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Brian Fredericks Gilmore	Date

A Biochemical Analysis of the Senataxin Protein

by

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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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The yeast protein Sen1 is essential for cell viability. Although its essential function is unknown, homology analysis has established a substantial sequence similarity between the C-terminal region of Sen1 and Superfamily I helicases (Steinmetz *et al.* 2006). Additionally, this region of helicase homology has been established as the same region required for cell viability. However, helicase activity has only been demonstrated in the Sen1 protein of *Schizosaccharomyces pombe* (Kim, *et al.,* 1999).

The human ortholog of Sen1, senataxin, is poorly understood. However, positional cloning studies have established that mutations in the senataxin-encoding SETX gene are linked to the neurodegenerative disorders AOA2 and ALS4. The positions of these mutations typically result in truncated proteins lacking the helicase homology domain or mutations within the helicase motifs, although other mutations have been identified (Bassuk *et al.* 2007). However, without additional knowledge of the biochemical function of senataxin, the functional effects of the mutations remain unknown. Thus, the effort to determine the function and significance of the Senataxin protein in humans is framed by the need to understand the functional significance of these disease-causing mutations.

Herein, we analyze the functional biochemistry of senataxin and provide some evidence that the protein exhibits helicase activity *in vitro* and that this activity is lost in disease-linked mutants. Additionally, our results indicate that senataxin may function as a nucleic acid-stimulated ATPase under specific conditions. However, these findings are

thus far inconclusive and additional studies are required to confirm the *in vitro* function of senataxin and how this relates to its essential *in vivo* role.

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I would like to thank Dr. Gray Crouse for serving on my defense committee, and Dr. Barry Yedvobnick for his continued support of me as a teacher, an evaluator and a mentor.

I extend my deepest appreciation to all the members of the Reines lab, particularly Tom O'Rourke and M. Harley Jenks. Through their technical expertise, experimental assistance and personal involvement they have contributed invaluably to my research experience at every turn.

Most of all, I would like to thank Danny Reines for his constant support, guidance and encouragement. I cannot overstate how valuable his mentorship has been in nurturing my curiosity and promoting my development as both a student and a scientist.

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Introduction

1. Helicases

Helicases are a well-studied, established class of enzymes that carry out the ATP-dependent unwinding of a nucleic acid double helix through the sequential destabilization of complementary base-pairing. The large scale-study of the amino acid sequences of a number of known helicases has led to the characterization of a number of conserved domains and specific functional motifs in both RNA and DNA helicases (Singleton *et al.,* 2007). ATP binding and hydrolysis induces structural changes that allow the protein to processively translocate along a nucleic acid, unwinding the duplex. Although the destabilization of the nucleic acid double helix is the characteristic activity of helicases, certain helicases are known to function in post-transcriptional RNA rearrangement or the disruption of RNA/DNA-protein interactions (Tanner and Linder, 2001).

Superfamily I helicases are perhaps the most thoroughly studied class of helicase enzymes (Singleton *et al.*, 2007). Consisting of both DNA and RNA helicases, and known to be both ATP and magnesium dependent, these enzymes are distinguished by eight highly conserved motifs known to be critical for helicase activity. These include motifs responsible for NTP-binding (including the so-called Walker motifs, conserved between NTPase enzymes), substrate binding and the coupling of NTP hydrolysis to helicase activity (Table 1). The conserved catalytic cores of these proteins are arranged into two domains with the conserved motifs oriented towards the cleft between the domains where NTP and nucleic acids are bound (Tanner and Linder, 2001).

Table 1: SF1 helicase motifs (adapted from Tanner and Linder, 2001)

Motif	Function
I (Walker A)	NTP-binding
Ia	Substrate-binding
Ib	Substrate-binding
II (Walker B)	NTP-binding via Mg^{2+}
III	Links NTP hydrolysis to enzymatic activity
IV	Substrate-binding
V	Substrate-binding
VI	Links NTP hydrolysis to enzymatic activity

2. Sen1

The *Saccharomyces cerevisiae* protein and putative helicase Sen1 is known to be essential for cell growth and viability (DeMarini *et al.,* 1992). Although the exact nature of that essential function remains unknown, it is presumed to lie in the role of Sen1 in the termination of small transcription units or the processing of RNA (Steinmetz *et al.,* 2006).

Initially thought to be an endonuclease involved in tRNA-splicing pathways (a function from which the protein derives its name—**s**plicing **en**donuclease) (DeMarini *et al.,* 1992), it is now thought that Sen1 is a nucleic acid helicase. Although this helicase activity had not previously been verified *in vitro*, it was supported by substantial sequence homology between the carboxy-terminal end of the Sen1 protein and conserved regions of known helicases such as *S. cerevisiae* Upf1 (Ursic *et al.*, 1997). This helicase homology domain contains several motifs conserved between Superfamily I helicases (Singleton *et al.*, 2007). Additionally, this C-terminal helicase homology domain has

been characterized as the minimum region required for cell viability (DeMarini *et al.,* 1992).

Many studies have analyzed the *in vivo* roles of Sen1, identifying several roles in transcription, particularly in transcription termination and subsequent RNA processing (Ursic *et al.*, 1997; Rasmussen and Culbertson, 1998; Steinmetz *et al.*, 2006). However, almost none have attempted to characterize the functional biochemistry of the Sen1 protein *in vitro* or confirm the presumed helicase activity. One exception to this is the work done by Kim *et al.* (1999) with the *Schizosaccharomyces pombe* homolog of Sen1. That research convincingly demonstrated that the *S. pombe* Sen1 is a helicase capable of unwinding the strands of a nucleic acid helix in vitro. It was found that the helicase activity was dependent on both ATP and magnesium and that the protein exhibited a 5'to 3' directionality. Through the use of a variety of different helicase substrates, Sen1 was shown to be capable of acting on either DNA/DNA or RNA/RNA homoduplexes as well as DNA/RNA heteroduplexes, with a clear preference for DNA/DNA substrates. In addition to functioning as an ATP-dependent helicase, Sen1 was found to exhibit nucleic-acid dependent ATPase activity. Another noteworthy finding was that while a purified, full-length Sen1 protein was enzymatically active, only the C-terminal region of the protein, which contains the helicase homology domain, is required for enzymatic activity. Although these findings clearly demonstrate that the *S. pombe* Sen1 protein exhibits helicase activity, that homolog is the only Sen1 protein for which helicase activity had been previously confirmed.

3. Senataxin

Senataxin, a 303 kDa protein encoded by the SETX gene, is the human ortholog of Sen1. Like its yeast counterpart, the C-terminal region of senataxin contains a domain with substantial sequence homology to Superfamily I helicases (Moreira *et al.*, 2004). In addition to this helicase homology domain, the N-terminal region of the protein contains a non-essential domain thought to function in protein-protein interactions *in vivo* (Chen *et al.*, 2006; Bassuk *et al.* 2007). Like Sen1, the presumed helicase activity of senataxin had not previously been verified *in vitro*. However, senataxin has been implicated in a wide range of *in vivo* roles which include transcriptional regulation, DNA damage repair and RNA processing (Suraweera *et al.*, 2009; Strong, 2010).

The studies described herein of the *in vivo* roles and *in vitro* function of senataxin are framed by its direct relevance to human health. Positional cloning studies have established that certain mutations in the SETX gene are linked to the neurodegenerative disorders Ataxia-Oculomotor Apraxia type 2 (AOA2) and Amyotrophic Lateral Sclerosis type 4 (ALS4) (Moreira *et al.*, 2004; Chen *et al.*, 2006; Bassuk *et al.*, 2007; Strong, 2010) (Figure 1). ALS4 is an autosomal dominant variant of familial ALS which leads to juvenile onset of progressive degeneration of motor neurons in the brain and spinal cord (Strong, 2010). Although muscle atrophy and spasticity are common between ALS variants, ALS4 differs from classical ALS in that it does not affect either respiratory muscles or patient longevity (Chen *et al.*, 2006). In contrast, AOA2 is an autosomal recessive, adult-onset disorder that leads to the progressive degeneration of the cerebellum, resulting in tremors and oculomotor ataxia (Bassuk *et al.*, 2007; Chen *et al.* 2006). Some of these disease-causing mutations have been further characterized and

shown to yield either nonfunctional full-length protein bearing mutations in critical helicase motifs or severely truncated senataxin protein missing some of the putative helicase domain (Moreira *et al.*, 2004). However, as the biochemical and *in vivo* functions of senataxin are still unknown, the functional consequences and true significance of these mutations remain unclear.

