Role of Ubiquitin like protein Ubl-5 (HUB1) in *Caenorhabditis elegans*

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A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali
April 2015

Certificate of Examination

This is to certify that the dissertation titled "**Role of Ubiquitin like protein Ubl-5 (HUB1) in *Caenorhabditis elegans***" submitted by *Ms. Ane Nishitha V* (Reg. No. MS10023) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Shravan Kumar Mishra and Dr. Kavita Babu at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Ane Nishitha V.
(Candidate)

Dated: April 24, 2015

In our capacity as the supervisors of the candidate's project work, we certify that the above statements by the candidate are true to the best of our knowledge.

Dr. Kavita Babu
Dr. Shravan Kumar Mishra
(Supervisors)

Acknowledgement

I would like to express my sincere gratitude to my supervisors Dr. Shravan Kumar Mishra and Dr. Kavita Babu for giving me an interesting project to work on as my master's thesis.

I extent my heartfelt thanks to Pallavi Sharma for guiding me in my project and helping me to troubleshoot whenever required. I would also like to thank my labmates for the support and encouragement during my thesis work.

I also take this opportunity to thank my friends who have been very supportive for the entire duration of my course work. Without these people accomplishing such a project would have been impossible. I thank my family members for being with me through my ups and downs, specially my dad who always motivates me to pursue my dreams.

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Notation

HIND - Hub1 interacting Domain

PCR - Polymerase Chain Reaction

DNA - Deoxyribo Nucleic Acid

RNA - Ribo Nucleic Acid

CGC - Caenorhabditis Genetics Centre

NGM - Nematode Growth Media

LB - Luria Broth

Y2H - yeast two hybrid

TAE - Tris Acetate EDTA

GST - Glutathione S Transferase

RT - Room Temperature

Contents

Abstract

Hub1, the non-canonical ubiquitin like protein is involved in alternative splicing of genes by interacting with the HIND (Hub-1 interacting Domain) containing proteins. In multicellular organisms, the pre-mRNA targets that require Hub1 for splicing are not yet known. Here in the multicellular nematode *C. elegans*, we report that Hub1 is required for the efficient splicing of the gene tos-1 (target of splicing). In *C. elegans* there are two putative proteins Prp38 and Snu66 that harbor HIND like sequence. However, the HIND of Prp-38 did not interact with Hub1 in yeast two hybrid assay. Additionally in *C. elegans*, Hub1 shows temporal expression during the development of the worm and is also essential for viability. Thus Hub1 role in RNA splicing seems to be conserved in this multicellular organism.

CHAPTER 1

INTRODUCTION

1.1 Introduction:

Splicing is one of the important processes in biological systems and was first discovered in a virus [1]. It is defined as the process whereby introns (non coding segments) are removed and exons (coding segments) are joined from the nascent RNA that has been just transcribed. The first transcript from the DNA contains both introns and exons and is known as pre-mRNA. This pre-mRNA undergoes splicing to give mRNA and is then transported to the cytoplasm to be translated into proteins. Splicing can be *cis* or *trans*. In most eukaryotes, spliceosomal splicing occurs in *cis*- (i.e) exons from the same pre-mRNA are joined together. However in some organisms like *C. elegans* and trypanosome parasite *trans* splicing is common. In *trans* splicing a common exon is being joined to a number of different and separately transcribed exons [1]. There is another regulated RNA processing which is alternative splicing.

Human genome consists of 25,000-30,000 genes even though there are at least 90,000 proteins made by human cell. Also humans and the small plant *Arabidopsis thaliana* have similar number of genes despite the complexity in human physiology [2]. This gene number paradox can be explained by alternative splicing.

