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Cloning And Biochemical Characterization Of The RecQ Helicase And Topoisomerase III In Schizosaccharomyces Pombe

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**Cloning and Biochemical Characterization of the RecQ Helicase and
Topoisomerase III in *Schizosaccharomyces pombe***

**An Honors Thesis Presented to
The Faculty of the Department of Biological Sciences
University of New England**

**in partial fulfillment of the
requirements for the Degree of
Bachelor of Science with Honors in Biology**

by

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Abstract

RecQ and Topoisomerase III proteins are both essential for proper chromosomal maintenance in species ranging from bacteria to humans. Due to their highly conserved nature throughout evolution, understanding their basic biochemical properties as well as their interactions together is important for understanding human health, disease, and even to provide potential evolutionary insight. *S. pombe* has been chosen as a model organism because little research has been done on the biochemical analysis of its RecQ homolog, Rqh1. This study shows developments in the biochemical characterization of a truncation protein of Rqh1 including AFM (Atomic Force Microscopy) Imaging, DNA binding, and ATP hydrolysis activity. New insight of the biochemical properties of Rqh1 will advance the development of a molecular model for Bloom's syndrome and provide a better understanding of the molecular aspects of other diseases associated with mutations in RecQ homologs.

Chapter I: Introduction

Genome instability can occur for a number of different reasons, and there are various diseases associated with this phenotype. One specific class of disorders results from mutations in proteins in the RecQ family of helicases. These diseases include Bloom's syndrome, Werner's syndrome, and Rothmund Thompson syndrome. Understanding the molecular basis of genome instability in loss of function or absence of RecQ helicase family proteins is likely to be beneficial for treating those diagnosed with diseases associated with mutations in RecQ proteins. Additionally, because of the cancer and aging phenotypes of RecQ associated diseases research in this area aids in understanding the mechanisms of cancer and aging in the general public. Previous research has been able to pinpoint binding domains of some of the RecQ proteins as well as preferential binding substrates, which does provide insight into protein activity, however little has been done in the area of *in vitro* assays of protein function. Although *in vivo* gene knockout and mutation data can be very useful for understanding the role RecQ proteins play in maintaining genome integrity, little headway has been made on precise mechanisms. Because RecQ proteins are involved in DNA metabolism events which require numerous proteins and their interactions, information in this area is still lacking. Developing a biochemical RecQ model would be extremely beneficial not only for obtaining a better understanding of RecQ properties and functions, but also for providing a better insight into the possible diseases mechanisms.

RecQ DNA Helicases

RecQ helicases exist in every bacterial and eukaryotic species that has been sequenced to date and have been highly conserved throughout evolution. (Killoran and

Keck 2006) (Fig. 1). The first RecQ protein was discovered in 1984 in a screen for *E. coli* mutants resistant to thymine starvation (Killoran and Keck 2006). These helicases unwind DNA in the 3'→ 5' direction (meaning they require a 3' single stranded DNA flanking region to bind) and function mainly to repress illegitimate recombination, resolve recombination intermediates, but can also initiate recombination (Heyer 2004). In addition, they may also be involved in the stress response mechanism for signaling stalled replication forks which suggests they are involved in a cellular defense mechanism against DNA damage (Heyer 2004).

E. coli and yeast have only one RecQ family member: RecQ in *E. coli*, Sgs1 in *Saccharomyces cerevisiae*, and Rqh1 in *Schizosaccharomyces pombe*. Humans, however, have at least five RecQ homologs, three of which are known to cause diseases when mutated which is not surprising given the nature of their activity. The five human homologs of *E. coli* RecQ are RECQ1, BLM, WRN, RECQ4, and RECQ5 (Ozgenç and Loeb, 2005). Of the five, mutations in BLM, WRN, and RecQ4 cause Bloom's syndrome, Werner's syndrome, and Rothmund-Thomson syndromes respectively (Fig. 2).

Diseases Associated with Mutations in Human RecQ Homologs

Bloom's Syndrome

Bloom's syndrome (BS) is a rare, autosomal recessive disorder which causes sensitivity to light, growth retardation, immunological disorders, and a predisposition to cancer at a relatively young age (Nicotera et al. 1989). Bloom's syndrome patients have a 100-fold increased risk for developing cancer, with average cancer onset at 25 years of age (Chester et al. 2006). Preliminary research in this area had shown Bloom's syndrome

to be a condition of oxidative stress; however, more recent research has been able to identify common mutations in BLM such as helicase mislocalization or loss of a nuclear localization signal to be the cause of the disease (Ramirez et al. 2007). This results in the inability for the suppression of cellular hyperrecombination in Bloom's syndrome cells (Ramirez et al. 2007). In addition, Bloom's syndrome cells exhibit high levels of ejected chromosomal material, called micronuclei (Chester et al. 2006). It is possible that oxidative stress first detected in Bloom's syndrome cells was a downstream effect as a result in defective BLM, which caused in mutations in mechanisms to neutralize reactive oxygen species. Because functions of BLM include the disruption of recombination intermediates such as D-Loops, Bloom's syndrome patients experience increased levels of recombination during double strand break repairs, DNA synthesis, or meiosis which ultimately results in genome instability (Ramirez et al. 2007). This genome instability is in turn tightly linked with cancer through the downstream effects of obtaining multiple chromosomal aberrations which in turn disrupts genes. Mutations in other proteins responsible for maintaining cellular genomic integrity such as tumor suppressors, or mutations in proteins that help manage oxidative stress are likely the cause of cellular and overall phenotypic effects.

Werner's Syndrome

Werner's syndrome (WS) is an autosomal recessive disease characterized by premature aging and genomic instability linked to a higher instance of cancer in affected people, as a result of mutations in the WRN protein. WRN has been shown to preferentially bind a variety of DNA structures including forked duplexes, 5' overhang duplexes, and Holliday junctions with varied preference at each of its domains (Kobbe et

al. 2003). Telomere dysfunction has recently been identified as the cause of genomic instability in Werner's syndrome cells (Crabbe et al. 2007). In addition to telomere dysfunction, Werner's syndrome cells show replicative defects, increased sensitivity to DNA damaging agents, and genomic instability (Kobbe et al. 2003) (Kipling et al. 2004). In culture, Werner's syndrome cells show poor growth and usually enter a senescent stage within a few population doublings, which is likely due to the activation of tumor suppressor pathways (Crabbe et al. 2007). Without functional WRN, cells are unable to signal short telomeres and this DNA damage is usually repaired by a nonhomologous end-joining pathway. This results in covalent fusion of chromosomes which can be observed using immunofluorescence during mitosis as anaphase bridges. Through subsequent cell division cycles, these fused chromosomes are broken during chromosomal segregation and result in genome instability, which signals cells to enter a permanent growth arrest. This is likely the reason why Werner's syndrome patients typically lack growth spurts and are relatively short in stature as adults. This accumulation of DNA damage is quite rapid because mutations in WRN can often result in a mutator phenotype, which speeds up the process of DNA damage (Kipling et al. 2004). One example of this is through a breakage fusion bridge cycle which occurs when broken chromosomes are replicated and then fuse together through non-homologous end joining. This dicentric chromosome is then pulled apart and additional breaks are introduced through subsequent cell division cycles.

Rothmund-Thompson's Syndrome

Rothmund-Thompson's syndrome (RTS) is a recessive disorder caused by a mutation in the RECQ4 gene and symptoms include growth deficiency, graying of hair,

juvenile cataracts, skin and skeletal abnormalities, and susceptibility to osteosarcomas and skin cancer (Ramirez et al 2007). In two thirds of Rothmund-Thompson's patients, the molecular basis of the diseases has been linked to truncations of RECQL4 (Ramirez et al. 2007). RECQL4 is different from BLM and WRN in that its nuclear localization and retention signals are located in the amino terminal portion of the protein, and that it lacks the RQC and HRDC domains required to bind and unwind nucleic acids (Burks et al. 2007). Data from knockout mice has proposed functional RECQL4 to be important in cell proliferation and development, and has shown a novel function in the initiation DNA replication (Burks et al. 2007). Rothmund-Thompson's syndrome cells have the inability to recover from oxidative stress and feature extreme DNA damage. This damage is sensed by cell cycle checkpoints and cells undergo growth arrest and have an overall decrease in DNA synthesis, both attributing to the growth deficiency phenotype.

Although Bloom's, Werner's, and Rothmund-Thompson's syndromes vary somewhat phenotypically, comparatively each results in predisposition to cancer, increased chromosomal aberrations, and sensitivity to DNA damaging agents resulting from mutations or deletions in specific RecQ proteins (Killoran and Keck 2006).

Structure of RecQ Helicases

RecQ helicases contain at least three functional domains including the helicase domain, which is unique to the RecQ family, the RecQC Domain (RecQ Conserved Domain), and the HRDC Domain (Helicase RNase D Conserved Domain), with the exception of WRN which contains an exonuclease in the N-terminal region (Khakhar et al. 2003). The helicase domain is conserved in all RecQ proteins, the RecQC and HRDC domains are found in most RecQ proteins, but are missing in a small group of others. For

example, human WRN lacks the RecQC and HRDC domains and human RecQ5 β lacks the HRDC domain (Killoran and Keck 2006).

Other regions of the protein have some variability, which explains the difference of preferential binding molecules of RecQ proteins. RecQ proteins will often bind other proteins or motifs that facilitate in the subcellular localization (Bachrati and Hickson 2003). This is important for protein-protein interactions, as there are numerous protein complexes formed during the events of replication, recombination, and repair. Examples of these interactions will be discussed subsequently in further detail.

