MECHANISM AND REGULATION OF CHROMATIN REMODELER, RSC

by

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A thesis submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Oncological Sciences

The University of Utah

May 2012

The University of Utah Graduate School

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ABSTRACT

RSC is a multiprotein complex that remodels the structure of chromatin and in turn regulates processes like transcription. The mechanism by which RSC remodels a nucleosome is still unclear. Although bulk solution experiments have added a growing body of evidence to the mechanism by which RSC remodels a nucleosome, the mechanistic properties of the remodeler is still under debate. In this work, we have constructed a system to study the mechanistic properties of a single molecule of RSC on a naked DNA template in real time and obtain its properties like rate of translocation, processivity, force generated by the translocase and rate of ATP turnover.

The SWI/SNF family of chromatin remodeling complexes contains conserved Actin-related proteins (ARPs). Genetic and biochemical evidence suggests that Sth1 might be regulated by two Actin-Related Proteins, ARP7 and ARP9. Genome wide suppressors of ARPs mapped to two domains in Sth1 called the post-HSA (Helicase/ Sant associated) domain and ATPase domain. In this work, dominant negatives of Sth1 were obtained that also localized to the post-HSA (Helicase/ Sant associated) domain. This suggests that the post-HSA domain might be involved in the regulation of Sth1.

Sth1 (301-1097) construct containing the suppressor mutations co-purified with ARPs were compared for their ATPase activity and K_M with wild type Sth1 (301-1097) Arps complex. The mutants showed higher ATPase activity than wild type, which was not due to their increased affinity for DNA.

Based on the fact that the loop formed by the action of a remodeler is negatively supercoiled, in this work, we also developed an assay to assess the torsion generated by ATP dependent chromatin remodelers on naked DNA. TetR-Sth1 (301-1146) fusion co-purified with ARPs successfully produced torsion in an ATP dependent manner as assayed by this assay. This assay also depends on the action of by *E. coli* topoisomerase I, which specifically relaxes negative supercoils and on TetR-TetO interaction which serves to anchor the motor protein and thus constrains the loop formed by it.

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ACKNOWLEDGMENTS

I would like to thank Dr. Bradley Cairns for being a great advisor, whose enthusiasm for science and timely intellectual contributions propelled my research forward. I truly admire his selflessness in guiding me with my applications. A number of people have contributed immensely in my journey towards this thesis - Heather, who taught me the basic lab protocols; Anjanabha, who taught me the ABCs of the chromatin field; Jacqui, my guru on protein purification; Kaede, who has always been my go-to person for trouble-shooting; Rachel and Arun, who helped me with my experiments during their rotations. I owe a lot to all my lab mates for making Cairns' Lab a fun place to work – it has truly been a wonderful journey. I'd like to convey a special thanks to the DNA Core Facility for quick synthesis of oligo nucleotides and sequencing of DNA.

1. INTRODUCTION

DNA is packaged into chromatin inside the cells. Chromatin consists of an array of the repeating unit called nucleosomes. Each nucleosome is made up of DNA wrapped around a core of histone proteins. This wrapping of DNA onto histone proteins occludes many sequences that are recognized by transcription factors that regulate transcription of genes. Thus, chromatin serves not only to achieve compaction but also a mechanism to control gene expression. In order for genes to be regulated, transcription factors have to bind to their target sequences. This is facilitated by two kinds of complexes-chromatin modifiers and chromatin remodelers. Chromatin modifying complexes add or remove covalent marks on the histone proteins that are recognized by transcriptional activators or repressors. Chromatin remodeling complexes reposition, eject or move nucleosomes in order to provide access to the DNA. The two complexes might also work in concert to restructure the chromatin and regulate the gene expression.

Chromatin remodelers are multiprotein complexes that restructure the chromatin by repositioning the nucleosomes. The mechanism by which these complexes remodel the chromatin is not yet fully understood. There are five different subfamilies of chromatin remodeling complexes in eukaryotes- SWI/SNF, ISWI, NURD/Mi-2/CHD, INO80 and SWR1 (Cairns, 2007). This work is centered on the chromatin remodeler in *S. cerevisiae,* RSC (remodels the structure of chromatin) which belongs to the SWI/SNF family of remodelers. RSC is an abundant and essential multisubunit complex in yeast

(Cairns et al., 1994; Cairns et al., 1996) and is conserved from yeast to humans. In *S. cerevisiae,* there are two SWI/SNF families of remodelers- SWI/SNF and RSC. Humans also possess two such remodelers – hSWI/SNF-A (also termed BAF) and hSWI/SNF-B (also termed PBAF) (Wang et al., 1996; Xue et al., 2000) while Drosophila has only one such complex termed Brm complex (Dingwall et al., 1995).

The SWI/SNF family of chromatin remodelers contain a conserved ATPase subunit (Vignali et al., 2000). The ATPase subunits of RSC (Sth1), SWI/SNF (Snf2), Brm (Brahma) and hSWI/SNF (hBrg1 or hBrm) contain highly similar domains which include the SNF2-like ATPase domain and the bromodomain (which recognizes the acetylated histone tails) (Eisen et al.,1995; Lusser, 2003). The fact that the ATPase domain is a conserved feature in these remodelers suggests that coupling ATP hydrolysis to remodeling might be a central theme for remodelers.

Apart from the catalytic subunit, the other conserved subunits in SWI/SNF family remodelers include the Actin Related Proteins (ARPs). RSC and SWI/SNF share the same actin related proteins. In fact, ARPs are the only subunits that are shared by these remodelers. The role for ARPs in chromatin regulation is largely unknown. This work revolves around understanding the mechanism of chromatin remodeling by Sth1, the catalytic subunit of RSC and its regulation by ARPs.

1.1 Mechanism of Chromatin Remodeling

Nucleosome consists of 146 bp of DNA wound around a core of histone octamer ~1.7 times. The crystal structure of the nucleosome solved by Luger et al. (1997) shows that the DNA makes contact with the nucleosome every 10 bp and resulting in 14 histone-DNA contacts on a nucleosome (Luger et al., 1997). A remodeler, in order to

displace the nucleosome from one position to another, has to break these 14 histone-DNA contacts. Earlier, one of the models predicted that the remodeler utilizes the energy from ATP hydrolysis to change the conformation of the histone octamer and thus eject the nucleosome (Workman and Kingston, 1998). However, given that energy is required to break each histone-DNA contact, it would be easier for the remodeler to break them in a sequential manner. This has evolved models like twist defect diffusion, bulge diffusion and translocation. The twist defect diffusion model hypothesizes that a local alteration to twist is made by the remodeler that is propagated around the octamer incrementally (Flaus and Owen-Hughes, 2003). DNA, being of helical structure, will be displaced due to the propagating twist defect. The other model, bulge diffusion, hypothesizes that DNA is bent, not twisted. That is, a remodeler would create bulges at the entry/exit site that would move on the nucleosome to the other end through diffusion (Längst and Becker, 2001).

A more recent model based on the observation that remodelers are DNA translocases is DNA translocation model. According to this model, the remodeler translocates DNA from a fixed internal site on the nucleosome in an ATP dependent manner, thereby causing a net displacement of the nucleosome it is anchored to. A study from our lab showed that RSC (or its catalytic subunit, Sth1) binds the nucleosome at an internal site located two turns from the nucleosome dyad. RSC then translocates the DNA from the proximal side to the distal side while remaining in a fixed position (Saha et al., 2005). Using linkers of various lengths, it was determined that RSC binds at a fixed site that is 50bp inside the nucleosome. These results were also confirmed by the restriction enzyme accessibility assay where strategically placed gaps prevented translocation and

thus prevented the exposure of the restriction enzyme sites (Saha et al., 2005). Also, the results from the triplex displacement assay suggest that RSC tracks on one of the strands in a 3'-5' direction. As a single nick in the tracking strand prevents RSC from displacing the triple helix, it is predicted that it has a step size of 1 bp (Saha et al., 2005). Incorporating these observations and from other studies, a model for the mechanism of chromatin remodeling by RSC has been recently conceived (see next section).

1.2 Remodelers and Helicases

The SWI/SNF family of remodelers falls under SF2 superfamily of helicases. This superfamily also houses DEAD/H box helicases, RecG helicases and Type I and III resctriction enzymes (Flaus et al., 2006). This includes NS3, which is a RNA helicase and Rad54, which is involved in Rad51-mediated homologous recombination. These are classified into the helicase superfamily as they possess classic helicase motifs in their ATPase domain - I, Ia, II, III, IV, V and VI. SF2, as well as SF1 members like PcrA, possess two characteristic RecA like domains. RecA is a protein involved in homologous recombination with domains required for DNA and ATP binding**.** These RecA like domains are conserved in the SF1 and SF2 helicases suggesting that they may have a role in binding to the DNA and ATP. Indeed, mutational studies have shown that the residues present in the interface between these domains are essential for ATP binding (Velankar et al., 1999).

Although the SF2 superfamily members exhibit similarity in domain structure, they do not exhibit dsDNA unwinding activity that is exhibited by helicases (Côté et al. 1994). This is expected as helicases unwind the DNA for processes like replication and so they possess a domain that wedges through the DNA, while the other enzymes have

different functions to perform that do not require duplex destabilization. However, in the recent past, many of these enzymes have been shown to be a DNA translocases (Saha et al., 2002; Jaskelioff et al., 2003; Whitehouse et al., 2003). RSC, SWI/SNF, ISWI and Rad54 translocate on the DNA in an ATP dependent manner (Saha et al., 2002; Whitehouse et al., 2003; Zofall et al., 2006; Jaskelioff et al., 2003).

The DNA translocation activity of RSC had been found by a series of elegant experiments – length dependent ATPase activity and triplex displacement assay (Saha et al., 2002). In the assay for length dependent ATPase activity, it was found that the ATPase activity is proportional to the length that the translocase travelled and so minicircles of DNA (similar to an infinite length) elicited maximum ATPase activity. In triplex displacement assay, the displacement of a third strand of DNA, bound to the duplex through weak Hoogsteen base pairs by the action of a translocase, is assayed. This elegant experiment gave us hints about the directionality of the translocase and its step size. By manipulating this technique using a nick in the duplex DNA at the triplex junction, it was also shown that RSC tracks along one strand on the DNA in a 3'-5' direction in an ATP dependent manner with a step size of 1 bp (Saha et al., 2005).

Over the years, several insights have been obtained from the crystal structures of PcrA, a SF1 helicase and Rad54, a SWI/SNF family enzyme involved in DNA repair (Velankar et al., 1999; Thoma et al., 2005; Durr et al., 2005). In PcrA, Wigley and colleagues have uncoupled the DNA translocation from the duplex unwinding activity (Soultanas et al., 2000). The translocase domains of PcrA and Rad54 are similar to that of Sth1. Based on this homology to PcrA and Rad54, ATPase domain of Sth1 can be divided into two subdomains: DNA binding domain and tracking domain. The co-

ordinated movement of these domains causes the remodeler to inch forward using a mechanism that will be described below.