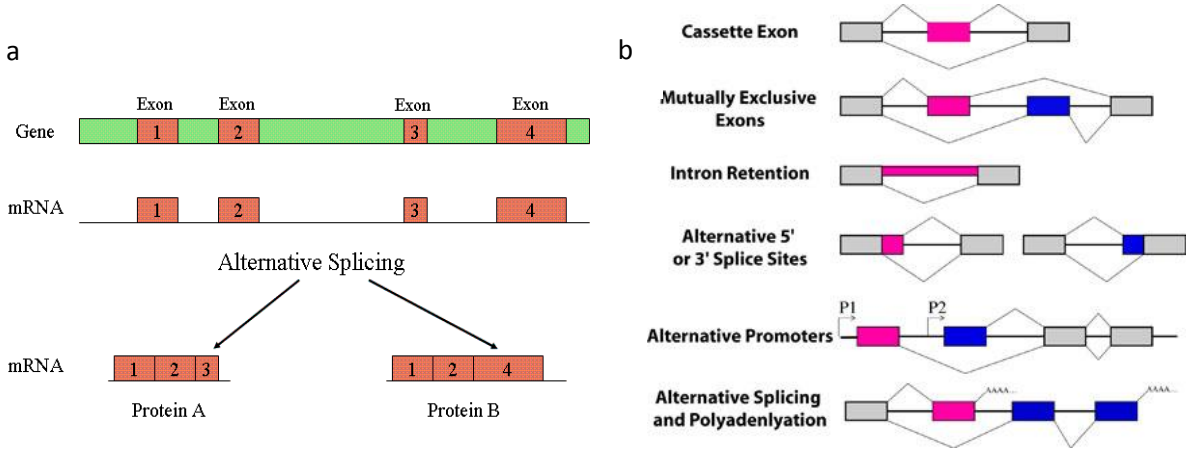
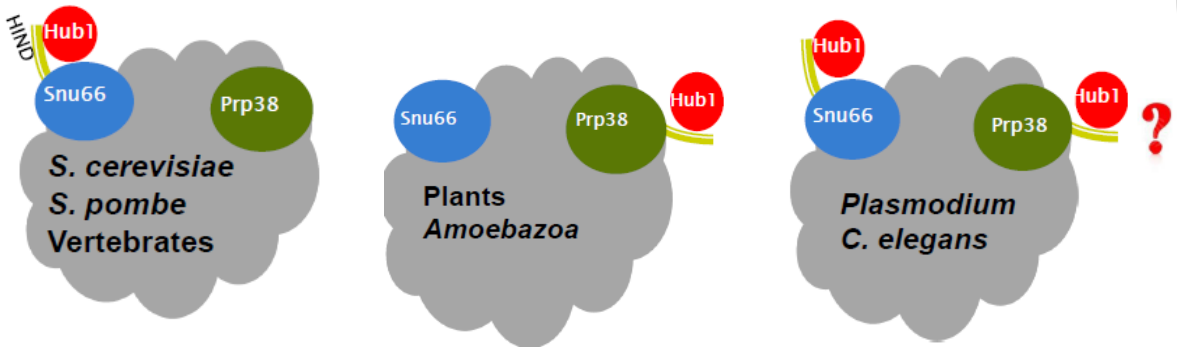


Figure 1.1: Schematic representation of a) alternative splicing of a gene that has four exons[13]. Exons are given in brown colour whereas introns in green. b) Different types of alternative splicing. Exons included in mRNA by alternative splicing are coloured and constitutive exons are shown in grey. Promoters are indicated as arrows and AAA for polyadenylation sites

Alternative splicing generates multiple isoforms of protein thus increasing the proteome of an organism [3]. It allows a single gene to encode for multiple proteins and thus increasing the complexity. Previous studies have shown that 40-60% of human genes are alternatively spliced. The various types of alternative splicing include alternative 5' splice sites, alternative 3' splice sites, exon skipping, intron retention, cassette exons and mutually exclusive exon[2,3].

Hub1, known as UBL5 in metazoans binds to certain splicesosomal proteins that possess a specific sequence known as HIND (Hub-1 interaction Domain). This sequence is about 18-20 aminoacids long and is conserved from Yeast to humans. Although Hub1, HIND sequence have been conserved, the protein that possess this HIND sequence varies. In case of Yeast and Vertebrates, the splicesosomal protein Snu66 has this sequence whereas in Plants it is Prp38. Intriguingly in *C. elegans* both these proteins possess this sequence[4].

a



b

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CeSnu66 320aa CePrp38 800aa

CeSnu66-HIND 299-319aa    DSMSIEETNKLRASLGLAPLE 21
CePrp38-HIND 50-71aa      EEREIAEANALRAKLGLAPLE 21
                          :.  .*  *:*  ***.*******
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Figure 1.2: a) Schematic depiction of the proteins interacting with Hub1 in various organisms. Yellow tail indicates HIND sequence[4]. b) Sequence similarity between Snu-66 & Prp-38 HIND region in *C. elegans*[4]. In Snu-66, HIND is present in N-terminus whereas in Prp-38 it is present in the C terminus.

Caenorhabditis elegans is a free-living, non-parasitic, transparent nematode which is found in soil environment. It's about 1-1.5 mm in length, transparent and has a life cycle of 3 days [6]. It was the first multicellular organism to have the entire genome sequenced. These attributes made this organism an excellent model to study genetic and behavioral analysis. They are seen either as hermaphrodites or as males. This natural mode of inbreeding by the self-fertilizing hermaphrodites and cross breeding with males made it convenient for genetics. The transparent body makes it easier to track cells and follow cell lineages. Its anatomical simplicity (>1000 cells), smaller genome (20 times larger than that of *E. coli* and 1/30 of that of human) are the other key features [6, 7].

