UNIVERSITY OF CALIFORNIA, SAN DIEGO

Cloning and Expression of Protein Tyrosine Kinases in the Medicinal Leech

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Bailey Zhao

Committee in charge:

Professor Eduardo Macagno, Chair
Professor Kathleen French
Professor Yimin Zou

2009

The Thesis of Bailey Zhao is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2009

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Foremost, I would like to express my deep and sincere gratitude to my advisor Dr. Eduardo Macagno for providing me with the opportunity to complete my Masters thesis in his lab; Dr. Macagno and Dr. Michael Baker for their continuous patience and guidance throughout this project. This thesis would not have been possible without their encouragement, support, and immense knowledge in leech biology. I would also like to thank Constantine Firme for providing technical assistance. Lastly, I would like to thank all the members of the Macagno Laboratory, past and present, for their assistance, support, and vast scientific knowledge.

ABSTRACT OF THE THESIS

Cloning and Expression of Protein Tyrosine Kinases in the Medicinal Leech

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Professor Eduardo Macagno, Chair

Some non-receptor protein tyrosine kinases (PTKs) are thought to play an important role in axonogenesis. I have partially cloned and sequenced two such PTKs found in *Hirudo medicinalis*: focal adhesion kinase (FAK) and Abelson kinase (Abl). Comparative analysis of the protein sequences of these two kinases reveal that they are

closer in homology to vertebrate species than to other invertebrates, such as insects. Whole-mount *in situ* hybridization (WISH) showed that leech FAK mRNA is strongly expressed in the ganglia of the central nervous system and nephridia, as well as potential expression in the bilateral Comb cells. Leech Abl mRNA is strongly expressed in the ganglia and nephridia across all trials, while some WISH trials show expression in the bilateral heart tubes, Comb cells, as well as sensilla clusters. Attempts to knock down expression of Abl have thus far been inconclusive.

INTRODUCTION

Intracellular signaling pathways play an important role in the regulation of cellular processes. Signal transduction can involve complex protein-protein interactions that may determine or amplify many downstream effects. In many instances, the phosphorylation state of serine, threonine or tyrosine residues on target proteins mediate changes in cellular growth, differentiation, or migration. For example, the SH2 (Src homology region 2) and PTB (phosphotyrosine-binding) domains are two well-studied protein modules that recognize and bind phosphotyrosine (Schlessinger & Lemmon, 2003). The SH2 and PTB modules are also involved in many intracellular signaling pathways such as the downstream effects on Ras, a protein that regulates cell development, differentiation and mitogenesis (Shoelson, 1997). Modular domains such as SH2 work with their respective protein kinases and phosphatases in order to localize proteins to specific cellular sites, control enzyme activity, organize multiprotein complexes as well as directly participate in signal transduction (Pawson & Scott, 1997). It is important to understand many of these mechanisms because dysfunctions in these transduction pathways can lead to a host of abnormalities and pathologies such as cancer, diabetes, immune deficiencies, and many others (Shoelson, 1997).

Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) are families of enzymes that work in conjunction with one another in cellular signaling processes that regulate directed neuronal growth and navigation among other physiological processes (Tonks, 2006). The PTKs regulate cell-to-cell communication

by using ATP as the phosphate donor to covalently modify tyrosine residues on target proteins (Hubbard, 2002). PTKs are divided into two different groups, receptor kinases, generally located on the plasma membrane, and non-receptor or soluble kinases, usually found in the cytoplasm. In humans there are 58 receptor tyrosine kinases and 32 non-receptor tyrosine kinases (Blume-Jensen & Hunter, 2001). The two kinases I have studied in this project, focal adhesion kinase (FAK) and Abelson kinase (Abl), belong to the non-receptor tyrosine kinase group (Hubbad, 2002; Lainer & Gertler, 2000).

By contrast, protein tyrosine phosphatases (PTPs), which also include both receptor protein tyrosine phosphatases (RPTPs) and non-receptor types, catalyze the dephosphorylation of phosphotyrosine residues and also play key roles in regulating signal transduction pathways (Tonks, 2006). Specifically, the Leukocyte common antigen-related (LAR) subfamily of RPTPs have been shown to be associated with a regulation of axon guidance and synaptogenesis (Johnson & Van Vactor, 2003). Past studies have shown that RPTPs, such as LAR, work together with PTKs such as FAK and Abl in regulating neurite outgrowth and controlling axon guidance behavior (Tonks, 2006; Wills et al., 1999). Figure 1A shows activated FAK as a downstream component for neurite outgrowth after the soluble ligand LARFN5C, which is a 99-residue ectodomain isoform of LAR, binds to the fibronectin domains on the extracellular segment of LAR (Yang et al., 2005).

LAR also plays a role in the activation of Trio guanine nucleotide exchange factor, which in turn enhances FAK activity (Medley et al., 2003). Activation of FAK

has been implicated in signal transduction events that lead to changes in cell adhesion and motility in addition to neurite outgrowth (Kolkova et al., 2000; Schaller, 2001). Figure 1B shows a diagram that represents potential downstream players of LARs for axon guidance, including *Drosophila* Abl (d-Abl) and Enabled (Ena). These two components act on the cytosolic side of the signal transduction cascade of LAR in *Drosophila* (Dlar). The mammalian homolog of Ena (Mena) functions in regulating assembly of the cytoskeletal microfilament actin, and plays a role in promoting actin polymerization at the leading edge in cell motility (Gertler et al., 1996; Lanier et al., 1999). Ena acts as a shared substrate between Dlar and Abl. Paired together with Dlar and d-Abl, Ena acts as a phosphorylation-dependent switch to control actin polymerization (Wills et al., 1999). Profilin is another actin-binding protein that interacts with Ena and d-Abl and is present in developing nervous systems; however, its role and specific interaction within this signaling cascade is still unclear (Wills et al., 1999).

It has been shown, using *in situ* hybridization (ISH), that two LAR-like RPTPs found in the embryonic leech, *Hirudo medicinalis* (Hm) HmLAR1 and HmLAR2, are expressed by central neurons. In particular, HmLAR2 mRNA has been detected in different subsets of central neurons and also within the growth cones of the peripheral glia-like Comb cells (Gershon et al., 1998). More recently, it was suggested that HmLAR2 has a role in modulating growth cone morphology as well as regulating focal adhesion complexes (Baker et al., 2008). Because many aspects of cell signaling pathways in relation to axon guidance in the developing nervous system are still not

well understood, there is a need to further investigate those gene products that have been implicated in this signal transduction cascade. Determining the expression patterns and functions of genes involved in the regulation of axon guidance can improve our understanding of growth cone motility and its role in axon guidance decisions in neural development.

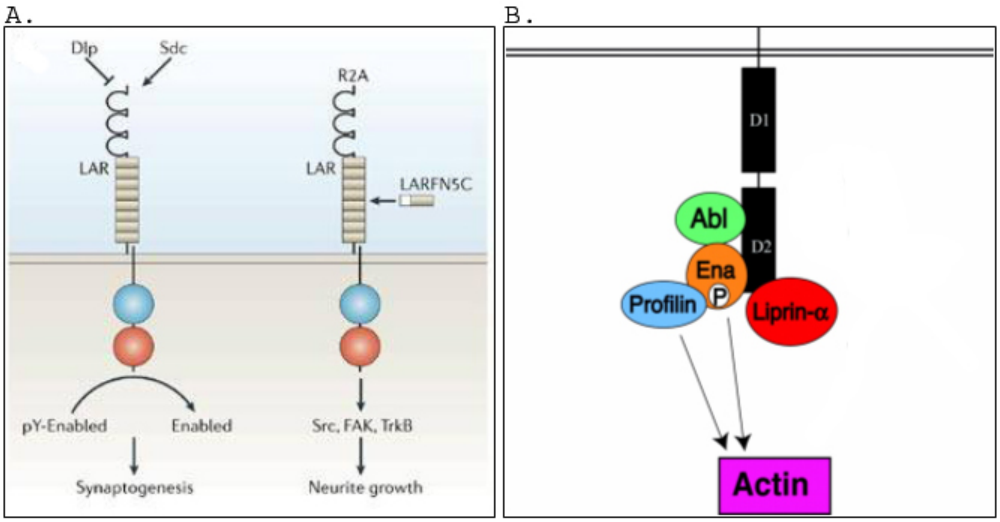

Figure 1. Proposed RPTP LAR cellular signaling pathways. (A) Regulation of receptor PTP function by ligands. On the left, the immunoglobulin domain can bind transmembrane protein syndecan (Sdc) to promote phosphatase action, while Dallylike (Dlp) protein suppresses phosphatase function. Ligand binding regulate Enabled, which regulates actin cytoskeleton assembly and disassembly in controlling synapse morphogenesis. On the right, soluble LARFN5C binds to the fibronectin domains; this leads to activation of FAK downstream in promoting neurite outgrowth. (B) Schematic diagram representing signal transduction cascades downstream of Dlar. The cytoplasmic domain of Dlar can bind to Liprin-alpha , a regulator of synapse formation. Dlar can also directly bind and dephosphorylate Abl and Ena. Ena is a shared substrate for Dlar and Abl and appears to form a phosphorylation-dependent switch controlled by the activity of Dlar and Abl. (Images from Tonks, 2006 (left) and Johnson & Van Vactor, 2003 (right).)

Most of the work in describing the relationship between Abl and LAR and their role in modulating substrate proteins has been carried out in the genetically amenable fruit fly. It was suggested that d-Abl kinase and Dlar phosphatase may have an antagonostic relationship in controlling growth cone guidance. This conclusion

was reached after a series of gene deletion experiments of both d-Abl and Dlar. D-Abl deletion mutants displayed CNS defects as well as a shorter intersegmental motor nerve b (ISNb) phenotype, which branched and failed to reach and innervate its target. This was in contrast to experiments with overexpression of d-Abl, which displayed a longer ISNb phenotype where the ISNb failed to branch and the axons grew past their target (Wills et al., 1999). Additionally, mutations that reduced Dlar levels showed the same phenotype as overexpression of d-Abl. Furthermore, *in vitro* experiments also showed that d-Abl binds to and phosphorylates the D2 cytosolic phosphatase domain of Dlar (also see Figure 1B), which suggests that they may regulate one another (Wills et al., 1999). In mouse models, mammalian Abl (c-Abl) and Abelson-related gene (Arg) mutants show depletions in leukocyte populations and behavioral defects, respectively (Tybulewicz et al., 1991; Schwartzberg et al., 1991). If both these genes are missing, mutant mice die of neural tube defects and actin cytoskeletal morphological deformities are seen in the neuroepithelium (Koleske et al., 1998).

Here I investigate putative PTKs that might be associated with the LAR pathway of cell motility and axon guidance in the leech. The leech is a useful model system for the examination of these kinases because of the extensive previous work done with HmLAR2 (Gershon et al., 1998; Baker & Macagno, 2000a,b; Baker et al., 2008). The leech homolog of Ena (Lena) was proposed to be a substrate for HmLAR1 and HmLAR2 intracellularly. RNAi knockdown of HmLAR1 using dsRNA in early embryos led to a hyperphosphorylation of Lena (Biswas et al., 2002). Investigation of leech Abl as another potential intracellular substrate of HmLARs could further

elucidate the role(s) of LAR in neuronal development. The previous association of LAR with Abl in the *Drosophila* model makes Abl a natural candidate to start with. Elucidation of leech Abl mRNA expression using whole-mount *in situ* hybridzation (WISH) show that the partial gene is localized to the ganglia in the CNS as well as bilateral sensilla in each ganglionic segment. Leech FAK mRNA expression revealed that the partial gene is localized to the CNS and nephridia, as well as the Comb cells in some cases. RNAi of leech Abl revealed no distinct morphological changes in serotonin antibody staining with dsRNA injections in the embryo; however, further experiments are required to determine if the dsRNA is able to reach and affect the neuronal cells that express Abl.

MATERIALS AND METHODS

Animals

Hirudo medicinalis embryos were obtained from a breeding colony, maintained at 23 °C and were also provided by Joyce A. Murphy, lab manager for Dr. William Kristan's lab at the University of California, San Diego. Leech embryos were staged following the same criteria as described by Fernandez and Stent (1982) and Reynolds et al. (1998).