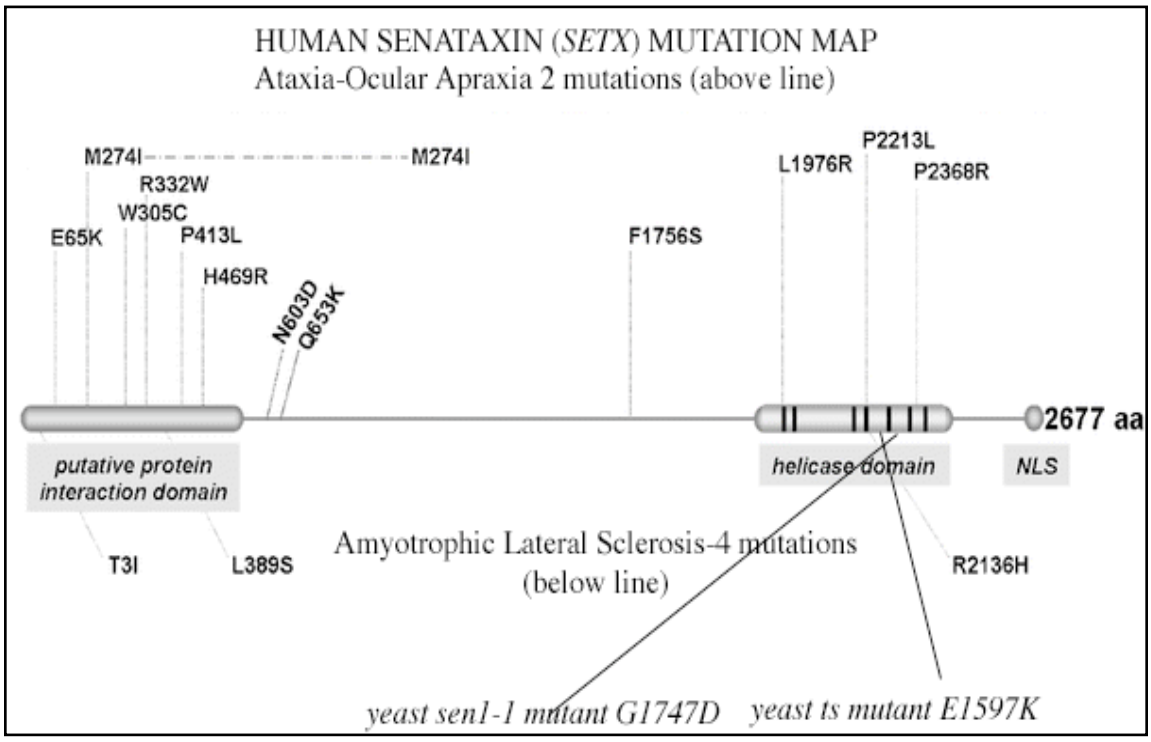

Figure 1: Modified from Bassuk et al. (2007). Mutations associated with AOA2 are shown above the line. Mutations associated with ALS4 are below. Additionally, the orthologs of two common yeast mutations are indicated.

4. Background

The involvement of the Reines lab in the study of Sen1 and Senataxin developed from an analysis of the unique transcription termination mechanism of the *S. cerevisiae*

IMD2 gene. Expression of IMD2, which encodes IMP dehydrogenase (IMPDH), an enzyme essential for the *de novo* synthesis of guanine, is regulated by cellular guanine levels (Figure 2). In high guanine conditions, transcription originates at any of several upstream initiation sites and is terminated at an intergenic terminator sequence. This produces short, unstable transcripts which are degraded by exosomes. However when guanine is scarce, transcription begins at a single, strong initiation site and reads through the IMD2 ORF, creating stable transcripts encoding the IMPDH protein and allowing for the *de novo* synthesis of guanine.

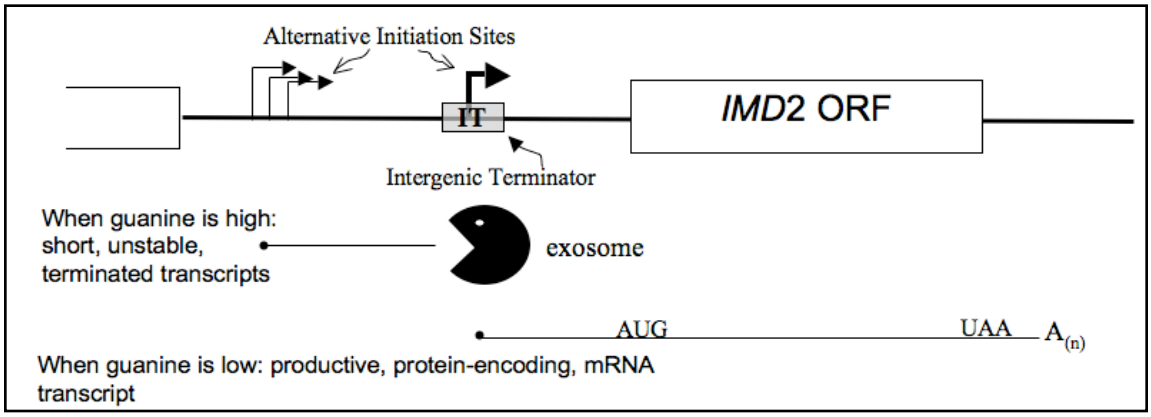

Figure 2: Schematic showing the guanine-dependent transcription termination system of IMD2 in *S. cerevisiae*.

In analyzing the genome-wide Sen1 function, it was found that the temperature-sensitive *sen1-E1597K* mutation prevented yeast from terminating transcription of short RNAs at upstream intergenic terminators (Steinmetz *et al.* 2006). In the case of IMD2, this prevented the production of these short, non-coding transcripts and resulted in read through into the downstream IMD2 ORF. These results suggest a role for Sen1 in the

transcription termination of small noncoding RNAs. However the precise role played by Sen1, whether it is destabilizing the DNA-RNA duplex, rearranging the RNA structure or promoting the dissociation of proteins from either the DNA or RNA, remains unclear. This potential *in vivo* role for the Sen1 protein prompted examination of the functional biochemistry of the Sen1 protein in order to characterize the activity of the enzyme and confirm the presumed helicase function. However, because of its more direct application to human health, the focus of this research was on the human ortholog of Sen1, senataxin.

5. Specific Aims

The goal of this project is to determine the functional biochemistry of the human senataxin protein through assays for helicase activity and ATPase activity in order to:

a) Demonstrate directly the helicase activity of the Senataxin protein for the first time.
b) Analyze the substrate specificity, and minimal reaction requirements of Senataxin in order to suggest a possible *in vivo* function of the protein.
c) Determine the functional consequences of relevant mutations known to cause the neurodegenerative disorders AOA2 and ALS4.

Methods and Materials

1. E. coli Expression Constructs

To facilitate production of large quantities of biochemically functional senataxin, a portion of the SETX gene was used to create a number of *E. coli* expression constructs.

1.1: pET28a-SETX

A pCMV-SPORT6 plasmid containing a cDNA clone of the SETX gene was obtained from Open Biosystems (accession BC032600). PCR was used to amplify a 2.4 kb region of this clone including the helicase homology domain (Figure 3). This PCR product was then digested with the restriction endonucleases SalI and NotI and inserted into a SalI and NotI digested pET28a plasmid (a Novagen *E. coli* expression system). Successful insertion was confirmed by sequencing.

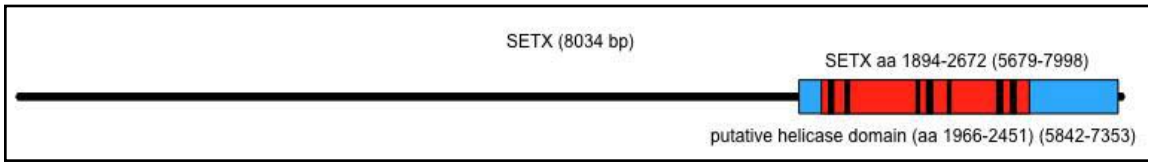

Figure 3: The human SETX gene. The amplified region (bases 5679-7998, encoding aa 1894-2672) is in blue and includes the helicase homology domain (bases 5842-7353, encoding aa 1966-2451) is in red. The seven conserved helicase motifs, as described by Singleton *et al.,* (2007) are indicated by black bands.