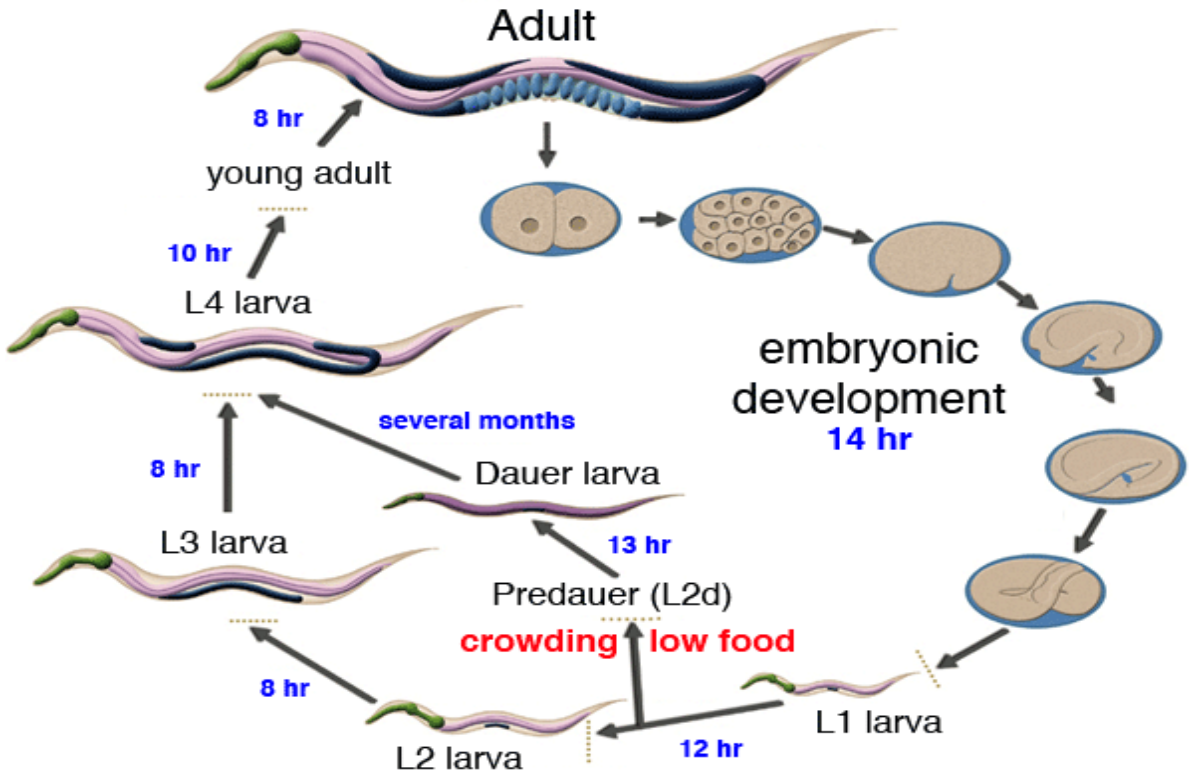


Figure 1.3: Life-cycle of *C. elegans* at 22° C. *C. elegans* develops through four larval stages and the duration for the development of each stage is indicated [13].

C. elegans has a life span of about 2-3 weeks under suitable living conditions. It takes three days to become an adult and a single animal can generate 300-350 progenies. *C. elegans* feeds on bacteria and develops through four larval stages. And alternative splicing plays an important role in worm development. In this animal 20% of the genes are alternatively spliced [3, 12] and 70% of the genes are trans-spliced.

Although Hub1 (Ubl-5) has been studied in various process like cell cycle progression, mitochondrial unfolded protein response [5], mRNA splicing, the genes that require Ubl-5 for splicing are not understood. So there were three objectives for this project: 1) to establish that in higher organisms Ubl-5 (Hub1) is indeed involved in splicing, 2) to find genes that require Ubl-5 for splicing in *C. elegans*, and 3) to understand the relevance of redundancy in the splicesosomal proteins that are interacting with Hub1 (Ubl-5) in *C. elegans*

CHAPTER 2

MATERIALS AND METHODS

2.1 *Cloning:*

Cloning involves the following steps: 1) PCR amplification of the gene to be cloned, 2) digestion of insert and the vector, 3) ligation, 4) transformation of the ligated product and 5) isolation of the ligated plasmid.

Prp38 was cloned in Yeast two Hybrid (pGADC1 and pGBDUC1) and GST (pGEX 5X1) vectors. The coding sequence length of the insert is 963bp and was cloned in these vectors. Also the region that code for the amino acids from 292 to 320 which contains the HIND part (around 90 bp) was cloned in these vectors.

Vector	Insert
pGADC1 (6667 bp)	Prp-38 entire length (963 bp)
	Prp-38 (90 bp)
pGBDUC1 (6000 bp)	Prp-38 entire length (963 bp)
	Prp-38 (90 bp)
pGEX 5X1 (4972 bp)	Prp-38 entire length (963 bp)
	Prp-38 (90 bp)

Table # 1: Vectors and the insert used for cloning. The size in base pairs (bp) is given in brackets.

2.1.1 Restriction Digestion and Ligation:

Restriction enzymes are enzymes that can cut the DNA at specific sites known as Palindromic sequence. They act as a defense mechanism against viruses and are harvested from them for research purposes. The recognition sequence is usually 4-8 nucleotide long. Because these enzymes cut the DNA within the sequence they are known as endonucleases. Once the vector is digested it is dephosphorylated to prevent self ligation. The enzyme ligase joins the vector and the insert. This ligated plasmid is then transformed to bacterial cells.

The yeast two hybrid vectors and the insert were digested with the enzymes BamHI and PstI. For GST vector, digestion was done with SalI and EcoRI.

Digestion: Reaction Volume 20 µL

Reagents	Amount
DNA	500ng to 1 µg
Buffer	2 µL
Enzymes	0.5 µL each
H_2O	to make up the volume

Table # 2: Reagents used for restriction digestion

Antarctic phosphatase was used to dephosphorylate the vectors

Dephosphorylation:

For a reaction volume of 25 µL

- 2.5 µL of buffer
- 0.5 µL of enzyme
- 20 µL of digested sample
- 2 µL of water was used

2.1.2 Transformation:

Transformation can be done by Chemical method or by Electroporation. Electroporation involves electric shock and making pores in the membrane of the bacteria. In chemical method heat shock is given combined with the use of Calcium to bring the negatively charged DNA molecules closer to the surface of the bacteria so that once the pores are created ligated plasmid can be actively taken inside the cell.

