Elucidation of the Mechanisms of Nucleosome Binding and Repositioning by a Chromatin Remodeler:

Monomeric ISWI Remodels Nucleosomes Through a Random Walk

By

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Submitted to the graduate degree program in Molecular Biosciences and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The regulation of chromatin structure is controlled by a family of molecular motors called chromatin remodelers. The ability of these enzymes to remodel chromatin structure is dependent on their ability to couple ATP binding and hydrolysis into the mechanical work that drives nucleosome repositioning. The goal of this work was to characterize quantitatively the nucleosome repositioning activity, and associated processes of nucleotide binding, DNA binding, and nucleosome binding, of the chromatin remodeler ISWI. ISWI is capable of repositioning clusters of nucleosomes to create well-ordered arrays or moving single nucleosomes from the center of DNA fragments toward the ends without disrupting their integrity.

The necessary first step in determining how these essential enzymes catalyze the repositioning of nucleosomes is to characterize both how they bind nucleosomes and how this interaction is regulated by ATP binding and hydrolysis. Toward this goal we monitored the interaction of the chromatin remodeler ISWI with fluorophore-labeled nucleosomes and DNA through associated changes in fluorescence anisotropy of the fluorophore upon ISWI binding to these substrates. We determined that one ISWI molecule binds to a 20 bp double stranded DNA substrate with an affinity of (18 ± 2) nM. In contrast, two ISWI molecules can bind to the core nucleosome with short linker DNA with stoichiometric macroscopic equilibrium constants $1/\beta_1$ = (1.3 ± 0.6) nM and $1/\beta_2$ = (13 ± 7) nM^2. Furthermore, in order to better understand the mechanism of DNA translocation by ISWI, and hence nucleosome repositioning, we determined the effect of nucleotide analogs on substrate binding by ISWI. While the affinity of ISWI to binding nucleosome

substrate with short lengths of flanking DNA was not affected by presence of nucleotides, the affinity of ISWI for binding DNA substrate is weakened in the presence of non-hydrolysable ATP analogs but not in the presence of ADP. Additionally, using standard electrophoresis assays we have monitored the ISWI-catalyzed repositioning of different nucleosome samples each containing different lengths of DNA symmetrically flanking an initially centrally positioned histone octamer. We find that ISWI moves the histone octamer between distinct and thermodynamically stable positions on the DNA according to a random walk mechanism. Through the application of a novel spectrophotometric assay for nucleosome repositioning we further characterized the repositioning activity of ISWI using short nucleosome substrates and were able to determine the macroscopic rate of nucleosome repositioning by ISWI. Additionally, quantitative analysis of repositioning experiments performed under various ISWI concentrations revealed that monomeric ISWI is sufficient to account for the observed repositioning activity as the presence of a second ISWI bound had no effect on the rate of nucleosome repositioning. We also found that ATP hydrolysis is poorly coupled to nucleosome repositioning suggesting that DNA translocation by ISWI is not energetically rate limiting for the repositioning reaction. This is the first calculation of a microscopic ATPase coupling efficiency for nucleosome repositioning and also further supports our conclusion that a second bound ISWI does not contribute to the repositioning reaction.

In conclusion, the characterization of the mechanism of nucleosome binding and repositioning by the chromatin remodeler ISWI presented in this dissertation provides a foundation for future studies aiming to understand how various regulatory elements influence the function of ISWI.

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DEDICATION

For Hikmat and Meral

الكلمات تعجز عن وصف مدى حبي لكما

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CHAPTER 1:

BACKGROUND

Nucleosomes: The Basic DNA Packaging Unit

DNA within our cells is packaged into a higher order structure called chromatin. The initial stage of packaging involves the wrapping of 147 bp of DNA ~1.65 times around a core of eight histone proteins forming the nucleosome (Figure 1.1) [1–3]. The histone octamer is composed of two of each of the histones H3, H4, H2B, H2A. The linker histone H1, along with other proteins, aids in the formation of more complex structures of the chromatin [1–3]. The length of the linker DNA between two nucleosome core particles varies among species and cell types with values ranging from 10-50 bp [1].

Within the nucleosome, the backbone of the wrapped DNA contacts the histone proteins every 10.2 bp forming several non-covalent, and mostly non-specific, interactions at each contact point. These interactions involve: hydrogen bonding between phosphate groups and main chain amide groups of amino acids; frequent hydrogen bonding and electrostatic interactions between oxygen atoms in the DNA backbone and basic side chains of histones; specific interactions between arginine side chains and bases within the minor groove; and electrostatic interactions between the N–terminus of each of the histones and phosphate groups on the DNA backbone [4]. It was shown that the bending and wrapping of the DNA around the histones induces changes in the basic properties of the DNA and that DNA sequences that are more accommodating of such distortions are preferred as nucleosome binding and positioning sequences [4,5].

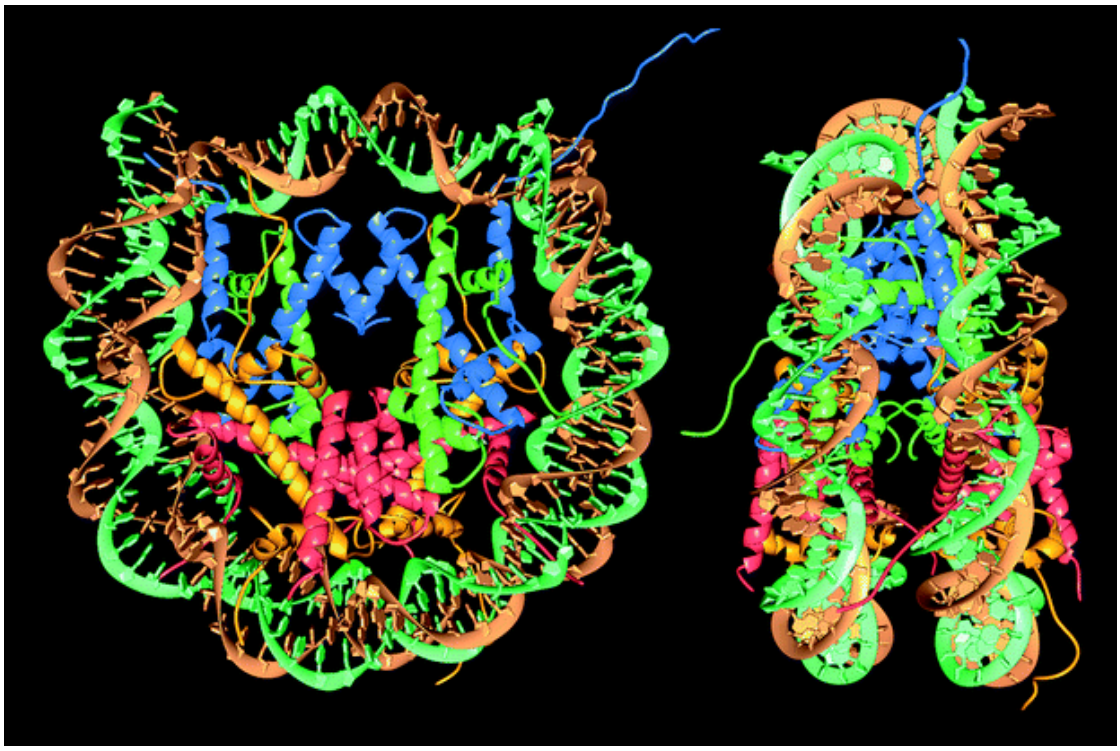


Figure 1.1: Crystal structure of the nucleosome core particle at 2.8 Å resolution. 146 bp DNA; blue: H3; green: H4; yellow: H2A; red: H2B. Modified from Luger *et al.* 1997, *Nature.*

The packaging of DNA into the higher order structures of the chromatin plays a protective as well as a regulatory role. For example, it can prevent access of DNA binding proteins to important DNA sequences (promoters and enhancers) and affect the activity of RNA polymerase thereby directly affecting the process of DNA transcription. Accessibility of the DNA replication and DNA damage repair machineries to DNA can also be hindered by the presence of nucleosomes along the DNA and by the altered properties of the wrapped DNA. Additionally, nucleosomes need to be re-deposited and repositioned following a new round of DNA synthesis. The rearrangement of chromatin structure in response to these processes can be achieved through two known mechanisms. One mechanism involves the epigenetic modification of the nucleosomes by chromatin modifying enzymes [6,7] (Phosphorylation, Methylation,

Acetylation, PARylation, SUMOlation, O-GlcNAcylation). The other mechanism involves an ATP-dependent rearrangement of the chromatin by a group of enzymes called chromatin remodelers [8–10]. The latter is the main focus of this dissertation.

Chromatin remodelers

Based on sequence and functional properties, chromatin remodelers are classified as part the Snf2 family of proteins, and in turn are part of the helicase superfamily II (SF-II) [11], which includes several DNA and RNA helicases as well as type I and III restriction enzymes[11]. Indeed, all chromatin remodelers share a highly conserved ATP-hydrolyzing domain which shares significant homology with other members of the Snf2 family such as helicases [11–13]. The ability of helicases to translocate along DNA is necessary for their double-stranded DNA unwinding activity [14–17]. Chromatin remodelers have been shown to lack helicase activity [18] but to retain the ability to translocate along free or nucleosomal DNA in an ATP-dependent manner, a property essential for their nucleosome repositioning activity [19–22].

Chromatin remodelers are further categorized into four subfamilies (ISWI, SWI/SNF, CHD, and INO80) based on additional domains that confer specific functional properties [11,23,24]; such as their ability to assemble, remove, transfer, space, or randomize nucleosomes as well as their ability to recognize histone modifications (Figure 1.2). The focus of this dissertation is the ISWI subfamily of chromatin remodelers.

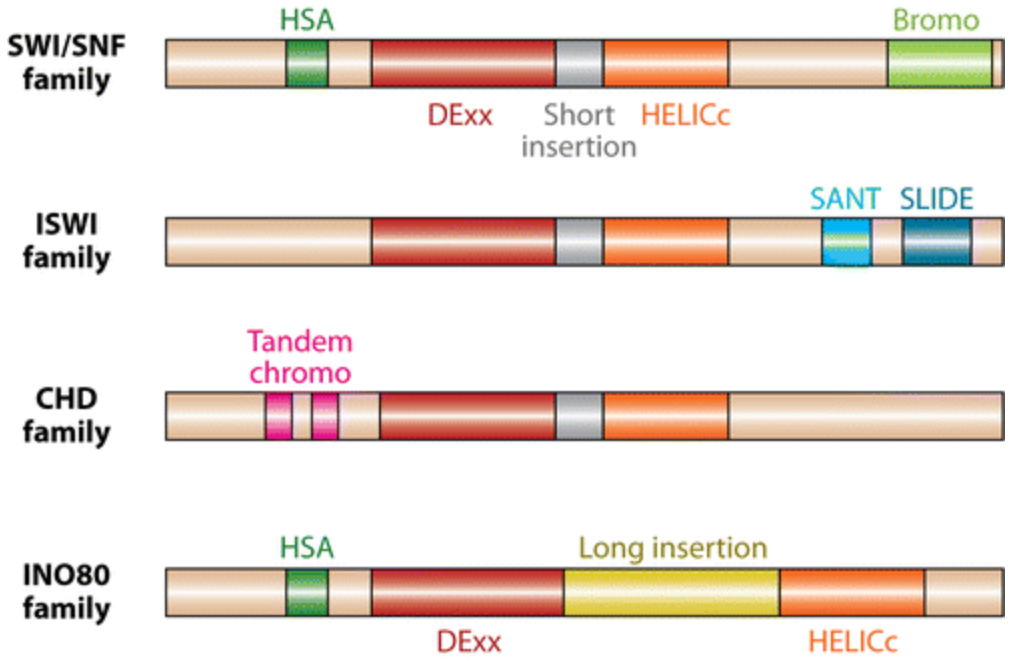


Figure 1.2: Chromatin remodeling subfamilies. Modified from Clapier and Cairns. 2009, *Annual reviews in Biochemistry*.

ISWI (Imitation SWItch)

The 135 kDa ISWI (Imitation SWItch) ATPase is a member of the ISWI subfamily of chromatin remodeling enzymes [25] and has been found to be required for cell viability in higher eukaryotes [26,27], ISWI homologs have also been identified in humans [28], *Drosophila melanogaster* [29], and *S. cerevisiae* [30]. In comparison to other remodelers, ISWI possess the unique property of being a conditional ATPase; DNA substrates cause only low levels of ATPase stimulation while nucleosomes allow for maximal stimulation of activity [29,31,32]. This suggests that ISWI recognizes specific motifs presented by the nucleosomes. Indeed, several studies have demonstrated that interactions between ISWI and histone tails, H4 tail in particular, are required for efficient ATPase stimulation and for the proper remodeling activity of ISWI complexes [31,33–36].

The N-terminal region of ISWI contains a highly conserved ATPase domain, while the C-terminal region of ISWI harbors the DNA binding module HSS (HAND-SANT (SWI/SNF, ADA, NCoR, TFIIIB)-SLIDE (SANT-like ISWI domain)). A recent study demonstrated that the N-terminal ATPase domain of ISWI was sufficient for the nucleosome repositioning activity of ISWI [37]. The ATPase domain was found to be an "autonomous" engine that has an intrinsic ability to establish ATPase simulating contacts with histone H4 tails and nucleosomal DNA and perform nucleosome remodeling [37]. It has been suggested that the C-terminal HSS domain serves a supporting role in the repositioning reaction where it contacts linker DNA, increases the affinity of ISWI to nucleosomes, enhances the remodeling efficiency and affects directionality of repositioning [37,38]. These results suggest a mechanism where the ATPase domain, independent of the HSS domain, is able to cause conformational changes in the nucleosome leading to weakened histone-DNA contacts and consequently nucleosome sliding. In addition, the ATPase domain of *Drosophila* ISWI is flanked by two regulatory modules, named AutoN and NegC. The sequence resemblance of AutoN to the H4 tail led to the proposal that AutoN prevents the activation of the ATPase domain in the absence of H4 tail. Also, the NegC was shown to regulate the DNA translocation activity of the ATPase domain. The results of a recent study suggest that these modules exert an auto-inhibitory effect on the ATPase function that is only relieved when the proper ISWI-substrate interactions are established [39].

Regulation of ISWI function:

The function of ISWI can be regulated by multiple factors that affect the targeting of ISWI to the chromatin, or influence the ATP consumption, nucleosome binding or nucleosome sliding activities of ISWI and consequently lead to a wide spectrum of cellular outcomes. One mode of regulation of ISWI function is through interactions with other noncatalytic proteins. ISWI interacts with other protein subunits to form three additional chromatin remodeling complexes: ACF (ATP-utilizing Chromatin assembly and remodeling Factor), CHRAC (CHRomatin Accessibility Complex), and WICH (WSTF-ISWI CHromatin remodeling complex) [40] (Figure 1.3). These highly conserved complexes were originally purified from *D. melanogaster* and the presence of the additional proteins within these complexes has been shown to modulate the nucleosome repositioning strategy and *in vivo* effects of ISWI activity [23,41–45]. **(**Table 1.1 list of subunits of chromatin remodeling complexes). Specifically, the presence of complex-specific noncatalytic subunits confer new properties to the ISWI-containing complex.

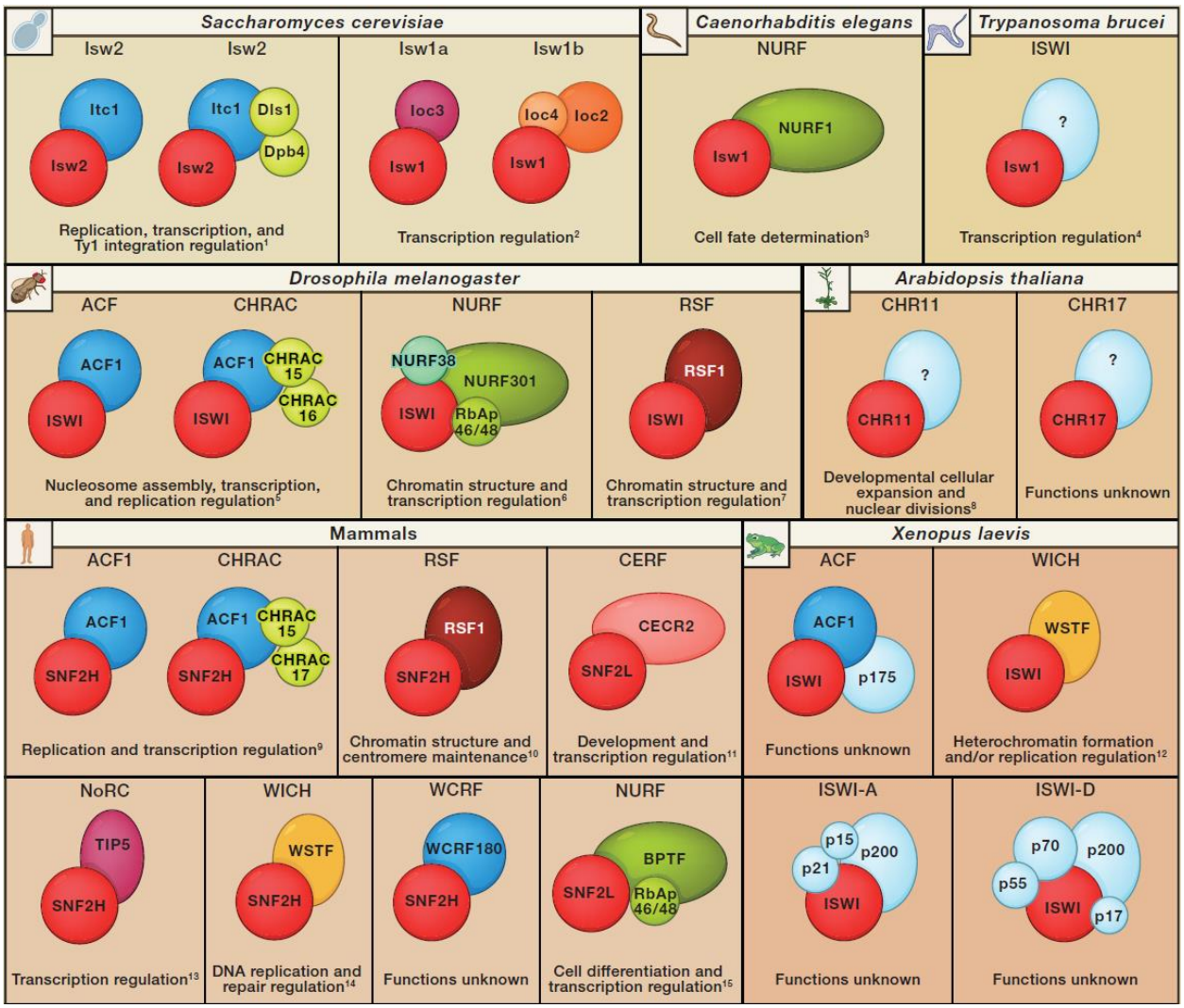


Figure 1.3: ISWI complexes. *Modified from Yadon et al. 2011, Cell.*

Family and composition		Organisms								
		Yeast			Fly			Human		
SWI/SNF	Complex	SWI/SNF		RSC	BAP		PBAP	BAF		PBAF
	ATPase	Swi2/Snf2		Sth1	BRM/Brahma			hBRM or BRG1		BRG1
	Noncatalytic homologous subunits	Swi1/Adr6			OSA/eyelid			BAF250/hOSA1		
							Polybromo BAP170			BAF180 BAF200
		Swi3		Rsc8/Swh3	MOR/BAP155			BAF155, BAF170		
		Swp73		Rsc6	BAP60			BAF60a or b or c		
		Snf5		Sfh1	SNR1/BAP45			hSNF5/BAF47/INI1		
					BAP111/dalao			BAF57		
		Arp7, Arp9			BAP55 or BAP47			BAF53a or b		
					Actin			β-actin		
	Unique	[a]		[b]						
ISWI	Complex	ISW1a	ISW1b	ISW2	NURF	CHRAC	ACF	NURF	CHRAC	ACF
	ATPase	Isw1		Isw2	ISWI			SNF2L	SNF2H[c]	
	Noncatalytic homologous subunits			Itc1	NURF301	ACF1		BPTF	hACF1/WCRF180	
						CHRAC14			hCHRAC17	
						CHRAC16			hCHRAC15	
					NURF55/p55			RbAp46 or 48		
	Unique	Ioc3	Ioc2, Ioc4		NURF38					
CHD	Complex	CHD1			CHD1	Mi-2/NuRD		CHD1	NuRD	
	ATPase	Chd1			dCHD1	dMi-2		CHD1	Mi-2α/CHD3, Mi-2β/CHD4	
	Noncatalytic homologous subunits					dMBD2/3			MBD3	
						dMTA			MTA1,2,3	
						dRPD3			HDAC1,2	
						p55			RbAp46 or 48	
						p66/68			p66α,β	
	Unique								DOC-1?	
INO80	Complex	INO80		SWR1	Pho-dINO80	Tip60		INO80	SRCAP	TRRAP/Tip60
	ATPase	Ino80		Swr1	dIno80	Domino		hIno80	SRCAP	p400
	Noncatalytic homologous subunits	Rvb1,2			Reptin, Pontin			RUVBL1,2/Tip49a,b		
		Arp5,8		Arp6	dArp5,8	BAP55		BAF53a		
		Arp4, Actin1			dActin1	Actin87E		Arp5,8	Arp6	Actin
		Taf14		Yaf9		dGAS41			GAS41	
		Ies2,6						hIes2,6		
				Swc4/Eaf2		dDMAP1			DMAP1	
				Swc2/Vps72		dYL-1			YL-1	
				Bdf1		dBrd8				Brd8/TRC/p120
				H2AZ,H2B		H2Av,H2B			H2AZ,H2B	
				Swc6/Vps71					ZnF-HIT1	
						dTra1				TRRAP
						dTip60				Tip60
						dMRG15				MRG15 MRGX
						dEaf6				FLJ11730
						dMRGBP				MRGBP
						E(Pc)				EPC1, EPC-like
						dING3				ING3
	Unique	Ies1,Ies3-5,Nhp10		Swc3,5,7	Pho			[d]		

[a]Swp82, Taf14, Snf6, Snf11.
[b]Rsc1 or Rsc2, Rsc3-5, 7, 9, 10, 30, Htl1, Ldb7, Rtt102.
[c]In addition, SNF2H associates respectively with Tip5, RSF1, and WSTF to form **NoRC**, **RSF**, and **WICH** remodelers.
[d]Amida, NFRKB, MCRS1, UCH37, FLJ90652, FLJ20309.

Table 1.1: List of members of chromatin remodeling complexes. *Modified from Clapier and Cairns. 2009, Annual reviews in Biochemistry.*

The Plant Homeo Domain, for example, present in noncatalytic subunits of ACF and NURF (NUcleosome Remodeling Factor) complexes provides an additional nucleosome binding site leading to an increase in the efficiency of repositioning [46–49]. Another example is the human CHRAC complex which contains two histone fold-containing proteins which were found to increase the efficiency of nucleosome repositioning by binding nucleosomal DNA as it exits the nucleosome [50]. Interestingly, the DNA sequence specific recruitment of *Drosophila* ISWI to promoter regions of certain genes was shown to be mediated by accessory subunits of the NURF complex. Unlike other subfamilies, ISWI lacks domains that allow it to recognize specific posttranslational modifications. However, it was shown that some ISWI complexes can be recruited to loci containing modified histones through modification-reading modules of the accessory subunits [23,51]. While some studies have shed light into the function of accessory subunits; the mode by which they regulate the nucleosome repositioning activity of ISWI, leading to various and sometime contradicting physiological outcomes, remains largely unexplored.

Another method of regulating the activity of remodelers is through covalent posttraslational modifications; two modifications have been reported to directly modify the ISWI motor. The histone acetyl transferase Gcn5 was shown to mediate the acetylation of lysine 753 of the HAND domain of ISWI both *in vitro* as well as *in vivo* [36]. However, the significance of this modification on the function of ISWI *in vivo* remains unresolved. A potential role for Poly-ADP-ribose polymerase 1 (PARP1) as an ISWI regulator was discovered through genetic screens aimed to identify factors interacting with ISWI in *Drosophila* [52]. Indeed, PAR (Poly-ADP-ribosylation) modification was found to occur on and modify ISWI function *in vitro* and *in vivo*

[53]. The occurrence and significance of this modification in higher eukaryotes is yet to be established.

Both the repositioning activity and the targeting of chromatin remodelers to the chromatin can also be influenced by posttranslationally modified histones. For example, the acetylation of lysine 12 and lysine 16 of H4 tails was shown to have inhibitory effects on *Drosophila* and yeast ISWI ATPase activity [31,33–35]. The Plant Homeo Domain of BPTF subunit of the NURF complex was shown to recognize trimethylated lysine 4 of H3 thereby recruiting ISWI to specific loci on the chromatin [49]. Furthermore, the recruitment of yeast ISWI to chromatin was shown to be dependent on di- and trimethylation of H3K4 [54]. Another example for ISWI regulation through histone modifications came from studies of *Xenopus* ISWI demonstrating a role for phosphorylation of serine 10 of H3 in regulating ISWI function during mitosis [25].

In addition to histone modifications, non-canonical histones were also shown to influence the activity of and interact with ISWI. H2A.Z, a variant of H2A, was found to increase the remodeling activity of ISWI *in vitro* [55]. Interestingly, this isoform was shown to be associated with centromeres [56], to play a role in chromosomal segregation, to affect stability and dynamics of nucleosomes [57,58], and to regulate transcription activation and repression [59]. Furthermore, the ISWI-containing complex, RSF (Remodeling and Spacing Factor), was shown to interact with H2A.Z exchange machinery and mediate the formation of heterochromatin in *Drosophila* [60]. Additionally, several studies suggest that the variant macroH2A regulates both ISWI binding and ISWI mediated nucleosome repositioning [61–63]. Interestingly, macro domains are generally

characterized for their ability to bind nicotinamide adenine dinucleotide (NAD) products such as PAR, a modification that occurs on ISWI itself [53]. This suggests a potential mode of recruitment of PAR-modified proteins to nucleosomes harboring this histone variant in a very specific manner. In addition, the WICH complex was shown to strongly interact with and play a role in modification of H2A.X in response to DNA damage [64]. Future studies will be required to identify and uncover the potential role of other accessory subunits, posttranslational modifications and histone variants in modulating the function of ISWI.