Based on observations from various labs on remodelers like SWI/SNF, RSC and ISWI and other enzymes like Rad54, NS3 and PcrA, in our current model, we hypothesize that the tracking domain translocates by one base while the DNA binding domain (DBD) is unbound. This is followed by a conformational change in the DBD making it step forward one helical turn and bind the DNA tightly. When it is returning to its original postion, the DBD pulls in 10 bp during which the tracking domain releases from the DNA. When the tracking domain resets, the pulled DNA is behind the tracking domain and in this process, the DNA is translocated by 11 bp. When applied to a nucleosome, RSC acts as a 1+10 ratchet pulling in DNA from the linker region into the dyad and then around the nucleosome (Cairns, 2007).

It is not known whether the DNA binding domain passively binds to the DNA and the tracking domain moves forward by 1bp or whether the DNA binding domain actively steps forward one helical turn and pulls the DNA and resets (Cairns, 2007). In fact, not much is known about these domains in remodelers as no structural information on them is available. Smith and Peterson (2005) have uncoupled the ATP hydrolysis from the remodeling activity of the Swi2/Snf2 ATPase domain (Smith and Peterson, 2005). But studies have not been carried out to uncouple the tracking activity from the DNA binding/ force generating activity (Fig. 1.1).

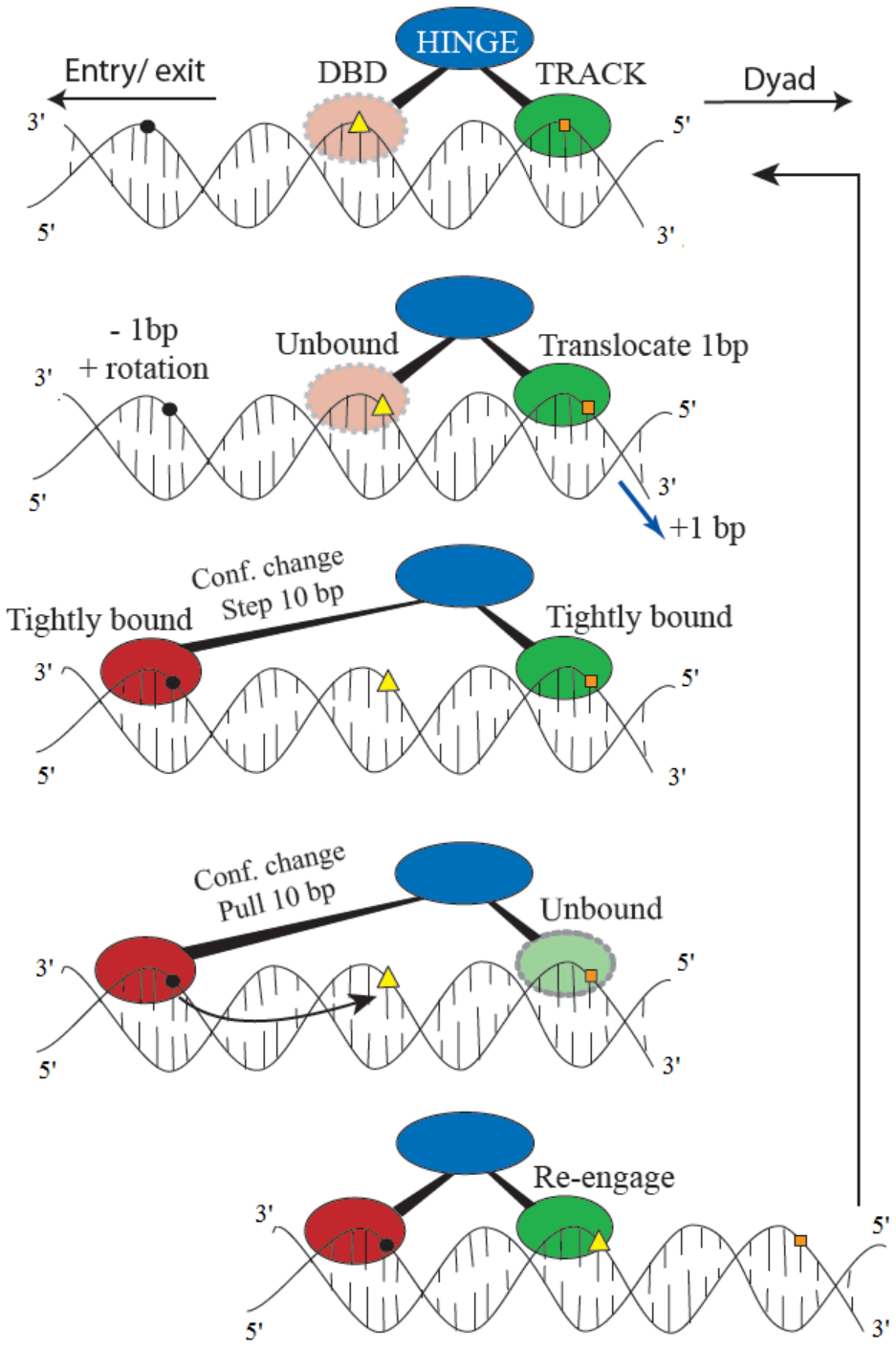


Fig. 1.1: Current Model for Nucleosome Remodeling by RSC (Adapted from Cairns, 2007)

1.3 Actin-Related Proteins in Chromatin Regulation

The presence of conserved actin related proteins (ARPs) in chromatin regulators has increased our interest in understanding the role played by them in these complexes. ARPs belong to the actin superfamily and show significant similarity to actin more in structure than sequence. They possess the conserved 'actin-fold', which is required for ATP hydrolysis and conformational changes in actin. *S. cerevisiae* possesses 10 ARPs, some of which are present in the cytoplasm and the rest in the nucleus. Cytoplasmic Arp1 is the only Arp known to polymerize into filament like structures (Schafer et al., 1994; Schafer and Schroer, 1999). Arp2 and Arp3 help in the actin assembly process in the cytoplasm (Dayel et al., 2001; Machesky et al., 1999; Mullins and Pollard, 1999). ARPs are numbered according to their similarity to actin, with Arp1 the most similar and Arp10 the least (Poch and Winsor, 1997; Schafer and Schroer, 1999). The ARPs present in the nucleus (Arps 4,5,6,7,8,9) exhibit limited similarity to actin. Interestingly, nuclear Arps are associated with complexes involved in chromatin regulation (reviewed in Olave et al., 2002). This includes some chromatin remodeling and chromatin modifying complexes, though not all. ARPs are present in the SWI/SNF, INO80 and SWR1 subfamilies of SNF2 family of remodelers and in the MYST family of histone acetyl transferases (HATs) (Cairns et al., 1998; Peterson et al., 1998; Shen et al., 2000 ; Krogan et al., 200; Kobor et al., 2004). The presence of ARPs as stable subunits in these complexes suggests that they might play a role in chromatin regulation.

Both SWI/SNF and RSC in yeast contain Arp7 and Arp 9 as their constitutive members (Peterson et al., 1998; Cairns et al., 1998). Arps are the only common subunits between these remodelers. Human SWI/SNF contains an ARP (Baf53) and β-actin itself

(Zhao et al., 1998). Similarly, the *Drosophila* Brm complex contains the ARP, Bap55 and β-actin itself ((Papoulas et al., 1998). Thus, SWI/SNF complexes contain either an ARP-ARP pair or an ARP-actin pair. INO80 contains Arp4, Arp5 and Arp8 along with β-actin (Shen et al., 2003). Likewise, SWR1 contains Arp4, Arp6 and β-actin as its components (Krogan et al., 2003; Kobor et al., 2004). The presence of ARPs is also conserved in the MYST family of histone acetyl transferases (Allard et al., 1999; Ikura et al., 2000). These are complexes that covalently add an acetyl group to particular lysine residue on the histone tails and sometimes on nonhistone proteins. The MYST family of HATs contains the MYST domain (MOZ, Ybf/Sas3, Sas2, Tip60) in their catalytic subunit (reviewed in (Carrozza et al., 2003)). This family contains the yeast NuA4 complex and its human ortholog hTIP60. The NuA4 complex contains Actin and Arp4 while the hTIP60 contains β-actin and Baf53 (Allard et al., 1999; Ikura et al., 2000).

Most of what was known about the the nature and role of ARPs was only from studies done on cytoplasmic ARPs like Arp2 and Arp3. Arp 2 and 3 participate in the actin assembly process by perceiving signals from WASp related proteins and promoting actin nucleation in an ATP dependent manner (Machesky et al., 1999; Dayel et al., 2001). Arp 2 and Arp3 heterodimerize and this function was shown to be important for actin filament formation (Dayel et al., 2001; Machesky et al., 1999; Mullins and Pollard, 1999). Like Arp2 and Arp3, Arp7 and Arp 9 have been shown to form stable heterodimers (Szerlong et al., 2003). Also, this heterodimerization is essential for their assembly into RSC (Szerlong et al., 2003). Similarly, Arp4 and actin require the presence of Arp8 to assemble into INO80 complex (Shen et al., 2003). However, in contrast to Arp2 and Arp3, which form an independent Arp2/3 complex, the Arps in chromatin

remodelers, associate with complex members for their assembly into the complex. In hSWI/SNF, Baf53 and β-actin require the presence of the ATPase subunit Brg1 for their assembly (Zhao et al., 1998). In yeast Swi/Snf, Arp7 and Arp9 are shown to associate with the catalytic subunit Swi2, where they form a minimal complex (Yang et al., 2007). Also, a large insertion in the ATPase subunit in Swr1 is required for the association of Arp6 (Wu et al., 2005). Recently, it has been shown in our lab that the Helicase and Sant Associated domain (HSA) in Sth1, the catalytic subunit of RSC, associates with Arp7 and Arp9 thus acting as a platform for the assembly of the Arps into RSC (Szerlong and Hinata, Unpublished data).

Also, in contrast to the ATP hydrolysis function exhibited by Arp2 and Arp3, studies with Arps 4, 7 and 9 show that they do not hydrolyse ATP for their function. Mutations predicted to destroy the ATP binding or hydrolysis activity had no effect, while mutations that affected their structure had serious effect on the function of Arps (Cairns et al., 1998; Stefanov et al., 2000; Zhao et al., 1998). This suggests that the structure of these Arps is essential for their function while ATP hydrolysis is not.

Arp7 and Arp9 are involved in transcription and chromatin regulation. This is shown by the null phenotypes of these Arps. Depending on the background of the yeast strain studied, Arps have been shown to be essential or important for growth. In S288C genetic background, *arp7*Δ and *arp9*Δ are essential for growth while in W303 background, *arp7*Δ and *arp9*Δ impair the growth of the cells conferring them a phenotypes common to *swi/snf* mutants (Cairns et al., 1998). In addition, they also exhibited Spt^- phenotype, which is common to phenotypes exhibited by genes involved in transcription and chromatin regulation (Cairns et al., 1998).

