references 35 and 48). In addition to ATP-dependent nucleosome remodeling, multisubunit complexes that can acetylate (12, 31, 39, 41) or methylate (6) histone and nonhistone proteins have the potential to directly modify chromatin structure and function.

A host of ATP-dependent chromatin remodeling complexes have been identified via biochemical fractionation of cell extracts, yeast genetics, or genome database mining (2, 5, 7, 17, 20, 23, 33, 44, 46, 47, 49, 50, 53, 55). A hallmark of these multisubunit complexes is that they contain a member of the SWI2/SNF2 subfamily of DNA-stimulated ATPases. Seventeen members of the SWI2/SNF2 family have been identified in the yeast genome (10, 38), and to date, four of these ATPases have been purified as subunits of distinct chromatin remodeling complexes (SWI-SNF [7]; RSC [5]; ISW1 and ISW2 [47]). Additional ATP-dependent remodeling complexes have been identified in *Drosophila* (ACF [17], CHRAC [49]), NURF ([4], and brm [33]), humans (hSWI-SNF [20], NURD [44, 53, 55], and RSF [25]), and frogs (Mi-2 [50]). Each of these complexes appears to catalyze a reaction in which the energy of ATP hydrolysis is used to weaken histone-DNA interactions which leads to an increase in nucleosomal DNA accessibility. In the case of the yeast SWI-SNF, *Drosophila* brm, and human SWI-SNF complexes, this reaction is required for transcriptional regulation of target genes in vivo (18).

Many of the in vitro studies that have focused on the mechanism of ATP-dependent remodeling have used mononucleosome substrates. In these cases, ATP-dependent remodeling is often scored as a disruption of the DNase I digestion pattern of rotationally phased nucleosomal DNA or an enhancement of transcription factor binding to nucleosomal sites. For yeast SWI-SNF, RSC, and human SWI-SNF complexes, the remodeled state of mononucleosomes is stable after removal of ATP or SWI-SNF (13, 16, 27, 42). Furthermore, this persistent disruption of mononucleosome structure is accompanied by formation of a novel, stably remodeled species that resembles a dinucleosome and retains a full complement of histones and DNA (27, 42; reviewed in references 18 and 45). In addition, under some conditions RSC can also generate persistent changes in mononucleosome accessibility by transferring the histone octamer onto acceptor DNA (28).

In contrast to studies with mononucleosomes, ATP-dependent remodeling of nucleosomal array substrates by yeast SWI-SNF and RSC leads to the formation of an unstable remodeled state that requires continuous ATP hydrolysis (24, 25; C. Logie, L. Boyer, and C. L. Peterson, unpublished data). In these studies, remodeling was monitored by quantifying the en-

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hanced kinetics of restriction enzyme digestion of a unique *SalI*/*Hin*cII site located within the central nucleosome of a positioned nucleosomal array. Addition of a remodeling enzyme leads to a 30- to 40-fold increase in digestion kinetics, but removal of ATP results in almost immediate reversal of the *SalI*/*Hin*cII restriction site to the occluded state.

The apparent differences in stability of the remodeled state between mononucleosome and nucleosomal array substrates might be due to the different assays used to detect remodeling events; alternatively, remodeling of mononucleosome substrates may yield novel, stable products that do not occur on nucleosomal arrays. Here we have tested these two possibilities by directly comparing the labilities of remodeled mononucleosomes and nucleosomal arrays by using a quantitative restriction enzyme coupled remodeling assay. Using this assay, we confirm that the remodeling of mononucleosome substrates by yeast SWI-SNF leads to a persistent accessibility of DNA that was previously occluded by a nucleosome, whereas the enhanced restriction enzyme accessibility of nucleosomal arrays appears to be more labile. We then show that the stable, accessible state of mononucleosomes correlates with the SWI-SNF-dependent movement of the histone octamer to the DNA ends. Surprisingly, SWI-SNF and ATP also lead to a randomization of nucleosome positions on the array substrate which persists after removal of ATP. Thus, SWI-SNF remodeling leads to persistent changes in nucleosome positioning on both mononucleosome and nucleosomal array substrates. However, whereas the randomization of nucleosomes within the array persists in the absence of ATP, the enhanced accessibility of nucleosomal restriction enzyme sites does not persist. We propose that SWI-SNF and ATP can establish a dynamic state of continuous nucleosome mobilization only on nucleosomal arrays and that this fluid chromatin state is required for enhanced restriction enzyme accessibility.

MATERIALS AND METHODS

Plasmid constructions. A partial *Eco*RI digestion was carried out on pCL7b (24) to release a DNA fragment encompassing five head-to-tail repeats of the 208-bp *Lyetechinus variegatus* 5S ribosomal DNA (rDNA) nucleosome positioning element. This *Eco*RI fragment was then cloned into the unique *Eco*RI site of pCL6 (24), to yield pCL113, where the last repeat bears the unique *SalI*/*Hin*cII site and is flanked by a unique *Pst*I site distal to the *Not*I site of pBS-SKII (+). To generate pCL114 (which contains a total of seven 5S repeats), a single, blunt-ended, *Eco*RI 208-bp *L. variegatus* 5S rDNA nucleosome positioning element was subcloned into the filled-in *Xba*I site of pCL113 to introduce a wild-type 208-bp rDNA repeat between the *Not*I site and the modified *SalI*/*Hin*cII site-bearing repeat of pCL113. pCL115 contains a single 5S repeat and was generated by fill-in of the *Sal*I site located in the polylinker of pCL6 so as to leave a unique *SalI*/*Hin*cII site at the predicted dyad axis of the single 5S repeat.

Reagent preparation and nucleosome reconstitutions. SWI-SNF and histone octamers were purified as described elsewhere (25). Apyrase was from Sigma (A-6410) and was diluted to a concentration of 1 U/μl as described elsewhere (16). Nucleosomal array DNA templates (*Not*I-*Eco*RV fragments derived from pCL7b [24], pCL113, or pCL114) were labeled by the Klenow polymerase fill-in reaction using [α-^{32}P]dCTP (6,000 μCi/mmol; Amersham). Linear nucleosomal arrays were reconstituted at a ratio of 1.0 to 1.3 octamers per 5S DNA repeat, and samples were characterized by *Eco*RI analysis as previously described (25). Circular minichromosomes were reconstituted as for the linear arrays, but in this case 2 μg of negatively supercoiled pCL115 and 2 μg of purified chicken histone octamers were assembled in 100-μl reactions (a histone/DNA ratio of ~1 octamer/150 bp).

Mono- and dinucleosomes were obtained by digestion of the appropriate labeled nucleosomal arrays with *Pst*I. The *Pst*I digestions were carried out 3 μg of the corresponding labeled nucleosomal array (1.5×10^6 cpm), 100 U of *Pst*I (New England Biolabs), 10 mM NaCl, 5 mM Tris-HCl, 1 mM $MgCl_2$, and 0.1 mM dithiothreitol (DTT) in a final volume of 200 μl. After 2 h at 37°C, the reactions were loaded on top of a 15-ml 10 to 30% linear glycerol gradient containing 1% bovine serum albumin (BSA), 0.2% phenylmethylsulfonyl fluoride, 0.1% Tween 20, 1 mM DTT, 10 mM Tris-HCl (pH 8.0), and 125 mM NaCl. The gradients were centrifuged for 17 h at 28,000 rpm in an SW28 rotor. The gradients were fractionated into 500-μl fractions, the position of the labeled DNA was determined by scintillation counting, and 1/30 of the radioactive fractions (~10^4 cpm) was analyzed on 4% native polyacrylamide gels. Mononucleosomes (216 bp) were detected in fractions 9 to 12; dinucleosomes (427 bp) were detected in fractions 14 to 19. Mononucleosomes reconstituted on the 427-bp DNA fragment were detected in fractions 9 to 12 of the dinucleosome gradient. For reconstitution of the 154-bp 5S mononucleosome, an *Nru*I-*Bam*HI fragment (154 bp) from pCL113 was used in a fast salt dilution reconstitution protocol (17). After the reconstitution procedure, mononucleosomes were purified through a 5-ml 5 to 30% linear glycerol gradient, and 150-μl fractions were collected, counted, and analyzed on a 4% native acrylamide gel.

Reaction conditions. For the coupled SWI-SNF reactions, reconstituted arrays, mononucleosomes, or dinucleosomes (0.3 to 2 nM, final DNA concentration) were mixed with 3 nM SWI-SNF complex and 10 U of restriction enzyme in a buffer containing final concentrations of 125 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl (pH 7.9), 100 μg of BSA per ml, and 3% glycerol. Where indicated, ATP was added to a final concentration of 1 mM, and 0.5 U of apyrase was added per 50 μl of reaction mixture. The reactions were incubated at 37°C. Under these conditions, removal of ATP by apyrase was complete in <2 min. At the indicated time points, an aliquot of the reaction was vigorously mixed for 10 s with 25 μl of Tris-EDTA and 50 μl of a 1:1 solution of phenol-chloroform. After this extraction, samples were treated with 1 mg of proteinase K per ml for 1 h at 37°C. The purified DNA fragments were resolved either by nondenaturing agarose gel electrophoresis in the presence of ethidium bromide or on 4% native acrylamide gels. For SWI-SNF remodeling reactions containing the circular minichromosomes, DNA topoisomers were resolved on 20-cm 1.75% agarose gels in 40 mM Tris (pH 8.0)–30 mM $NaPO_4$–1 mM EDTA at 40 V for 2 days (13), followed by Southern blotting and probing with pCL115 sequence. The fractions of topoisomers and of cut and uncut DNA were obtained by phosphorimager analysis using the ImageQuant software.

MNase digestion. Reconstituted arrays at a concentration of 0.8 nM were digested with either 1, 2, or 4 U of micrococcal nuclease (MNase; Sigma) per ml in 50 mM NaCl–10 mM Tris-HCl–2.5 mM $MgCl_2$–0.25 mM $CaCl_2$–1 mM DTT–0.1 mg of BSA per ml at 37°C for 10 min. ^{32}P-labeled DNA was digested under the same conditions at a concentration of 10 pM. For nucleosome protection experiments, 3 nM arrays was digested with 500 U of MNase. Purified DNA was analyzed on a 1% agarose gel or a 4% acrylamide gel (acrylamide-to-bisacrylamide ratio of 30:0.8).

RESULTS

SWI-SNF-dependent enhancement of restriction enzyme accessibility requires the continuous presence of ATP. To quantify the accessibility of nucleosomal DNA in the context of nucleosomal arrays, we have developed a biochemical assay where nucleosome remodeling activity is coupled to restriction enzyme activity such that remodeling is revealed as an enhancement of restriction enzyme cleavage rates (24). In our previous studies, the central nucleosome of an 11-mer nucleosomal array contained a unique *SalI*/*Hin*cII site located at the predicted dyad axis of symmetry (24–26, 37, 54). Restriction enzyme kinetics are biphasic in this system; the first phase is rapid and reflects the fraction of restriction sites that are not occluded by a nucleosome (due primarily in our assays to nucleosomes that occupy minor translational positions; see Discussion and also references 24 to 26). The second phase is very slow and reflects a dynamic equilibrium between the occluded and open nucleosomal DNA states (Fig. 1B) (24, 37). Addition of SWI-SNF and ATP stimulates the second phase of *SalI*/*Hin*cII digestion 20- to 30-fold, but in contrast to the persistent remodeling of mononucleosome substrates, the enhancement of nucleosomal array digestion by *Sal*I or *Hin*cII requires continuous ATP hydrolysis (24, 25).

To investigate whether these kinetics of SWI-SNF remodeling are unique to the central nucleosome of an array, we constructed new DNA templates where the nucleosome positioning sequence marked by the *SalI*/*Hin*cII site was located within the last or second to last position of the array (Fig. 1A). We then reconstituted these new DNA templates into nucleosomal arrays and compared the kinetics of nucleosomal array remodeling by SWI-SNF with the rates of remodeling of our original array template (Fig. 1B). Addition of SWI-SNF to remodeling reactions resulted in a dramatic enhancement of restriction enzyme activity for all three nucleosomal array substrates; preincubation of the arrays with SWI-SNF for 1 h prior to restriction enzyme addition also resulted in enhanced cleav-

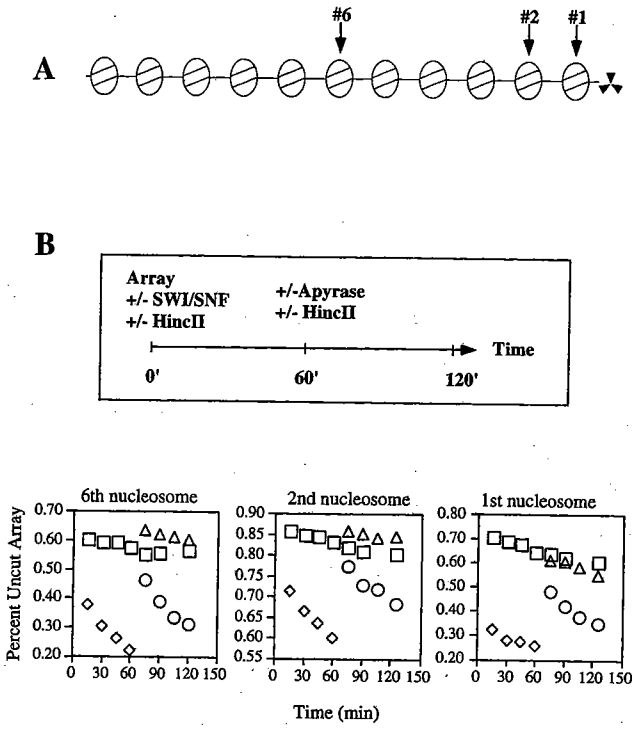

FIG. 1. Remodeled nucleosomes do not accumulate in the context of linear nucleosomal arrays. (A) Schematic representation of the nucleosomal DNA templates used for the coupled restriction enzyme–SWI-SNF remodeling assay. Each template is composed of head-to-tail repeats of a 5S rDNA nucleosome positioning sequence from *L. variegatus*. The first, second, or sixth nucleosome is tagged by a unique *SalI*/*Hin*cII restriction site. (B) The nucleosomal arrays were incubated with *Hin*cII (□), SWI-SNF (○, △), or both (◇). After 1 h, *Hin*cII (○) or *Hin*cII and apyrase (△) were added to the reaction to test for accumulation of remodeled template or for ATP dependence, respectively. Cleavage rates were quantified as described in Materials and Methods. Similar results were obtained in three separate experiments.

age rates; preincubation of the arrays with SWI-SNF for 1 h followed by coaddition of restriction enzyme and apyrase (to enzymatically remove ATP) resulted in cleavage kinetics that were identical to reactions where SWI-SNF was omitted from the reaction (Fig. 1B). These results indicate that novel, accessible nucleosomes do not accumulate during the preincubation with SWI-SNF and ATP regardless of their positions within the array.

Remodeling of closed circular nucleosomal arrays. In contrast to our studies with linear nucleosomal arrays, Kingston and colleagues have reported that SWI-SNF-dependent remodeling of closed circular nucleosomal arrays, as assayed by a decrease in the number of constrained negative supercoils, does not require continuous ATP hydrolysis (i.e., a persistent change in minichromosome structure) (13, 16, 42). To test whether the topology of the nucleosomal array influences the stability of the remodeled state, as assayed by restriction enzyme digestion, we reconstituted an average of seven nucleosomes onto a ~3-kb plasmid DNA template that contains a single 5S nucleosome positioning sequence harboring a unique *SalI*/*Hin*cII site (Fig. 2). This array was then subjected to a remodeling/reversal experiment identical to those displayed in Fig. 1. In the absence of SWI-SNF, we found that 57% of the plasmid was linearized during the 30-min *Hin*cII digestion, which reflects the fraction of *Hin*cII sites not occluded by histone octamers (Fig. 2B, lane 1). Addition of SWI-SNF and ATP resulted in 90% of the plasmid being cleaved (Fig. 2B, lane 2). However, preincubation of the circular nucleosomal array with SWI-SNF for 30 min, followed by removal of ATP with apyrase and subsequent exposure to *Hin*cII, resulted in only 49% cleavage (Fig. 2B, lane 3). Thus, as we observed for linear nucleosomal arrays, the SWI-SNF-dependent stimulation of *Hin*cII cleavage of circular nucleosomal arrays requires continuous ATP hydrolysis.