Physiological functions of chromatin remodelers

The different subfamilies of chromatin remodelers contribute to a broad spectrum of functions and outcomes *in vivo.* It is not surprising that the presence of the above mentioned extensive network of factors regulating the function of the basic ISWI motor allows this remodeler to play a central role in several processes inside the cell. For example, several studies have established a role for chromatin remodeling complexes in DNA replication; ISW2, an ISWI containing complex in yeast, has been shown to play a role in DNA replication initiation and progression [65]. Human ACF and WICH complexes facilitate heterochromatin DNA replication through their association with PCNA (Proliferating Cell Nuclear Antigen) [66, 67]. Additionally, ACF and CHRAC complexes were shown to regulate the spacing of newly deposited nucleosomes following DNA replication [68]. Moreover, ISWI complexes have also been shown to play a role in chromosome structure and compaction as mutations in the Drosophila NURF complex genes were associated with depletion of the linker histone H1 from the chromatin and a significant decompaction of the male X chromosome and some decondensation of mitotic chromosomes [26,69,70]. A role for chromatin remodelers in chromosomal segregation has also been

suggested [71,72]. Indeed, human ISWI has been shown to mediate the loading of cohesion [72]. Interestingly, ISWI was shown to be a microtubule binding protein and the lack of ISWI causes defects in microtubule assembly and chromosomal segregation during anaphase in *Xenopus* egg extracts. Furthermore, the WSTF subunit of the WICH complex, was shown to play a role in DNA repair by phosphorylating tyrosine 142 and thereby mediating the cross talk with serine 139 phosphorylation of H2A.X in response to DNA damage [64].

ISWI was found to play a role in both transcription repression and activation. For example, repressor-mediated recruitment of yeast ISW2 complex to certain promoters contributes to repression of the respective genes [73]. Also contributing to gene repression is the interaction of yeast ISW2 and *Drosophila* ISWI with RPD3, a histone deacetylase (HDAC) [74]. ISWI was also found to inhibit transcription re-initiation by removing TATA box binding protein (TBP) from some promoters [75,76]. Gene specific transcription initiation was shown to be regulated through Interactions of the *Drosophila* NURF, ACF and human RSF complexes with various transcription factors *in vivo* [69,77–81].

Chromatin remodeling and disease

Heterozygous deletion of several genes including the gene encoding the transcription factor WSTF, a subunit required for the recruitment of the WICH complex to heterochromatic replication sites, is associated with Williams-Beuren syndrome [66,82]. Moreover, heterozygous mutations affecting the expression levels of the ATPase CHD was shown to be associated with CHARGE (Coloboma of the eye, Heart defect, Atresia choanae, Retardation of growth/development, Genital and Ear abnormality) syndrome in humans [83]. Mutations in genes

required for coupling of DNA excision repair to transcription, such as Cockayne syndrome protein B (CSB), a SWI/SNF ATPase give rise to Cockayne syndrome [84,85]. Cockayne syndrome patients suffer from UV sensitivity and growth and neurological defects. Mutations in the SNF2 related ATPase, ATRX, are associated with α-thalassemia X-linked mental retardation (ATRX) syndrome [86,87].

Members of the CHD subfamily have been shown to positively regulate the tumor suppressor genes p16 and p19 [88]. Loss of members of the CHD subfamily was shown to be associated with neuroblastoma [88]. Furthermore, overexpression of the metastasis associated proteins (MTA) subunits of the NuRD complex was shown to be associated with metastasis and invasive behavior of several cancers [89]. Moreover, *Drosophila* NURF was shown to repress the expression of genes controlled by the JAK/STAT pathway. The lack of NURF was found to be linked to abnormal activation of STAT target genes causing the overproliferation of blood cells leading to melanotic tumors [69,78].

Interestingly, the human SWI/SNF subfamily BAF complex has been shown to interact with tumor suppressors and oncoproteins (MLL, c-MYC, RB and BRCA1) [90–92]. Additionally, mutations in several subunits of the human BAF complex have been identified in prostate, ovarian, breast, lung and pancreatic cancer in addition to several pediatric malignancies [90–92]. Specifically, mutations in Brg1, the catalytic subunit of BAF, have been reported in various types of cancer. Furthermore, the noncatalytic subunit, SNF5, was shown to play roles in tumor suppression, genomic stability and cell cycle regulation [90–92].

Future studies will be required to uncover the downstream effects of the cancer-associated chromatin remodeling mutations and how certain mutations are associated with specific types of cancer.

Understanding the mechanism of nucleosome repositioning

The ability of remodelers to reposition nucleosomes relies on their ATP-dependent translocation along the nucleosomal DNA. This activity causes the sliding of the histone octamer relative to the associated DNA. While many details remain unresolved, several advances have been made towards understanding the process of nucleosome sliding. Current models were proposed and supported by evidence derived from several studies conducted thus far (reviewed in [8,23,37]).

The loop propagation model suggests that the translocation of the motor causes the DNA to loop out on the surface of the histone octamer. The loop is then resolved through propagation around the octamer resulting in sliding of the histones along the DNA. On the other hand, the twist-diffusion model assumes that translocation of the remodeler on the DNA causes twists in the DNA leading to partial distortions of histone-DNA contacts. These distortions then propagate from one DNA segment to another and then exit from the other end of the nucleosome leading to histone sliding in the process. (Figure 1.4). The ATPase domain of ISWI was shown to be an intrinsic translocase that forms contacts with both the nucleosomal DNA and the H4 tail, as discussed earlier [37]. On the other hand, the C-terminal HSS domain mainly contacts the linker DNA and was shown to serve a supporting role in enhancing the affinity of nucleosome binding and the efficiency of the repositioning process. Both the loop propagation and twist-diffusion

models require the ATPase domain to be stably anchored to the nucleosomes. In the case of ISWI, the H4 tail provides an anchor point for the remodeler two helical turns from the dyad. ATP hydrolysis cycle by ISWI is likely accompanied by conformational changes that are communicated to the HSS domain potentially causing it to change contacts with the linker DNA. The HSS domain can either passively allow the DNA to enter the nucleosomes or alternatively actively pump the DNA into the nucleosomes.

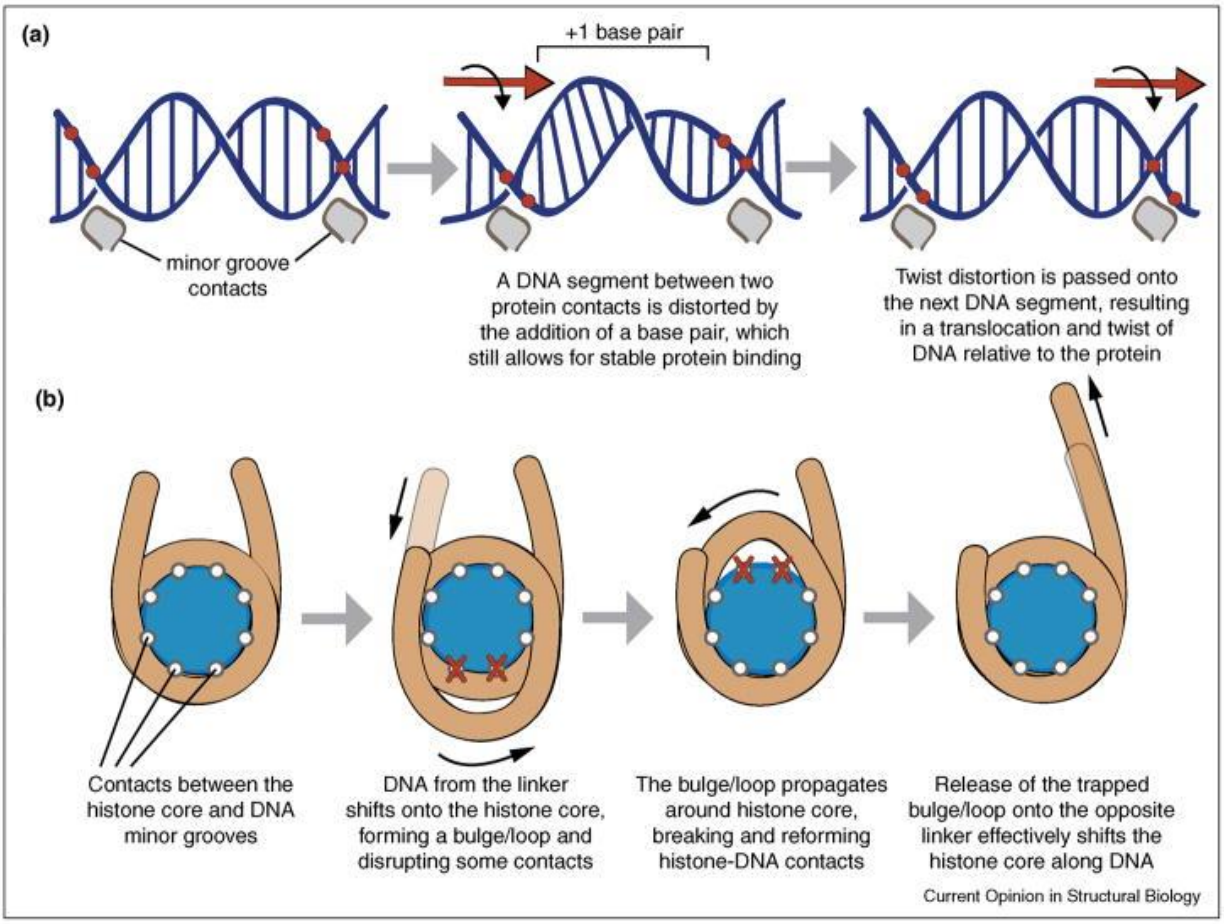


Figure 1.4: **Models of nucleosome sliding. A)** Twist-diffusion model. **B)** Loop propagation model. Modified from G. D. Bowman, 2010, *Current opinion in structural biology.*

Despite the development of these two models, the details of the repositioning mechanism remain unclear and several questions regarding the function of remodelers still persist. For example, what is the rate of nucleosome repositioning by ISWI? How many basepairs is the octamer moved per step? How much ATP is consumed in the process? Also of interest is to establish how the basic mechanism of the ISWI motor is regulated by various factors to cause the various physiological outcomes observed *in vivo*. Another challenge is to identify downstream targets of chromatin remodelers that are affected by cancer-associated mutations and alterations of remodelers. The resolution of these questions requires that the fundamental properties of the ISWI motor to be studied.

The goal of my dissertation was to provide insights into the mechanism of chromatin remodeling through an in depth quantitative characterization of the nucleosome binding and repositioning properties of the molecular motor subunit, ISWI. In addition, this work aimed to explore potential mechanisms of ISWI regulation through various factors. To achieve these goals I studied the equilibrium binding properties of recombinantly expressed ISWI to free DNA and mononucleosome substrates. I found that two ISWI molecules interact with the nucleosome with high affinity. Contrary to previous studies I demonstrate that ISWI can bind to nucleosomes with very short linker DNA and that the length of the extranucleosomal DNA has no effect on the interaction of ISWI with the nucleosomes as evident by measurements of the affinity to nucleosomes or nucleosome stimulated ATPase activity of ISWI. These findings are significant and suggest that re-evaluation of current models for ISWI:nucleosome interaction is required, especially considering that many of the current proposed models of nucleosome repositioning by

ISWI rely on previous findings proposing a central role for linker DNA length sensing-capabilities of ISWI in the repositioning mechanism.

In order to gain further understanding into the regulatory mechanism of ISWI function, I explored the effect of the ATP hydrolysis cycle on the interactions of ISWI with nucleosome and DNA substrates. I found that binding of ISWI to ATP analogs reduces the affinity of the motor to binding free DNA. Interestingly, I found that the binding affinity of ISWI to nucleosomes was unaffected by nucleotides. Taken together, these findings suggests that while the interactions of ISWI with extranucleosomal DNA might be regulated through the ATPase cycle, ISWI remains stably anchored to the nucleosomes.

Moreover, using a novel real time fluorescence-based repositioning assay along with traditional gel-based repositioning assays, I characterized the nucleosome repositioning activity of ISWI. I found that ISWI remodels the nucleosome through a random walk mechanism. Furthermore, and using parameters determined from analysis of equilibrium binding studies, I found that even though two ISWI molecules can bind, only one ISWI motor is responsible for the observed nucleosome repositioning activity. Additionally, through analysis of the nucleosome stimulated ATPase activity of ISWI, I found that ATP hydrolysis is poorly coupled to octamer movement, requiring the consumption of hundreds of ATP molecules.

In conclusion, this dissertation represents an extensive characterization of the basic function of a molecular motor providing a solid foundation for future studies aiming to explore

the regulatory effects of additional factors on the activity of the ISWI motor and consequently leading to various outcomes inside the cell.

CHAPTER 2 :

EXPERIMENTAL PROCEDURES

Recombinant ISWI expression and purification: cDNA coding for *Xenopus Laevis* ISWI (A kind gift from Dr. Paul Wade) was amplified using PCR with primers containing a BglII restriction site at the 5' end and an EagI restriction site at the 3' end and then cloned into pCR4-TOPO vector (Invitrogen). The insert was then further subcloned into BamHI/NotI-digested pPIC3.5-CBP-Xpress-zz yeast expression vector. All recombinant constructs were confirmed by sequencing. The ISWI containing recombinant construct was then transformed into GS115 strain of *Pichia pastoris* yeast through electroporation followed by recombinant ISWI expression according to manufacturer protocol (Invitrogen). Briefly, cells were grown in buffered glycerol complex media (Invitrogen) until $O.D._{600}$= 10, the cells were then resuspended in buffered methanol media and allowed to shake for 6 hr at 30 ºC to induce protein expression. Cells were harvested by centrifugation at 3000x g for 5 min at 4 ºC. Cell paste was loaded into a syringe, dispensed into liquid nitrogen and stored at -80 ºC until use. Mixtures of dry ice and frozen yeast cells were mechanically lysed followed by addition of lysis buffer (50 mM Tris (pH 8.0), 300 mM NaCl, 0.1 % Triton® X-100, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10% glycerol, 10 mM PMSF, 1.3 mM β-Mercaptoethanol) and centrifugation at 15,000x g for 30 min at 4 ºC. Supernatant containing 1 mM $CaCl_2$ was then incubated with Calmodulin Sepharose 4b resin (GE Healthcare) for 4 hrs. CBP tagged ISWI was then eluted using 10 mM EGTA-containing buffer. Collected elutions were examined using a 8% SDS-PAGE analysis and ISWI-containing fractions were further purified using heparin column (GE Healthcare) followed by buffer exchange (20 mM HEPES (pH 7.8), 100 mM NaCl, 10 mM $MgCl_2$, 5% glycerol, 0.5 mM DTT)

and stored at -80 ºC. Protein concentration was determined through measurements of the A_{280} and the extinction coefficient and further confirmed using Bradford assay. Subsequent analysis using dynamic light scattering confirmed that ISWI was monomeric under these solution conditions. ISWI activity and properties were not affected by the presence of affinity tags.

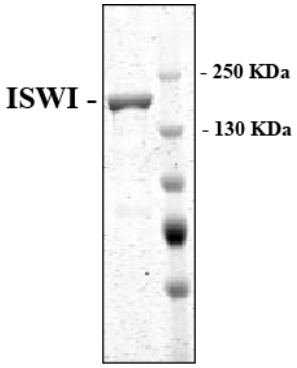


Figure 2.1: **Purified Recombinant ISWI protein**. Sample analyzed using 8% SDS gel and stained with coomassie brilliant blue.

Nucleosomes reconstitution reactions: pET28 Plasmids containing untagged H2A, H2B, H3 and H4 (A kind gift from Dr. Bradley Cairns) were transformed into BL21 (DE3) pLys. Recombinant yeast histone expression, purification and octamer assembly were performed according to a modified version of the previously described protocol[93,94]. DNA fragments containing the 147 bp Widom 601 high affinity nucleosome positioning sequence [95] (Sequence-containing plasmid is a kind gift from Timothy J. Richmond) and additional length of flanking DNA was amplified using large scale PCR followed by purification of the amplified fragment. Either non-labeled primers or Alexa488 end-labeled primers (IDT, Coralville, IA) were used to reconstitute the mononucleosome substrates with the desired fluorophore label and flanking

DNA length. Samples containing a mixture of DNA fragments and histone octamer in high salt buffer (10 mM Tris (pH 7.5), 2 M KCl, 1 mM EDTA, 0.05% Tween-20, 10 mM β-ME) were subjected to slow gradient dialysis against low salt buffer (10 mM Tris (pH 7.5), 50 mM KCl, 1 mM EDTA, 0.05% Tween®-20, 10 mM β-ME) using peristaltic pumps as described previously [93,94]. Reconstituted mononucleosomes were evaluated using a 5% native polyacrylamide-bisacrylamide gel (60:1) at 100 V in 0.25x Tris-Borate-EDTA (TBE) buffer followed by either staining using SYBR® gold or exposed for fluorescence and imaging using a Typhoon imager (GE Healthcare). Percent of free DNA remaining in the final reconstituted reactions was estimated from DNA standard curves. Nucleosome concentration was determined by measuring the A_{260} and further concentrated using a centrifugational filter (100,000 MWCO, Millipore).

The Widom 601 nucleosome positioning sequence (marked in bold is the primary nucleosome position, the secondary nucleosome position is flanked by grey highlight [96].

1 cgggatccta atgaccaagg aaagcatgat tcttcacacc gagttcatcc cttatgtgat

61 ggaccctata cgcggccgcc **ctggagaatc ccggtgccga ggccgctcaa ttggtcgtag**

121 **acagctctag caccgcttaa acgcacgtac gcgctgtccc ccgcgtttta accgccaagg**

181 **ggattactcc ctagtctcca ggcacgtgtc agatatatac atcctgt**gca tgtattgaac

241 agcgaccttg ccggtgccag tcggatagtg ttccgagctc cc

Nucleosome and DNA binding studies: A 20 bp 5'-FITC or Alexa488-labeled double stranded DNA substrate (5'CCATGTCCATGGATACGTGG 3') (IDT) was titrated with increasing concentrations of ISWI in reaction buffer (10 mM HEPES (pH 7.0), 20 mM KCl, 10 mM $MgCl_2$, 4% glycerol, 0.1 mg/ml BSA, 0.5 mM DTT) at 25 ºC. ISWI binding to this DNA substrate was

measured by monitoring changes in the anisotropy of the fluorophore using a Syergy2 fluorescence spectrophotometer (BioTek) set at 485 nm excitation and monitoring emission at 520 nm. In order to test the effect of nucleotide analogs on ISWI-DNA interactions, similar experiments were performed in the presence of varying total concentrations of ADP (Sigma-Aldrich), ATP-γ-S (Roche), or AMP-PNP (Roche) already present in solution prior to ISWI titration. Binding of ISWI to 5'Alexa488-labeled nucleosomal substrates was performed under the same conditions. All concentrations are indicated in the figures and figure legends.

Electrophoretic mobility shift assay: Reactions containing 50 nM nucleosomes were incubated with increasing concentrations of ISWI (12-300 nM) in reaction buffer (10 mM HEPES (pH 7.0), 20 mM KCl, 10 mM $MgCl_2$, 4% glycerol, 0.1 mg/ml BSA, 0.5 mM DTT) for 30 min at 25 ºC. The reactions were then analyzed using 5% native polyacrylamide-bisacrylamide gel (60:1) run at 100 V in 0.25x TBE buffer followed by staining using either SYBR gold or detecting Alexa488 fluorescence using a Typhoon imager depending on the utilized nucleosomal substrate.

Gel based repositioning assays: 10 nM ISWI was incubated with 50 nM nucleosome substrates in reaction buffer (10 mM HEPES (pH 7.0), 20 mM KCl, 10 mM $MgCl_2$, 4% glycerol, 0.1 mg/ml BSA, 0.5 mM DTT) at 25 ºC. Repositioning reactions were initiated by the addition of 1 mM ATP and allowed to proceed for specific times before stopping by the addition of quenching solution containing EDTA and competitor plasmid DNA. The reactions were then analyzed using 5% native polyacrylamide-bisacrylamide gel (60:1) at 100 V in 0.25x TBE buffer followed by staining using either SYBR gold and visualized using a Typhoon imager (GE healthcare).

Anisotropy based repositioning assays: Varying ISWI concentrations ranging from 5-20 nM (specific concentrations listed in Figure legends) were incubated with 10 nM of Alexa488-labeled nucleosome substrates in reaction buffer (10 mM HEPES (pH 7.0), 20 mM KCl, 10 mM $MgCl_2$, 4% glycerol, 0.1 mg/ml BSA, 0.5 mM DTT) at 25 ºC. Reactions were initiated by the addition of 1 mM ATP and the movement of the octamer was detected by monitoring changes in the fluorescence anisotropy of the fluorophore using a Syergy2 fluorescence spectrophotometer (BioTek) set at 485 nm excitation and monitoring emission at 520 nm.

ISWI ATPase activity assay: To screen for effective non-hydrolyzable ATP analogs, reactions containing 250 nM of 50 bp DNA substrate were incubated with 500 nM ISWI in reaction buffer (10 mM HEPES (pH 7.0), 20 mM KCl, 10 mM $MgCl_2$, 4% glycerol, 0.1 mg/ml BSA, 0.5 mM DTT) at 25 ºC. Additional sets of reactions contained either 1 mM of ADP, AMP-PNP or ATP-γ-S. The reactions were initiated by addition of 1 mM unlabeled ATP containing 7.5 µCi ^{32}P -α-ATP. Aliquots were withdrawn at specific time points and mixed with equal volumes of 0.5 M EDTA to stop the reaction. In order to separate ADP from ATP species, reactions were analyzed using thin liquid chromatography PEI-Cellulose plates (EMD chemicals) in 0.6 M potassium phosphate (pH 3.4) buffer and quantified using a Typhoon Phosphor imager. Studies of nucleotide concentration-dependent inhibition of nucleosomes-stimulated ATPase activity (Figure 3.9) contained 50 nM ISWI along with 250 nM 10NF5 nucleosomes and increasing concentrations of nucleotide ranging from 50-500 µM. Reactions were initiated by adding 200 µM ATP containing ^{32}P-α-ATP. Reactions were stopped and analyzed as described above. See Tables and Figure legends for specific concentrations of all other nucleosome repositioning-

associated ATP consumption experiments. The ATPase rate for each nucleosome substrate was determined from a linear fit of the data.

Data analysis: Mathematical models and quantitative analysis of equilibrium binding of ISWI to DNA and nucleosomes (developed together with Dr. Christopher J. Fischer and Mr. Koan Briggs) are presented in Appendix I. Mathematical models and quantitative analysis of the nucleosome repositioning activity and associated ATP consumption by ISWI (conducted together with Dr. Christopher J. Fischer) are presented in Appendix II.

CHAPTER 3:

Quantitative Determination of ISWI Binding to Nucleosomes and DNA shows Allosteric Regulation of DNA Binding by Nucleotides

INTRODUCTION

ISWI is capable of translocating along both single- and double-stranded DNA [20]. The ability of chromatin remodelers to translocate along DNA is fundamental to their nucleosome repositioning activity [10,20,22,97]. During these processes of DNA translocation and nucleosome repositioning the remodeler continually experiences repeated cycles of ATP binding, ATP hydrolysis, release of ADP and inorganic phosphate, and possibly additional conformational changes [19,22,98–100]. However, processive translocation and efficient movement along the DNA, and hence effective repositioning of nucleosomes, requires that the remodeler alternate between a DNA-bound and a DNA-unbound state while simultaneously maintaining contact with the histone octamer. Identifying how the binding of ISWI to both DNA and nucleosomes is allosterically regulated by ATP binding and hydrolysis is essential not only for determining the catalytic cycle associated with nucleosome repositioning, but also in understanding how DNA translocation and nucleosome repositioning are regulated.

Furthermore, a quantitative characterization of the equilibrium binding of ISWI to both DNA and nucleosomes under conditions of known stoichiometry is required to determine the kinetic mechanism of nucleosome repositioning by ISWI (*i.e.*, the associated microscopic and macroscopic rate constants, the stoichiometry of the active oligomeric state, *etc*.). Indeed, currently accepted models for nucleosome repositioning by ISWI were based on the results of experiments performed with nucleosome substrates with increasing lengths of flanking DNA [20,101,102] and under conditions that had been shown to increase the stoichiometry with which ISWI

complexes bind to these substrates [103–105]. Variations in the stoichiometry of the remodeler:nucleosome interaction in these experiments may have contributed to the apparent sensitivity of the repositioning rate of these enzymes to the length of the flanking DNA. In addition, and perhaps because of this, studies aimed at obtaining quantitative descriptions of remodeler-nucleosome binding or allosteric regulation of these interactions unfortunately yielded conflicting reports [99,102,104,105].

Here we report our determination of the equilibrium constants associated with ISWI binding to DNA, mononucleosomes, and nucleotides. Utilizing a fluorescence-anisotropy based assay, we quantitatively investigated the equilibrium binding of ISWI to fluorophore-labeled DNA and to a nucleosomal substrate with short flanking DNA and found that ISWI can bind to these substrates with high affinity. Furthermore, in order to better understand the mechanisms of DNA translocation and nucleosome repositioning by ISWI, we determined the effect of nucleotide analogs on substrate binding by ISWI. Interestingly, we found that while the affinity of ISWI binding to nucleosome substrates with short flanking DNA is not affected by the presence of nucleotides, the binding of ISWI to DNA is weakened in the presence of non-hydrolysable ATP analogs but not by ADP. These results suggest that high affinity, non-nucleotide-regulated contacts between ISWI and histones form an anchor about which DNA translocation by ISWI results in nucleosome repositioning. Furthermore, we demonstrate that the affinity with which ISWI interacts with nucleosomes is independent of the length of the flanking DNA. These findings are further discussed in context of the current knowledge of nucleosome binding and repositioning by ISWI and ISWI-containing complexes.

RESULTS

DNA binding studies of ISWI

ISWI is able to translocate along both single- and double-stranded DNA, a trait necessary for its nucleosome repositioning activity [20,97]. However, a quantitative description of ISWI's ability to bind to and translocate along DNA is required for further delineation of the role of DNA translocation in the mechanism of nucleosome repositioning by ISWI. Perhaps more importantly, since ISWI has been demonstrated to bind to DNA flanking the nucleosome core particle [20,105] identifying the affinity for ISWI-DNA interactions, and how these interactions are affected by the presence of nucleotides, is critical for the interpretation of data obtained in nucleosome repositioning experiments with ISWI.

