Probing the role of exon 1(of *crh-1 e*-CREB homolog isoform e) in the regulation of its own expression in *C.elegans*

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

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Certificate of Examination

This is to certify that the dissertation titled "Probing the role of exon 1(of crh-1 e- CREB homolog isoform e) in the regulation of its own expression in C.elegans" submitted by Mr. Maruthi Prasad (Reg. No. MS12077) 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.

Dr. Kavita Babu (Supervisor)

Prof. Anand Bachhawat

Dr. Rachna Chaba

Dated: April 21, 2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of 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.

Maruthi Prasad

(Candidate)

Dated: April 21, 2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Kavita Babu

(Supervisor)

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I thank my mother from the bottom of my heart for all that is me; special thanks to my brother for his company and friendship.

Maruthi Prasad

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Abstract

Uncovering and dissecting the roles of cellular and molecular players involved in learning and memory has been an intriguing issue in neuroscience. The nuclear protein CREB is well established to mediate the aforementioned function in diverse animals. Using the invertebrate model *Caenorhabditis elegans*, our group previously dissected a novel role of a particular CREB homolog isoform *crh-1e* (of *C.elegans*) in long-term associative memory.. Preliminary qRT-PCR data revealed a strikingly significant lower expression of this particular isoform in comparison to the other *crh-1* isoforms in the nematode *C.elegans*. Herein, we investigated the regulatory mechanism underlying expression of *crh-1e* isoform. We uncover a suspected role played in part by the first exon of the *crh-1e* isoform to underlie this observation using confocal imaging and qRT-PCR analysis on transgenic worm strains.

Chapter 1:

Introduction

1.1 Basic Theory:

Learning and ability to modify behavior based on past experience provides significant advantage in survival of an animal. The nervous system of animals constantly surveys and receives cues from the environment, integrate and process the signals into information which are then encoded in neurons as strengthened/modified synaptic connections (thereby enabling rapid retrieval). Additionally in complex organisms, the nervous system also serves as the co-ordination and central control center of the animal's activities and physiologies. Of recent, there is emerging scientific literature uncovering the lesser known functions of the nervous system's role in effecting immune responses and even basal stress responses of the tissues that it innervates.[1,2]

The invertebrate model *Caenorhabditis elegans* was initially introduced as a model organism for genetic and developmental studies by Sydney Brenner in the 1970s owing to its many advantages for laboratory study[3, 4].The model organism is currently widely utilized in neurobiology, aging, developmental and molecular biology studies uncovering crucial processes and mechanisms that are widely conserved among complex animals as well. The nervous system of the nematode is in particular well studied, with regard to neuronal connections/circuitry mapping, development and the behavioral output.

Learning, memory and behavioral plasticity are important traits in animals that facilitate organisms to make choices that aid their survival and life processes. Learning and behavioral plasticity in the soil dwelling *Caenorhabditis elegans* should be of primal importance as this could potentially mediate aversive behaviors to toxins, pathogens and detrimental stresses such as UV or heat. Hence associative learning would better its chances of survival and fitness in the wild. Extensive understanding of *Caenorhabditis elegans'* neuro-anatomy, development and genetics make it a good candidate to study organismal learning and memory[5]. Quite a few associative and non-associative assays that measures the worms'

movement/ chemotactic/ mechanosensory response patterns to external cues have been developed. These assays and studies have established the capacity of the nematode to learn, habituate and get sensitized to the stimuli in different contexts. This behavior provides with the opportunity to study and dissect the genetic and physiological players of this behavior.

The nuclear protein CREB and memory:

The cAMP Response Element-Binding protein (CREB) transcription factor modulates the transcription of genes with cAMP responsive elements in their promoters. CREB (cAMP response element binding protein) is a transcription factor. CREB binds to cAMP response element (CRE) sites in the regulatory region of a gene. CREB can upregulate or downregulate gene expression by acting as an activator or a repressor. CRE sites contain a highly conserved nucleotide sequence 5'-TGACGTCA-3'. CRE sites are generally found upstream of genes within promoter regions. Increases in the concentration of either calcium or cAMP can trigger the phosphorylation and activation of CREB[6, 7, 8].