The resulting construct, pET28a-SETX, placed SETX under an IPTG-inducible *E. coli* promoter and produced a 90 kDa protein containing the minimal helicase homology domain as well as both N- and C-terminal His_6-tags to allow for purification by nickel chromatography (Figure 4).

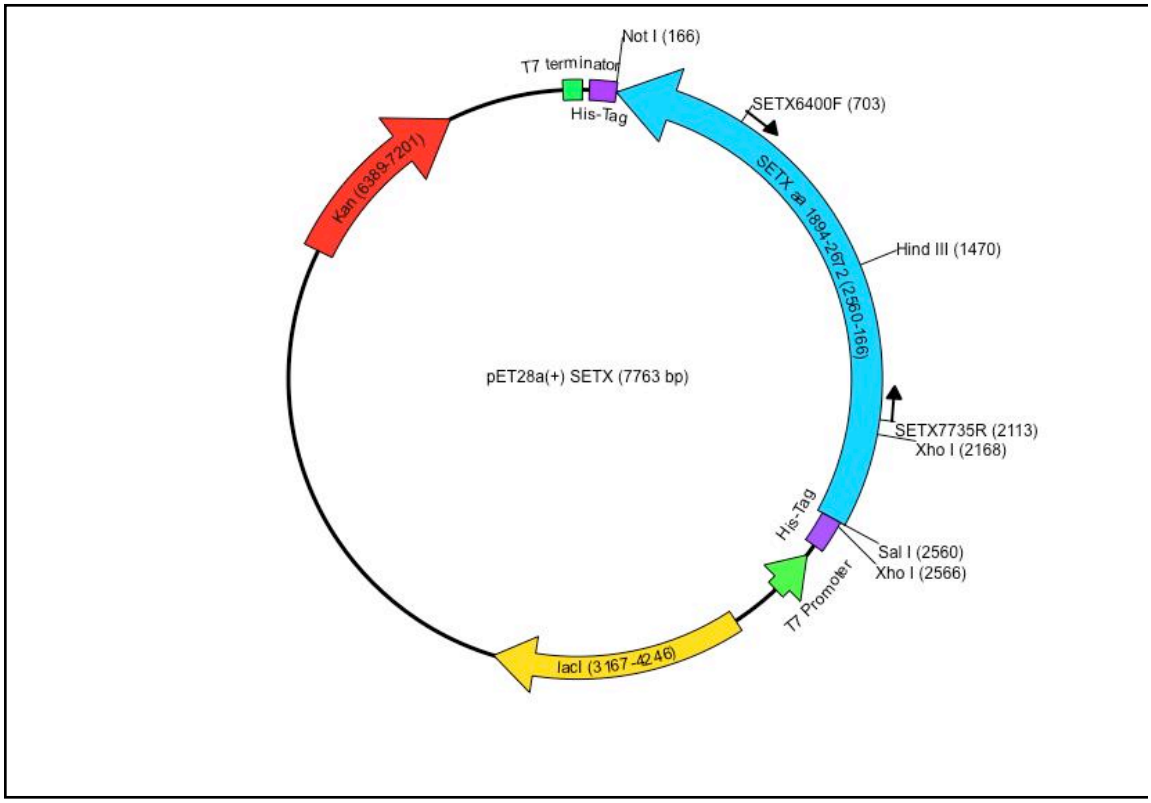

Figure 4: The pET28a-SETX Expression construct

1.2: pGEX-SETX

An alternative expression vector was created by M.H. Jenks and D. Reines to allow for affinity purification on glutathione sepharose. This plasmid, pGEX-SETX was constructed by removing the SETX sequence from pET28a-SETX by

SalI and NotI digestion and inserting it into a SalI/NotI digested pGEX vector. This expression plasmid yields a 120 kDa protein containing the putative helicase domain of senataxin and a glutathione-S-transferase (GST) tag. Treatment with Tobacco Etch Virus (TEV) protease leads to removal of the GST tag, altering the size of the protein and producing a visible mobility shift after SDS-PAGE.

1.3: Generation of Patient Mutants

Site-directed mutagenesis was performed on the pGEX-SETX expression plasmid by M.H. Jenks and D. Reines in order to generate a number of previously-characterized senataxin mutants for biochemical analysis. These include mutations associated with the neurodegenerative disorders AOA2 and ALS4 as well as the human ortholog of a temperature sensitive yeast mutation (Table 2).

Table 2: SETX Mutants and Effects

Mutant	**Source**	**Effect**	**Citation**
E2188K	SETX Ortholog of yeast sen1 E1597K	temperature-sensitive missense	Steinmetz and Brow, 2006
P2213L	AOA2 patient	missense	Moreira *et al.,* 2004
D2207V	AOA2 patient	missense	Bernard *et al.,* 2009
K2382E	AOA2 patient	missense	Bernard *et al.,* 2009
R2414Q	AOA2 patient	missense	Bernard *et al.,* 2009

2. Protein Production and Purification

The pET28a-SETX and pGEX-SETX plasmids were used to transform *E. coli* of the CodonPlus strain. This strain over-expresses tRNAs to allow for more efficient expression of human proteins. Transformed cells were grown at 30ºC to an OD_{600} of 0.5-0.7 AU and then treated with 0.5 mM Isopropyl β–D-1-thiogalactopyranoside (IPTG) at 30ºC for 3 hours to induce production of recombinant senataxin. Cells were collected by centrifugation and sonicated to yield a protein extract for purification.

2.1: Purification from pET28a-SETX

A protein extract from IPTG-induced, pET28a-SETX-transformed *E. coli* was loaded onto an Invitrogen ProBond nickel column. This column was then washed with low-imidazole buffer. A gradient up to 0.5 M imidazole was used to elute the protein from the column. The resulting fractions were then analyzed by SDS-PAGE. Those fractions containing a clear band at 90 kDa were then subjected to additional purification by ion-exchange chromatography.

Peak SETX fractions were loaded onto a Diethylaminoethyl (DEAE) column. This column was eluted with a gradient from 0.005 M NaCl to 0.5 M NaCl. Peak protein fractions were then analyzed by SDS-PAGE.

2.2: Purification from pGEX-SETX

A protein extract from pGEX-SETX-transformed *E. coli* was incubated on a column of glutathione-Sepharose™ resin overnight at 4ºC. After incubation and washing, the column was eluted with a series of 1 mL glutathione buffer washes

at 4ºC. The resulting eluates were collected and measured for protein concentration by Bradford Assay. Peak fractions were then TCA precipitated and analyzed by SDS-PAGE.

Production and purification of GST-SETX protein from the pGEX-SETX plasmid was carried out by M.H. Jenks and T. O'Rourke of the Reines lab. Optimization of this protocol has been ongoing in order to reduce known contaminants and increase protein yield.

3. Helicase Assay

The assay for helicase activity in which recombinant senataxin proteins are tested for their ability to processively unwind the two strands of a nucleic acid duplex, is a modification of the protocol used by Kim *et al.*, (1999) in studying the helicase activity of the *S. pombe* Sen1 homolog.

3.1: Protocol

Purified, recombinant senataxin proteins were incubated with a helicase substrate (see section 3.2) at 31ºC. Reactions were performed in 10 mM Tris-Cl at pH 7.5 with or without 5 mM $MgCl_2$ and with or without 5 mM ATP. After 30 minutes (unless otherwise indicated), reactions were stopped with 40 mM EDTA and 1% SDS. The stopped reactions were subjected to polyacrylamide gel electrophoresis on a non-denaturing gel to distinguish double-stranded substrate molecules from single-strand products of helicase activity. Gels were dried and

exposed to film at -80ºC. Minor variations to this protocol were used to characterize the activity, substrate specificity and minimum reaction requirements of senataxin.