The primary function of the helicase domain is to couple nucleotide triphosphate (NTP) hydrolysis in order to separate nucleic acid duplexes. Interestingly, it has been proposed that the helicase domain may actually possess a base-flipping activity in order to process rigid substrates with restricted backbone flexibility (Killoran and Keck 2006). Like many other helicases, RecQ proteins contain the seven most commonly conserved helicase motifs (I, Ia, II, III, IV, V, VI) to unwind DNA (Killoran and Keck 2006). Although the motif roles are not very well defined, these helicase motifs are typically involved in binding to the phosphate backbone or bases of DNA (Killoran and Keck 2006). Additionally, both Bloom's and Werner's syndrome can result from mutations in the helicase domain. Typical mutations in the helicase domain cause changes in size or polarity, which are predicted to cause a destabilization of the domain resulting in disease phenotypes (Killoran and Keck 2006).

The RecQC domain is the second largest conserved domain in the RecQ family of helicases. It provides structural stability, metal cofactor binding, facilitates structure specific nucleic acid binding, and aids in protein-protein interactions (Killoran and Keck

2006). This region of the protein is often the location for mutations and truncations in BLM, causing the Bloom's syndrome phenotype, as well as mutations in the budding yeast, *Saccharomyces cerevisiae* Sgs1 which result in DNA damage sensitivity.

The HRDC domain is primarily important for the structure specific nucleic acid binding of various substrates and provides an area for protein-protein interactions to take place (Killoran and Keck 2006).

Rqh1, the *S. pombe* yeast homolog has all of the functional domains humans have including the RecQC Domain (RecQ Conserved Domain), and the HRDC Domain (Helicase RNase D Conserved Domain RecQ C terminal domain) with the exception of the WRN exonuclease (Fig. 4). Research has shown Rqh1 to function in a manner most similar to that of the human homolog BLM (Raji and Hartsuiker 2006).

Functions of RecQ Helicases

RecQ proteins have been shown to function as part of the S-phase checkpoint, which is a cellular mechanism for monitoring and assuring accurate DNA replication as well as the removal of DNA damage (Bennet and Keck 2004). In *S. cerevisiae* and *S. pombe*, the loss of function in RecQ homologs results in sensitivity to inhibitors of DNA replication and cells fail to execute normal cell cycle progression following recovery from induced S-phase arrest (Davies et al. 2004). This is consistent with the model in which RecQ helicases act to restore DNA replication following S-phase arrest, and therefore prevent subsequent genomic instability (Davies et al. 2004). This subsequent genomic instability often occurs through breakage fusion bridge cycles, which may occur when chromosome segregation proceeds prior to the resolution of sister chromatid exchanges.

The general mechanism by which RecQ homologs work to suppress illegitimate recombination and thereby maintaining genome integrity is through their helicase activity. Illegitimate recombination is typically defined as recombination resulting in chromosomal aberrations and includes non-homologous recombination, homeologous recombination, and single strand annealing. DNA replication and recombination pathways are tightly linked events of DNA metabolism, although not always considered to be. For example, replication forks which have stalled due to DNA damage can be rescued through recombinational events which ensure completion of the DNA replication process (Cox et al. 2000). Unwinding of recombination intermediates by RecQ proteins inhibits recombination by preventing the instance of restarting of a stalled fork through recombination. RecQ helicases are responsible for stabilizing replication forks, the removal of recombination intermediates, and may even function in the signaling of stalled forks (Heyer 2004). Recombination is clearly advantageous in some situations, however in excess it results in loss of heterozygosity (LOH) and contributes to genome instability.

RecQ proteins additionally aid in replication by unwinding secondary structures preceding a replication fork which might otherwise stall fork progression. An example of this is the ability of WRN to aid DNA polymerase δ in the replication through four-stranded DNA stabilized by G quartets (G4DNA) and hairpin structures. This explains the slower elongation rates of polymerases in human Werner's syndrome and Bloom's syndrome cells (Bennet and Keck 2004). RecQ proteins may also play a crucial role in stabilizing DNA polymerases by maintaining the forked structure during replication and repair. For example, Sgs1 has been shown to play a direct role in the binding of both

polymerases α and ϵ at stalled replication forks (Bennett and Keck 2004). In the absence of Sgs1, the level of DNA polymerases at replication origins is reduced between two and four fold (Bennett and Keck 2004). The consistent localization of Sgs1, BLM, and WRN proteins sites of DNA synthesis during replication, particularly in areas of DNA damage or stalled replication forks, signifies the conserved role of RecQ proteins in replication (Bennett and Keck 2004).

Interactions of RecQ Helicases with other Proteins

RecQ proteins have been shown to interact with a number of other proteins involved in the DNA replication, recombination, and repair pathways. Among these proteins are POT1 (a telomeric single stranded DNA binding protein), ATM, PCNA (proliferating cell nuclear antigen), RPA, and DNA polymerases δ , ϵ , and α (Opresko et al. 2005) (Beamish et al. 2002) (Bennett and Keck 2004).

For example, Both BLM and WRN have shown to bind to POT1 (Protection of Telomeres 1), the only human telomere specific single stranded DNA binding protein identified thus far (Opresko et al. 2005). This shows obvious implications in the disease mechanisms of WRN specifically, which has been proposed to be a disease associated with telomere dysfunction as the cause of genomic instability (Crabbe et al. 2007).

BLM has additionally been shown to interact with ATM (ataxia telangiectasia mutated) and other damage checkpoint proteins contributing to the role in stalled replication forks which are signaled similarly to DNA damage (Ho et al. 2006)

Topoisomerase III

Topoisomerases are highly conserved proteins that function to catalyze topological rearrangements in the structure of DNA and are required for many aspects of

DNA metabolism including replication, recombination, and repair (Mankouri and Hickson 2006). They function to cleave strands of DNA where topological issues such as supercoiling, tangling, or catenation have occurred, and they allow DNA strands to unwind in order to relieve tension before resealing the cut strand. There are two classes of topoisomerases, Type I or Type II, distinguished by their ability to cleave either one or two strands of a DNA duplex (Berger et al. 1998). In addition, there are two classes of Type I topoisomerases, A or B based on differences in their amino acid sequences and reaction mechanisms (Berger et al. 1998). Topoisomerase III is a Type IA enzyme, ATP independent, requires divalent metal ions (preferably Mg^{2+}), and is primarily responsible for inducing negatively supercoiled DNA (Gangloff et al. 1999) (Berger et al. 1998). Topoisomerases also include the DNA gyrases.

Like RecQ helicases, topoisomerases homologs have been identified in humans, mice, yeast, and bacteria (Harmon et al. 2003). In addition to providing genomic stability in both prokaryotes and eukaryotes requiring RecQ helicase activity, TopoIII enzymes are required for maintaining genome integrity (Harmon et al. 2003). Due to the fact that there are structural and sequence differences between prokaryotic and eukaryotic topoisomerases, they have been significant for cancer therapy and antibiotic development. Topoisomerase inhibitors range from the common laboratory antibiotic nalidixic acid to the class of fluoroquinolones which are often prescribed as chemotherapy drugs designed to interfere with topoisomerase activity.

Regulating DNA topology is clearly essential for adequate genomic maintenance and cells mutant for TopoIII display a wide range of phenotypes including an altered cell cycle, hyperrecombination, abnormal gene expression, and chromosomal instability

(Gangloff et al. 1999). Additionally, null mutations in mouse TopoIII α are embryonically lethal, and in *S. pombe* they lead to aberrant chromosome segregation which is lethal as well (Harmon et al. 2003).

Interactions between RecQ and Topoisomerase III

RecQ and Topoisomerase III have been shown to interact, specifically during DNA metabolism. Mutations in Rqh1 (*S. pombe* RecQ homolog) have been shown to rescue the lethality associated with Top3 mutations (Ahmad and Stewart 2005). Based on this and other data showing the rescuing of Top3 mutations through the removal of genes involved in homologous recombination, it has been suggested that Rqh1 processes DNA to form an intermediate substrate that is subsequently resolved by the action of Top3 (Ahmad and Stewart 2005). Without Top3, it appears this DNA substrate is lethal to the cell, indicating the importance of RecQ and Topoisomerase III interaction.

Understanding the biochemical characteristics of both Rqh1 and Topoisomerase III in *Schizosaccharomyces pombe* will be beneficial for a better understanding of RecQ human homolog mutation disease progression, for obtaining a clearer scientific knowledge of DNA replication, recombination, and repair, and will hopefully provide an *in vitro* model which can be used to test other nuclear targeted proteins for interaction with RecQ and Topoisomerase III proteins.

Chapter II: Experimental Procedures

Reagents

All chemicals used were reagent grade and were purchased from Sigma unless otherwise noted. Aqueous solutions were prepared with deionized, glass-distilled water.

Nucleic Acids

Oligos were purchased from BioSyn. TopoIII N terminal TOPO3-N 5'-CAC CAT GCG CGT CCT ATG TGT TGC TGA AAA AAA C-3' ; TopoIII C terminal TOPO3-C 5'-CTA GGT TTG CGG TTC ATT ATG ACT CAT AAG GTA-3' ; RecQ N terminal primer RECQ-N 5'-CAC CAT GAC AGT AAC GAA AAC AAA CCT TAA TCG TC-3' ; RecQ C terminal RECQ-C 5'-TTA ACG ATA ATT TTG CTT AAC CAT GGG ATG TAT G-3' ; RecQ N terminal partial truncation RECQ-353 5'-CAC CAT GTC GCA CTC AAC ATC TAG CTC AAA CGT CCC TCG T-3' ; RecQ N terminal partial truncation RECQ-1078 5'-CAC CAT GCC GGA GTC TAC CGT GAA AGA AAA TAG CAC TCG GCC A-3' ; RecQ without N terminus RECQ-1483 5'-CAC CAT GGA TGA TCC CAT GCT TTC GTA TCC TTG GTC-3' (Fig. 3, 4).