Previous studies with circular nucleosomal array substrates detected a persistent decrease in plasmid linking number due to a combined incubation of the arrays with SWI-SNF, ATP, and topoisomerase I (13, 16, 42). One possibility is that SWI-SNF-dependent changes in the topology of minichromosomes are persistent, whereas the enhanced accessibility of nucleosomal DNA within a single nucleosome requires continuous ATP hydrolysis. Alternatively, inclusion of topoisomerase I during the remodeling reaction may trap the remodeled state irreversibly (e.g., by inducing nucleosome loss). To investigate this latter possibility, we monitored the changes in minichromosome topology under conditions where we could temporally separate topoisomerase I and SWI-SNF activities. Figure 2C shows that addition of calf thymus topoisomerase I to our reconstituted minichromosomes resulted in the appearance of ~15 discernible topoisomers after deproteination and agarose gel electrophoresis (Fig. 2C, lane 2). Quantification of the intensity of the topoisomers indicated that an average of seven nucleosomes had been reconstituted onto this 3.2-kb plasmid (peak topoisomer has a linking number of 8 [about 50% saturation]). This degree of saturation correlates well with the percentage of nucleosomal *Hin*cII sites (Fig. 2B). Incubation of the minichromosome with topoisomerase I, SWI-SNF, and ATP resulted in a redistribution of the topoisomers corresponding to a loss of about two constrained supercoils per plasmid (Fig. 2C, lane 4; the peak topoisomer has a linking number of 6). When apyrase was added after the incubation with SWI-SNF and topoisomerase I, the pattern of topoisomers was not greatly altered, indicating that the effect of SWI-SNF action was persistent in this assay as previously observed (Fig. 2C, lane 5) (13, 16, 42). However, if the minichromosomes were incubated for 30 min with SWI-SNF and ATP, and topoisomerase I was added after treatment of the SWI-SNF reaction with apyrase, then the topoisomer distribution became similar to that in the control reactions that lacked SWI-SNF (peak topoisomer has a linking number of 8) (Fig. 2C, lane 6). In fact, there is an increase in the proportion of topoisomers that appear to contain 11 to 15 constrained supercoils, which may reflect topoisomerase I-induced DNA knotting of stable SWI-SNF-nicked array DNA complexes (Fig. 2C, lane 6; see reference 9). Importantly, apyrase does not inhibit topoisomerase I activity (data not shown). We conclude that only the topoisomerase I-relaxed and remodeled state has a stable change in topology after removal of ATP. In contrast, in the absence of topoisomerase I, SWI-SNF-induced changes in minichromosome topology rapidly collapse (reverse) after removal of ATP.

Persistent remodeling of isolated di- and mononucleosomes. In contrast to nucleosomal arrays, several studies have reported persistent alterations in mononucleosome accessibility due to SWI-SNF action (13, 16, 27, 42). Moreover, novel, "stably remodeled species" that resemble dinucleosomes were formed from mononucleosome substrates (27, 42). In light of the above results, we wished to study remodeling on mono- and dinucleosomes, using the coupled remodeling-restriction enzyme assay to test whether it could also detect a stable remodeled species. To obtain pure di- and mononucleosomes, we engineered nucleosomal array DNA templates where a *Pst*I site flanks the ultimate or the penultimate (*SalI*/*Hin*cII-tagged)

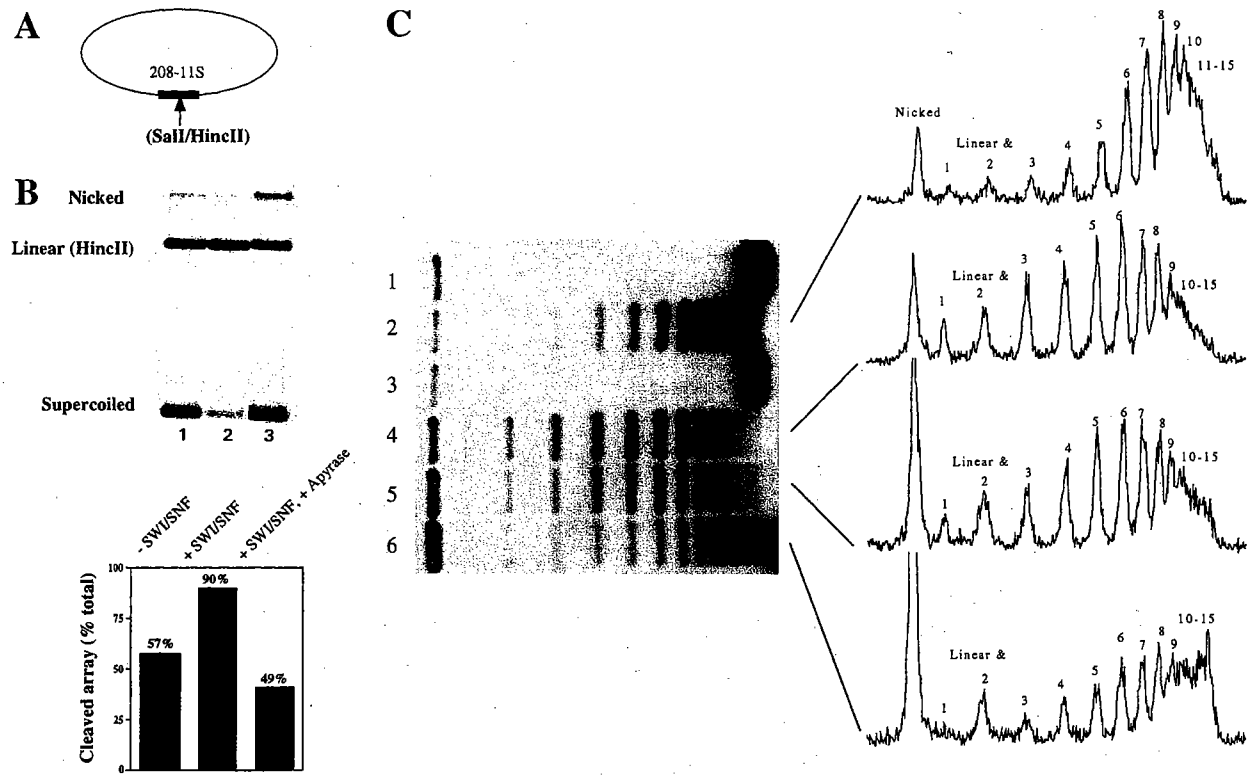

FIG. 2. Remodeled nucleosomes do not accumulate on closed circular nucleosomal arrays. (A) Nucleosomal arrays were reconstituted on negatively supercoiled closed circular plasmids bearing a unique *SalI*/*Hin*cII site on a single 5S nucleosome positioning sequence. (B) The closed circular arrays were subjected to *Hin*cII digestion for 30 min followed by a 30-min incubation with (lane 2) or without (lane 1) SWI-SNF. Alternatively, the arrays were incubated for 30 min with SWI-SNF, followed by removal of ATP with apyrase and digestion with *Hin*cII for 30 min (lane 3). (C) A supercoiling assay was performed on the closed circular nucleosomal arrays (lane 1) in order to detect remodeling as changes in plasmid linking number. Addition of calf thymus topoisomerase I resulted in the appearance of approximately 15 bands after deproteination and agarose gel electrophoresis (lane 2). Incubation of the arrays with SWI-SNF and ATP for 30 min had no effect on topology (lane 3). Addition of SWI-SNF, ATP, and topoisomerase I resulted in a redistribution of the topoisomers (lane 4). Removal of ATP by addition of apyrase after 30 min of incubation in the presence of SWI-SNF plus topoisomerase did not affect the distribution of topoisomers (lane 5). Incubation of the arrays with SWI-SNF and ATP for 30 min followed by apyrase and then topoisomerase resulted in a topoisomer distribution similar to that in lane 2 (lane 6). Quantification of the autoradiograph indicates that for lanes 2 and 6, the predominant band corresponds to a linking number of 8; for lanes 4 and 5, the predominant band corresponds to a linking number of 6. Note that apyrase treatment of the circular array results in an increase in the nicked plasmid form (B, lane 3; C, lanes 5 and 6). Apyrase-induced plasmid nicking does not affect *Hin*cII activity or the relative distribution of topoisomers upon topoisomerase I treatment of the array (data not shown).

nucleosome positioning sequence (Fig. 3A and data not shown). Nucleosomal arrays were reconstituted on these templates and digested with *Pst*I, and the products of the restriction reaction were fractionated on glycerol gradients to isolate homogeneous 216-bp mono- or 427-bp dinucleosome particles.

Isolated mono- or dinucleosomes were used as substrates in restriction enzyme digests in the presence or absence of SWI-SNF. In the absence of SWI-SNF complex, 24% of the mononucleosomes were rapidly cleaved by *Sal*I, indicating that this population contains accessible *Sal*I sites (Fig. 3A). The remaining 76% of the mononucleosomes were cleaved with the slow digestion kinetics diagnostic of nucleosomal *Sal*I sites (data not shown). In contrast, when SWI-SNF and ATP were included in the reaction, 90% of the mononucleosomes were cleaved by *Sal*I during a 30-min incubation. No stimulation of mononucleosome digestion was observed if ATP was omitted from the reactions (data not shown). In contrast, preincubation of the mononucleosomes with SWI-SNF and ATP, followed by coaddition of apyrase and *Sal*I, resulted in 67% cleavage of the mononucleosomes (Fig. 3A). Identical results were obtained when, after the preincubation step, the binding of SWI-SNF to the mononucleosome substrate was competed for by addition of a 20-fold excess of chicken erythrocyte oligonucleosomes (data not shown). Similar results were also obtained with the dinucleosome substrates (data not shown). Thus, in contrast to our results with nucleosomal array substrates (Fig. 1 and 2), the majority of the SWI-SNF-dependent enhancement of mono- or dinucleosome accessibility, as assayed by *Sal*I digestion, does not require continuous ATP hydrolysis.

Previous studies have used mononucleosome substrates assembled onto short DNA fragments (e.g., 154 bp [7, 16]), and thus we wished to confirm that our restriction enzyme assay would also be able to detect persistent remodeling of these types of mononucleosome substrates. To address this issue, we reconstituted 154-bp mononucleosomes using the reconstitution method described by Imbalzano et al. (16) (see Materials and Methods; Fig. 3B). In this case only 10% of the *Hin*cII sites were cleaved after 30 min of digestion in the absence of SWI-SNF. Incubation of these 154-bp mononucleosomes with SWI-SNF and ATP led to 50% cleavage after the 30-min incubation. Finally, if the mononucleosomes were preincubated with SWI-SNF and ATP, and then apyrase and *Hin*cII were added, 26% of the sites were cleaved (Fig. 3B). Although the remodeled state of the 154-bp mononucleosomes was clearly less stable than for the 216-bp mononucleosomes, much of the stimulation of *Hin*cII digestion due to SWI-SNF action was persistent in the absence of continuous ATP hydrolysis.

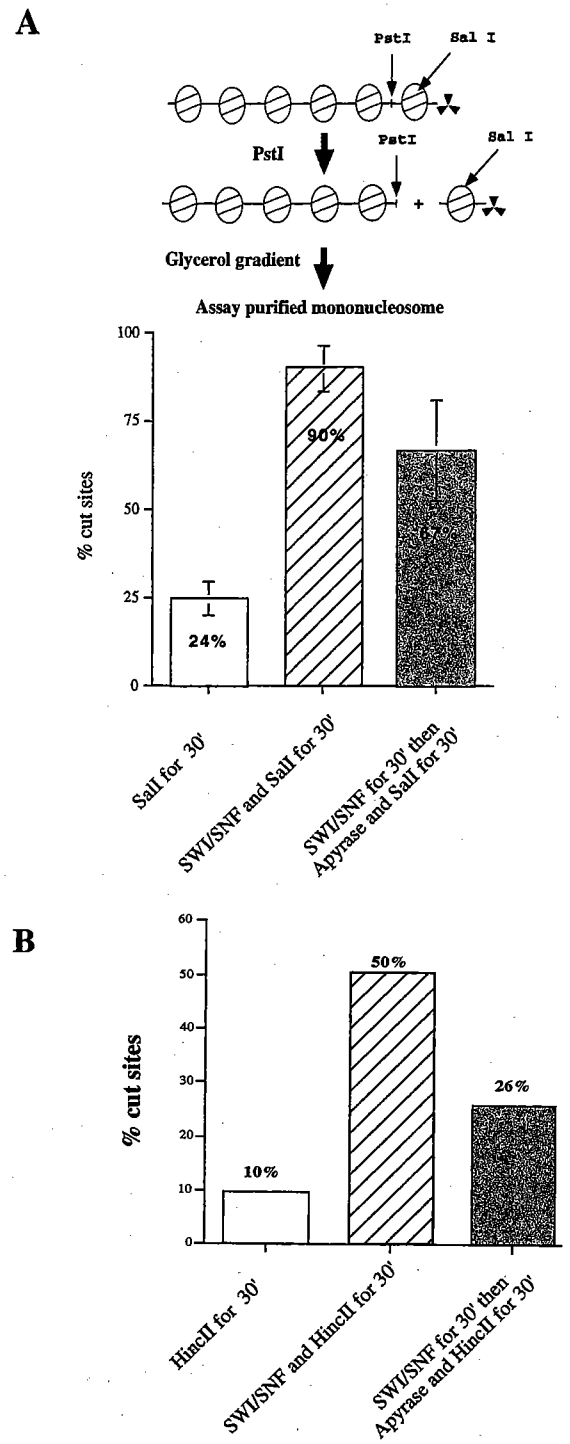

FIG. 3. SWI-SNF remodeling of isolated mononucleosomes leads to persistent DNA accessibility. (A) Purified mononucleosomes, obtained following *Pst*I digestion of a 6-mer nucleosomal array, were treated with SWI-SNF, *Sal*I, and/or apyrase as indicated. (B) Mononucleosomes assembled on a 154-bp DNA fragment were used as substrates in the same assays as panel A. Similar results were obtained in two additional, independent experiments.

SWI-SNF remodeling of mononucleosomes results in a protection of the ends of the DNA. Our data indicate that SWI-SNF-dependent remodeling of mononucleosomes leads to a more stable, accessible reaction product than does SWI-SNF remodeling of nucleosomal arrays. One possibility that we considered is that SWI-SNF action might lead to translational movements of histone octamers. A prediction of this hypothesis is that the stable, increased accessibility of the *Hinc*II site should be accompanied by an increased protection of DNA elsewhere on the template. To test this idea, we digested a 216-bp mononucleosome with two additional restriction enzymes, *Bam*HI and *Nco*I, whose sites are located 9 and 23 bp, respectively, from the ends of the DNA template (Fig. 4A). In the absence of SWI-SNF, we find that 65 to 90% of the *Nco*I and *Bam*HI sites were freely accessible, consistent with the known preferred position of the 5S nucleosome (Fig. 4B). Upon incubation of the mononucleosomes with SWI-SNF for 30 min, followed by addition of apyrase, we observe an increased protection of the *Nco*I and *Bam*HI sites (25 and 50% increase in proportion of occluded sites, respectively [Fig. 4C]). This contrasts with the *Hinc*II sites, of which 25% were rendered more accessible by SWI-SNF action and subsequent removal of ATP (Fig. 3 and 4B). Similar results were observed if the binding of SWI-SNF to the mononucleosome substrate was competed for by addition of a 20-fold excess of chicken erythrocyte oligonucleosomes (data not shown). These experiments strongly imply that SWI-SNF remodeling can result in altered translational positioning of histone octamers on DNA. Furthermore, the dramatic increase in protected restriction sites near the ends of the DNA fragment indicate that DNA ends may act as sinks where SWI-SNF remodeled nucleosomes preferentially accumulate.

To further investigate the SWI-SNF-dependent protection of mononucleosomal DNA, we determined the kinetics of *Bam*HI cleavage in the presence or absence of SWI-SNF and in the presence or absence of continued ATP hydrolysis. In the absence of SWI-SNF, 72% of the *Bam*HI sites were rapidly cleaved, and only 18% of the *Bam*HI sites were cleaved at a rate diagnostic of nucleosomal DNA (Fig. 4D). Thus, the majority of histone octamers do not appear to be positioned over the *Bam*HI site. After a 30-min incubation with SWI-SNF and ATP, 60% of the mononucleosomes were more resistant to subsequent *Bam*HI cleavage, reflecting the possible movement of the histone octamer (time zero; Fig. 4D). If apyrase was added after the 30-min preincubation to remove ATP, mononucleosomes were digested by *Bam*HI at the low rate that is diagnostic of nucleosomal DNA; in contrast, if ATP hydrolysis was allowed to continue, SWI-SNF was able to remodel the newly occluded *Bam*HI sites as assayed by the increased kinetics of *Bam*HI digestion (Fig. 4D). These results support our view that SWI-SNF action can lead to movement of histone octamers to the DNA ends, and furthermore that these remodeled nucleosomes represent cannonical nucleosomes that can inhibit the accessibility of DNA as well as serve as new substrates for SWI-SNF remodeling.