We monitored the binding of ISWI to DNA using a fluorescence anisotropy based assay [106,107]. Previous studies have shown that ISWI is unable to bind either a 15 bp or a 18 bp DNA substrate but is able to bind to a 23 bp DNA substrate [20,106]. Furthermore, both 32 bp and 35 bp DNA substrates have been shown to accommodate more than one bound ISWI [20,106]. In order to avoid the possibility of multiple ISWI being bound to the DNA we therefore used a fluorophore-labeled 20 bp double stranded DNA. The titration of this DNA substrate with increasing concentrations of ISWI resulted in an increase in the fluorescence anisotropy of the fluorophore (Figure 3.1) [106,107]; similar increases were detected whether the DNA was labeled with FITC or Alexa488. This increase in the fluorescence anisotropy is consistent with the formation of an ISWI-DNA complex. Through simultaneous global least squares analysis of equilibrium binding isotherms conducted at two different total DNA concentrations (10 nM and 25 nM) using Scheme AI.1 (Appendix I) we determined that the simplest model consistent with the data was a

1:1 stoichiometry with an affinity of $1/\beta_1$=(18 ± 2) nM (Figure 3.1 and Table 3.1). This result is also in agreement with previous reports of contact and occluded site sizes of 15 bp to 23 bp for ISWI [20] and with an affinity of approximately 15 nM for ISWI binding cooperatively to a 35 bp DNA substrate [106].

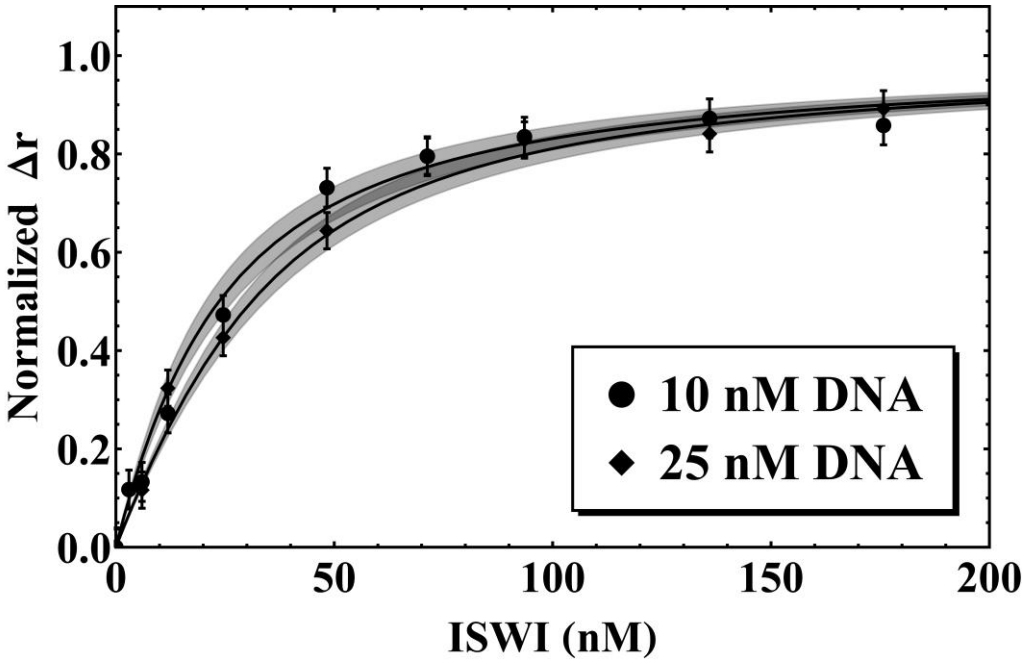


Figure 3.1: Fluorescence anisotropy measurements (Δr) of ISWI binding to DNA. A 20 bp FITC-labeled DNA substrate (●10 nM and ♦25 nM) was titrated with ISWI concentrations ranging from 6-183 nM and changes in fluorescence anisotropy were monitored. Isotherms were analyzed using Scheme AI.1 (as described in Appendix I). The solid line represents the fit of the data to this scheme which returned a value of $1/\beta_1$ = (18 ± 2) nM. Shaded area represents 68% confidence limits of the fit.

Model	A	B	C
Equations	$P + D \overset{\beta_1}{\rightleftarrows} PD$	$P + D \overset{\beta_1}{\rightleftarrows} PD$ $P + PD \overset{\beta_2}{\rightleftarrows} P_2D$	$P + D \overset{\beta_1}{\rightleftarrows} PD$ $PD + D \overset{\beta_2}{\rightleftarrows} PD_2$
$1/\beta_1$ (nM)	18 ± 2	4 ± 4	24 ± 8
$1/\beta_2$ (nM)	N.A.	20 ± 20	70 ± 70
Variance of fit	3.5×10^{-5}	6.6×10^{-5}	3.3×10^{-5}

Table 3.1: Results of analysis of equilibrium DNA binding isotherms in the absence of nucleotides using different models. In these equations P denotes ISWI, D denotes the DNA substrate, and β_1 and β_2 are overall macroscopic equilibrium constants. The stoichiometries for the ISWI:DNA interactions in these models are 1:1 (Model A), 2:1 (Model B), and 1:2 (Model C). Simultaneous global analysis of equilibrium binding isotherms measured at two different DNA concentrations (10 nM and 25 nM) resulted in the parameters listed in the table. Comparison of the variance of the fit for these analyses indicates that using Model B provided the worst agreement with the data and that using either Model A or Model C resulted in fits of similar quality. Although the affinity for the first ISWI:DNA binding event ($1/\beta_1$) determined using Model C is within uncertainty of the value determined using Model A, the affinity of the second DNA binding event ($1/\beta_2$) is poorly constrained, indicating that this parameter cannot be determined from the data. Because of this we believe that Model C is not appropriate and therefore favor Model A. Therefore, we argue that ISWI binds to this DNA substrate with a 1:1 stoichiometry with an associated affinity of (18 ± 2) nM.

Nucleosome binding studies of ISWI

Previous native gel-based binding studies demonstrated that only low levels of binding were reported for ISWI [20,105] and ISWI-containing complexes ACF [101,103,104] and ISW2 [108–110] interacting with nucleosomes containing no flanking DNA. On the other hand, multiple ISWI-nucleosome complexes were detected upon interaction of ISWI with nucleosomal substrates with longer stretches of flanking DNA [20,43,105]. Taken together these results indicate both that flanking DNA affects the affinity of ISWI for nucleosome binding and that the presence of long flanking DNA on the nucleosome might provide an additional ISWI binding site that may not be in direct contact with the histones. Since any mechanistic study of the nucleosome repositioning activity of a remodeler requires the determination of the oligomeric state associated with the remodeler-nucleosome interaction we sought to quantify the stoichiometry and affinity of the ISWI-nucleosome interaction.

In order to accomplish such an analysis, we reconstituted mononucleosomal substrates using the high affinity Widom 601 nucleosome positioning sequence [95]. This sequence contains 147 bp which allows for the positioning of the histone octamer to one major site on the DNA fragment [95,111]. The positioning of the nucleosomes reconstituted with yeast histones and the 601 sequence or the significantly weaker 5S nucleosome positioning sequence has been demonstrated by several groups through nuclease digestion assays[112–118].

In order to minimize the possibility for ISWI to bind to only the flanking DNA, and not contact the histones, we restricted the length of the flanking DNA to less than 23 bp, the approximate occluded site size for ISWI-DNA binding [20,106]. In our initial experiments we

monitored the binding of ISWI to a nucleosome substrate with 10 bp of DNA flanking from one side of the nucleosome core particle and 5 bp flanking the other side; we refer to this substrate as 10N5. The binding of ISWI to a non-fluorophore-labeled 10N5 was monitored using a native gel electrophoretic mobility shift assay (EMSA). As shown in Figure 3.2 A, upon ISWI titration we detect the formation of two major slow mobility bands consistent with ISWI bound to each 10N5 in a 2:1 stoichiometry at saturation. It is worth noting that EMSA experiments that we performed using fluorophore-labeled nucleosomes yielded the same stoichiometry of ISWI-nucleosome binding, eliminating the possibility of fluorophore effects on stoichiometry.

In order to confirm this stoichiometry and to more readily determine the equilibrium constants associated with the binding of ISWI to nucleosomes we monitored the binding interaction using a fluorescence anisotropy-based assay [106] and a nucleosome substrate where either one or both 5' ends of the flanking DNA were labeled with Alexa488; we refer to these substrates as F10N5 and F10N5F respectively. In these experiments the titration of the nucleosome substrates with increasing concentrations of ISWI resulted in an increase in the fluorescence anisotropy of the fluorophore, consistent with the formation of ISWI-nucleosome complexes (Figure 3.2 B) [106,107]. The simplest model consistent with the observed 2:1 stoichiometry of ISWI binding to these substrates is shown in Scheme AI.2. Through the global least squares analysis using Scheme AI.2 of equilibrium binding isotherms collected with two different total F10N5F concentrations we determined the associated overall equilibrium constants to be $1/\beta_1 = (1.3 \pm 0.6)$ nM and $1/\beta_2 = (13 \pm 7)$ nM^2.

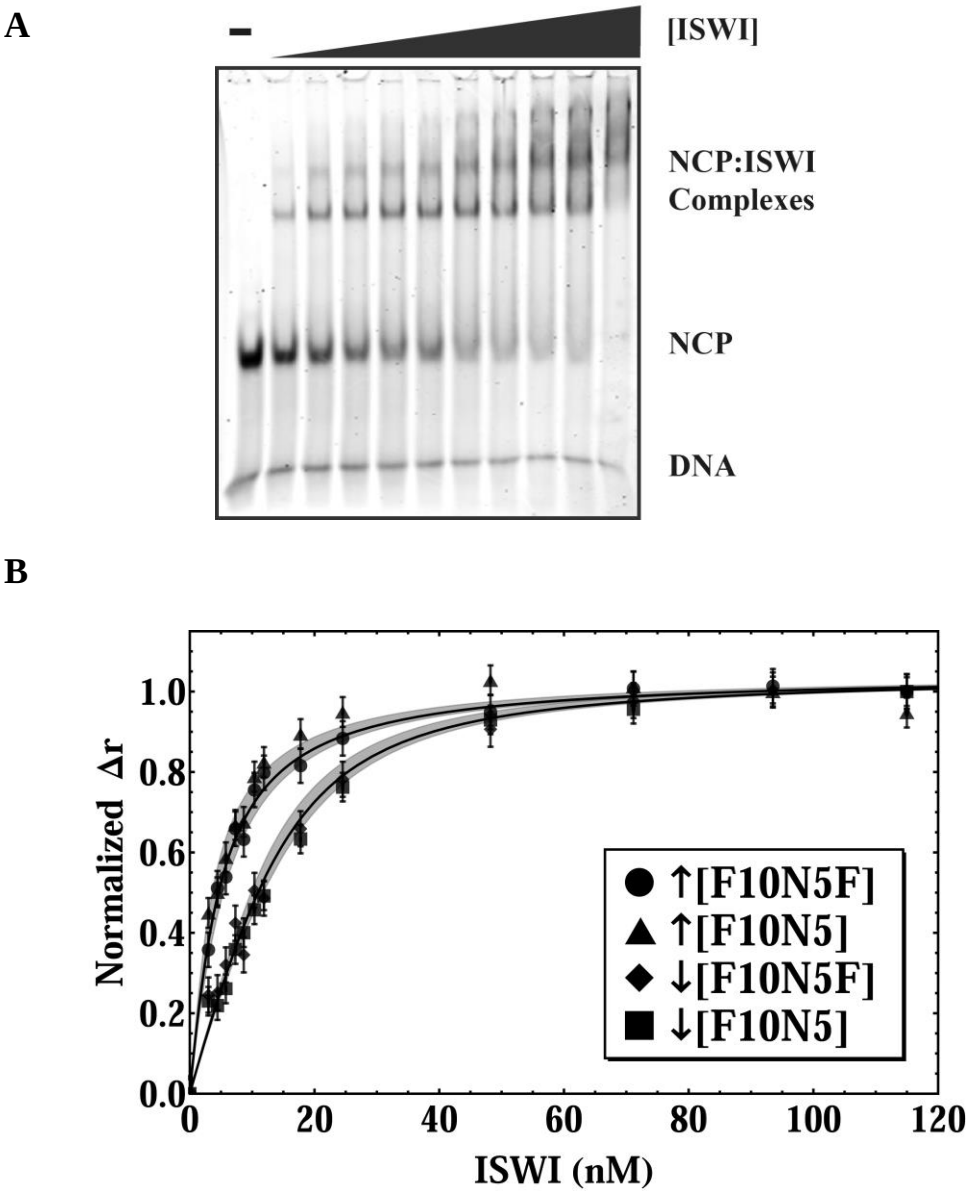


Figure 3.2: Fluorescence anisotropy measurements (Δr) of ISWI binding to nucleosome substrates. (A) Electrophoretic mobility shift assay performed by titrating a non-labeled 10N5 nucleosome substrate (50 nM) with increasing ISWI concentrations (0, 25, 50, 60, 75, 80, 100, 125, 150, 175, 200 nM). Samples were analyzed using a 5% TBE-acrylamide native gel. Gels were stained using a DNA staining dye and imaged using a Typhoon imager. Independent experiments showed that high molecular weight smearing is caused by interaction of ISWI with free DNA present (< 2%) in the reconstituted nucleosome sample (See Figure 3.4). **(B)** Fluorescence anisotropy measurements of ISWI binding to doubly labeled Alexa 488 (F10N5F) and singly labeled Alexa 488 (F10N5) nucleosomal substrates. Nucleosomes at 2.5 nM (down arrow) (●, ▲) and 10 nM (up arrow) (♦, ■) were titrated with increasing concentrations of ISWI ranging from 3 nM to115 nM. Equilibrium binding isotherms were analyzed using Scheme AI.2 as described in Appendix I. The solid line represents the fit of the data to this scheme which returned values of $1/\beta_1 = (1.3 \pm 0.6)$ nM and $1/\beta_2 = (13 \pm 7)$ nM^2. Shaded area represents 68% confidence limits of the fit.

Using these parameters we simulated the species distribution for the equilibrium binding of ISWI to F10N5F nucleosomes (Figure 3.3). As indicated in Figure 3.3 cooperativity in the binding of ISWI to this substrate, if it exists, is weak. Additional models, including one postulating that ISWI can exist as a dimer in solution and thus bind the substrate as either a monomer or a dimer, were also tested but were not consistent with the binding isotherms (see Table 3.2).

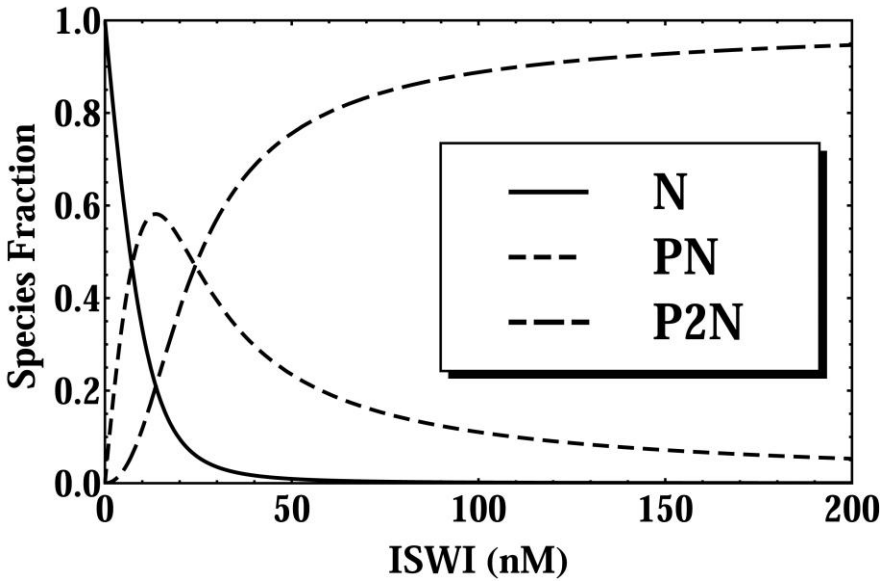


Figure 3.3: Nucleosome fractions. Computer simulations according to Scheme AI.2 of the fraction of free nucleosome (N), singly-bound nucleosome (PN), and doubly-bound nucleosome (P_2N) species present as a function of the concentration of ISWI. In these simulations the total nucleosome concentration was 10 nM and the values of $1/\beta_1 = (1.3 \pm 0.6)$ nM and $1/\beta_2 = (13 \pm 7)$ nM^2 were taken from the analysis of the data in Figure 3.2.

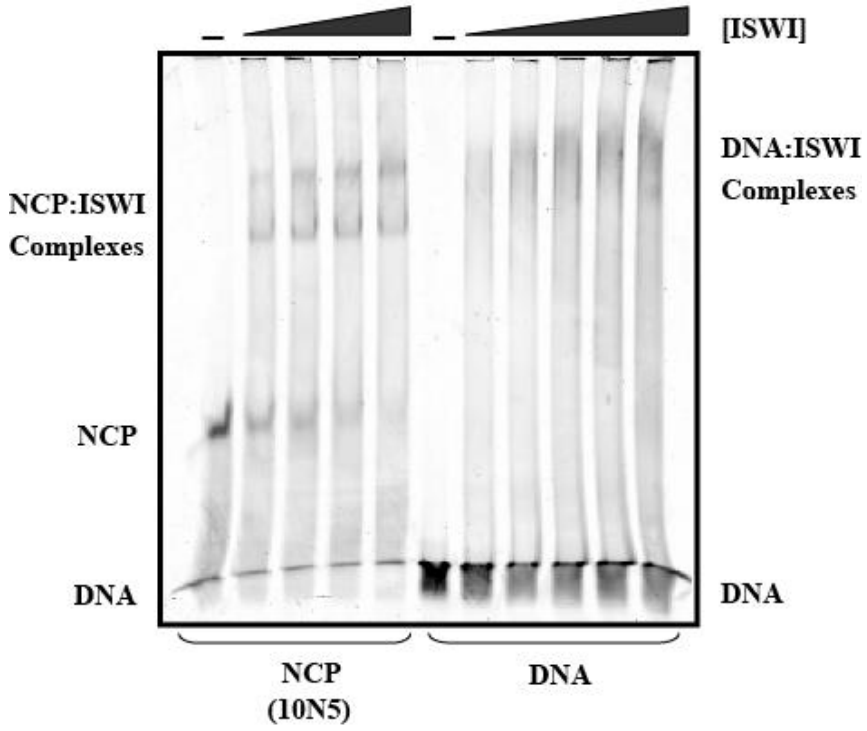


Figure 3.4: ISWI binding to 10N5 nucleosomes and DNA. EMSA performed by titrating a 10N5 nucleosome substrate (50 nM) or DNA substrate of the same length (161 bp) with increasing concentrations of ISWI (0, 100, 125, 150, 200 nM). Samples were analyzed using a 5% TBE-acrylamide native gel. Gels were stained using a DNA staining dye and imaged using a Typhoon imager.

ISWI:NCP	1:1	2:1	3:1
$1/\beta_1$ (nM)	4.6 ± 1.2	1.3 ± 0.6	1.6 ± 0.9
$1/\beta_2$ (nM^2)	N.A.	13 ± 7	10 ± 20
$1/\beta_3$ (nM^3)	N.A.	N.A.	160 ± 140
Variance	2.70×10^{-5}	1.53×10^{-5}	1.47×10^{-5}

Table 3.2: Results of analysis of different stoichiometries of ISWI binding to nucleosomes. β_1, β_2 and β_3 are overall macroscopic equilibrium constants. The stoichiometries for the ISWI:NCP interactions in these models are 1:1, 2:1and 3:1. Simultaneous global analysis of equilibrium binding isotherms resulted in the parameters listed in the table. Comparison of the variance of the fit for these analyses indicates that using a 1:1 binding model provided the worst agreement with the data and that using either 2:1 or 3:1 model resulted in fits of similar quality. However, the affinity of the second and third ISWI binding event ($1/\beta_2$ and $1/\beta_3$) are poorly constrained. Therefore, we argue that ISWI binds to NCP substrate with a 2:1 stoichiometry.

ISWI binding to DNA and nucleosomes in presence of ADP and ATP analogs

We repeated our equilibrium binding studies in the presence of ADP and non-hydrolyzable ATP analogs to investigate the effect of the ATP hydrolysis cycle on DNA and nucleosome binding. Such information is critical for the proper modeling of the nucleosome repositioning activity of ISWI (Chapter 4). In order to determine the proper analog for these experiments, we measured the DNA-stimulated ATPase activity of ISWI in the presence of ADP and the non-hydrolyzable ATP analogs, ATP-γ-S and AMP-PNP; in these experiments the concentration of the ADP or ATP-analog was equal to the concentration of the ATP in solution. We found that ATP-γ-S was the most effective in competing with ATP for binding to ISWI as demonstrated by its ability to inhibit the ATPase activity of ISWI (Figure 3.5).

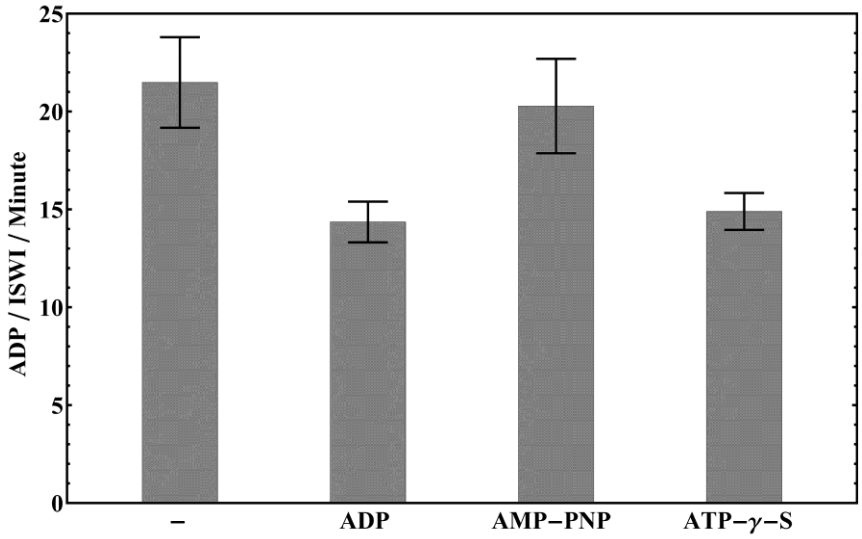
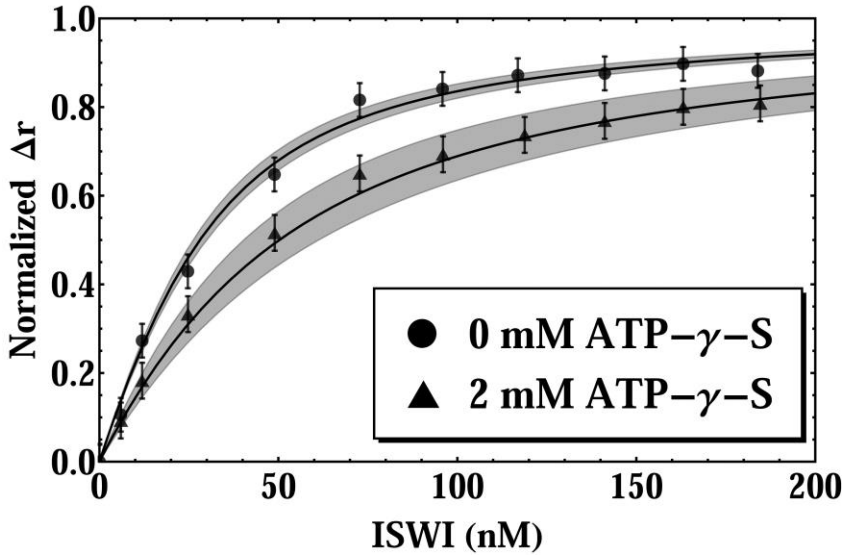


Figure 3.5: **Nucleotide analog screening.** 500 nM ISWI was incubated with 250 nM DNA and 1 mM nucleotides (ADP, AMP-PNP or ATP-γ-S). Reactions were initiated by the addition of 1 mM ATP spiked with ^{32}P-α-ATP and were allowed to proceed for 30 minutes before stopping and analyzing using thin liquid chromatography.

Similarly, we found that at 1:1 equimolar concentrations ADP was effectively competing with ATP for ISWI binding and consequently inhibiting ISWI ATPase activity. Next, we performed equilibrium DNA binding studies in the presence of concentrations of ADP and ATP-γ-S ranging from 0.5 mM to 2 mM. The presence of ADP had no effect on ISWI-DNA interactions (Figure 3.6 B). In contrast, in the presence of ATP-γ-S the affinity of ISWI to DNA was reduced significantly (Figure 3.6 A). We globally fit the equilibrium DNA binding isotherms in the presence of this analog using Scheme AI.1. This analysis returned values of $1/\beta_A$ = (140 ± 30) μM, $1/\beta_{A,1}$ = (390 ± 70) μM and $1/\beta_{1,A}$ = (42 ± 8) nM, indicating that the affinity of ISWI for binding DNA is reduced by a factor of 3 in the presence of ATP-γ-S. It is worth noting that our estimate of β_A = (140 ± 30) μM is consistent with a recent report of K_m = (150 ± 50) μM for the steady-state ATPase activity of *Drosophila* ISWI in the presence of DNA [37]. We observed a similar decrease in the affinity of DNA binding in the presence of ATP-γ-S in additional experiments conducted with a 60 bp DNA substrate (Figure 3.7), confirming that this effect is not a DNA length effect (*i.e.*, resulting from partial contact of ISWI with the DNA).