Cloning of Rqh1 and Topoisomerase III

Cloning of the gene sequences from *Schizosaccharomyces pombe* genomic DNA was been conducted via PCR methods. PCR products were integrated into pET 100-D expression vectors via a Topo cloning kit (Invitrogen). Positive clones were identified via colony PCR and restriction digestion methodologies. Rqh1 N-terminal truncations were constructed using primers designed for truncation in order to increase chances of yielding protein expression, as N-terminal truncations have been shown to increase protein solubility (Hyo-Jin et al. 2003).

PCR Reactions

Reactions were performed in 1x pfx reaction buffer, with 0.5mM forward primer, 0.5mM reverse primer, 0.05mM *S. pombe* genomic DNA template, 0.5 units Pfx polymerase (Invitrogen), 2.0 mM MgSO₄, and brought to 20 μL deionized H₂O. Thermocycler Parameters were as follows: 95°C for 3 min, 30 cycles of: 95°C for 30 s, 52°C for 30 s, 68°C for 3 min 30 s, and a final extension at 68°C for 10 min. PCR products were electrophoresed in 1% agarose gels and stained for 20 min in (0.1%) ethidium bromide to visualize.

Colony PCR

Single colonies were randomly picked from plates and added to 25 μL reactions containing: 20.5 μL sd H₂O, 2.5 μL 10x Taq buffer, 250 mM dNTP (each), 0.5mM forward primer, 0.5mM reverse primer, 0.05mM, and 0.4 units Taq polymerase (New England BioLabs). Thermocycler Parameters were as follows 95°C for 3 min, 30 cycles of: 95°C for 30 s, 56°C for 30 s, 72°C for 3 min 30 s, and a final extension at 72°C for 10 min and visualization methods were as previously mentioned.

Topo-cloning into pET 100-D vectors

Cloning reactions contained 1 μL fresh PCR product, 1 μL NaCl solution, 2 μL sterile H₂O, and 1 μL Topo vector (Invitrogen). 3 μL of reaction mix was added to cells, heat shocked at 42°C for 30 s, and transferred immediately to ice. 250 μL SOC medium was added, cells were allowed to shake 1 hr at 37°C, and plated on AMP plates.

Plasmid DNA Isolation

Plasmid DNA isolation was as previously described (Maniatis et al. 1989)

Protein Expression

Expression of desired proteins was conducted by transforming recombinant pET100-D vectors isolated from positive clones into Rosetta Gami *E. coli* cell lines (Novagen). Positive transformants were then identified using ampicillin and chloramphenicol or streptomycin resistance. Cell lines expressing recombinant protein were then identified via Western Blot Analysis. Six-liter cultures were then inoculated using positive cell lines expressing desired protein and were grown to an optical density of 0.5 at 37°C. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was then added to cultures to 0.3 mM along with a temperature reduction to 18°C in order to induce expression of soluble recombinant proteins under the transcriptional control of a T7 RNA promoter.

Western Blotting

Western Blots were performed in order to analyze protein expression, according to standard procedures. Denaturing polyacrylamide gel electrophoresis was performed in duplicate on all samples. Gels include both a resolving gel (10 mL containing 5.65 mL deionized H₂O, 2 mL acrylamide, 2.25 mL 1.5M Tris pH 8.8, 0.1 mL 10% SDS, 0.2 mL 10% APS and 20 μ L TEMED), and a stacking gel (4 mL containing 3.0 mL deionized H₂O, 500 μ L acrylamide, 500 μ L 0.5 M Tris pH 6.8, 40 μ L 10% SDS, 80 μ L 10% APS, and 8 μ L TEMED) and electrophoresis was performed using a Bio-Rad electrophoresis apparatus in a Tris-Glycine buffer system (25 mM Tris, 250 mM glycine (pH 8.3), 0.1% SDS). One gel was stained with Coomassie Blue in order to visualize total protein content and the second gel was transferred to nitrocellulose for immunoblotting. The transfer took place in Tris-Glycine buffer containing 20% methanol at 500 milliamps for 1 hr. Nitrocellulose was then blocked in 5% bovine serum albumin (BSA) at room

temperature for 1 hr and incubated overnight in a primary antibody (Rabbit Anti-Flag) (Invitrogen). Blots were washed with 1x TBST (150 mM NaCl, 10 mM Tris-HCl, 0.1% TWEEN® -20, pH 7.5) three times, two 1 min washes followed by 1 five min wash. A secondary antibody (Goat Anti-Rabbit) (Invitrogen) conjugated to horseradish peroxidase was then used for identification of positive protein expression.

Protein Purification

Initial purification of Rqh1 truncation 3 (Rqh1-3) was done via Immobilized Metal Ion Affinity Chromatography (IMAC) and cation exchange chromatography (Fig. 5). IPTG induced chemically competent Rosetta Gami cells were suspended in lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 2 mM B-ME, 1 mM PMSF, 20 mM imidazole, and deionized H₂O) and then sonicated on ice with Branson Sonifier 150 until adequate lysis had occurred as detected by Bradford Reagent (Bio-Rad Protein Assay Dye Reagent). Cell lysates were then centrifuged for 60 min at 15,000 rpm at 4°C. Supernatants (crude cell extract) were then subject to IMAC chromatography and protein was eluted from the column with imidazole-containing elution buffer (196 mL H₂O, 2 mL 1 M Tris pH 8.0, 10 mL 5 M NaCl, 150 µL B-ME, and 0.702 g imidazole). Protein-containing elution fractions were then dialyzed into DC-100 (10 mM Tris pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 1 mM B-ME, and 10% glycerol) for cation exchange chromatography on SP sepharose. Proteins were eluted using DC-100, DC-600, and DC-1000. DC buffers are identical except for the NaCl concentration which is designated in the buffer name (mM).

Gel Mobility Shift Assays

PhiX174 DNA (virion or RFII) was mixed with varying concentrations of Rqh1 protein in the indicated buffers in a total volume of 20 μ l and incubated at 25°C for 15 minutes. Sample buffer (4 μ l of 50 mM Tris (pH 7.5), 50% glycerol, and 0.2% bromphenol blue) was added to each sample, the binding reactions were subsequently subjected to electrophoresis through a 1.0% agarose gel in TAE buffer (40 mM Tris acetate, 2 mM EDTA (pH 8.0) at 80 V for 3 hours and were visualized by ethidium bromide staining.

ATPase Hydrolysis Assay

Rqh1 Truncation #3 (Rqh1-3) ATP hydrolysis was measured via an indirect assay in which ATP regeneration is coupled to the oxidation of NADH. This regeneration system involves the phosphorylation of ADP to ATP by pyruvate kinase at the expense of phosphoenolpyruvate. The pyruvate generated in this reaction is subsequently reduced to lactate by lactate dehydrogenase with the concomitant oxidation of NADH to NAD⁺. NADH concentration is measured via absorbance at 340nm, reaction conditions are established such that the rate limiting step is ATP hydrolysis. The constant regeneration of ATP allows monitoring the ATP hydrolysis rate over the entire course of the assay. Unless otherwise indicated, the assays were conducted at 25°C with Rqh1 protein concentrations ranging of either 0.3624 μ M, 0.7248 μ M or 1.4496 μ M and single strand DNA concentration of 1 μ M with respect to nucleotides. Reactions took place in 1mL volume of the ATPase reaction buffer containing 20 mM Tris pH 6.8, 90 mM potassium acetate (KOAc), 10 mM magnesium acetate, 6 units/ml pyruvate kinase, 6 units/ml lactate

dehydrogenase, 2.3 mM phosphoenolpyruvate, and 0.23 mM NADH. The absorbance data were collected using an Ocean Optics USB4000 spectrophotometer.

Scanning Probe Microscopy

Rqh1 was diluted to 7.8 μ M for binding reactions. Double strand DNA (Phi-X174) was purchased from New England Biolabs. 20 μ L samples were prepared by adding 14 μ L deionized H₂O, 2 μ L One-Phor-all buffer, 2 μ L ssDNA, and 0.1208 μ M Rqh1. One-Phor-all buffer (OPA 10x) was purchased from Pharmacia and contains 5mM Tris-acetate pH 7.6, 50mM sodium acetate, 5mM magnesium acetate. Samples incubated for 5 minutes, and then directly adsorbed onto freshly cleaved muscovite mica (Ted Pella Inc.) and incubated for another 5 minutes. The sample was rinsed with 1.0 mL deionized H₂O and dried with either nitrogen or helium and subsequently imaged with a Digital Instruments Nanoscope IIIa AFM using silicon nitride tips in TappingmodeTM and beginning with 5.12 (scan rate- .2 nm/sec) nanometer scan widths, and incrementally narrowing the range to 1 μ m (scan rate - 1nm/sec) and 512 nm scan widths (scan rate - 2nm/sec). A second, high-resolution tip was also utilized to take images below 1 nm, which have proven to image at atomic resolutions.

Protein Concentration

Rqh1-3 protein concentration was determined using Beer's Law, an A280 spectrometer reading, and an extinction co-efficient for the truncation of 74,565 as predicted by ProtParam (Gasteiger et al. 2003).

Chapter III: Results

Cloning of Rqh1 and Topoisomerase III from *S.pombe*

Rqh1 truncations #1, #2, #3, and full length protein were cloned via PCR from *Schizosaccharomyces pombe* genomic DNA (Fig. 6). Aliquots were reserved and subject to agarose gel electrophoresis and visualized with ethidium bromide staining. Although progress has been made in the scientific study of knockout or mutations versions of RecQ homologs in cell lines, little has been done on the biochemistry of Rqh1.