DH5α cells were used as competent cells and ligated plasmids were chemically transformed.

Chemical transformation:

1. Took 20-40 µL of DH5α competent cells and added 1-5 µL of the ligated product
2. Kept it in ice for 30 mins
3. Gave heat shock (42^{o} C) for about 30 seconds

4. Immediately placed the tubes in ice for 5 mins
5. Added 200 µL of LB and incubated it for an hour at 37^{o} C
6. The samples were plated on antibiotic plates (here the vectors are resistant for ampicillin) and incubated at 37^{o} C overnight

2.1.3 Plasmid Isolation:

Bioneer plasmid extraction kit was used to isolate plasmids

1. Grew the transformed bacteria in LB (Luria Broth) medium overnight
2. Pelleted the cells at 7830 rpm for 4 mins
3. Discarded the supernatant
4. Added 250 µL of P1 buffer (contains RNAse) and vortexed the tubes
5. Added 250 µL of lysis buffer and inverted it gently. Transfered it to 1.5 µL tube
6. Added 350 µL of neutralizing buffer (solution turned turbid because of protein accumulation) and centrifuged it at maximum speed for 10 mins
7. Transfered the supernatant to an empty column and centrifuged it for 60 seconds at maximum speed. Discarded the flowthrough
8. Then added 500 µL of wash buffer to the column and centrifuged it at maximum speed for 60 seconds. Discarded the flowthrough
9. Repeated step 8
10. Gave an empty spin for 60 seconds at maximum speed to remove all residual ethanol
11. Added 20 µL of water and kept it in room temperature (RT) for 5 mins
12. Centrifuged it for 60 seconds at 13000 rpm

2.2 Worm maintenance:

Ubl-5 mutant worms that were maintained in heterozygous condition were obtained from CGC. Both the mutant (VC3368) strain and the wild type were maintained in 25^{o} C. *E. coli* strain OP50, which is a uracil auxotroph was given as a food source [8]. 60mm NGM plates were used for normal maintenance of worms and 90mm plates were used when the large quantities of worms were required.

2.2.1 Genotyping of the Ubl-5 mutant strain:

- Picked one worm in 2 µL solution of lysis buffer and proteinase K using platinum wire worm pick.
- Lysed the worms and released the genomic content by heating it to 65^{o} C for 60-90 mins.
- Inactivated proteinase K by heating it to 95^{o} C for 15 mins
- Amplified the gene using PCR

Lysis buffer composition:

- 200mM NaCl
- 100mM Tris-HCl (pH 8.5)
- 50mM EDTA (pH 8.0)
- 0.5 % SDS

Three primers were designed for the genotyping of the Ubl-5 mutant strain VC3368. Two forward primers and one common reverse primer. The forward primer that was designed in the deleted region doesn't show a band if the worm is a homozygous mutant.

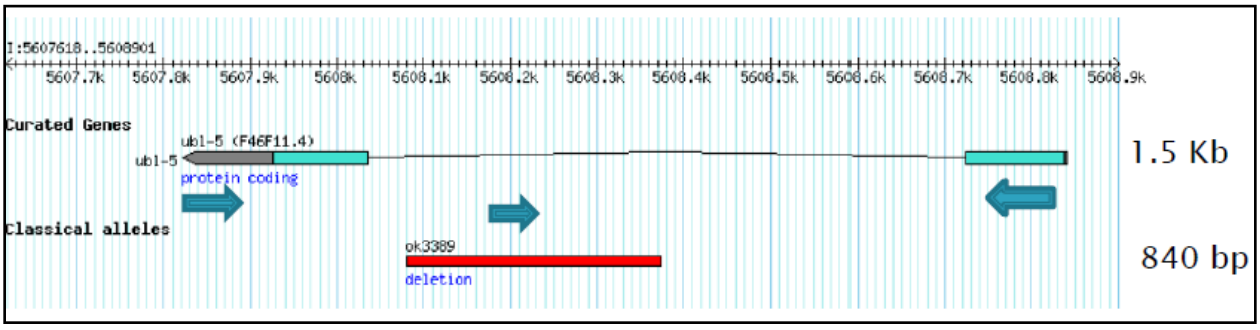


Figure 2.1: Ubl-5 (HUB1) transcript and the deleted portion. Red line indicates the deleted region in the mutant (modified from reference 14). Arrows indicate the regions where primers were designed to confirm the mutation.

