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The Differential Splicing of the SOCS2 5'UTR, a Gene Involved in Successful Central Nervous System Axon Regeneration in Xenopus laevis

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The Differential Splicing of the SOCS2 5'UTR, a Gene Involved in Successful Central Nervous System Axon Regeneration in *Xenopus laevis*

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Abstract

The amphibian *Xenopus laevis* has the ability to regenerate axons of its optic nerve even after metamorphosis. From previous studies done in our lab, we found that Suppressor of Cytokine Signaling 2 (SOCS2) could be involved in the complex pathway of genes regulating nervous system development and regeneration. 3' and 5' Rapid Amplification of cDNA Ends (RACE) revealed that the 5' untranslated region (UTR) of SOCS2 contains two splice forms. One splice form contained a previously unidentified 68 base pair exon, which will be referred to as 'Exon 2', which we hypothesized is involved in post-transcriptional regulation of SOCS2. I performed *in situ* hybridization on retina sections of the regenerating and non-regenerating eye 12 days after optic nerve crush to test whether Exon 2 is specifically expressed during optic nerve regeneration. I observed that expression of the splice form containing Exon 2 increased in the retina. This suggests that Exon 2 does play a role in the regulation of expression of SOCS2 during regeneration. RT-PCR and qPCR were performed to study the expression differences of the two splice forms at 3 days and 7 days after optic nerve crush. These data, when combined with the *in situ* hybridization data, suggest that the 5'UTR of SOCS2 is differentially expressed relative to the stages of regeneration. The form of the SOCS2 5'UTR that contains Exon 2 is expressed more in the intermediate to late stages of optic nerve regeneration, whereas the form that lacks this exon is associated with the early stages. Based on these observations, I hypothesized that the 5'UTR with Exon 2 or without it could be functioning as an internal ribosome entry site (IRES), to facilitate translation of SOCS2 protein under stress conditions where cap-dependent translation is suppressed. To test this *in vivo*, I have created a bicistronic fluorescent protein reporter plasmid that contains the SOCS2 5' UTR sequences with and without Exon 2. *In vitro* transcribed mRNA from two control constructs was injected into *X. laevis* embryos. Assaying for expression indicated that this method can be used to determine IRES activity. mRNA from the experimental constructs containing the SOCS2 5'UTR will be injected into embryos for confirmation or denial of my hypothesis. From my study I hope to better understand the regulatory mechanisms of the SOCS2 5'UTR during axon regeneration in *X. laevis*.

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Table of Contents

Introduction

The optic nerve, part of the central nervous system (CNS), connects the eye to the brain. In anamniotes when the optic nerve is injured, it regenerates and restores the axonal connections. The molecular mechanisms underlying this phenomenon are only partially understood. This makes members of the anamniotes a useful group in which to study these mechanisms (Sperry, 1944). The South African claw-toed frog, *Xenopus laevis* has been the model organism of choice to study optic nerve regeneration for many years (Gaze, 1959). CNS regeneration is an area of interest because in amniotes, damage to the axons of the optic nerve is permanent. The CNS axons of amniotes, including mammals, do not regenerate after damage. In non-regenerative axons, the portion of the injured neuron that is part of the greater cell body makes the axon retract. The retracting region of axon is the retraction bulb, which inhibits axonal outgrowth. A glial scar forms a physical barrier to regeneration and the local glia express growth inhibitory compounds (Vajn et al., 2013).

In *X. laevis*, after sustaining damage to the optic nerve, retinal ganglion cells (RGCs) begin generating new axons at 3 days (Zhao and Szaro, 1994). The axons grow along the periphery of the optic tract until they reach the brain approximately 18 days after the nerve injury. The process ends after several months, when normal vision has been restored (Szaro et al., 1985). Previous experiments have implicated Suppressor of Cytokine Signaling 2 (SOCS2) as part of the CNS axonal regeneration process in tadpoles (Gibbs et al., 2011).