SWI-SNF action alters the translational positioning of mononucleosomes. To verify that the persistent aspect of SWI-SNF-mediated mononucleosome remodeling is due to a repositioning of histone octamers, we used a nucleosome mobility assay where populations of mononucleosomes with heterogeneous translational positions are resolved by virtue of their different electrophoretic properties on native polyacrylamide gels (29). First, we used the strategy outlined in Fig. 3B to isolate mononucleosomes reconstituted onto a 427-bp DNA fragment that contains two 5S nucleosome positioning sequences. This substrate is nearly identical to the 416-bp mononucleosome described by Meersseman et al. (29); as this group previously observed (29), these purified mononucleosomes resolve into eight electrophoretically distinct species on 4% native acrylamide gels (Fig. 5, lane 1). Two of the species make

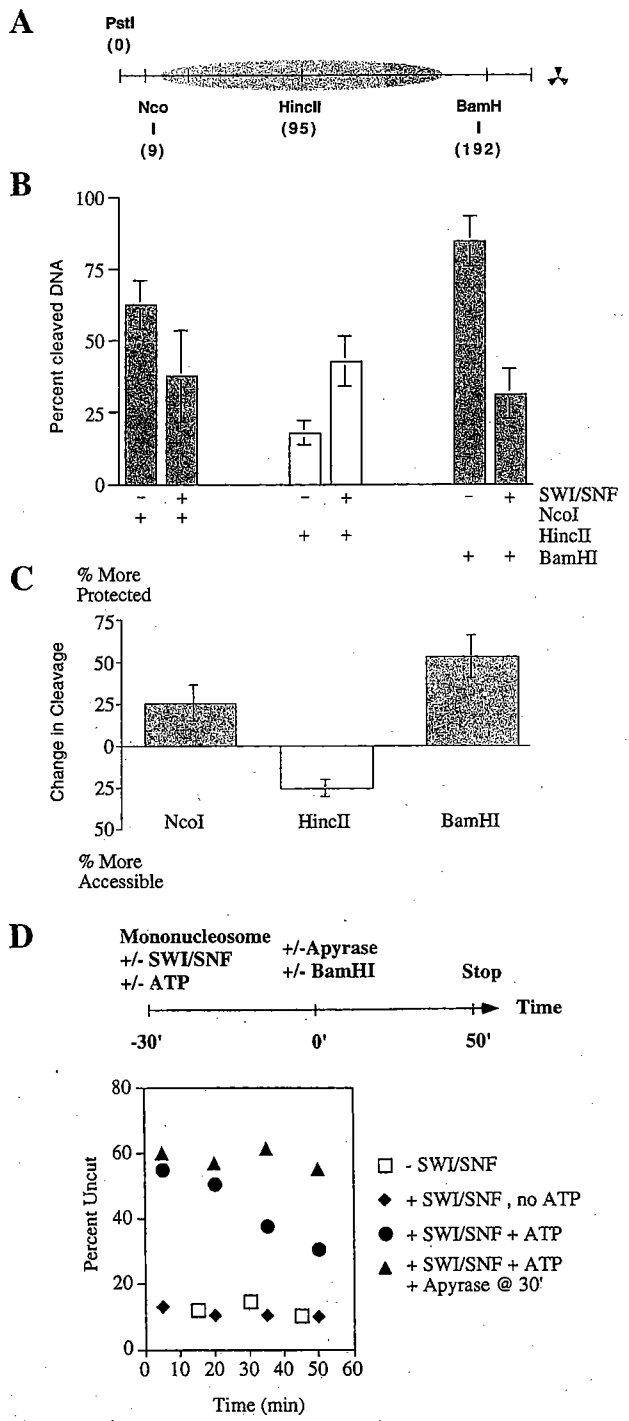

FIG. 4. SWI-SNF action leads to increased protection of DNA ends. (A) Schematic of the 216-bp mononucleosome substrate. (B) The ends of the DNA fragment are more protected after a 30-min incubation with SWI-SNF. The graph represents the percentage of cleaved nucleosomal DNA after restriction enzyme digestion without SWI-SNF (−) or after a 30-min incubation with SWI-SNF followed by removal of ATP with apyrase (+). Error bars represent the standard deviation from at least three experiments. (C) Difference in percent nucleosomal DNA cleaved by the restriction enzyme in the absence (−) and presence (+) of SWI-SNF (see panel B). (D) Time course of *Bam*HI DNA cleavage. Mononucleosomes were preincubated for 30 min in the absence of SWI-SNF (□), in the presence of SWI-SNF without ATP (◆), or in the presence of SWI-SNF and ATP (●, ▲). Reactions containing SWI-SNF and ATP were then incubated with (▲) or without (●) apyrase, *Bam*HI was added to all reactions, and the amount of cleavage was determined throughout a 50-min time course.

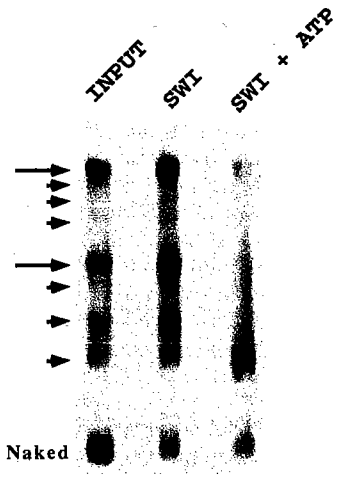

FIG. 5. SWI-SNF action alters the translational positioning of a mononucleosome. Mononucleosomes reconstituted onto a 416-bp DNA fragment that contains two copies of a 5S nucleosome positioning sequence were electrophoresed on a 4% native polyacrylamide gel for 8 h. Arrows denote eight electrophoretically distinct particles that reflect different nucleosome translational positions. Mononucleosomes were incubated for 30 min at 37°C in the absence of SWI-SNF (lane 1), in the presence of SWI-SNF but in the absence of ATP (lane 2), and in the presence of both SWI-SNF and ATP (lane 3). Prior to loading of samples, all three reactions received a 100-fold molar excess of unlabeled chicken oligonucleosomes to compete for binding of SWI-SNF to the mononucleosome substrate. Note that SWI-SNF and ATP shifts the distribution of mononucleosome particles to the faster-migrating species without leading to an increase in the amount of free, naked DNA. Similar results were obtained in at least three additional experiments.

up ~60% of the total population (Fig. 5, lane 1), and the fastest-migrating species represent octamers located close to the DNA ends (29). In the absence of ATP, exposure to SWI-SNF had no effect on the distribution of species (Fig. 5, lane 2). In contrast, addition of SWI-SNF and ATP resulted in a quantitative switch of >90% of the total population to the faster-migrating species (Fig. 5, lane 3). Incubation of these mononucleosomes with SWI-SNF and ATP did not increase the amount of free DNA (Fig. 5, compare lanes 2 and 3), and these remodeled mononucleosomes still cosedimented in glycerol gradients with mononucleosomes that had not been remodeled (data not shown). Furthermore, prolonged incubation of the mononucleosomes (up to 2 h) resulted in the same pattern (data not shown), suggesting that the reaction rapidly reaches an equilibrium where the distribution of nucleosome translational positions is strongly biased towards histone octamers occupying end positions.

SWI-SNF action mobilizes histone octamers within positioned nucleosomal arrays. The observed difference in the stability of the enhanced restriction enzyme accessibility for mononucleosome and nucleosomal array substrates might be due to an inhibition of nucleosome mobility within an array context. In this case, the remodeled state on arrays may represent novel, accessible nucleosomal structures whose maintenance requires continuous ATP hydrolysis. On the other hand, the remodeling of nucleosomal arrays might also reflect the ATP-dependent movement of histone octamers. In this case, continuous ATP hydrolysis might be needed to maintain changes in nucleosome positions that create an accessible *Hinc*II/*Sal*I site. To test this latter possibility, we first monitored the accessibility of the *Bam*HI and *Nco*I restriction sites located adjacent to the *Sal*I/*Hinc*II-marked central nucleosome (Fig. 6A). In the absence of SWI-SNF, 65% of the *Nco*I sites and 90% of the *Bam*HI sites were accessible to restriction

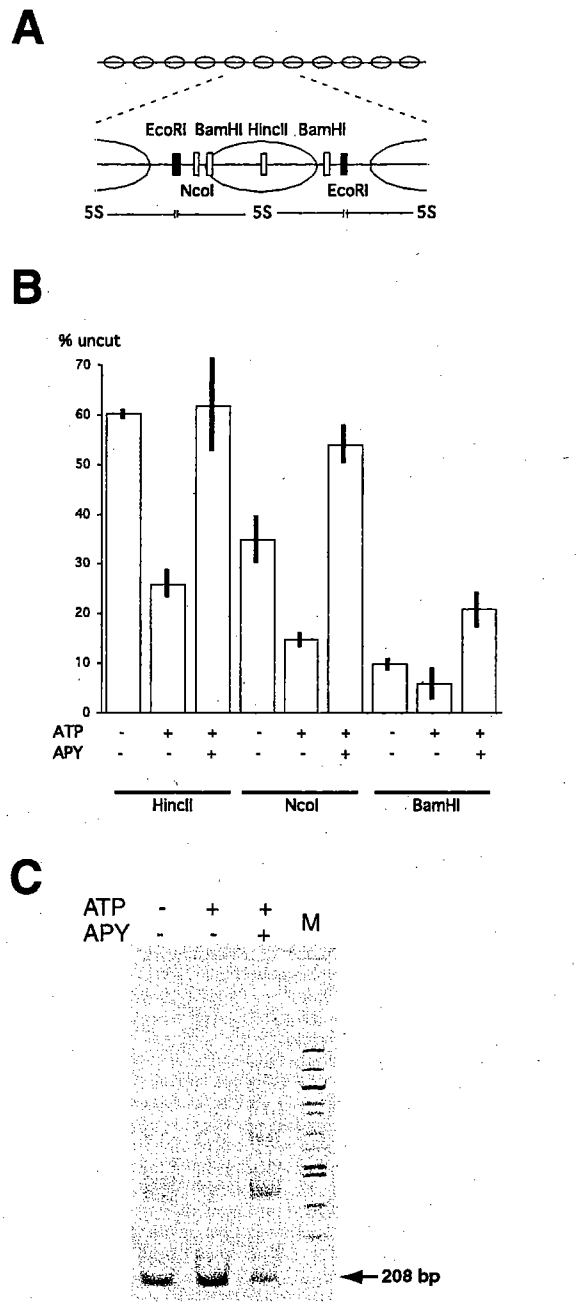

FIG. 6. SWI-SNF-dependent remodeling changes nucleosome positions within reconstituted arrays. (A) Schematic representation of the DNA template showing positions of restriction endonuclease sites unique to the central nucleosome (open bars). *Eco*RI sites (filled bars) are located in the linker region between nucleosomes in every 5S repeat. (B) Change in the accessibility of restriction endonuclease sites at the nucleosome dyad (HincII) and at the linker region (NcoI and BamHI) following SWI-SNF reaction. The bars represent percentages of uncut arrays after restriction endonuclease cleavage of reconstituted arrays in the presence of ATP and/or SWI-SNF as indicated. In apyrase (APY) experiments, the SWI-SNF reaction was stopped with apyrase and restriction enzyme was then added. Error bars represent the standard deviation from at least three experiments. (C) SWI-SNF reaction results in a decrease in accessibility of linker regions throughout nucleosomal arrays. The *Eco*RI digestion of reconstituted nucleosomal arrays was carried out as in panel B; DNA was isolated and analyzed in a 4% acrylamide gel. Gel was stained with Vistra Green (Amersham) and scanned with a Molecular Dynamics Storm scanner. The limit digestion product (208-bp 5S DNA) is marked with an arrow. The 232-bp product represents the *Hin*cII/*Sal*I-marked nucleosome. Larger products represent partial digestion products. Lane M contains a 100-bp DNA ladder (New England Biolabs).

enzyme (Fig. 6B), which is consistent with the major translational frame of the 5S nucleosome and is similar to what we observed for the 216-bp 5S mononucleosome (Fig. 4). However, in contrast to our results with the mononucleosome substrates, addition of SWI-SNF and ATP led to a further increase in the accessibility of *Nco*I and *Bam*HI sites (Fig. 6B). If the nucleosomal arrays were preincubated with SWI-SNF and ATP, and then apyrase was added with the restriction enzyme, an increased occlusion of the *Bam*HI and *Nco*I sites (25% change for *Nco*I and 15% change for *Bam*HI [Fig. 6B]) resulted. These results suggest that the enhanced accessibility of the *Hin*cII, *Bam*HI, and *Nco*I sites requires continuous ATP hydrolysis, and furthermore, as we observed for mononucleosome substrates, that SWI-SNF may stably alter nucleosome positioning within the array.

To investigate the accessibility of DNA adjacent to every nucleosome within the array, we analyzed the accessibility of the *Eco*RI sites that are located between each of the 11 5S DNA repeats (Fig. 6A). In the absence of SWI-SNF, *Eco*RI digestion of the nucleosomal array yields primarily the 208-bp 5S DNA limit product, as well as some di- and trinucleosome-size partial digestion products (Fig. 6C). This result is consistent with the majority of histone octamers occupying positions between *Eco*RI sites. When arrays are incubated with SWI-SNF and ATP, we observe a small increase in *Eco*RI cleavage which is best visualized by a decrease in the amount of di- and tri-nucleosome sized DNA fragments (Fig. 6C). However, when arrays are preincubated with SWI-SNF and ATP for 30 min, followed by addition of apyrase and *Eco*RI, accessibility of the *Eco*RI sites is dramatically reduced as visualized by a decrease in the amount of 208-bp DNA product and a large increase in the amount of partial digestion products (Fig. 6C). This SWI-SNF-dependent decrease in DNA accessibility following removal of ATP is similar to the changes in *Nco*I and *Bam*HI accessibility at the central nucleosome and are consistent with the hypothesis that SWI-SNF might alter nucleosome positioning within the array.

To further investigate persistent changes in nucleosome positioning within the array due to SWI-SNF action, we performed partial MNase digestions. MNase cleavages occur only within the linker regions between nucleosomes; thus, in the absence of SWI-SNF, MNase digestion reveals a repeating pattern of cleavages and protections indicative of a positioned array of 11 nucleosomes (Fig. 7A). Addition of SWI-SNF and ATP disrupts the positioned nucleosomal array, yielding digestion products that are nearly identical to those of the unassembled 5S DNA template (Fig. 7A; note that MNase cleavage of the 5S array DNA yields a repeating pattern of cleavages and protections that is the inverse of the nucleosomal pattern). Furthermore, this disruption of the positioned 5S array is persistent and does not require continuous ATP hydrolysis (Fig. 7A, +APY lanes). This effect of SWI-SNF remodeling represents changes in translational positioning of nucleosomes rather than nucleosome loss, as the remodeled arrays still cosediment with control arrays on glycerol gradients (Fig. 7B), and they still contain nucleosomes that protect ~150 bp of DNA after extensive digestion with MNase (Fig. 7C). Thus, these results indicate that SWI-SNF remodeling of 5S arrays is associated with a dramatic randomization of nucleosome translational positions. In the presence of SWI-SNF and ATP, this randomized state is associated with an enhanced restriction enzyme accessibility throughout the array. In contrast, the subsequent inactivation of SWI-SNF remodeling activity leads to a randomized array where many restriction enzyme sites are persistently occluded.

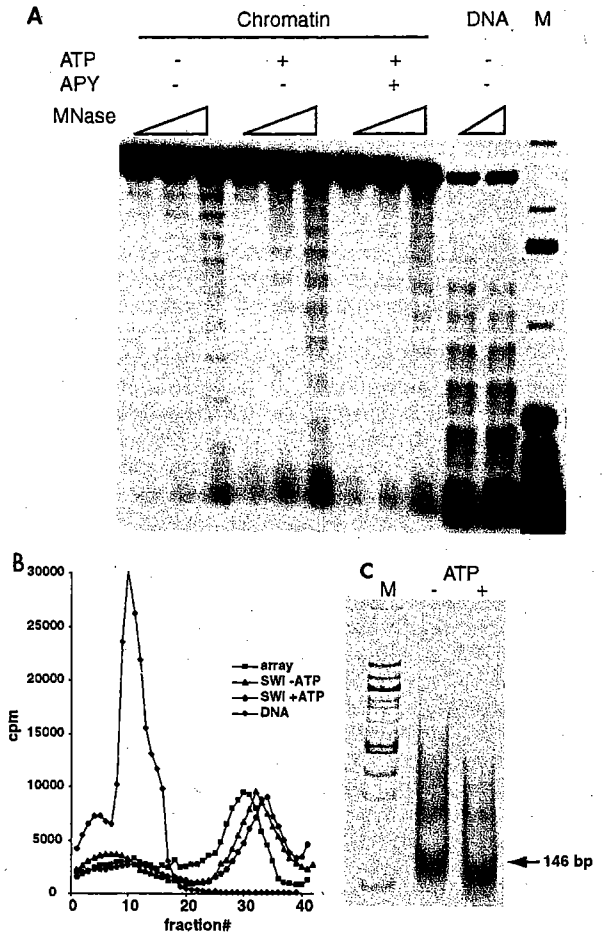

FIG. 7. Persistent randomization of nucleosome positions within reconstituted arrays as a result of SWI-SNF-dependent remodeling. (A) MNase digestion of reconstituted 11-mer arrays. [α-^{32}P]dATP-labeled reconstituted nucleosomal arrays were digested with increasing amounts of MNase in the presence of SWI-SNF and either in the presence or absence of ATP before or after addition of apyrase (APY). DNA was isolated and analyzed on a 1% agarose gel. Lane M, ^{32}P-labeled 1-kb DNA ladder (Gibco BRL). Note that 5S array DNA also exhibits a repeating pattern of MNase cleavages that is the inverse pattern of nucleosomal arrays. (B) SWI-SNF-dependent remodeling does not change the sedimentation properties of nucleosomal arrays. Shown are 10 to 40% linear glycerol gradient profiles of naked DNA and reconstituted nucleosomal arrays either before or after SWI-SNF-dependent remodeling. ^{32}P-labeled reconstituted arrays were incubated in the presence of ATP and/or SWI-SNF for 30 min, loaded on top of the gradient, and centrifuged in an SW28 rotor (Beckman) at 33,000 $\times$ *g* for 16 h. Fractions of 0.4 ml were collected from the top of the gradient and counted by scintillation. (C) Nucleosomes within remodeled arrays protect ~150 bp of DNA. Nucleosomal arrays were incubated in the presence of SWI-SNF with or without ATP, the reaction was stopped with apyrase, and remodeled arrays were digested with a high concentration of MNase. DNA was isolated, fractionated in a 4% acrylamide gel, and visualized by staining with Vistra Green. The position of the 146-bp nucleosomal DNA is marked with an arrow. Lane M, 100-bp DNA ladder.