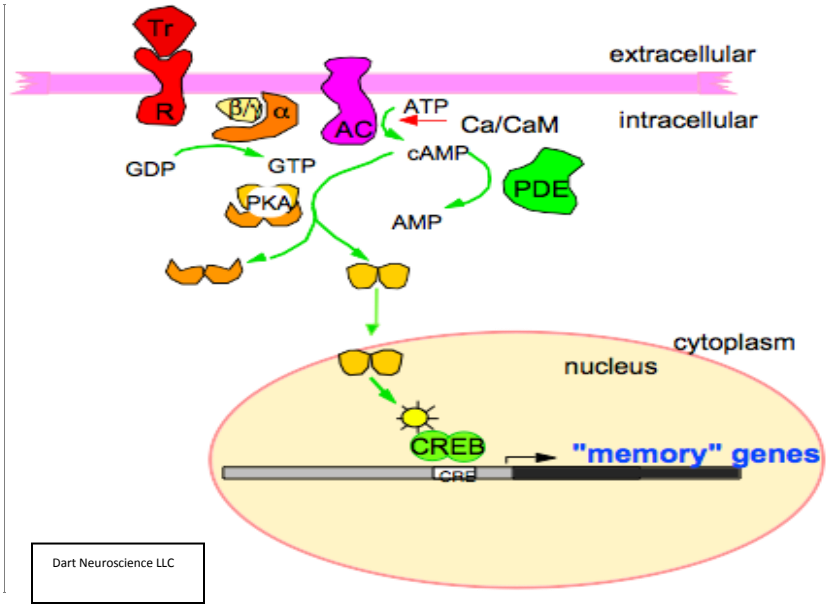


Figure 1 CREB Nuclear protein

The role of CREB transcription factor and its role in long-term memory formation and neuronal plasticity have been well established in model systems as diverse as fruit flies,

Aplysia and mice. CREB activity has been shown to impact synaptic restructuring- widely believed to be important in memory formation[7]. The activation of CREB and subsequent memory formation also extends to humans, as in neurodegenerative diseases such as Alzheimer's, CREB signaling pathway is negatively impacted. And hence, subsequent therapies to normalize CREB activity seem to ameliorate neurodegeneration and cognition[9].

CREB Homolog isoform *crh-1* in *C.elegans*:

The *crh-1*genein *C.elegans* encodes the homolog of the CREB nuclear protein.*crh-1* is involved in the transcription of CRE-sequence dependent genes activated by a CaM kinase cascade [10].Deletion of the *crh-1* gene in *C.elegans* exhibits defects in memory retention and have variation in the transient neuronal calcium levels[11]. Thus the CREB homolog isoform *crh-1* plays a role in influencing neuronal associative learning and memory in the nematode *C.elegans.*

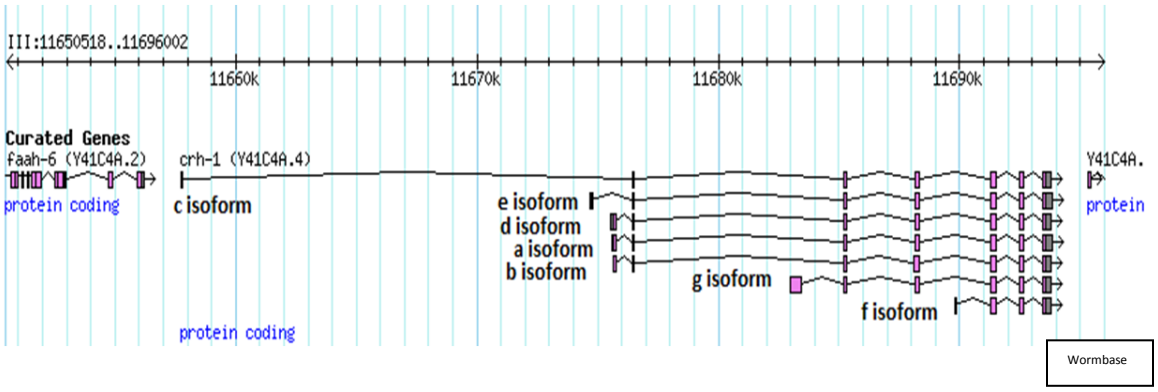


Figure 2 Isoforms of the *crh-1* gene

The *crh-1* gene encodes 7 protein isoformsCRH-1a-g.Given the established role of the *crh-1* gene in associative learning and memory, our group previously sought to discern as to if memory related functions of CRH-1 can be attributed to any specific isoform.