SOCS2 is one of the eight members of the SOCS protein family (Hilton et al., 1998). Its expression in the cell is induced by stimulation from hormones and other cytokines. SOCS2, like the other members of the family, is able to regulate the cytokine-dependent Janus Kinase and Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway in several

systems *in vitro* (as reviewed by Rico-Bautista et al., 2006). SOCS2 is an important component of many cell activities such as negatively regulating growth hormone signaling (Metcalf et al., 2000) and immune responses to infection (Machado et al., 2006).

SOCS2 appears to play an important role in the development of the nervous system. In the mouse nervous system, SOCS2 expression is high during fetal development, especially during the process of neurogenesis and dendritic outgrowth (Polizzotto et al., 2000). Deletion of the *SOCS2* gene in mice leads to a 30-40% decrease in the density of neurons, and inducing neural stem cells to differentiate while having *SOCS2* knocked-out results in 50% fewer neurons than in the control group. If mice neural stem cells are modified to overproduce SOCS2, more neurons are produced (Turnley et al., 2002). SOCS2 has also been implicated in the process of ocular dominance plasticity during development (Rietman et al., 2012). Besides influencing neurogenesis during development, SOCS2 also has an effect on neurite outgrowth. Neural cells that overexpress SOCS2 show more neurite extension, with increases in the amount and the length of the neurites (Goldshmit et al., 2004).

SOCS2 also appears to play an important role in regenerative CNS axonal outgrowth in anamniotes such as *Xenopus laevis*. In *X. laevis, SOCS2* mRNA expression increases in the hindbrain of the tadpole under conditions that allow regeneration of spinal cord axons, and under conditions inhibiting axon regeneration in the spinal cord, *SOCS2* mRNA levels decrease (Gibbs et al., 2011). Preliminary experiments suggest that during optic nerve regeneration, SOCS2 protein expression increases in the retinal ganglion cell layer (RGCL) as well as other retinal layers, and when SOCS2 is knocked down in the eye, optic nerve regeneration is markedly reduced (unpublished data).

To further study the role and function of SOCS2 in regeneration it is important to know the entire sequence and the gene structure of *SOCS2*. Manual curation of predicted genes in the *X. laevis* genome indicates that as many as 65-70% of the predicted mRNA sequences are incomplete, with many sequences in the database missing alternatively spliced products, as well as segments of the 5'UTR and 3'UTR. In my thesis, I describe our discovery of a 68 base pair exon in the 5'UTR of *SOCS2* that had not been previously identified and discuss the implications of this finding for the upregulation of SOCS2 expression at the protein and mRNA levels during optic axon regeneration.

Materials and Methods

Surgery and Total RNA Recovery

For optic nerve crush, juvenile albino *Xenopus laevis* frogs (<4 months post-metamorphosis) were anesthetized [0.1% ethyl 3-aminobenzoate (MS222), Sigma-Aldrich] and the right optic nerve was crushed at the orbit as described previously (Zhao and Szaro, 1994). All procedures involving animals were performed in accordance with the University at Albany Institutional Animal Care and Use Committee.

For recovery of RNA for analysis, the right, operated eye; left, unoperated eye; and brain of each frog were collected at 3 or 7 days after optic nerve crush. Using a Polytron PT-1000, each tissue collected was homogenized in Buffer RLT (Qiagen) or guanidine isothiocyanate (GITC)-containing buffer (Ananthakrishnan and Szaro, 2008). Total RNA was extracted from homogenate using an RNeasy Mini Kit (Qiagen) or cesium chloride ultracentrifugation as described previously (Ananthakrishnan and Szaro, 2008).

In situ hybridization

For analysis of mRNA expression by *in situ* hybridization, anesthetized frogs were dissected and perfused with 4% paraformaldehyde and processed for cryosectioning to yield transverse sections with a thickness of 20 μm. These sections each contained both sides of the head, as described previously (Gervasi et al., 2003).