Topoisomerase III was successfully cloned from *Schizosaccharomyces pombe* genomic DNA. Although topoisomerases vary significantly between prokaryotes and eukaryotes, human topoisomerase III α has been shown to have a high amino acid sequence homology to yeast topoisomerase III (Ng et al. 1999).

Positive PCR reactions of Rqh1 full length, truncations, and Topoisomerase III as identified by ethidium bromide staining of agarose gel electrophoresis were subject to Topoisomerase dependent ligation into pET 100D vectors (Invitrogen). Ligation reaction products were subsequently transformed into Topo cells and vectors were transformed into chemically competent Rosetta Gami Cells (Invitrogen). Detection of plasmid presence was verified through antibiotic resistance as well as colony PCR (data not shown).

Protein Expression and Purification

Small scale expression of Topoisomerase III in a 10 mL culture of Rosetta Gami cells was performed to identify positive expressing cell lines via Western Blotting. In addition, we have achieved successful small scale expression of Rqh1-3 (Fig. 7). A positive transformant was then used to inoculate a large scale culture. Positive clones

were identified via Western Blotting and were grown in six liters for purification of reagent quantities of recombinant Rqh1-3 (Fig. 8). Cells were lysed via sonication and clarified extracts were subject to nickel affinity chromatography. Rqh1-3 was subsequently eluted with 200 mM imidazole and dialyzed into storage buffer. Purity was determined via SDS PAGE gel electrophoresis, followed by staining with Coomassie blue (Figure 8 A). Western blotting was subsequently employed to verify that the purified protein was Rqh1-3 (Figure 8 B).

Identification of nucleoprotein complexes containing Rqh1-3 bound to single stranded and double stranded DNA

In order to assess the ability of the Rqh1-3 protein to bind ssDNA and dsDNA a gel mobility shift assay utilizing phage DNA from Phi-X 174 as the experimental lattice was employed. Here, a 75 μM nucleic acid lattice (with respect to nucleotides) was titrated with Rqh-1 from 4.0 μM to 20.0 μM in four μM increments. The resulting complexes were subsequently electrophoresed in an agarose gel and visualized via staining with ethidium bromide. Results demonstrate that Rqh1 binds both ssDNA and dsDNA (Figure 9). However there appears to be distinct binding modes for the two DNA lattices. The Rqh1-3:ssDNA complex displays a continuous decrease in mobility as more protein is titrated onto the lattice, suggesting that saturation of the lattice does not occur within the experimental range of Rqh1-3 concentration (Figure 9 A). This would appear to indicate that the Rqh1-3:ssDNA binding site size is less than four nucleotides per monomer of Rqh1-3. However, the Rqh1-3:dsDNA complex appears to have no further shift after lane three (Figure 9 B). This would suggest a saturation concentration between

8 and 12 μM Rqh1-3. This would correlate to a binding site size of approximately 7-10 nucleotides per Rqh1-3 monomer.

Preliminary AFM imaging of Rqh1-3

In an effort to obtain structural information regarding the nature of these Rqh1-3 nucleoprotein complexes, we have undertaken scanning probe microscopic analysis of Rqh1-3 bound to double stranded DNA. Here we present evidence that Rqh1-3 binds dsDNA and appears to form filaments on duplex DNA (Figure 11). When naked double stranded DNA (Figure 11 A) is compared to dsDNA incubated with Rqh1-3 (Figure 11 C) a distinct difference in the appearance and diameter of the duplex is apparent. When compared to the structure of Rqh1-3 in solution (Figure 11 C), it appears that the difference in structural properties could be due to interaction with the Rqh1-3 protein. In comparing the two images, there is in fact a significant difference in the width of the DNA fibers when Rqh1-3 is present (Table 1, Table 2).

ATPase Activity of Rqh1-3

Given the thermodynamic barrier associated with the role of a helicase protein in DNA strand separation, a thorough understanding of its ATP hydrolytic properties can be useful in the elucidation of helicase mechanism. Here we employ an indirect assay that couples the hydrolysis of ATP to the oxidation of NADH in a pyruvate kinase dependent ATP regenerating system. In this assay oxidation of NADH was determined to be dependent on the concentration of Rqh1-3 in the reaction (Figure 10). We subsequently varied the ATP concentration to determine the kinetic properties of Rqh1-3 ATP hydrolysis. Figure 12 summarizes these experiments; a Michaelis-Menten plot (Figure 12 A) was constructed to determine the kinetic parameters K_M and V_{max} . This data was

replotted in a Lineweaver-Burke plot for easier visualization of these data (Figure 12 B). Finally, a Hill plot was constructed to assess whether Rqh1-3 displays cooperativity in its interaction with ATP (Figure 12 C). These data are summarized in Table 3. Due to the slope of the line on the Hill plot being very close to that of 1 at 1.05, this indicates Rqh1-3 binds ATP in a noncooperative fashion. Overall, it appears that Rqh1-3 displays a potent DNA activated ATPase activity and is in fact active despite its N-terminal truncation.

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Chapter IV: Discussion

Understanding the biochemical properties of proteins involved with suppressing illegitimate recombination and aiding in the replication process is clearly advantageous for gaining a better perspective of diseases associated with mutations or loss of function, as well as broadening the depth of knowledge in this developing field.

We have obtained a biochemically active truncation of *Schizosaccharomyces pombe* Rqh1 and were able to identify some of its biochemical properties. Specifically, this Rqh1 truncation protein (Rqh1-3) hydrolyzes ATP in a DNA dependent manner, as no hydrolysis was detected in the absence of ATP (data not shown). The rate of ATP hydrolysis by the Rqh1 truncation appears to be protein concentration dependent, as shown in Figure 10. The fastest rate of ATP hydrolysis was measured with the reaction containing 1.4496 μM of Rqh1-3 at about 41 $\mu\text{M}/\text{min}$. In addition, using a Hill Plot we have been able to determine that RecQ binds ATP in a noncooperative manner (Fig. 12 C). This is due to a slope of 1.05 which is also referred to a Hill coefficient (nH) (Table 3). This kinetic data we have obtained will be useful for comparing the activity of Rqh1 to other known and identified DNA helicases in order to obtain a better understanding of how RecQ proteins unwind DNA complexes in order to suppress illegitimate recombination.

Additionally, we have successfully completed initial analysis of the Rqh1-3 DNA binding properties. Through gel mobility shift assays we have been able to visualize differences in binding of Rqh1-3 to single and double stranded DNA (Figure 9 A, B). One possible mechanism for by which Rqh1-3 binds differently to single versus double stranded DNA is due to possible plasmid supercoiling by the double stranded viral

plasmid. This is one possible explanation for the greater shift seen in single stranded viral plasmid with increasing concentrations of Rqh1-3. It is possible Rqh1-3 is affecting the single stranded DNA structure differently than the double stranded DNA. Future investigation in this area will involve determining DNA binding properties for this Rqh1 truncation with other DNA substrates including Holliday Junctions, forked duplexes, 3' overhangs, and other possible recombination intermediates.

To date we have cloned Topoisomerase III from *Schizosaccharomyces pombe* and hope to continue research in this area to fully express and purify an active form of Topoisomerase III in order to assess its interactions with Rqh1. Because the proposed Topoisomerase III binding site is in the N-terminus of Rqh1 and we have only expressed an N-terminal truncation protein, future expression work will hopefully yield a full length Rqh1 protein with which we can assess Topoisomerase III binding.

By cloning Topoisomerase III and expressing Rqh1-3, we have opened up an avenue for research in this area on the fission yeast, *Schizosaccharomyces pombe*, where before the area was lacking. Coupled with biochemical system already in place for the analysis of homologous recombination, this will allow detailed biochemical analysis of role of Rqh1 in the suppression of homologous recombination. This will directly relate to the mechanism of carcinogenesis in Bloom's syndrome as this disease has been linked to excessive sister chromatid exchange. Overall, we have essentially begun the construction of an *in vitro* model for carcinogenesis in Bloom's syndrome.

Although progress has been made in cellular genetics involving human RecQ homologs, mutagenic research on the cellular level is limited due to the necessity of functional RecQ homologs and other proteins involved with DNA metabolism. These

limitations, however, can be overcome through the utilization and implication of biochemical approaches for solving these types of problems. This research has provided the basis for a more detailed study of Rqh1 and Topoisomerase III as well as for future studies involving other RecQ homologs or other proteins involved with DNA metabolism.