2.3 RNA isolation:

The following protocol was used for isolating RNA from *C. elegans.*

1) Collected worms from 3-10 plates (For L1-L2 stages more worms are required to get a good concentration of RNA) in 400µL of Trizol
2) Frozen and thawed it for 3 times
3) Added 200 µL of Trizol. Mixed and incubated for 5 minutes at room temperature (RT)
4) Centrifuged the tubes at 14000 rpm for 10 min at 4^{o} C.
5) Removed the supernatant and added 100 µL of Chloroform. Incubated for 2-3 min at RT
6) Transfered to phase-lock columns and centrifuged at 12000 rpm for 15 mins at 4^{o} C (Before using phase-lock columns gave an empty spin for 30 seconds at 12000 rpm)
7) Added 150 µL of chloroform and repeat step 6.
8) Transfered the aqueous layer to a new tube and added equal amount of isopropanol
9) Incubated for 10 mins at RT
10) Centrifuged at 14000 rpm for 10 mins at 4^{o} C. Discarded the supernatant.
11) Then washed with 100 µL of 70% ethanol and gave a spin at 10000 rpm for 5 mins at 4^{o} C.
12) Removed supernatant and dried in laminar hood for 30-60 mins.
13) Once it dried added around 20 µL of H_2O and heated it for 5 mins at 60^{o} C

2.4 cDNA preparation :

Complemantary DNA (cDNA) was being prepared using Roche Kit (for normal PCR) and Takara (for Real Time PCR)

<u>cDNA synthesis using Roche Kit:</u>

- RNA-500ng to 1µg
- Random hexamer primer-2 µL
- H_2O to make up the volume to11.4 µL

- Keep it at 65^{o} C for 10 mins
  - Buffer- 4 µL
  - RNAse inhibitor- 0.5 µL
  - dNTP mix- 2 µL
  - DTT- 1 µL
  - Reverse Transcriptase -1.1 µL
- Keep it in 55^{o} C for 10-30 mins
- Then at 85^{o} C for 5 mins

cDNA synthesis using Takara primescript:

  - 5X Mastermix
  - RNA- 500 ng to 1 µg
  - H_2O to make up the volume
- 37^{o} C for 15 mins
- 85^{0} C for 5 seconds
- 4^{o} C

2.5 Polymerase Chain Reaction:

Polymerase Chain reaction is a technique used in molecular biology to amplify single or few copies of DNA sequences across several orders of magnitude [(9)]. The basic principle of PCR is that it multiplies DNA copy number exponentially. This exponential increase is accomplished by polymerases which are enzymes that can bring together individual nucleotides to form long molecular strands.

Steps involved:

- Denaturation: DNA denaturation was done in 95^{o} C.
- Primer annealing: This temperature depends on the melting temperature of the primers. The primers that were used for various reactions in this project have a melting temperature ranging from 52 ^{o}C to 62 ^{o} C.
- Elongation: Temperature used for this step was 72^{o} C.

PCR was used for genotyping the strains, amplifying the gene of interest (Prp-38), and also to identify genes that show splicing defect when Ubl-5 (HUB1) was knocked out. Phusion reagent kit was used for amplifying Prp-38 and Red Tag mix was used for genotyping of Ubl-5 and for identifying genes that show defects in splicing.

2.6 Real time PCR:

Real time PCR or quantitative PCR is a modification to the Polymerase Chain Reaction where the progress of the reaction is observed in real time. It allows for the detection and quantification of the PCR product in real time Detection of products in real-time can be performed by two methods 1) by using fluorescent dyes that intercalate the double stranded DNA, 2) by using specific DNA probes consisting of fluorescently labeled reporters [(11)]. The advantages of real-time PCR include: 1) it enables to perform quantitative analysis of gene expression, 2) it allows calculating the efficiency of the reaction precisely, 3) it gives a look in to the reaction, thereby aiding to know what all reactions have worked.

Takara Primescript reagent Kit was used for real-time PCR

Reagents	**Amount**
SYBR *Premix Ex Taq II* (2X)	12.5 µL
PCR forward primer	1 µL
PCR reverse primer	1 µL
cDNA	2 µL
dH_2O	8.5 µL
Total	25 µL

Table # 3: Reagents used for real-time PCR

2.7 Agarose gel electrophoresis:

The following procedure was followed to make 1% agarose gel

- Weighed 1 g of agarose and mixed it with 100 µL of 1X TAE buffer
- Heated it till the entire agarose got solubilized
- Added 4 µL of EtBr
- Placed the comb in the gel tray and poured the gel
- Once the gel got solidified, removed the comb and loaded the gel in the gel tank
- Then loaded the samples in the wells and ran it at 110 V

2.5 % gel was used to detect and purify Prp-38 90bp region.

CHAPTER 3

RESULTS AND DISCUSSION

3.1) CePrp-38 did not interact with CeHUB1 (Ubl-5)

Prp-38 was cloned in yeast two hybrid vectors pGADC1 and pGBDUC1 using the enzymes BamHI and PstI. After transformation of the ligated plasmid, restriction digestion was performed to confirm positive clones containing Prp-38 insert. XhoI cuts the insert Prp-38. So when digesting with XhoI and AflII, pGADC1 Prp-38 gives two bands around 1500bp and around 5Kb. The vector alone (pGADC1) gets linearized. For pGBDUC1, digesting with XhoI gives two bands one around 6Kb and another around 300 bp if insert is being cloned.