In order to study the function of Arps in RSC, spontaneous suppressors of *arp7*Δ and *arp9*Δ single mutants and *arp7*Δ *arp9*Δ double mutant were isolated in W303 background and were named *mra1* (modify the requirement for ARPs). The *mra1* mutations were able to restore wild type growth to some extent at 28°C on rich media plates (Szerlong et al., 2003). Later, using a linkage mapping technique involving a synthetic genetic array (SGA), the *mra1* mutations were found to be linked to Sth1. They were called *mra1-1*, *mra1-2* and *mra1-3* as they are all suppressors of ARPs and were linked to the same locus. Later, these mutations were characterized to be single amino acid substitutions in Sth1. These substitutions were also tested in isolation and were found to suppress *arp*Δ phenotypes showing that they are necessary and sufficient for suppressing the need for ARPs. By comparing the sequences of WT Sth1 and Sth1 with *mra1*mutations, *mra1-1* and *mra1-2* (different alleles of Sth1 that modify the requirement of ARPs) were found to be substitutions of two different amino acids of Sth1 – N384K and L680M (Szerlong and Hinata, Unpublished data).

In addition, the most remarkable fact is that all *mra1* mutations cluster to just two regions in Sth1 named the post-HSA domain (which follows the HSA domain in sequence) and a small region in the ATPase domain**.** The N384K mutation localized to the post-HSA domain while the L680M substitution localized to the ATPase domain of Sth1. It was also shown that the region in the ATPase domain that these suppressors localized to is a conserved feature in Snf2 family proteins (Szerlong and Hinata unpublished data). These data suggest that ARPs might be interacting with two domains in Sth1- the post HSA domain and the ATPase domain, and thus might be regulating it.

In order to study the effect of ARPs in the biochemical property of RSC, RSC lacking Arp7 and Arp9 was purified from yeast and its ATPase, nucleosome remodeling activities were studied. RSCΔ7/9 showed ATPase and translocation activity on dsDNA similar to wild type levels. It was also able to remodel a nucleosome as assayed by restriction enzyme accessibility (Szerlong et al., 2003). But, a caveat to these experiments was that RSC was purified from an *arp*Δ strain that also possessed the *mra1* mutation. Thus it was not known if the effects of the lack of Arps were nullified by the suppressor mutation. Recently, RSCΔ7/9 containing the wild type Sth1 showed reduced ATPase activity of about two fold (Szerlong, Hinata, unpublished data).

In summary, ARPs are conserved in many remodelers and modifiers. All SWI/SNF family remodelers, including RSC, contain ARPs (Cairns et al., 1998; Peterson et al., 1998). Also, there is strong genetic and biochemical evidence pointing to the hypothesis that ARPs might regulate Sth1(Szerlong et al., 2003, Szerlong and Hinata, unpublished data). Thus, in our aims, we set out to understand how ARPs might regulate the properties of Sth1.

1.4 Single Molecule Studies on RSC

Bulk solution experiments have enabled us to understand the fundamentals of the way chromatin remodelers reposition the nucleosome. However, in order to refine the basic mechanism by incorporating the properties of the remodeler like force generated by the remodeler, its processivity and speed, we have to study the action of a single molecule of the remodeler in real time. Single molecule studies have been performed on RSC on both nucleosomal templates and on naked DNA (Zhang et al., 2006; Lia et al., 2006). Each of these utilizes a different monitoring technique – action of RSC on

nucleosomal template monitored using optical tweezers and on naked DNA using magnetic tweezers.

Both the techniques monitored the decrease in the end-end distance due to the formation of a loop as a result of the remodeling activity of RSC on their respective templates. In the magnetic tweezers technique, as employed by Lia et al. (2006) a single stretch of DNA is anchored on one end to a glass slide and the other is stretched using a magnetic bead. The bead is kept in a magnetic field which not only allows the molecule to be stretched but also to rotate. Thus, through this technique it was possible to monitor the decrease in length of the DNA due to remodeler action. Also, as the rotation of the bead can be controlled, this technique also monitored the supercoiling of the loop which gave a measure of the twist associated with remodeler action (Lia et al., 2006). In the optical tweezers technique used by Zhang et al. (2006), a single piece of nucleosomal DNA was stretched between two beads, one of which was held by an optical trap while the other was attached to a pipet tip. The pipet tip can move with respect to the fixed optical trap. When RSC forms loops on the nucleosomal surface, there is a shortening of the length of the DNA which is measured by the system. However, this system does not measure the supercoiling associated with the formation of a loop (Zhang et al., 2006).

Both the above said systems gave an idea on the size of the loop formed by RSC, the force against which it can translocate, and its rate of translocation. However, the results obtained due to these systems were quite different from each other. While in the magnetic tweezer technique, RSC was found to form loops of average size ~420bp against a force of 0.3 pN translocating at the rate of 200 bp/s, in the optical tweezer technique, it was found to form loops of average size of 105 bp, in a moderate force

range of 3-7 pN, with a translocation rate of 13 bp/s. One possible reason for this difference is that they both are measured at different tension ranges with the remodeler translocating faster and better in a lower force range. The other reason could be that they are measured on different templates – the former on naked DNA and the latter on nucleosomal templates. However, the mechanistic properties of the remodeler are still under debate.

To control for these factors, we have initiated single molecule studies of RSC on naked DNA template. Thus, we will be able to obtain a true estimate of the mechanistic parameters of RSC.

1.5 Rationale and Aims of this Research

Our current model predicts that the DNA binding domain and the tracking together remodel a nucleosome. But we currently lack information on the nature of these domains. Based on homology to helicases like PcrA, we think that the force generation domain might be different from the tracking domain. Thus, we wanted (1) to test if we can uncouple the tracking and force generation activity of the translocase.

It has been shown earlier in the lab that RSC is an ATP dependent dsDNA translocase. Sth1 also exhibits DNA translocation activity when present by itself or as RSC complex. So, one interesting question is, how does the translocation property of Sth1 alone compare to that of RSC? Is the motor activity of Sth1 regulated? Two of the possible regulators of Sth1 are the Arp7 and Arp9 (Actin Related Proteins). ARPs are conserved in many remodelers. Arp7 and Arp9 bind to Sth1 and genome wide suppressors of Arp7/9 null map to Sth1. Thus, there is strong evidence pointing to the

hypothesis that Arp7 and Arp9 might regulate Sth1. Therefore, we wanted (2) to test if the ARPs regulate Sth1 by purification and testing of the *arp*Δ suppressor (*mra1* mutant).

Also, in order to study the mechanistic properties of a remodeler, it will be best to analyze the single molecule of remodeler in real time. Single molecule studies on RSC have already been done, though the use of different templates might explain the controversies in the field. Therefore, to obtain the true parameters of the remodeler properties like its rate of translocation, we wanted (3) to develop a method for single molecule studies of RSC on naked DNA template.

There are assays developed to measure the ATPase activity, DNA translocation activity, tracking activity, remodeling activity, etc. of the translocase. But, we wanted (4) to develop a method to assay for torsion generated by the translocase.

2. MATERIALS AND METHODS

2.1 Screen for Dominant Negatives

The screen involved random mutagenesis of Sth1 by Polymerase Chain Reaction (PCR) using Taq DNA polymerase, which has the highest error rate. The primers spanned region containing the HSA domain, post- HSA domain and the ATPase domain of Sth1. The randomly mutagenized PCR product is purified, cut with the restriction enzymes BglII and BsaBI and cloned into a clean vector bearing full length Sth1, which is also cut with BglII and BsaBI by homologous recombination. The PCR product and the linearized vector are transformed into the yeast strain 608 (leu2Δ0 lys2Δ0 ura3Δ0) and plated on to Sc-Ura plates. The colonies obtained are replica plated onto Sc-Ura and Sc-Ura-Met. Sc-Ura selects for the plasmid and Sc-Ura-Met selects for the dominant negatives. The colonies that are viable on Sc-Ura plates but are inviable or sick on Sc-Ura-Met plates are selected.

In order to confirm the dominant negative phenotypes exhibited by the selected plasmids, they are isolated from the yeast strain using Zymoprep kit. They are transformed into *E. coli* and amplified. They are then retransformed into the same yeast strain, 608 using a high efficiency yeast transformation protocol and plated onto Sc-Ura plates. The transformants are checked for their dominant negative phenotypes by plating onto Sc-Ura and Sc-Ura-Met plates and confirmed using the viability test.

In order to make sure that the dominant negative phenotype is not due to a mutation in the Met25 gene, the transformants containing the dominant negatives are grown on YPD (rich media) plates, which makes the cells lose the plasmids. Also, as a second round of selection, as the plasmids contain a ura marker, the cells are plated onto plates containing 5FOA (5-fluoroorotic acid) that select for cells that have lost the plasmid. Thus their viability is checked on Sc + 5FOA and Sc +5FOA–Met plates. Mutation in the *met25* gene will make the cells remain sick in the Sc +5FOA–Met plates. Thus, if the cells are alive on Sc +5FOA–Met plates, then the mutation is linked to the plasmid.

2.2 Cloning and Expression of WT Sth1, Sth1^{N384K} and Sth1^{L680M} (301-1097) Construct

pRS316 STH1 plasmids bearing N384K and L680M mutations were cut with BsaI and BsaBI and the fragment containing the mutations were subcloned into CDFDuet-1 vector (Novagen) bearing a STH1 (301-1097 a.a) construct with a 10X His tag at the N terminus (Wittmeyer, J.), which is the plasmid used for expressing wild type Sth1 (301-1097). The sequenced plasmids are transformed into codon^{+} BL21 cells along with RSFDuet vector containing ARP7 and ARP9 and selected on appropriate antibiotic plates.

The expression of wt and mutant Sth1 (301-1097) Arps complex is induced by autoinduction method (Studier, F.W).

2.3 Purification of the WT and Mutant Sth1 (301-1097) Arps Complex

The cells were resuspended in twice the volume of lysis buffer (50mM Phosphate buffer, pH 7.5, 300mM NaCl, 10% glycerol, 0.5mM β-ME and 1X protease inhibitors).

Lysozyme is added to a final concentration of 1mg/ml and incubated for 10-30 minutes. The cells were then sonicated at 50% duty cycle for 30-40 seconds and chilled on ice after every cycle. The lysate was centrifuged at 20,000g for 20 minutes.

The supernatant was bound to Ni-NTA beads (Qiagen) at an imidazole concentration of 20 mM and incubated for 2 hours at 4 ºC and washed with the wash buffer (20mM Tris pH 7.5, 100mM NaCl, 10% glycerol, 0.5mM β-ME, 30mM Imidazole and 1X protease inhibitors) in batch format. The beads were washed thoroughly to lower the salt content. The complex is eluted from Ni beads using equal volumes of elution buffer (20mM Tris pH 7.5, 100mM NaCl, 10% glycerol, 0.5mM β-ME, 250 mM Imidazole and 1X protease inhibitors).

The eluted complex was loaded onto heparin column equilibrated prior to loading. The filter-sterilized and degassed buffers used were Buffer A (20mM Tris pH 7.5, 10% glycerol, 0.5mM β-ME and 1X protease inhibitors) and Buffer B (20mM Tris pH 7.5, 1M NaCl, 10% glycerol, 0.5mM β-ME and 1X protease inhibitors). The complex eluted at salt concentration around 450mM NaCl.

The eluted complex was pooled, concentrated and run through a gel filteration column equilibrated with the sizing buffer (20mM Tris pH 7.5, 200mM NaCl, 10% glycerol, 0.5mM β-ME and 1X protease inhibitors). The fractions were collected, pooled and frozen in liquid nitrogen. The amount of the complex was determined using A_{280} and by coomasie staining of SDS-PAGE gels.