Molecular Cloning

The sequences of interest were cloned from cDNA made from leech embryos of mixed ages to be used for constructing in-situ hybridization RNA probes as well as bioinformatics analysis. The primers used in the polymerase chain reaction (PCR) to generate the DNA sequences were designed from the sequences found in the leech EST libraries. In addition to manually designing primers, two software programs, FastPCR and Primer3, were used to facilitate easy primer design. FastPCR generates a large list of primers to choose from based on optimal melting temperature, GC%, and also the size of the product. Primer3 allows for manipulation of the design parameters, such as desired primer size, melting temperature, GC%, etc. Additional optimization of the PCR required testing of the optimal melting temperature, size of the primers as well as amount of the reagents used in the PCR reaction, such as magnesium chloride and cDNA template. A list of the primers used in all the PCR

reactions can be found in the appendix. A 400 base pair piece of LAR1 acted as a positive control in many of the PCR reactions.

Total RNA was extracted from leech embryos of mixed ages by completely homogenizing whole embryos with TRIzol reagent (Invitrogen, Carlsbad, CA). The resulting RNA was then cleaned up by performing a liquid-liquid phenol-chloroform extraction, followed by a chloroform extraction. Embryonic genomic complementary DNA (cDNA) was reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA). Abelson kinase was initially amplified by polymerase chain reaction (PCR) using the primers 5'-GAGTGGACGAGTGTCAAGCA-3' and 5'-AACCTTCCCATCAAACCTCA-3'. The sequence was extended by using a reverse degenerate protein tyrosine kinase primer 5'-ATAGGAATTCCAWRGGACCAVACRTC-3' provided by Michael Nitabach. The sequence was further extended by 3' rapid amplification of cDNA ends (RACE) using the First Choice RNA ligase mediated-RACE kit (Ambion, Austin, TX), and full-length sequences cloned by PCR using specific primers.

PCR was performed with 0.5 µl Taq polymerase (Invitrogen, Carlsbad, CA) in 50 µl reactions containing 10 µM each dNTP, 2 µM $MgCl_2$, 0.2 µM each primer, 5 µl Taq buffer, 38.5 µl sterile water, and 1 µl cDNA template (1 ng). The following conditions were used: initial denaturation step at 94 °C for 3 mins, 35 cycles of amplification at 94 °C for 1 min, primer annealing temperature for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products were run on a 1% agarose gel. Bands of the expected size were excised and extracted using a gel-extraction kit

(Promega, Madison, WI) and cloned using TOPO TA Cloning Kit, pCR-2.1 TOPO Vector (Invitrogen, Carlsbad, CA) in accordance with manufacturer protocol. Another vector, pBluescript SK+ was also used. The DNA was commercially sequenced by Eton Bioscience, Inc. (San Diego, CA) by using the standard M13, T7, and T3 primers.

In order to extend the FAK sequence in the 5' direction, two methods were used: 5' RACE PCR and a 5' degenerate PCR. The idea for the 5' RACE takes advantage of an adaptor sequence that is ligated onto the 5' end after it is treated with CIP (calf intestine phosphatase) to remove the free 5' phosphates and TAP (tobacco acid pyrophosphatase) to remove the 5' mRNA cap. The challenge in successfully completing a 5' RACE is designing GSP (gene-specific primers) that will work in the nested PCR because knowledge of where the 5' end starts and stops is required in such designs. Not surprisingly, the 5' RACE PCR failed to further extend the FAK sequence in the 5' direction. However, 5' degenerate PCR was successful in extending the FAK sequence in the 5' direction. In order to run this PCR, a 5' degenerate primer needed to be constructed using the homologous, conserved region of the 5' end of the focal adhesion kinases found in the previously mentioned organisms. The degenerate primers were designed from multiple sequence protein alignments with the help of a software program called CODEHOP, which stands for consensus-degenerate hybrid oligonucleotide primers. An input of local multiple alignments (referred to as blocks) of a group of related protein sequences in a FASTA or CLUSTAL format generates an output of primers based on degeneracy. Primers

were chosen based on the highest score, which correlated with lower degeneracy because degeneracy measures the number of different sequences specified by the primer (see appendix).

Bioinformatics Analysis

BLAST searches were done using National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov). The nucleotide sequence was analyzed by using the BLASTx program. The resulting translated protein sequence was then compared against the top five hits, which were prioritized by their E value, with the most significant scores having the lowest E value.

Analysis of the protein structure of the kinases began with a protein BLAST search on NCBI to look at the putative conserved domains as well as the distribution of blast hits found in other organisms. The PROSITE protein domain database on the ExPASy proteomics server of the Swiss Institute of Bioinformatics (SIB) was another tool used to further elucidate the specific profile and pattern hits of the sequences. The results are distinguished between profile hits, which focus on protein domains and pattern hits, which are characterized by shorter amino acid stretches of binding/active site motifs.

The SDSC Biology WorkBench (http://workbench.sdsc.edu/) is an online web-based tool that has a CLUSTALW program, which was used to complete the multiple sequence alignment on the sequences from the different organisms found from the NCBI protein BLAST. The output data from the CLUSTALW alignment program

was then used to construct a rooted dendrogram, which illustrates the sequence similarity based on distance measure.

In situ hybridization

Embryos, staged E7 to E9 (46% to 48%) were anesthetized in 8% ethanol in artificial pond water and dissected by opening the cryptolarval membrane dorsally and removing the yolk sac. The embryos were pinned out flat with small pieces of tungsten wire onto Sylgard dishes in Wenning's solution (40 mM malic acid, 4 mM KCl, 10 mM succinic acid, 10 mM Tris-HCl, 1.8 mM $CaCl_2$, pH 7.4) with 8% ethanol. They were fixed in 4% paraformaldehyde (PFA) in PBS and 50 mM EDTA for 30 minutes at room temperature. The embryos were rinsed twice for 1 minute each in PBS and permeabilized with 0.5 mg/ml Pronase E (Sigma Aldrich, St. Louis, MO) in 5X TE (10 mM Tris-Cl, 1 mM EDTA at pH 8) for 2-5 minutes depending on the age of the embryo, then washed three times for 1 minute in PBS plus 2 mg/ml glycine. Embryos were then post-fixed as above for 10 minutes at room temperature and then washed five times for 5 minutes each in PBS plus 0.1% Tween 20 (PBT) on the shaker. The PBT was replaced with two washes with 2X SSC plus 0.3% CHAPS detergent (SSCHAPS) for 10 minutes. The embryos were unpinned and placed in a 1.5 ml Eppendorf tube with 500 µl of SSCHAPS and 500 µl of hybridization buffer (50% formamide, 5X SSC, 0.1 mg/ml heparin, 1X Denhardt's solution, 0.1% Tween 20, 0.1% CHAPS) for 10 minutes at room temperature.

Previous cDNA clones were linearized and digoxigenin (DIG)-labeled antisense cRNA probes were transcribed from *Abl* using a commercial kit (Roche Applied Sciences, Indianapolis, IN). Embryos were pre-hybridized in 200 µl of hybridization solution (HB) and incubated in 55 °C for 1 hour and then hybridized overnight at 55 °C with 500 ng/ml cRNA probe in hybridization solution. As a negative control some ganglia were hybridized with DIG-labeled sense cRNA probe. After hybridization, the ganglia were washed through the following series at 55° C: 1x 20 minutes 500 µl pre-warmed HB, 1x 20 minutes 250 µl pre-warmed HB and 250 µl SSCHAPS (1:1), 3x 20 minutes pre-warmed 500 µl SSCHAPS, 1x 30 minutes 100 µl SSCHAPS with 10 µg/ml RNase A at 37° C, 3x 20 minutes pre-warmed 500 µl SSCHAPS at 55° C. Embryos were then washed twice at room temperature in 500 µl PBT.

Embryos were repinned on Sylgard plates for antibody staining. Non-specific binding sites were blocked with normal goat serum (NGS) after the solution was heat-inactivated at 65° C for 1 hour. The embryos were blocked with 5% NGS in PBT at room temperature on a shaker for 1 hour and then incubated with an anti-digoxigenin antibody that was diluted 1:5,000 in 5% NGS in PBT overnight at 4 °C. Embryos were then washed for 3 hours at 20 minute intervals with PBT on the shaker at room temperature. Subsequent antibody staining was detected using NBT/BCIP tablets following the manufacturer's instructions.

RNA Interference (RNAi)

Electrodes were prepared using thin-wall glass capillary tubes approximately 1.0 MM x 0.75 MM in size, which were heated and pulled in the middle. The RNA or saline control preparations were prepared using two methods. The first method mixed the RNA or saline with 0.1% Fast Green, injected into the capillary tubes via thin pipette tips and then centrifuged down. The second method utilized a liposomal agent with a cationic polymer called in vivo-jetPEI (Polyplus Transfection SA, Illkirch, FR). In theory, the in vivo-jetPEI would act as a delivering agent, much like endosomes that would fuse with cell membranes and release the RNA content into the cytoplasm. RNA concentrations of 0.1-1.0 µg/µl were mixed with 15% glucose and 1.3% methylene blue solution before vortexing and adding 0.1-1 µl of the in vivo-jetPEI to the solution. The mixture was vortexed and incubated for 15 minutes at room temperature. Like the first method, the solution was injected into the electrodes and centrifuged down. Ideally, E7 embryos are selected for and injected under the germinal plate and above the yolk sac with the solution. Each electrode carries around 3 µl of solution and about a third is injected into each animal for a 1-1.5 µl injection volume. The solution was ejected from the pulled capillary tube using a 10 ml syringe, which was connected by rubber tubing to a micro capillary. The embryos were then placed in separate wells filled with pond water and set aside for the next 48 hours in the dark.

Immunohistochemistry

In preparing the embryos for antibody staining for fluorescent visualization, dissection of the embryos followed the same protocols as previously described for in situ hybridization with the following modifications: After dissection, the embryos were fixed in 4% PFA for one hour at room temperature, washed three times for 15 minutes each in wash buffer (PBS with 0.5% Triton-X), treated for 1 hour in blocking buffer (wash buffer with 10% goat serum) and incubated overnight with primary antibodies in the blocking buffer at 4 °C. Primary antibodies, which were obtained commercially, consisted of α-tubulin (Sigma Aldrich, St. Louis, MO) prepared in mouse and α-5HT (ImmunoStar, Hudson, WI) prepared in rabbit at a dilution of 1:400. Lan3-2 primary antibodies (provided by Zipser) were prepared at a dilution of 1:200. The embryos were then washed the following day for at least 5 hours in 20 minute intervals with wash buffer. The secondary antibodies, which were also commercially obtained, were conjugated with a fluorescent molecule: α-mouse Alexa568, which was red for tubulin or Lan3-2 and α-rabbit Alexa488, which was green for serotonin (Invitrogen, Carlsbad, CA). The preparations were blocked and incubated overnight at 4 °C with the same procedures as the primary antibodies (diluted 1:400). After 3 hours of wash the following day with wash buffer, the preparations were mounted and examined with a spinning-disc confocal microscope and imaged using the MetaMorph image acquisition and analysis software program.

RESULTS AND DISCUSSION

Search for putative kinases

The protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) superfamily of enzymes work in complementary fashion in order to regulate cellular signaling pathways. Specifically, leukocyte antigen related (LAR) phosphatases have been shown to be associated with Abelson (Abl) protein tyrosine kinases in regulating growth cone behavior and axon guidance in *Drosophila* (Willis *et al*., 1999). The search for putative kinases that might be associated with LAR protein tyrosine phosphatases in the leech began with a keyword search of "kinase" in the Leechmaster database. The Leechmaster database is composed of sequences from two EST libraries. One library was constructed from whole embryos at different developmental stages and the other was constructed from adult CNS ganglia. Clones in the database were sequenced by Genoscope, the University of Iowa, and the Joint Genome Institute (JGI). The search for sequences matching the word "kinase" yielded a total of 974 hits. Of those sequences, only 23 matched additional keywords "Abelson," "Abl," "focal adhesion kinase," or "FAK." The longest sequence hits were isolated and the shorter, fragmented contigs (contiguous sequences) were disregarded. After filtering all the resulting sequence hits, there was a tyrosine-protein kinase abl-1 that was 228 base pairs and a focal adhesion kinase that was 427 base pairs. The Abelson kinase sequence was identical to a portion of a 432 base pair sequence that was obtained from the previous work of Jeanette Kuhn in the laboratory.

Molecular cloning and sequence extension

The FAK was cloned at an expected length of 427 base pairs. The Abl was cloned at an expected length of 432 base pairs. All of the clones were commercially sequenced to confirm their authenticity as the original sequence found in the EST libraries. After comparison of the cloned sequences to homologous translated protein sequences in other organisms, it was determined that these were not full length gene sequences.

Focal Adhesion Kinase (FAK)

The corresponding FAK proteins found in other organisms were found to be around 1,000 amino acids in length. By comparing the query sequence to the subject sequence, it was determined that the cloned FAK sequence was at the 3' end of the whole gene sequence and that there was a portion of the 5' end missing. The organisms taken into consideration in the comparisons were: *Danio rerio* (zebrafish), *Marsupenaeus japonicus* (kuruma shrimp), *Pan troglodytes* (common chimpanzee), *Drosophila virilis* (fruit fly), and *Lytechinus variegates* (sea urchin). The degenerate PCR was successful in generating a 1.6 kb product (see appendix) from a previous length of only 427 base pairs.