A



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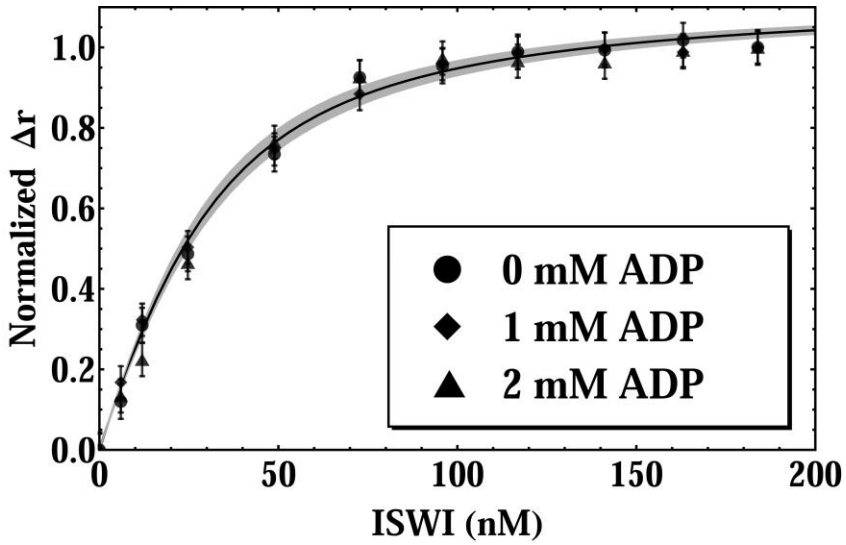


Figure 3.6: Fluorescence anisotropy measurements (Δr) of equilibrium ISWI binding to DNA in the presence of nucleotides. (A) Equilibrium binding to a 20 bp FITC labeled DNA substrate (25 nM) in the presence of ATP-γ-S. These data were analyzed using Scheme AI.1 as described in Appendix I. The solid lines in the figure represent the fits of the data to this scheme which returned values of $1/\beta_A$= (140 ± 30) mM, $1/\beta_{A,1}$= (390 ± 70) mM and $1/\beta_{1,A}$ = (42 ± 8) nM. Shaded area represents 68% confidence limits of the fit. **(B)** Equilibrium binding to a 20 bp FITC labeled DNA substrate (25 nM) in the presence of ADP. The solid line in this figure represents the fit of equilibrium DNA binding data collected in the absence of nucleotide.

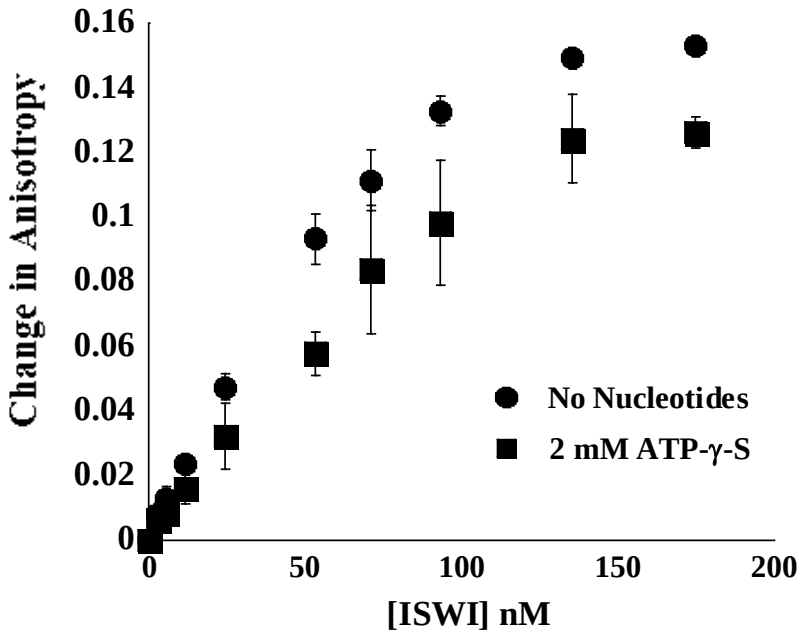
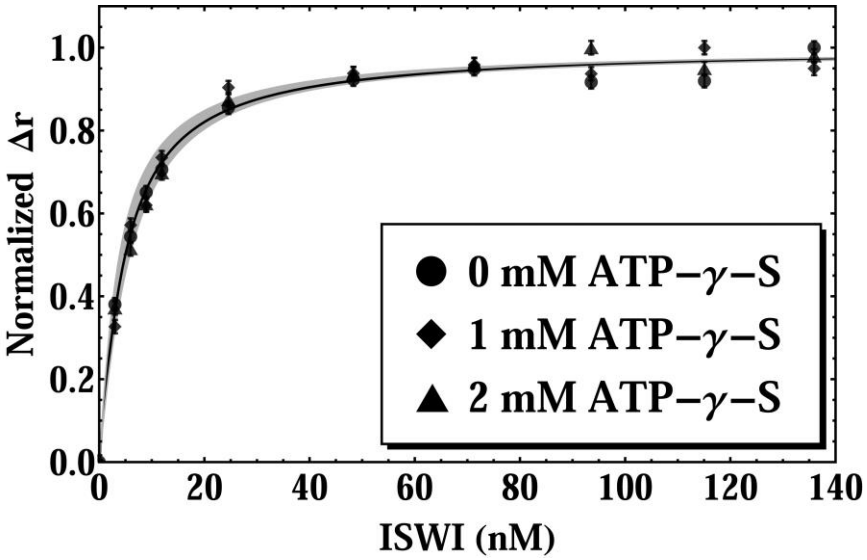


Figure 3.7: Fluorescence anisotropy measurements (Δr) of ISWI binding to 60 bp DNA. A 60 bp FITC labeled DNA substrate (25 nM) was titrated with ISWI concentrations ranging from 3-175 nM and changes in fluorescence anisotropy were monitored without (●) or in the presence of 2 mM ATP-γ-S (■).

Interestingly, as shown in Figure 3.8 A and B, the affinity of nucleosome binding by ISWI was independent of the presence of ADP and ATP-γ-S. To confirm that ISWI can still bind ATP-γ-S and ADP when bound to a nucleosome we performed nucleosome-stimulated ATPase assays in the presence of increasing concentrations of ATP-γ-S or ADP. We found that both nucleotides inhibit the ATPase activity of ISWI in a concentration dependent manner, demonstrating the ability of nucleosome bound ISWI to bind to these nucleotides (Figure 3.9 A, B).

A



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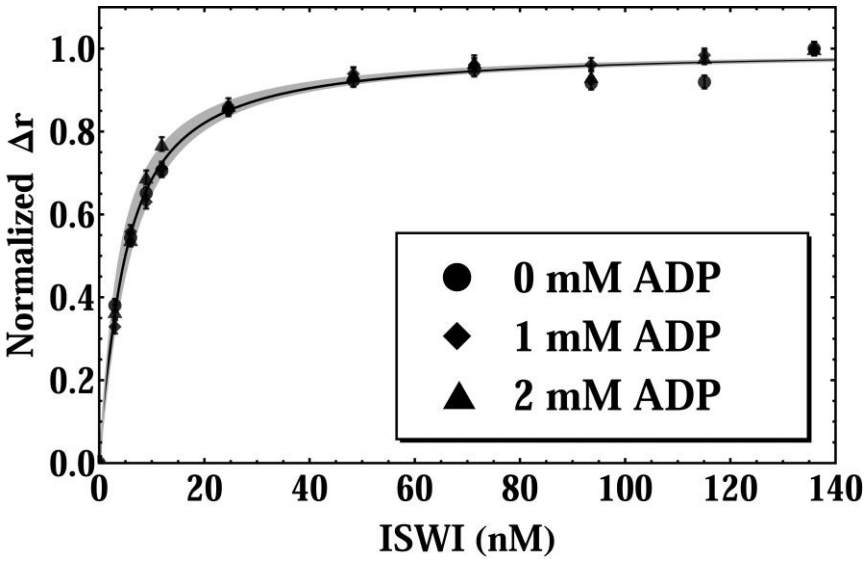

Figure 3.8: Fluorescence anisotropy measurements (Δr) of equilibrium ISWI binding to nucleosomes in the presence of nucleotides. (A) Equilibrium binding to an Alexa 488 labeled 10N5 nucleosome substrate in the presence of ATP-γ-S. **(B)** Equilibrium binding to an Alexa 488 labeled 10N5 nucleosome substrate in the presence of ADP. The solid lines in panel are the fits of the equilibrium nucleosome binding data collected in the absence of nucleotides.

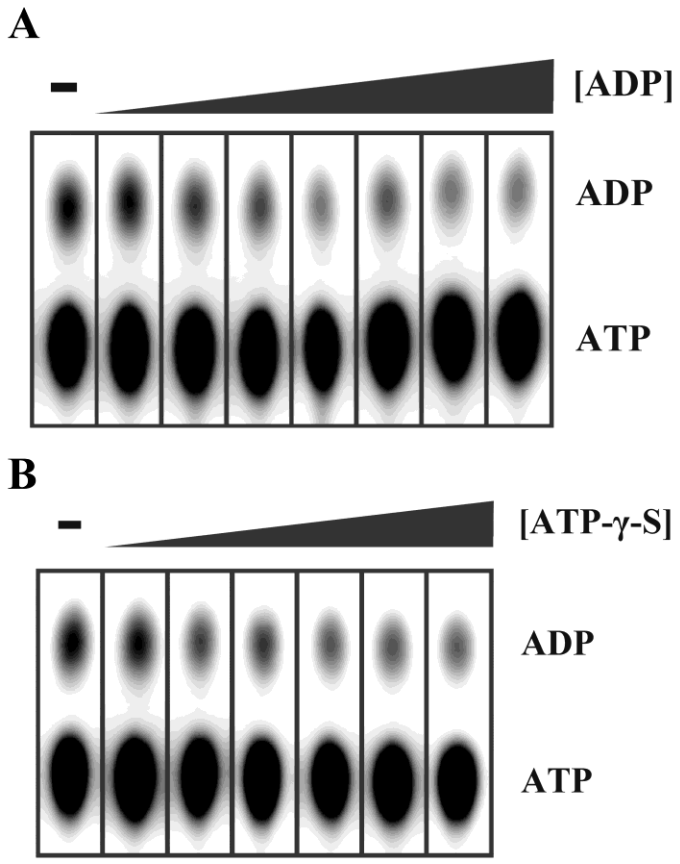

Figure 3.9: Nucleosome stimulated ATPase-nucleotide competition assay. 50 nM ISWI incubated with 250 nM 10N5 nucleosomes in the presence of increasing concentrations of **(A)** ADP or **(B)** ATP-γ-S ranging from 50 mM to 500 mM. Following the addition of 200 μM ATP spiked with ^{32}P-α-ATP the ATPase reaction was allowed to proceed for 30 minutes before being quenched and analyzed using thin layer chromatography.

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ISWI binding to nucleosome substrates with long flanking DNA

Recent studies of the nucleosome repositioning activity of the ISWI-containing remodeling complex ACF have suggested that the dependence of the affinity of ACF for nucleosomes on the length of the DNA flanking the core particle results in ACF generating evenly spaced nucleosome arrays [101–103]. Similarly, the affinity of the ISWI-containing complex ISW2 for nucleosome binding has been shown to increase with increasing length of the DNA flanking the nucleosome core particle, with a minimum length of 20 bp required for any binding and optimal binding requiring at least 60 bp of DNA [102]. Because of these results we sought to determine whether ISWI has different affinity for binding to nucleosomal substrates with longer flanking DNA and whether the binding to these substrates is regulated by nucleotides in a manner that is similar to the regulation observed for our free DNA substrate.

To address this question we redesigned our previous nucleosomal substrate by increasing the length of the flanking DNA on one side from 5 bp to 18 bp; this new substrate is denoted F10N18F. We chose this length of flanking DNA to minimize the possibility of an additional ISWI binding to the flanking DNA alone (*i.e.*, not in contact with the histones); furthermore a similar length of flanking DNA was shown by photochemical cross-linking assays to be contacted by the catalytic subunit (Isw2) of ISW2 complex [108] and is a length that is below ISWI-DNA occluded site size [20]. We monitored ISWI binding to this substrate using the same native gel analysis and fluorescence anisotropy assay; the resulting data suggest that ISWI binds to this substrate with the same affinity and stoichiometry as the F10N5F substrate (Figure 3.10 A, B).

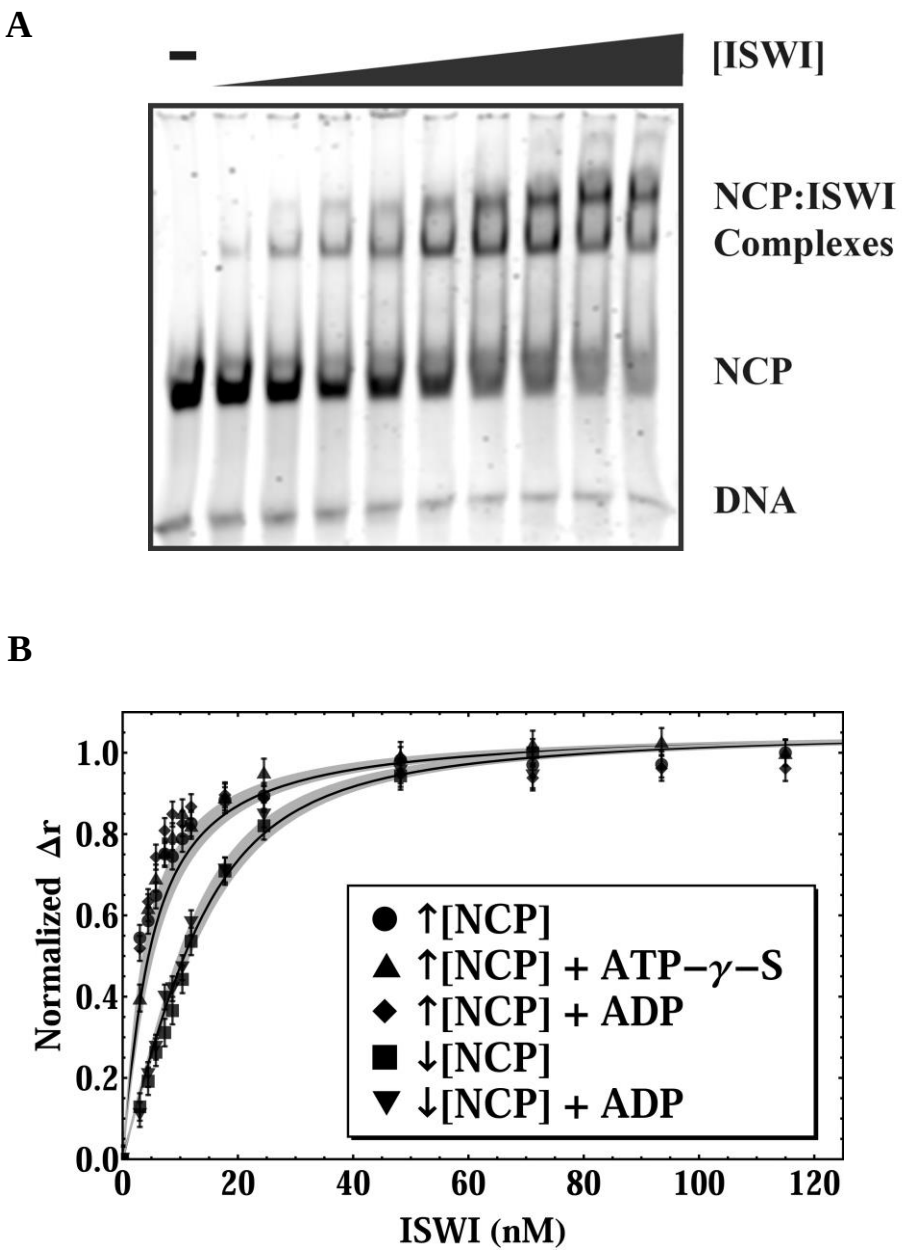


Figure 3.10: ISWI binding to nucleosome substrate with long flanking DNA. **(A)** EMSA performed by titrating a 10N18 nucleosome substrate (50 nM) with increasing concentrations of ISWI (0, 12, 25, 50, 75, 100, 125, 150, 175, 200 nM). Samples were analyzed using a 5% TBE-acrylamide native gel. Gels were stained using a DNA staining dye and imaged using a Typhoon imager. **(B)** Fluorescence anisotropy measurements (Δr) equilibrium binding of ISWI to Alexa 488 labeled 10N18 nucleosomes in the presence of 2 mM nucleotides. In order to more readily determine the effect of ADP on ISWI binding, two different concentrations (2.5 nM (down arrows) (●,▲, ♦) and 10 nM (up arrows) (■,▼)) of the 10N18 substrates were used in the associated binding experiments. The solid line in this panel represents the fit of the equilibrium nucleosome binding data collected in the absence of nucleotides.

Binding studies performed with 10N24 and with symmetrical substrates (18N18 or 24N24) yielded similar outcomes (Figure 3.11 A, B). Similar to the F10N5 substrate, the presence of nucleotides had no effect on the affinity of ISWI to the F10N18 substrate (Figure 3.11 B) or the F10N24 substrate (Figure 3.11 A). In contrast, when using a substrate with very long flanking DNA, 5N71, we found additional ISWI can be accommodated as demonstrated in our EMSA experiment (Figure 3.12). Overall these findings suggest that the presence of additional flanking DNA is not required for stable ISWI binding or the nucleotide regulation of the binding to the nucleosome core as evident when comparing the binding to 10N5 to the 10N18 substrates. Furthermore, the presence of very long flanking DNA can provide an additional binding site for an ISWI molecule that may be regulated by nucleotides.

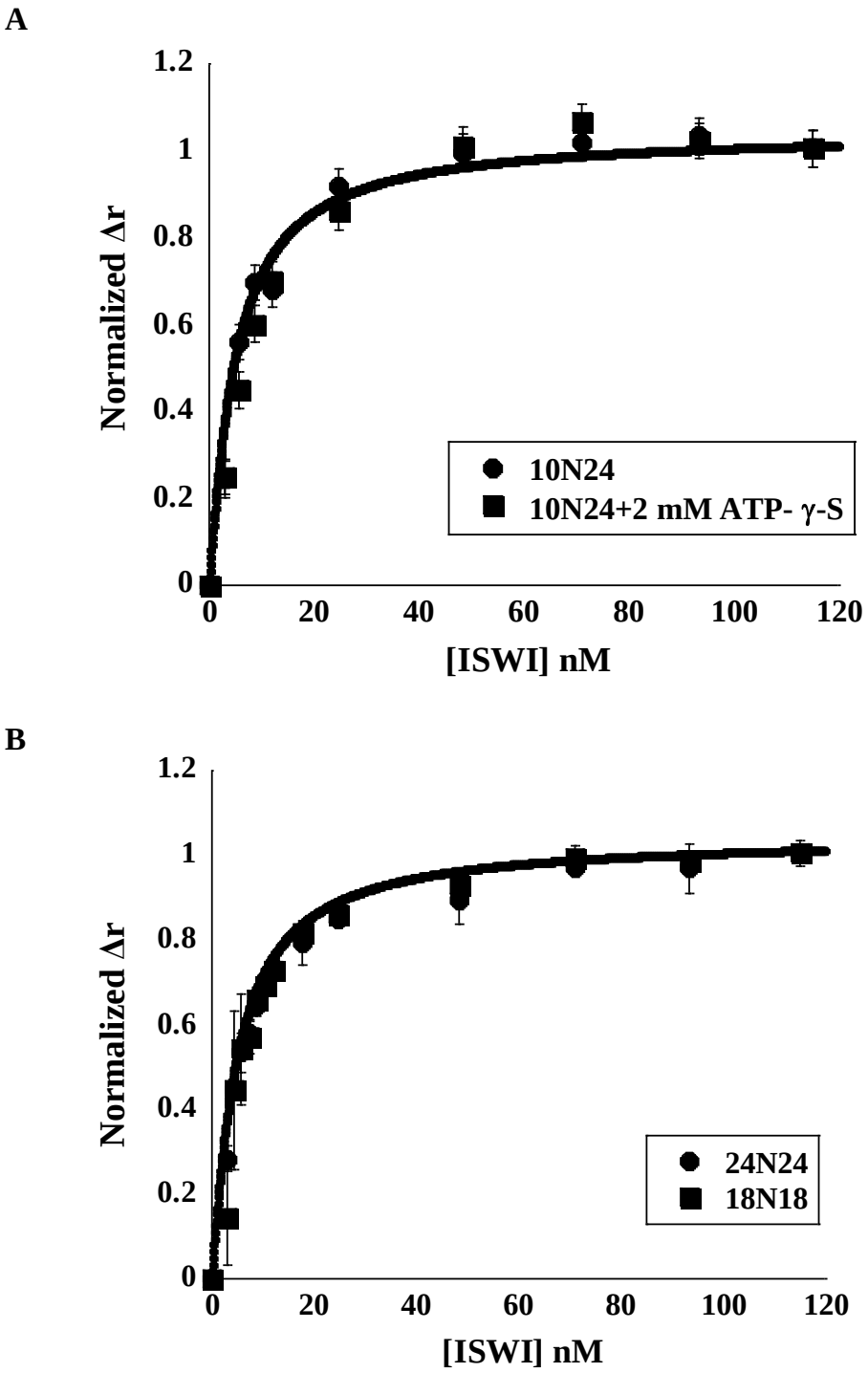


Figure 3.11: Fluorescence anisotropy measurements (Δr) of ISWI binding to F10N24, F18N18F and F24N24F nucleosomes. A) Fluorescence anisotropy measurements of ISWI binding to fluorophore labeled F10N24 (2.5 nM) without nucleotides (●) or with 2 mM ATP-γ-S (■). **B)** Fluorescence anisotropy measurements of ISWI binding to fluorophore labeled F18N18F (■) or F24N24F (●) (2.5 nM). Equilibrium binding isotherms were analyzed using Scheme AI.2 as described in Appendix I. The solid line represents the fit of the binding data using 10N5 nucleosomes to this scheme.

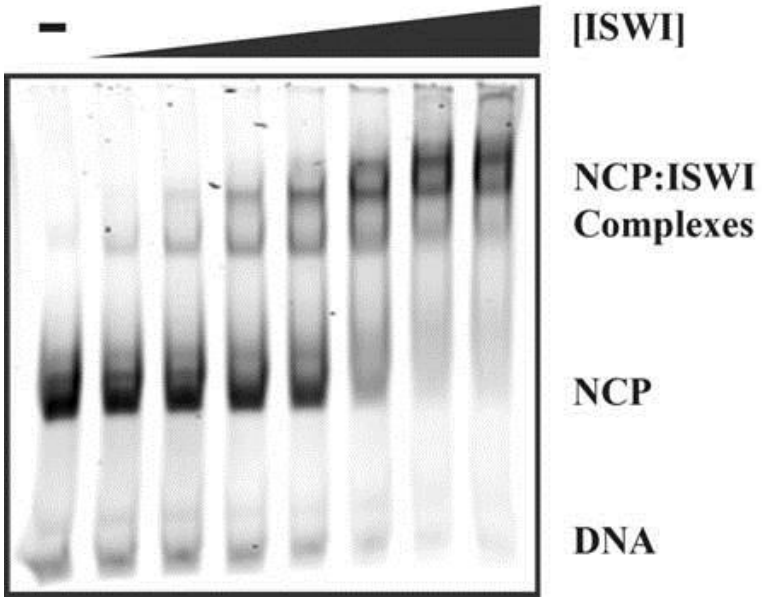


Figure 3.12: ISWI binding to nucleosome substrate with long flanking DNA. Electrophoretic mobility shift assay performed by titrating a non-labeled 5N71 nucleosome substrate (50 nM) with increasing concentrations of ISWI ranging from 12 nM to 300 nM. Samples were analyzed using a 5% TBE-acrylamide native gel. Gels were stained using a DNA staining dye and imaged using a Typhoon imager.

CONCLUSIONS

The ability of ISWI to translocate along DNA in an ATP dependent manner is necessary for its nucleosome repositioning activity [20,22]. During these processes, the enzyme undergoes continual rounds of ATP binding, hydrolysis and product release. For further delineation of the role of DNA translocation in the mechanism of nucleosome repositioning by ISWI, we quantitatively characterized the DNA and nucleosome binding properties of ISWI. Furthermore, in order to understand the role of ATP binding and hydrolysis cycle in regulating translocation, we quantified ISWI binding to DNA and nucleosome substrates in the presence of nucleotide analogs.

ISWI binding to DNA substrate

The simplest model consistent with our studies of the equilibrium binding of ISWI with a 20 bp double-stranded DNA substrate is a 1:1 interaction with an equilibrium constant of $1/\beta_1$ = (18 ± 2) nM (Scheme AI.1). This result agrees with previous studies showing that while *Drosophila* ISWI is unable to bind either a 15 bp or an 18 bp double-stranded DNA with detectable affinity [20,106], binding to a 23 bp DNA substrate was observed [20]. *Drosophila* ISWI has also been shown to bind a 35 bp DNA in a cooperative manner, indicating that more than one ISWI molecule binds to this substrate, with a reported $K_{1/2}$ of 15 nM [106]. Consistent with our model, these data suggest that the contact and occluded site sizes for DNA binding are between 18 and 23 bp. Interestingly, an apparent weaker DNA binding affinity ($K_{1/2}$) for SNF2h was determined from analysis of its DNA-stimulated ATPase activity [102]. While these results suggest that ISWI and SNF2h have different intrinsic affinities for DNA binding, the weaker affinity for DNA binding by SNF2h might also result in part from it being determined indirectly through

DNA stimulated ATPase assays [102]. Furthermore, this affinity was found to vary with DNA length from >1400 nM for 10 bp DNA to 4 nM for 100 bp DNA [102]. It is not surprising that the apparent affinity determined from these experiments would increase with increasing DNA length since the rate of DNA binding will scale with the number of DNA binding sites, and hence with the length of the DNA [19,119]. It is worth noting that although ISWI binds double-stranded DNA more tightly than the SWI/SNF chromatin remodeler RSC (K_d ~ 140 nM [107]), the k_{cat} for ISWI is much lower than for RSC [107] and may suggest a constraint related to the catalytic domain common to both ISWI and RSC.

ISWI binding to nucleosomal substrates

Our EMSA studies of the equilibrium binding of ISWI to nucleosomal substrates with very short flanking DNA, 10N5, demonstrated that ISWI binds to this substrate with a 2:1 stoichiometry. Through subsequent global analysis of anisotropy-based equilibrium binding studies of ISWI to fluorophore-labeled nucleosomal substrates, we determined the associated overall equilibrium constants to be $1/\beta_1 = (1.3 \pm 0.6)$ nM and $1/\beta_2 = (13 \pm 7)$ nM^2. Increasing the length of the flanking DNA to 24 bp from one side did not affect the affinity or the stoichiometry of ISWI binding, while further increasing the length to 71 bp provides an additional binding site leading to the binding of an additional ISWI.