All purification steps were done in the presence of protease inhibitor cocktail containing 2 µg/ml chymostatin, 2 µM pepstatin A, 0.6 µM leupeptin, 2 mM benzamidine, and 0.5 mM phenylmethylsulphonyl fluoride.

2.4 DNA-dependent ATPase Assay

The ATPase activity of the complexes is measured by a colorimetric assay that is described by Cairns et al. (1994). pBSKS plasmid was used for all studies. Except for K_M measurements, the DNA was added to final concentration of 20ng/μL. The complex was added at a Sth1 concentration of 200 ng and reaction was done at 30 ºC for 30 minutes. The absorbance of the phosphomolybdate complex formed due to the DNA dependent ATPase activity of the complex was read at 650 nm.

For K_M measurements, pBSKS was added at different nucleotide concentrations ranging from 0 to 50 μM (0, 0.2, 0.4, 0.8, 1, 2, 3, 5, 10, 20, 30, 40, 50 μM) incubated with 200 ng of Sth1 and the assay was repeated as described above.

2.5 Cloning, Expression and Purification of the TetR-Sth1 Fusion Complex with ARPs

TetR is amplified by PCR from pWH610 (Ettner et al., 1996) using Pfu DNA polymerase. The PCR product is purified and inserted into MCS1 (multiple cloning site) of CDFDuet vetor used for expression in *E. coli* between NdeI and KpnI sites. The other copy of TetR was fused to 2 different constructs of Sth1 (both starting at a.a 301 but ending at a.a 1097 or 1146) with two different linkers between the fusion. The linker lengths were chosen, at random, to be 8 and 20 amino acids. They contained alternating Glycines and Alanines to allow maximal conformational flexibility. The fusion was done by PCR amplification of Sth1 (301-1097/1146) using *Pfx* DNA polymerase. The reverse primer also had a Flag tag. This PCR product was purified and inserted in MCS2 of CDFDuet-1 between BamHI and NotI. Then, TetR was PCR amplified from pWH610 using Pfx DNA polymerase with the reverse primer containing the linker. The purified

PCR product was cut with NcoI and BglII and inserted in the CDF Duet vector containing Sth1 (generated as described above) between NcoI and BamHI.

This vector was expressed in BL21 codon$^+$ cells along with Arp7 and Arp9 and the protein production was induced by autoinduction (described above). The complex was purified using Ni-NTA, FLAG and gel purification columns as described earlier. The proteins were resolved in SDS-PAGE and stained by Coomassie brilliant blue. The concentration of the complex was calculated using A_{280}.

2.6 Template DNA for Single Molecule Studies

Oligos containing TetO site were designed with sites for nicking by the nicking endonuclease N.BbVCIA (New England Biolabs) 15 bases from either side of TetO. The nicking sites were either designed on the same strand on either side of TetO or on opposite strands. These oligos containing the nicking sites and TetO between them were gel purified, annealed, cut and cloned into pRS413Met25 (Mumberg et al., NAR, 1994) between NdeI and MScI.

The intact template for single molecule studies was generated by linearizing pRS413Met25 containing the TetO site. The nicked templates were obtained by nicking the above said plasmid with the nicking endonuclease, N.BbVCIA for 20 minutes at 37ºC and linearized using BseYI. The products were checked on a 1.2% agarose gel and purified by chloroform extraction.

2.7 Assay for Torsion

1μg of pUC19 containing TetO (Saha.A.) was used as the template for this assay. This was relaxed using *E. coli* Topoisomerase I, which relaxes negatively supercoiled

DNA. The reaction mixture is set up with 1μg of the above said relaxed template DNA, 500 ng of the fusion complex, 8 units of *E. coli* Topoisomerase I (NEB) in 1X NEB buffer 4 (20mM Tris-acetate, 10mM Magnesium acetate, 100mM NaCl, 1mM dithiothreitol, pH 7.9), BSA (final concentration of 1 μg/ml). ATP was added at a final concentration of 1 mM. The reaction mixture was incubated at 37 ºC for 1 hour and then heat inactivated at 65 ºC for 20 minutes. The DNA is extracted by phenol: chloroform and run on a 1.2% agarose gel (without ethidium bromide) for 18 hours at 20 V. It is post-stained with Ethidium bromide and visualized under UV light. In reactions containing Tetracycline, Tetracycline hydrochloride (Sigma) was added at a final concentration of 1mM to the reaction mixture.

3. RESULTS

3.1 Screen for Dominant Negatives of Sth1 Yielded Mutations Localized to the Post-HSA Domain

Sth1 translocates on the DNA and exhibits dsDNA dependent ATPase activity in isolation. In order to understand the mechanism by which Sth1 remodels a nucleosome, we wanted to obtain mutants that uncouple its various activities like tracking, force generation, torsion and ATPase activity. Studies on these mutants, then, will inform us about the role played by various domains of Sth1 in the chromatin remodeling process.

To dissect out the motor functions, we performed a screen for dominant negatives of Sth1. We targeted the entire Sth1 for want of mutants that would help uncouple the above said motor properties. These mutants might include those that are defective only for their ATPase activity, or only for their tracking activity or those that possess ATPase and tracking activity but are defective in force generation activity.

We adopted the method of random mutagenesis using PCR and cloned the product under Met25 promoter and screened for dominant negatives of Sth1 by replica plating. As Sth1 is an essential gene in *S. cerevisiae,* a viability test after the induction of Met25 promoter was used to screen dominant negatives (Fig. 3.1A). Also, in order to test whether the mutation that caused the dominant negative phenotype was linked to the plasmid, we grew them in rich media and selected for those that have lost their plasmid and then tested them for viability on plates lacking methionine. If the mutation was in the

Met25 gene, then the cell will die on plates lacking methionine even after losing the plasmid (Fig. 3.1B). All candidates grew well in plates lacking methionine showing that the mutation was shown to be linked to the plasmid and not to the Met25 region. Dominant negatives of Sth1 were obtained and were confirmed by isolating the plasmid, retransforming and checking their viability on plates lacking methionine (Fig. 3.1C).

Surprisingly, sequencing the whole of the *sth1* showed that all dominant negatives carried mutations in the post-HSA domain (Table 3.1). One of the dominant negative mutations (STH1-1) resulted in a stop codon that terminates the Sth1 protein at 402 a.a (Table 3.1). This region also encompasses the post-HSA domain. Some of these dominant negatives carried additional mutations in the ATPase region but in these cases, the mutations in the post-HSA domain were conserved suggesting that the dominant negative phenotype is associated with the mutations in the post-HSA domain. In addition, the allele specificity of the dominant negative phenotype was ascertained by further studies in the lab (Hinata, K.) The localization of the dominant negatives to the post-HSA domain was consistent with the fact that a dominant negative phenotype, previously observed in the lab, was due to deletion of a stretch of 8 amino acids in the post-HSA domain of Sth1 (Szerlong, H). This deletion, referred to as ΔDL, is in fact used as a positive control for this experiment.

It was remarkable how a dominant negative screen of Sth1 yielded mutations only in the post-HSA domain and not a single one in the ATPase domain. In order to ascertain that the screen was complete, we determined the phenotype of a mutant that was reported to be defective for ATPase activity by Du et al. (1998) in the screen that we used. The K501R mutant was cloned similarly under Met25 promoter and was tested if it behaves

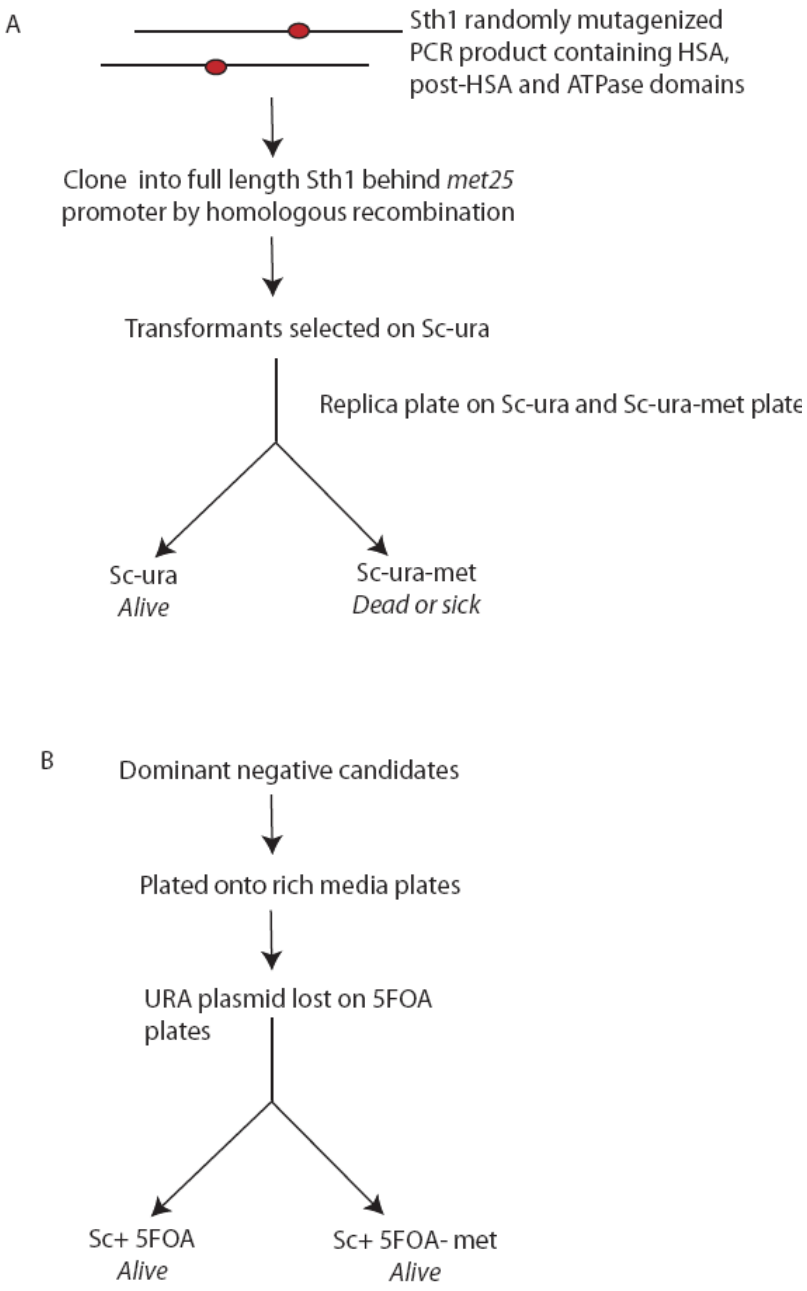


Fig. 3.1. Screen for Dominant Negatives of Sth1. A. Scheme of the screen for dominant negatives of Sth1 by PCR mutagenesis and replica plating. B. Scheme for the confirmation that the mutation is linked to the plasmid and not to the *met25* gene. C. Dominant negatives of Sth1.