3.2: Substrates

Helicase activity was characterized using double-stranded nucleic acid substrates. These substrates consisted of a short, radiolabeled oligonucleotide annealed to a longer, complementary oligonucleotide. The first such substrate used the single-stranded M13 phage genome annealed to a 17-base, γ-^{32}P-labelled oligonucleotide. However, this initial substrate failed to consistently enter the gels used to resolve the assays and its use was discontinued. The primary substrate used, M13 (-20) + M13 5'OHR, consisted of a 17-base DNA oligonucleotide M13(-20) (sequence GTAAAACGACGGCCAGT) annealed to a complementary 27-base DNA oligonucleotide, M13 5'OHR (sequence TTTTTTTTTTACTGGCCGTCGTTTTAC) resulting in a substrate that was double-stranded for a stretch of 17-bp with a 10-base 5'-overhang (Figure 5). Other substrates with 3'-overhangs and no overhangs were also used to test the directionality of the helicase. The shorter strand was 5'-end labeled with γ-^{32}P ATP using polynucleotide kinase while the longer strand was left unlabeled.

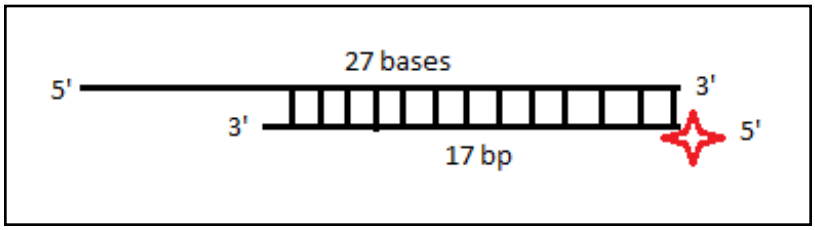

Figure 5: A representative substrate: M13(-20) + M135'OHR. A 17-base DNA oligonucleotide is annealed to a complementary 27-base DNA oligonucleotide creating a 17-bp region of double-stranded substrate and a 10-base single-stranded 5' overhang. γ-^{32}P is represented by an asterisk.

4. ATPase Assay

Recombinant senataxin protein was assayed for the ability to cleave the phosphodiester bonds of ATP, a known function of ATP-dependent enzymes. Purified senataxin was incubated with γ-^{32}P-labeled ATP at 31ºC for 30 minutes. This reaction was carried out in 10 mM Tris-Cl at pH 7.5 with or without 5 mM $MgCl_2$ and stopped with 40 mM EDTA and 1% SDS. Additional nucleic acid cofactors were used to analyze the nucleic-acid dependence of this activity. These cofactors included unlabeled M13(-20) + M135'OHR helicase substrate (Figure 5), salmon sperm DNA or yeast total RNA. Stopped reactions were subjected to PAGE on a non-denaturing gel in order to physically separate the labeled ATP from the hydrolyzed phosphate. Gels were then exposed to film.

Results

When protein extracts from pET28a-SETX-transformed *E. coli* induced with IPTG were analyzed by SDS-PAGE and compared to non-induced protein extracts, a clear induction of a 90 kDa protein, thought to be recombinant senataxin, was observed. In order to separate the senataxin from endogenous proteins, the protein extracts were subjected to nickel-affinity chromatography. This first chromatographic step resulted in some purification of the putative senataxin protein from the crude extract (Figure 6; compare lanes 5-11 to lane 2), but failed to remove many of the contaminating *E. coli* proteins. Those protein fractions that contained the greatest amount of putative-senataxin and the fewest contaminants (Figure 6, lanes 5, 6 and 7) were pooled and subjected to additional chromatography on a DEAE column. SDS-PAGE of the protein fractions from this second round of chromatography indicated some additional, but still incomplete purification of senataxin (Figure 7; compare lanes 6-8 to lanes 2 and 3).

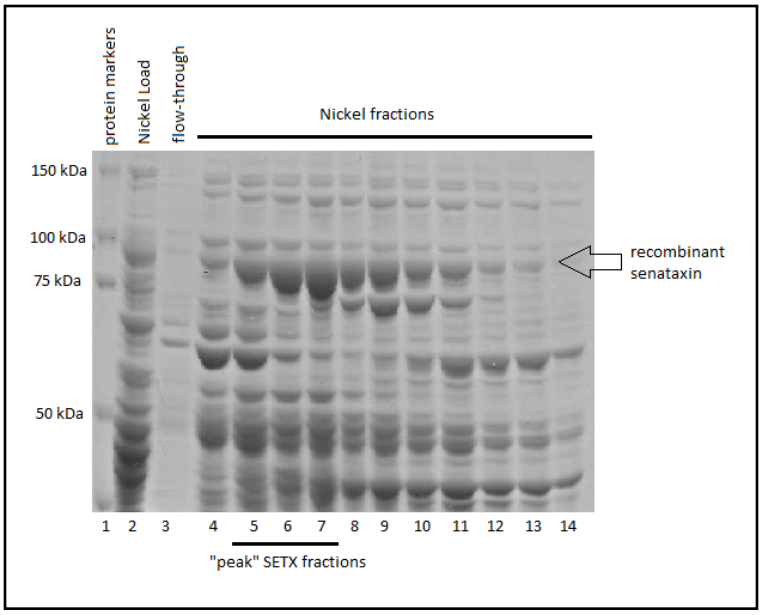

Figure 6: SDS-PAGE of the peak protein fractions resulting from nickel-affinity purification of the pET28a-SETX transformed *E. coli* protein extract. A heavy band between 75 and 100 kDa, not present in untransformed cells, is the senataxin protein. Three peak senataxin fractions (lanes 5-7, marked as "peak" SETX fractions) were pooled and further purified.

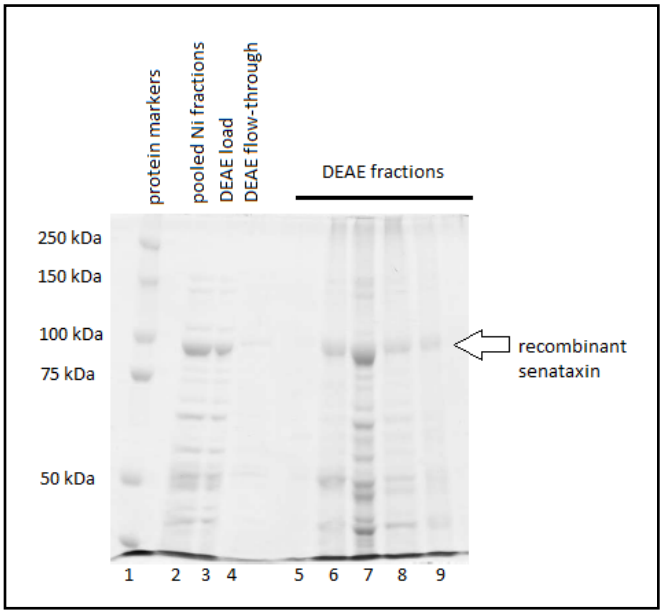

Figure 7: SDS-PAGE of the peak protein fractions resulting from DEAE chromatography of peak nickel-purified fractions. A clear enrichment of senataxin is seen in the DEAE fractions, particularly in lane 7.

The protein fractions resulting from this DEAE-chromatography were subjected to helicase assays. Early tests indicated that a peak of helicase activity correlated with the presence of the 90 kDa protein assumed to be the recombinant senataxin (Figure 8, lanes 4, 5, 6 and 7). Additional helicase assays verified this co-chromatographing of helicase activity and recombinant senataxin (data not shown).

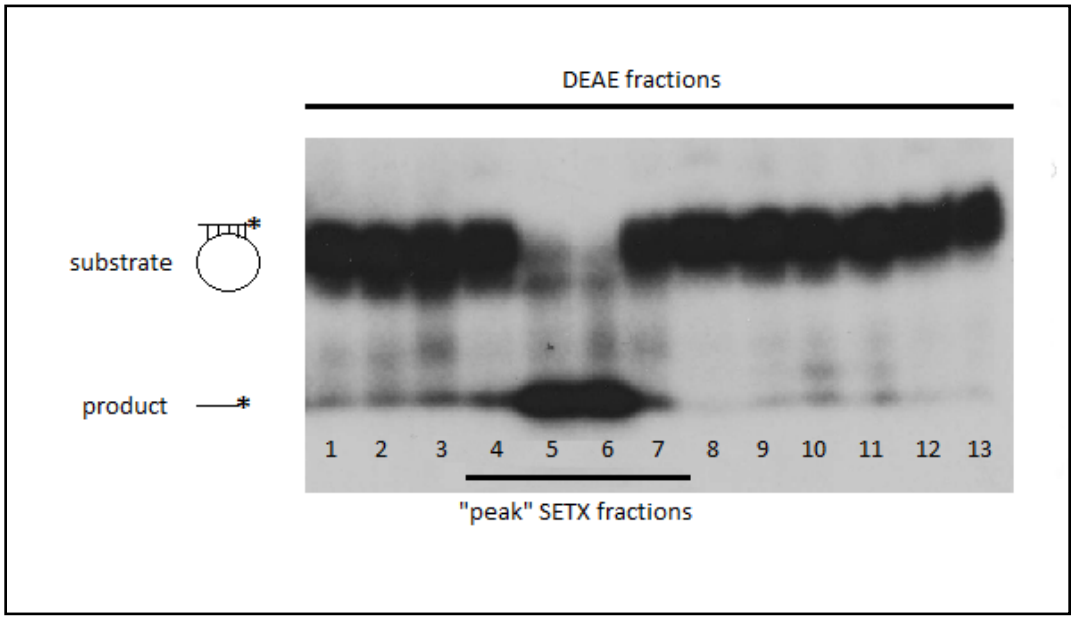

Figure 8: Helicase assay demonstrating the correlation of helicase activity and the presence of the 90 kDa recombinant senataxin protein (marked as “peak” SETX fractions). The M13 circular helicase substrate was used for this assay.