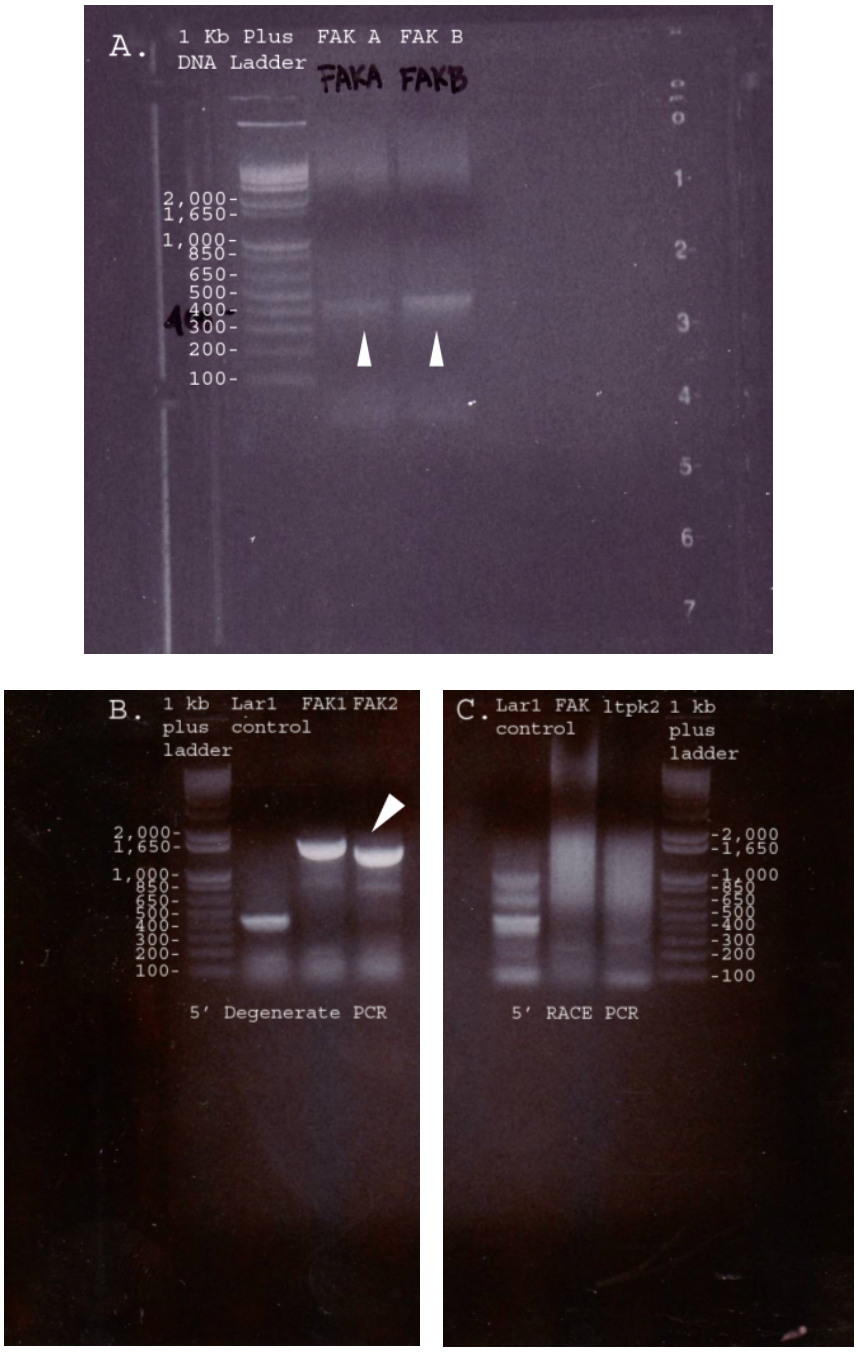

Figure 2. Molecular cloning and sequence extension of focal adhesion kinase (FAK). (A) Molecular cloning confirmed the length and sequence of FAK, which originally was 427 bp. FAK A and B were the same product confirmed by sequencing. (B) 5' degenerate PCR. FAK2 lane was extracted, purified, and sequenced. FAK1 used degenerate primers that bound slightly further upstream on the 5' end. (C) 5' RACE failed to generate any specific sequences. 5' RACE was also attempted on ltpk2, which is an insulin receptor kinase that has similar domain structures to FAK – the RACE PCR failed as well. A 400 bp piece of Lar1 acted as controls for both PCRs for quality assurance of the template.

Abelson Kinase (Abl)

Comparison of Abl kinase in the leech to putative orthologs found in other organisms revealed that the small leech 432 base sequence is closer to the 5' end of homologous sequences, which indicates that extension in the 3' direction is necessary to obtain the full length transcript. Comparable Abl proteins were found to range from 4.0-4.5 kb in length. The translated amino acid alignment showed strong homology (defined by the lowest E values) to several organisms: *Acyrthosiphon pisum* (pea aphid), *Pediculus humanus corporis* (human body louse), *Tribolium castaneum* (red flour beetle), *Apis mellifera* (honey bee), and *Nasonia vitripennis* (wasp). The leech sequences aligned to about 100-150 amino acids downstream of the 5' end. Abelson kinases show two significantly conserved domains: an SH2 domain and a protein tyrosine kinase domain. A protein blast showed that these two putative conserved domains were detected, but the protein tyrosine kinase domain was incomplete. The first attempt at a 3' degenerate PCR failed to generate any products. The primers used in the first degenerate PCR were designed against the conserved regions near the 3' end found in those organisms mentioned above. A second 3' degenerate PCR was more successful after using a set of degenerate primers that were designed by Michael Nitabach when he was a PhD student in our laboratory. These primers were specific to the protein tyrosine kinase domain. This doubled the length of the original sequence from 432 base pairs to 916 base pairs. An additional PCR with a new set of primers confirmed the sequence identity to be specific to the leech, as seen in Figure 3B. Finally, a 3' RACE PCR was conducted to attempt further extension of the

sequence in the 3’ direction. The RACE was successful in extending the sequence from 916 base pairs to 1.6 kb. A second 3’ RACE further extended the sequence to 2.1 kb, which currently stands as the longest piece of Abelson kinase found in the leech. The 2.1 kb product was obtained after the *in situ* probes and dsRNA were made from the 1.6 kb sequence.

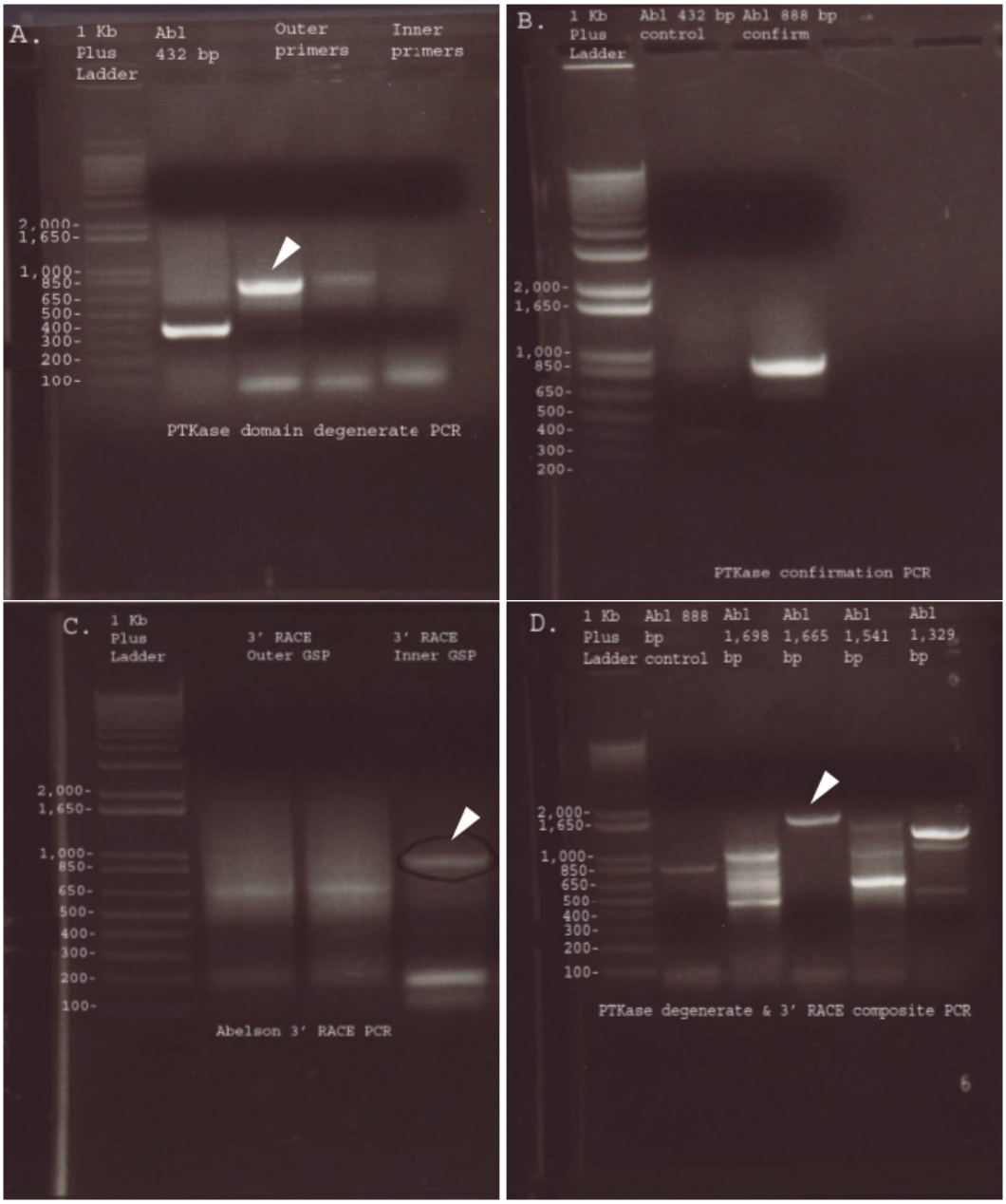

Figure 3. Molecular cloning and sequence extension of Abelson (Abl) kinase. (A) From an original length of 432 bp in lane 1, Abl was extended to 916 bp using degenerate protein tyrosine kinase primers. Lane 2 and 3 were the same and lane 4 was the nested PCR, which failed to generate any band. (B) After the 916 bp band was excised and sequenced, a confirmation PCR was run with a new set of primers that generated a smaller 888 bp product. This product was excised and sequenced and confirmed to be identical to the 916 bp product. (C) Designing primers to the middle of the sequence, a 3' RACE generated a product around 1 kb. (D) The two sequences obtained from the two methods of extension were combined into one composite sequence. Primers were designed to this composite sequence and a PCR was done to confirm authenticity of the gene in leech cDNA. Products of different lengths were tested to confirm the optimal primers.

Bioinformatic analysis of FAK and Abelson clones

The two domain superfamilies that were detected in FAK by the protein BLAST search on NCBI were the protein tyrosine kinase (PTK) domain and the focal adhesion targeting (FAT) region. These two domains are separated by a region of about 150 amino acids. The protein tyrosine kinase domain is incomplete, which suggests that further extension in the 5' direction is required for completion of the whole sequence (roughly 3,000 bases). Structurally, the FAT region is at the C-terminal end of focal adhesion kinase and the crystal structure reveals that it forms a four-helix bundle, which binds to the focal adhesion protein paxillin (Hayashi *et al*., 2002). The FAT region has been shown to play a critical role in the function of the kinase by localizing and regulating cell adhesion and also the phosphorylation of paxillin (Shen *et al*., 1999).