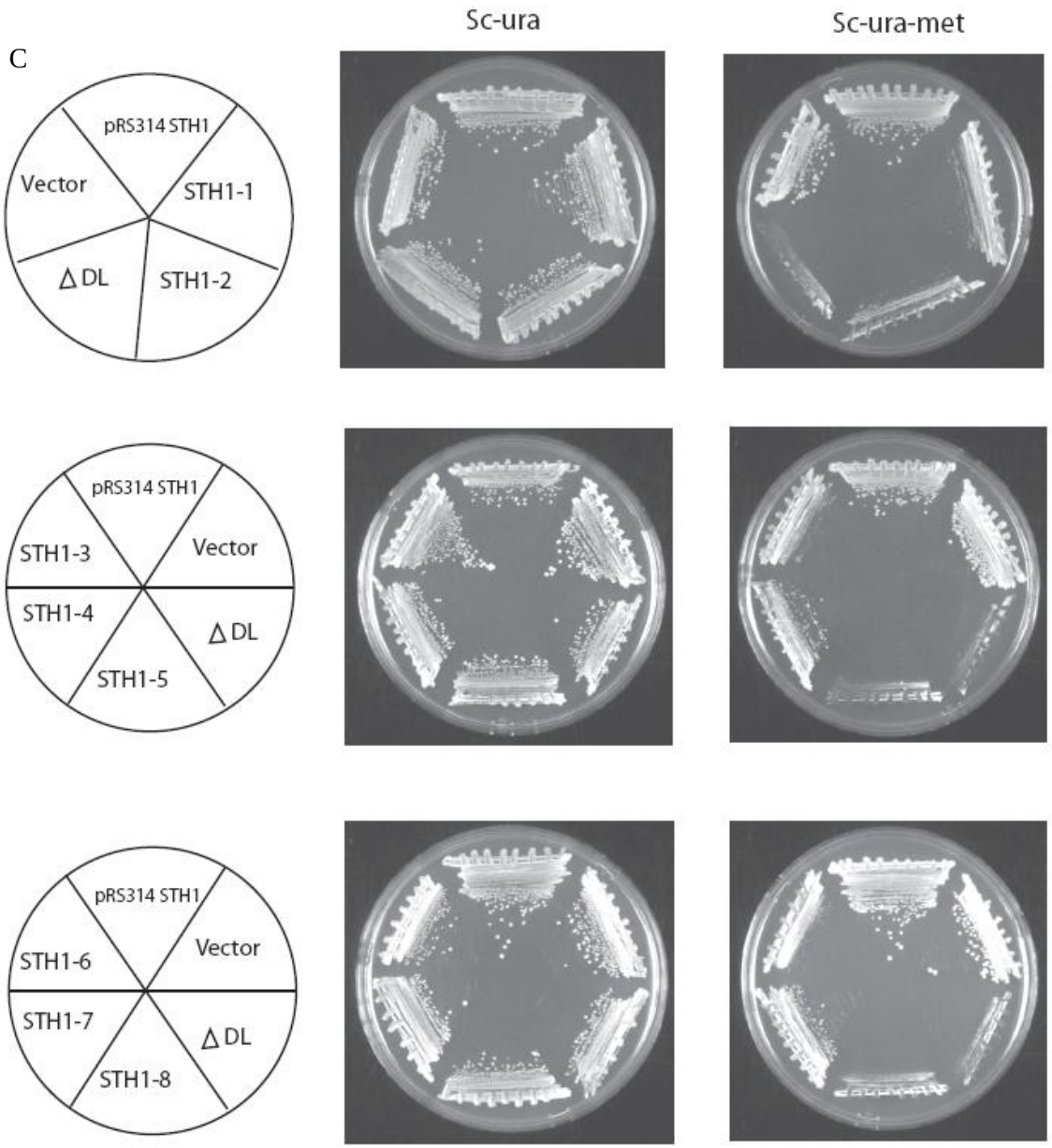


Fig.3.1 continued. pRS314.STH1 contains a single copy of *sth1* behind *met25* promoter in the plasmid pRS314. Vector denotes pRS314 plasmid containing *met25* promoter. ΔDL represents a deletion in the post-HSA domain of Sth1 which was shown to exhibit a dominant negative phenotype (used as positive control). The dominant negative phenotypes obtained in this screen are denoted as STH1-(1-8).

Table 3.1: Identification of the Dominant Negative Mutations. The mutations causing the dominant negative phenotype were identified as amino acid changes in the post-HSA domain and the ATPase domain of Sth1 based on sequence comparison with wild type.

	Mutation in the HSA domain	Mutation in the post-HSA domain	Mutation in the ATPase domain
STH1-1	___	Resulted in stop codon terminating protein at 402 a.a	___
STH1-2	___	S383P	T628A
STH1-4	___	S416P	___
STH1-5	___	L392P	___
STH1-6	___	S383P	___
STH1-8	___	L392P	M835I

as a dominant negative. Sth1K501R grew on plates lacking methionine and thus did not phenocopy the dominant negatives.

3.2 Purification of Sth1 (301-1097) bearing *mra1* Muations with Arps and Comparison of the ATPase Activities of WT and Mutant Sth1 (301-1097) Arps Complex

We wanted to characterize a role for ARPs in RSC and to test if ARPs regulate the motor function of Sth1. Earlier, RSC lacking Arp7 and Arp9 was purified from yeast. On biochemical analysis, it showed similar levels of ATPase activity and remodeling activity to that of wild type RSC. But, as purifying RSC lacking Arp7 and Arp9 from yeast demanded the presence of *mra1* mutations in Sth1 in order for the strain to grow, the RSCΔ7/9 possessed the *mra1* mutation in Sth1. Thus, it was not possible to determine if the ARPs are not required for the ATPase activity of RSC or whether the *mra1* mutations suppressed the effect of ARPs on the activities of RSC.

In order to assess the true effect of ARPs on the motor function of RSC, we decided to use a simpler system. As Sth1, the catalytic subunit of RSC translocates on DNA in an ATP dependent manner and capable of remodeling by itself, we decided to purify Sth1 harboring those mutations from *E. coli* and compare its activities with wild type Sth1. We decided to purify Sth1 bearing *mra1-1,* identified as N384K mutation in the post-HSA domain of Sth1 and *mra1-2,* identified as L680M mutation in the ATPase domain. This is because among the suppressors, these mutations showed the best rescue of the growth defect conferred by ARPs at 28°C.

It has been observed in our lab that the Sth1 construct (301-1097 a.a) comprising of the HSA, post- HSA and ATPase domain behaved well during purification and had ATPase activity (Wittmeyer, J., unpublished observation). So we decided to use this construct for purifying wild type Sth1 and Sth1 containing the *mra1* mutations. In this whole experiment, Sth1 refers to this construct spanning from amino acids 301 to 1097. Also, co-expression of Arp7 and Arp9 with Sth1 in *E. coli* helps in keeping Sth1 soluble (Wittmeyer, J., unpublished observation). Thus Sth1 (wild type and *mra1*) was cloned into *E. coli* expression vector CDF duet and co-expressed with Arp7 and Arp9, which were present on a RSF duet vector. Autoinduction was used to induce the protein production resulting in over-expression of the proteins, Sth1 and ARPs. Wild type and mutant Sth1 bearing 7X His tag was co-purified with Arp7 and Arp9 using Ni-NTA resin, Heparin and size exclusion chromatography to yield a Sth1-Arp7-Arp9 complex (Fig. 3.2A). Attempts to isolate Sth1 (wild type and mutant) alone without the ARPs failed as Sth1 shows a tendency to aggregate when present in isolation.

WT Sth1, Sth1^{N384K} and Sth1^{L680M}, each co-purified with Arp7 and Arp9 were tested for their ATPase activities using a color development assay using malachite green. The Sth1^{N384K}-Arps complex showed ~1.9 fold increase in ATPase activity while the Sth1^{L680M}-Arps complex showed ~2.7 fold higher ATPase activity when compared with WT. This assay was repeated several times and a consistent difference in the ATPase activities of the wild type and mutants was observed (Fig. 3.2B).

3.3 K_M of Wild Type and Mutant Sth1 (301-1097) Arps Complex

One reason for the higher ATPase activity displayed by the mutant Sth1- Arps is that it might possess higher affinity for DNA than wild type. In order to test this

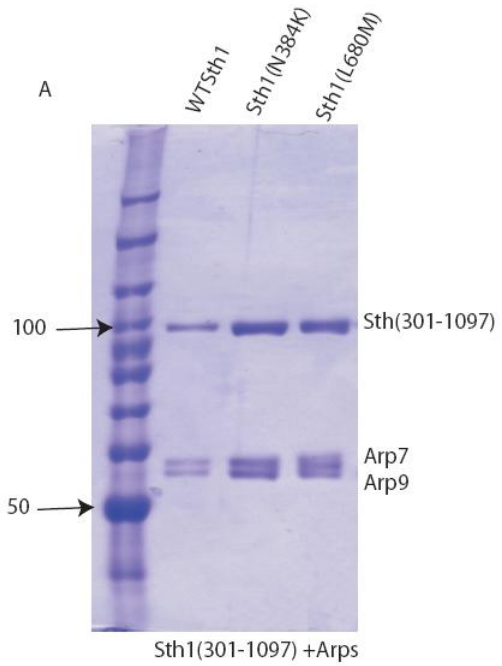
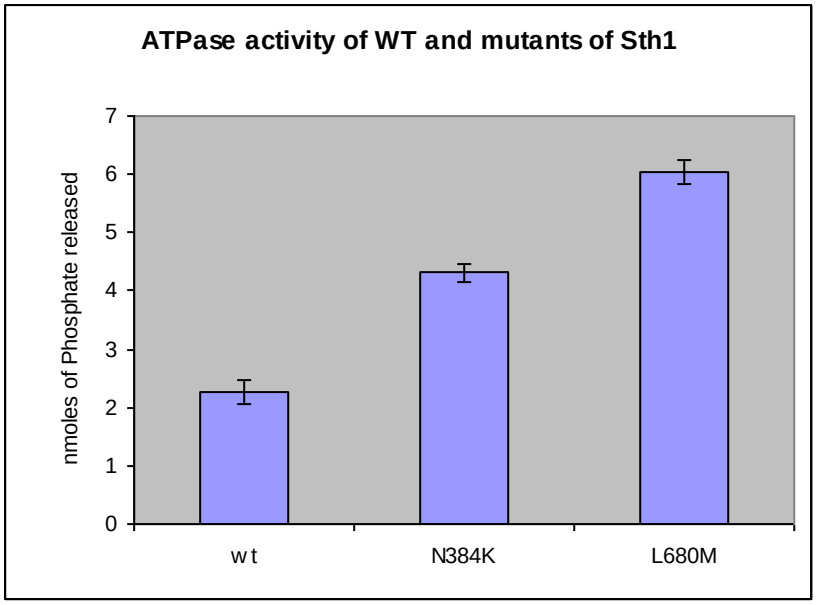


Fig. 3.2 Purification of WT and Mutant Sth1 (301-1097) Arps Complex and Assay of their ATPase Activity and K_M Studies. A. SDS-PAGE gel of the purified complex of WT Sth1 (301-1097), $Sth1^{N384K}$(301-1097) and $Sth1^{L680M}$ (301-1097) with Arp7 and Arp9. B. ATPase activity of the WT and mutant Sth1 (301-1097) Arps complex assayed by malachite green assay for nanomoles of phosphate released by 200 ng of Sth1 in 30 minutes. C. The Michalis-Menton constant for WT and mutant Sth1 (301-1097) Arps complex assayed by using varying concentrations of pBSKS (ranging from 0-50μM) in 30 minutes. V_{max} for each concentration was repeated thrice and average K_M from 3 different data sets for each sample is reported here.