Previous studies of equilibrium nucleosome binding by ISWI have presented conflicting results regarding the capability of ISWI to bind nucleosome core particles that lack flanking DNA [20,105,120]. One possibility for these differences is in the sequence used to reconstitute the nucleosomes; indeed, it is known that different positioning sequences give rise to different

dynamic nucleosome states [121]. Similarly, measurements of affinity to nucleosomes determined indirectly through ATPase assays showed that SNF2h binds to nucleosomes with short (< 20 bp) flanking DNA with affinities ranging from 25 nM to > 250 nM [102,122]. These studies argue that the presence of additional noncatalytic subunits, namely Acf1, is required for efficient binding to nucleosomes with very short flanking DNA [102]. In comparison to our observed stoichiometry, a negative stain electron microscopy study showed that two SNF2h molecules are bound to a nucleosomes substrate with 60 bp of flanking DNA. It is worth noting that, unfortunately, in these images the flanking DNA was not visible. Although it was suggested that this might be a result of flanking DNA flexibility or that DNA is occupied by one of the bound SNF2h molecules [104]. Other native gel studies have shown that multiple ISWI molecules can bind to a nucleosome substrate with 36 to 64 bp of flanking DNA [44,105].

Studies conducted with other ISWI containing complexes, such as the yeast ISW2, have shown that the affinity of nucleosome binding is dependent upon the length of the flanking DNA with a minimum of 20 bp required for stable binding [108]. Affinities of ISW2 for nucleosome binding similar to what we report here for ISWI required more than 70 bp of flanking DNA [108], suggesting that interactions mediated by the non-catalytic protein subunits in the ISW2 complex to the flanking DNA might be contributing to the observed affinity. Unfortunately more quantitative comparisons between these results are complicated by the fact that conflicting estimates of the stoichiometries for ISW2 binding to various nucleosome substrates (0N20, 0N67, 0N70, 0N109) have been reported [108,123,124]. Naturally any ambiguity in the stoichiometry of the interaction of ISW2 with these substrates complicates estimates of the associated affinity.

Nucleotide regulation of ISWI binding to DNA

We investigated the effect of ATP hydrolysis cycle on DNA binding, and found that while ADP has no effects on the binding affinity of ISWI to DNA, ATP analogs weakened the interaction between ISWI and DNA. The binding of other members of the SF-II superfamily to single- and double-stranded DNA was shown to be modulated by the ATP hydrolysis cycle, and that this allosteric effect is central to the processive DNA translocation activity of these enzymes [125–128]. Our observation that ADP binding by ISWI has no regulatory effects on DNA binding by ISWI is also consistent with a previous study of *Drosophila* ISWI [106]. However, studies that characterized how nucleotides allosterically regulate the DNA binding affinity of ISW2 have yielded conflicting results: while one study showed that ADP reduced the DNA binding affinity of ISW2 [109], another study showed that ADP had no affect on DNA binding affinity [123]. The authors suggested that this discrepancy results from differences between recombinant and native preparations of ISW2 [123]. Furthermore, our observations are different from the regulation that was reported for the SWI/SNF subfamily chromatin remodeler RSC [107]. This suggests a difference in the mechanisms of DNA translocation by ISWI and RSC which might contribute to the differences in the proposed models of their nucleosome repositioning activities [21,22,97].

Nucleotide regulation of ISWI binding to nucleosomes

We also characterized the effect of nucleotide binding on nucleosome binding by ISWI and found that the binding of ISWI to nucleosomes with flanking DNA ranging from 5-24 bp in length was unaltered by ADP or ATP analogs. In agreement with our observations for ISWI, neither ATP-γ-S nor ADP affect the nucleosome binding affinity of ISW2 [109,123], however the authors conclude from restriction mapping experiments that slight changes in contacts with the

nucleosomes occur in the presence of ATP analogs [109]. Interestingly, a recent study has demonstrated that only a small percent (1-3%) of the nucleosome bound SNF2h *in vivo* was affected by ATP levels [129]. More striking are differences between the nucleotide-mediated regulation of nucleosome binding by ISWI and SNF2h. The affinity of SNF2h for binding a 0N40 nucleosome has been shown to increase in the presence of an ATP-analog and decrease in the presence of ADP [104]. It is worth mentioning that the length of the flanking DNA used in these experiments is beyond both the contact and occluded site sizes of DNA binding by ISWI [20,105,106] and that additional ISWI complexes have been observed for nucleosomes with comparable lengths (36 bp to 64 bp) of flanking DNA [105]. Thus, the presence of an additional SNF2h binding site on the flanking DNA was possible in these experiments. Since SNF2h is known to bind DNA with affinities comparable to those for core nucleosome binding [122] and if the binding of SNF2h to DNA is regulated by nucleotides similar to ISWI, the presence of an additional SNF2h binding site on flanking DNA would lead to the overall observation of nucleotide mediated regulation of nucleosome binding by ISWI or SNF2h .

Naturally, it is also possible that, although highly conserved, ISWI and SNF2h proteins from different species display distinct behaviors. Indeed, the allosteric effect of nucleotides on DNA binding was found to vary when comparing helicases from different superfamilies with very similar structures [125–128,130–132]. Nevertheless, without an independent determination of the stoichiometry of SNF2h or ISWI binding to these nucleosome substrates a determination of the mechanism through which nucleotide binding allosterically regulates nucleosome binding is problematic. Indeed, as demonstrated in Chapter 4 such information is critical for correct modeling of nucleosome repositioning activity of ISWI.

Implications for nucleosome repositioning

Both SNF2h and human ACF are believed to function as dimers [103,104] and that dependency of ATP hydrolysis on the length of the flanking DNA [101] along with the allosteric regulation by nucleotides controls which subunit of the dimer is active [104]. These models were, unfortunately, proposed based on experiments performed under conditions of unknown stoichiometry and from conflicting results, suggesting a need for reevaluating these models.

Our observation that a single nucleosome, with very short length of extranucleosomal DNA, can accommodate up to two bound ISWI enzymes, the binding of which is not regulated by nucleotides, raises several questions regarding the nucleosome repositioning activity of such a complex: if two ISWI enzymes were bound simultaneously to the same nucleosome would only one or both be active during repositioning? Does any crosstalk occur between the two ISWI during repositioning? In other words, do the two enzymes work independently or concertedly? In light of the difficulty in interpreting nucleosome repositioning data in the absence of information about the stoichiometry with which the remodeler binds the nucleosome substrate, the resolution of these questions would require measuring the nucleosome repositioning activity of ISWI under conditions of known bound stoichiometry and information regarding nucleotide regulation. Therefore, in order to resolve some of these questions we used parameters obtained from our nucleosome binding studies presented here to properly characterize the nucleosome repositioning activity of ISWI (Chapter 4).

CHAPTER 4:

ISWI Remodels Nucleosomes Through a Random Walk

INTRODUCTION

ISWI has been shown to have basal nucleosome binding and repositioning activities independent of its association with other complexes; however, the nucleosome repositioning strategy of ISWI appears to change when it is in these different complexes [23,41,42,44,45,133] from creating well-spaced arrays to completely random nucleosome spacing. Additionally, the directional bias of histone repositioning, either towards or away from thermodynamically favored positions on the associated DNA, varies among these complexes [44,47,77,101,102,134]. Naturally, understanding the nucleosome repositioning activity of the fundamental ISWI motor is essential to understanding how the activities of these varied chromatin remodeling complexes are differentiated and thus how the function of ISWI is regulated by the other interacting proteins in these complexes. The elucidation of these regulation mechanisms will then allow for the determination of how these different complexes are used by the cell to achieve different chromatin reorganization outcomes *in vivo*.

Many experiments have been conducted to understand the regulatory mechanisms underlying the remodeling activity of ISWI and ISWI-containing complexes. The results of recent studies have suggested that the rate of nucleosome repositioning by Snf2h, the human ISWI homolog, is dependent upon the length of flanking DNA on each side of the nucleosome core and that the interaction of Snf2h with nucleosomes is allosterically regulated by the binding of nucleotides [20,101,102]. Taken together these data form the basis of the hypothesis that the

coupling of the nucleotide regulation and flanking DNA length sensing properties modulates the repositioning activity and directionality of this remodeler [104]. However, in our previous work we have shown that nucleosome binding by ISWI is neither regulated by nucleotide binding nor by length of linker DNA (Chapter 3). These results suggest that ISWI is stably anchored to the nucleosome core with high affinity and that the allosteric regulation by nucleotides may not play the dominating role in modulating the nucleosome repositioning activity of ISWI. Consistent with this conclusion are the results of two recent reports re-evaluating the proposed role of the C-terminal DNA binding domains of ISWI and the related chromatin remodeler Chd1 in the nucleosome repositioning activities of these enzymes [37,115]. In contrast to the widely accepted view, the results of these studies demonstrated that the ATPase domains of these two related chromatin remodelers are both sufficient to reposition nucleosomes. Therefore, neither energy transduction nor conformational changes between the ATPase and the DNA binding domains of these enzymes is directly required for their nucleosome repositioning activity. Instead, the DNA binding domain might affect the affinity of DNA binding and consequently the directionality and efficiency/processivity of nucleosome repositioning. Taken together, these data demonstrate that several questions still persist regarding the mechanism of nucleosome repositioning by ISWI.

Here we report the characterization of the ability of ISWI to reposition various nucleosomal substrates using both a gel-based assay as well as a novel fluorescence anisotropy-based assay. We found that nucleosome repositioning by ISWI generated a distinct distribution of histone octamer translational positions. Furthermore, analysis of time courses of ISWI repositioning nucleosome substrates with limited lengths of DNA, and hence limited translational positions, was consistent with ISWI remodeling the nucleosomes through a random

walk mechanism. Our characterization of nucleosome binding (Chapter 3) was utilized in additional analysis of repositioning time courses observed with nucleosomes containing shorter lengths of flanking DNA. This analysis revealed that even though two ISWI can bind to a nucleosome, the presence of a second ISWI monomer bound to the nucleosome did not affect the rate at which the nucleosome was repositioned, suggesting that a monomeric ISWI is sufficient to obtain the observed repositioning activity. We also found that ATP hydrolysis is poorly coupled to nucleosome repositioning suggesting that DNA translocation by ISWI is not energetically rate limiting for the repositioning reaction.

RESULTS

ISWI distributes the nucleosomes into distinct translational positions

We began our characterization of the nucleosome repositioning activity of ISWI using central or asymmetric nucleosome substrates reconstituted with the Widom 601 high affinity positioning sequence [113–117] in a native gel electrophoresis assay. In this assay, the length of DNA flanking either side of the histone octamer or, equivalently, the position of the octamer on the DNA, affects the mobility of the nucleosome on the gel; with centrally positioned nucleosomes displaying the slowest gel mobility [44,135,136]. The ISWI catalyzed repositioning of three nucleosomes with symmetric lengths of flanking DNA (51, 71 and 91 bp) extending on both sides of the nucleosome core (N) are shown in Figure 4.1. As shown in Figure 4.1, we found that ISWI changed the distribution of octamer locations on the DNA from initially being primarily centered on the DNA to being spread over a series of translational positions.

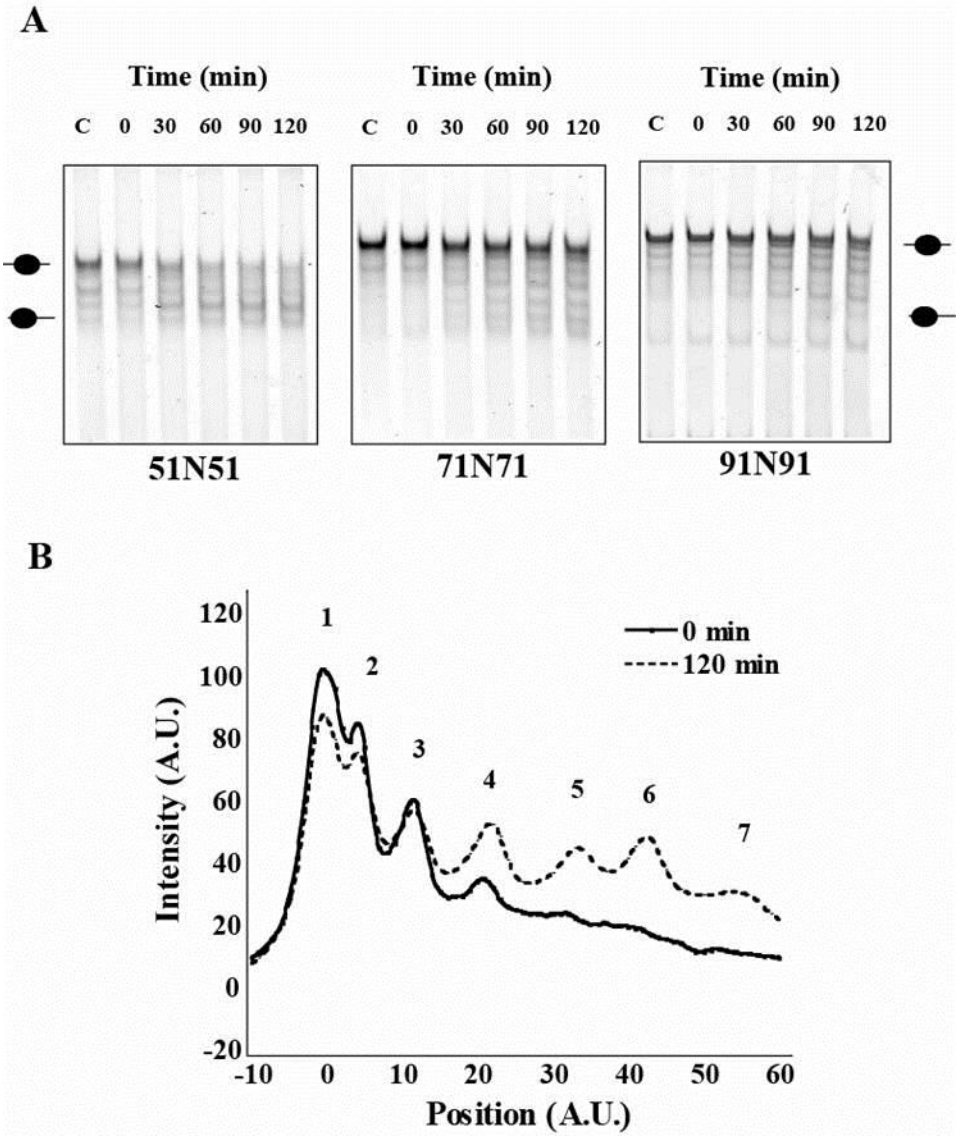


Figure 4.1: Native gel-based repositioning of various nucleosome substrates by ISWI. **A)** Repositioning of 51N51, 71N71 and 91N91 nucleosomes (50 nM) by ISWI (25nM). ISWI and nucleosomes were incubated together at 25° C repositioning reactions were initiated by addition of 1 mM ATP. Reactions were stopped at the indicated time points by the addition of stopping buffer and resolved using a 5% TBE-acrylamide native gel. The first lane in each gel, denoted by "C", is a control reaction lacking ISWI and allowed to proceed for 120 minutes before being stopped. Gels were stained for DNA and imaged as indicated in experimental procedures (Chapter 2). **B)** Analysis of changes in translational positions overtime for 91N91 nucleosome substrate.

Furthermore, the number of apparent translational positions was dependent on the total length of the flanking DNA. From a linear analysis of the number of apparent translational positions as a function of the length of the flanking DNA we determined that a new position was associated with each 12 bp of additional flanking DNA. Similarly, ISWI was able to reposition asymmetrical nucleosome substrates away from their original position into a similar distribution of distinct translational positions (Figure 4.2 A). Additionally, repositioning reactions with different asymmetrical substrates demonstrated the ability of ISWI to move the octamer in both directions along the DNA (Figure 4.2 A and B).

The ATPase activity of ISWI was linear over the entire repositioning reaction time (Figure 4.3). Altering the repositioning assay conditions by increasing the concentration of ISWI or the continuous titration of additional ISWI and ATP into the reactions also did not affect the final distribution of octamer positions. Thus, this distribution appears to be a stable dynamic equilibrium of the possible translational positions for the histone octamer on the DNA.

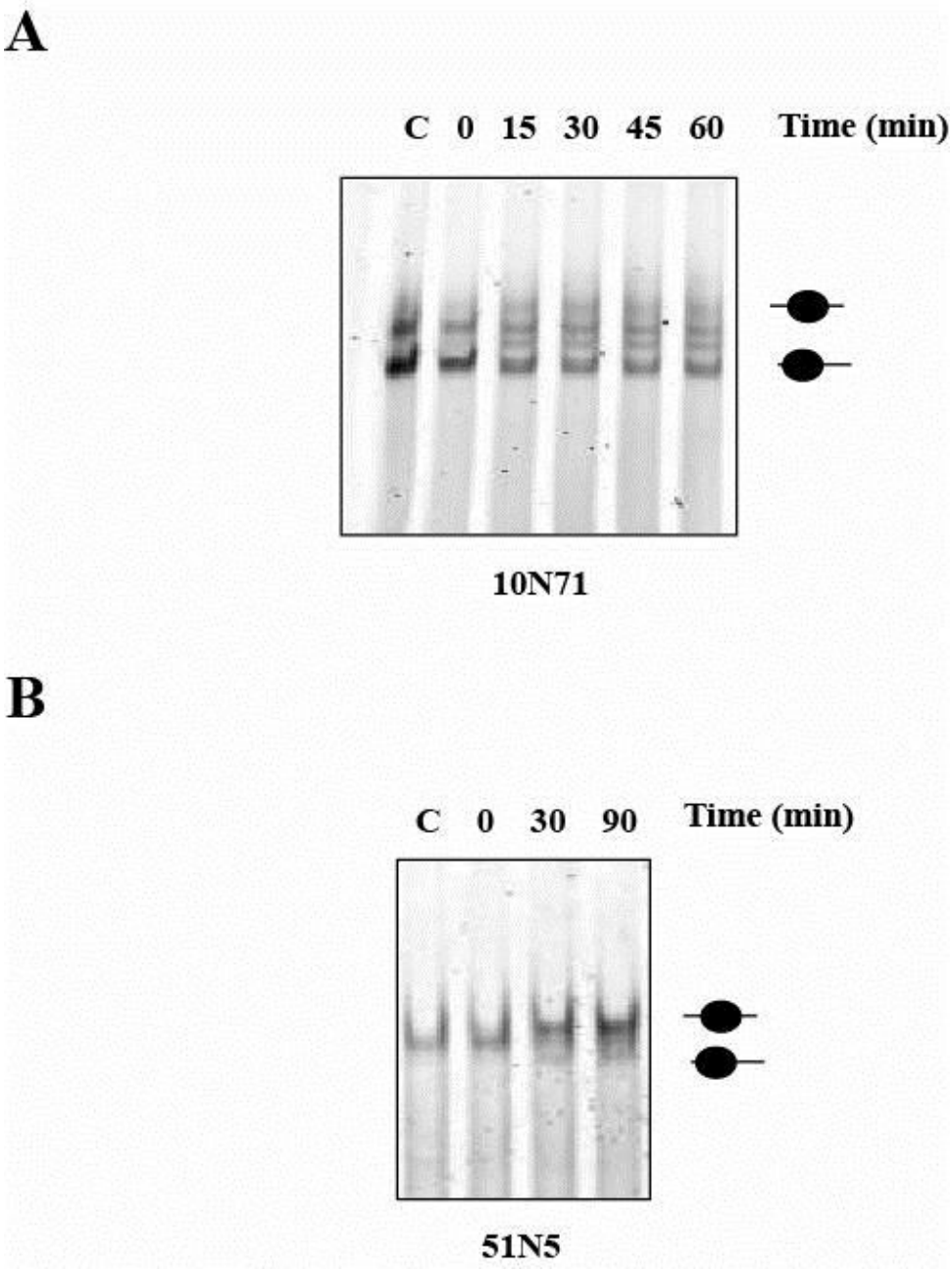


Figure 4.2: Native gel-based repositioning of asymmetrical nucleosome substrates (51N5) and (10N71). Repositioning of **A)** 51N5 and **B)** 10N71 nucleosomes (50 nM) by ISWI (10 nM). ISWI and nucleosome were incubated together at 25° C and the repositioning reaction was initiated by the addition of 1 mM ATP. Reactions were stopped at various time points by the addition of stopping buffer and resolved using a 5% TBE-acrylamide native gel. The first lane in each gel, denoted by "C", is a control reaction lacking ISWI and allowed to proceed for 90 minutes before being stopped. Gels were stained for DNA and imaged as indicated in experimental procedures (Chapter 2).

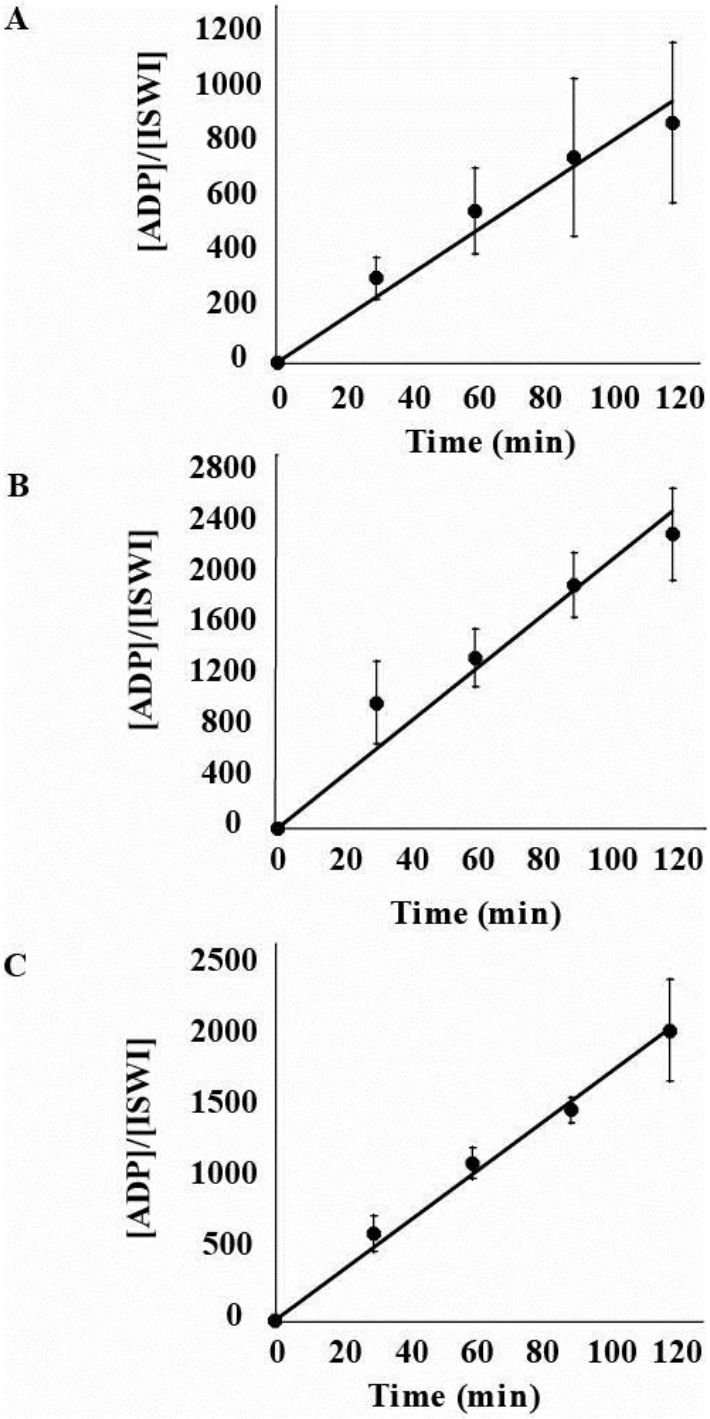


Figure 4.3: Nucleosome stimulated ATPase activity of ISWI. 200 nM of **A)** 51N51, **B)** 71N71, or **C)** 91N91 nucleosomes were incubated with 100 nM ISWI at 25° C. Reactions were initiated by addition of 1 mM cold ATP spiked with ^{32}P -α-ATP. Aliquots were stopped at various time points and analyzed using thin layer chromatography followed by imaging using a Typhoon imager.

ISWI remodels the nucleosomes through a random walk

The final dynamic equilibrium of histone octamer positions on the DNA is consistent with ISWI moving the octamers between these defined positions through a random walk mechanism [137]. Specifically, the processivity with which ISWI moves the octamers is so low that the location of an octamer is shifted, on average, only to the nearest translational position before ISWI dissociation. In subsequent ISWI binding there is no "memory" of the previous direction of translocation and so there is equal probability of the octamer moving in either direction [137]. In order to simplify the determination of the microscopic rate constants associated with this mechanism we sought to analyze the ISWI catalyzed repositioning of nucleosome substrates with only one or two possible translational positions for the histone octamer on the flanking DNA.

We also developed a novel assay for monitoring nucleosome repositioning in which the effect of histone octamer position on the motion of the flanking DNA is measured. In this assay, the movement of the histone octamer toward the end of the DNA constricts the motion of the DNA and thus increases the anisotropy of a fluorophore attached there, similar to how the movement of the counterweight along the pendulum of a metronome changes the frequency of the metronome's oscillation (Figure 4.4).

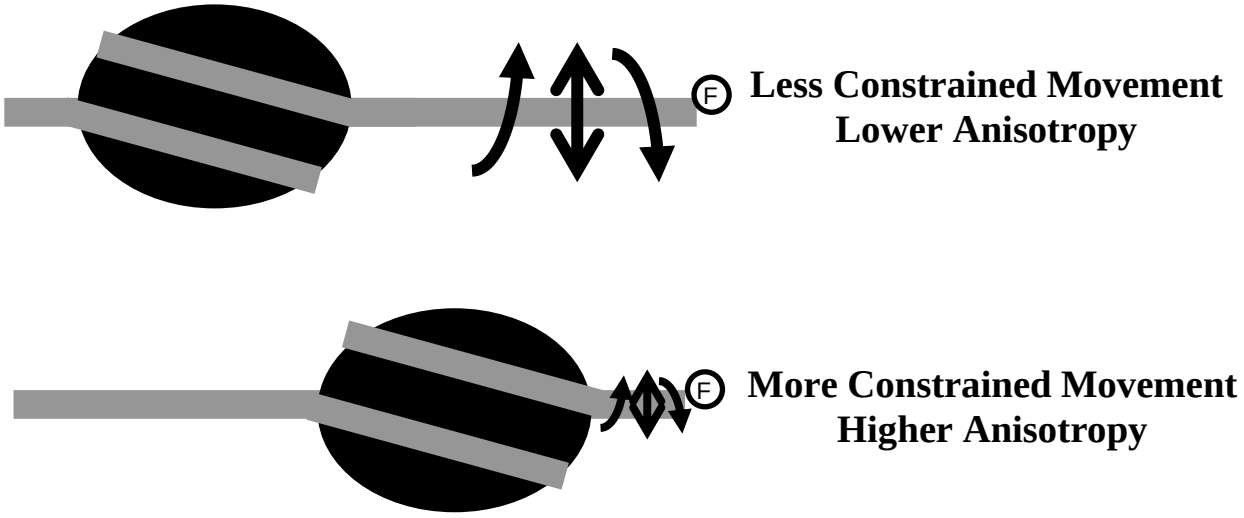


Figure 4.4: Molecular Metronome. The twisting and bending modes of the DNA (gray) will become more constrained as the length of the DNA is changed through repositioning of the octamer (black). This can be monitored as a change in the anisotropy of a fluorophore (F) attached to the end of the DNA.