Protein BLAST analysis on the FAK query sequence revealed the best matches and highest homology with the following organisms: *Danio rerio*, *Drosophila virilis*, *Drosophila mojavensis*, *Apis mellifera*, *Drosophila willistoni*, *Lytechinus variegatus*, *Acyrthosiphon pisum*, *Xenopus tropicalis*, *Rattus norvegicus*, *Canis familiaris*. Of these organisms, FAK in the leech had the highest protein homology to the zebrafish, *Danio rerio*. By comparing the two proteins' domains, it was apparent that they share the same conserved protein tyrosine kinase domain as well as the FAT region; however, the complete zebrafish sequence shows an additional B41 domain upstream near the 5' end. B41 (Band 4.1 homologues) are also known as ezrin/radixin/moesin (ERM) protein domains, which are known to play a role in forming crosslinks between

actin filaments and plasma membranes (Tsukita *et al*., 1999). The PROSITE protein domain database refers to these domains as FERM (F representing the B41 group of proteins). All the *Drosophila* hits were grouped under the highest hit, *Drosophila virilis* in order to broaden range of comparable organisms for the dendrogram. The dendrogram in figure 4B shows that alignment of the protein sequences groups FAK found in the leech closer to that of vertebrates than invertebrates. This idea that annelids are closer in relation to vertebrate species than invertebrate species is not a novel one. There have been systematic analyses of annelid genomes that revealed that the genomes of certain marine worms were closer in relation to the human genome than those of insects or nematodes when comparing intron numbers and positions (Fedorov et al., 2006). BLAST analysis of the multiple sequence alignment revealed that most of the conserved residues aligned in the protein tyrosine kinase domain and the focal adhesion targeting domain. To summarize these results, FAK in the leech is more homologous to FAK sequences in vertebrate organisms. Comparison of these protein sequences reveals a missing B41 region of the 5' end of FAK in leech, which warrants further extension.

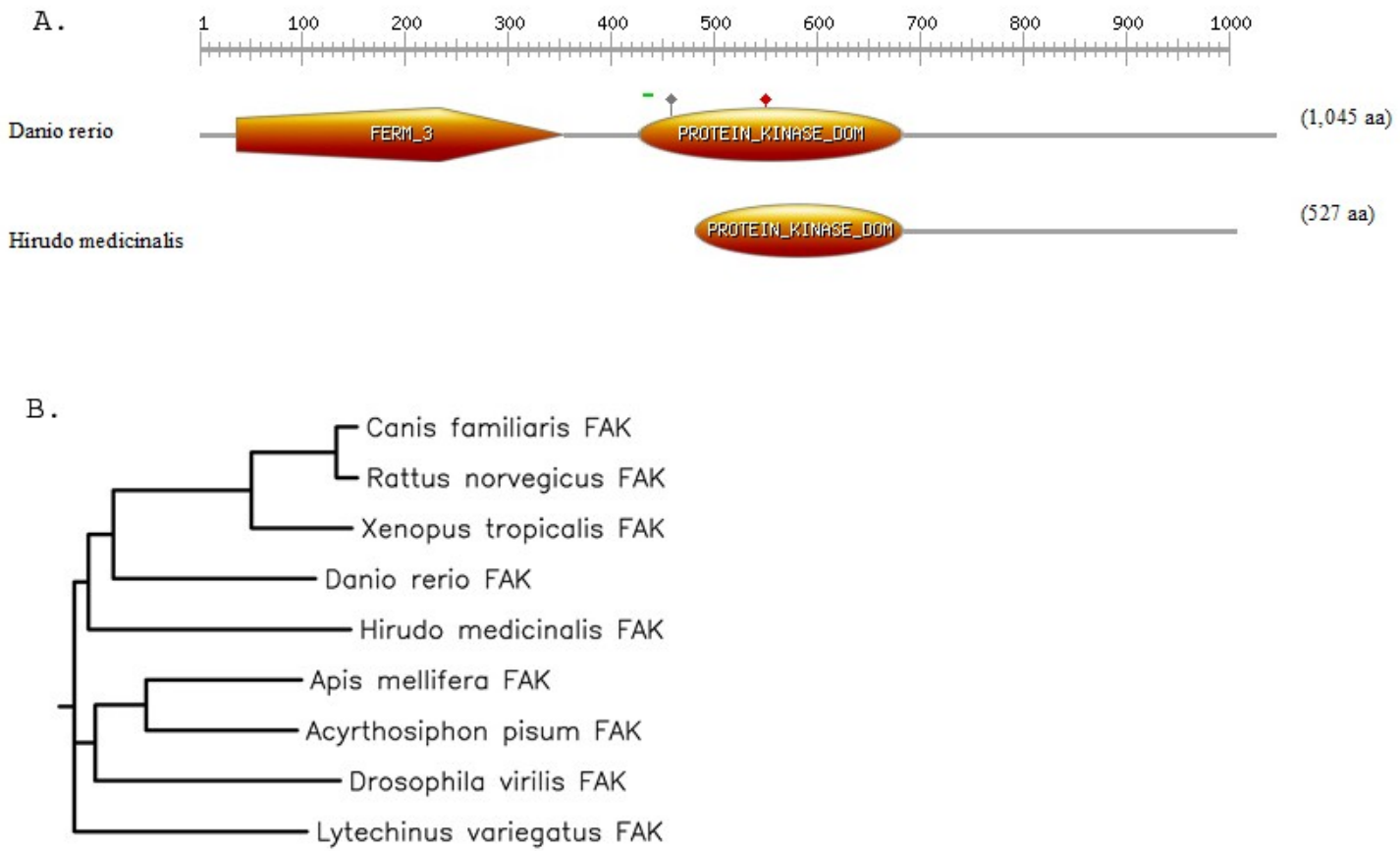

Figure 4. Comparison of FAK in different species. (A) Leech FAK with partial protein tyrosine kinase domain compared to the closest homology match in zebrafish. Zebrafish FAK contains a B41 (FERM_3) domain upstream near the 5' end. Figures generated by PROSITE database protein query. (B) A dendrogram displaying the homology of leech FAK and the four vertebrate animals as well as the four invertebrate organisms. Leech FAK has the closest homology with the zebrafish FAK proteins based on CLUSTALW multiple protein sequence alignment using protein and alignment tools on SDSC Biology WorkBench.

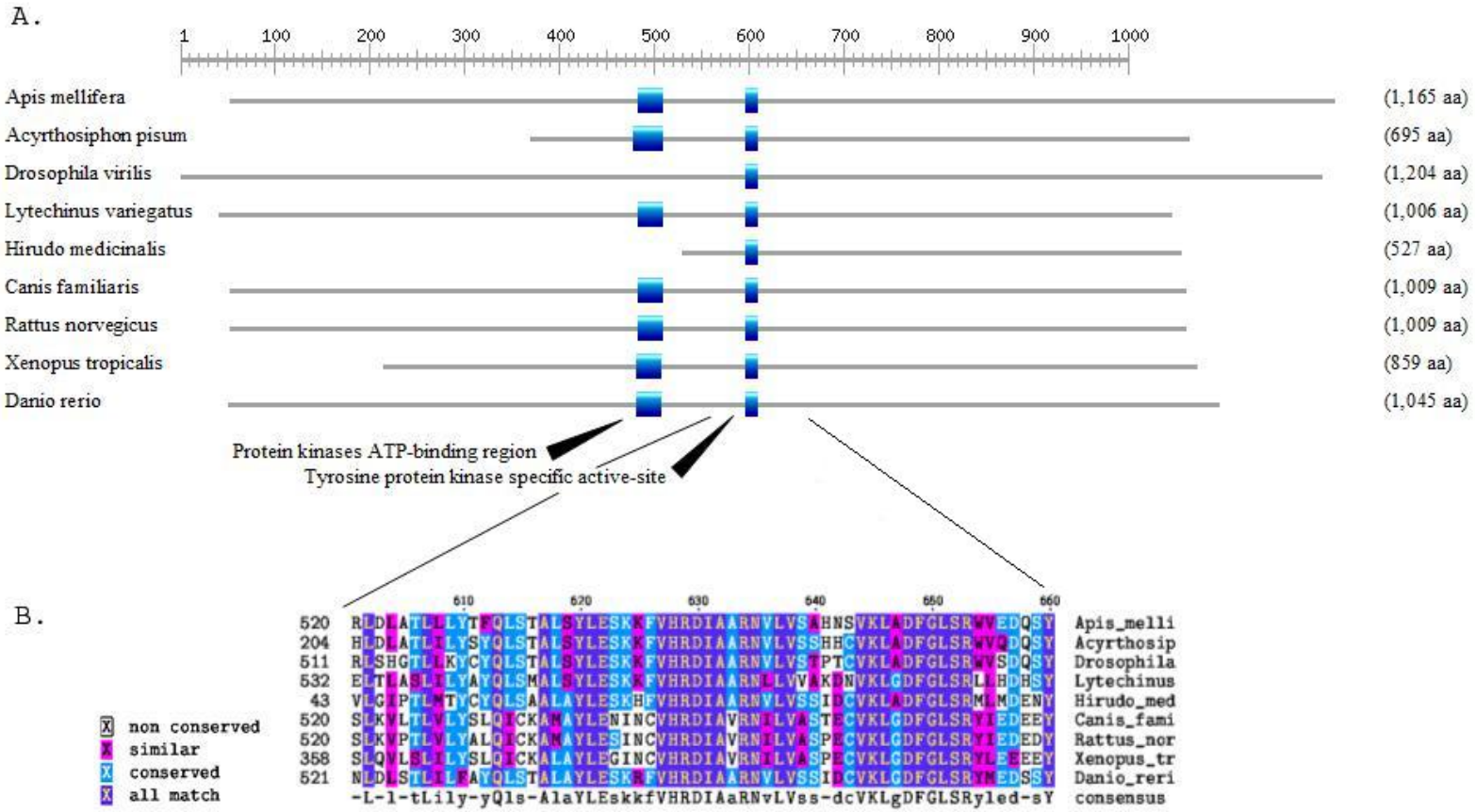

Figure 5. Multiple sequence alignment showing pattern hits in FAK in other species. (A) A comparison of the specific pattern hits in the different FAKs show that FAK in leech is specifically missing the ATP-binding region in the protein tyrosine kinase domain (PTK). Pattern hits showcase smaller stretches of amino acids that resemble active/binding sites, whereas profile hits look for themes in domains. (B) A closer look at the consensus sequence of the conserved region in the protein tyrosine kinase active site. The number on the left of the sequence documents the amino acid of that sequence, while the numbers on top track the relative amino acid based on the starting length of the longest sequence (in this case, *Drosophila virilis*).

Analysis of the protein structure of Abl kinase revealed an SH2 domain and a protein tyrosine kinase domain as shown in Figure 4A. The two domains are adjacent to one another and are found near the 5' end of the gene. The protein tyrosine kinase domain is similar to the one found in FAK and is classified as part of a superfamily of protein kinases with catalytic activity. In mammalian cells, c-Abl is localized to the nucleus in fibroblasts and in the cytoplasm in neurons. Structurally, c-Abl proteins and their related homologues share an SH2 domain that binds phosphorylated tyrosine residues and an SH3 domain that binds proline-rich domains at the N-terminus. Lastly, the long C-terminal tail contains the sequence that directs localization to the nucleus as well as export signals to control cellular localization of the protein (Shaul *et al*., 2005).

NCBI protein BLAST analysis showed a distribution of 100 blast hits on the query sequence for Abl, with the highest homology matching the following organisms: *Acyrthosiphon pisum, Tribolium castaneum, Pediculus humanus corporis, Nasonia vitripennis, Apis mellifera*. Among these organisms, leech Abl shared the most homology with the pea aphid, *Acyrthosiphon pisum*; however, there was a discrepancy when leech Abl had a better match with *Mus musculus* when the sequences were placed in the CLUSTALW program. This discrepancy could be due to differences between algorithms in a basic local alignment search tool and a clustering program. Comparing the protein domain structure to the highest homology hits from the NCBI BLAST, the leech Abl sequence contains many of the same motifs found in the other Abl kinases with the exception of a missing SH3 domain. Many of the other Abls also

showed binding pockets for phosphotyrosine as well as a hydrophobic binding pocket that is a feature of SH2 domains. In addition, the SH2 domain is located upstream and adjacent to the protein tyrosine kinase domain; however, they are both found around 200 amino acids downstream of the 5' end. This suggests that a 600 base pair portion of the 5' end is still missing from Abl in leech, which includes the SH3 domain and a small fragment of the SH2 domain. Abl in leech is also missing the long tail extending in the 3' direction.

For the multiple sequence alignment, *Drosophila melanogaster* was included because much of the work with Abelson kinase and its association with LAR phosphatases were performed in that species. CLUSTALW analysis showed that the amino acid sequences aligned in the conserved domain regions for SH2 and protein tyrosine kinase only. The dendrogram in figure 6B reveals that the Abl in leech is more similar to the protein found in the leukemia virus and the common house mouse than any of the other organisms. The fact that the sequence obtained is still incomplete must be taken into consideration when comparing these amino acid alignments.