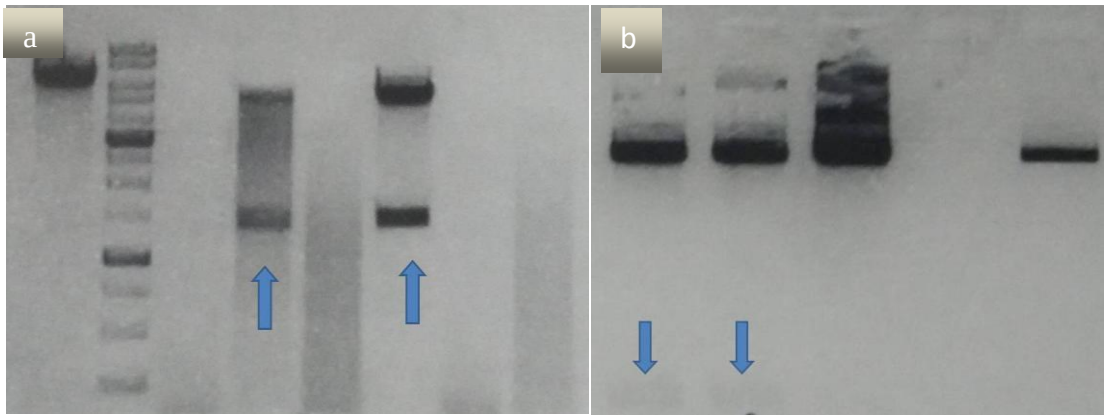


Figure 3.1: Positive clones for the Y2H vectors: a) vector pGADC1: lane 1 vector, lane 2 1Kb ladder, lane 3-8 ligated clones. b) Vector pGBDUC1: lane 1-4 ligated clones, lane 5 vector alone. Arrows indicate positive clones.

Yeast two hybrid [1*] was done to check interaction of Prp-38 with Ubl-5 (Hub-1). In the control plate (-leu -ura) histidine was supplied and hence growth was observed. In the selection plate (-His -leu -ura) no exogenous histidine was supplied and hence the organism survives only when these two proteins Prp-38 and Ubl-5 (Hub1) interact.

1* Yeast two hybrid was done by Kiran Kumar Reddy

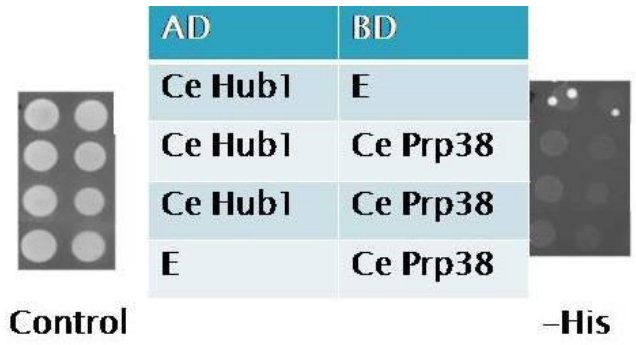
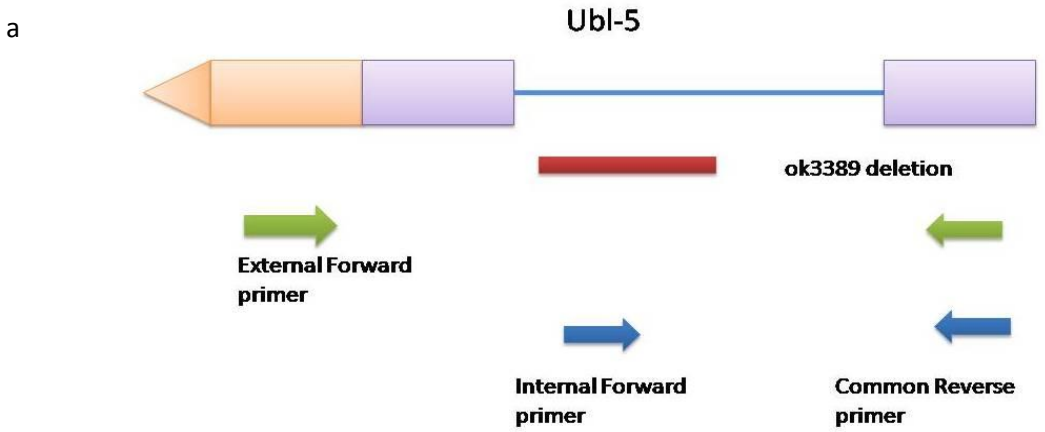


Figure 3.2: Interaction of *C. elegans* Hub1 with Prp-38 (full length) in yeast two hybrid assay [1*]. AD stands for activating domain and has leucine marker while BD stands for binding domain and has uracil marker. Left panel shows Ce Hub1 with Ce Prp-38 on control plates (-leu -ura). The right panel shows the interaction on –His plate (-leu –ura –his).

GST pull down assay was also done to check the interaction. But in both the cases, we couldn't observe interaction of Prp-38 with Ubl-5. This might be because 1) of the absence of endogenous environment which might affect protein structure or 2) these two proteins doesn't interact.

3.2) Ubl-5 expression increases during L3-L4 stage:

Hub1 (ubl-5) is essential for the survival in *S. pombe* [(4)] . Since Ubl-5 is involved in various proceses we speculated that the worms mutant for Ubl-5 is embryonic lethal. But the homozygous worms developed till the L3 stage after which they started dying. Genotyping was done to confirm that homozygous mutant worms have deleted copy of Ubl-5.