B



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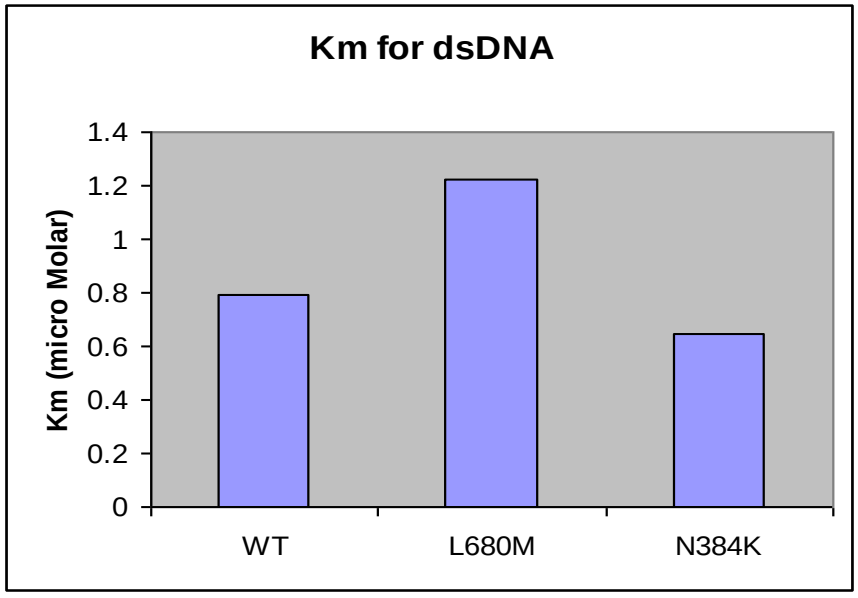

Fig. 3.2 continued.

possibility, the K_M of both mutant and wild type Sth1-Arps complex was determined. The K_M of wild type Sth1-Arps complex was found to be 0.79 µM while that of Sth1L680M-Arps complex was 1.22 µM and that of Sth1N384K-Arps complex was 0.65 µM (Fig. 3.2C). The lower the K_M, it is interpreted that the affinity of the protein for that substrate is higher. Since there was only a modest difference in the K_M between the mutants and the wild type, the increased ATPase activity displayed by the mutant is not due to an increased affinity of the mutant for DNA.

3.4 Single Molecule Studies on Naked DNA Template

To understand the intrinsic properties of the remodeler RSC like how much force is generated by the translocase, its rate of translocation, processivity, its ATP turnover rate and also probe into whether other members of RSC regulate the translocation properties of RSC, we decided to resort to single molecule studies of RSC. Although a single molecule of RSC has been studied on nucleosomal template using the optical tweezers technique and on naked DNA template using magnetic tweezers, its mechanistic properties are still under debate. In order to understand the above said properties, we decided to construct a system that would allow us to analyze the action of a single molecule of RSC on a naked DNA template using the optical tweezers technique.

In this technique, the translocation by RSC is monitored by the decrease in the end-end distance due to the formation of a loop. But, in order to form loops and cause shortening of the length, RSC has to anchor itself onto a fixed site and them pump the DNA. In the study by Zhang et al. (2006), the nucleosome provided a site for RSC to anchor itself, which will be absent in the case of a naked DNA template. Thus, in order

for us to observe the loop formation mechanism, there should be another site on the DNA to which the motor can anchor and then translocate.

To achieve this, the strategy was to fuse Sth1 to the Tetracycline repressor protein, TetR. DNA template to be used for single molecule studies will possess a Tetracycline operator site, TetO. TetO-TetR interaction is one of the strongest interactions known. Thus, in this system, TetR will bind to TetO serving to anchor Sth1 (present by itself or in complex with other proteins or as RSC). When Sth1 translocates on the DNA, the loop formed will be constrained between the TetR and the translocase resulting in the shortening of the length of the DNA between the beads.

A longer construct of Sth1 (301-1146) behaved similar to Sth1 (301-1097) construct, which was used in previous experiments. Thus we decided to construct TetR-Sth1 fusions of both the constructs. Also, we did not have any information on the length of the amino acid linker that should be put between TetR and Sth1. It should be long enough to allow conformational flexibility but should not be too long as it will be proteolysed. Thus, we constructed the fusion with two varying lengths of linker – 8 amino acid long and 20 amino acid long. We obtained the clones of four different versions of TetR with Sth1 – two containing Sth1 (301-1097) construct, and two containing Sth1 (301-1146) construct each set containing two linker lengths. We successfully purified the TetR with Sth1 (301-1146) with an eight amino acid linker separating them.

Also, it is known that TetR binds to TetO when present as a dimer. Thus, the clones described above all have one copy of TetR with 7X His tag and another copy fused with Sth1 constructs bearing a Flag tag. They were expressed in *E.* coli along with

the ARPs, Arp7 and Arp9 in order to obtain soluble proteins. The heterodimer consisting of TetR and TetR-Sth1 was co-purified with Arp7 and Arp9 using Ni-NTA resin, Flag resin and the oligomeric status of the fusion complex was checked using size exclusion chromatography (Fig. 3.3A). This purified complex was assayed for its ATPase activity and sent for single molecule analysis along with the DNA template described below. As expected, purification of TetR-Sth1 without ARPs resulted in formation of aggregates.

The DNA template containing a TetO site was created by cloning in an oligo containing well characterized TetO into a plasmid and linearizing the plasmid (Fig. 3.3B, Lanes 3 and 8). The ends would be attached to the beads and this template will be used for single molecule analysis.

If the loop formed by the translocase develops so much of torsional stress, then this would impair the single molecule studies as the remodeler would fall off to relieve the stress. In order to avoid this, we also constructed, along with intact DNA, templates that bear nicks in them on either side of the TetO site. The nicks are placed either on the same strand or on opposite strands (Fig. 3.3B, Lanes 2 and 7). These nicked templates will serve to relieve any torsional stress that accumulates during translocation by allowing free rotation of one strand with respect to another. Lanes 4, 5, 9 and 10 of Fig. 3.3B bear appropriate controls for the analysis of the status of nicked and linearized templates using agarose gel electrophoresis.

3.5 Development of an Assay for Torsion

Previously developed assays monitor the translocation properties of RSC or Sth1. The triplex displacement assay and the DNA length dependent ATPase assay measure the tracking activity of RSC or Sth1. The restriction enzyme accessibility assay measures the

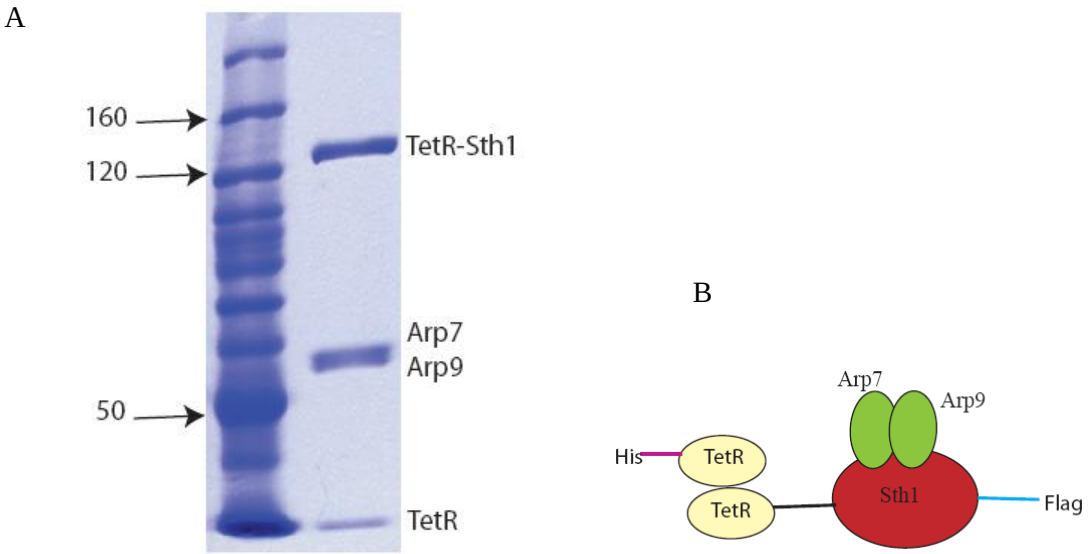

Fig. 3.3 Reagents and Scheme for Single Molecule Studies. A. SDS-PAGE gel of purified TetR-Sth1(301-1146) fusion in complex with Arp7 and Arp9. B. Cartoon showing TetR-Sth1(301-1146) heterodimerizing with TetR co-purified with ARPs. C. Templates for the single molecule analysis. TetO site is cloned into pRS413.Met25 plasmid with or without sites for nicking one strand. BseY1 is used for linearizing the plasmid and Nb. BbVCIA is used to specifically nick the plasmid on one strand. Lanes 2-5 deal with plasmid that has nick sites on both strands on either side of TetO. Lanes 7-10 deal with plasmid that has nick sites on the same strand on either side of TetO. D. Cartoon depicting the single molecule studies that will be carried with the TetR-Sth1(301-1146) fusion with Arps complex using the optical tweezer technique. The fusion complex is expected to translocate in an ATP dependent manner leading to the generation of loops that cause the shortening of the end-end length of the template DNA.