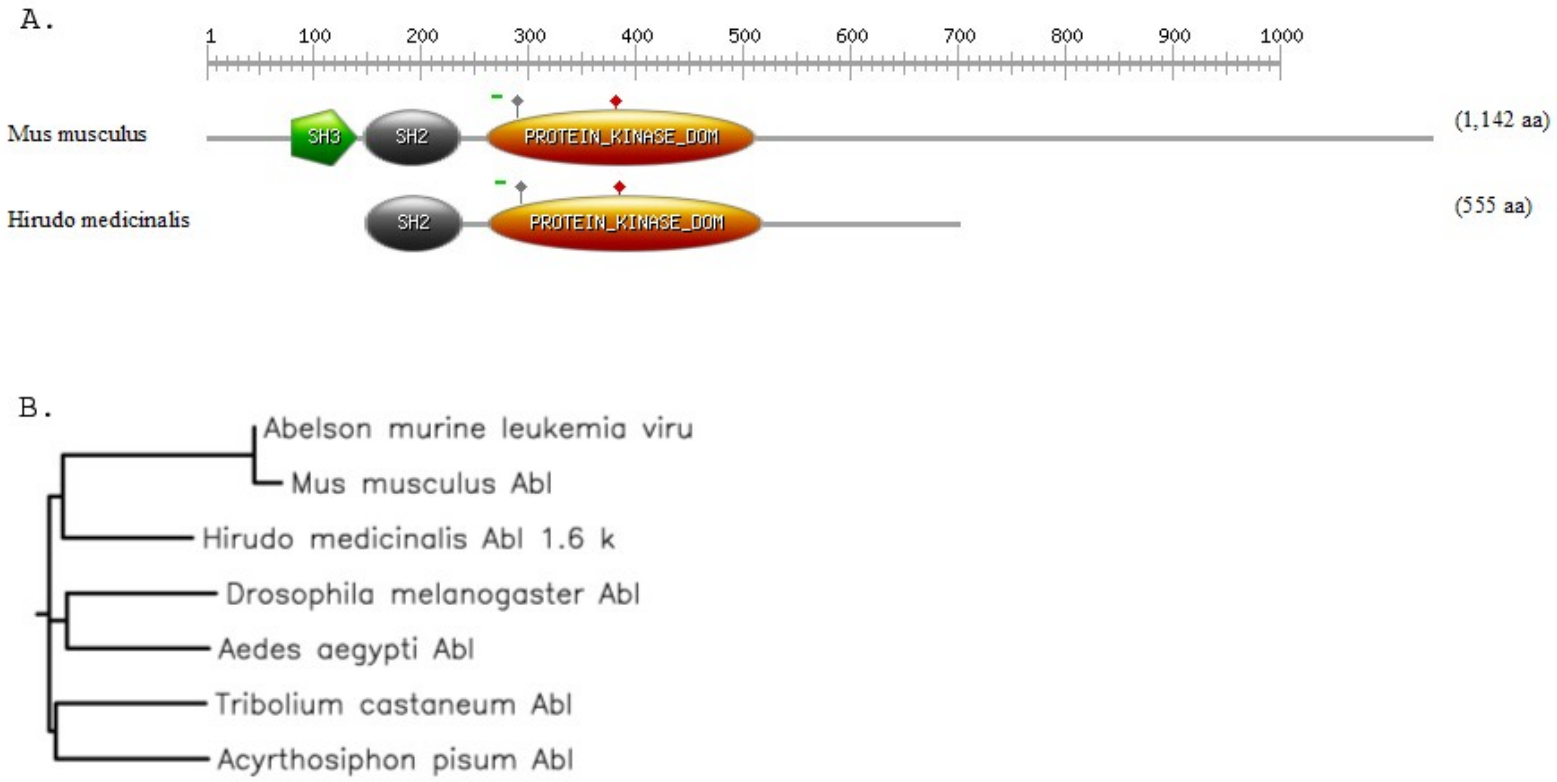

Figure 6. Comparison of Abelson in different species. (A) Leech Abl with a complete protein tyrosine kinase domain match to the next closest living organism, the mouse. The leech Abl is missing a small piece of the SH2 domain and also the SH3 domain upstream near the 5' end. It is also missing a long piece of the 3' tail. Figures generated by PROSITE database protein query. (B) A dendrogram displaying the homology of leech Abl to the mouse and Abelson murine leukemia virus. While Abl displays closer homology to the virus, this may be due to the fact that it is missing an SH3 domain near the 5' end. The next closest match is the common house mouse, *Mus musculus*.

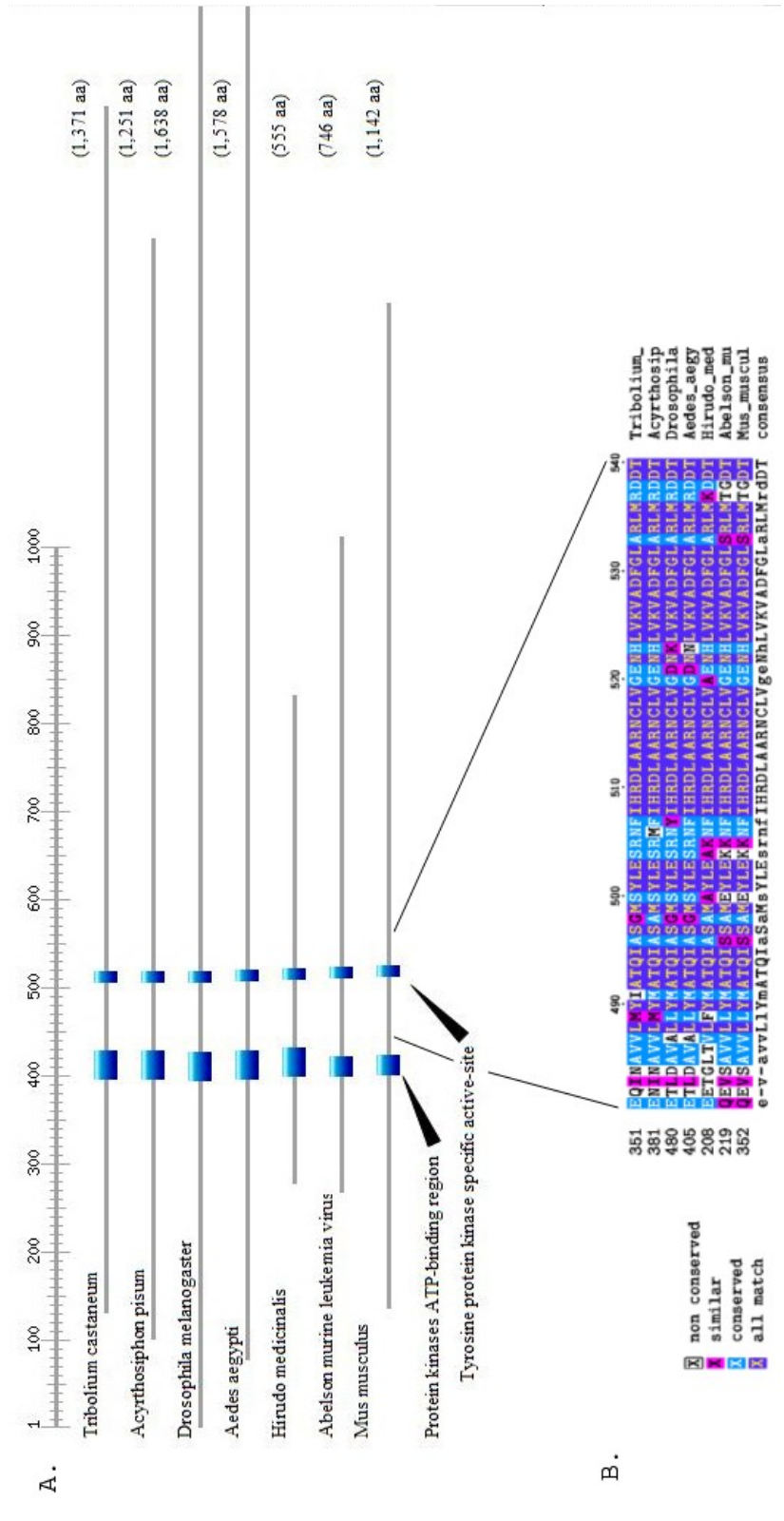

Figure 7. Multiple sequence alignment showing pattern hits in Abl in other species. (A) A comparison of the specific pattern hits in the different Abelsons show that Abl in leech missing the long 3' tail as well as a short segment of the 5' end. (B) A closer look at the consensus sequence of the conserved region in the protein tyrosine kinase active site. The number on the left of the sequence documents the amino acid of that sequence, while the numbers on top track the relative amino acid based on the starting length of the longest sequence (in this case, *Drosophila melanogaster*).

Whole-mount *in situ* hybridization localizes expression of kinase transcripts

The optical transparency of the developing leech embryo enables whole-mount *in situ* hybridization (WISH) as the method to elucidate the expression pattern of both FAK and Abl genes. WISH can provide a spatial as well as temporal pattern of expression as development of the embryo proceeds (taking approximately 30 days from cocoon deposition to free-swimming juvenile animal). Linearization of different plasmids that contain the sequence of interest led to transcription of either sense control or antisense digoxigenin-labeled RNA probes, as determined by the choice of RNA polymerase used (located at each end of the plasmids multiple cloning site). Despite the fact that different plasmids should theoretically produce the same RNA probe, results varied depending on the type of plasmid used to construct the RNA probe. Probes that were made from the plasmid pBluescript seemed to generate cleaner *in situ* results in terms of level of background compared to similar probes that were constructed from TOPO or litmus vectors. The latter also have problems in terms of producing a similar pattern of expression using the sense control, which in theory should not show an expression pattern at all (other than background). The background noise is also seen to be very high using these probes. Despite these challenges in constructing clean and reliable RNA probes, 40% of the *in situ* trials produced good results that clearly demonstrate the patterns of expression in certain structures in the leech embryo.

The expression profile for FAK protein showed strong neuronal staining in the ganglia of the central nervous system as well as the nephridia (Figure 8). This was

compared to leech synaptobrevin (Bruns *et al.*, 1997) as a positive control, which is known to label all central and peripheral neurons (personal communication, Eduardo Macagno). A quarter of the *in situ*s also revealed expression in the Comb cells (Figure 9), which are found bilaterally in each ganglionic segment near the ventral surface of the body wall in younger stages of embryogenesis (Jellies and Kristan, 1988, 1991). This finding proved initially to be exciting because *Hm*LAR2 is expressed in the growth cones of the Comb cells (Gershon *et al.*, 1998). However, repeated trials of *in situ* hybridizations never provided enough consistent results to say conclusively that there is significant expression of FAK protein in the Comb cells. This is due to the fact that the sense control probes showed similar expression patterns to the antisense probe as well as the fact that expression in the Comb cells can be seen in some trials, but not others. Darker staining in the connectives for the antisense probe is seen as a subtle difference as seen in Figure 8B. Early trials of WISH with FAK utilized probes that were only 400 base pairs in length and encompassed the FAT sequence region. One explanation for localized expression in the Comb cells could be generalized expression of that conserved domain by other tyrosine kinases in these cells. In addition, repeated WISH trials with probe constructed from the newly synthesized longer 1.6 kb sequence still showed high background noise and the lack of a clean control result.

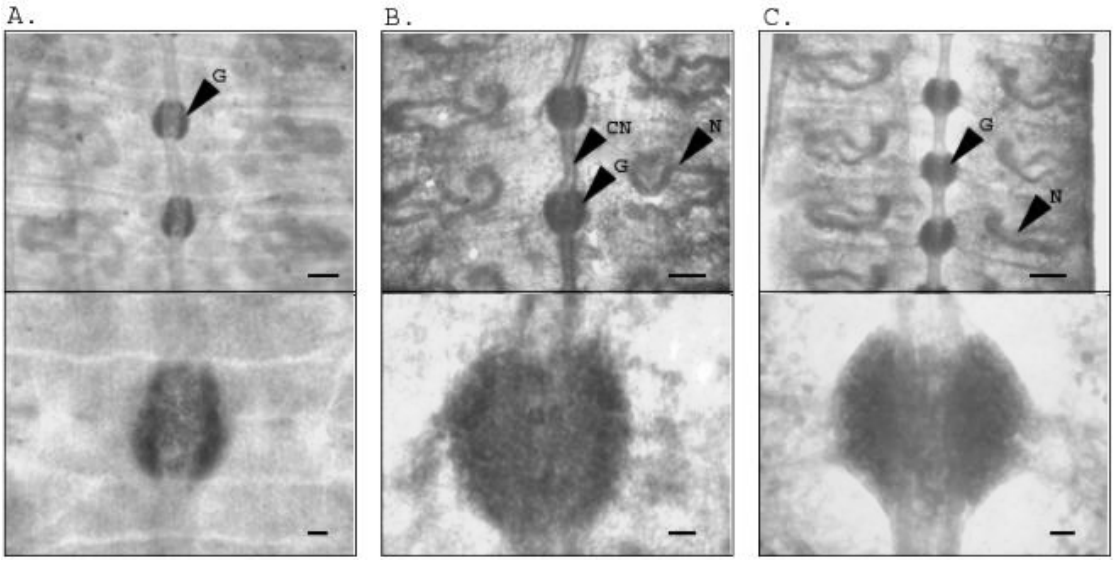

Figure 8. WISH expression profile of FAK on embryos aged at E7 (46%). (A) Leech synaptobrevin (SBV) positive control expression in the ganglia (G). Light staining is also seen in the nephridia (N). The background noise is very low. (B) Antisense probe made from elongated 1.6 kb sequence showing expression in the ganglion, but with high background noise. Dark staining also appears in the nephridia as well as the connectives (CN) between each ganglion. (C) Sense control probe made from the same sequence showing the same expression pattern in the ganglion and nephridia. Background noise appears to be lower than the antisense probe. Scale bars: top boxes, 100 μm; bottom boxes, 10 μm.