However, when *E. coli* were transformed with empty pET28a vector and the resulting protein extract was purified and analyzed, similar patterns of activity were observed (Figure 9). This finding suggests that the observed helicase activity was due, at least partially, to the presence of co-chromatographing *E. coli* helicases.

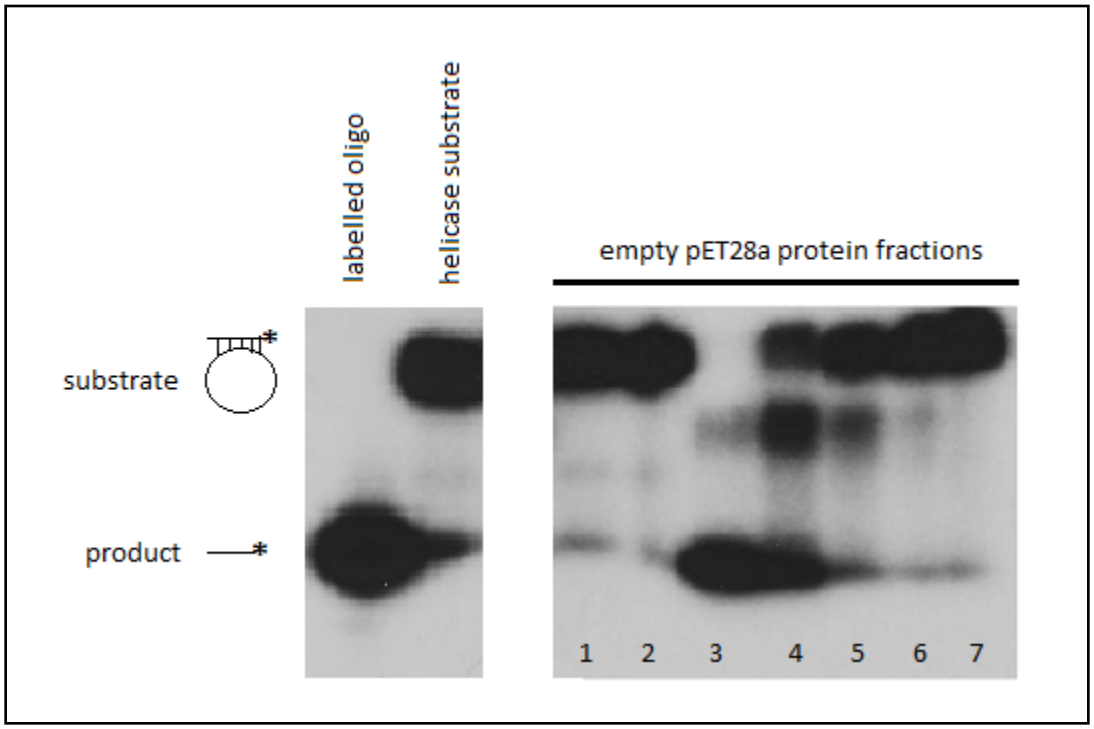

Figure 9: Helicase assay of purified protein extract from empty pET28a-transformed *E. coli* demonstrating the presence of contaminating helicase activity in protein fractions comparable to those used in Figure 8. The labeled oligo/helicase substrate were run on the same gel as markers.

Although nickel affinity chromatography failed to reliably produce purified senataxin, M.H. Jenks and T. O'Rourke were successful in generating another expression construct capable of producing recombinant senataxin linked to a glutathione-S-transferase (GST) tag via a TEV protease recognition site. The identity of this GST-tagged senataxin, which could be highly enriched by purification on glutathione sepharose columns, was confirmed by treatment with TEV protease, which removed the 30 kDa GST tag and caused a clear mobility shift of the protein (Figure 10). Because of their relative purity, these glutathione-purified proteins, produced by T. O'Rourke and M.H. Jenks, were the subject of all future helicase assays.

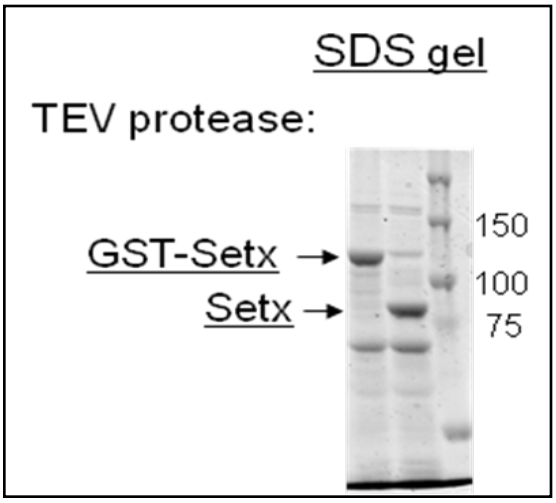

Figure 10: SDS-PAGE demonstrating the mobility shift caused by removal of the GST tag from the GST-SETX protein (M.H. Jenks).

Early helicase assays performed by M.H. Jenks and D. Reines strongly suggested that this recombinant senataxin was a functional helicase exhibiting 5' to 3' directionality and a preference for DNA substrates. These findings agree with the results described by Kim *et al.* (1999) in their studies with *S. pombe* Sen1. These preliminary results prompted an analysis of the minimum reaction requirements of the protein.

Although helicases are known to be dependent upon both magnesium and ATP, when senataxin was assayed for magnesium and ATP-dependence its activity initially appeared to be independent of either cofactor (Figure 11; lanes 1 and 2). However, treatment with EDTA prevented helicase activity (Figure 11; lane 5), indicating that the enzyme was magnesium-dependent and that magnesium, required by the polynucleotide kinase used to ^{32}P label the substrate, was carried over into the helicase reactions. It was also found that additional ATP inhibited helicase activity (Figure 11; lane 3), but that the

addition of excess magnesium was able to overcome this inhibition (Figure 11; lane 4). This suggested that excess ATP was capable of chelating magnesium.

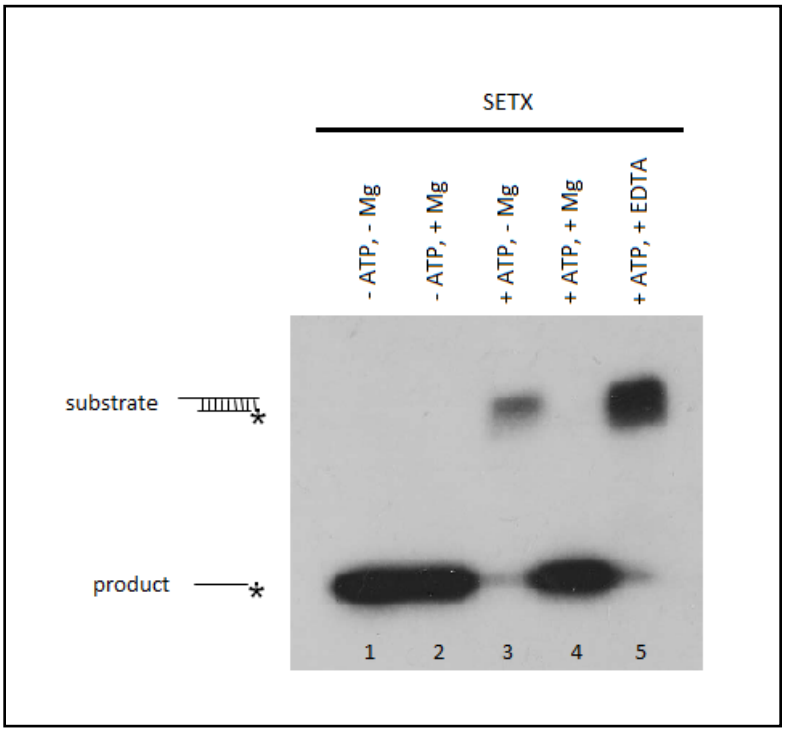

Figure 11: Helicase Assay demonstrating reaction requirements of the senataxin protein. Although additional magnesium is not required for activity, chelation of contaminating magnesium by either EDTA or excess ATP prevents substrate unwinding. Activity appears to be independent of ATP.