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References

- Ahmad F, Stewart E. 2005. The N-terminal region of the *Schizosaccharomyces pombe* RecQ helicase, Rqh1p, physically interacts with Topoisomerase III and is required for Rqh1p function. *Mol. Gen. Genomics* 273:102-114
- Bachrati CZ, Hickson ID. 2003. RecQ helicases: suppressors of tumorigenesis and premature aging. *Biochem J.* 374:577-606
- Beamish H, Kedar P, Kaneko H, Chen P, Fukao T, Peng C, Beresten S, Gueven N, Purdie D, Lees-Miller S, Ellis N, Kondo N, Lavin MF. 2002. Functional link between BLM defective in Bloom's syndrome and the ataxia-telangiectasia-mutated protein, ATM. *Journal of Biological Chemistry* 277:30515-30523
- Berger JM, Fass D, Wang JC. 1998. Harrison SC. Structural similarities between topoisomerases that cleave one or two DNA strands. *Proc. Natl. Acad. Sci.* 95:7876-7881
- Bennett RJ, Keck JL. 2004. Structure and Function of RecQ DNA Helicases. *Critical Reviews in Biochemistry and Molecular Biology* 39:79-97
- Burks LM, Yin J, Plon SE. 2007. Nuclear import and retention domains in the amino terminus of RECQL4. *Gene* 391:26-38
- Chester N, Babbe H, Pinkas J, Manning C, Leder P. 2006. Mutation of the Murine Bloom's Syndrome Gene Produces Global Genome Destabilization. *Molecular and Cellular Biology* 26:6713-6726
- Cox MM, Goodman MF, Kreuzer KN, Sherratt DJ, Sander SJ, Marians KJ. 2000. The importance of repairing stalled replication forks. *Nature* 404:37-41
- Crabbe L, Jauch A, Naeger CM, Holtgreve-Grez H, Karlseder J. 2007. Telomere dysfunction as a cause of genomic instability in Werner Syndrome. *PNAS* 104:2205
- Davies SL, North PS, Dart A, Lakin ND. 2004. Hickson ID. Phosphorylation of the Bloom's Syndrome Helicase and Its Role in Recovery from S-Phase Arrest. *Molecular and Cellular Biology* 24(3):1279
- Dhillon KK, Sidorova J, Saintigny Y, Poot M, Gollahon K, Rabinovitch PS, Monnat RJJ. 2007. Functional role of the Werner syndrome RecQ helicase in human fibroblasts. *Aging Cell* 6:53-56
- Gangloff S, Massy BD, Arthur L, Rothstein R, Fabre F. 1999. The essential role of yeast topoisomerase III in meiosis depends on recombination. *The EMBO Journal* 18(6): 1701-1711

- Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel R.D, Bairoch A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis *Nucleic Acids Res.* 31:3784-3788
- Harmon FG, DiGate RJ, Kowalczykowski SC, 1999. RecQ Helicase and Topoisomerase III Comprise a Novel DNA Strand Passage Function: A Conserved Mechanism for Control of DNA Recombination. *Molecular Cell* 3:611-20
- Harmon FG, Brockman JP, Kowalczykowski SC. 2003. RecQ Helicase Stimulates Both DNA Catenation and Changes in DNA Topology by Topoisomerase III. *The Journal of Biological Chemistry* 278(43):42668-42678
- Heyer WD. 2004. Damage Signaling: RecQ Sends an SOS to You. *Current Biology* 14:R895-897
- Ho CC, Siu WY, Lau A, Chan WM, Arooz T, Poon RY. 2006. Stalled replication induces p53 accumulation through distinct mechanisms from DNA damage checkpoint pathways. *Cancer Research* 66(4):2233-41
- Hyo-Jin P, Ju-Youn C, Young-Mo S, Chul H, Sung-Joo KY, Seongman K, Hyangshuk R. 2003. Antigenicity of the region encoded by exon8 of the human serine protease, HtrA2/Omi, is associated with its protein solubility. *Biotechnology Letters* 25: 1597-1603
- Killoran MP, Keck JL. 2006. Sit down, relax and unwind: structural insights into RecQ helicase mechanisms. *Nucleic Acids Research* 34(15):4098-4105
- Kobbe VC, Thomas NH, Czyzewski BK, Pavletich NP, Bohr VA. 2003. Werner Syndrome Protein Contains Three Structure-specific DNA Binding Domains. *Journal of Biochemistry* 278:52997-53006
- Khakhar RR, Cobb JA, Bjergbaek L, Hickson ID. 2003. Gasser SM. RecQ Helicases: Multiple Roles in Genome Maintenance. *Trends in Cell Biology* 13:493-499
- Kipling D, Davis T, Ostler EL, Faragher RGA. 2004. What can progeroid syndromes tell us about human aging? *Science* 305:1426-1431
- Mankouri HW, Hickson ID. 2006. Top3 Processes Recombination Intermediates and Modulates Checkpoint Activity after DNA Damage. *Molecular Biology of the Cell* 17:4473-4483
- Ng SW, Liu Y, Hasselblatt KT, Mok SC, Berkowitz RS. 1999. A new human topoisomerase III that interacts with SGS1 protein. *Nucleic Acids Research* 27(4):993-1000

Nicotera TM, Notaro J, Notaro S, Schumer J, Sandberg AA. 1989. Elevated Superoxide Dismutase in Bloom's Syndrome: A Genetic Condition of Oxidative Stress. *Cancer Research* 49:5239-5243

Opresko PL, Mason PA, Podell ER, Lei M, Hickson ID, Cech TR, Bohr VA. 2005. POT1 Stimulates RecQ Helicases WRN and BLM to Unwind Telomeric DNA Substrates. *The Journal of Biological Chemistry*. 280(37):32069-32080

Opresko PL, Laine JP, Brosh RM, Seidman MM, Bohr VA. 2001. Coordinate Action of the Helicase and 3' to 5' Exonuclease of Werner Syndrome Protein. *The Journal of Biological Chemistry* 276(48):44677-44687

Ozgen A, Loeb LA. 2005. Current advances in unraveling the function of the Werner syndrome protein. *Mutation Research* 577:237-251

Petkovic M, Dietschy T, Freire R, Jiao R, Staglijar I. 2005. The human Rothmund-Thomson syndrome gene product RECQL4, localizes to distinct nuclear foci that coincide with proteins involved in the maintenance of genome stability. *Journal of Cell Science* 118:4261-4269

Pichierri P, Franchitto A, Mosesso P, Palitti F. 2001. Werner's Syndrome Protein Is Required for Correct Recovery after Replication Arrest and DNA Damage Induced in S-Phase of Cell Cycle. *Molecular Biology of the Cell* 12:2412-2421

Raji H, Hartsuiker E. 2006. Double-strand break repair and homologous recombination in *Schizosaccharomyces pombe*. *Yeast* 23:969

Ramirez CL, Cadinanos J, Varela I, Freije JMP, Lopez-Otin C. 2006. Human progeroid syndromes, aging and cancer: new genetic and epigenetic insights into old questions. *Cellular and Molecular Life Sciences* 64:156

Sambrook J, Fritsch EF, and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3

Tables

Width (nm)						
Double Stranded DNA	13.1	16.5	12.7	11.2	13.1	11.7
Double Stranded DNA + Rqh1-3	25.1	21.4	26.1	23.4	22.8	21.3

Table 1. AFM Measurements of DNA Strands with or without Rqh1-3.

ANOVA	Average	Variance	P-value
Double Stranded DNA	13.05	3.455	2.1E-06
Double Stranded DNA + Rqh1-3	23.43333	3.418667	

Table 2. Anova Single Factor analysis of significant difference between DNA strand widths with or without Rqh1-3.

KM (mM)	VMAX ($\mu\text{M}/\text{min}$)	K2 (min^{-1})	K2/KM ($\text{min}^{-1}/\text{mM}$)	nH
3.08	62.93	43.412	14.095	1.05

Table 3. Kinetic Parameters of Rqh1 Catalyzed ATP Hydrolysis.

Figure Legends

- Fig. 1 A schematic diagram of selected members of the RecQ family of DNA helicases (Bennett and Keck 2004)
- Fig. 2 Human diseases associated with mutations in RecQ homologs and the possible disease causing mechanisms. Adapted from Ramirez et al. 2007.
- Fig. 3 (A) DNA sequence of Rqh1 showing the primer regions, Dead-like helicase domain, HelicC domain, and the HRDC domain. Primer regions are highlighted in order beginning with RecQ-N and continuing with RecQ-353, RecQ-1078, RecQ-1483, RecQ-C. (B) Rqh1 truncations of the N-terminus resulting from designed primers.
- Fig. 4 (A) Amino acid sequence of Rqh1 protein showing Dead-like helicase, HelicC domain, and HRDC domain. (B) Proposed structure of Rqh1 as predicted by SMART amino acid sequence analysis (Gasteiger et al. 2003).
- Fig. 5 Standard Affinity Column Chromatography
- Fig 6 (A) A PCR of positive Rqh1 clones detected on an agarose gel. From left to right: Ladder, Truncation #4, Truncation #4, Truncation #3, Truncation #3, Truncation #2, Truncation #2. (B) Positive clones run on an agarose gel and stained with ethidium bromide. From left to right, Ladder, Full length Rqh1, Topoisomerase III.
- Fig. 7 A Western Blot showing small-scale expression of a Rqh1-3 clone. Wells were loaded with boiled cell extracts and lanes from left to right are Ladder, clones 4-3, 1-6, 2-7, 3-4, and 4-5. The first number in each clone indicates which Rqh1 truncation it is and the second number indicates from which plate it was taken from. An arrow indicates the presence of a band in the 3-4 clone lane.
- Fig. 8 (A) A coomassie gel of Rqh1 purification. Lanes from left to right are Ladder, Crude Extract, Flow Through, Wash, and Elution fractions. A 96 kD band signifies the presence of Rqh1-3. (B) A Western blot probed with antibodies to Rqh1-3. Lanes from left to right are Ladder, Crude Extract, Flow Through, Wash, and Elution fractions. A band indicated by the arrow signifies the presence of 96 kD protein in the Elution fraction lane.
- Fig. 9 DNA Binding Assay. (A) An agarose gel showing Rqh1-3 binding to single stranded DNA.
(B) An agarose gel showing Rqh1-3 binding to double stranded
- Fig. 10 Rate of ATP hydrolysis is dependent upon Rqh1 concentration. ATP hydrolysis was measured via an indirect assay where the Rate of ATP hydrolysis of Rqh1 is correlated to NADH oxidation.

Fig. 11 Atomic force microscopy images. (A) Image of naked double stranded DNA. (B) An image of Rqh1-3. (C) Double stranded DNA in the presence of Rqh1-3.

Fig. 12 (A) Michaelis Menten plot to determine K_m and V_{max} . (B) A Lineweaver Burke Plot which allows visual representation of K_m and V_{max} . (C) Hill Plot of Rqh1 catalyzed ATP hydrolysis yields a slope (NH) of 1.05 indicating uncooperative binding.