DISCUSSION

In this study, we carried out experiments aimed at reconciling two seemingly contradictory experimental observations concerning the persistence of the remodeled nucleosome state induced by yeast SWI-SNF and related ATP-dependent remodeling complexes (8, 13, 16, 24, 27, 42). First, when the remodeling activity of SWI-SNF and RSC was analyzed on mononucleosome substrates, the remodeled, accessible state (detected by restriction enzyme cleavage, transcription factor binding, or DNase I digestion patterns) appeared to persist for over 30 min upon removal of ATP or SWI-SNF from the reaction (8, 13, 16, 27, 42). However, when SWI-SNF, RSC, NURF, CHRAC, or Mi-2 activity was analyzed on nucleosomal array substrates, remodeling (detected by restriction enzyme digestion) seemed to be reversible (24, 32; Logie et al., unpublished data). The experiments presented here suggest that the stable, accessible state that is detected on mononucleosomes is due to SWI-SNF-induced movement of histone octamers to the DNA ends. Furthermore, the apparent reversibility of nucleosomal array remodeling is a misnomer since SWI-SNF action leads to persistent randomization of nucleosome positions within the array. Our data are consistent with a recent study on yeast SWI-SNF by Whitehouse et al. (51) and with two studies which showed that chromatin remodeling complexes that contain the ISWI ATPase can induce nucleosome mobility (14, 21).

Nucleosome mobilization: mononucleosomes versus arrays. Why does the SWI-SNF-dependent enhancement of restriction enzyme digestion appear to be reversible on nucleosomal array substrates? For each 5S repeat within an array, nucleosomes assume a major translational position that is present in at least 50% of the population. The remaining nucleosomes assume multiple, minor translational positions that differ from the major frame by multiples of 10 bp (34). Thus, the 5S array substrate is a heterogeneous population where 60 to 75% of the arrays contain a central nucleosome positioned at or near the major translational position (containing an occluded *SalI*/*Hin*cII site), and the remaining arrays contain a central nucleosome that occupies several different minor translational frames (characterized by an accessible *SalI*/*Hin*cII site). MNase analysis (Fig. 7) indicates that SWI-SNF and ATP rapidly redistribute these nucleosome positions such that nucleosomes are randomly positioned. In the presence of ATP, this randomized state is also associated with enhanced accessibility of restriction enzyme sites throughout the array. When ATP is subsequently removed from the reaction, nucleosomes remain randomly positioned as determined by MNase analysis, but there is an increased occlusion of restriction enzyme sites compared to arrays incubated with SWI-SNF and ATP. Thus, continual ATP hydrolysis is required to maintain enhanced restriction enzyme accessibility but not to maintain random nucleosome positions. This is in contrast to the 216-bp mononucleosome substrate, where SWI-SNF action leads to an accessible *SalI*/*Hin*cII site that persists after removal of ATP. We propose that SWI-SNF and ATP leads to a state of constant redistribution of nucleosome positions within an array and that this fluid chromatin state favors restriction enzyme accessibility. When ATP is removed from the array assay, the continual redistribution of nucleosomes terminates, and a random pattern of nucleosome positioning is frozen. In this case, the randomized array is associated with a decreased accessibility of restriction enzyme sites throughout the array. For restriction enzyme sites located within the minor translational frames of the starting substrate (*Eco*RI, *Nco*I, and *Bam*HI), SWI-SNF action, followed by removal of ATP, leads to increased occlusion of these sites compared to the starting array. Furthermore, in the case of the *SalI*/*Hin*cII site, inactivation of SWI-SNF leads to a level of occlusion that is fortuitously similar to the starting substrate, making the remodeling reaction appear to be reversible. Furthermore, and most important, although SWI-SNF creates changes in nucleosome positions on all substrates which then persist in the absence of continuous ATP hydrolysis, SWI-SNF does not appear to create stable, novel nucleosomes that have enhanced DNA accessibility.

We note that in our studies we have monitored the persistence of the remodeled state for only short time periods (<30

min) following inactivation of SWI-SNF. Previous studies have indicated that the remodeled state of mononucleosomes is at least partially reversible after more extended incubations, and it is expected that nucleosomes will eventually reestablish the preferred translational positions within the 5S arrays (32). Thus, it is not clear whether any feature of the remodeled state is truly stable or if there are differences in the rate of reversibility between remodeled mononucleosomes and nucleosomal arrays.

Role of octamer mobilization in remodeling of 154-bp mononucleosomes. Given that persistent disruption of mononucleosomal DNA (as assayed by restriction enzyme digestion) appears to correlate with the translational movement of the histone octamer, it seems surprising that SWI-SNF action can lead to a stable disruption of mononucleosomes that are reconstituted on very short DNA fragments (e.g., 154 bp). On these substrates the *Hin*cII site is located ~75 bp from the nucleosomal edge, and thus a movement of the octamer to the end of the DNA fragment will still leave the *Hin*cII site buried within the nucleosome. One possibility is that SWI-SNF remodeling of 154-bp mononucleosomes yields novel reaction products that are not generated on nucleosomal arrays or on mononucleosomes with longer stretches of linker DNA. Alternatively, we favor a model in which SWI-SNF induces movement of the histone octamer off the end of the DNA fragment, leading to a histone octamer with less than 147 bp of DNA wrapped onto its surface. This type of reaction may not be favored; consistent with this view, remodeling of the 154-bp mononucleosomes does lead to fewer accessible *Hin*cII sites after SWI-SNF inactivation compared to the 216-bp mononucleosome (Fig. 3). Previous studies have shown that reconstitution of histone octamers onto a 145-bp DNA fragment can lead to the preferential assembly of only 128 bp of DNA (40). Furthermore, visualization of SWI-SNF remodeling by electron microscopy indicates that nearly 20 bp of DNA is lost from a remodeled nucleosome (3). If SWI-SNF generates remodeled mononucleosomes that contain a significant number of unoccupied DNA binding sites, then these particles may show a propensity to self-associate via histone-DNA interactions. This model may provide a simple explanation for the previously described novel reaction product that behaves biochemically as a dinucleosome (27, 42).

Is ATP-dependent remodeling equivalent to histone octamer mobilization? Our results indicate that nucleosome remodeling by SWI-SNF and related enzymes leads to dramatic changes in nucleosome positioning. One simple model posits that remodeling is equivalent to octamer mobilization and that changes in nucleosome positions are responsible for the enhanced binding of transcription factors or activity of restriction enzymes. In this case, the energy of ATP hydrolysis might be used to directly move histone octamers, perhaps by "screwing" DNA over the octamer surface as suggested by Varga-Weisz and Becker (48). Furthermore, in this model the disruption of DNase I digestion patterns might not reflect changes in DNA path around the octamer as previously suggested (8), but disruption might be due to octamer movement and subsequent exposure of nucleosome-free DNA. In this case, disruption of the DNase I ladder would represent a mixture of species, where on average 50% of all sequences would be more or less nucleosome-free.

Alternatively, SWI-SNF-like enzymes might use the energy of ATP hydrolysis to generate a high-energy intermediate where DNA-histone contacts have been disrupted but the translational frame of the histone octamer on DNA has not yet been altered. This activated state may also constrain fewer negative supercoils due to the weakened histone-DNA interactions (see below). A similar activated intermediate consisting of weakened histone-DNA contacts has been proposed for remodeling catalyzed by ISWI-containing complexes (1, 17). This high-energy state might then decay into stable changes in nucleosome positions. In this model, the preferred stable outcome for mononucleosomes appears to be histone octamers at the DNA ends, but for nucleosomal arrays, where DNA ends do not flank each nucleosome, remodeling leads to a more random positioning of nucleosomes. Consistent with this high-energy-intermediate model, human SWI-SNF is still proficient at ATP-dependent remodeling of immobilized substrates which contain histone octamers cross-linked to nucleosomal DNA (22).

Stability of the remodeled state as a function of chromatin topology. Kingston and colleagues have used a minichromosome topology assay to monitor the persistence of nucleosomal array remodeling by human SWI-SNF complex (13, 16, 42). In this remodeling assay, nucleosomes are reconstituted onto a closed circular DNA template in the presence of topoisomerase I, and these substrates are then incubated with SWI-SNF and ATP in the presence of topoisomerase I. In these reactions, ATP-dependent remodeling by human SWI-SNF results in the loss of a large number of constrained supercoils, and these topological changes persist for several hours after inactivation or removal of the remodeling enzyme. Based on these results, it was proposed that the stability of remodeled minichromosomes (as detected by these changes in topology) reflects a novel remodeled nucleosome species. In contrast, we hypothesized that the persistent nature of the remodeled product detected in the topology assay might be due to the combined incubation of minichromosomes with SWI-SNF and a topoisomerase, rather than a property inherent to the SWI-SNF remodeling reaction. To test this possibility, we designed a remodeling experiment where the activities of SWI-SNF and topoisomerase I were temporally uncoupled (Fig. 2). Whereas coincubation of SWI-SNF and topoisomerase I led to a change in minichromosome topology that persisted in the absence of ATP or SWI-SNF, incubation of the minichromosome with SWI-SNF and ATP, followed by removal of ATP and subsequent addition of topoisomerase I, eliminated the stable change in topology. Thus, persistent changes in minichromosome topology requires the combined action of SWI-SNF and topoisomerase I. Although the nature of this persistent change is not clear, it is possible that topoisomerase I traps a transient change in DNA topology that is induced during the remodeling reaction. In the absence of topoisomerase I, this change in topology would collapse, due either to the artificially high level of supercoiling on the minichromosomes or to an inherent reversibility of the remodeling reaction. Alternatively, the combined action of SWI-SNF and a topoisomerase might lead to more efficient eviction of histone octamers from circular chromatin.

Stability of the remodeled state in vivo. Two studies have recently addressed the issue of continuous versus transient requirement of the yeast SWI-SNF complex in vivo (4, 43). In both cases, inactivation of SWI-SNF led to the rapid loss of gene expression, indicating that SWI-SNF is continuously required to maintain activated levels of gene expression. Previously it had been proposed that this continuous requirement for SWI-SNF observed in vivo might reflect the activity of other remodeling complexes (such as RSC) that might use the energy of ATP hydrolysis to reestablish a repressive chromatin structure (45). The data presented here, however, imply that there is no a priori need to invoke a balance between positively and negatively acting ATP-dependent remodeling complexes

since termination of the nucleosome remodeling reaction leads to reappearance of bona fide, DNA-occluding nucleosomes.

Our data also provide a simple model to explain how SWI-SNF can be required for transcriptional repression of some genes in vivo (15). As we observe here, SWI-SNF might use the energy of ATP hydrolysis to alter the positions of nucleosomes surrounding *cis*-acting regulatory sites in vivo. In some cases, these movements might enhance DNA accessibility (either by continuous action of SWI-SNF or by placing DNA sites between nucleosomes), but in other instances SWI-SNF action might lead to the occlusion of DNA sites that are required for gene expression. In fact, SWI-SNF action might first act to promote accessibility of DNA to key transcription factors, but inactivation or loss of SWI-SNF from the target gene would help reestablish a repressed state by imposing inhibitory nucleosome positions.

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The first two authors contributed equally to this work.

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Rad54p Is a Chromatin Remodeling Enzyme Required for Heteroduplex DNA Joint Formation with Chromatin*

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In eukaryotic cells, the repair of DNA double-strand breaks by homologous recombination requires a RecA-like recombinase, Rad51p, and a Swi2p/Snf2p-like ATPase, Rad54p. Here we find that yeast Rad51p and Rad54p support robust homologous pairing between single-stranded DNA and a chromatin donor. In contrast, bacterial RecA is incapable of catalyzing homologous pairing with a chromatin donor. We also show that Rad54p possesses many of the biochemical properties of *bona fide* ATP-dependent chromatin-remodeling enzymes, such as ySWI/SNF. Rad54p can enhance the accessibility of DNA within nucleosomal arrays, but it does not seem to disrupt nucleosome positioning. Taken together, our results indicate that Rad54p is a chromatin-remodeling enzyme that promotes homologous DNA pairing events within the context of chromatin.

Chromosomal DNA double-strand breaks (DSBs)[1] arise through exposure of cells to harmful environmental agents such as ionizing radiation or mutagenic chemicals (radiomimetics, alkylating agents, etc.). DSBs can also be caused by endogenously produced oxygen radicals, by errors in DNA replication, or as obligatory intermediates during programmed cellular processes, such as meiosis or V(D)J recombination (1–3). Cell survival and maintenance of genome integrity depend on efficient repair of DSBs, because unrepaired or misrepaired DSBs may lead to mutations, gene translocations, gross chromosomal rearrangements, or cellular lethality.

Several pathways for repairing DSBs have evolved and are highly conserved throughout eukaryotes. Homologous recombination (HR) is a major pathway of DSB repair in all eukaryotes and has a distinct advantage over other mechanisms in that it is mostly error-free. In organisms ranging from yeast to human, HR is mediated by members of the *RAD52* epistasis group (*RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, and *XRS2*). Accordingly, mutations in any one of these genes result in sensitivity to ionizing radiation and other DSB-inducing agents (2). The importance of the HR pathway in maintaining genome integrity is underscored by the fact that mutations in each one of its critical factors have been correlated with chromosomal instability-related ailments, including ataxia telangiectasia-like disease, Nijmegen breakage syndrome, Li Fraumeni syndrome, as well as various forms of cancer (4).

In vivo and *in vitro* studies have suggested the following sequence of molecular events that lead to the recombinational repair of a DSB. First, the 5′ ends of DNA that flank the break are resected by an exonuclease to create ssDNA tails (5). Next, Rad51p polymerizes onto these DNA tails to form a nucleoprotein filament that has the capability to search for a homologous duplex DNA molecule. After DNA homology has been located, the Rad51-ssDNA nucleoprotein filament catalyzes the formation of a heteroduplex DNA joint with the homolog. The process of DNA homology search and DNA joint molecule formation is called "homologous DNA pairing and strand exchange." Subsequent steps entail DNA synthesis to replace the missing information followed by resolution of DNA intermediates to yield two intact duplex DNA molecules (6).

The homologous DNA pairing activity of Rad51p is enhanced by Rad54p (7). Rad54p is a member of the Swi2p/Snf2p protein family (8) that has DNA-stimulated ATPase activity and physically interacts with Rad51p (7, 9, 10). Because of its relatedness to the Swi2p/Snf2p family of ATPases, Rad54p may have chromatin remodeling activities in addition to its established role in facilitating Rad51p-mediated homologous pairing reactions. In this study we show that Rad51p and Rad54p mediate robust D-loop formation with a chromatin donor, whereas the bacterial recombinase, RecA, can only function with naked DNA. Furthermore, we find that the ATPase activity of Rad54p is essential for D-loop formation on chromatin and that Rad54p can use the free energy from ATP hydrolysis to enhance the accessibility of nucleosomal DNA. Experiments are also presented to suggest that chromatin remodeling by Rad54p and yeast SWI/SNF involves DNA translocation.

EXPERIMENTAL PROCEDURES

DNA—All DNA manipulations were carried out using standard methods (11). Oligonucleotides were obtained from Operon Technologies (Alameda, CA). Plasmid pXG540 and T4 EndonucleaseVII used in the cruciform extrusion experiments were a kind gift of Dr. T. Owen-Hughes.