Surprisingly, putting back CRH-1c and CRH-1e isoforms under pan neuronal promoter *prab-3*could rescue the associative memory defect in the worm[12].

Given this observation of the involvement CRH-1e isoform in learning, a natural curiosity arose as to observe the relative expression levels of the various *crh-1* isoforms in the worm.

qRT-PCR was carried out to quantify the relative mRNA levels of the various isoforms of the *crh-1* gene in the wild type (WT) animal. This experiment revealed a stark difference in the mRNA level of the *crh-1 e* isoform relative to the other isoforms (Figure 3).Hence, the main study of this thesis project was to investigate as to why we observe such a low expression of the *crh-1 e* isoform in the animal.

This led to DNA sequence analysis of the *crh-1e* gene. We hypothesized a role played in part by the first *exon* (*exon-1*) of the *crh-1 e* isoform in the regulation of its expression.

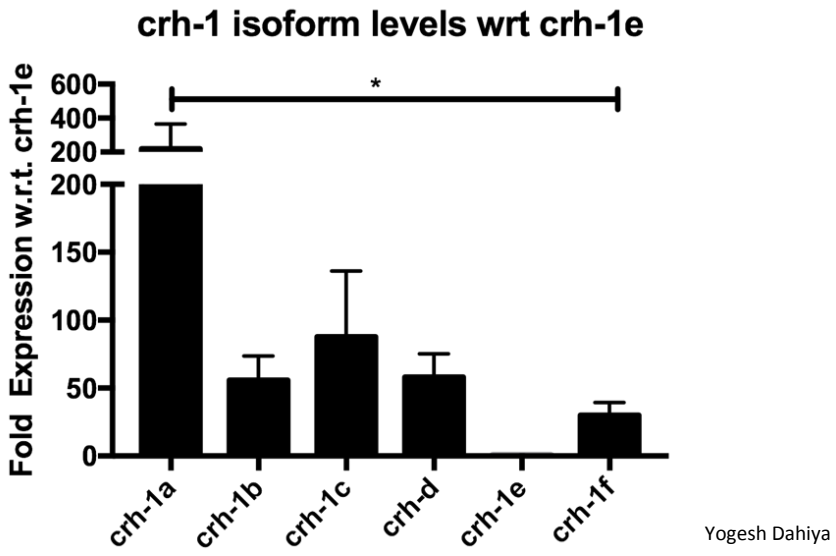


Figure 3 Relative mRNA levels of the *crh-1* isoforms in *C.elegans*

1.2 Experimental Methods

Maintenance of worms

C. elegans is maintained in the laboratory on Nematode Growth Medium (NGM) agar which has been aseptically poured into Petri plates[3]. Plates are seeded with bacterial strain *E.coli* OP50, which serves as food source for *C. elegans*. To seed the plate, a drop of OP50 culture is dropped at the centre and spread using a glass rod spreader. Then plates were incubated at 37° C for 12-13 hours. Worms were then reared on such plates at 20° C.

Strains:

Wild type animals used are *C. elegans* strain N2. The transgenic strains used were p*rab-3*:: *GFP*; p*rab-3*::*exon-1:mCherry*;p*crh-1e*::GFP and p*crh-1e*::*exon-1:mCherry*.(wherein exon-1 refers to the first coding exon of the *crh-1e* isoform transcript).

Creation of fluorescent transgenic reporter strains:

Cloned transgenes can be microinjected into the gonad of *C.elegans* leading to the creation of transgenic progeny. This mode of transgenesis is a powerful genetic toolbox in *C.elegans* research. This method has led to the visualization of gene expression patterns using fluorescent reporter proteins. These injected trangenes remain as extrachromosomal arrays or occasionally integrated into the genome of the progeny.[14, 15].