Initially we characterized the repositioning activity of ISWI using a double fluorophore labeled F18N18F substrate. The addition of ISWI and ATP causes an increase in anisotropy as a function of time, while the addition of ISWI only or ATP only had no effect on the anisotropy (Figure 4.5). Furthermore, no effect on the anisotropy value was observed when using ADP or non-hydrolyzable ATP analogs (Figure 4.6 A). This suggests that these changes in anisotropy require the presence of ISWI and both the binding and hydrolysis of ATP. These results are therefore consistent with the change in anisotropy being associated with the movement of the

histone octamer. Additionally, changes in anisotropy are not observed when a 181 bp fluorophore-labeled DNA, of comparable length as the DNA used to reconstitute the F18N18F nucleosomes, was used as the substrate in the reaction (Figure 4.6 B). This is also consistent with movement of the octamer by ISWI being responsible for the observed time-dependant changes in anisotropy, rather than the binding or movement of ISWI. It is worth mentioning that this assay allows us to monitor changes in the total population of nucleosomes as a function of time and that the change in anisotropy we observe is an average of all species present in solution.

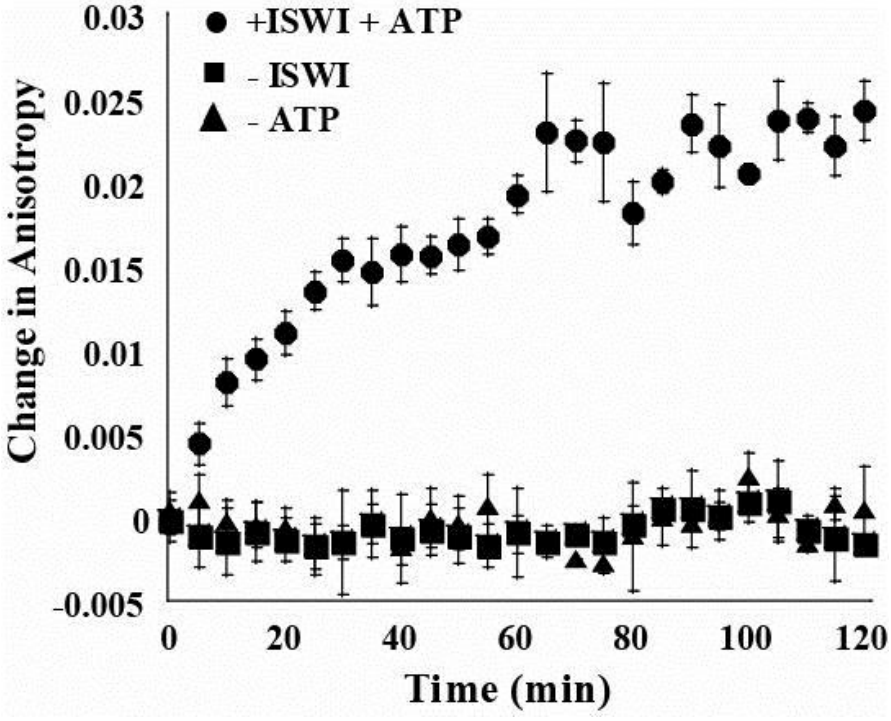


Figure 4.5: Fluorescence anisotropy-based repositioning of F18N18F by ISWI. Measurements of changes in anisotropy (Δr) of 10 nM of fluorophore labeled F18N18F nucleosome incubated with 10 nM ISWI and 1 mM ATP (●), or without ISWI (■), or without ATP (▲).

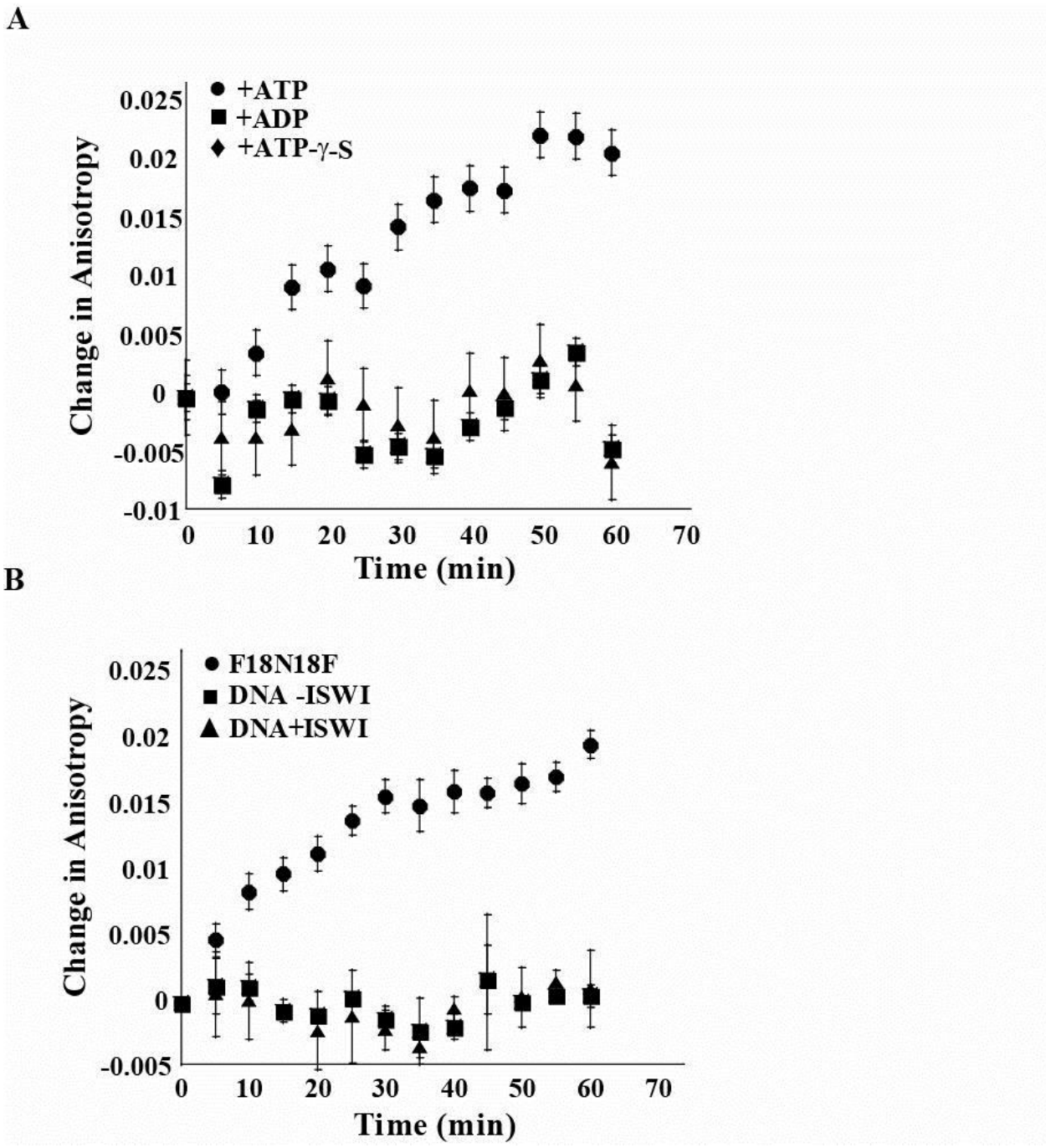


Figure 4.6: Requirement of octamer and ATP for the observed changes in fluorescence anisotropy-based assay. A) Measurements of changes in anisotropy (Δr) of 10 nM of fluorophore labeled 5N18F nucleosome incubated with 10 nM ISWI and 1 mM ATP (●), or 10 nM ISWI and 1 mM ADP (■), or 10 nM ISWI and 1 mM ATP-γ-S (♦). **B)** Measurements of changes in anisotropy of 10 nM of fluorophore labeled F18N18F nucleosome (●) or 10 nM fluorophore labeled 181 bp DNA without ISWI (▲) or with 10 nM ISWI (■) and 1 mM ATP.

Using this molecular metronome assay we then monitored the repositioning of nucleosome substrates with 18 or 24 bp of flanking DNA; these lengths of flanking DNA were chosen such that these substrates would be expected to have one or two, respectively, translational positions for the histone octamer on the flanking DNA. Experiments conducted with 10 nM ISWI and 10 nM nucleosomes are shown in Figure 4.7. As demonstrated in Figure 4.7 the apparent rate of repositioning of F24N24F nucleosomes is slower than the rate of repositioning of F18N18F nucleosomes; these rates are $(0.012 \pm 0.003)\ s^{-1}$ and $(0.031 \pm 0.003)\ s^{-1}$, respectively, as determined from a single-exponential fit of the time courses. This change in repositioning rate is not a function of differences in the stoichiometry or affinity with which ISWI binds these substrates as these are identical for these nucleosomes as demonstrated in Chapter 3. Rather, it is likely a simple consequence of the F24N24F substrate having more translational positions for the histone octamer than the F18N18F substrate. This is consistent with our native gel-based repositioning experiments using long non-labeled nucleosome substrates demonstrating that the rate of repositioning is for 51N51 is faster than that of 71N71 and 91N91 (Figure 4.1 A).

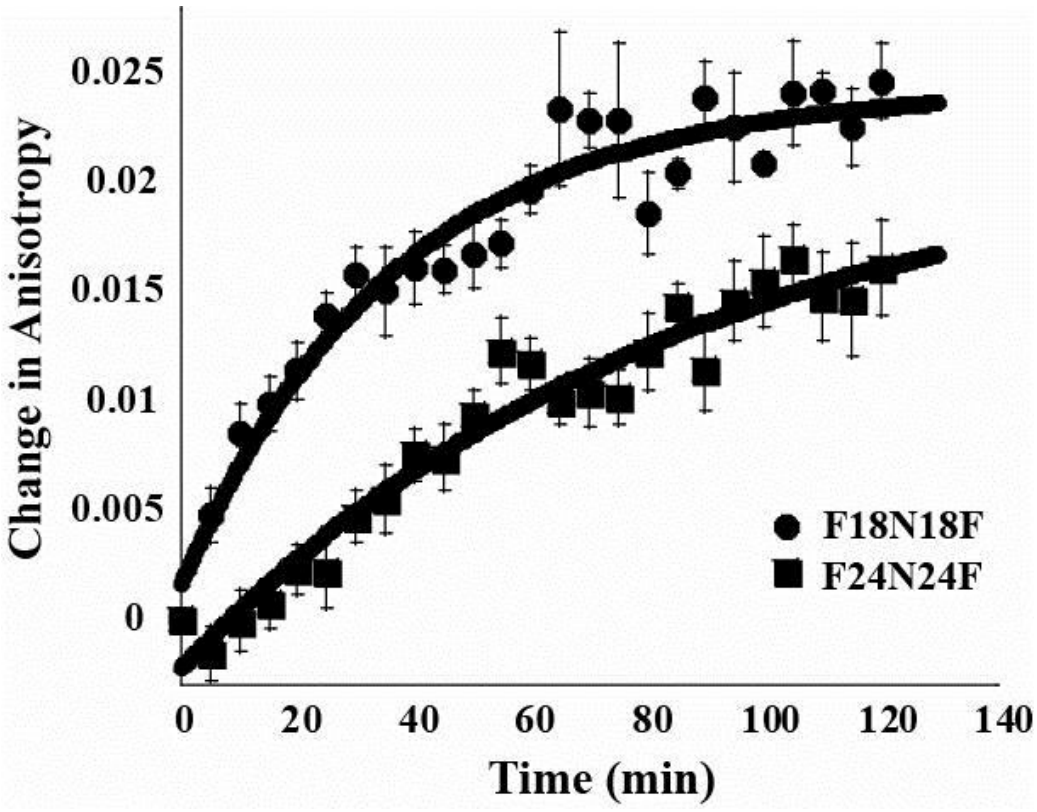


Figure 4.7: Fluorescence anisotropy-based repositioning of F18N18F and F24N24F. Measurements of changes in anisotropy (Δr) of 10 nM of fluorophore labeled F18N18F (●) or 24N24 (■) nucleosomes incubated with 10 nM ISWI and 1 mM ATP. Solid line represents a single-exponential fit of the data.

To test this hypothesis further we monitored the ISWI-catalyzed repositioning of F18N18F and F24N24F nucleosomes at several different concentrations of ISWI and subsequently analyzed the individual time courses for each nucleosome substrate separately to determine the number of octamer translational positions for each nucleosome substrate (Appendix II). The results of this analysis (Table 4.1 and 4.2) demonstrate that the best fit of the time courses is associated with F18N18F nucleosomes having one translational position on the flanking DNA and F24N24F nucleosomes having two. This is consistent with our previous estimate of ~12 bp being required for each translational position.

[ISWI] nM	Equation	k_{obs} (min^{-1})	Variance of Fit
5	II.19	0.0050 ± 0.0008	1.36×10^{-6}
	II.25	0.0283 ± 0.0019	1.78×10^{-6}
10	II.19	0.0157 ± 0.0016	2.70×10^{-6}
	II.25	0.058 ± 0.005	5.03×10^{-6}
15	II.19	0.022 ± 0.002	3.02×10^{-6}
	II.25	0.075 ± 0.008	4.17×10^{-6}
20	II.19	0.023 ± 0.006	1.65×10^{-6}
	II.25	0.089 ± 0.017	2.32×10^{-6}

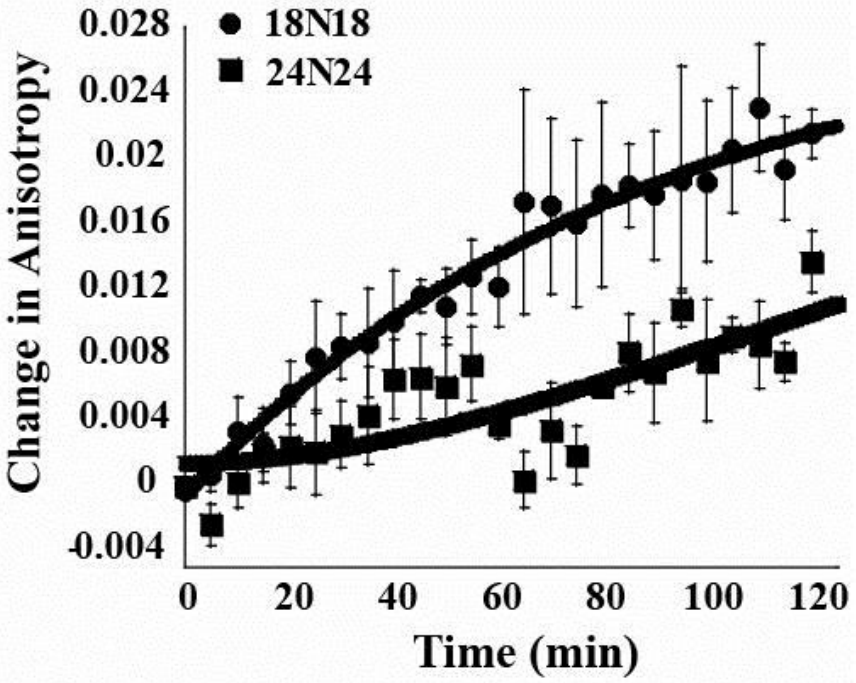
Table 4.1: Results of analysis of F18N18F nucleosome using Equation (II.19) and Equation (II.25) (Appendix II). According to the variance of the fit, Equation (II.19) best describes repositioning data obtained using the F18N18F nucleosome substrate.

[ISWI] nM	Equation	k_{obs} (min^{-1})	Variance of Fit
5	II.19	0.0089 ± 0.0018	6.02×10^{-6}
	II.25	0.019 ± 0.007	5.93×10^{-6}
10	II.19	0.0029 ± 0.0013	2.31×10^{-6}
	II.25	0.024 ± 0.002	1.61×10^{-6}
15	II.19	0.0038 ± 0.0012	1.50×10^{-6}
	II.25	0.026 ± 0.002	1.11×10^{-6}
20	II.19	0.015 ± 0.003	2.52×10^{-6}
	II.25	0.052 ± 0.007	1.92×10^{-6}

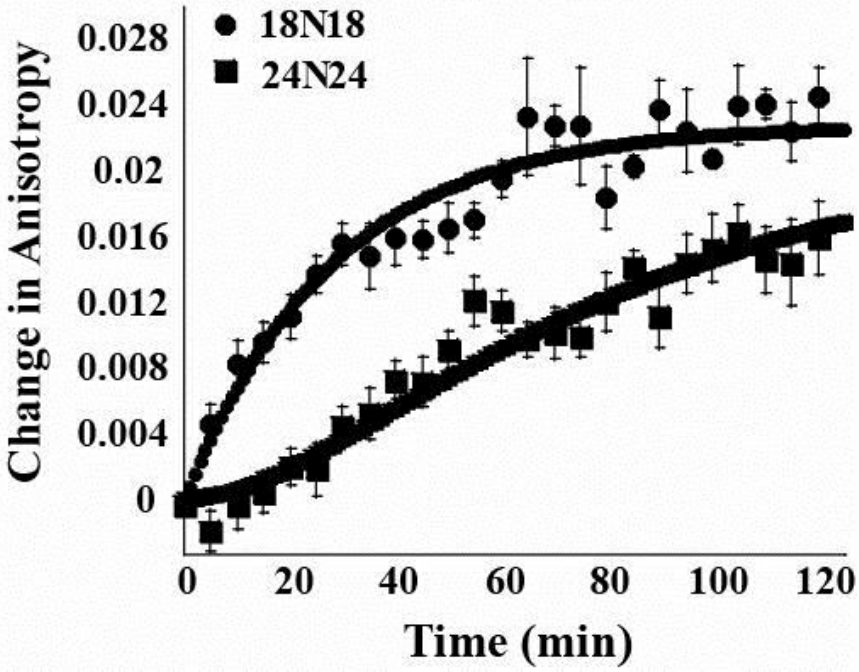
Table 4.2: Results of analysis of F24N24F nucleosome using Equation (II.19) and Equation (II.25) (Appendix II). According to the variance of the fit, Equation (II.25) best describes repositioning data obtained using the F24N24F nucleosome substrate.

We next performed global analysis of the repositioning time courses which includes both nucleosome substrates, F18N18F and F24N24F, together at each ISWI concentration. In this analysis we assumed that the macroscopic rate of repositioning was constant for both substrates, but that the number of translational positions was different for each substrate. This analysis took advantage of the fact that the affinity and stoichiometry of ISWI-nucleosome binding is not affected by nucleotides as shown in Chapter 3 (Appendix II for analysis). This analysis provided a good description of the data, as judged by the variance of the fits, and furthermore demonstrated that the apparent rate of repositioning increased with increasing ISWI concentration (Figure 4.8 and Table 4.3).

A


18N18
24N24
Change in Anisotropy
0.028
0.024
0.02
0.016
0.012
0.008
0.004
0
-0.004
0
20
40
60
80
100
120
Time (min)

B


18N18
24N24
Change in Anisotropy
0.028
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0.012
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Time (min)

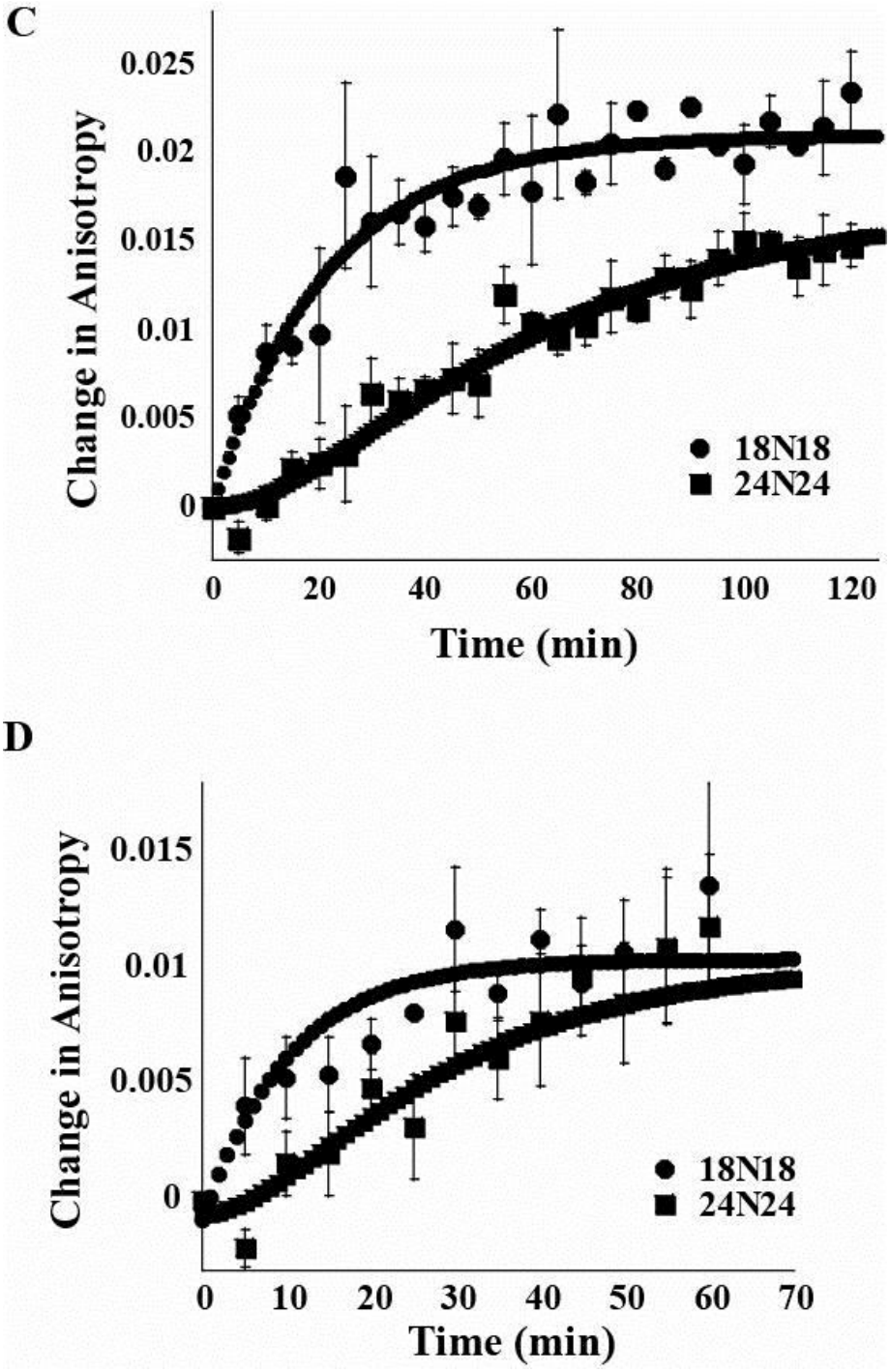


Figure 4.8: Fluorescence anisotropy-based repositioning of F18N18F and F24N24F in the presence of various ISWI concentrations. Measurements of changes in anisotropy (Δr) of 10 nM of F18N18F (●) or F24N24F (■) incubated with **A)** 5 nM ISWI, **B)** 10 nM ISWI, **C)** 15 nM ISWI or **D)** 20 nM ISWI. Reaction was started by the addition of 1 mM ATP. Isotherms were analyzed as described in Appendix II; solid line represents the fit of the data.