The oligonucleotide used for triplex formation was TFO (triplex-forming oligonucleotide) (5′-TTCTTTTCTTTCTTCTTTCTTT-3′). To

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[1] The abbreviations used are: DSB, double-strand break; HR, homologous recombination; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; TFO, triplex-forming oligonucleotide; DTT, dithiothreitol; BSA, bovine serum albumin; ATPγS, adenosine 5′-*O*-(thiotriphosphate); AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; Mnase, micrococcal nuclease.

generate pMJ5, the annealed oligonucleotides TFOB5 (5′-TCGAGAAGAAAAGAAAGAAGAAAGAAAC-3′) and TFOB3 (5′-TCGAGT TTCTTTCTTTCTTTTCTTC-3′) were ligated to the product of a *Xho*I digestion carried out on pCL7c (12). This yielded a pBluescript SKII (−) plasmid containing 5 head-to-tail repeats of the 208-bp *Lytechinus variegatus* 5S rDNA nucleosome positioning element flanked by a TFO-binding site. The DNA template (208–11) for reconstituting nucleosomal arrays for the ATPase, remodeling, and Mnase assays consists a *Not*I-*Hin*dIII fragment derived from pCL7b (12), containing 11 head-to-tail repeats of a 5S rRNA gene from *L. variegatus*, each one possessing a nucleosome positioning sequence. The sixth nucleosome is tagged by a unique *Sal*I restriction site.

Reagent Preparation—Recombinant yeast Rad51p, Rad54p, rad54K341Ap, and rad54K341Rp were overexpressed in yeast and purified as previously described (7). SWI/SNF purification was as described (13). Histone octamers were purified from chicken erythrocytes as described by Hansen *et al.* (14). Octamer concentrations were determined by measurements of A_{230} (15). Nucleosomal array DNA templates (pXG540, pMJ5, or 208–11) were labeled by the Klenow polymerase fill-in reaction using [α-^{32}P]dCTP (3000 μCi/mmol, Amersham Biosciences). Nucleosomal arrays were reconstituted by salt dialysis as previously described (13), and the nucleosome saturation was determined to be 60–80% by digestion analysis.

D-loop Reactions—Oligonucleotide D1 (90-mer) used in the D-loop experiments has the sequence: 5′-AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT-3′, being complementary to positions 1932–2022 of pBluescript SK(−) replicative form I DNA. Oligonucleotide D1 was 5′ end-labeled with ^{32}P using [γ-^{32}P]dATP and polynucleotide kinase, as described (7). Buffer R (35 mM Tris-HCl, pH 7.4, 2.0 mM ATP, 2.5 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, and an ATP-regenerating system consisting of 20 mM creatine phosphate and 30 μg/ml creatine kinase) was used for the reactions; all of the incubation steps were carried out at 30 °C. Rad51 (0.8 μM) and Rad54 (120 nM) were incubated with radiolabeled oligonucleotide D1 (2.4 μM nucleotides) for 5 min to assemble the presynaptic filament, which was then mixed with naked pBluescript replicative form I DNA (38 μM base pairs) or the same DNA assembled into chromatin (38 μM base pairs). Chromatin assembly was monitored by following topological changes as well as measuring the degree of occlusion of a unique *Eco*RI restriction site close to the D1 sequence. Substrates were estimated to be ~80% saturated with nucleosomes. The reactions containing RecA protein (0.8 μM) were assembled in the same manner, except that they were supplemented with an additional 12.5 mM $MgCl_2$ at the time of incorporation of the duplex substrates. At the indicated times, 4-μl portions of the reactions were withdrawn and mixed with an equal volume of 1% SDS containing 1 mg/ml proteinase K. After incubation at 37 °C for 5 min, the deproteinized samples were run in 1% agarose gels in TAE buffer (40 mM Tris-HCl, pH 7.4, 0.5 mM EDTA) at 4 °C. The gels were dried, and the radiolabeled DNA species were visualized and quantified by PhosphorImager analysis (Personal Molecular Imager FX, Bio-Rad).

ATPase Assay—Recombinant yeast Rad54p (1 nM) was incubated at 30 °C or 37 °C with 5 nM of either naked 208–11 dsDNA or reconstituted nucleosomal arrays in the presence of 100 μM ATP, 2.5μCi [γ-^{32}P]dATP (6000 μCi/mmol, Amersham Biosciences), 2.5% glycerol, 0.1% Tween 20, 20 mM Tris-HCl, pH 8.0, 200 μM DTT, 5 mM $MgCl_2$, 100 μg/ml BSA. For the DNA length-dependence assays, 5 nM Rad54p, 5 nM Rad51p, or 10 nM SWI/SNF were used. Oligonucleotides (random N-mers ranging from 10–100 nucleotides in length) were PAGE-purified to ensure length homogeneity (Integrated DNA Technologies, Inc., Coralville, IA). Samples were taken after 2, 5, 15, and 30 min and resolved by TLC. The proportion of liberated ^{32}P-pyrophosphate was determined using the Molecular Dynamics PhosphorImager and ImageQuant Software. ATPase assays were independently repeated 3 times, yielding very similar results.

Cruciform Formation Assay—Cruciform formation assays were performed as previously described (16). Briefly, 8 ng of *Ava*I-linearized pXG540 (either naked, N, or nucleosomal, C) were incubated with various concentrations of Rad54, Rad51, or rad54 K341A and 0.15 mg/ml EndoVII (except where noted), in the presence of 10 mM Hepes, pH 7.9, 50 mM NaCl, 3 mM $MgCl_2$, 5% glycerol, 0.1 mM DTT, 1 mM ATP (except where noted), 3 mM phosphoenolpyruvate, and 20 units/ml pyruvate kinase for 30 min at 30 °C. The products were resolved in 1.2% agarose gels and visualized with Sybr Gold staining (Molecular Probes, Eugene, OR) followed by analysis with ImageQuant software.

Chromatin-remodeling Reaction—For the coupled SWI/SNF-or Rad54-*Sal*I reactions, reconstituted 208–11 nucleosomal arrays (~1 nM final concentration) were preincubated at 37 °C for 20 min with 2.5units/μl *Sal*I in a buffer containing (final concentrations) 50 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 3 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 1 mM DTT, 10 mM Tris-HCl, pH 8.0, 100 μg/ml BSA, and 3% glycerol. Nucleosomal arrays were ~80% saturated with nucleosomes. Buffer, 2 nM SWI/SNF complex, or various concentrations of recombinant Rad51p and Rad54p were added and samples were taken at the indicated time points, vigorously mixed for 10s with 25 μl TE and 50 μl 1:1 solution of phenol/chloroform. The purified DNA fragments were resolved by electrophoresis in 1.2% agarose gels in the presence of 50 μg/ml ethidium bromide. The gels were then dried on 3MM Whatman paper. The fraction of cut and uncut DNA was determined by PhosphorImager analysis using a Molecular Dynamics PhosphorImager and ImageQuant software. Experiments were repeated independently at least 3 times, which yielded very similar results.

Micrococcal Nuclease Digestion—15 nM reconstituted 208–11 nucleosomal arrays were incubated at 37 °C with 2 nM SWI/SNF, 100 nM Rad54p, or buffer, in the presence of 2 mM ATP, 5 mM NaCl, 2.5 mM Tris-HCl, pH 8.0, 0.25 mM $MgCl_2$, 0.3 mM $CaCl_2$, 3 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 1 mM DTT, 10 μg/ml BSA, 0.5% glycerol. After 20 min, 0.0005 units of Micrococcal Nuclease (Worthington) was added to the reaction, and aliquots were taken at the indicated time points and then treated for 20 min with 2 μg/μl proteinase K and extracted twice with a 1:1 solution of phenol:chloroform. The resulting digestion products were resolved by electrophoresis in 2% agarose gels, run at 2.5 volts/cm for 12 h. The gels were fixed, dried, and analyzed using a Molecular Dynamics PhosphorImager and ImageQuant Software.

Triple-helix Displacement Assay—Triple-helix formation was performed as described (17). Briefly, equimolar concentrations (100 nM) of *Ssp*I-linearized pMJ5 and ^{32}P-labeled TFO were mixed in buffer MM (25 mM MES, pH 5.5, 10 mM $MgCl_2$) at 57 °C for 15 min and left to cool to room temperature overnight. The resulting triplex was either used directly or reconstituted into nucleosomal arrays. To introduce nicks into the DNA, pMJ5 was exposed to various concentrations of DNaseI (Promega, Madison, WI) for 2 min at 37 °C, the reactions were stopped with 5 mM EDTA, vigorously mixed for 10 s with a 1:1 solution of phenol/chloroform, ethanol-precipitated, and resuspended in water. The degree of nicking introduced by DNaseI treatment was assessed by electrophoretic analysis of native and heat-denatured samples (in the presence of 15% formamide) on denaturing 1.3% agarose gels, followed by Sybr Gold Stain (Molecular Probes, Eugene, OR).

The triplex-containing substrates (5 nM) were incubated at 30 °C with 5 nM recombinant Rad54 protein or SWI/SNF complex, in a buffer containing 35 mM Tris-HCl, pH 7.2, 3 mM $MgCl_2$, 100 μg/ml BSA, 50 mM KCl, 1 mM DTT, 3 mM phosphocreatine, 28 μg/ml creatine phosphokinase, and where noted, 3 mM ATP. Samples were taken at the indicated time points, the reactions were quenched with GSMB buffer (15% (w/v) glucose, 3% (w/v) SDS, 250 mM 4-morpholinepropanesulfonic acid, pH 5.5, 0.4 mg/ml bromphenol blue), and analyzed in 1.2% agarose gels (40 mM Tris acetate, 5 mM sodium acetate, 1 mM $MgCl_2$, pH 5.5) at 10 volts/cm for 1.5 h at 4 °C. Gels were fixed in 5% acetic acid, 50% methanol for 1 h, and dried. The proportion of bound and free TFO was determined using a Molecular Dynamics PhosphorImager and ImageQuant Software.

RESULTS

Rad51p and Rad54p Promote DNA Pairing with a Chromatin Donor—Repair of a DSB by homologous recombination begins with the invasion of a double-stranded, homologous donor by a Rad51-ssDNA nucleoprotein filament, also referred to as the presynaptic filament. This strand invasion reaction is typically monitored *in vitro* by following the Rad51p-dependent formation of a D-loop between a radiolabeled oligonucleotide and a homologous double-stranded DNA donor (Fig. 1*A*). In this case, efficient D-loop formation also requires the ATPase activity of Rad54p. *In vivo*, however, the search for homology and strand invasion involves a homologous donor that is assembled into chromatin. Given that the Rad54p ATPase shows sequence relatedness to known chromatin remodeling enzymes, it was of considerable interest to examine the ability of Rad54p to promote Rad51p-dependent D-loop formation with a nucleosomal donor.

Fig. 1 shows the results of D-loop assays that use either a circular, naked DNA donor or this same circular DNA assembled into nucleosomes. Consistent with previous studies, the

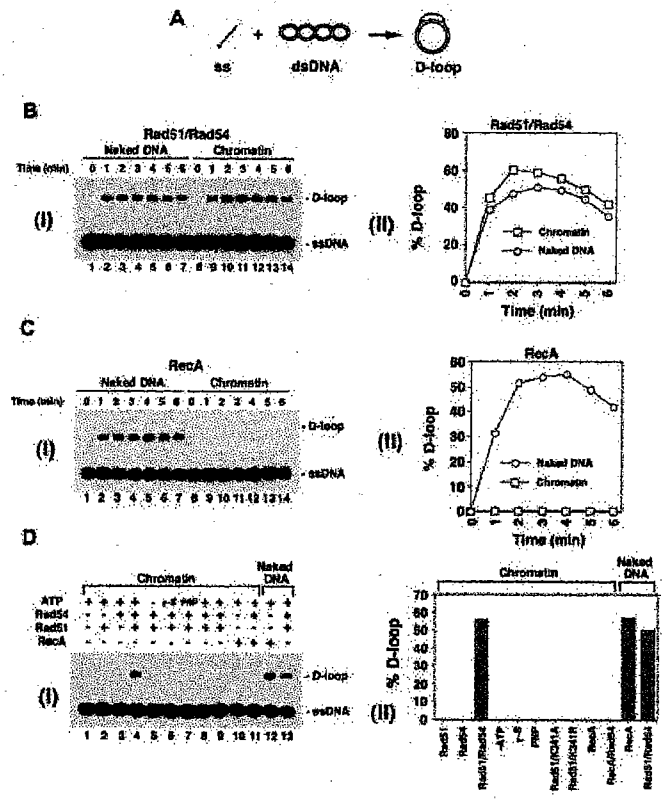

FIG. 1. **Rad51p and Rad54p promote efficient DNA strand invasion with chromatin.** *A*, schematic of the D-loop reaction. A radiolabeled oligonucleotide (*ss*) pairs with a homologous duplex target (*dsDNA*) to yield a D-loop, which, after separation from the free oligonucleotide on an agarose gel, is visualized and quantified by PhosphorImager analysis of the dried gel. *B, panel I* shows D-loop reactions mediated by Rad51p and Rad54p with the naked homologous duplex (*Naked DNA*) and the homologous duplex assembled into chromatin (*Chromatin*). The results from the experiments in *panel I* are graphed in *panel II*. The *ordinate* refers to the proportion of the homologous duplex converted into D-loop. *C, panel I* shows D-loop reactions mediated by RecA with the naked homologous duplex (*Naked DNA*) and the homologous duplex assembled into chromatin (*Chromatin*). The results from the experiments in *panel I* are graphed in *panel II*. *D, panel I* shows D-loop reactions in which Rad51p and RecA were used either alone or in conjunction with Rad54p or rad54 mutant variants with the naked homologous duplex (*Naked DNA*) and the homologous duplex assembled into chromatin (*Chromatin*), as indicated. ATP was omitted from the reaction in *lane 5*, and ATPγS (*γS*) and AMP-PNP (*PNP*) replaced ATP in *lanes 6* and *7*, respectively. The reactions in *lanes 8* and *9* contained ATP, but Rad54p was replaced with the ATPase-defective variants rad54 K341A (*KA*) and rad54 K341R (*KR*), respectively. The results from the experiments in *panel I* are summarized in the bar graph in *panel II*.

combination of yeast Rad51p and Rad54p led to rapid and highly efficient D-loop formation on the naked DNA donor (Fig. 1*B*). A similar level of D-loop formation was also obtained when the bacterial recombinase RecA was used in these assays with naked DNA (Fig. 1*C*). Surprisingly, assembly of the circular donor into chromatin had no effect on the efficiency of D-loop formation by Rad51p and Rad54p (Fig. 1*B*). D-loop formation on the chromatin donor required ATP hydrolysis by Rad54p, because nonhydrolyzable ATP analogs (ATPγS and AMP-PNP) were unable to substitute for ATP (Fig. 1*C*, *panel I*, *lanes 6* and *7*), and two ATPase-defective mutant variants of Rad54p, rad54K341Ap and rad54K341Rp (18), were inactive (Fig. 1*D*). In contrast to reactions that contained Rad51p/Rad54p, the activity of RecA was completely eliminated when the donor was assembled into nucleosomes (Fig. 1*C*). Furthermore, addition of Rad54p to the RecA reaction did not rescue D-loop formation on chromatin (Fig. 1*D*, *panel I*, *lane 11*). Thus, the eukaryotic recombination proteins have the unique capability of performing the DNA strand invasion reaction with a chromatin donor.

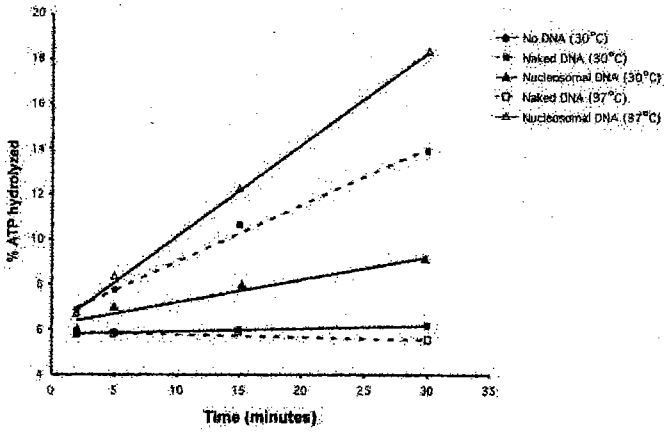

FIG. 2. **Nucleosomal DNA protects Rad54p from thermal inactivation.** ATPase assays. 1 nM Rad54p was incubated at 30 °C with no DNA (*circles*), 5 nM naked (*closed squares*) or nucleosomal dsDNA (*closed triangles*), or at 37 °C with 5 nM naked (*open squares*) or nucleosomal dsDNA (*open triangles*). Samples were taken after 2, 5, 15, and 30 min.

Nucleosomal DNA Protects Rad54p from Thermal Denaturation—The ATPase activity of Rad54p is required for many of its biological functions *in vivo* and for enhancing Rad51p-mediated homologous DNA pairing reactions *in vitro*, both on naked DNA (18) and on chromatin (Fig. 1*D*). Given the latter finding, we were interested in determining whether chromatin influences the ATPase activity of Rad54p.