Fluorescent Reporter gene fusions: Germline transformation techniques allow for the rapid creation of transgenic animals in *C.elegans.* Moreover, the transparency of the animal provides an excellent platform to observe/visualize gene expression patterns using reporter fluorescent fusion proteins *in vivo.*

In the ensuing set of experiments, we utilized fluorescent transcriptional reporters; Transcriptional reporters consist of a promoter fragment from a gene of interest driving the

expression of a fluorescent protein. Typically, promoter fragments of a few kilobases immediately upstream of the start codon contain a significant portion of the cis-regulatory information necessary to provide a tentative expression pattern of the endogenous gene under study[16].

Figure 4 Architecture of transcriptional reporter gene constructs[18]

prab-3 used was 1.3 kb upstream of translation start site while pcrh-1e was 2.7 kb upstream of translation start site.

Confocal Laser Scanning microscopy and imaging:

The fluorescent reporter transgenic worms were imaged using the fluorescent laser scanning confocal microscope. It is an optical imaging technique for increasing optical resolution and contrast by of adding a spatial pinhole at the confocal plane of the lens to eliminate out-of-focus light. It enables the reconstruction of three-dimensional structures from sets of images obtained at different depths (optical sectioning) within a thick specimen[19].

Quantitative Reverse Transcriptase PCR:

Quantitative real time PCR is a technique used to quantify DNA molecules above or below a certain amount by PCR. Two methods used for detection of PCR products are:

1.Non-specific fluorescent dyes (SYBR Green) cDNA probes

2.Sequence specific DNA probes consisting of oligonucleotides labelled with fluorescent reporter.

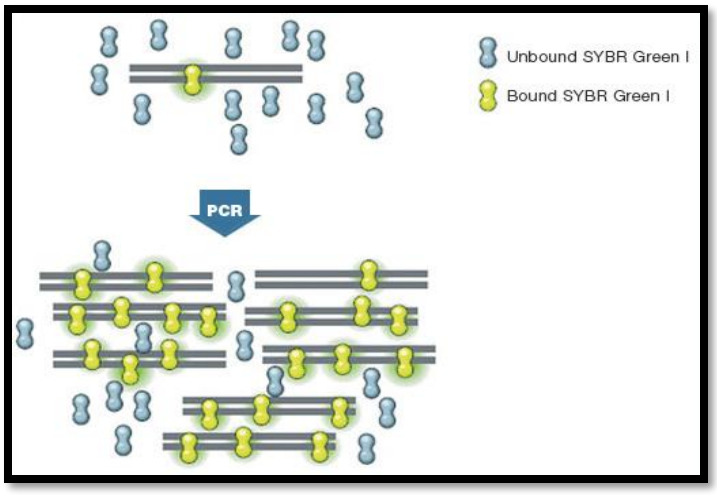


Figure 5 SYBR Green for qRT-PCR[17]

Quantitative real time PCR to measure gene expression: Gene expression in cell can be measured by copies of mRNA present in it. mRNA is first reverse transcribed into cDNA with reverse transcriptase. A fluorophore (SYBR Green) along with cDNA, primers, deoxy ribonucleotides, buffer and DNA polymerase are added in a PCR reaction to measure generation rate of the specific product.

Experimental Schematic:

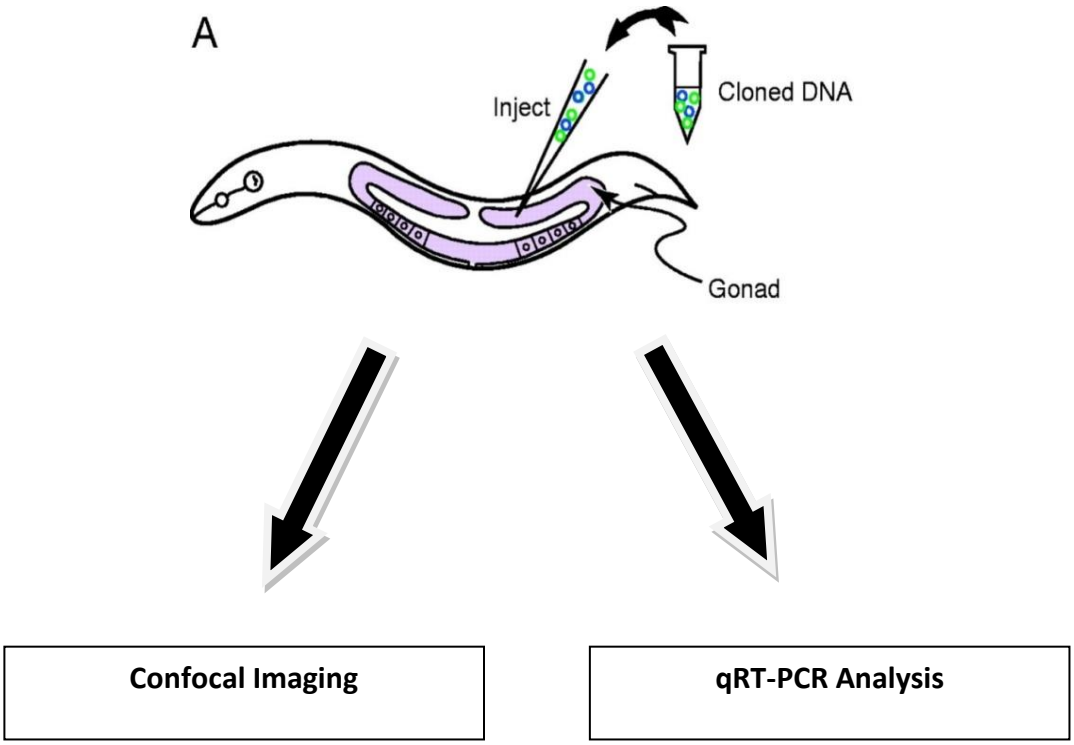


Figure 6 Experimental Schematic

Results:

Fluorescent reporter strains imaging:

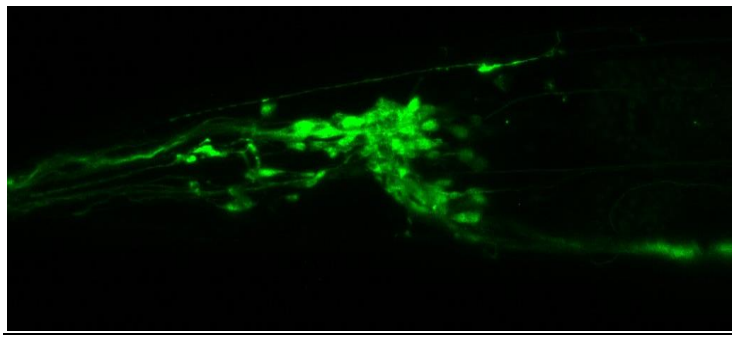
The 2 transgenic fluorescent worm strains

i. *rab-3* strain harboring the transgenes *prab-3::GFP* and *prab-3::exon-1:mCherry*
ii. *crh-1e* strain harboring the transgenes *pcrh-1e::GFP*and *pcrh-1e::exon-1:mCherry*

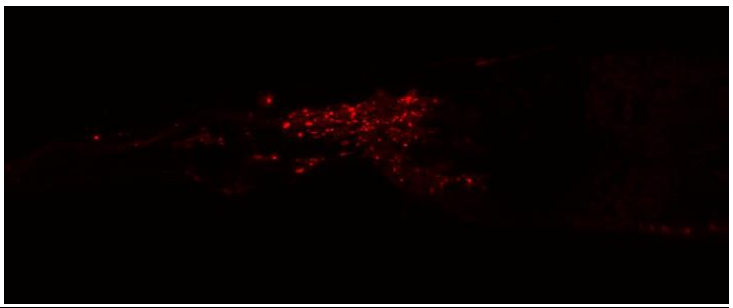
were imaged using the fluorescent confocal laser scanning microscopy.