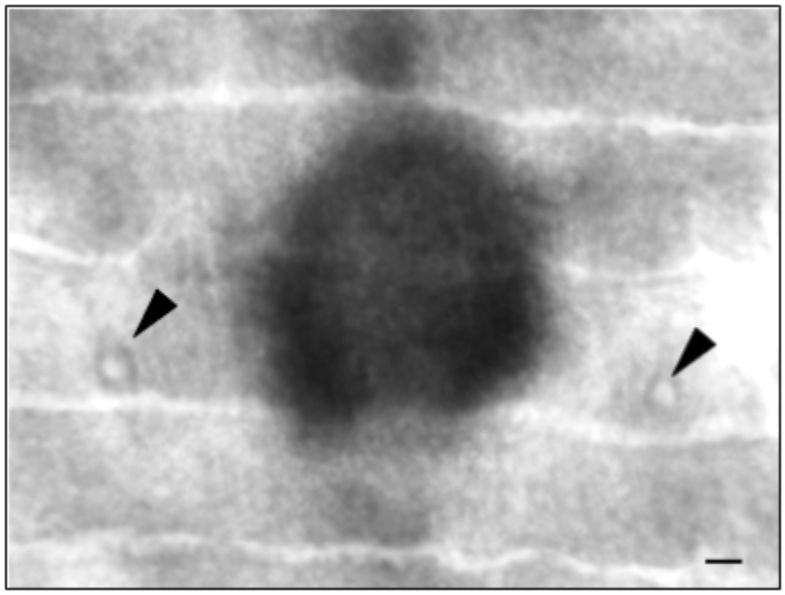

Figure 9. WISH expression of FAK in comb cells. Ganglion out of focus due to focus on ventral cross section. Scale bar, 10 μm.

In contrast to the problems in the development of a reliable sense control probe for FAK, the Abl WISH probes proved to be more successful in generating consistent results. While initial WISH showed high background in many of the preps, later probes showed cleaner results as well as changes in the expression patterns, which may be attributable to the longer length of the sequence. An initial WISH screen focused on localization of spatial expression as well as a temporal expression profile of Abl in embryos at various developmental time points. WISH was done on embryos aged 48 hours apart starting at E7 (46%) and progressing to E9 (48%) and E11 (49%). Differences in spatial expression patterns could not be distinguished from embryos of these three age groups. Abl was primarily found to be pan-neuronally expressed in the ganglia of the CNS, the nephridia, and also along bilateral muscles. Expression of Abl is also detected in the Comb cells, but results are not reproducible and seem to be inconsistent. The temporal expression trials were done with probes made from the 432 base pair sequence.

Table 1. Abl WISH expression in different structures at different ages

Age (%)	**CNS (ganglia)**	**Nephridia**	**Bilateral muscles**	**Comb cells**
E7 (46%)	+++	+++	++	-
E9 (48%)	+++	+++	++	+/-
E11 (49%)	+++	+++	++	+/-

In situs done with 432 bp probe. Staining is strong in the CNS and nephridia, but less pronounced in the bilateral muscles across all age groups. Expression in the Comb cells was seen in E9 and E11 age groups, but results were varied (as denoted by a +/-).

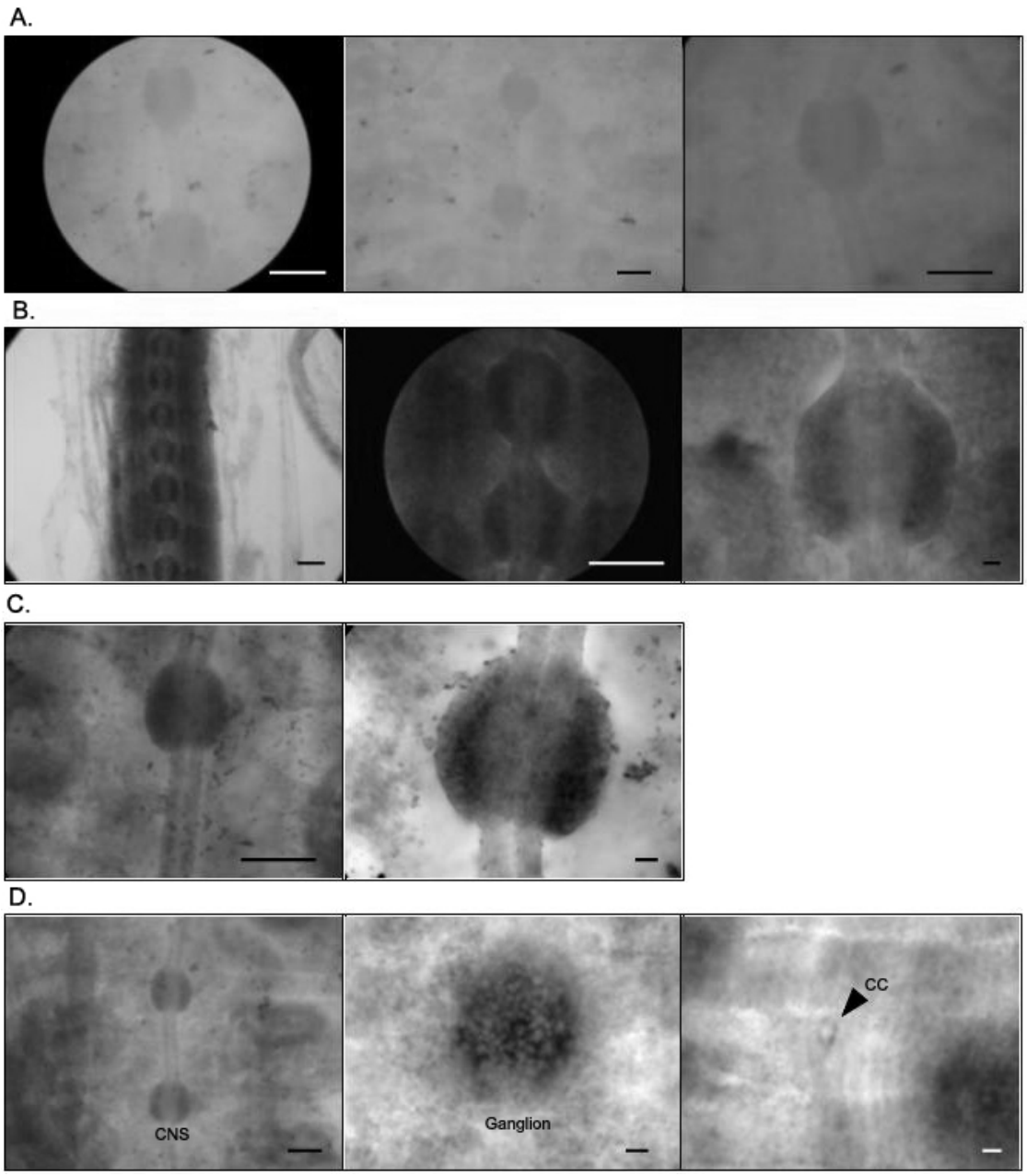

Figure 10. WISH expression profile of Abl at various developmental time points with 432 bp probe. (A) Sense negative control probe displays no specific staining of any tissue. Scale bars, 100 μm. (B) E7 (46%) embryo shows staining of the CNS. Scale bars: 100 μm; 100 μm; 10 μm. (C) E9 (48%) embryo shows staining of the ganglia of the CNS. Scale bars: 100 μm; 10 μm. (D) E11 (49%) embryo shows staining in the ganglia as well as the Comb cell (CC). Scale bars: 100 μm; 10 μm; 10 μm.

WISH analysis with probes made from the 916 base pair sequence showed similar expression patterns in the ganglia, nephridia, and also among surface cells along the bilateral muscular heart tubes. This finding is interesting since *Hm*LAR1 was found to be expressed in the lateral heart tubes at embryonic day 10 (Gershon *et. al*, 1998). Distinct cell bodies of neurons in the CNS can be distinguished upon closer magnification of a single ganglion (Figure 10D). Expression is also seen in the Comb cells, but again, these profiles vary from one WISH to another. Of all the trials conducted, the Comb cells can be distinguished in 25% of the preps. The latest Abl probe was constructed from the 1.6 kb sequence and shows markedly different patterns of expression. Granular staining in the CNS is still clear and distinct; however, expression and staining in the bilateral heart tubes disappears. Distinct bilateral clusters can also be seen in these new WISH trials. These clusters are that of the second segmental sensillum (Figure 11C). These sensillar neurons are thought to include mechanoreceptors that are sensitive to touch or pressure, as well as photoreceptors. The change in the patterns of expression might be attributed to the difference in length of the RNA probe. Since the different lengths contain different areas of the sequence that might house conserved domains, it might be possible to have a more specific area of hybridization as evident in the latest WISH than compared to more generalized expression seen with earlier WISH. This might be due to the fact that conserved domains such as the protein tyrosine kinase domain is expressed broadly in many cell signaling pathways, while extension in the 3' end narrows where expression of Abl is found. One factor that also may need to be taken

into consideration is the method of probe construction. The RNA probe is transcribed from DNA that was amplified from cDNA from embryonic leeches from the colony in the laboratory. An issue at hand is that in-house breeding might be yielding a hybrid between two species that are present in the colony, *Hirudo medicinalis* and *Hirudo verbana*. The mixing of different species may produce offspring that have subtle differences in genotype. Thus, probes made from the cDNA from one group of embryos may be different from one made from another group.

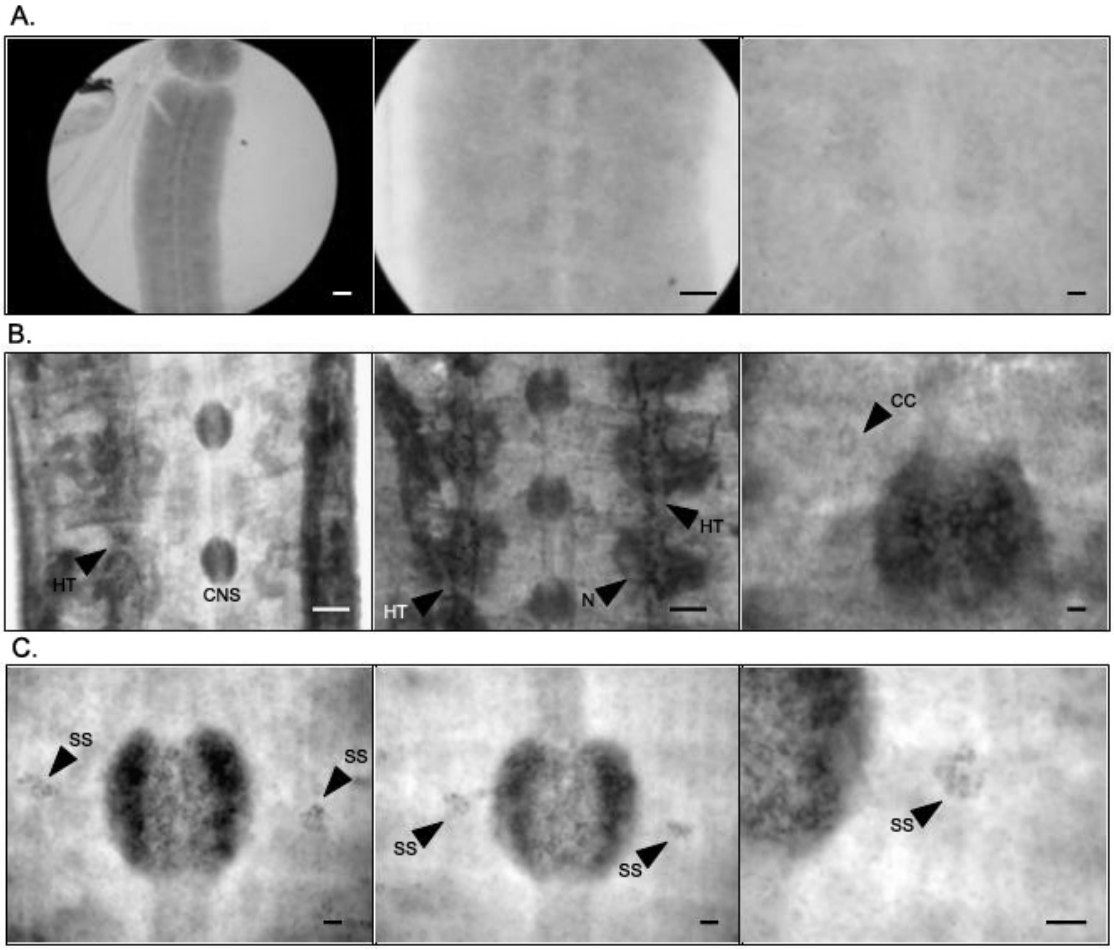

Figure 11. WISH expression patterns of Abl. (A) Sense negative control probe shows no specific staining in any tissue. Scale bars: 100 μm; 100 μm; 10 μm. (B) 916 bp antisense probe clearly labels the heart tubes (HT), CNS and also the nephridia (N) in the first two panels. In the third panel, the comb cell (CC) is present as well as cell bodies inside the ganglion. The third panel is a close-up of a ganglion from the same CNS as the second panel. The first and second panel feature different embryos. Scale bars: 100 μm; 100 μm; 10 μm. (C) 1.6 kb antisense probe clearly labels granular cells in the CNS as well as the bilateral sensilla clusters. Scale bars, 10 μm.