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Figures

Fig. 1

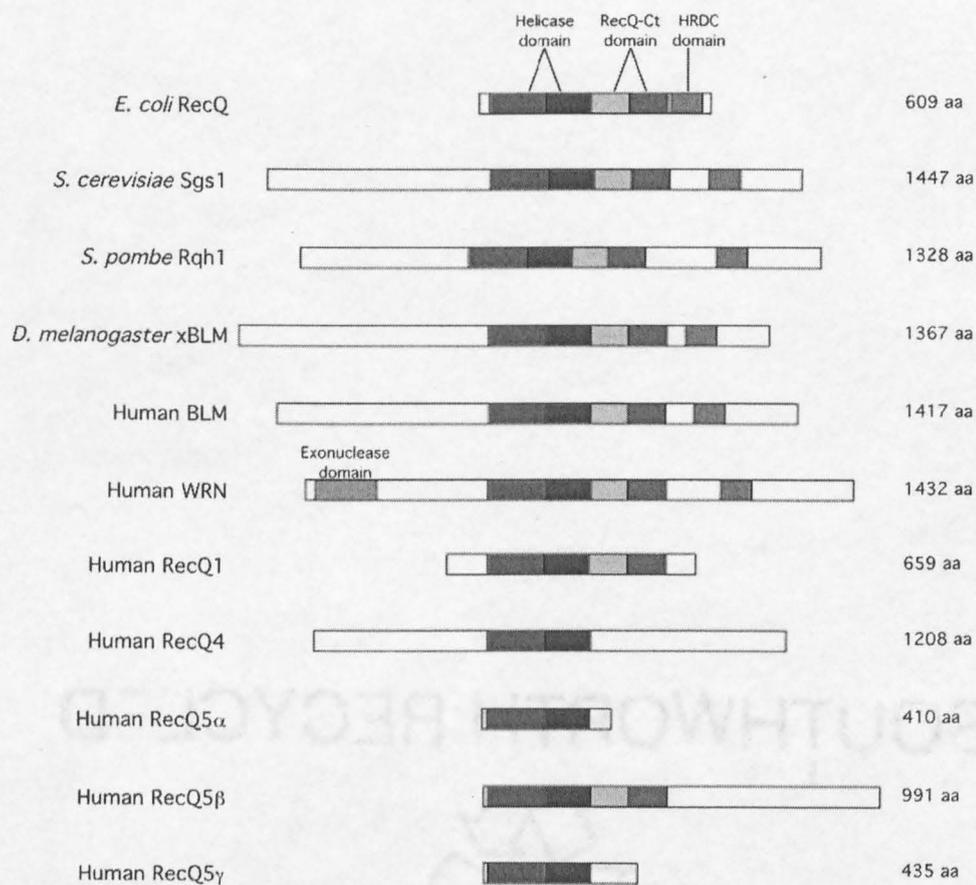


Fig. 2

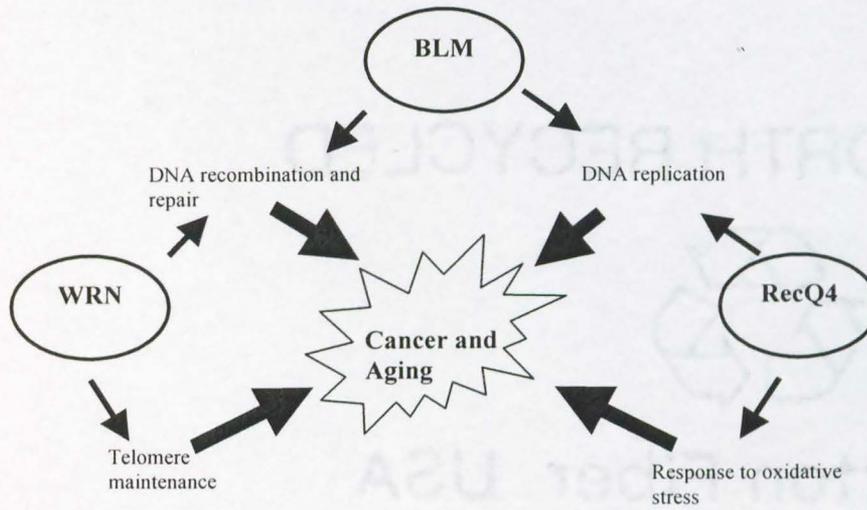


Fig. 3

A

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Primers RecQ-N, RecQ-353, RecQ-1078, RecQ 1483, RecQ-C
Dead-like helicase
HeliCc domain
HRDC domain

B

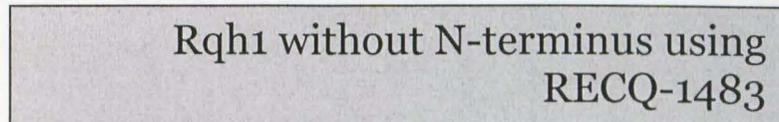
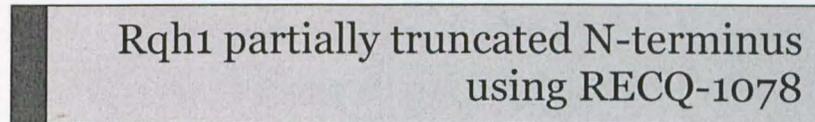
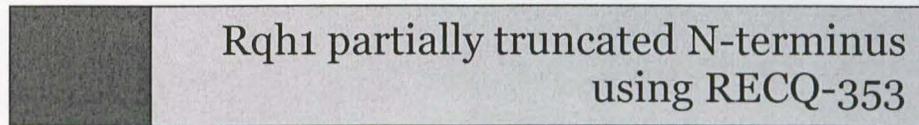
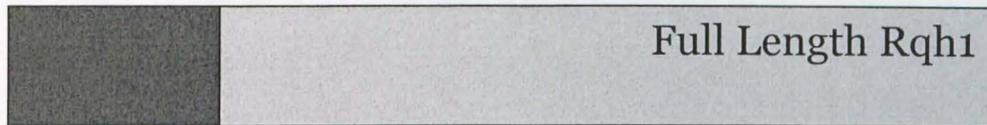


Fig. 4

A

Rqh1 Amino Acid Sequence

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A I D L T E E H Q S S S L N N L K W K D V E G P N I L K P I K K I
A V P A S E S E E D F D D V D E E M L R A A E M E V F Q S C Q P L
A V N T A D T T V S H S T S S S N V P R S L N K I H D P S R F I K
D N D V E N R I H V S S A S K V A S I S N T S K P N P I V S E N P
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N N D N N A I E K R D S A S P T P S S V S S Q I S I D F S T W P H
Q N L L Q Y L D I L R D E K S E I S D R I I E V M E R Y P F S S R
F K E W I P K R D I L S Q K I S S V L E V L S N N N N S N N N N G
N N G T V P N A K T F F T P P S S I T Q Q V P F P S T I I P E S T
V K E N S T R P Y V N S H L V A N D K I T A T P F H S E A V V S P
L Q S N I R N S D I A E F D E F D I D D A D F T F N T T D P I N D
E S G A S S D V V V I D D E E D D I E N R P L N Q A L K A S K A A
V S N A S L L Q S S S L D R P L L G E M K D K N H K V L M P S L D
D P M L S Y P W S K E V L G C L K H K F H L K G F R K N Q L E A I
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A K L R N D Y G L K A W H Y H A G L E K V E R Q R I Q N E W Q S G
S Y K I I V A T I A F G M G V D K G D V R F V I H H S F P K S L E
G Y Y Q E T G R A G R D G K P A H C I M F Y S Y K D H V T F Q K I
I M S G D G D A E T K E R Q R Q M L R Q V I Q F C E N K T D C R R
K Q V L A Y F G E N F D K V H C R K G C D I C C E E A T Y I K Q D
M T E F S L Q A I K L L K S I S G K A T L L Q L M D I F R G S K S
A K I V E N G W D R L E G A G V G K L L N R G D S E R L F H H L V
S E G V F V E K V E A N R R G F V S A Y V V P G R Q T I I N S V L
A G K R R I I L D V K E S S S K P D T S S R S L S R S K T L P A L
R E Y Q L K S T T A S V D C S I G T R E V D E I Y D S Q M P P V K
P S L I H S R N K I D L E E L S G Q K F M S E Y E I D V M T R C L
K D L K L L R S N L M A I D D S R V S S Y F T D S V L L S M A K K
L P R N V K E L K E I H G V S N E K A V N L G P K F L Q V I Q K F
I D E K E Q N L E G T E L D P S L Q S L D T D Y P I D T N A L S L
D H E Q G F S D D S D S V Y E P S S P I E E G D E E V D G Q R K D
I L N F M N S Q S L T Q T G S V P K R K S T S Y T R P S K S Y R H
K R G S T S Y S R K R K Y S T S Q K D S R K T S K S A N T S F I H
P M V K Q N Y R Stop