We observed relatively lower mCherry fluorescence intensity when compared to the GFP fluorescence intensity in both the transgenic strains. This hints at a role for the *exon-1*(of the *crh-1e* isoform) in regulating the expression levels of the mCherry protein. However, a major caveat in this experiment is the variable fluorescent emission intensities of mCherry in comparison to GFP. Hence, experiments in which the *exon-1*of the *crh-1e* is cloned upstream of GFP also need to be carried out, and to check if we could replicate the results below.

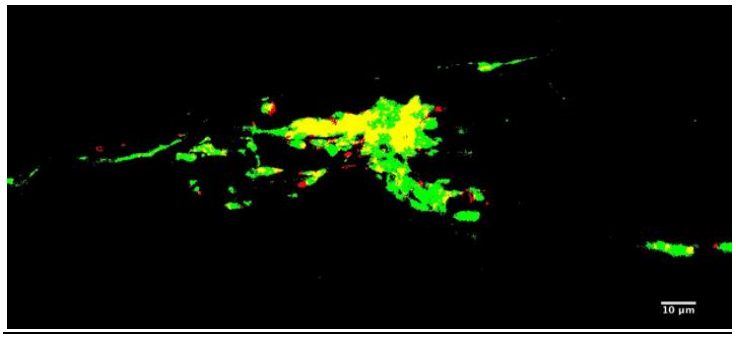
rab-3 strains:



Picture 1:* *prab-3::GFP

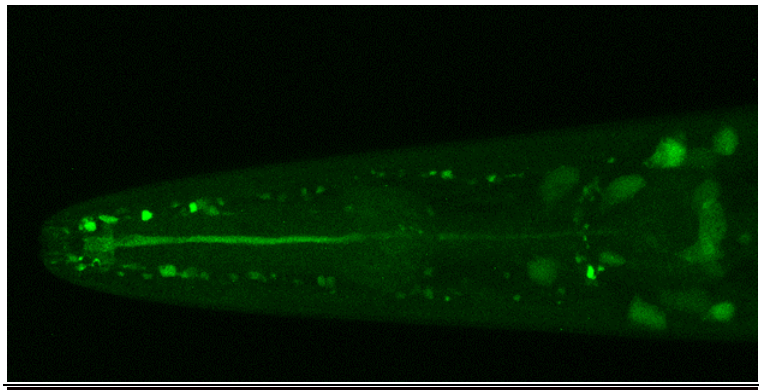


Picture 2:* *p-rab3::exon1:mCherry

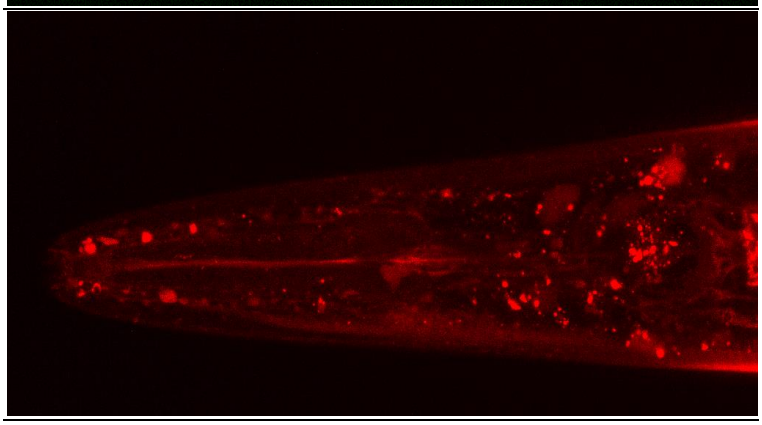


*Picture 3: **Binary overlay***

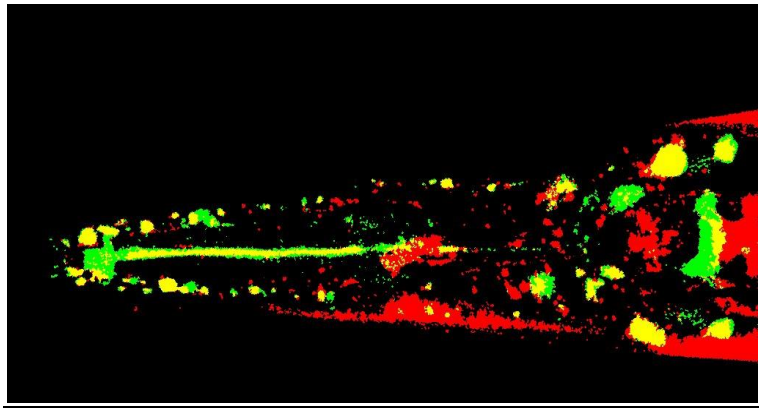
***crh-1 e* strains:**



*Picture 4: **pcrh-1e :: GFP***



*Picture 5: **pcrh-1e:: exon 1: mCherry***



*Picture 6: **Binary overlay***

qRT- PCR results:

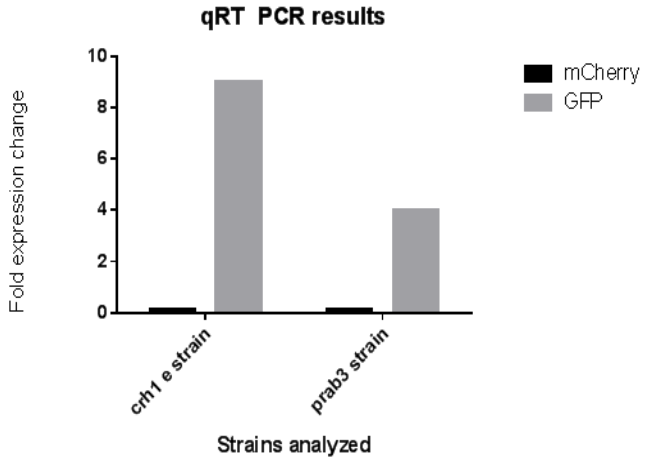


Figure 7 qRT PCR results

To obtain a more rigorous result in examining the role of *exon-1* of the *crh-1e*isoform in regulating the expression of *mCherry* , we carried out a qRT-PCR analysis to compare the relative mRNA transcript levels of *mCherry* and *GFP* in the transgenic worm strains.

We found a significantly lower mRNA level of *mCherry* transcript relative to *GFP* transcript levels. This result also hints at a role played by the *exon-1* of the *crh-1e*isoform in gene expression regulation.

Chapter 2

Summary & Conclusions

2.1 Concluding Remarks:

Given the novel role of the involvement of *crh-1e*in associative memory in *C.elegans*[12], delineating the regulation of its tightly controlled expression, is of interest in understanding its mechanism. Could there be a relationship between the regulatory mechanism of*crh-1e*'s expression and its role in mediating synaptic plasticity and learning in *C.elegans* needs investigation. Our preliminary data hint at a case propounding a regulatory mechanism underlying the expression levels of *crh-1e* isoform at the DNA and/or mRNA level. The qRT PCR results suggest a role for the exon 1 of *crh-1e* isoform in the regulation of its expression. More corroborative experiments involving the replication of the above experiments with exon 1 upstream of GFP are required to conclusively settle for a role played by exon 1 in *crh-1e* expression.

Given the role of this particular isoform in learning and associative memory[12], it could be that the regulatory mechanism could be repealed on/during associative learning. The exact regulatory mechanism remains to be probed and defined. We hypothesize the involvement of epigenetic modifications to the DNA/miRNAs/ circular RNAs to be at play here. This needs further investigation and elucidation.

2.2 Future Outlook:

1. Experiments to be performed in which the *exon-1* is cloned upstream of GFP – and further confocal imaging and qRT-PCR need to be done to see if we could **replicate** the above results.
2. Probe for putative **miRNA mediated expression regulation** of the *crh-1 e* isoform.
3. DNA sequence/ histone (Epigenetic) modifications at the *crh-1e* site have to be probed.
4. Probe the involvement of circular RNAs in regulating *crh1-e* isoform expression.

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