As shown in Fig. 2, both naked DNA (*solid squares*) and chromatin (*solid triangles*) stimulated the ATPase activity of Rad54p at 30 °C, with naked DNA being somewhat more effective. At the low protein concentrations at which these assays were performed (1 nM), purified Rad54p is extremely temperature-labile and is rapidly inactivated at 37 °C (Fig. 2; Ref. 36). Thus, as expected, the ATPase activity of Rad54p was not detectable in the presence of naked DNA when the reactions were performed at 37 °C (Fig. 2, *open squares*). Importantly, when the reaction was carried out in the presence of chromatin (*open triangles*), the rate of ATP hydrolysis at 37 °C was even greater than the rate obtained in reactions conducted at 30 °C. Importantly, there was no measurable ATPase activity associated with the nucleosomal arrays in the absence of Rad54p, and BSA, free histones, and replication protein A were unable to stimulate the DNA-stimulated ATPase activity of Rad54p at 37 °C. These results indicate that nucleosomal DNA is uniquely able to protect Rad54p from thermal inactivation, and these data suggest that Rad54p may physically interact with nucleosomes.

Rad54 Generates Unconstrained Superhelical Torsion in Nucleosomal DNA—A number of chromatin remodeling complexes that contain Swi2/Snf2-related ATPases have been shown to alter chromatin structure by generating superhelical torsion in DNA and nucleosomal arrays (16). Indeed, the ability to introduce superhelical stress may represent a primary biomechanical activity of all Swi2/Snf2-like ATP-dependent DNA motors, and this activity is likely to be crucial for catalyzing alterations in chromatin structure. Previous studies have shown that Rad54p can also generate both negative and positive supercoiled domains in dsDNA, and it has been suggested that this activity reflects the tracking of Rad54p along DNA (19, 20).

We investigated whether Rad54p is able to introduce superhelical torsion on nucleosomal substrates, using a cruciform extrusion test that has been used for examining other chromatin remodeling enzymes (Fig. 3*A*). In this assay, superhelical

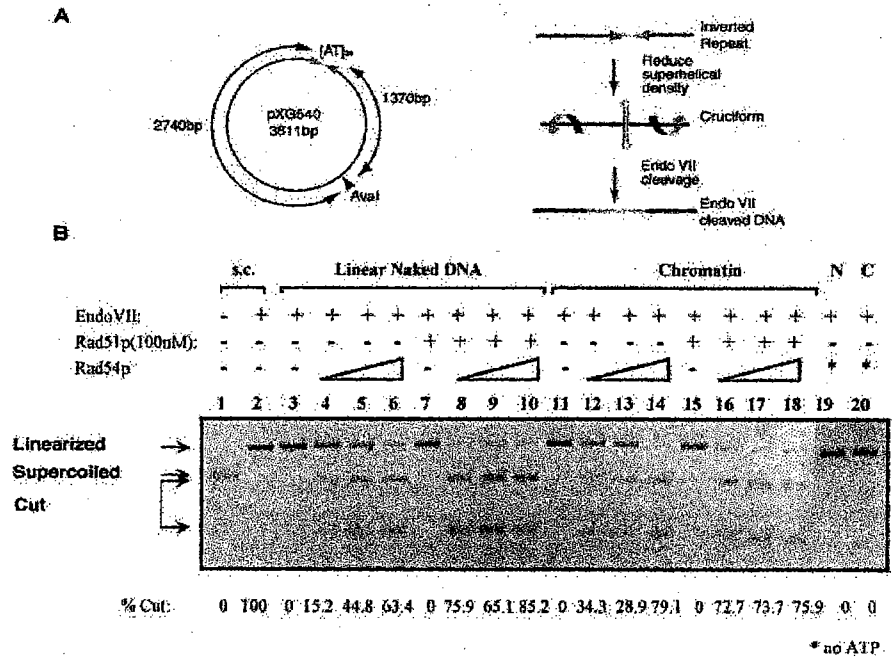

FIG. 3. **Rad54 generates superhelical torsion on nucleosomal DNA.** *A*, schematic illustration of the cruciform extrusion assay. A linearized plasmid (*pXG540*) containing an inverted repeat sequence is incubated with T4 Endonuclease VII, a highly selective junction resolving enzyme, and Rad54p, in the presence of ATP. Rad54p increases the local unconstrained superhelical density, resulting in the extrusion of a cruciform structure, which is recognized and cut by Endo VII. Adapted from Havas *et al.* (16). *B*, results of a typical cruciform formation assay. Supercoiled (*lanes 1, 2*), *Ava*I-linearized pXG540 DNA (*lanes 3–10, 19*), or nucleosomal pXG540 (*lanes 11–18, 20*) was incubated with 12.5, 25, or 50 nM Rad54p as indicated, in the presence or absence of 100 nM Rad51p. EndoVII was omitted in *lane 1*. ATP was omitted in *lanes 19* and *20*. The numbers below each lane (*% cut*) represent the percentage of pXG540 molecules cleaved by EndoVII. *s.c.*, supercoiled substrate; *C*, chromatin substrate; *N*, naked linear DNA substrate.

torsion leads to extrusion of a cruciform that is then recognized and cleaved by bacteriophage T4 endonuclease VII that has high specificity for this DNA structure (16). Consistent with previous studies (19), Rad54p action generates torsional stress on a linear, dsDNA substrate (*N*) which leads to cruciform extrusion (Fig. 3*B*, *lanes 4–6*). Importantly, Rad54p was able to generate torsional stress on the nucleosomal substrate (*C*) with comparable efficiency (*lanes 12–14*). The addition of 100 nM Rad51p greatly stimulated the ability of Rad54p to promote the formation of cruciform structures on both naked and nucleosomal substrates (compare *lanes 4* and *8*, and *12* and *16*, respectively. Also note the decreased levels of linear template in *lanes 16–18*). Importantly, Rad51p fails to support cruciform formation by itself (*lanes 7* and *15*). As expected, the generation of torsional stress required ATP (*lanes 19* and *20*). Furthermore, the ATP hydrolysis mutant variant rad54 K341A was inactive in these assays (data not shown). These data indicate that Rad54p, like other Swi2/Snf2 family members, uses the free energy from ATP hydrolysis to alter DNA topology and that nucleosomal arrays constitute excellent substrates for this activity.

Rad54p Can Disrupt a DNA Triple Helix—How Rad54p introduces topological stress in nucleosomal DNA is unclear. Previously, we suggested that superhelical torsion might result from translocation of Rad54p along the DNA double helix (19). Recently, chromatin remodeling by the yeast RSC complex (which contains the Swi2/Snf2-related ATPase, Sth1p) has been shown to involve ATP-dependent DNA translocation (21). To further evaluate the ability of Rad54p to translocate on DNA, we used a DNA triple-helix-displacement assay that was originally developed to follow the translocation of a type I restriction endonuclease along DNA (17). The substrate used (see Fig. 4*A*) consists of a radioactively labeled oligonucleotide (TFO*) bound via Hoogsteen hydrogen bonds to the major groove of a 2.5-kb linear dsDNA. Translocation of a protein along the DNA displaces the triplex, which can be detected as dissociation of the radioactive TFO* from the DNA triplex. Fig. 4*B* shows typical levels of triplex displacement in the absence or presence of Rad54p or yeast SWI/SNF. Both Rad54p and ySWI/SNF were able to efficiently displace a preformed triplex from both naked (*squares*) and nucleosomal (*triangles*) substrates in an ATP-dependent manner. Similar results were

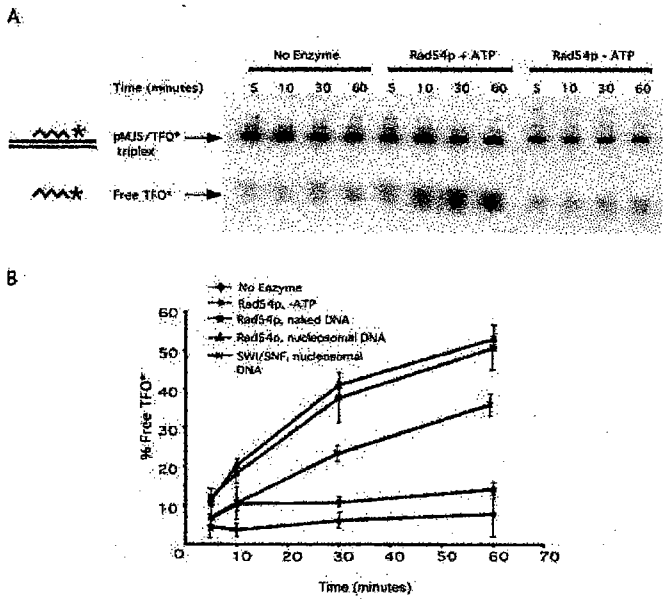

FIG. 4. **Rad54p action displaces a preformed triplex.** *A*, typical results obtained with naked triplex-containing substrate. The upper band corresponds to the duplex-bound TFO*; the lower band corresponds to free TFO*. Reactions contained 5 nM triplex substrate and 5 nM Rad54p. *B*, percentage of free TFO* in four or more experiments were averaged and plotted as a function of time. Note that triplex displacement from the nucleosomal template occurs at equal efficiency to that of naked DNA.

obtained when the TFO*-bound substrate contained single-strand nicks (data not shown), strongly suggesting that the displacement of the TFO* reflects translocation of Rad54p and ySWI/SNF and that it is not due simply to the generation of torsional stress. Thus, yeast RSC (21), ySWI/SNF, and Rad54p (Fig. 4) all share the ability to use the free energy from ATP hydrolysis to disrupt triplex DNA.

Rad54p Has ATPase Kinetics Diagnostic of a DNA-translocating Enzyme—The "inch-worm" model for DNA translocation, originally envisioned (22) for DNA helicases and later modified by Velankar *et al.* (23), proposes that the translocating enzyme progresses along the contour of the DNA in steps of

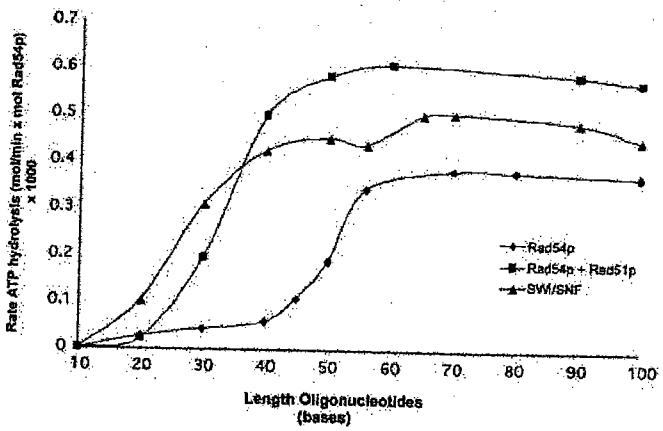

FIG. 5. **Rad54 has ATPase kinetics typical of a unidirectional DNA translocating enzyme.** The rate of ATP hydrolysis by 5 nM Rad54p (*diamonds*), 5 nM Rad54p + 5 nM Rad51p (*squares*), or 10 nM SWI/SNF (*triangles*) was measured in the presence of 50 μM ssDNA (n-mers) oligonucleotides of different lengths. The average values from 3 independent experiments were plotted. Rates were determined from experiments with at least four time points.

a single base, and each step requires the hydrolysis of one ATP molecule. This model predicts that the rate of ATP hydrolysis of a unidirectional DNA translocating enzyme will depend on the length of the DNA (24).

To investigate whether Rad54p has ATPase properties characteristic of a unidirectional DNA translocating enzyme, the rate of ATP hydrolysis was measured in the presence of saturating amounts of single-stranded oligonucleotides ranging from 10 to 100 nucleotides in length (Fig. 5). For comparison, we also monitored the ATPase activity of yeast SWI/SNF (*triangles*). In the case of Rad54p, oligonucleotides shorter than 40 bases failed to stimulate the ATPase activity of Rad54p (*diamonds*), whereas oligonucleotides between 40 and 70 bases led to a stimulation of ATPase activity that was proportional to DNA length. For oligonucleotides longer than 70 bases, the ATPase activity no longer increased with the DNA length. When Rad51p was added to these reactions (*squares*), shorter oligonucleotides became more effective in promoting ATP hydrolysis, and the overall activity was enhanced. Likewise, the ATPase activity of ySWI/SNF (*triangles*) was also proportional to the DNA length, with a plateau reached at 60 bases.

These results are fully consistent with both Rad54p and ySWI/SNF coupling ATP hydrolysis to unidirectional translocation, in which the rate of DNA binding is slower than the rate of DNA translocation (21). In this case, no ATP hydrolysis is observed with very short substrates, presumably because a minimum DNA length is required for Rad54p or ySWI/SNF to bind and to translocate before reaching an end and releasing the DNA. When the substrate is ~30–40 nucleotides in length, Rad54p and ySWI/SNF readily bind the substrate, and more extended translocation events take place. The rate of ATP hydrolysis is fairly constant with DNA substrates longer than 60–70 nucleotides, reflecting the possibility that Rad54p and ySWI/SNF have little processivity, and thus they release their substrate after ~60–70 bases regardless of the total length of the DNA molecule. Although the triphasic kinetics of ATPase activity are consistent with a DNA-translocation mechanism, it remains a possibility that the longer single-stranded oligonucleotides exhibit more extended secondary structures that are either more proficient at binding Rad54p (or SWI/SNF) or stimulating its ATPase activity.

Rad54 Is an ATP-dependent Chromatin Remodeling Enzyme—Cairns and colleagues (21) proposed that short-range translocation events may be the key feature of chromatin remodeling enzymes, leading to a "pumping" of DNA across the surface of the histone octamer, which then results in enhanced DNA accessibility and nucleosome movements. To investigate whether Rad54p might also enhance the accessibility of nucleosomal DNA, we used an assay in which nucleosome remodeling activity is coupled to restriction enzyme activity such that remodeling is revealed as an enhancement of restriction-enzyme cleavage rates (12). This assay uses a nucleosomal array substrate in which the central nucleosome of an 11-mer array contains a unique *Sal*I site located at the predicted dyad axis of symmetry (see Fig. 6*A*). In the absence of a remodeling enzyme, the rate of *Sal*I cleavage is very slow (Fig. 6*A*, *solid diamonds*), whereas addition of a remodeling enzyme, such as yeast SWI/SNF, leads to enhanced digestion (Fig. 6*A*, *solid squares*). When Rad54p was added to the remodeling reactions, *Sal*I digestion was also dramatically enhanced (*solid circles*, *triangles*), although a higher concentration of this protein (50 nM) was required to achieve a rate of digestion comparable with that of reactions that contained yeast SWI/SNF (2 nM, *squares*). However, when Rad51p (50 nM) and Rad54p (50 nM) were both present in the reaction, much higher levels of remodeling were attained (*open circles*). Note that Rad51p has no intrinsic chromatin remodeling activity (*open diamonds*). The stimulation of the Rad54p chromatin remodeling activity by Rad51p is congruent with previous studies showing that Rad51p enhances the rate of ATP hydrolysis and DNA supercoiling by Rad54p (19, 25, see Fig. 3). Thus, the above data indicate that Rad54p is sufficient for chromatin remodeling activity but that the combination of Rad51p and Rad54p constitutes a more potent remodeling machine.

Rad54p Does Not Induce Significant Nucleosome Mobilization—A number of chromatin remodeling complexes that contain a Swi2/Snf2-related ATPase (ySWI/SNF, dCHRAC, dNURF, and xMi-2) can use the energy of ATP hydrolysis to move nucleosomes in cis (26–30). To investigate whether Rad54p can also catalyze nucleosome mobilization, ^{32}P-end-labeled nucleosomal arrays were incubated with buffer, ySWI/SNF, or Rad54p, and nucleosome positions were mapped by micrococcal nuclease (Mnase) digestion (Fig. 6*B*). Mnase can only cleave DNA between nucleosomes, which leads to a periodic ladder of digestion products indicative of a positioned 11-mer nucleosomal array (Fig. 6*B*). Consistent with our previous studies, incubation with ySWI/SNF (2 nM) and ATP leads to a complete disruption of the Mnase digestion pattern, indicative of nucleosome sliding (Fig. 6*B*, *left panel*; also see Ref. 28). In contrast, addition of Rad54p (100 nM) and ATP had very little effect on the cleavage periodicity (Fig. 6*B*, *right panel*). Likewise, addition of both Rad51p and Rad54p (100 nM each) to these assays did not change the Mnase digestion profile (data not shown). Importantly, these experiments used concentrations of ySWI/SNF and Rad54p that yielded similar levels of chromatin remodeling in the restriction enzyme accessibility assay (Fig. 6*A*). Thus, although Rad54p can enhance the accessibility of nucleosomal DNA to restriction enzymes, this activity does not appear to reflect large scale rearrangement of nucleosome positions.