Dead-like helicase

HeliCc domain

HRDC domain

B



SOUTHWORTH RECYCLED



25% Cotton Fiber USA

Fig. 5

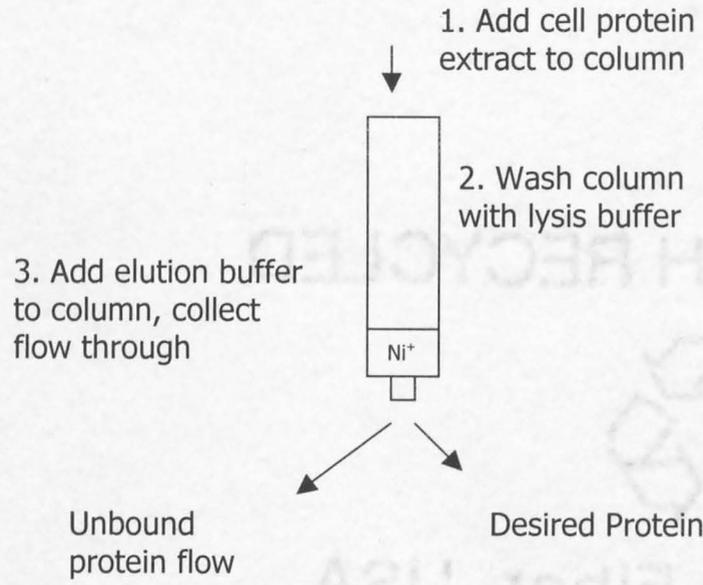


Fig. 6

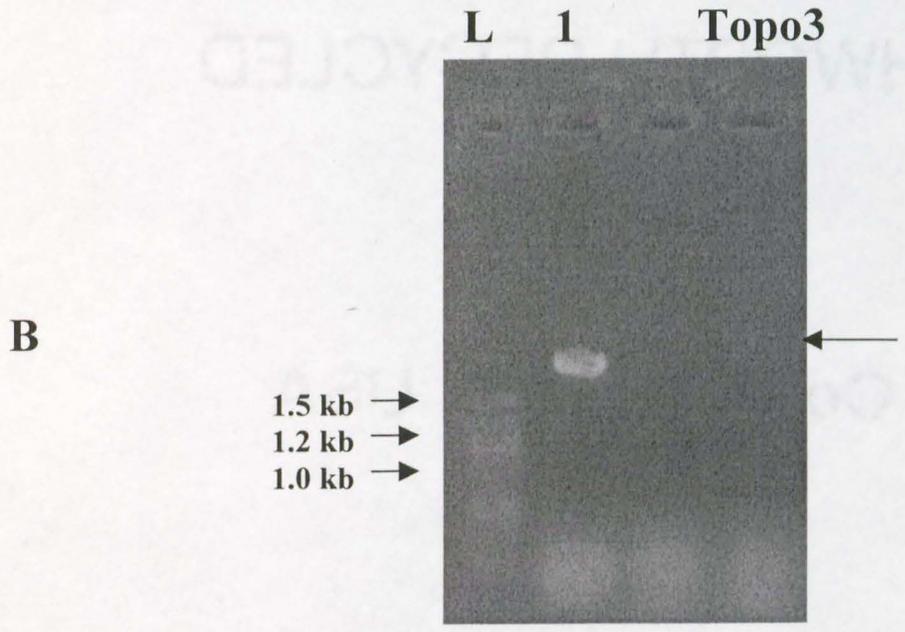
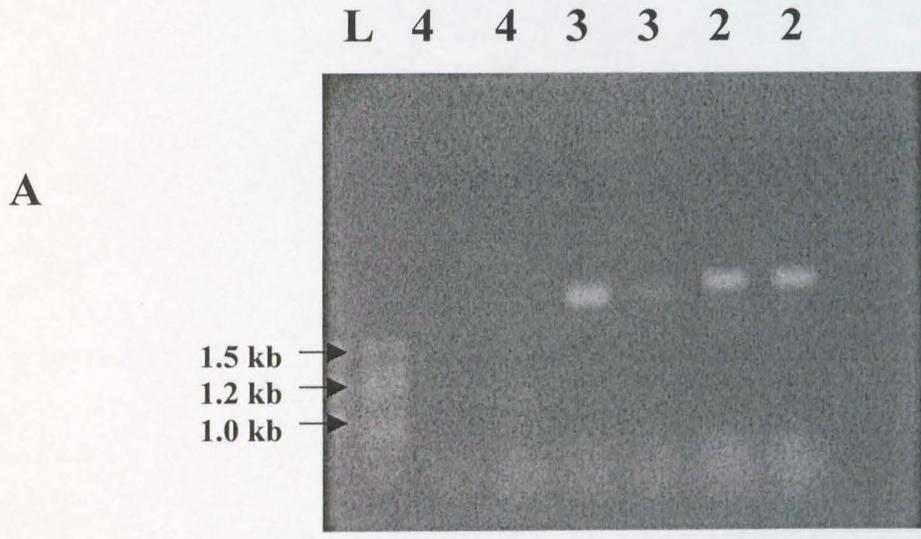


Fig. 7

L 4-3 1-6 2-7 3-4 4-5

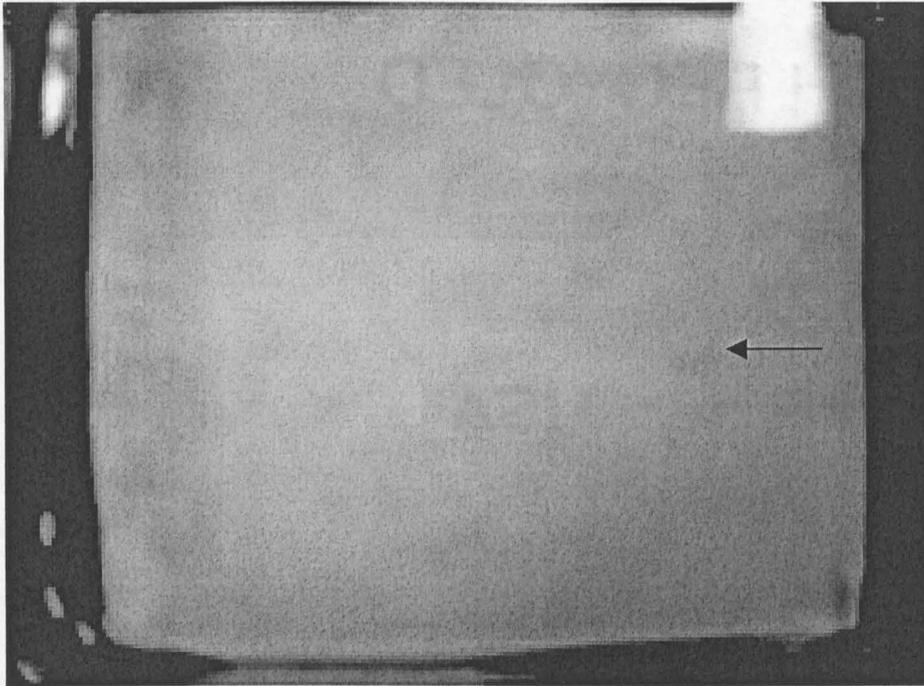


Fig. 8

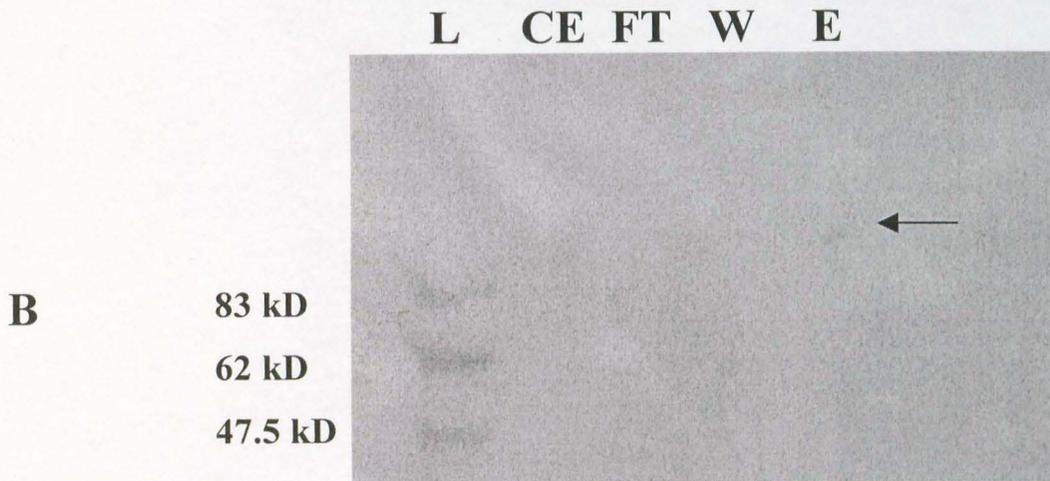
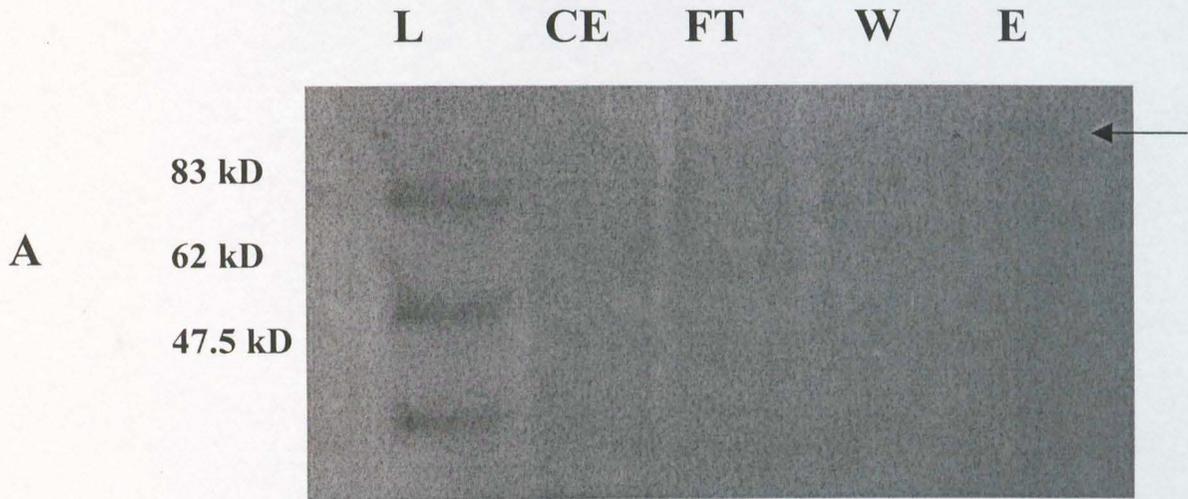