[ISWI] nM	k_r (min^{-1})	Variance of fit
5	5.65×10^{-3}	3.63×10^{-6}
10	1.78×10^{-2}	2.55×10^{-6}
15	2.31×10^{-2}	2.17×10^{-6}
20	4.87×10^{-2}	2.27×10^{-6}

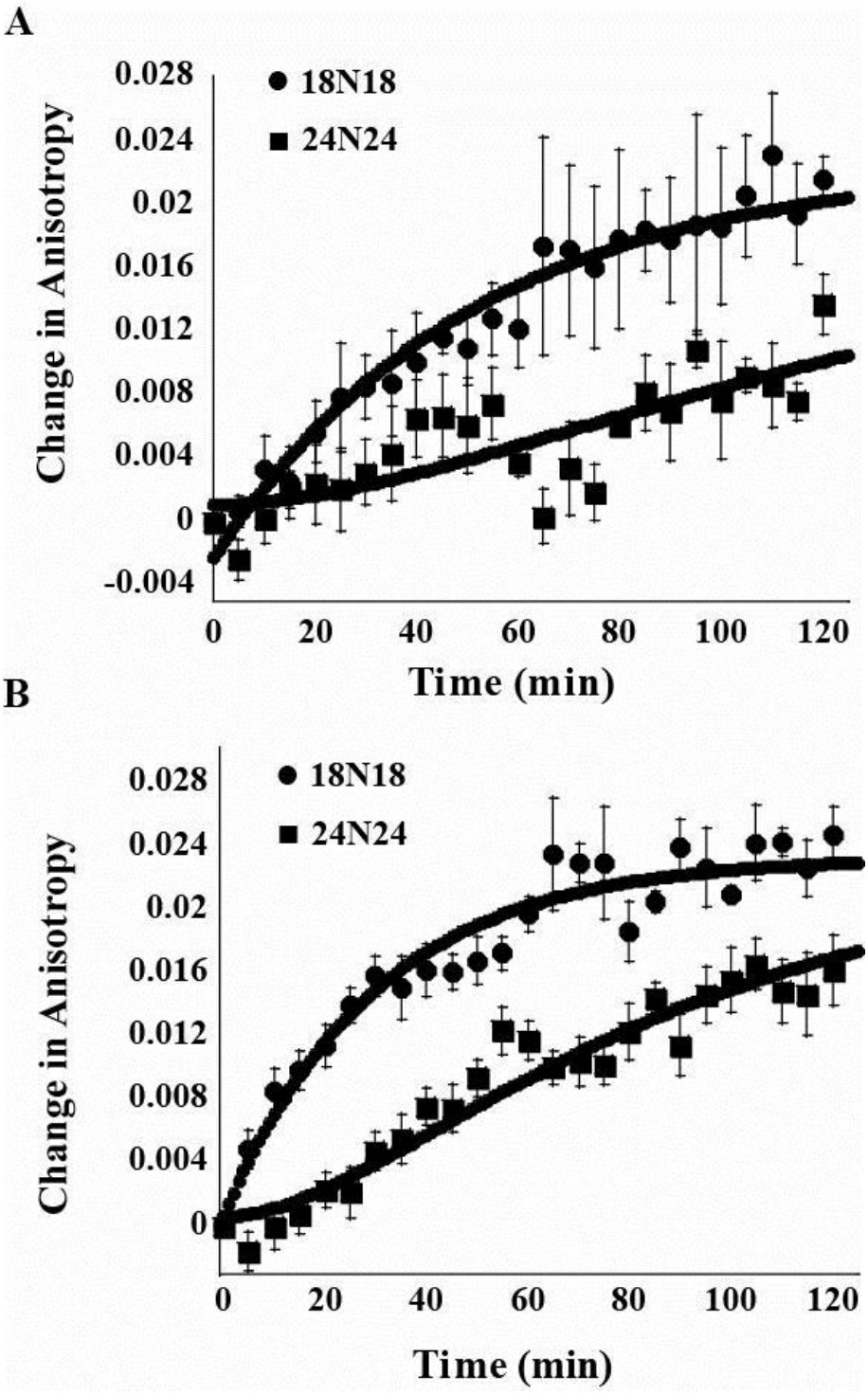
Table 4.3: Results of global analysis for F18N18F and F24N24F together at each ISWI concentration according to Equation (II.19) for F18N18F and Equation (II.25) for F24N24F.

The binding of a second ISWI does not affect the rate of nucleosome repositioning

We characterized the equilibrium binding of ISWI to nucleosomes and demonstrated that two ISWI can bind to nucleosome substrates with limited lengths of flanking DNA (5-24 bp). Using the determined stoichiometric binding constants we are able to determine the fraction of nucleosomes bound with a single ISWI and the fraction bound with two ISWI (Table 4.4); we denote these species as PN and P_2N, respectively. We then performed additional global analysis including all of our nucleosome repositioning time courses together with these species fractions as additional constraints to determine the repositioning activity of each species (Figure 4.9). The result of this analysis is summarized in Table 4.5; the best fit of the data is associated with a model in which both species have the same repositioning rate. Thus, the presence of a second ISWI monomer bound did not affect the rate at which the nucleosome was repositioned, suggesting that a single ISWI monomer is sufficient to obtain the observed repositioning activity.

[ISWI] nM	PN	P_2N	PN+P_2N
5	0.36480	0.028516	0.39332
10	0.54939	0.11795	0.66735
15	0.57744	0.24722	0.82466
20	0.52668	0.37790	0.90458

Table 4.4: Determination of fraction of nucleosomes bound with one or two ISWI at various ISWI concentrations using equilibrium constants determined in our binding studies. P=ISWI; N=nucleosomes; PN=nucleosome with one ISWI bound; P_2N= nucleosome with two ISWI bound; PN + P_2N= total nucleosomes bound.


A
0.028
0.024
0.02
0.016
0.012
0.008
0.004
0
-0.004
Change in Anisotropy
18N18
24N24
0
20
40
60
80
100
120
Time (min)
B
0.028
0.024
0.02
0.016
0.012
0.008
0.004
0
Change in Anisotropy
18N18
24N24
0
20
40
60
80
100
120
Time (min)

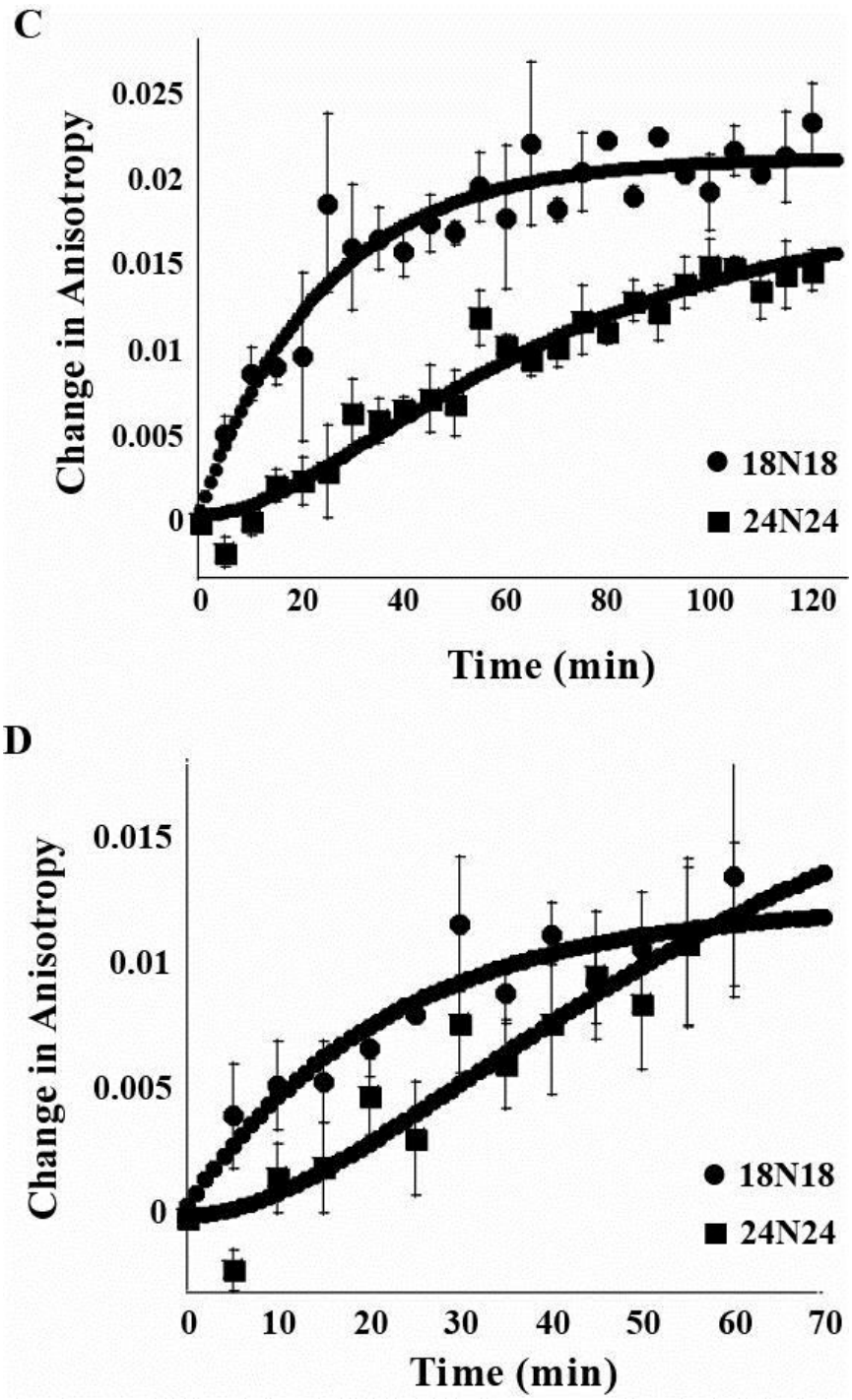


Figure 4.9: Results of global analysis of measurements of changes in Anisotropy. Measurements of changes in anisotropy (Δr) of 10 nM of F18N18F (●) or F24N24F (■) incubated with **A)** 5 nM ISWI, **B)** 10 nM ISWI, **C)** 15 nM ISWI or **D)** 20 nM ISWI. Reaction was started by the addition of 1 mM ATP. Solid lines represent fits resulting from global analysis of the different nucleosome substrates and concentrations using nucleosome species fractions as additional constraint. Detailed information regarding the analysis of these isotherms is described in Appendix II.

Affinities	Species	k_r (min^{-1})	Variance of fit
$1/\beta_1$ = 1.26 nM $1/\beta_2$ = 13.92 nM^2	PN	0.030 ± 0.002	3.34 x 10^{-6}
	P_2N	0.13 ± 0.01	3.37 x 10^{-6}
	PN + P_2N	0.0247 ± 0.0018	2.96 x 10^{-6}
$1/\beta_1$ = 1.04 nM $1/\beta_2$ = 11.94 nM^2	PN	0.029 ± 0.002	3.34 x 10^{-6}
	P_2N	0.137 ± 0.011	3.48 x 10^{-6}
	PN + P_2N	0.0240 ± 0.0018	2.97 x 10^{-6}
$1/\beta_1$ = 1.62 nM $1/\beta_2$ = 20.32 nM^2	PN	0.032 ± 0.002	3.30 x 10^{-6}
	P_2N	0.138 ± 0.011	3.30 x 10^{-6}
	PN + P_2N	0.0256 ± 0.0019	2.96 x 10^{-6}

Table 4.5: Results of simultaneous global analysis for all F18N18F and F24N24F repositioning time courses at different nucleosome binding affinities (affinities were determined using the stoichiometric binding constants determined in our biding studies and varied based on uncertainties determined therein) using Equation (II.19) and Equation (II.25) for the F18N18F and F24N24F substrates, respectively. P=ISWI; N=nucleosomes; PN=nucleosome with one ISWI bound; P_2N= nucleosome with two ISWI bound; PN + P_2N= total nucleosomes bound. As shown from the variances of the fits, the PN + P_2N species model is the best.

ATP hydrolysis is weakly coupled to octamer movement

Our observation that the PN and P_2N species have identical repositioning rates prompted us to question what role is played by the second bound ISWI in the repositioning reaction. We sought to answer this question by determining the ATPase activity associated with each species of bound ISWI during repositioning. We began by measuring the ATPase activity of ISWI at four different ISWI concentrations (50 nM, 250 nM, 500 nM, and 800 nM) in the presence of 250 nM of nucleosomes (10N5, 18N18 and 24N24). Although the observed ATPase rate was dependent upon the concentration of ISWI, as expected, there was no significant difference in the ATPase rates between the three nucleosome substrates at each ISWI concentration. Using the determined stoichiometric binding constants (Chapter 3) we determined the fraction of PN, P_2N, and P species present in each of these ATPase reactions. We then used these species fractions to determine the ATPase rate associated with each species (Table 4.6). As shown in Table 4.6, the ATPase activity of the P_2N species is equal to that of the PN species within the uncertainty of the analysis.

Using the rate of repositioning determined from our global analysis of repositioning time courses measured at different ISWI concentrations, (0.0247 ± 0.0018) min^{-1}, we can calculate from Table 4.6 the efficiency at which ISWI couples ATP hydrolysis to octamer movement. From this calculation we determined that (890 ± 110) ATPs are hydrolyzed for each translational step of the octamer by ISWI.

Affinities	Species	k_{ATP} (ADP/min)	Average k_{ATP} (ADP/min)	Coupling Efficiency (ADP/step)	Coupling Efficiency (ADP/bp)
$1/\beta_1$ = 1.26 nM	PN	17 ± 5	22 ± 2	890 ± 110	74 ± 9
$1/\beta_2$ = 13.92 nM^2	P_2N	23 ± 3			

Table 4.6: Determination of nucleosome stimulated ATP hydrolysis rates associated with different ISWI-nucleosome species. Analysis was performed using Equation II.27.

CONCLUSIONS

The mechanism of nucleosome repositioning by chromatin remodelers remains incompletely understood. Elucidating how ISWI repositions nucleosomes requires knowledge of the stoichiometry, the affinity and the fraction of ISWI bound to the nucleosomes during the repositioning reaction. In Chapter 3 we presented these determined parameters and demonstrated that two ISWI are bound at equilibrium with high affinity to nucleosome substrates with short lengths of flanking DNA and that the binding of ISWI to these substrates is not affected by nucleotides or the length of flanking DNA. These results, together with our determination of the equilibrium constants for nucleosome binding, allow us to predict the fraction of ISWI bound to the nucleosomes during repositioning at various ISWI concentrations.

ISWI remodels the nucleosomes through a random walk

Using native gel-based repositioning assays we observed that the chromatin remodeler ISWI is able to move histone octamers away from their initial location at the high affinity

positioning sequence and generate a distribution of octamer positions when repositioning both central and asymmetrical nucleosome substrates with long lengths of extranucleosomal DNA (51, 71 and 91 bp). These findings are consistent with previous studies that demonstrated the ability of ISWI to generate a distribution of remodeled nucleosome products [101,102]. Furthermore, movement of the octamer in both directions along the DNA was evident by the ability of ISWI to reposition a variety of asymmetrical nucleosome substrates with different initial octamer positions. Interestingly, from a linear analysis of the number of apparent translational positions as a function of the length of the flanking DNA we determined that a new position was observed for each ~12 bp of additional flanking DNA; this spacing is consistent with periodicity of histone:DNA contacts within the high affinity positioning sequence (~10 bp [138,139]). The observed distribution of translational positions may therefore be influenced by both the inherent step size of the enzyme ISWI and the underlying DNA-nucleosome interactions. The possibility exists that other intermediate species are created by the remodeler during repositioning, but we are unable to detect these species because they are unstable and collapse into positions that are more thermodynamically favorable [140].

The distribution of histone octamers into a dynamic equilibrium of translational positions on the DNA is consistent with ISWI moving the octamers between these defined positions through a random walk mechanism. In order to confirm this and more readily analyze this remodeling behavior, we sought to study the repositioning activity of ISWI by means of a novel anisotropy-based repositioning assay using nucleosome substrates with short lengths of flanking DNA. Specifically, we designed centrally positioned nucleosomes with lengths of flanking DNA predicted by our native gel analysis to be short enough to accommodate only one or two octamer

translational positions. Subsequent analysis of repositioning time courses for fluorophore-labeled F18N18F or F24N24F nucleosome substrates using a random walk model demonstrated that the best fit is associated with F18N18F nucleosomes having one translational position on the flanking DNA (one on each side of the nucleosome positioning sequence) and F24N24F nucleosomes having two translational positions on the flanking DNA (consistent with our estimate of ~12 bp being required for each observed translational position). We also globally analyzed the repositioning data of F18N18F and F24N24F together at several ISWI concentrations and found that the rate of repositioning increased with increasing ISWI concentration as expected.

ISWI repositions the nucleosomes as a monomer

We demonstrated that two ISWI can bind to a nucleosome substrate with short flanking DNA (Chapter 3). Previous studies demonstrated that two SNF2h monomers can bind to a nucleosome and that the repositioning activity of this SNF2h "dimer" is regulated by the effect of nucleotide binding on nucleosome binding affinity together with the flanking DNA length-sensing capability of SNF2h. However, we demonstrated that the nucleosome binding affinity of ISWI is not affected by nucleotide binding or the length of the flanking DNA. In order to explore the role of the two ISWI in the observed nucleosome repositioning activity, we used the determined stoichiometric binding constants to quantify the fraction of nucleosomes bound with a single ISWI and the fraction bound with two ISWI. These values were used as additional constraints in a global analysis of all repositioning data where we found that the presence of a second ISWI monomer bound did not affect the rate at which the nucleosome was repositioned, suggesting that a monomer is sufficient for the observed repositioning activity.

ATP hydrolysis by ISWI is poorly coupled to nucleosome repositioning

We determined the efficiency at which ISWI couples ATP hydrolysis to octamer movement. Consistent with what has been observed previously, we found that ATP binding and hydrolysis is poorly coupled to octamer movement, specifically that this movement requires the consumption of hundreds of ATP molecules [102]. If we assume a size of ~12 bp for each translational movement of the octamer then moving the octamer 1 bp requires the hydrolysis of (74 ± 9) ATP. As the ATP coupling efficiency for DNA translocation by other DNA translocases, including chromatin remodelers, is between 0.5 ATP/bp and 3 ATP/bp [141–143] our result argues against the possibility that DNA translocation by ISWI is energetically rate limiting for the repositioning reaction. Therefore, we believe that the poor coupling efficiency of ATP hydrolysis to octamer movement results either from significant hydrolysis being associated with futile repositioning or from a significant ATP consumption requirement associated with the initiation of repositioning. It is worth noting that the former might be an indication of several abortive attempts to move the octamer occurring prior to each successful repositioning event. A low probability of successful repositioning associated with ISWI binding is consistent with the poor template commitment previously reported for SNF2h [102]. Additionally, we found that the rate of ATP hydrolysis of the PN species is the same as the rate of the P_2N species; this is consistent with our global analysis of repositioning data suggesting that a monomer is responsible for the observed nucleosome repositioning activity.

CHAPTER 5:

Discussion and Future perspective

Our findings show that ISWI binds nucleosomes with 5 or 10 bp of flanking DNA with very high affinity, and that additional lengths of flanking DNA are not required for further enhancement of the stability of the binding to the nucleosome core particle. The finding that free DNA but not nucleosome binding is allosterically regulated by nucleotides might suggest a model in which ISWI stably contacts structures that are present on the nucleosome but not on free DNA. This agrees with the nucleosome repositioning model for ISWI put forward by Längst and Becker [43], with previous published data showing that the ATPase activity of ISWI is maximally stimulated in the presence of nucleosomes but not free DNA [29,31,32], and with previously published data demonstrating that interactions between ISWI and the H4 tail regulate ISWI's ATPase activity [31,33–36]. On the other hand, it is possible that ISWI bound to the nucleosome core still interacts weakly with the flanking DNA and that those interactions are regulated by nucleotides. However, even if those weaker contacts with the flanking DNA are present and regulated by nucleotides in a manner similar to the regulation of binding to free DNA, the weak nature of those contacts in comparison to the interactions made with the nucleosome core would still result in no observed nucleotide mediated regulation of nucleosome binding. This possibility is in agreement with our observations that increasing the length of the DNA up to 24 bp has no effect on the affinity of ISWI to the nucleosome core, and that the binding to this longer substrate is also not regulated by nucleotides.

Since ISWI has two domains which are known to interact with both DNA and nucleosomes (the ATPase domain and the C-terminal HAND-SANT-SLIDE (HSS) domain [120]) determining the origin of the differences in how ISWI binds to nucleosomes and DNA requires resolution of how these two domains interact with these substrates. Interestingly, recent studies revealed that the ATPase domain of *Drosophila* ISWI and Chd1 serves as the basic motor for these enzymes and is sufficient for the repositioning activity of these remodelers and furthermore that the C-terminal DNA interacting domain of these enzymes may serve more of a regulatory role in the nucleosome repositioning process such as determining directionality, binding specificity and processivity of the repositioning process [37,115].

Consistent with this model are the results of recent single molecule studies of nucleosome repositioning by the ISW2 complex. These studies reported that changes in the direction of nucleosome movement could occur after at least ~7 bp of DNA translocation by wild type ISW2 [38,144]. Interestingly, the fraction of nucleosomes undergoing these changes in directionality increased significantly (from 6% to 54%) in repositioning experiments with an ISW2 complex containing mutations that compromised the interaction of the C-terminal DNA binding SLIDE domain with extranucleosomal DNA [38,144]. While these mutations were shown to not affect the affinity of ISW2 binding to nucleosomes or the interaction of its ATPase domain with the nucleosomes, DNA fingerprinting showed that the interactions of this ISW2 mutant with flanking DNA were altered and that the repositioning activity was significantly affected [38,144]. The fact that nucleotide binding has an effect on ISWI-free DNA interaction but no effect on the affinity of ISWI to nucleosomes (Chapter 3) may suggest a role for the ATPase cycle in regulating the interactions with flanking DNA and hence the repositioning activity of ISWI.

Similar to the effects of compromising mutations in the SLIDE domain of ISW2 on repositioning efficiency and directionality of nucleosome translocation; the ATP binding and hydrolysis cycle of ISWI may contribute to the random walk behavior that we observe. Future mutagenesis and deletion studies aiming to dissect the details of the binding to DNA/nucleosomes, nucleotide-mediated allosteric regulation and potential cross-talk between the different domains will be of great interest and will contribute further to the understanding of the role of each domain in regulating the nucleosome binding and repositioning activity of ISWI.

It is also possible that other non-catalytic subunits associated with ISWI in ISWI-containing complexes play a role in the allosteric regulation of nucleosome binding by those complexes. Conformational changes in ISWI upon ATP binding and hydrolysis can be also translated to these protein subunits causing pronounced changes in their interaction with flanking DNA. Similarly, changes in ISWI conformation occurring when in complex with these subunits may alter the interactions between ISWI with DNA and/or nucleosomes. Indeed, it has been demonstrated that non-catalytic proteins within the ISW2 complex contact the flanking DNA and that those contacts can extend as far as 53 bp [108,124] and that additional subunits within the ACF complex and the CHRAC complex appears to modulate the nucleosome binding activities of these complexes [102,145]. Future studies that directly compare the nucleosome binding and its associated allosteric regulation for ISWI-containing complexes are required to further resolve these issues.

Our findings that ISWI remodels the nucleosomes through a random walk mechanism and that, even though one nucleosome can accommodate two ISWI molecules, only a monomer

is required for the observed ATP hydrolysis and nucleosome remodeling activity raises several questions. For example, why is the second bound ISWI molecule to be inactive for repositioning? What happens when one of the two monomers dissociates? How is the direction of octamer movement determined? What is the significance of having two ISWI bound *in vivo*? How is this regulated inside the cell?

While it is not immediately clear what determines which monomer is responsible for the repositioning activity, it is possible that the binding of one ISWI monomer causes a conformational change in nucleosome structure or affects the binding of the second monomer rendering only one of the monomers active for repositioning. The binding orientation of the active monomer may in turn determine the direction of the octamer movement (as discussed below). Further mutagenesis and structural studies will be required to address these speculations. It is worth noting that estimates of the total concentration of SNF2h and nucleosomes human cells argues that the predominant bound species *in vivo* is monomeric SNF2h [129]. Nonetheless, remodeling complexes, such as WCRF and human CHRAC contain multiple ISWI subunits [146,147] further emphasizing the need to determine how multiple ISWI bound to the same nucleosome coordinate their nucleosome repositioning activity.

The random walk movement of the octamer back and forth among various translational positions requires a continuous change in the directionality of octamer movement along the DNA. Indeed, and as discussed earlier, changes in directionality of octamer movement have been reported for ISW2 [38,144]. The directionality of octamer movement is likely determined by the orientation with which ISWI is bound to the nucleosome; *i.e.* perhaps each binding site is

associated with one direction of translocation/repositioning. Such changes in the directionality of octamer movement can then potentially be achieved through the dissociation and re-binding of ISWI to another binding site on the nucleosome in a different orientation randomly and independently of the previous binding. The presence of a distribution of remodeled species suggests that the rate at which ISWI dissociates from the nucleosomes is much faster than the rate at which it moves the octamer between translational positions. This conclusion is consistent with previous competition experiments demonstrating that SNF2h dissociates from the nucleosomes at a faster rate than the repositioning taking place and is therefore not rate limiting to the repositioning process [102]. It is worth mentioning that one factor contributing to the repositioning rate we observe could be the altered nucleosome dynamics due to the use of the high affinity sequence to reconstitute the nucleosomes [121].

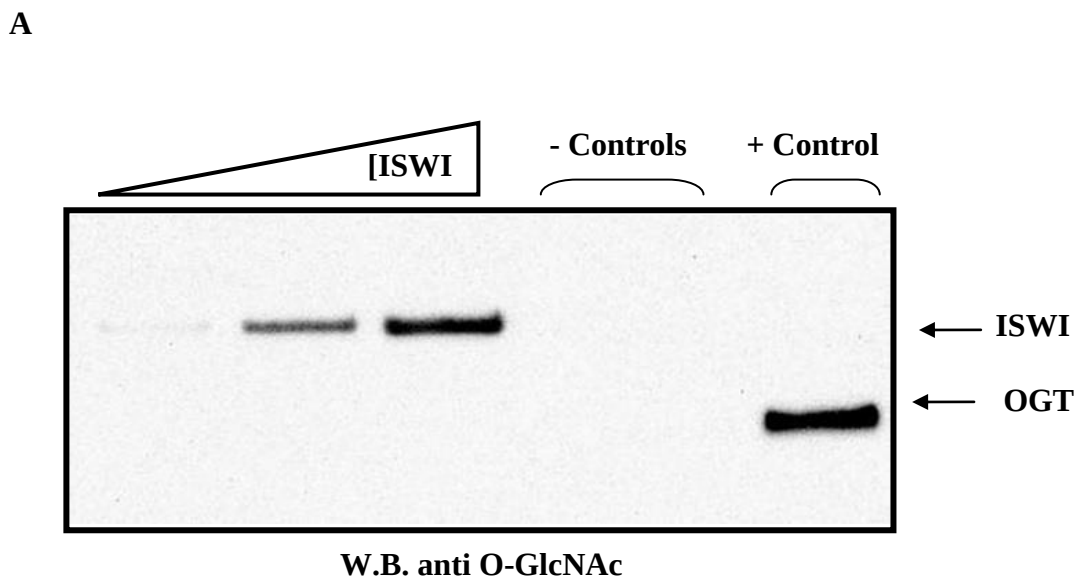
The ability of ISWI to remodel the nucleosome through a random walk presents the motor with the flexibility to be regulated through multiple cellular mechanisms. These mechanisms could influence the efficiency, targeting and directionality of ISWI and consequently leading to the various physiological outcomes observed with ISWI and associated complexes. One method of controlling the accessibility to a binding site on the nucleosome, and thereby regulating octamer movement directionality, could be through histone modifications. These posttranslational modifications could influence the activity of the motor subunit directly, such as acetylation of H4 tails [31,33–35]. Alternatively, histone modifications might be recognized by noncatalytic subunits of ISWI-containing complexes. One example is the recruitment of the NURF complex was shown to be mediated through trimethyated lysine 4 of H3 recognition by the BPTF subunit [49].