C

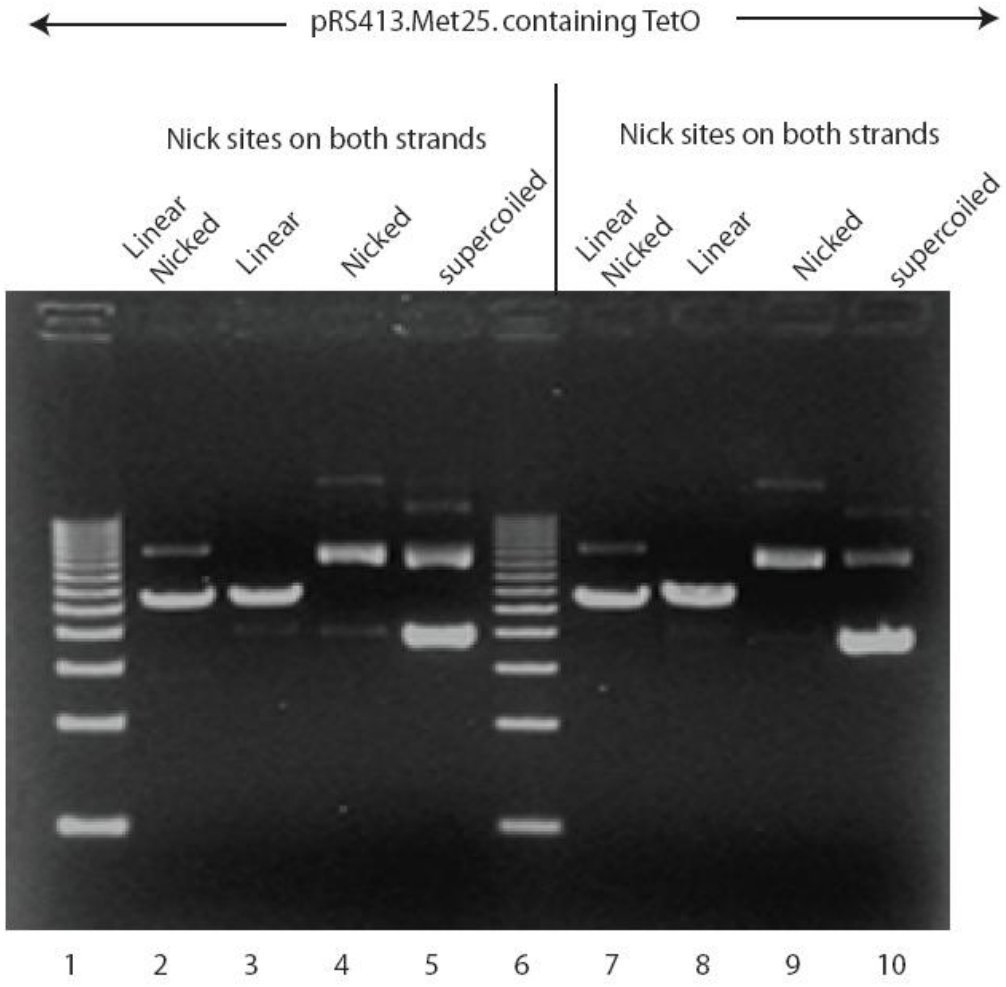

Fig. 3.3 continued

D

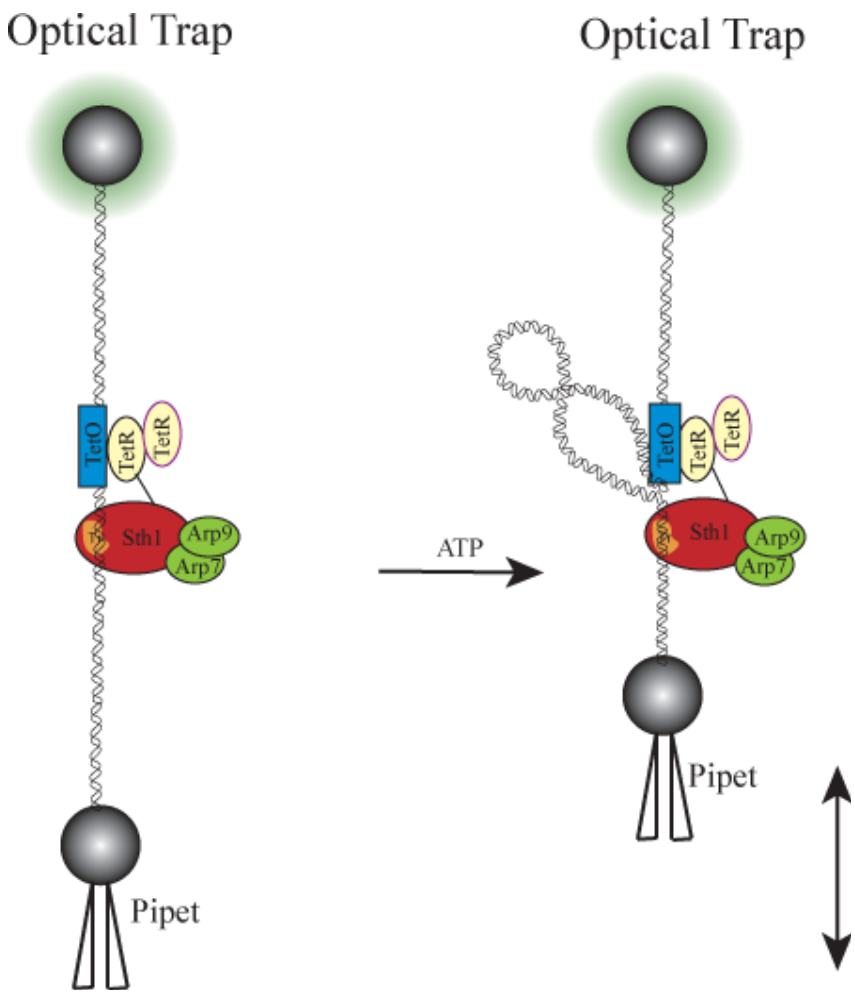

Fig. 3.3 continued

remodeling activity of RSC or Sth1. But these assays do not measure the torsion generated by RSC or Sth1. We have successfully developed an assay for torsion. The underlying principle behind this assay is that the constrained loop formed by the motor activity of RSC on nucleosomal templates is negatively supercoiled. Thus, if the above mentioned complex of TetR-Sth1 fusions with Arps was used on a plasmid DNA containing the TetO site, the loop formed between TetR and Sth1 due to the translocation of Sth1 will be negatively supercoiled. Thus, if we start with a relaxed plasmid, due to the conservation of linking number, the rest of the plasmid will be positively supercoiled. When this reaction is done in presence of *E. coli* topoisomerase I, which specifically relaxes negative supercoils, and the DNA extracted, the positive supercoils distribute throughout that plasmid molecule and thus the end product will be positively supercoiled.

The TetR-Sth1 (301-1146) fusion protein in complex with Arp7 and Arp9 was tested for its ability to generate torsion using the above said assay. TetR-Sth1 fusion with Arps when incubated with a relaxed plasmid containing TetO successfully generated torsion and converted the relaxed into positively supercoiled plasmid in an ATP dependent manner (Fig. 3.4, Lanes 5 and 6; Lane 9). Also, the interaction between TetR and TetO is required to constrain the negative supercoils to the loop. When tetracycline is added, TetR falls off TetO and no loop is formed. Thus in the presence of tetracycline, the fusion complex does not convert the relaxed plasmid into a supercoiled plasmid (Fig. 3.4, Lanes 7 and 8). Consistent with this, we also found that TetR-Sth1 fusion with Arps complex does not generate any torsion on a plasmid lacking a TetO site. This assay was *E. coli* topoisomerase I dependent as the activity of this enzyme is required for changing the linking number of the plasmid (Fig. 3.4, Lane 11). Also, no loop is generated in the

A

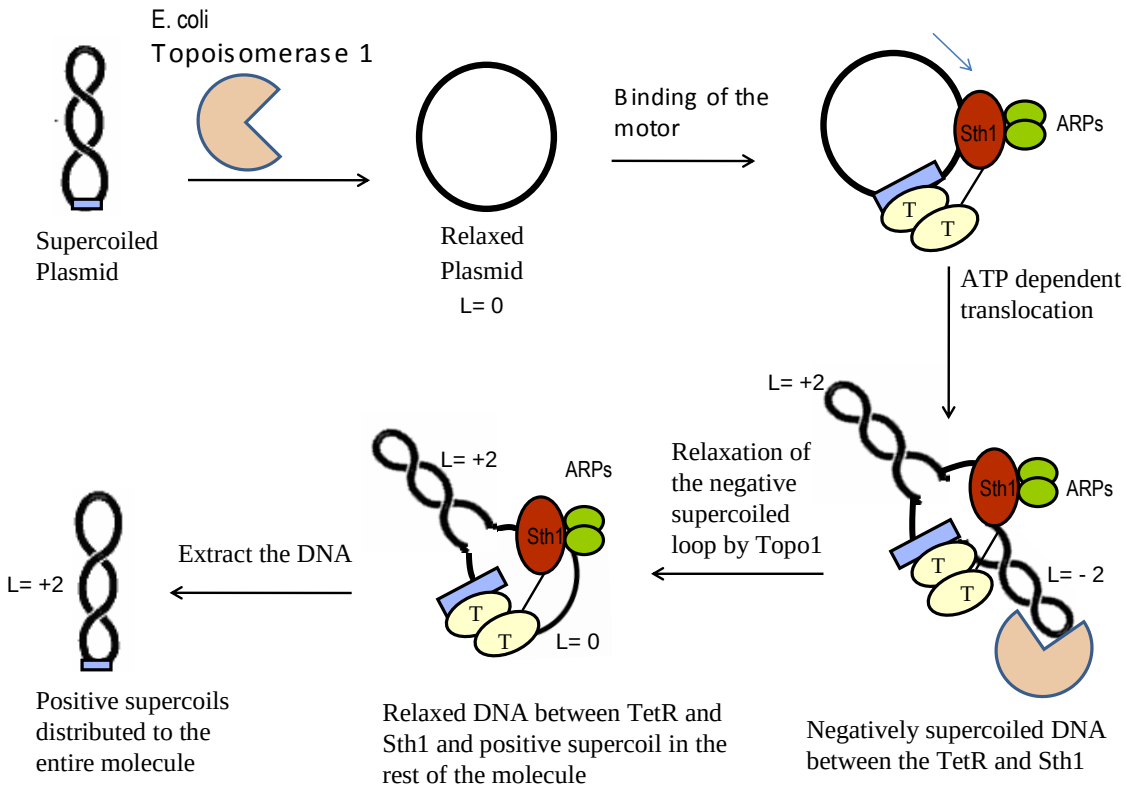

Fig.3.4 Assay for Torsion and its Schematic Representation. A. Cartoon depicting the scheme of the torsion assay. B. Agarose gel electrophoresis showing the conversion of relaxed pUC19 plasmid containing a TetO site into a supercoiled one by TetR-Sth1(301-1146) fusion- Arps complex in an ATP-dependent, TetR-TetO interaction dependent, *E. coli* Topoisomerase I dependent manner.