Fig. 9

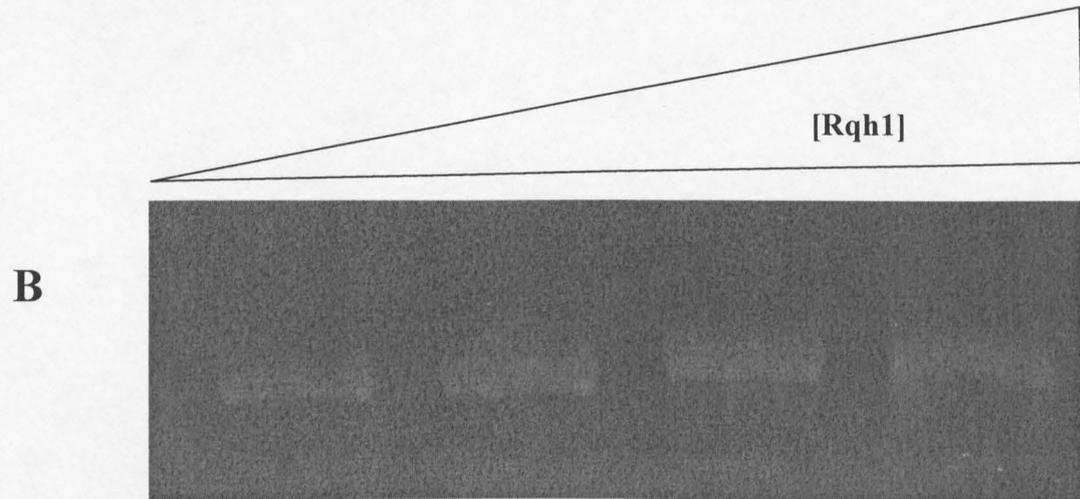


Fig. 10

Rate of ATP Hydrolysis with Varying [Rqh1] (μM)

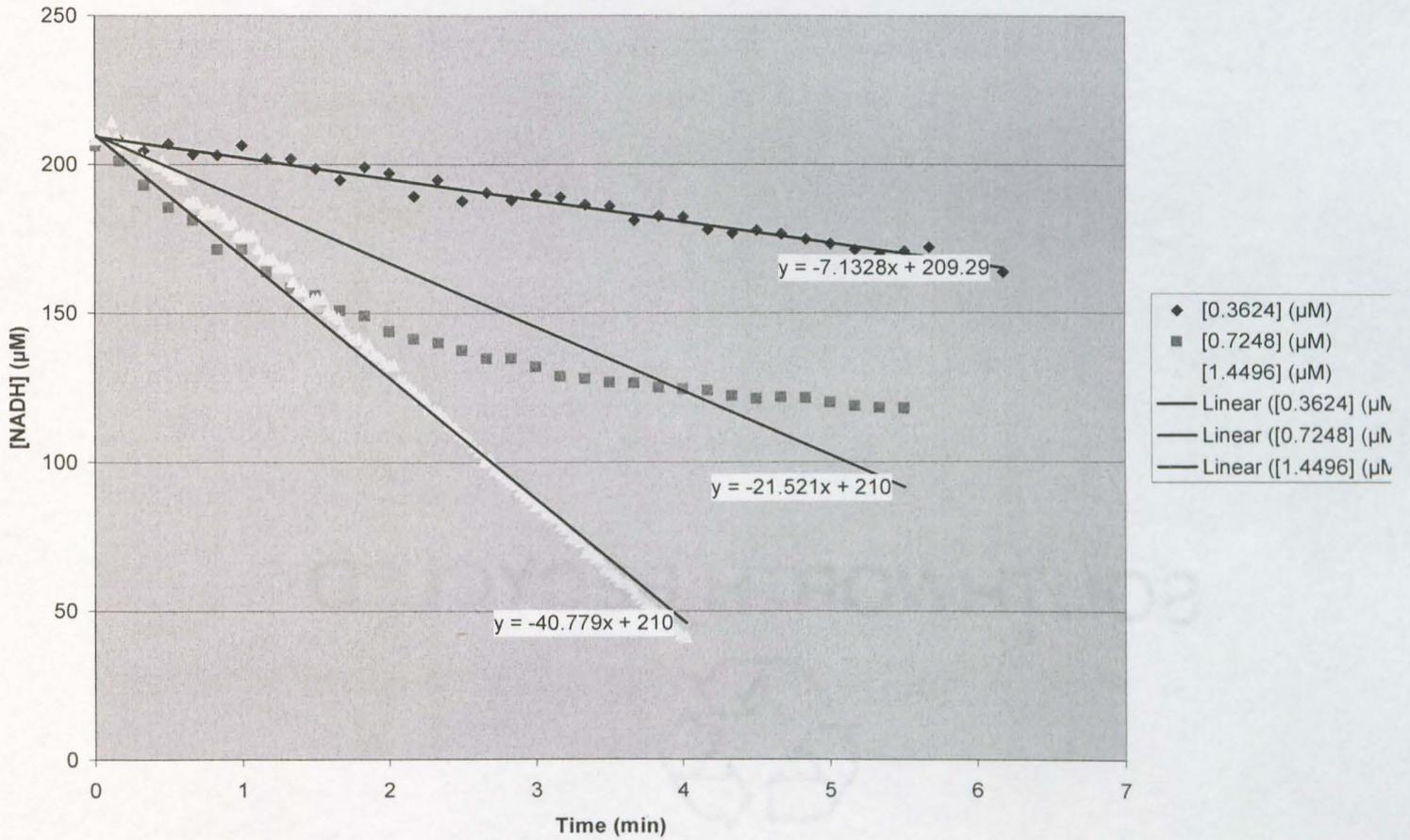
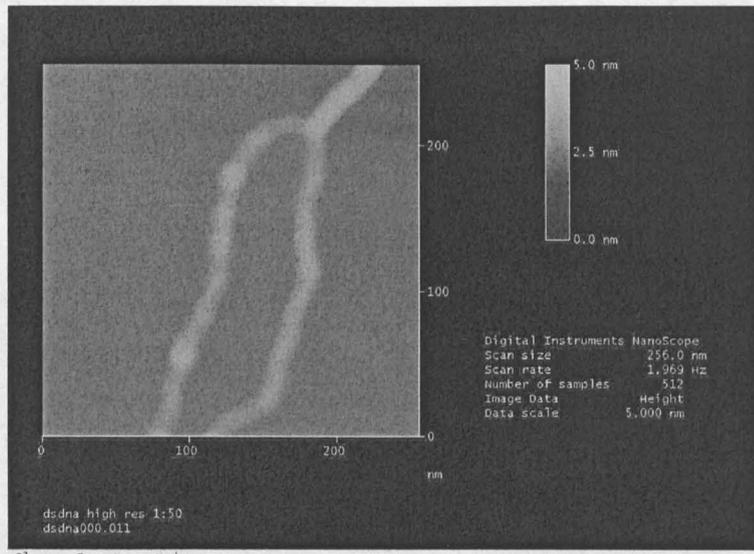
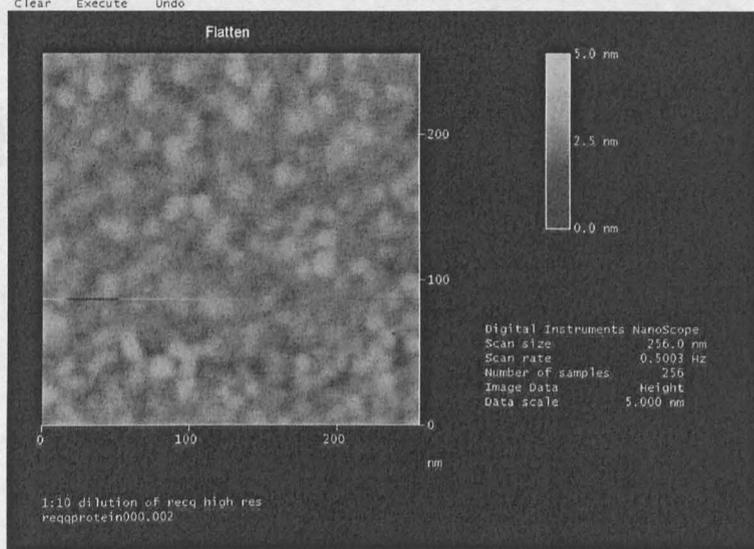


Fig. 11

A



B



C

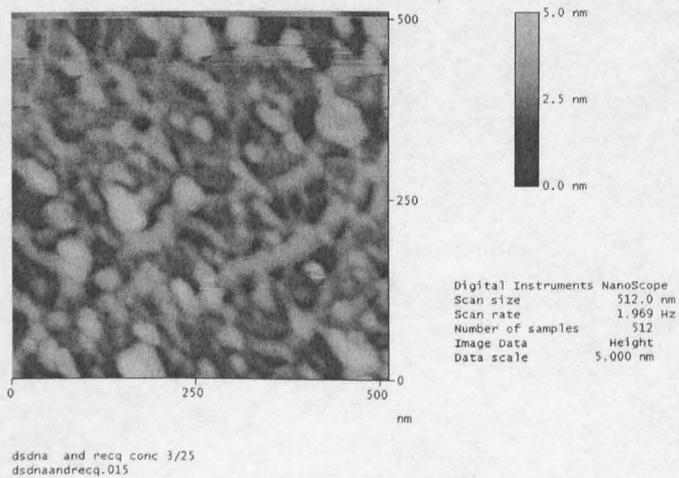


Fig. 12