DISCUSSION

In eukaryotes, chromatin presents an accessibility dilemma for all DNA-mediated processes, including gene transcription and DNA repair. Although much progress has been made on identifying the enzymes that remodel chromatin structure to facilitate transcription, less is known of how the DNA-repair machinery gains access to damaged DNA within chromatin (reviewed in Ref. 31). In particular, it has not been clear how the recombinational repair machinery can locate short regions of DNA homology when those DNA donor sequences are assembled into chromatin. Here we have shown that the yeast re-

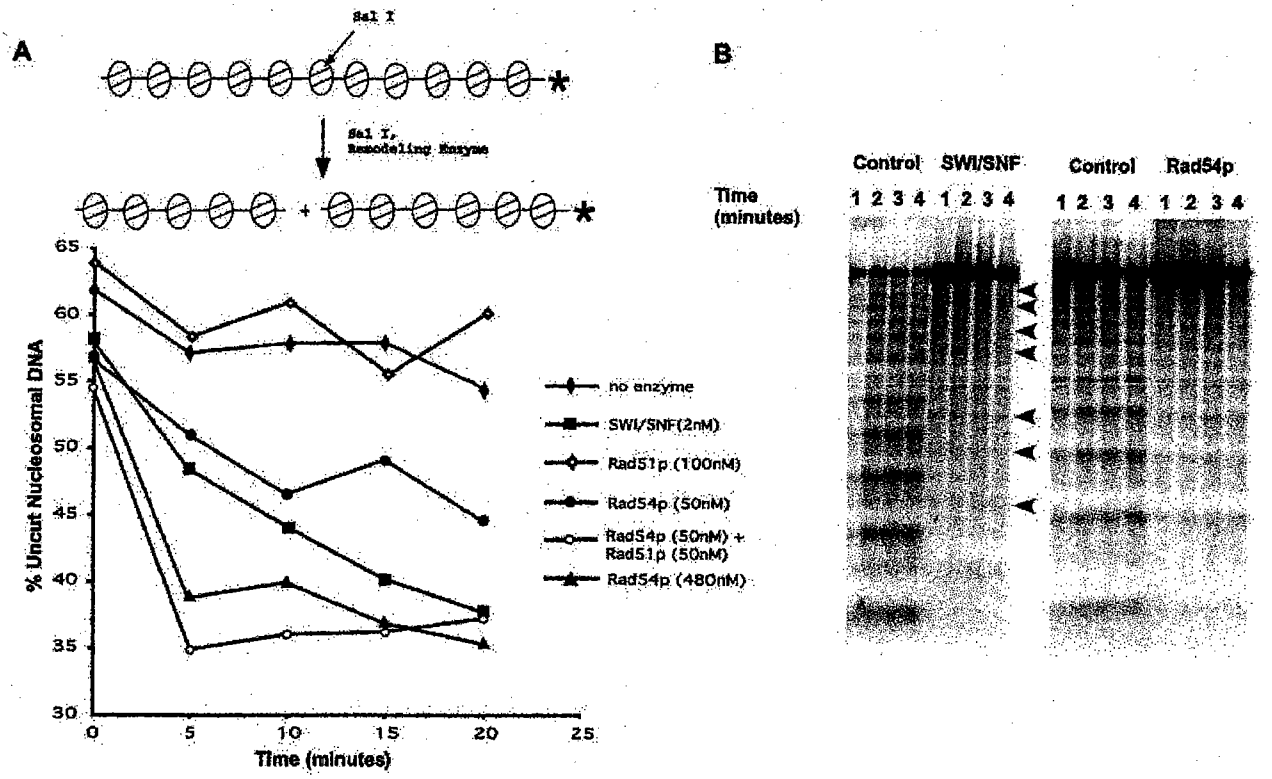

FIG. 6. **Rad54 is an ATP-dependent chromatin remodeling enzyme.** *A*, various concentrations of recombinant Rad54p were tested for chromatin-remodeling activity in a coupled remodeling-restriction enzyme cleavage assay. The nucleosomal substrate was incubated with 50 nM (*closed circles*) or 480 nM Rad54p (*triangles*), 100 nM Rad51p (*open diamonds*), 50 nM Rad54p + 50 nM Rad51p (*open circles*), 2 nM ySWI/SNF (*squares*), or buffer (*closed diamonds*). *B*, 208–11 reconstituted nucleosomal arrays were incubated at 37 °C with 2 nM SWI/SNF, 100 nM Rad54p, or buffer (control lanes). Aliquots were treated with Mnase for the indicated times. The *arrowheads* in the *left panel* indicate the alternate banding pattern as a result of SWI/SNF-induced nucleosome movement.

combination proteins, Rad51p and Rad54p, are sufficient to promote heteroduplex DNA joint formation with chromatin. In contrast, the bacterial recombinase RecA is completely inactive with a chromatin donor. The unique capacity of the eukaryotic machinery to contend with chromatin likely reflects the chromatin-remodeling activity of Rad54p, in which the free energy from ATP hydrolysis enhances the accessibility of nucleosomal DNA. Strand invasion with chromatin may also require a specific interaction between Rad51p and Rad54p because the chromatin remodeling activity of Rad54p does not facilitate RecA-dependent D-loop formation with chromatin (Fig. 1*D*). Recently, Alexiadis and Kadonaga have reported that the *Drosophila* Rad51 and Rad54 proteins can also facilitate strand invasion with chromatin (35).

How Does Rad54p Remodel Chromatin Structure?—Several studies have shown that SWI/SNF-like chromatin remodeling enzymes can perform two separable reactions: 1) they can use the free energy from ATP hydrolysis to enhance the accessibility of nucleosomal DNA and 2) they can use this free energy to mobilize nucleosomes in cis (reviewed in Ref. 32). Recent work from Cairns and colleagues have suggested that both of these activities may be caused by ATP-dependent "pumping" of DNA into the nucleosome (21). In this model, small amounts of DNA translocation might lead to transient exposure of small "loops" of DNA on the surface of the histone octamer, whereas larger quantities of DNA "pumped" into the nucleosome would lead to changes in nucleosome positions. Our data support this model, as we find that both yeast SWI/SNF and Rad54p, like yeast RSC (21), can disrupt a DNA triplex in an ATP-dependent reaction, presumably by translocation of DNA along the surface of the enzyme or by translocation of the enzyme along the DNA. Furthermore, the ATPase activities of ySWI/SNF and Rad54p are sensitive to DNA length, which is diagnostic of DNA-translocating enzymes (21).

Although ySWI/SNF and Rad54p can both enhance the accessibility of nucleosomal DNA, only ySWI/SNF appears to be proficient at changing nucleosome positioning. This result suggests that the precise mechanism of chromatin remodeling by Rad54p may be distinct from that of ySWI/SNF. For instance, Rad54p may only be able to pump small amounts of DNA across the histone octamer surface. Alternatively, Rad54p may translocate along DNA, rather than pumping DNA into the nucleosome. In this model, Rad54p may "pull" the Rad51-ssDNA nucleoprotein filament along the chromatin fiber, leading to changes in nucleosomal DNA topology and DNA accessibility. Such a DNA tracking mechanism might play a key role in facilitating both the search for homology as well as the strand invasion step.

Multiple Roles for Rad54p during Homologous Recombination—Our results suggest that Rad54p is an extremely versatile recombination protein that plays key roles in several steps of homologous recombination. Recently, we found that Rad54p is required for optimal recruitment of Rad51p to a double strand break *in vivo*, and likewise Rad54p can promote formation of the presynaptic filament *in vitro* by helping Rad51p contend with the inhibitory effects of the ssDNA-binding protein replication protein A.[2] Several studies over the past few years have also shown that the ATPase activity of Rad54p plays key roles subsequent to formation of the presynaptic filament. For instance, Rad54p is required for the Rad51p-nucleoprotein filament to form a heteroduplex joint DNA mol-

[2] B. Wolner, S. Van Komen, P. Sung, and C.L. Peterson, submitted for publication.

ecule, even when the homologous donor is naked DNA (Fig. 1*A*; see also Refs. 7, 18, 33). In this case, it has been proposed that Rad54p might use the free energy from ATP hydrolysis to translocate along DNA, which facilitates the homology search process. This DNA-translocation model is fully consistent with our findings that Rad54p can displace a DNA triplex and that the ATPase activity of Rad54p is proportional to DNA length. Rad54p also stimulates heteroduplex DNA extension of established joint molecules (34). Finally, we have shown that Rad54p is required for Rad51p-dependent heteroduplex joint molecule formation with a chromatin donor. In this case, our results suggest that the ATPase activity of Rad54p is used to translocate the enzyme along the nucleosomal fiber, generating superhelical torsion, which leads to enhanced nucleosomal DNA accessibility. It seems likely that this chromatin remodeling activity of Rad54p might also facilitate additional steps after heteroduplex joint formation. Future studies are now poised to reconstitute the complete homologous recombinational repair reaction that fully mimics each step in the repair of chromosomal DNA double strand breaks *in vivo*.

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Chromatin and transcription: histones continue to make their marks

Mariela Jaskelioff and Craig L. Peterson

Chromatin architecture is modulated by a large number of enzymes, resulting in the proper regulation of transcription, replication, cell cycle progression, DNA repair, recombination and chromosome segregation. The structure, regulation and coordination of these enzymatic activities were the main topics of discussion at The Enzymology of Chromatin and Transcription Keystone Symposium held in Santa Fe, NM (March 10–16, 2003).

Chromatin fibres are the natural substrates for all DNA-mediated processes, and thus it is not too surprising that a variety of evolutionarily conserved enzymes modulate the architecture of chromatin, allowing the precise temporal and spatial regulation of genetic processes. These enzymes, the focal point of this Keystone Symposium organized by Shelly L. Berger (Wistar Institute, Philadelphia, PA) and Jerry L. Workman (Pennsylvania State University, State College, PA), can be divided in two major classes: first, those that introduce covalent modifications on histone amino- or carboxy-terminal 'tail' domains; second, those that use the free energy derived from ATP hydrolysis to actively disrupt nucleosomal structure.

With the exception of one plenary session devoted to the mechanism of action of the ATP-dependent remodelling enzymes, many of the presentations centred on the coordinated action of histone-modifying enzymes during the transcription process and the various roles that the subsequent histone 'marks' play in establishing on or off states of gene expression.

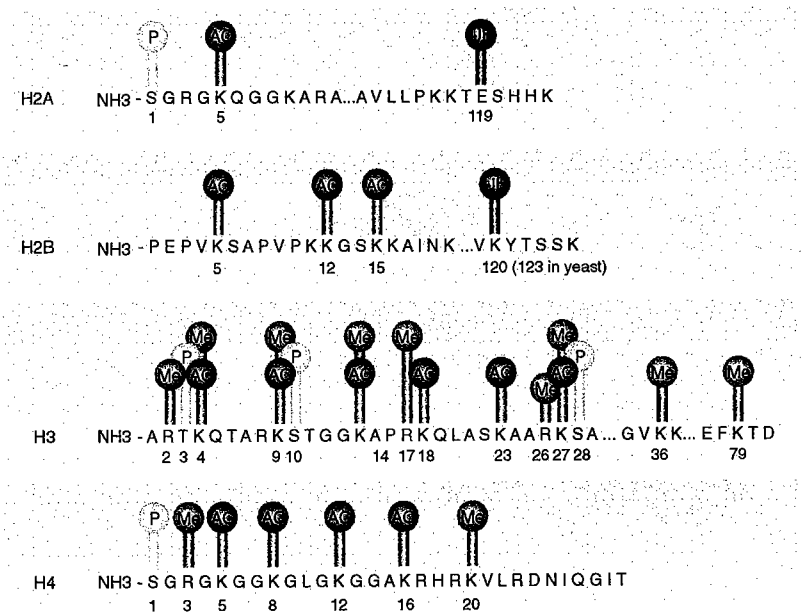

Figure 1 **Potential sites of post-translational modification on nucleosomal histones. Many modification patterns have been closely linked to unique biological outcomes. Ac, acetylation; Me, methylation; P, phosporylation; Ub, ubiquitination.**

Histone methylation comes to centre stage

In his keynote address, C. David Allis (University of Virginia, Charlottesville, VA) proposed that histones are major carriers of epigenetic information and that covalent modifications on the histone N-terminal tails function as master on/off switches that determine whether a gene is active or inactive. Histone tails are subject to a wide range of post-translational modifications (Fig. 1). Although in past years, histone acetylation on lysine residues has received the lion's share of attention, remarkably, histone acetylation was not a major topic of discussion at this meeting. In its place, histone methylation permeated nearly every session. Methylation can have multiple effects on chromatin function, depending on the specific lysine and the level of modification (that is, mono-, di-, or tri-methylation of a single lysine). For instance, H3-K9 di-methylation and H3-K27 tri-methylation are both largely associated with gene silencing and heterochromatin formation[1], whereas methylation of H3-K4, H3-K36, or H3-K79 is associated with active chromatin. Tony Kouzarides (University of Cambridge, Cambridge, UK) also presented evidence that the number of methyl groups on a single lysine is associated with distinct chromatin states. Whereas several inactive genes are marked by H3-K4 di-methylation, the transition to transcriptional competence correlated with H3-K4 tri-methylation[2]. Thus, it is clear that the site specificity of lysine methylation, as well as the number of methyl groups attached to a particular lysine, can have distinct consequences for transcription.

With few exceptions, histone methylation is catalysed by proteins that contain a conserved SET domain flanked by cysteine-rich pre-SET and post-SET domains. Steve Gamblin (Institute for Medical Research, London, UK), Raymond Trievel (NIH, Bethesda, MD) and Xing Zhang (Emory University, Atlanta, GA) each presented crystallographic structures of the catalytic domains from three different methyltransferases — SET7/9, LSRT and DIM-5, respectively — in complex with their substrate. A hallmark of each structure was a narrow, doughnut-like hole at the catalytic core where methyl transfer occurs. The peptide substrate binds on one side of the channel and the methyl donor occupies the opposite face. Once the lysine is docked into

the active site, one hydrogen from the ε-NH_3 protrudes through the hole to the opposite face where the transfer reaction takes place. In the case of a mono-methylase, SET7/9, the target ε-NH_3 group is rigidly bound in the active site such that only a single methyl-transfer event can occur[3]. In contrast, the ε-NH_3 group has greater freedom of rotation in the active site of di- or tri-methyltransferases (LSMT, DIM-5), which allows for additional methylation events[4,5]. Remarkably, Xing Zhang (Emory University) demonstrated that a tri-methylase or a mono-methylase could be converted to a di-methylase simply by changing one of the amino acids that controls the hydrogen-bonding environment of the ε-NH_3 group.

Combinatorial histone modifications: regulating the marks

To date, there are still no reports of a *bona fide* 'demethylase', and thus it seems that histone methylation is a relatively stable chromatin mark that can only be lost by successive rounds of DNA replication or by replication-independent histone replacement. David Allis suggested that cells may use reversible serine/threonine phosphorylation to regulate the biological outcomes of lysine methylation[1]. His 'methyl/phospho switch' hypothesis argues that a phosphorylation event on a histone tail would regulate the binding of an effector protein to an adjacent methylated residue. In support of this model, he presented numerous examples in which a serine or threonine is found adjacent to a methylation site (lysine) throughout the sequence of histone proteins. As a proof of principle, David Allis presented evidence that phosphorylation of Ser 10 of a histone H3 peptide blocks the ability of heterochromatin protein 1 (HP1) to bind its cognate substrate, di-methylated H3-K9.