RNAi knock-down of Abelson kinase

Following the validation of the expression patterns of Abl, the next step was to try to elucidate the function of Abl through RNAi knock-downs using double-stranded RNA. It has been previously shown that RNAi knock-down of *Hm*LAR2 via dsRNA injection into the comb cell induces growth cone collapse and loss of filopodia (Baker and Macagno, 2000). Additionally, the *Hm*LAR2 RNAi phenotypes display cross-

over errors and zigzagging of processes. If Abl works in tandem with LAR as has been shown in *Drosophila*, then one might expect RNAi knock-down of Abl to produce a phenotype that displays the opposite effect of growth cone collapse in the comb cell.

Knock-down trials were conducted using double-stranded RNA transcribed from the 1.6 kb DNA sequence. The quality of the RNA was measured to be high, with optical densities 260/280 and 260/230 both over 2.0. Procedures were taken to carefully remove any possible protein contaminants through aqueous-organic liquid extraction. The concentration of the RNA used for the first set was 0.3 μg/μl and at least 1.0 μg/μl thereafter. Figure 11 shows a final gel electrophoresis test that was done to confirm that the RNA of interest was indeed in the solution that was injected.

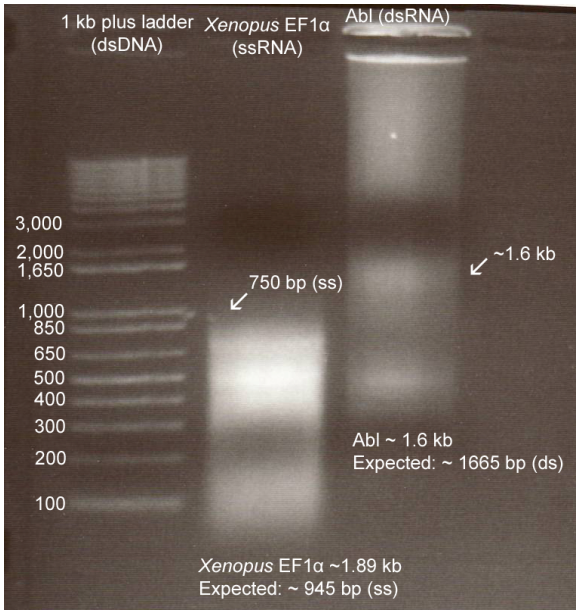

Figure 12. Confirmation gel of Abl dsRNA. The first lane is a positive control for the RNA transcription. The product is slightly lower than the expected 945 bp because the transcription reaction was not optimized for that strand of RNA. The second lane shows Abl at the expected length of 1.6 kb. The residual band at 400 bp could be nonspecific binding or premature termination of transcription.

Injections were conducted on E7 (46%) embryos using pulled thin glass capillaries tubes. The site of penetration of the tip of the electrode was at the posterior tail under the germinal plate, which is between the body of the animal and the yolk sac, where approximately 1-2 μl were injected. Embryos were dissected 5 days after the first set of injections, but were dissected 48 hours after injections in the subsequent sets due to high mortality rate. SiRNA injections were also done in conjunction with the dsRNA to optimize the experiments and compare if the efficiency of one was greater than the other. Fluorescent antibody staining was done with anti-serotonin after dissection, which provides a good view of a subpopulation of neurons in the ganglia as well as the lateral axons that extend out peripherally. Serotonin staining also shows the connectives very nicely. In addition, it provides a basic layout of the CNS, which has an advantage over anti-tubulin, which shows a complex network of peripheral axon projections. The third ganglion from the tail was imaged in all the dissected embryos because the segments closest to the site of injection should show the most promise in terms of phenotype change.

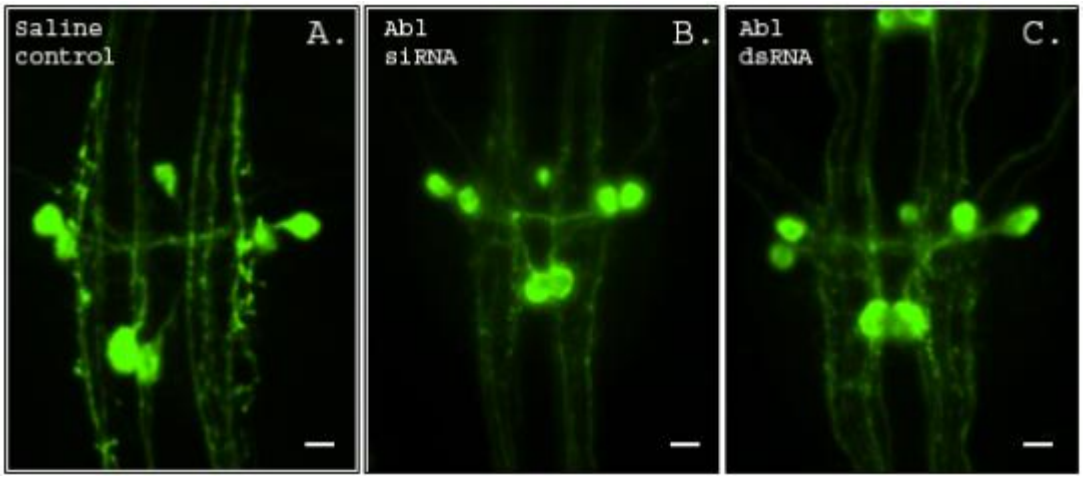

Figure 13. Serotonin staining of 3rd ganglia from the tail 5 days after RNAi. (A) Saline control injection with liposome. (B) Abl siRNA injection with liposome. (C) Abl dsRNA injection as liposomes. Scale bars, 10 μm.

Table 2. RNAi Trials

Condition	Total injected	Total survived
Saline control without liposome	8	2
Saline control with liposome	4	2
Frog GADPH siRNA control (from kit) with liposome	12	6
LAR1 siRNA control with liposome	6	2
Abl dsRNA without liposome	8	3
Abl dsRNA with liposome	4	3
Abl siRNA without liposome	8	3
Abl siRNA with liposome	22	10
Total	72	31

Figure 13 shows no apparent change in phenotype. Subtle changes can be seen in the density of fascicles in the bilateral connectives, but no major change can be conclusively argued. A total of 12 embryos were used in the set, which divided 4 embryos to each injection group. The embryos had a 75%, 50%, and 25% mortality rate among the saline control, siRNA, and dsRNA injections respectively after 5 days.

Care was taken to inject each embryo in the same manner and with the same amount of solution (around 1 μl); however, potential error can arise in terms of the health of the embryo and also the damage caused by the trauma of the injection. Using a liposomal agent was a measure taken in order to increase the efficiency of transporting the RNA through the cell membrane and into the cytoplasm. In order to increase the confidence that the RNA was actually getting into the cells, the following control experiment was done to ensure that this was occurring. By using a DNA plasmid with the GFP gene under control of leech actin promoter, cells should fluoresce if the liposomes actually passed through the cell membrane and into the nucleus. If this event can occur, one can be fairly confident that the liposomes carrying RNA can deliver the RNA into the cytoplasm of the cell. Figure 14 shows expression of GFP in some cells found in the body wall, near the site of injection. However, neuronal cells in the CNS did not appear to express any EGFP. This suggests that lack of delivery of the RNA into the cytoplasm of neuronal cells might be the reason that there was no detectable change in neuronal phenotype.

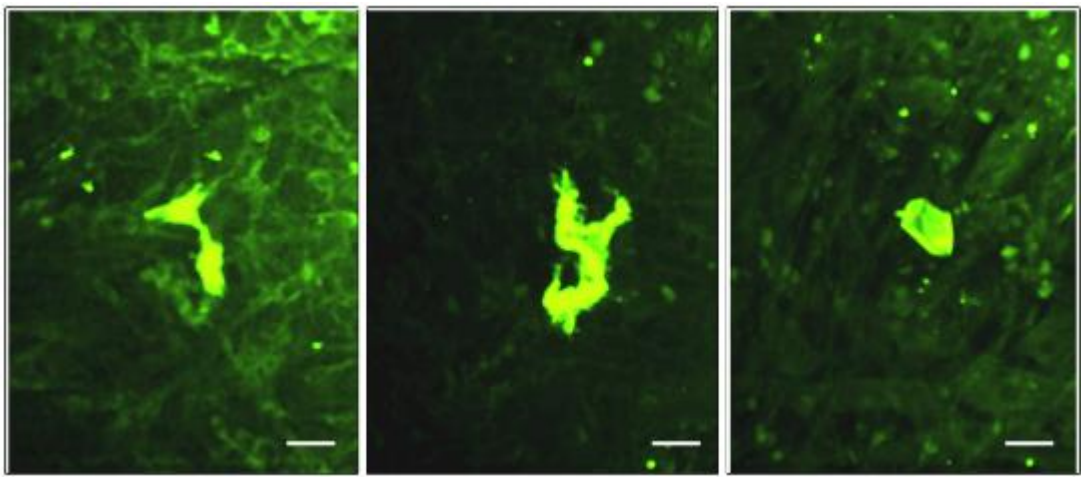

Figure 14. Cells in the body wall expressing EGFP near the site of injection. Scale bars, 10 μm.

Five total sets of injections showed no morphological difference in phenotype between the control groups and the RNAi groups despite trying to enhance the efficiency of RNA delivery through use of liposomes and also increasing the concentration of the RNA. Subsequent injections also replaced the saline control injection with a frog GADPH RNA and also LAR1. The last set of injections also were stained with the monoclonal antibody Lan3-2, which stains the axons projecting from the sensilla to the central nervous system, cells that are shown to express the leech Abl kinase in Figure 11C. A change in phenotype could be reflected in errors in the morphology of the central axonal terminals, but comparison of control to RNAi groups showed no distinctive differences. One possible explanation for RNAi failing to show a distinctive phenotype could be that the Abl gene I cloned is incomplete and that the whole sequence is required to produce an effective RNAi knock-down. Another possibility is that this particular kinase sequence cannot be knocked down by RNAi. In fact, RNAi does not work equally as well in all cell types. For example, systemic administration of dsRNA has been shown to be ineffective in knocking down expression in neuronal cells in *C. elegans* (Kennedy *et al.,* 2004). To test this possibility, an additional experiment needs to be performed to express and subsequently knock down Abl in cell culture, but that also requires a complete protein sequence. Future goals are to complete the whole sequence for Abl in addition to testing if Abl is truly being knocked down in these RNAi experiments. Possible methods to test the latter are shooting RNA coated gold particles into the area of interest, or single-cell injections into the comb cells, as well as electroporation.