Digoxigenin-labeled cRNA probes were synthesized [DIG RNA labeling kit (SP6/T7), Roche] using the plasmid generated from 5'RACE containing the *SOCS2* 5'UTR with the Exon 2 sequence. Probe hybridization and visualization, using alkaline phosphatase-conjugated antibodies to digoxigenin, were performed as described previously (Gervasi et al., 2003). Sections were imaged on a Leitz Laborlux S compound microscope using a 40X Plan ApoChromat, 0.65NA objective and a Nikon DS-Ri1 camera.

3'Rapid Amplification of cDNA Ends

Juvenile *Xenopus laevis* eye oligo-d(T) selected cDNA was used as the template for a 3' Rapid Amplification of cDNA Ends (RACE) (Frohman et al., 1988). The primers and nested primer (Gene-Specific Primer 2) sequences are in Table 1. The 3'RACE products were cloned into a pGEM T-Easy Vector according to the manufacturer's procedures (Promega). The plasmids were then introduced into Subcloning Efficiency™ DH5α™ Competent Cells or MAX Efficiency® DH5α™ Competent Cells (Invitrogen) by transformation through heat shock. The resulting colonies were screened for those containing the insert using X-gal – IPTG induced blue/white screening. The plasmids were purified using PureYield™ Plasmid Miniprep System (Promega) from selected colonies following the manufacturer's procedures. The sequences of the 3'RACE products were obtained by priming at the SP6 or T7 promoters in the plasmid (Genewiz).

cDNA Synthesis and 5'RACE

For 5'RACE, total RNA from *Xenopus laevis* stage 40 embryos was extracted using an RNeasy Mini Kit (Qiagen). The resultant total RNA was used to synthesize the first strand of cDNA using a gene-specific primer (Primer A) targeting specifically the SOCS2 mRNA sequence (Table 1), and SuperScript® III Reverse Transcriptase (Invitrogen), following the manufacturer's procedures. The reverse transcription product was then purified with Wizard® SV Gel and PCR Clean-Up System (Promega). A poly-A tail was attached to the 3' end of the SOCS2 cDNA sequence using Terminal Transferase (New England Biolabs).

This first strand cDNA was mixed with adaptor oligo-d(T) primer and GoTaq® Green Master Mix (Promega) using Gene-Specific Primer B (Table 1) for a PCR reaction that would be the first round of 5'RACE. The PCR product was used as the template for the second round of 5'RACE. The PCR product was mixed with Gene-Specific Primer C and Adaptor Primer (Table 1). The resultant cDNA was separated by agarose gel electrophoresis (Davis et al., 1994) and the resultant DNA smears were excised, eluted, and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), following the manufacturer's recommended procedures. This gel-purified PCR product was then cloned into pGEM T-easy vector, propagated, and sequenced as was done for the 3'RACE products.

Preparation of plasmids for *in vitro* transcription of RNA for expression in *Xenopus*

The initial plasmid to construct the bicistronic fluorescent protein reporter was a modified pGEM-3Z Vector (Promega) that was previously used (Lin and Szaro, 1996) in our laboratory. This modified vector contained Green Lantern Green Fluorescent Protein (glGFP) and the rabbit β-globin 3'UTR inserted in the HindIII site on the vector. We added an AflII restriction site after the stop codon of glGFP and a ClaI site before the rabbit β-globin 3'UTR by performing a PCR

with Elongase® Enzyme (Invitrogen) using appropriate primers (Table 1). The coding sequence of the red fluorescent protein td-Tomato was amplified from pRSET-B (Life Technologies) cloning vector to attach an AflII site at its 5' end and a ClaI site at its 3'end.

The UTR of *X. laevis SOCS2* was excised from plasmids previously made in the 5'RACE and cloned into the glGFP/td-Tomato AflII site. IRES sequence in the pIRES2-dsRed2 (Clontech) was also inserted into the AflII site as positive controls. Accuracy of all constructs was confirmed by sequencing (Genewiz).