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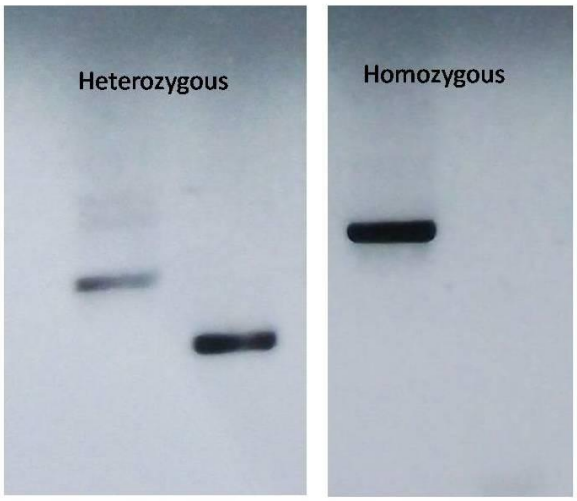


Figure 3.3: a) Cartoon showing Ubl-5 gene: exons are represented by purple boxes and the line represents the intron. Red line indicates deleted region. Green arrows indicate external primers and blue arrows indicate internal primers (modified from reference 14). b) Genotyping of the strain VC3368 for Ubl-5. Lanes 1,2 show heterozygous mutant worms and lane 3,4 show homozygous mutant worms. In lane 1 & 3, Ubl-5 amplified using external primer. Lane 2 & 4 amplified using internal primer.

No internal band was observed in homozygous mutant confirming that VC3368 homozygous mutant worms possess deletion in the Ubl-5 gene. Multiple worms showed similar result.

This raise the question of temporal expression of Ubl-5. So we proceeded with the quantification of Ubl-5 expression in different stages of the worm using Real time PCR. RNA was isolated from various stages of Wild-type worms. Actin was used as the control.

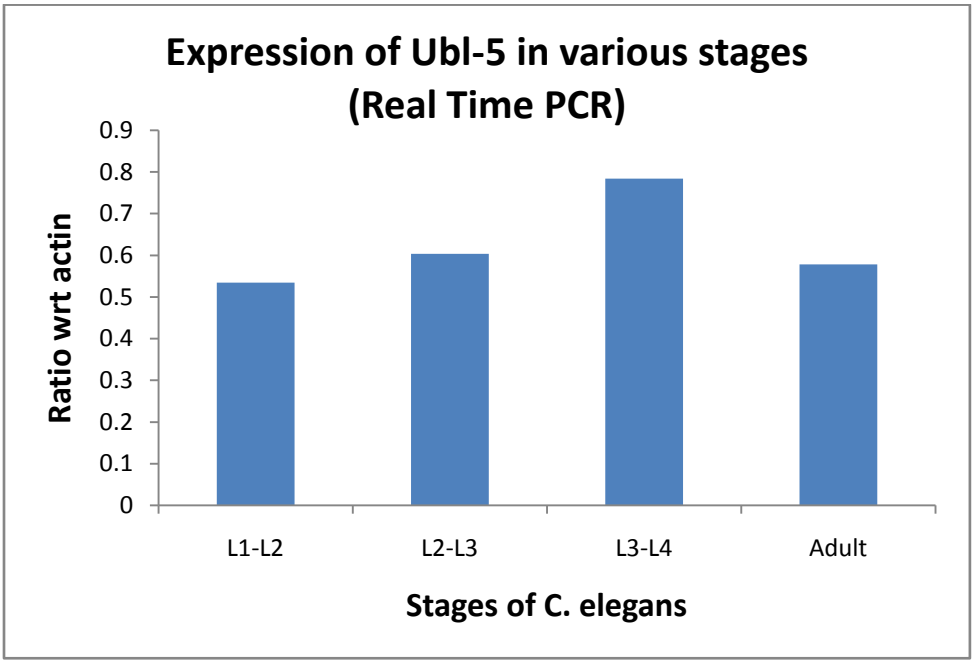


Figure 3.4: Real-time PCR showing the Ubl-5 expression during various stages of *C. elegans* life cycle

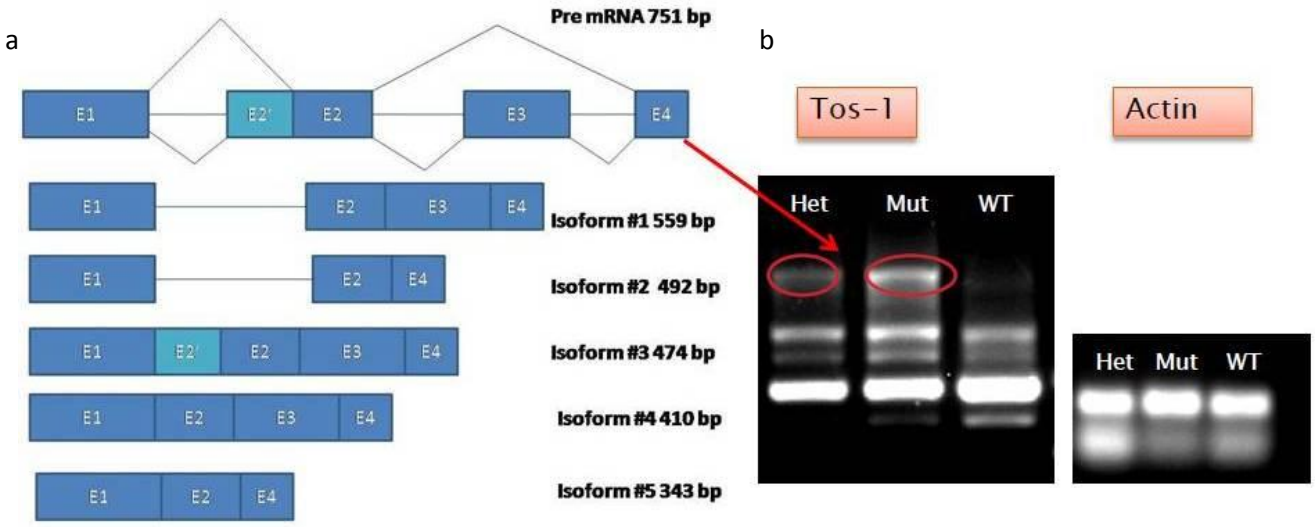
Real-time data was obtained for both actin and Ubl-5 in every stage of worm development. Then the ratio between actin and Ubl-5 was calculated and plotted in Excel.