The ability of the catalytic subunit ISWI to reposition nucleosomes in a random walk fashion might also be modified and regulated by other binding subunits as part of larger complexes. Indeed, not only is the final outcome of repositioning activity and remodeled products different between free ISWI and ISWI-containing complexes, but also the repositioning outcome was shown to greatly vary among different ISWI-containing complexes [44,47,77,101,102,134]. Interestingly, the directional bias of histone repositioning, either towards or away from thermodynamically favored positions on the associated DNA, varies among these complexes [44,47,77,101,102,134]. These additional proteins could read histone modifications, recognize histone variants, interact with regulatory element-binding proteins, or directly recognize regulatory sequences. These noncatalytic subunits could therefore play an important role in influencing the directionality of octamer movement by recruiting ISWI to a specific site on the nucleosomes. Noncatalytic partners might also influence the rate of repositioning by ISWI. Consistent with this, the two histone fold-containing proteins in the human CHRAC complex were found to increase the efficiency of nucleosome repositioning by binding DNA as it exits the nucleosome [50]. Also, noncatalytic subunits of ACF and NURF complexes were shown to increase the efficiency of repositioning by providing an additional attachment to the nucleosome [46–49]. Future studies elucidating the detailed mechanism by which these subunits regulate the repositioning activity of ISWI would be of great interest.

Studies monitoring nuclear SNF2H found that this remodeler associates with the chromatin only transiently during G1/2 phase and that efficient nucleosome repositioning requires additional signals that stabilizes the interaction of SNF2H with the chromatin observed during S phase [129]. Along with alterations to histone modifications and changes in availability of

noncatalytic subunits mentioned above, these signals could be modifications of ISWI itself in response to cell cycle or other stimuli such as DNA damage or cellular stress. Only two posttranslational modifications were shown to occur directly on the motor subunit of ISWI, PARylation and acetylation. While the significance of ISWI acetylation *in vivo* remains unclear, PARylation was shown to inhibit the nucleosome binding and ATPase activity of ISWI *in vitro* and to affect the association of ISWI with the chromatin in *Drosophila* [53]. Along with PARylation, our group identified two additional novel posttranslational modifications of ISWI (SUMOylation and O-GlcNAcylation). Interestingly, we found that O-GlcNAcylation inhibited the ATPase activity of ISWI *in vitro.* (Figure 5.1). Future studies will be needed to elucidate the importance of these modifications in regulating the function of ISWI inside the cell.

Recent advances in whole genome sequencing have enabled the identification of cancer associated mutations of chromatin remodelers. Recent sequencing studies demonstrating that 20 % of all human cancers are associated with mutations in SWI/SNF chromatin remodeling complexes suggest a role for this complex in tumor suppression [148–150]. Interestingly, recent identification of fusion proteins integrated into chromatin remodeling complexes as the only genetic abnormality in certain types of pediatric cancer suggests a role for mutations in chromatin remodelers as driving mutations rather than playing a supporting role [151–153]. These findings further emphasize the need for future studies aiming to identify pathways and downstream targets that are affected by these mutations.



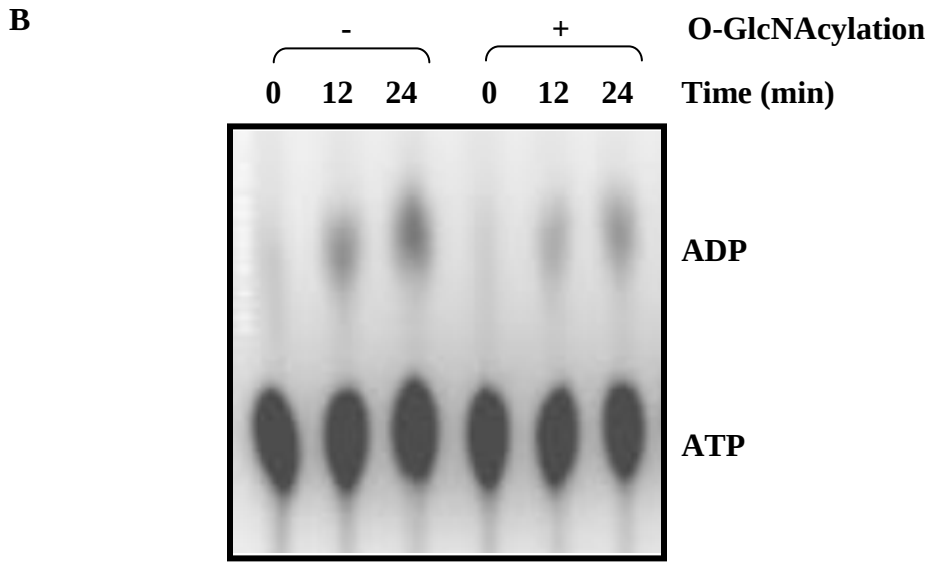


Figure 5.1: Inhibition of the ATPase activity of ISWI by O-GlcNAc. A) Increasing concentrations of ISWI (470, 940 and 1400 ng) were incubated with OGT (100 ng) and UDP-GlcNAc. Samples were analyzed using western blot analysis using antibodies against O-GlcNAc modification. OGT self modification (at high concentrations 940 ng) is shown as a positive control. Negative controls are reactions lacking either OGT or substrate. **B)** Thin layer chromatography analysis of ISWI ATP consumption demonstrate a 36% inhibition of the ATPase activity of ISWI by O-GlcNAc modification.

APPENDIX I:

Quantitative data analysis of equilibrium binding of ISWI to DNA and nucleosomes.

The work presented here is by Dr. Christopher J. Fischer and Mr. Koan Briggs.

The simplest model consistent with our measured equilibrium ISWI-DNA binding isotherms is a 1:1 binding model; analysis of these isotherms to alternative models is shown in Table 3.1. The simplest model consistent with the equilibrium binding of ISWI, DNA, and nucleotides is shown in Scheme AI.1. In Scheme AI.1 a single ISWI (P) can bind to a single DNA molecule (D) with a stoichiometric macroscopic equilibrium constant β_1, ISWI can bind to nucleotide with a stoichiometric macroscopic equilibrium constant β_A, and a complex of ISWI and nucleotide (PA) can bind to DNA with a stoichiometric macroscopic equilibrium association constant $\beta_{1,A}$. This model is in agreement with previous mutagenesis studies showing that ISWI contains only one nucleotide binding site [37,109].

Initial EMSA experiments demonstrated a stoichiometry of 2 ISWI bound to each nucleosome thus providing an initial estimate for the stoichiometry in the analysis of equilibrium ISWI-nucleosome binding isotherms monitored using the fluorescence anisotropy based assay; analysis of these isotherms to alternative models is shown in Table 3.2. The simplest model consistent with this data and stoichiometry is shown in Scheme AI.2. In this model a single ISWI (P) can bind a single nucleosome (N), to form a singly-bound complex (PN) with an associated stoichiometric macroscopic equilibrium constant β_1 and two ISWI can bind a single nucleosome to form a doubly-bound complex (P_2N) with an associated stoichiometric macroscopic equilibrium constant β_2. Simultaneous global analyses of equilibrium binding isotherms to the analytical expressions associated with Scheme AI.1 and Scheme AI.2 were

performed using Mathematica (Wolfram Research) to determine the equilibrium constants and anisotropy signal changes associated with the PD (for DNA binding) or PN and P_2N (for nucleosome binding) species; these analytical expressions were also determined using Mathematica (not shown). Separate independent analysis of these equilibrium binding isotherms through simultaneous implicit analysis using Conlin [154] produced identical results. For this implicit fitting the following equations were used for Scheme AI.1:

$$[P_{total}] = [P] + \beta_1[P][D] + \beta_A[P][A] + \beta_{1,A}[P][A][D] \quad \text{(I.1)}$$

$$[D_{total}] = [P] + \beta_1[P][D] + \beta_{1,A}[P][A][D] \quad \text{(I.2)}$$

$$[A_{total}] = [P] + \beta_A[P][A] + \beta_{1,A}[P][A][D] \quad \text{(I.3)}$$

In these equations $[P_{total}]$, $[D_{total}]$, and $[A_{total}]$ are the total concentrations of ISWI, DNA, and nucleotide, respectively, in solution, β_1, β_A, $\beta_{1,A}$, and $\beta_{A,1}$ are the stoichiometric macroscopic equilibrium constants as defined in Scheme AI.1, [P] is the concentration of free ISWI, [D] is the concentration of free DNA, and [A] is the concentration of free nucleotide. The observed change in anisotropy can be determined from free concentrations of ISWI, DNA and nucleotide using the following equation

$$f = s*\left(\frac{[P][D]}{[D_{total}]}\right)\left(\beta_1 + C*\beta_{1,A}\beta_A[A]\right) \quad \text{(I.4)}$$

In Equation (I.4) s is the signal change associated with the formation of the PD complex and C is the ratio of the signal change associated with the formation of the PDA complex to that associated with the formation of the PD complex.

For equilibrium nucleosome binding the implicit fitting equations are:

$$[P_{total}] = [P] + \beta_1[P][N] + 2\beta_2[P][P][N] \quad \text{(I.5)}$$

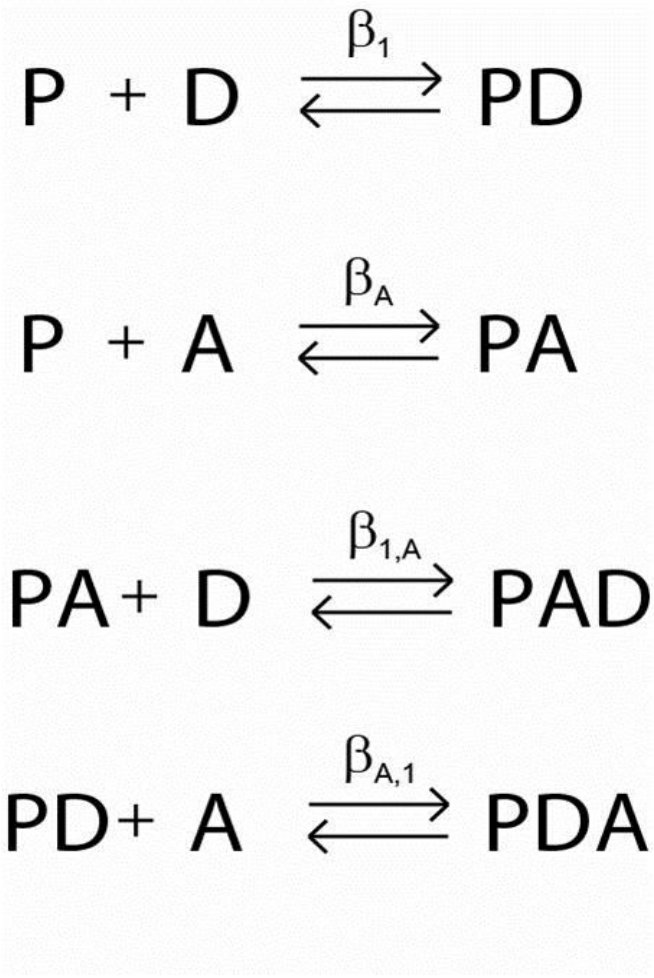
$$[N_{total}] = [N] + \beta_1[P][N] + \beta_2[P][P][N] \quad \text{(I.6)}$$

In these equations $[P_{total}]$ and $[N_{total}]$ are the total concentrations of ISWI and nucleosomes, respectively, in solution, β_1 and β_2 are the stoichiometric macroscopic equilibrium binding constants as defined in Scheme AI.2, [P] is the concentration of free ISWI, and [N] is the concentration of free nucleosomes. The observed change in anisotropy can be determined from free concentrations of ISWI and nucleosomes using the following equation

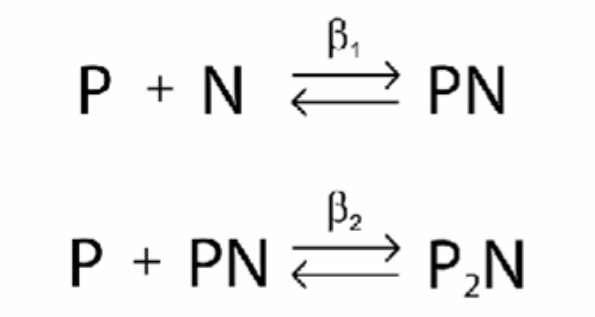
$$f = s * \left(\frac{[P][N]}{[N_{total}]} \right) \left(\beta_1 + C * \beta_2 [P] \right) \quad \text{(I.7)}$$

In Equation (I.7) s is the signal change associated with the formation of the PN complex and C is the ratio of the signal change associated with the formation of the P_2N complex to that associated with the formation of the PN complex.

Unless otherwise noted all traces presented in the figures have been normalized to the final asymptotic value of the anisotropy change as determined from this analysis. Finally, unless otherwise noted all uncertainties represent 68% confidence intervals (±1 standard deviation) as determined by Monte Carlo analysis



Scheme AI.1: ISWI (P) binding to DNA (D) and the nucleotide analog (A). β_1, β_A, $\beta_{1,A}$ and $\beta_{A,1}$ represent the stoichiometric macroscopic equilibrium constants.



Scheme AI.2: ISWI (P) binding to nucleosomes (N). β_1 and β_2 represent the stoichiometric macroscopic equilibrium constants.

APPENDIX II:

Quantitative data analysis of the nucleosome repositioning activity and associated ATP consumption by ISWI.

The work presented here is by Dr. Christopher J. Fischer.

We begin by considering a model in which there are three possible locations for the histone octamer on the DNA; this model is depicted in Scheme AII.1. In this model these locations (R_i) relative to the central position are denoted by a subscript. Thus, R_0 denotes a nucleosome at the central position and $R_{\pm1}$ denotes a nucleosome that is one translational position away from the central position. Because the affinity of DNA binding by the histone octamer is likely strongest at the central position we will assume different rate constants for the movement of the octamer away and toward this location. We define k_1 to be the rate constant for the movement away from R_0 and k_2 to be the rate constant for the movement toward R_0. Note that since there are two pathways for repositioning from R_0 the rate constant for each pathway is denoted as $k_1/2$ in Scheme AII.1.

The differential equations associated with this scheme are

$$\frac{d}{dt}\left[R_{-1}\right] = \frac{k_1}{2}\left[R_0\right] - k_2\left[R_{-1}\right] \tag{II.1}$$

$$\frac{d}{dt}\left[R_0\right] = -2\left(\frac{k_1}{2}\right)\left[R_0\right] + k_2\left[R_{-1}\right] + k_2\left[R_{+1}\right] \tag{II.2}$$

$$\frac{d}{dt}\left[R_{+1}\right] = \frac{k_1}{2}\left[R_0\right] - k_2\left[R_{+1}\right] \tag{II.3}$$

If we assume that a fraction *f* of the octamers is initially at R_0 and (1-*f*)/2 at R_{+1} and R_{-1}, then the solution of these differential equations is

$$\frac{[R_0](t)}{\sum_i [R_i](t)} = \frac{1}{k_1 + k_2}\left(k_2 + \left(f\left(k_1 + k_2\right) - k_2\right)e^{-(k_1+k_2)t}\right) \quad \text{(II.4)}$$

$$\frac{[R_{-1}](t)}{\sum_i [R_i](t)} = \frac{[R_{+1}](t)}{\sum_i [R_i](t)} = \frac{1}{2\left(k_1 + k_2\right)}\left(k_1 + \left(k_2 - f\left(k_1 + k_2\right)\right)e^{-(k_1+k_2)t}\right) \quad \text{(II.5)}$$

Thus, the apparent rate constant for the approach to the steady-state equilibrium is

$$k_{app} = k_1 + k_2 \quad \text{(II.6)}$$

As expected, it is not possible to determine values for k_1 and k_2 from the rate at which the system approaches its steady state.

The repositioning reaction is coupled to the hydrolysis of ATP. If we assume that each movement of a histone octamer is associated with *c* ATP molecules being hydrolyzed (and thus *c* ADP molecules being formed) the equation for the rate of change of the concentration of ADP is

$$\frac{d}{dt}[ADP] = 2c\left(\frac{k_1}{2}\right)R_0 + ck_2\left(R_{+1} + R_{-1}\right) \quad \text{(II.7)}$$

The solution to this equation is given in Equation II.8.

$$[ADP](t) = \frac{c}{k_{obs}^2}\left(f\left(k_1^2 - k_2^2\right) - k_1k_2 + k_2^2 - e^{-k_{obs}t}\left(k_1 - k_2\right)\left(k_{obs}f - k_2\right) + 2k_1k_2k_{obs}t\right) \quad \text{(II.8)}$$

According to Equation II.8 the time course of ADP production will consist of a burst phase, with associated rate constant k_{obs}, followed by a steady state phase. The magnitude of the burst phase is directly proportional to the difference between k_1 and k_2. If the rates of repositioning are the

same regardless of octamer position (*i.e.*, if $k_1=k_2=k$) then Equation II.8 simplifies to Equation II.9.

$$[ADP](t) = ckt \tag{II.9}$$

Scheme AII.1 can be modified to include the binding and dissociation of ISWI from the nucleosome as shown in Scheme AII.2. In this scheme the histone octamer can exist in two states R and NR. In the R state the octamer is bound by ISWI and thus is capable of being repositioned. In the NR state the octamer is not bound by ISWI and thus cannot be repositioned. The rate constant for the dissociation of ISWI from the nucleosome is k_d and the rate constant for ISWI being binding to the nucleosome is k_b; in this representation k_b is a composite rate constant which includes contributions from the concentration of ISWI present in the solution. As previously demonstrated it is not possible to determine independent estimates of the microscopic rate constants for repositioning (k_1 and k_2 in Scheme AII.1) so we will assume that they are equal (denoted as k_r in Scheme AII.2). The differential equations associated with Scheme AII.2 are

$$\frac{d}{dt}[R_0] = -2\left(\frac{k_r}{2}\right)[R_0] - k_d[R_0] + k_b[NR_0] + k_r[R_{+1}] + k_r[R_{-1}] \tag{II.10}$$

$$\frac{d}{dt}[R_{+1}] = \frac{k_r}{2}[R_0] - k_r[R_{+1}] - k_d[R_{+1}] + k_b[NR_{+1}] \tag{II.11}$$

$$\frac{d}{dt}[R_{-1}] = \frac{k_r}{2}[R_0] - k_r[R_{-1}] - k_d[R_{-1}] + k_b[NR_{-1}] \tag{II.12}$$

$$\frac{d}{dt}[NR_i] = k_d[R_i] - k_b[NR_i] \tag{II.13}$$

For simplicity, we will assume that all of the protein is bound initially at either R_0 or NR_0 and we will use the variable K to denote the fraction of the protein bound initially at R_0; 1-K is thus the fraction initially bound at NR_0. Since the presence of nucleotide does not affect the affinity of

ISWI for binding nucleosomes these fractions will remain constant throughout the repositioning reaction. Thus,

$$k_b = \left(\frac{K}{1-K}\right)k_d \tag{II.14}$$

The solutions to Equations II.11 through II.13 can be simplified if we assume that the rate of dissociation of ISWI from the nucleosome is faster than the rate of repositioning (*i.e.*, $k_d >> k_r$). This assumption is consistent with the poor substrate commitment demonstrated by ISWI-containing chromatin remodelers for nucleosome repositioning (refs). With this assumption we have

$$\frac{[R_0](t)}{\sum_i [R_i](t)+[NR_i](t)} = \frac{K}{2}\left(1+e^{-2Kk_r t}\right) \tag{II.15}$$

$$\frac{[R_{+1}](t)}{\sum_i [R_i](t)+[NR_i](t)} = \frac{[R_{-1}](t)}{\sum_i [R_i](t)+[NR_i](t)} = \frac{K}{4}\left(1-e^{-2Kk_r t}\right) \tag{II.16}$$

$$\frac{[NR_0](t)}{\sum_i [R_i](t)+[NR_i](t)} = \left(\frac{1-K}{2}\right)\left(1+e^{-2Kk_r t}\right) \tag{II.17}$$

$$\frac{[NR_{+1}](t)}{\sum_i [R_i](t)+[NR_i](t)} = \frac{[NR_{-1}](t)}{\sum_i [R_i](t)+[NR_i](t)} = \left(\frac{1-K}{4}\right)\left(1-e^{-2Kk_r t}\right) \tag{II.18}$$

The total population of octamers at the $i = \pm 1$ positions is given by Equation II.19.

$$\frac{[R_{+1}](t)+[R_{-1}](t)+[NR_{+1}](t)+[NR_{-1}](t)}{\sum_i [R_i](t)+[NR_i](t)} = \frac{1}{2}\left(1-e^{-2Kk_r t}\right) \tag{II.19}$$

Thus, the repositioning reaction approaches its steady state solution with an apparent rate constant of

$$k_{app} = 2Kk_r \tag{II.20}$$

The rate of ADP production associated with repositioning is

$$[ADP](t) = ck_r Kt \tag{II.21}$$

As expected, the rate of ADP production is linearly dependent upon the fraction of bound nucleosomes. Similarly, if there were two octamer positions available on each side of the central position then

$$\frac{[R_0](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{K}{4} e^{-2Kk_r t} \left(1 + e^{-Kk_r t}\right)^2 \tag{II.22}$$

$$\frac{[R_{+1}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{[R_{-1}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{K}{4}\left(1 - e^{-2Kk_r t}\right) \tag{II.23}$$

$$\frac{[R_{+2}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{[R_{-2}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{K}{8} e^{-2Kk_r t} \left(1 - e^{-Kk_r t}\right)^2 \tag{II.24}$$

The total population of octamers at the $i = \pm 2$ positions is given by Equation II.25.

$$\frac{[R_{+2}](t) + [R_{-2}](t) + [NR_{+2}](t) + [NR_{-2}](t)}{\sum_i [R_i](t) + [NR_i](t)} = \frac{e^{-2Kk_r t}}{4}\left(1 - e^{-Kk_r t}\right)^2 \tag{II.24}$$

The repositioning reaction is now biphasic with

$$k_{app,1} = Kk_r \tag{II.25}$$

$$k_{app,2} = 2k_{app,1} = 2Kk_r \tag{II.26}$$

However, the rate of ADP production is still given by Equation II.21.

The simplest model consistent with our 3 state random walk model for the ISWI catalyzed repositioning of an 18N18 nucleosome is shown in Scheme AII.1. In Scheme AII.1 R_i denotes the population of nucleosomes bound with ISWI with histone octamers in the i^{th} translational position; $i = 0$ denotes the central position, defined by the NPS, and $i = \pm 1$ denotes positions 1 translational step away from the central position. NR_i denotes the population of nucleosomes with histone octamers in the i^{th} translational position, but without ISWI bound. The rate constant for ISWI dissociation from nucleosomes is denoted by k_d and the rate constant for ISWI binding nucleosomes is denoted by k_b. The macroscopic rate constant for octamer movement between translational positions is denoted by k_r. The equation for the time dependence of the population of octamers at the $i = \pm 1$ positions is given by Equation (II.19).

The variable K in Equation (II.19) denotes the fraction of ISWI bound initially at R_0. Similarly, for repositioning of 24N24 nucleosomes in which there are two positions for the histone octamer on either side of the central position, defined by the NPS, the equation for the time dependence of the population of octamers at the $i = \pm 2$ positions is given by Equation (II.25).

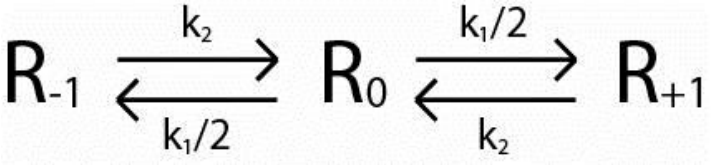
All ATPase time courses were simultaneously globally analyzed using Equation (II.27).

$$[ADP](t) = \left(k_{ATP,PN}[PN] + k_{ATP,P_2N}[P_2N]\right)t \qquad \text{(II.27)}$$

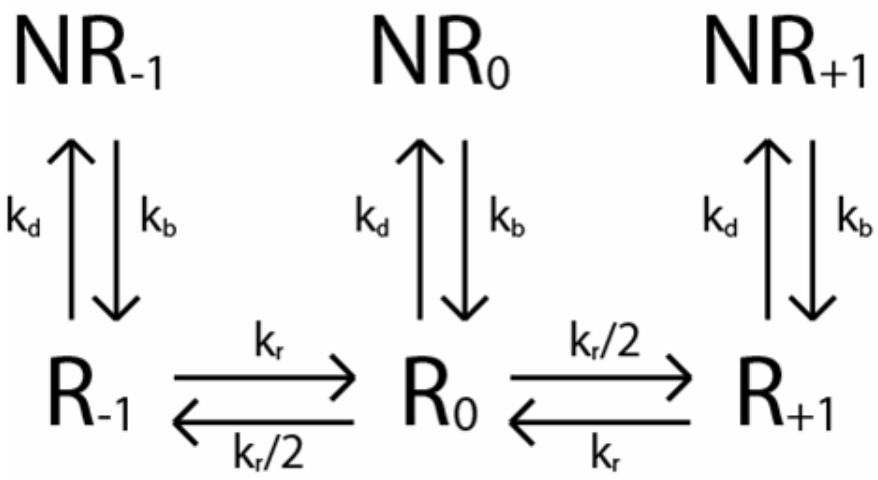
In Equation (II.27) $k_{ATP,PN}$ and k_{ATP,P_2N} are the steady state rates of ATP hydrolysis for the PN and P_2N states, respectively.

Analysis of repositioning time courses using Equation (II.19) and Equation (II.25) and ATPase time courses using Equation (II.27) was performed using Conlin [107]. Unless otherwise noted all traces presented in the figures in this manuscript have been normalized to the final

asymptotic value of the anisotropy change as determined from this analysis. Finally, unless otherwise noted all uncertainties represent 68% confidence intervals (±1 standard deviation) as determined by Monte Carlo analysis.



Scheme AII.1: R*i* denotes the population of nucleosomes bound with ISWI with histone octamers in the i^{th} translational position; $i = 0$ denotes the central position, defined by the NPS, and $i = \pm 1$ denotes positions 1 translational step away from the central position. k_1 and k_2 represent the microscopic rate constants for repositioning.



Scheme AII.2: NR_i denotes the population of nucleosomes with histone octamers in the i^{th} translational position, but without ISWI bound. The rate constant for ISWI dissociation from nucleosomes is denoted by k_d and the rate constant for ISWI binding nucleosomes is denoted by k_b. The rate constant for octamer movement between translational positions is denoted by k_r.

APPENDIX III:

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