In vitro Transcription and Embryo Microinjection

Plasmids were linearized (Sal*1*) and transcribed *in vitro* (mMessage mMachine SP6 kit; Ambion) for injection into single blastomeres of two-cell stage, periodic albino *X. laevis* embryos of either sex, as described by Gervasi and Szaro (2004).

qRT-PCR

RT-PCR and qPCR was performed as described previously (Ananthakrishnan et al., 2008; Liu and Szaro, 2011), with minor modifications. *GAPDH* was used as an endogenous control for qPCR. This was performed using TaqMan Gene Expression Master Mix (Applied Biosystems), using 1 μl of cDNA template, 250 nM TaqMan probe, and 900 nM each forward and reverse primers (Tables 1 & 2). Data were collected using an ABI Prism 7900HT Sequence Detection System (software version 2.3) and analyzed by the comparative C_T method (Schmittgen and Livak, 2008) Statistical comparisons between two samples were made using two-tailed Students t tests, as indicated in text.

Table 1. Primers

Experiment	Primer Name	Direction	Sequence (5' → 3')
3'RACE	Adaptor	Reverse	GACTCGAGTCGACATCGA
	Gene-Specific 1	Forward	GTGGCTGGTGAAGCCACTATACA
	Gene-Specific 2	Forward	CCGTCCTTACAGCATCTCTGTAGA
5'RACE	Poly d(T)-Adaptor	Forward	GACTCGAGTCGACATCGA(17)
	Adaptor	Forward	GACTCGAGTCGACATCGA
	Gene-Specific A	Forward	TTCGATAAGATGGACAACACTGTC
	Gene-Specific B	Forward	GTTCCTTCTGGAGCATCTTGCAAC
	Gene-Specific C	Forward	GAGCTCTCCCATAGACTGAGCGAT
In situ hybridization	Exon 2	Forward	GACTAAAAAGAAGTCAATGC
		Reverse	TTGTGCTCTGTGGTGATACG
qPCR	Exon 2	Forward	ACATTCAAAGATTCGCACGACTAA
		Reverse	TGCTCTGTGGTGATACGTTCCT
	No Exon 2	Forward	AGAGACAGGCGAGCAGATCAG
		Reverse	CGCTTGGCGTATCTTGGAG
IRES Bicistronic Reporter Construct	pGEM3z-glGFP	Forward	GTGACAATCGATTGAGAACTTCAGGGTGAG
		Reverse	GTGACACTTAAGTCACTTGTACAGCTCGTC
	td-Tomato	Forward	GTGACACTTAAGATGGTGAGCAAGGGCG
		Reverse	GTGACAATCGATTTACTTGTACAGCTCGT
	SOCS2 5'UTR	Forward	GTGACACTTAAGTTTACCAGATATGGGGAG
		Reverse	GTGACACTTAAGTTGACAGTGGCGTGCGC
	HCV IRES	Forward	GTGACACTTAAGGGCGACACTCCACCATAG
		Reverse	GTGACACTTAAGGGCGGTTTTTCTTTGAGG
	DsRed IRES	Forward	ATACTTAAGGCCCCTCTCCCTCCCCCC
		Reverse	GTGGCGCTTAAGTGTGGCCATATTATCATC

Table 2. TaqMan Probes

Probe Name	Sequence (5' → 3')
Exon 2	6FAM-TCAATGCAGAGCTGTGGAACCTCCTCA-TAMRA
No Exon 2	6FAM-TTCAAAGATTCGCACGGTGAACAA-TAMRA