As expected there was an increase in the level of Ubl-5 during L3-L4 stage. This might be the reason why the worms homozygous for Ubl-5 died after this stage. This is just a preliminary result and it has to be rechecked. It is not yet clear whether this lethality is due to the involvement of Ubl-5 in other processes or whether Ubl-5 is required for the splicing of genes that are essential for the development of the worm in the later stages. Experiments to identify novel genes that are being spliced by Ubl-5 at this stage are ongoing.

3.3) Role of Ubl-5 in alternative splicing:

The homozygous mutant worms started dying in their late L3s and there was an increase in the expression of ubl-5 during L3. So we collected homozygous, heterozygous and wild type worms during L2-L3 stage. Isolated RNA, prepared cDNA and then screened for alternatively spliced genes using PCR. Actin was used as the Control.

We identified a gene tos-1, which showed positive in the screen. Tos-1 (target of splicing-1) has been studied extensively in case of alternative splicing. It has four exons and five isoforms of which isoform 4, the most abundant [(12)]. Eventhough the function of tos-1 is not yet understood it serves as an excellent platform to study alternative splicing as it posses intron retained, exon skipping isoforms.



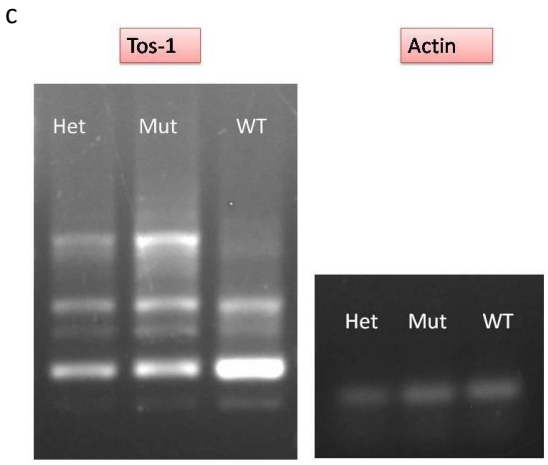


Figure 3.5: Alternative splicing of tos-1 gene. a) Schematic representation showing the different isoforms of tos-1 gene (modified from reference 12). To the right, the size of each isoform is given in bp (base pairs). b) splicing of tos-1 gene when Ubl-5 is: mutated in single copy (Het), double copy mutation (Mut) and Wild type (WT) condition. Arrow shows the band corresponding to unspliced form of tos-1. Circles indicate the accumulation of unspliced variant in Heterozygous (Het) and Homozygous (Mut) mutant. c) Experiment was repeated to confirm the result.

In case of mutant, unspliced form of tos-1 was accumulating whereas in wild type this band could not be observed. Also the isoform that retains intron1 seems to accumulate in the homozygous mutant. The band with a greater intensity corresponds to the fourth isoform, where all the introns are spliced. And this isoform seems to accumulate in wild type worms. These observations clearly suggest that Ubl-5 is required for the splicing of tos-1 gene.

CHAPTER 4

CONCLUSION

Eventhough the highly conserved HIND region is present in Prp-38, we couldn't observe any interaction with Hub1 in yeast two hybrid and pull down assays. The use of heterologous system might be a possible reason for this observation. *C. elegans* model system has not been exploited for biochemical assays. However certain *in vivo* studies like immunoprecipitation can be performed to further check the interaction[15].

C. elegans mutant worms homozygous for Ubl-5 did not survive after L3 stage. On analysing the expression of Ubl-5 during various stages of worm development it appears that temporal expression of Ubl-5 might be the reason for this phenotype. However future experiments need to confirm this result.

In the screen for alternatively-spliced genes that require Ubl-5 for proper splicing, we identified tos-1 (target of splicing) to have splicing defect when Ubl-5 is mutated. Accumulation of the isoform that retains the intron in the homozygous mutant clearly indicates the role of ubl-5 in alternative splicing. However every isoform has to be isolated and sequenced to obtain greater insights in this case. Experiments to identify other novel genes are ongoing.

Altogether these results suggest that Ubl-5 plays an important role in alternative splicing in this multicellular nematode.

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