In order to analyze the ATP-independence of the helicase activity, the protein samples were treated with apyrase, an enzyme which hydrolyzes ATP. A helicase assay performed on these apyrase-treated proteins indicated that this treatment still failed to inhibit helicase activity (data not shown). This apparent ATP-independence prompted an analysis of the ATP-hydrolyzing capability of the senataxin protein.

In addition to an ATP-dependent helicase, Kim *et al.* (1999) demonstrated that *S. pombe* Sen1 was a nucleic-acid dependent ATPase. Initial assays supported the idea that senataxin was able to effectively hydrolyze ATP even in the absence of nucleic acid cofactors (Figure 12; compare lane 1 to lanes 3 and 4). Double-stranded RNA cofactors were found to be ineffective in stimulating this activity (Figure 12; lanes 5 and 6). However, the presence of double-stranded DNA increased ATPase activity two to three-fold (Figure 13; compare lane 1 to lanes 2 and 3).

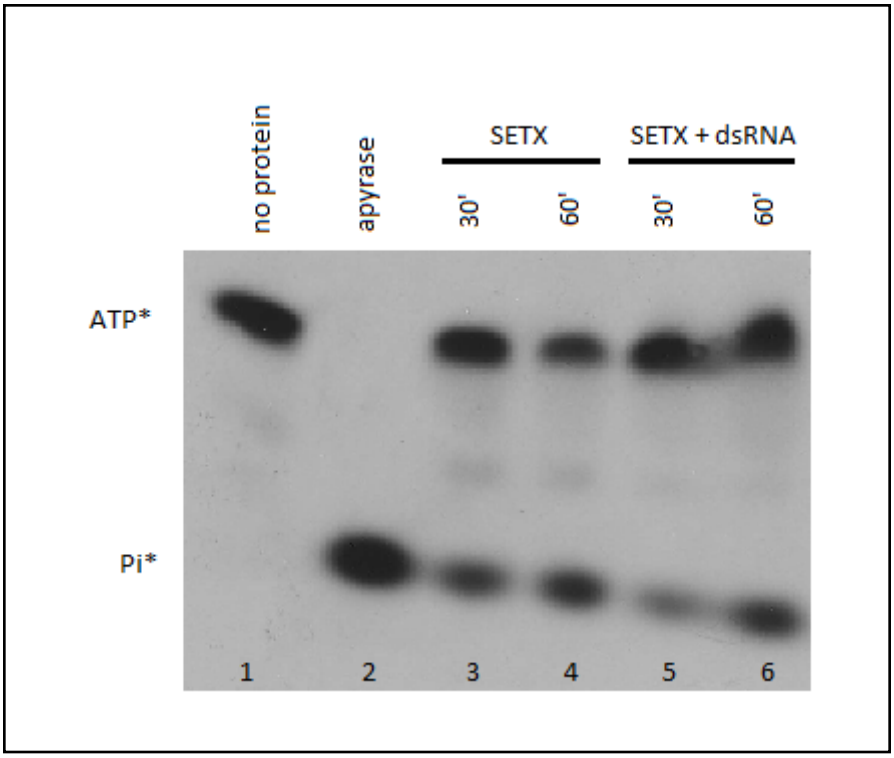

Figure 12: ATPase assay demonstrating the hydrolysis of ATP by senataxin. There is an increase of radiolabeled phosphate produced as the reaction time increases. Double-stranded RNA cofactors, in this case tRNA isolated from *S. cerevisiae*, does not stimulate activity.

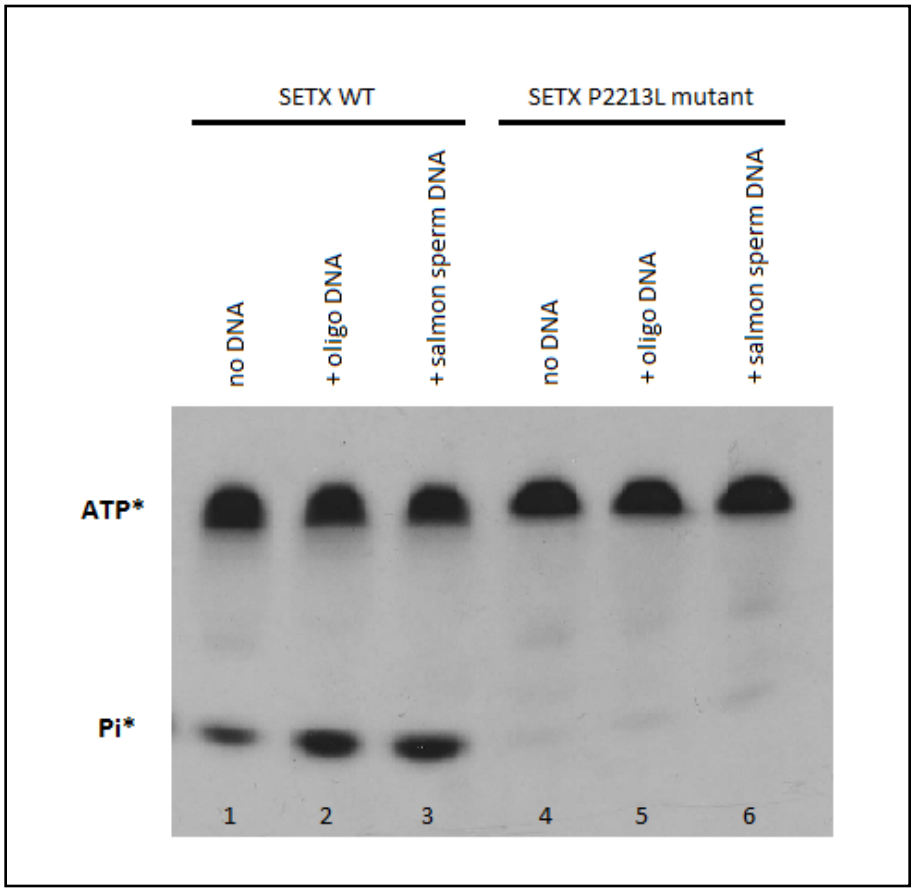

Figure 13: Assay demonstrating the stimulation of ATPase activity in wildtype senataxin by the presence of double-stranded DNA cofactors, either M13(-20)+5'OHR helicase substrate or salmon sperm DNA. This activity is not present in the disease-linked SETX-P2213L mutant.

Both wildtype and a mutant version of senataxin, SETX-E2188K (the human ortholog of a temperature-sensitive yeast mutant), exhibited this ATPase activity as well as stimulation of activity by DNA cofactors (Figure 14; compare lanes 1 and 2 to lanes 4 and 5). However the disease-linked SETX-P2213L mutant, which lacked helicase activity (M.H. Jenks), failed to demonstrate the ability to hydrolyze ATP effectively (Figure 13; lanes 4-6). Double-stranded DNA cofactors were unable to stimulate ATPase activity in this mutant (Figure 13; lanes 4-6; Figure 14, lanes 3 and 6).