Results

The *SOCS2* mRNA Sequence had Incomplete 3' and 5'UTRs

The 3'RACE using eye cDNA extended the 3'UTR of *SOCS2* mRNA 508 nucleotides downstream of the NCBI database sequence (Figure 1). The 3'UTR had the transcription termination and polyadenylation sequence AAATAA that was lacking in the mRNA sequence present in the database. The UTR did not have a long open reading frame. This sequence was one result out of twenty, the other 19 sequences were identical to the current database sequence, indicating that the poly-d(T) adaptor primer was mispriming from the string of 10 A nucleotides at the end of the database sequence. This may explain why the 3'UTR was previously undetected.

```
5'- CCGTCCTTAC AGCATCTCTG TAGAATGACT GTCAACAAAT GTACCAACAA AATCGATGAA 60
    TTGCCATTAC CAATGAGACT GAAAGAGTAT ATTACAGAAT ACCGTTACCA TGTATAGATG 120
    TTTTGCTGAA CATCAGTTAA GCATTGGGAA CATCTTCTTC CAAATATAGA AAAAAAAAAG 180
    TTAATTATCA GTTACCTGCA CCGTTCTAAG TGGTTAAAAT GATCAGTGTC GTTTCATTTC 240
    AGTAAGAATT TACTTGATGG CAATGAAAAA CTTCACAAAA CACATCGCTT TTGCAGCACT 300
    TCTTAAATCC ACTTTATCTT CTACTTTTTT TCACTTCAAT GAAATCATGG GGATCAGTAT 360
    GCTCTGATTT TTGAGCATTG GATGTTGATC AAATGCATAA TCTGACAGGC CAAAAAGTAT 420
    TTTGCTTTTC CAGTTCCTGT GTTTCCGCGT GTATGCTGCC TGACAATAAT GGATTAAAGT 480
    GGCCAAAGTT CACTTCGGTG CCAAAGAAAC TGTGTATCCC ATCTTTCAGC CAGTAATTTC 540
    AGACCCTGGG TCTTTATTGT TGCACTAAAA TTGCCTCAGA AAATCGAAAT TTAGCTGATG 600
    GGATCAAATA CGTGACTAAT GGGATCAAGG TTTTTTTTTG TCTGTCTACT GTGCAAAGCT 660
    GAAATAATTA CCGTATTCAG AAAAAAAAAA AAAAATCGA TGTCGACTCG AGTC -3'
```

Figure 1. The DNA sequencing revealed a ~550 bp 3'UTR that contained a transcription termination and polyadenylation signal. *All 3'RACE sequences contained some or all of the known SOCS2 sequence (yellow and green). One sequence contained a 505 bp extension of the known sequence after the stop codon (un-highlighted). It also had the transcription termination and polyadenylation signal (light blue). All sequences contained Gene-Specific Primer 2 and Adaptor Primer (gray).*

The 3'UTR sequence of *SOCS2* correlates to the sequence of Scaffold 5925 from base pairs 1810221 to 1809658 on the – strand using GBrowse 7.2 (XenBase). There are no introns in the 3'UTR genome sequence, which immediately follows the coding sequence. Within the coding

sequence, there is one intron that is 43,484 bp in length. The intron separates the first 151 bp of the coding domain (1854314-1854164) from the remaining 458 bp (1810679-1810222).

5'RACE using Stage 40 *X. laevis* embryo cDNA revealed two different splice forms of the 5'UTR, one splice form containing a 68 bp exon (Figure 2B) and the other lacking this exon (Figure 2A). Both forms of the 5'UTR were extended upstream of the NCBI database sequence. These results were the only forms of the UTR found. None were found that exactly matched the *SOCS2* 5'UTR found in the database sequence.