CONCLUSION

My investigation of two leech phosphotyrosine kinases, FAK and Abl, showed that these two kinases exhibited strong expression in the CNS, but expression was less consistent and evident in the Comb cells. It has been shown that HmLAR1 and HmLAR2 are localized to neurons as well as other types of cells. Specifically, HmLAR2 was found on the filopodia of the Comb cells (Gershon et al., 1998). Previous genetic work in *Drosophila* suggests that d-Abl is a suppressor of Dlar function and that they play antagonistic roles in the specification of the intersegmental nerve route of a subset of axons (Wills et al., 1999a). Consistent positive expression in the Comb cells would provide a strong basis for suggesting that leech Abl or FAK might be potential antagonists in a pathway for actin assembly and neurite outgrowth (Figure 1).

RNAi knock-down could not be confirmed through injections under the germinal plate. Delivery of dsRNA into the cells is one obstacle. Another challenge is that some genes work well, while others do not and specific sequences might be required and is critical for knocking down signal. Members of the RPTP and PTK families can also have partially redundant functions so that the absense of one member does not produce strong phenotypes (Desai et al., 1997). Although the exact function of leech Abl has yet to be determined, future experiments should include biolistic delivery of coated gold particles with dsRNA, which might reduce the amount of tissue damage as well as allowing for RNAi in selected segments and central ganglia of the leech embryo (Shefi et al., 2008). Elucidating the function of these PTKs such

as Abl remains not only to be an important step in understanding the role of these kinases in neuronal outgrowth in the developing leech, but also how they fit into the larger picture of cellular signaling pathways.

APPENDIX

FAK Primers

427 bp PCR product:

FAK-F (5’ – TAAACATTTCGAGGACCGAGG – 3’)

FAK-R (5’ – GCAACAGAAGCCTCCTTTCTTGTGGTGCC – 3’)

1.6 kb degenerate PCR product:

Block A-F-Outer (5’ – ATGAGGCAGTTCGACCAYCCNCAYAT – 3’)

Block B-F-Inner (5’ – CAAGAGGTTCGTCCACAGAGAYATHGCNGC – 3’)

Block J-R-Inner (5’ – TADSTYTACCGGGTGTTCCACGA – 3’)

Block K-R-Outer (5’ – CTRCGNTTYTTGGACGACCTGCAGTAGC – 3’)

FAK Sequence

ATGAGGCAGTTCGACCACCCGCACATCACTCAACTTATTGGCGTCTGCTCT

AAAGATCAGGTGCTTATTGTCATGGAACTAGCAGAACTGGGAGAGATGAG

GAGTTTCCTTCAAGAGAACCGACACGTGCTCGGCATTCCGACCCTGATGAC

GTACTGCTATCAACTGAGCGCCGCACTGGCTTATTTGGAATCGAAGCATTT

CGTGCACAGAGACATAGCGGCGAGAAACGTCTTGGTGTCCTCGATCGATT

GCGTCAAACTTGCAGACTTCGGGTTGTCGAGGATGTTGATGGACGAGAAC

TACTACACAGCTTCGATCGAAGGAAAACTGCCAATAAAGTGGATGGCACC

AGAGTCGATAAATTACAGAAGATTTACTTCGGCCAGCGACGTGTGGATGT

TCGGTGTTTGCATGTGGGAAATTCTTTCCTACGGCACCAAGCCCTACTTCG

GGGTGAAGAACAGCGACGTGATCACGAGGATCGAAATGGGCGAGAGGCT

CCCTCTGCCCGAGATGTGTCCACCGAGCTTTTACAACCTCATGTGTCATTG

TTGGGCTTACATTGCAGAAGAGAGGCCTAACTTTACTCTGCTTCAGAAGTT

AATTTGTGATGCACTTTCACAAGAAAGGTGCGATTCTTCGCTTCCGTCCTG

GATGGTTGAAAATTGCATTCAAGCGCACGACAAACCTCCCACAAAAAGCT

CAGAGGGTGCTCCCAGCAAACAGTCGGGCGGCTTCAACGGCCTCGCCATT

TCTACGCCGAACCTGCCGAACGTCCCGAACGGTGCTTTCGACCTGGCGACG

TGGTCTCGTCGCAGGGAATGGATCGATGCAGATCTCCAGGATTCTTTGGGG

AGGGGTGGAAGGTGCTCCAGCGACAACATCCTCGACAAGAGAGATGAAGT

TCAATTCGCCAATTGCCCCGAAGGTTTCTACAGCGAGATAAATTCCCCCCA

TACACCTGGAAGTAGTGCCACAAACATTTTGCATCATCAGAAGCTGCAGA

GTTGTCGAGATGAAGCATGGTTGCAATCGGTGGAGAGTAGAACAACTCCT

AAGAAGCAAGATGAATGCAGGACAGCTGGCTTGGTGCCCTCATCAACATT

GCACCAGTCTCTGAGAAAGTCCATGCCCGATGAATTCTTCAAGTCCATAAA

CATTTCGAGGACCGAGGACAAAGTTTACGAAGCCACGAAGGGGCTCATTG

CATCAGTGCTTAATCTCACCTCGATGACAAAATCCAAGCACTTTGAGAAGA

CCTATGCTCAGCTTGCCATGGTTGTTGGTGGGTGTTTGAAGCAACTGCTTG

GTTTGACTGAAAAAGAAATGTTGGATGAAGGCAAGGAAGAGATTCAAATG

GCTCAAAAGCTGCTCGTTTCAGACATGGCCAATCTCGTCAAGACGACAAA

GCTTGCAGATTCAAACAGGGCCACCATTCTCGACGAACAATATCGTAAGA

ACATGCTCCAAGCTGCTCACGTACTTGCTGTGGATTCAAAAAACTTTCTTG

AAACTGTGGACTGCATCAGAAAATACCAAATATTCACCATTGGCACCAAA

GAAAGGAGGCTTCTGTTGC

Abl Primers

432 bp PCR product:

Abl1-F (5’ – CCATTGTCAGGCTCAGGACCAGTT – 3’)

Abl1-R (5’ – CAAAACTAGGGAGTGGACGAGTG – 3’)

888 bp PCR product:

Abl2-F (5’ – TCCATGGTCCAGTATCCAGGAGTA – 3’)

Abl2-R (5’ – AGACGTCTGATTTTGTTGAGAACC – 3’)

916 bp PTK degenerate PCR product:

Abl1-F (5’ – CCATTGTCAGGCTCAGGACCAGTT – 3’)

PTKase-R (5’ – ATAGGAATTCCAWRGGACCAVACRTC – 3’)

3’ RACE PCR:

Abl3’RACE1-R-Outer (5’ – AAGTGGCCGACTTTGGCTTGGCA – 3’)

Abl3’RACE1-R-Inner (5’ – AGGACGACACCTACACTGCCCA – 3’)

Abl3’RACE2-R-Outer (5’ – AGGTCTCGCTTACAACAGGTTCTC – 3’)

Abl3’RACE2-R-Inner (5’ – AGACGTCTGGTCCCTTTGGAA – 3’)

3’ RACE confirmation PCR:

Abl3-F (5’ – GCCCTTTCCATGGTCCAGTA – 3’)

Abl-1698bp-R (5’ – GAAGAAATGCAGGAGGAGGAGG – 3’)

Abl-1665bp-R (5’ – CCGAGACATCGGTGTCTGGA – 3’)

Abl-1541bp-R (5’ – GCTTCGAGACTGGCTGGCAG – 3’)

Abl-1329bp-R (5’ – CTGCCCATGGTTGCACCATC – 3’)

Abl 1.6 kb 3' RACE PCR:

Abl3-F (5' – GCCCTTTCCATGGTCCAGTA – 3')

Abl3'RACE3-R-Outer (5' – CTCCACCCACAAGGACAAGTTCC – 3')

Abl3'RACE-R-Inner (5' – TTCAGGGATCAGATGGTGCAACC – 3')

Abl3'RACE-R-SH2PTK (5' - CACCTCCAGTATCCCGTGTCCAA – 3')

Abl 2.1 kb 3' RACE PCR:

Abl3'RACE2.1-F (5' – TCCATGGTCCAGTATCCAGGAGTA – 3')

Abl 3'RACE2.1-R (5' – TACCATATCGTGCTGTCGACG – 3')

Abl Sequence

GCCCTTTCCATGGTCCAGTATCCAGGAGTAAGGCAGAATATTTGCTTAGCA

GTGGAATTAATGGCAGCTTCTTGGTGAGGGAGAGCGAGAGCTGTCCGGGC

CAATTGTCCATATCACTGAGGTTTGATGGGAAGGTTTATCACTATCGAATT

TATCAAGATGAAGCTGAGACTAAGATGTTTGTGACCACCGAGAACAAGTT

CACAACCCTGGCCGAGTTGATTCACTTCCACTCGAAGCAGTCGGCAGGCCT

GGTGGTTCACCTCCAGTATCCCGTGTCCAAGAAGACTCAACCTCTTGCCTT

TGGAACTGGTCCTGAGCCTGACAAATGGGAGATTGAGAGGACAGACATTC

AAATGAAGCATCAGCTTGGCAGTGGACAGTATGGAGATGTATACGAAGGT

CTCTGGTTGTCTAGGAATATCACAGTTGCTGTCAAGACACTCAAGGAGGA

GACGATGGTGTTGAAGGATTTCTTGGAGGAGGCAGCTGTTATGAAGGAGA

TGAAGCATCCAAATCTTGTGCAATTGCTGGGTGCTTGCACGCTGGAACCTC

CGTTCTTCATCATCACCGAGTTCATGAGCAAAGGAAACCTTCTGGACTTCC

TCAGGAATTGTTCAGAGGAGGAGACTGGCCTGACAGTTCTGTTCTACATGG

CAACTCAGATTGCATCGGCCATGGCCTATTTGGAAGCCAAGAATTTCATTC

ACAGAGATCTGGCTGCCCGCAATTGTCTGGTAGCAGAGAACCACCTCGTG

AAAGTGGCCGACTTTGGCTTGGCAAGGCTGATGAAGGACGACACCTACAC

TGCCCATGCCGGGGCAAAGTTCCCCATCAAATGGACTGCACCGGAAGGTC

TCGCTTACAACAGGTTCTCAACAAAATCAGATGTTTGGGCCTTTGGCATTT

TGCTGTGGGAACTTGCTACTAGGGGCCAGTCACCATACCCTGGTGTGGACC

TCACAGAAGTTTACCATCTGCTTGAGAATGGGTATCGAATGGAAATGCCA

CCTGGATGTCCAGTGAATGTCTACAACCTCATGACCTACTGTTGGAGATGG

GAACCTCAGGAAAGACCTTCCTTCAAGGAAATCCTTCACCTTCTCGACAAC

ACACTTCTGGCATCGAGCCAGTGTGCTGACAAGGATTCAGATTCTGTTGAG

GAGATGGAAATGAAAAGGAGCGGCCATTTTCATTCGGAAAATAGCACTTC

GCCTGGATCCACGCTAAGCTCTTTTGGCAGCAAGCACTCAAAACTGAGCCT

AAAGAAAGCTCCAACTCCACCCACAAGGACAAGTTCCTTCAGGGATCAGA

TGGTGCAACCATGGGCAGGGGAGGAAGAAGGAATGGACCAACAGATTTC

ATCTTGCGACCGAAGTTACTCTAGCAAGAGTCACAGCACCGTCATGTCTGA

GTCAGTTCTGAGCAGATCGAAGCCTCACTGGTCTAATGAGGCACTTCTCAG

TGAATCTTTTAGCGACCACGTGCGATCTCAAAGCAGCAGTGATGATCTGCT

TTCGACTTCTGCCAGCCAGTCTCGAAGCGGCAGTTTCGACAGGATGCTCAT

GAGTCGCAAGGCTAGTCACGAACACGTTCCTGCCTCTCTTGTGGACTCCTC

GAATGATCAGCAGGAGCTCGGTCACAAGAGTCCAGACACCGATGTCTCGG

ATTCTTCTTTTCCTCCTCCTCCTGCATTTCTTCTAGAAGAACAGAAAAACTT

TGTCCCTATACCTTCAAAAAGAATGACCCAAAAGGTTAAAGAGAAATCCA

ATATTCCATCTGGCAGCCTCAACAGACGTCAAACTGCCATGGCCAGAGAG

GTTTTCGAGGAGGAAACCTTCACTGATTCTGGCCATTCCTCCCCTGCTGTT

GGTTTCCTGAGTGGAAATGCTTCAGGAATTCCTGTCAGTAAGGAATCCTTG

CCTCCAAATAGTCATTGTATCACCATACCCAAAAGAGCTGCCACCATGACC

TGCAAGGAAGAACTTGTCGAGGTCCTCGGAAGGCGTCGACAGCACGATAT

GGTAAAGTGCGATGAAGTAAAAAATGCTCCACTGGAAAAACCCATGACAA

ATTCCAATGCTCCCTACCTCAGCCTCATC

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