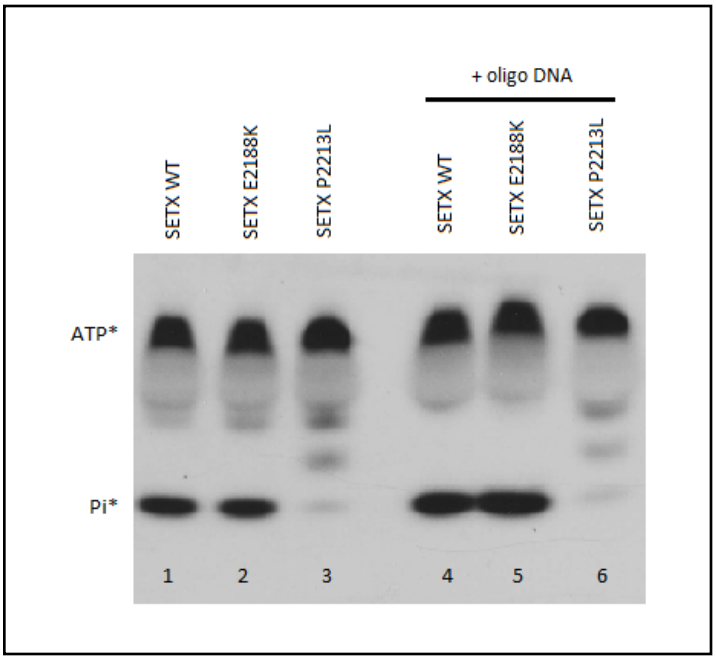

Figure 14: ATPase assay of wildtype, temperature-sensitive and disease-linked mutant senataxin proteins. Wildtype and E2188K temperature-sensitive senataxin exhibit clear ATPase activity and an approximately two-fold stimulation of activity by double-stranded DNA cofactors. The disease-linked and putative loss-of-function P2213L is inactive regardless of the presence of DNA cofactors.

A side-by-side analysis of the ATPase activity of several different recombinant senataxin preparations, prepared by M.H. Jenks and T. O'Rourke, appeared to indicate that the ATPase activity was present in wildtype senataxin and the temperature-sensitive E2188K mutant, but absent from the disease-linked P2213L, D2207V, K2382E and R2414Q mutants (data not shown). However, this initial assay was not normalized for protein concentration. When this assay was repeated with equal amounts of each purified recombinant senataxin protein, it was found that ATPase activity was present at similar levels in all preparations regardless of any SETX mutations (Figure 15).

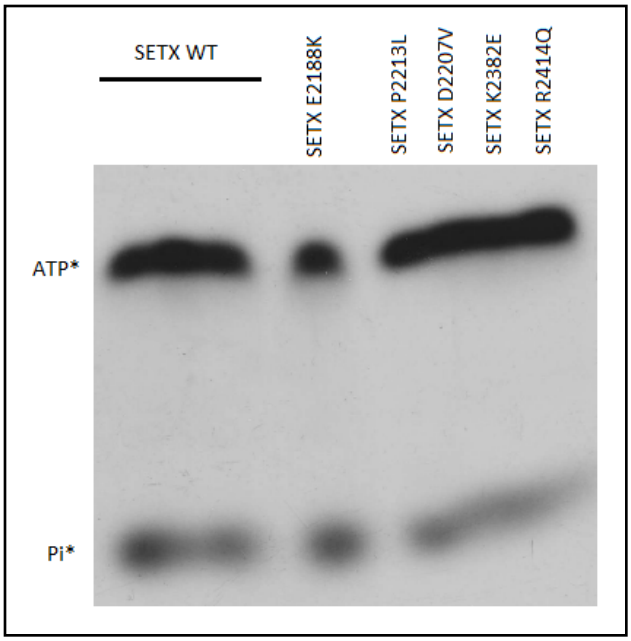

Figure 15: ATPase assay scanning multiple mutant and wildtype preps of senataxin. The mutants include both disease-linked patient mutants and SETX-E2188K, the human ortholog of the temperature-sensitive SEN1-E1597K yeast mutant. All samples demonstrate some, relatively consistent level of helicase activity, regardless of the presence or nature of mutations.

The presence of this ATPase activity in senataxin protein preparations that contained mutations in their nucleotide binding motifs indicated that it might be the result of a co-chromatographing contaminant, present in mutant and wildtype senataxin samples. One contaminant known to co-purify with GST-fusion proteins produced in *E. coli* is the 70 kDa heat shock protein HSP70, an ATPase encoded by the *E. coli* gene dnaK (Hengen, 1997). SDS-PAGE of the assayed senataxin protein found that a 70 kDa protein consistently co-purified with both mutant and wildtype samples (Figure 10). These samples were subjected to DEAE chromatography, which partially separated the 70 kDa contaminant from senataxin. An ATPase assay was used to test if this contaminant was the source of ATP hydrolysis. It was found that the contaminant had

some ability to hydrolyze ATP (Figure 16; compare lane 1 to lane 4), but that this activity was less than that observed in senataxin protein preparations (data not shown). Addition of either warmed (treated at 65ºC) or boiled (treated at 95ºC) bovine serum albumin (BSA), which should stimulate the chaperone function of the putative heat shock protein and thus its ATPase activity, failed to induce activity (Figure 16).

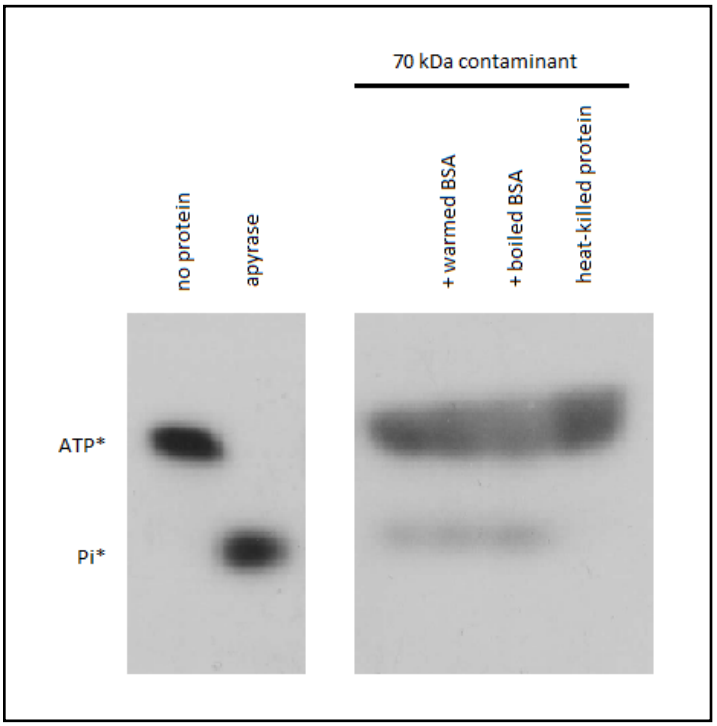

Figure 16: ATPase assay of wildtype and mutant senataxin preparations compared to an isolated contaminant, thought to be HSP70. Activity is consistent between all samples, but is not stimulated by either molten or denatured BSA. Heat killing at 95ºC eliminates activity.

Conclusions

In studying *S. pombe* Sen1, Kim *et al.* (1999) demonstrated that the C-terminal domain of the protein, containing a number of conserved helicase homology motifs, exhibited nucleic acid helicase activity *in vitro*. In these studies, a recombinant protein, containing the minimal helicase homology domain of the human Sen1 ortholog, senataxin, was produced in *E. coli* and purified using various chromatography methods.

A His_6-tagged recombinant senataxin, purified by both nickel-affinity and ion-exchange chromatography, was found to co-chromatograph with a number of *E. coli* proteins. Although early helicase assays demonstrated helicase activity correlating with the presence of the 90 kDa recombinant senataxin, further studies indicated that activity was present even when the senataxin protein was not. This data suggests that the observed helicase activity was due to the presence of one or more contaminating *E. coli* helicases.

An alternative version of the recombinant senataxin protein marked with a GST-tag was purified over a glutathione sepharose column with relatively few contaminants. Initial assays performed by M.H. Jenks and D. Reines, indicated that this protein was a helicase exhibiting a 5’ to 3’ directionality and a clear preference for DNA substrates. Although the activity of this protein was expected to be magnesium and ATP-dependent and the magnesium requirement was confirmed, it was found that the helicase function was independent of ATP. This unexpected ATP-independence prompted an analysis of the ATP-hydrolyzing capability of the senataxin protein.

Early assays indicated that recombinant senataxin exhibited the expected DNA-stimulated ATPase activity. It was also found that RNA cofactors were incapable of stimulating this activity, which appeared to be present in both wildtype and temperature-sensitive proteins, but absent in several proteins linked to the neurodegenerative disorder AOA2. This, together with the work of M.H. Jenks which demonstrated a loss of helicase function in disease-linked mutants, supported the idea that these mutations resulted in a loss of helicase function.