Whereas the control of histone methylation by adjacent phosphorylation is a newly emerging paradigm, other types of combinatorial histone modifications are better established. For instance, several presentations, including those from Mary Ann Osley (University of New Mexico, Albuquerque, NM), Karl Henry (Wistar Institute, Philadelphia, PA), Brad Bernstein (Harvard Medical School, Boston, MA) and Ali Shilatifard (St. Louis University, St. Louis, MO) demonstrated that ubiquitination of H2B-K123 is required for subsequent methylation of H3-K4 and H3-K79 (refs 6–8). All three of these marks are important for transcriptional activity. Similarly, Robert Roeder (Rockefeller University, New York, NY) presented evidence from *in vitro* studies for a multistep model for p53-mediated *in vitro* transcription, in which methylation of H4-R3 by protein arginine methyltransferase 1

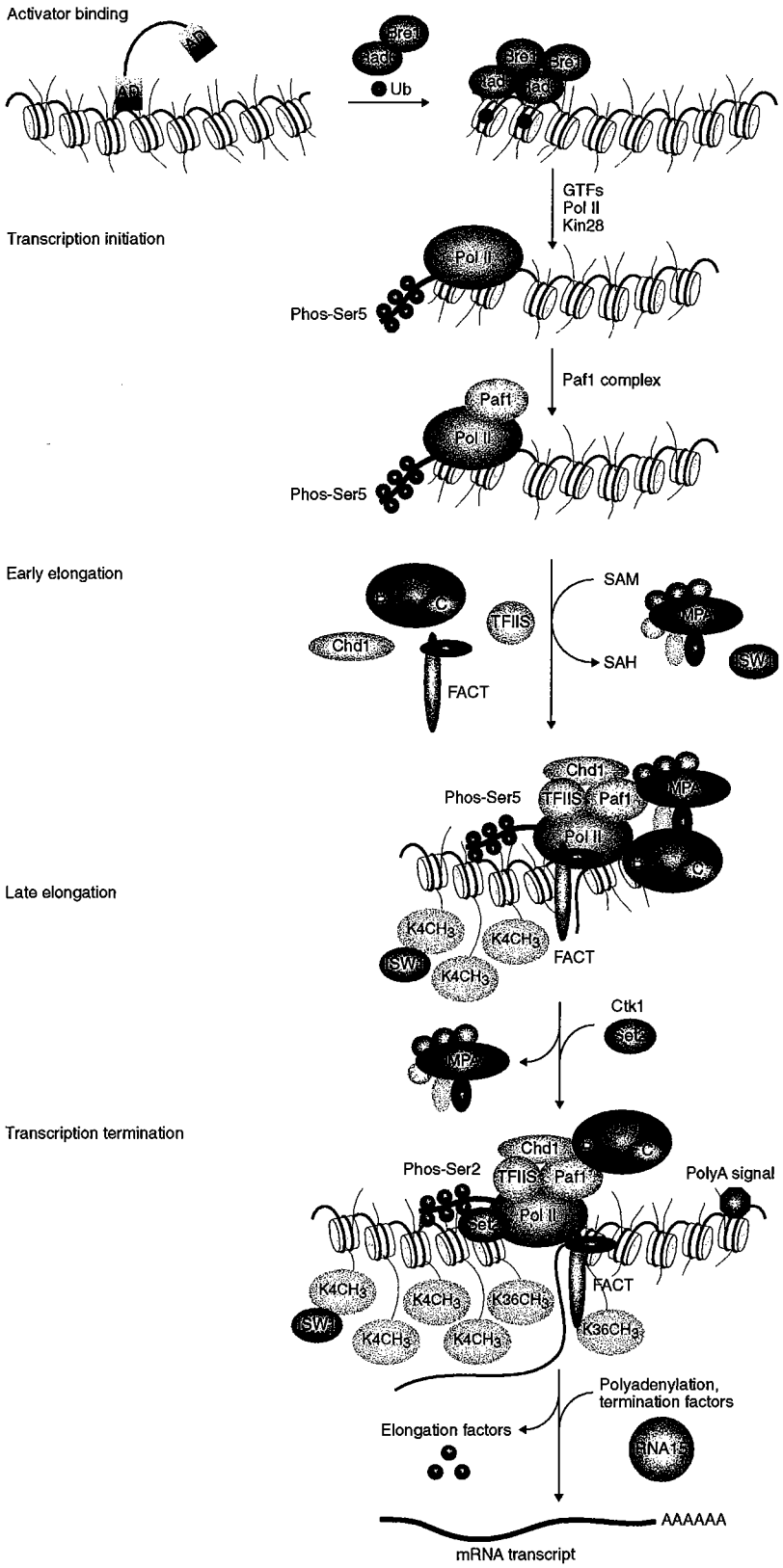

Figure 2 **Transcriptional elongation by RNAPII on chromatin substrates. Rad6p/Bre1p are recruited by transcriptional activators to ubiquitinate H2B. The RNAPII CTD repeats are phosphorylated on Ser 5 by Kin28p. As the RNAPII complex progresses, Ser 5 is dephosphorylated and Ser 2 is phosphorylated by Ctk1p. The H3 K36-specific methyltransferase Set2p associates with the ongoing complex, promoting late elongation. When a polyadenylation site is reached, Ser 2 is dephosphorylated by Fcp1p and most elongation factors dissociate from the RNAPII complex. Polyadenylation and termination factors associate with the RNAPII complex, resulting in the release of the polyadenylated mRNA transcript. We thank Ali 'the fisherman' Shilatifard for help with this figure.**

(PRMT1) stimulates CBP/p300 acetylation of H4-K5, K8, K12 and K16, which in turn promotes the methylation of H3-R2, R17 and R26 by another PRMT family member, CARM1. The integrated action of these histone-modifying enzymes correlates with strong transcriptional activation of the p53-dependent gene. Cellular chromatin immunoprecipitation (ChIP) assays confirmed the stepwise recruitment of these cofactors and accompanying histone modifications in response to activation of a p53-dependent gene by ultraviolet irradiation. Similarly, Michael Stallcup (University of Southern California, Los Angeles, CA) showed that oestrogen-dependent transcription also required the concerted action of PRMT1/CARM1/p300, recruited to the oestrogen-responsive promoter by GRIP-1/ER-activating complex[9]. Finally, Ronen Marmorstein (Wistar Institute, Philadelphia, PA) presented the structural basis for why phosphorylation of H3-S10 enhances the ability of Gcn5p to acetylate H3-K14 *in vitro* and at some sites *in vivo*[10]. He found that the phosphorylated serine induces H3 adjustments both local and distal to H3-S10, resulting in a more optimal anchoring of H3 for K14 acetylation.

How do histone modifications exert their effects?

A large number of presentations focused on how histone marks exert their biological effects. In theory, site-specific histone modifications might influence chromatin function through two mechanisms: first, by affecting nucleosome–nucleosome interactions that control the folding of nucleosomal arrays; second, by promoting or disrupting the chromatin binding of non-histone proteins that link the covalent modification to a biological outcome. Addressing the first potential mechanism has been problematic because of the technical inability to reconstitute nucleosomal arrays that harbour homogeneous and site-specific modifications. In response to this problem, we presented our recent success at developing a native chemical ligation strategy permitting the reconstitution of nucleosomal arrays that harbour a wide range of site-specific histone modifications, including serine phosphorylation, lysine acetylation and lysine methylation[11].

A great deal of progress is being made in the identification of proteins that bind to histone tails harbouring specific marks. Several talks discussed the binding of the heterochromatin component HP1 to the H3 tail dimethylated at K9. Similarly, Yi Zhang (UNC Chapel Hill, NC) presented *in vivo* and *in vitro* studies demonstrating that the *Drosophila melanogaster* and mammalian Polycomb complexes interact with histone H3 tri-methylated at K27. In both of these cases, the binding of effector proteins to methylated H3-K9 or H3-K27 promoted gene silencing[12]. Chromatin marks that are associated with transcriptionally active genes may also exert their effects by influencing the binding of chromatin remodelling factors. Although not discussed at this meeting, acetylated lysines can interact with bromodomains found within subunits of SWI/SNF-like chromatin remodelling complexes[13]. Additionally, Tony Kouzarides presented compelling evidence that methylation of H3-K4 promotes the binding of the yeast Isw1p ATPase. Thus, histone modification provides a means to recruit enzymes that disrupt chromatin structure (for example, Isw1p) or to recruit proteins that further package the chromatin fibre into inaccessible states (for example, HP1).

Heterochromatin formation and silencing

The establishment and maintenance of transcriptional silencing by heterochromatic structures was discussed at length. Michael Grunstein (UCLA, Los Angeles, CA) provided evidence supporting a stepwise model for telomeric heterochromatin assembly in budding yeast. His results indicated that the Rap1p protein, which is localized to the T_{1-3} telomeric repeats, recruits Sir4p, which in turn recruits the histone-binding protein Sir3p and the histone deacetylase Sir2p. The NAD^+-dependent deacetylation of H4-K16 by Sir2p then allows the spreading of these three Sir proteins from the telomere[14]. Work presented by both Michael Grunstein and Masami Horikoshi (University of Tokyo, Tokyo, Japan) indicated that the advancement of this silencing structure into euchromatic regions is blocked by the action of Sas2p, a histone acetyltransferase that targets H4-K16 and counteracts the action of Sir2p[15,16]. In fact, Sas2p seems to have a global role in the maintenance of H4-K16 acetylation. Ann Ehrenhofer-Murray (Max-Planck Institute, Berlin, Germany) reported that Sas2p is part of the yeast SAS-I complex, which interacts with the chromatin assembly factor CAF-1 and is recruited to DNA replication forks to re-establish H4-K16 acetylation on newly assembled chromatin[17].

A number of speakers discussed the roles of HP1 in gene silencing, heterochromatin formation and chromosome segregation. In *Drosophila*, HP1 is associated with pericentric and telomeric regions, a region along the arm of the 4th chromosome and approximately 200 other euchromatic sites. *In vitro*, HP1 prefers to bind to a histone H3 tail dimethylated at K9, and *in vivo* HP1 generally (but not always) colocalizes with this histone mark. Lori Wallrath (University of Iowa, Iowa City, IA) demonstrated that the artificial tethering of a LacI–HP1 fusion protein to an ectopic location could cause the long range silencing of reporter genes[18]. Remarkably, the tethered HP1 did not result in H3-K9 methylation. Sally Elgin (Washington University, St. Louis) then discussed the fact that HP1 requires other gene products to effect heterochromatin-based gene silencing, and that one of these factors, HP2, colocalizes with HP1 at many loci[19]. This HP1-based silencing system is not used solely for establishment of heterochromatin domains, as Frank Rauscher III (Wistar Institute, Philadelphia, PA) provided an example of HP1 recruitment to specific promoters by the KAP-1 corepressor. KAP-1 coordinates histone deacetylation, histone methylation and HP1 deposition to effect silencing by the KRAB-ZFP superfamily of transcriptional repressors. Interestingly, this highly localized silencing structure was mitotically stable and was maintained for more than 50 population doublings, even in the absence of the DNA-bound KRAB repressor[20].

Perhaps the most highly anticipated talks of the symposium were those presented by Shiv Grewal (CSHL, Cold Spring Harbor, NY) and Robin Allshire (Wellcome Trust Centre for Cell Biology, University of Edinburgh), who discussed their recent studies on the role of the RNA interference (RNAi) machinery in establishing and maintaining heterochromatin structures at the fission yeast mating-type locus and centromeres[21,22]. Both groups demonstrated that a short piece of centromeric repetitive DNA was sufficient to establish RNAi-dependent, heterochromatin-based gene silencing at an ectopic location. Furthermore, RNAi was shown to be required to recruit the histone H3-K9 methyltransferase, Clr4, the fission yeast homologue of HP1, Swi6 and cohesion pro-

teins. Shiv Grewal then described recent work indicating that the fission yeast homologue of the Sir2 deacetylase is also required for establishing heterochromatin-based gene silencing at centromeres and the mating-type locus. Robin Allshire also presented data indicating that repression of some euchromatic genes may be regulated by the RNAi machinery/Clr4/Swi6. Thus, it seems that the RNAi-dependent establishment of heterochromatin may represent a more general means for creating silent states at euchromatic loci.

The transcriptional cycle

In contrast to previous meetings of this kind, very few presentations focused on the regulated formation of the transcription pre-initiation complex, but rather transcriptional elongation by RNA polymerase II was the centrepiece of discussion. Work from a number of groups indicated that transcriptional elongation involves an enormous number of regulatory factors (Fig. 2). As expected, several talks focused on the how RNAPII contends with the nucleosomal barrier to elongation. Danny Reinberg (UMDNJ, Piscataway, NJ) presented compelling evidence for association of the FACT complex with elongating polymerase in *Drosophila* (in collaboration with J. Lis) and yeast cells. Mechanistic studies by his group (and in collaboration with V. Studitsky) indicated that FACT facilitates nucleosomal elongation by destabilizing one of the histone H2A/H2B dimers. Robert Kingston (Harvard Medical School, Boston, MA) also presented *in vivo* evidence that the human SWI/SNF ATP-dependent remodelling complex is recruited to the hsp70 promoter by heat shock transcription factor, where SWI/SNF is then central to transcriptional elongation.

Several presentations focused on the role of multisite phosphorylation of the heptapeptide repeats in the C-terminal domain (CTD) of the largest subunit of RNAPII as a means for recruiting various elongation factors. For instance, Stephen Buratowski (Harvard Medical School, Boston, MA) discussed how phosphorylation of Ser 5 within the CTD of early elongation complexes results in recruitment of the mRNA-capping enzyme and the Set1p-containing histone H3-K4 methyltransferase complex, COMPASS (Fig. 2). Similarly, Ali Shilatifard showed that recruitment of COMPASS to this elongating form of RNAPII involved its interaction with the PAF1 complex, which is known to associate with the elongating form of RNAPII. He also showed that the PAF1 complex controls the histone methyltransferase activity of Dot1p, thereby linking methylation of H3-K79 to transcriptional elongation[23] (See News and Views by Bryan Turner on page 390 of this issue). Work presented by Tony Kouzarides and Antonin Morillon (University of Oxford, Oxford, UK) indicated that the elongation role of the COMPASS methyltransferase complex may involve recruitment of the Isw1p ATP-dependent chromatin remodelling enzyme, as Isw1p binds to an H3 tail that is di- or tri-methylated at K4, and *ISW1* is required *in vivo* for a normal distribution of RNAPII on coding regions. As elongation by RNAPII progresses, Ser 2 on the CTD is phosphorylated by the Ctk1p kinase. Results presented by both Brian Strahl (UNC, Chapel Hill, NC) and Ali Shilatifard indicated that phosphorylation of Ser 2 may release the COMPASS complex and result in recruitment of Set2p, which methylates H3-K36. Why H3 methylation sites change during the stages of RNAPII elongation is not known.

Ubiquitin-dependent proteolysis and transcription

Several presentations described examples of transcriptional regulation by ubiquitin-dependent recruitment of proteosomal components. Talks by Thomas Kodadek (UT Southwestern, Dallas, TX) and Michael Hubner (EMBL, Heidelberg, Germany) described how the functioning of either yeast Gal4p or the mammalian oestrogen receptor are controlled by ubiquitin-dependent recruitment of the 19S APIS proteasomal subcomplex. In the case of Gal4p, Kodadek presented evidence that Gal4p may be ubiquitinated when bound at the *GAL1* promoter, and that subsequent recruitment of a 19S APIS proteasomal subcomplex might regulate Gal4p DNA binding or control Gal4-RNAPII holoenzyme interactions[24]. Hubner presented an integrated model for the cyclic turnover of oestrogen receptor α on responsive promoters. In this model, both unliganded and liganded oestrogen receptor α, as well as E3 ubiquitin ligases and the 19S APIS complex, cycle on and off an oestrogen response element *in vivo*. Subsequent ubiquitination and proteasome-mediated removal of oestrogen receptor α from the promoter are instrumental for maintaining oestrogen signalling. Consequently, inhibition of the proteasome results in the loss of oestrogen receptor α-mediated transcription, whereas transcriptional inhibition prevents oestrogen receptor α degradation by the proteasome[25]. Thus, both of these studies suggest that dynamic regulation of activator degradation or function may control transcriptional activation.

Transcriptional regulation by ubiquitin-dependent events does not seem to be restricted to the gene-specific activators. Jesper Svejstrup (Cancer Research UK London Research Institute, London, UK) discussed their identification of a yeast factor called DEF1, which seems to be a novel elongation factor that is required for ubiquitin-dependent degradation of RNAPII in response to DNA damage[26]. On a related note, Joan Conaway (Stower Institute, Kansas City, MO) described their ongoing studies characterizing the Elongin B/C ubiquitin ligase complex. She described yeast two-hybrid and biochemical studies, which show that Elongin B/C interacts directly with the Med8p subunit of the mammalian mediator complex. In fact, she showed that purified preparations of mammalian mediator contain sub-stoichiometric levels of Elogin B/C and that mediator has ubiquitin ligase activity[27]. What the targets for this activity might be in the transcription initiation or elongation complex are not known.

The future

As researchers continue to pry their way into the mysteries of chromatin and transcriptional control, the possibilities for regulation seem endless. A multitude of histone modifications control recruitment of chromatin remodelling enzymes, as well as proteins that influence the higher-order folding of chromatin fibres. It now seems that millions of Daltons of protein are not only bound at the transcription initiation site, but that even larger protein assemblies travel down the gene with the elongating polymerase. And yet, although the meeting described many new factors and new regulatory paradigms, many old topics went virtually unmentioned. For instance, linker histones and histone variants are general features of the intrinsically heterogeneous chromatin fibre, but almost no mention was made of how these factors control the solution state behaviour and function of chromatin fibres. Recent *in vivo* studies from Belmont and colleagues indicate that transcription actually occurs on enormous, 100–400-nm thick chromatin fibres[28]. How can this fibre accommodate the many proteins involved in the initiation and elongation of transcription? And finally, as we noted at the start of this report, chromatin structure and histone modifications are commonly thought to imprint an epigenetic code on transcriptional patterns. However, we still have little knowledge of how chromatin states are propagated after passage of a replication fork. It is clear that we still have lots of work to do before all of the mysteries are solved. □

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