B

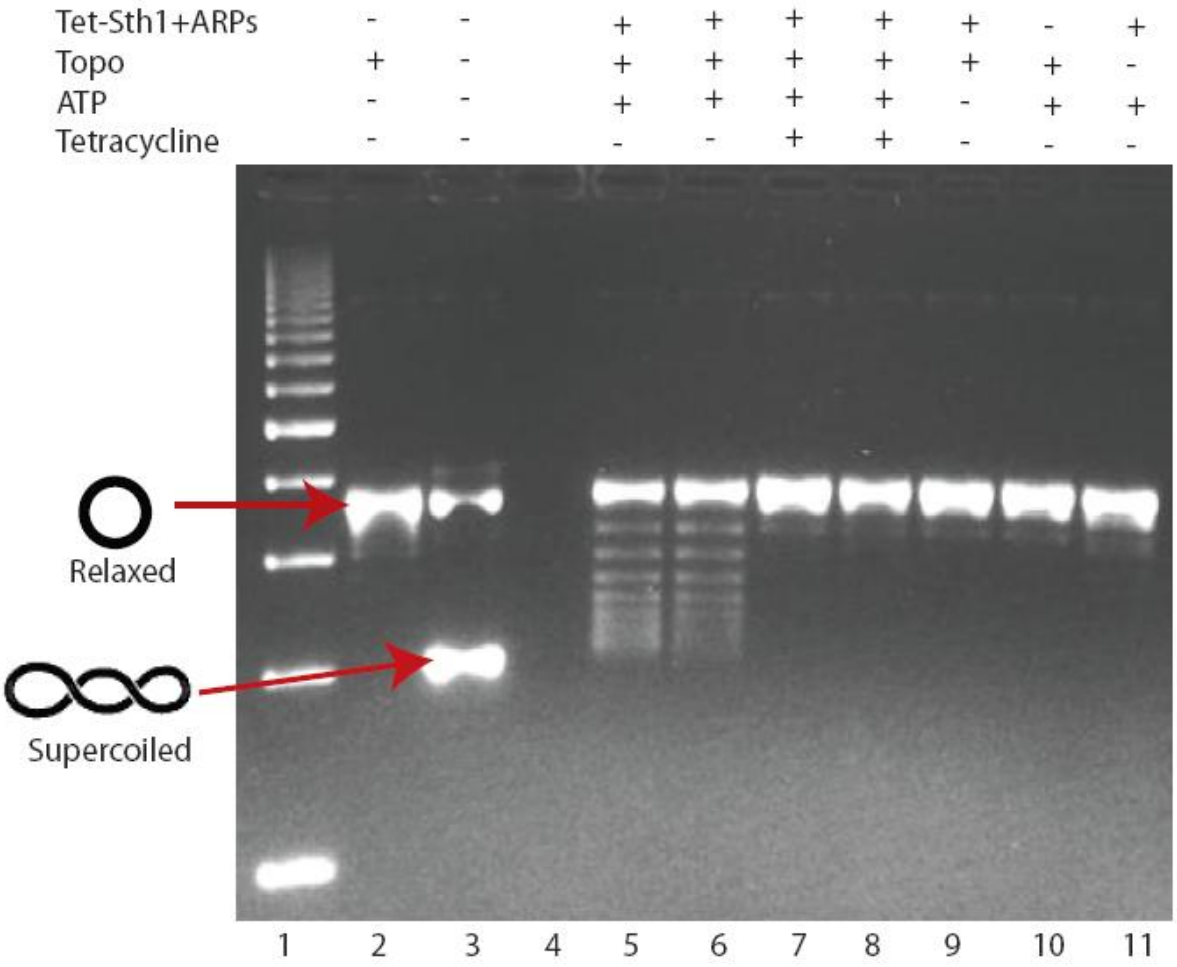

Fig. 3.4 continued

absence of the fusion complex showing that this assay is specifically measuring the torsion activity of the motor (Fig. 3.4, Lane 10).

4. DISCUSSION

In our screen for dominant negatives, we expected that we would obtain mutants that would help us uncouple the various motor properties like tracking, force generation and ATPase activity. That is, if a mutant is able to track in an ATP dependent manner but not generate force, or if a mutant hydrolyses ATP but does not track, then the domain and the amino acid residues responsible for generating force or tracking can be determined. In the absence of a crystal structure of RSC, it would help us understand the mechanism of nucleosome remodeling by RSC. However our screen for dominant negatives yielded mutations that were only localized to the post-HSA domain. While this does not tell us anything about the domains responsible for the motor function of Sth1, it still suggests that the post-HSA domain is important for the functioning of Sth1. Previous evidence in our lab suggests that the 5 of the 10 suppressors of *arp*Δ localized to the post-HSA domain. It is surprising that both *arp*Δ suppressors and dominant negatives of Sth1 localize to the same domain. More intriguing is the fact that some of the suppressors and dominant negatives are mutations of the same amino acid. For instance, when Leucine 392 is mutated to Valine, it acts as a suppressor of *arp*Δ while when it is mutated to Proline, it acts as a dominant negative. As Proline is an amino acid that is known to disturb the structure, it can be inferred that the structure of the post-HSA domain might be important for regulating the activity of Sth1. This is also consistent with the fact that deleting a stretch of amino acids from D385 to L392 in the post-HSA domain, referred to

as ΔDL, confers a dominant negative phenotype to Sth1 (Szerlong, H., unpublished observation). This suggests that the structure of the post-HSA domain is essential to the functioning of Sth1.

However, it is, indeed, quite surprising that there was no dominant negative mutation obtained in the ATPase domain. One possible reason is that the post-HSA domain is very important in regulating the function of Sth1. Another alternative is that the phenotypes of the dominant negatives of the post-HSA domain are stronger than that of the ATPase domain mutants that they are over-represented in the screen. A way to overcome this limitation is to target the ATPase domain alone for random mutagenesis. This will help us obtain mutants that are useful for understanding the motor properties of Sth1. Another possibility is that Met25 is a leaky promoter and so the dominant negative mutants in the ATPase domain die even before replica plating. This can be overcome if Gal promoters are used instead of Met as they can be tightly regulated.

In order to check if ATPase mutants behave as dominant negatives but did not show up as our screen was incomplete, we checked the phenotype of $Sth1^{K501R}$, which has been shown to be defective for ATPase activity (Du et al., 1998). We found that it did not phenocopy the dominant negatives. But before we assume that the mutation is not a dominant negative mutation, its ability to assemble into the RSC complex needs to be tested. This is because, in the dominant negative screen used, there exists a wild type copy of Sth1 in the strain. Thus when the mutant Sth1 does not assemble into RSC complex efficiently, the wild type Sth1 might compete against the mutant and rescue the cell from death.

In order to test if the mutations that suppressed the requirement for ARPs *in vivo* also suppressed the need for ARPs *in vitro*, we intended to purify WT Sth1 and compare its properties with $Sth1^{N384K}$ and $Sth1^{L680M}$ lacking ARPs. However, expression or purification of Sth1 in isolation resulted in its aggregation. Therefore, we had to co-purify the wt and mutant Sth1 with Arp7 and Arp9. Nevertheless, comparison of the ATPase activity of the wild type and mutant complex was also informative. The ATPase activity of the $Sth1^{N384K}$-Arps complex was 1.9 fold and $Sth1^{L680M}$-Arps complex showed ~2.7 fold higher than that of wild type. This is interesting because RSC lacking Arps containing wild type Sth1 was purified recently and its ATPase activity was tested (Szerlong and Hinata, Unpublished data). On comparison with wild type RSC (containing ARPs), RSC lacking ARPs showed a reduced ATPase activity by twofold. The presence of the suppressor mutations increases the ATPase activity of Sth1 (a construct, in this study) even in the presence of ARPs. This suggests that ARPs might be involved in reducing the ATPase activity of Sth1 while the suppressor mutation might alleviate this effect of ARPs on Sth1. However, to test this hypothesis, we need to compare the ATPase activity of Sth1 containing these suppressor mutations in the presence and absence of Arps. This will only tell us whether the increased ATPase activity is solely due to the effect of the suppressor mutations.

Whatever may be the case, we sought out to understand the reason behind the increased ATPase activity of the mutant. On comparing the K_M of the wild type Sth1-Arps complex with the mutants, we inferred that the increased ATPase activity of the mutant is not due to its increased affinity for DNA. The other possible reason why the mutant might possess increased ATPase activity might be because of its increased affinity

for ATP. This can be tested by comparing the affinity of WT Sth1-Arps and mutant complexes for ATP. If the mutants possess a lower K_M, they have higher affinity for ATP and that might explain their increased ATPase activity. However, another alternative is that they burn more ATP to travel short distances than wild type Sth1-Arps. This can be tested using a DNA length dependent ATPase assay as reported in Saha et al. (2002).

Single molecule studies are powerful ways to understand the mechanistic aspects of remodeling. Similar studies in other enzymes have provided deep insights into their mechanism. In the case of NS3, single molecule studies using optical tweezers at varying concentrations of ATP suggested that NS3 translocates on RNA in a series of substeps each of which requires ATP (Dumont et al., 2006). The results obtained from single molecule studies of RSC will help us refine the existing mechanisms of chromatin remodeling. The results obtained by single molecule studies on RSC using optical tweezers method by Zhang et al. (2006) were quite different from those obtained by Lia et al. (2006) using magnetic tweezers. This might because of the differences in the nature of the technique and the substrates used. For instance, studies using magnetic tweezers were done at a tension below 1 pN and on naked DNA template while the tension used in optical tweezers technique spanned from 1 to 12 pN and done on nucleosomal templates. Thus, this study aims to resolve these differences as a system for studying a single molecule of RSC on naked DNA has been developed.

Also, single molecule studies of RSC will also help us understand how RSC is regulated. RSC, as well as its catalytic subunit Sth1, displays ATP-dependent DNA translocation activity. Thus if the isolated catalytic subunit can translocate on the nucleosome by itself, what are the roles for the other subunits of the RSC complex? In

order to understand this, we aim to study the mechanistic parameters of Sth1 in isolation using the single molecule technique and compare them with that of the whole complex RSC. As ARPs are possible regulators of Sth1, single molecule studies of the minimal complex of Sth1 with Arps will give us hints on the effect of ARPs on the motor function of Sth1.

Remodelers have been shown to possess the ability to generate superhelical torsion in DNA. The cruciform extrusion assay was the only assay that was developed to monitor the twisting activity of remodelers (Havas et al., 2000). This assay is based on the extrusion of a cruciform structure formed by inverted $[AT]_n$ during negative superhelical stress and recognition of this cruciform structure by a specific enzyme EndoVII. We have developed another assay for torsion exploiting the negatively supercoiled nature of the loop formed due to the motor function of a remodeler and the specific relaxation of negative supercoils by *E. coli* Topoisomerase I. This assay can be used for screening reagents before they are studied using expensive but sophisticated techniques like single molecule studies.

5. CONCLUSION AND FUTURE DIRECTION

The central question in the chromatin remodeling field is how remodelers couple the energy of ATP hydrolysis to reposition the nucleosome. Our current model for chromatin remodeling is based on the observation that remodelers are ATP dependent DNA translocases. Elegant experiments have helped us understand the directionality of the translocase, its step size etc. But those properties that are difficult to be understood from bulk solution experiments remain unknown. In this work, we have developed a system to study the properties of a single molecule of RSC on naked DNA in real time using optical tweezers. This study will help us determine the rate at which RSC translocates on a nucleosome, its ATP turn over rate and processivity. Together, it will help us refine the existing mechanisms of chromatin remodeling of RSC to the finest detail. Also, a simple assay through which the torsion generated by the motor can be measured has been developed in this work.

Actin Related Proteins (ARPs) are conserved in many remodelers. The presence of actin-related proteins in chromatin regulating complexes is intriguing, although a role for these proteins in these multisubunit complexes is not known. This work has added growing evidence to our hypothesis that actin related proteins regulate the activity of Sth1. We obtained dominant negatives of Sth1 localized to a domain in Sth1 to which suppressors of ARPs also mapped to in a previous study. Thus, this region might be responsible for the regulation of the motor activity of Sth1 by ARPs. Further biochemical

studies of these mutants in the presence and absence of ARPs will shed light on how ARPs regulate the motor properties of Sth1.

RSC, as well as its isolated ATPase subunit, are DNA translocases. So this raises the following questions: How do the translocation properties of RSC compare with that of isolated Sth1? Also, as ARPs seem to possible regulators, how do the motor properties of Sth1-Arps complex compare with that of RSC? The results from single molecule studies of Sth1 in complex with Arps (this thesis) coupled with future studies on Sth1 in isolation will help us determine the difference in their mechanistic properties.

Also, based on homology to Rad54, a DNA translocase whose crystal structure is known, mutants in key residues of Sth1 that interact with DNA can be isolated. Biochemical studies of these mutants will help elucidate the mechanism by which RSC translocates the DNA on a nucleosome. This shall subsequently enable a better understanding of the fundamentals of chromatin remodeling by RSC.

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