However, further analysis of multiple protein samples indicated that this activity varied in both wildtype and mutant proteins. This finding suggested that the activity was, at least in part, due to some consistent contaminant and not the senataxin protein. When both wildtype and disease-linked mutant protein were compared to a sample enriched for a known 70 kDa contaminant thought to be the heat shock protein HSP70 (possibly over-expressed by *E. coli* due to the stress of induced protein production), it was found that this contaminant exhibited some ATPase activity. However, it is still unclear whether this 70 kDa contaminant is entirely responsible for the observed activity or simply supplements the activity of senataxin.

Although early helicase assays supported the *in vitro* helicase function of senataxin, recent studies have called that role into question. Work done by K. Van Bortle and D. Reines demonstrated that some helicase products co-migrate with a radiolabeled NMP, which would be the product of nuclease activity, and that senataxin was capable of generating both products. This suggests that the senataxin samples assayed for helicase activity are either contaminated by a nuclease or that senataxin is a nuclease itself. A precedent for proteins exhibiting both helicase and nuclease activities has been

established by the studies of certain DNA repair enzymes such as DNA2 (Lee *et al.*, 2000; Bae and Seo, 2000).

In addition to the aforementioned problems with co-chromatographing contaminants, there has been a noticeable loss of recombinant protein in the purification process due to insolubility. Insolubility is a known complication from producing large recombinant proteins in *E. coli*. Thus, the future directions of this project are focused on reliably producing more soluble senataxin of greater purity.

An additional future direction is the study of the recombinant senataxin protein *in vivo*. To this end, expression constructs will be created using the SETX gene fragment used in previous SETX expression vectors. This fragment will be inserted into the plasmid pRS315GPD, producing a yeast expression vector, pRS315GPD-SETX (Figure 17). This plasmid, through the use of the TDH3 promoter, will allow for the constitutive expression of an untagged, recombinant senataxin protein. A similar construct, intended as a control, will be constructed by inserting a comparable fragment of the wildtype SEN1 gene into the pRS315GPD plasmid, producing another expression vector, pRS315GPD-SEN1 (Figure 18). These expression vectors will be used to transform *S. cerevisiae* with a temperature sensitive mutation in SEN1 (SEN1-E1597K) in order to determine whether the enzymatic core of the recombinant protein can substitute for the temperature-sensitive absence of functional endogenous Sen1. The findings of this experiment should indicate whether the recombinant senataxin used for helicase assays retains its essential *in vivo* function. This result, along with further studies of the *in vitro* biochemistry of the protein, should indicate the relation, if any, between the essential and putative helicase functions of the protein.

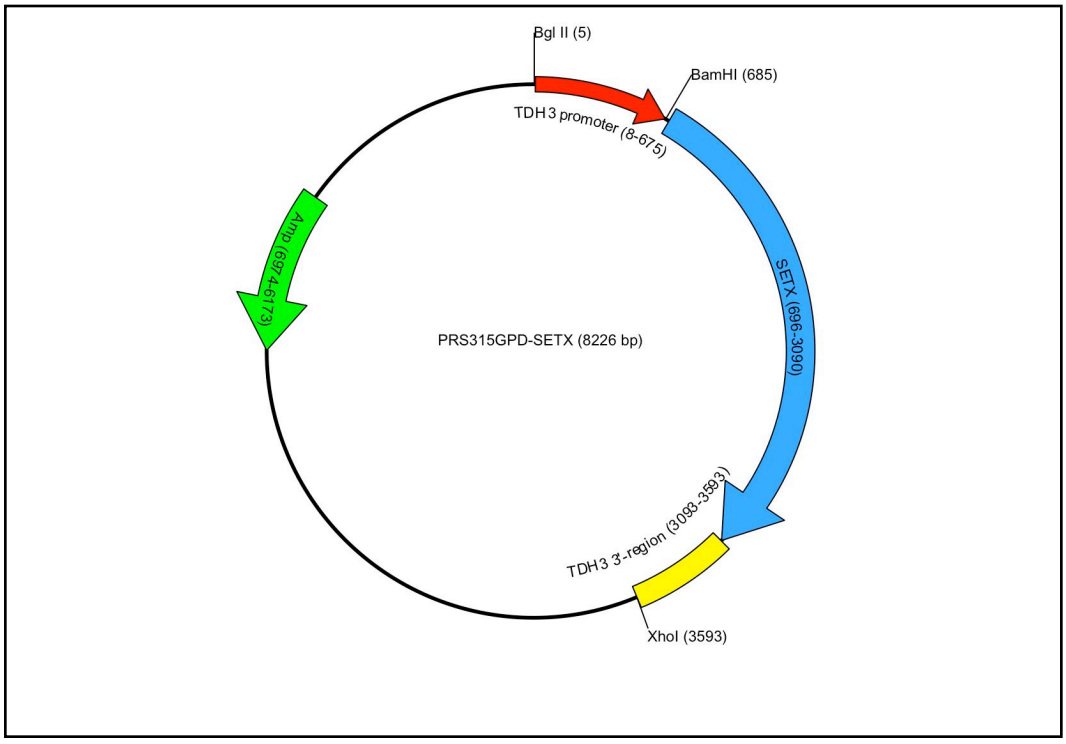

Figure 17: The pRS315GPD-SETX yeast expression vector

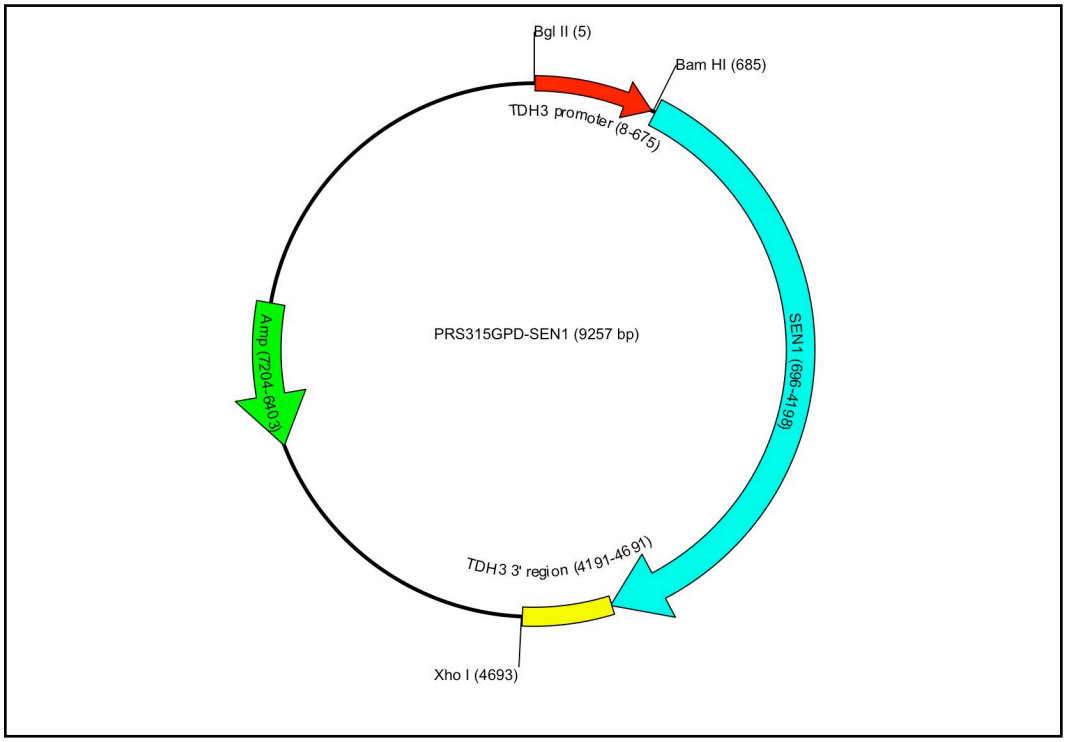

Figure 18: The pRS315GPD-SEN1 yeast expression vector

In conclusion, unequivocal proof of the helicase activity of senataxin has proven elusive; some evidence has been provided to support the *in vitro* role of senataxin as a nucleic-acid helicase under specific conditions, but those findings are not definitive and additional assays using new helicase substrates and a further-purified senataxin protein will be required to determine the *in vitro* function of senataxin and how that relates to its essential *in vivo* role.

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