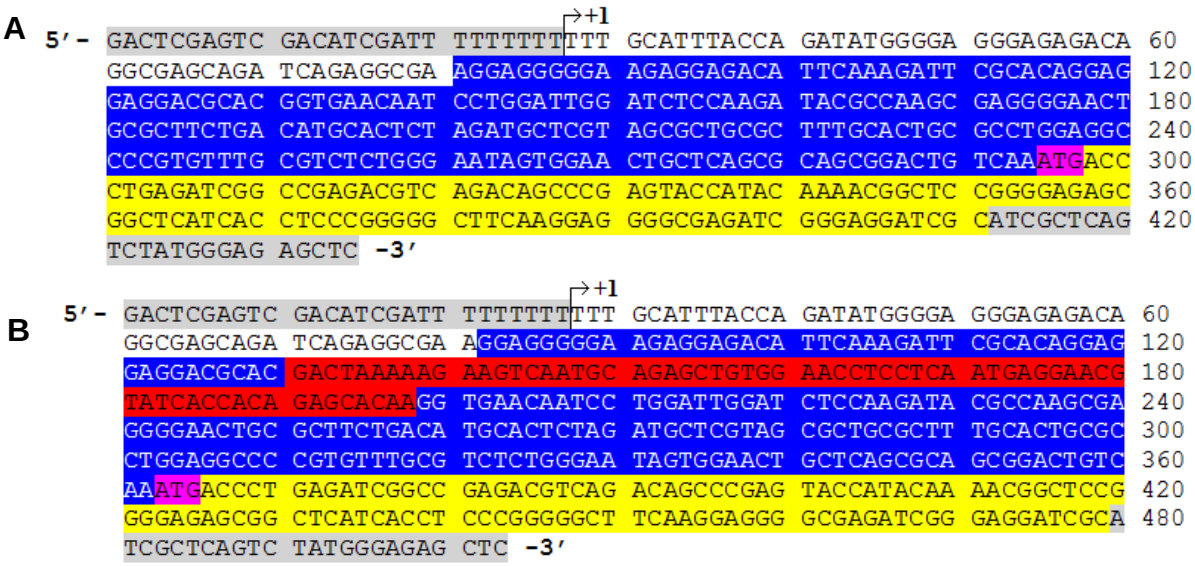

Figure 2. Two versions of the SOCS2 5'UTR were found in Stage 40 embryo cDNA. *The 5'UTR was extended 53 bp (un-highlighted) upstream of the current database sequence (blue). (A) One form is only extended upstream. (B) Another result had a 68 bp section (red) of the 5'UTR that did not match known SOCS2 and was within the database sequence. The known SOCS2 5'coding domain sequence was highlighted in yellow. The Adaptor Primer and Gene-Specific Primer C are in gray. The start codon for SOCS2 (purple) was found in all sequences.*

Nevertheless, both versions of the *SOCS2* 5' end corresponded to sequences within a *X. laevis* genome scaffold. The scaffold, Scaffold 5925, was the same one used to verify our 3'RACE results. The first section of the 5'UTR correlated to the nucleotides 1857511-1857408. The 68 bp section of the *SOCS2* 5'UTR in red (Figure 2B) matched a sequence in the scaffold at 1856758-186690. There were 650 nucleotides separating these sequences in the scaffold, and

another 2,212 bp intron separated the ~70 bp from the rest of the *SOCS2* 5'UTR located at nucleotides 1854476-1854315. It was determined based on these results that the *SOCS2* 5'UTR is split into three exons, with the ~70 bp exon being optional. The first 151 nucleotides of the *SOCS2* coding domain were found immediately following the 1854476-1854315 region of the 5'UTR.

The 3' and 5' RACE verified the sequence of *SOCS2*. Some corrections were made to the 3' and 5'UTRs, but no coding domain errors were found in the NCBI database. Using Scaffold 5925, the introns and exons were mapped in Figure 3. Based on their respective order in the *SOCS2* mRNA, exons were named sequentially with Exon 2 being a cassette exon, or an exon that could be included or excluded based upon splicing (Figure 4).

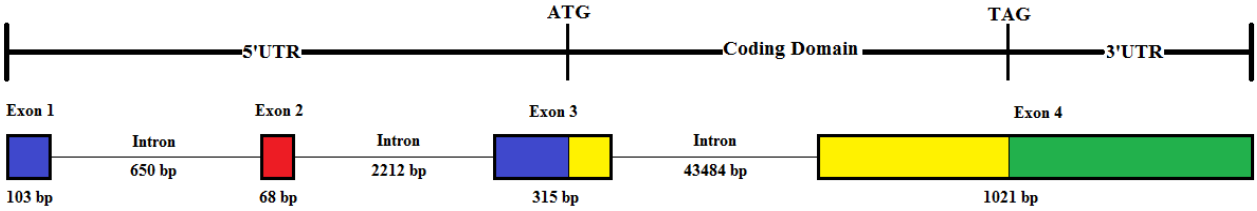

Figure 3. All SOCS2 sequences matched to sequences within Xenbase Scaffold 5925. *The scaffold determined what 3'and 5'RACE results corresponded to which exons of the SOCS2 mRNA and where in the* X. laevis *genome introns were located.*

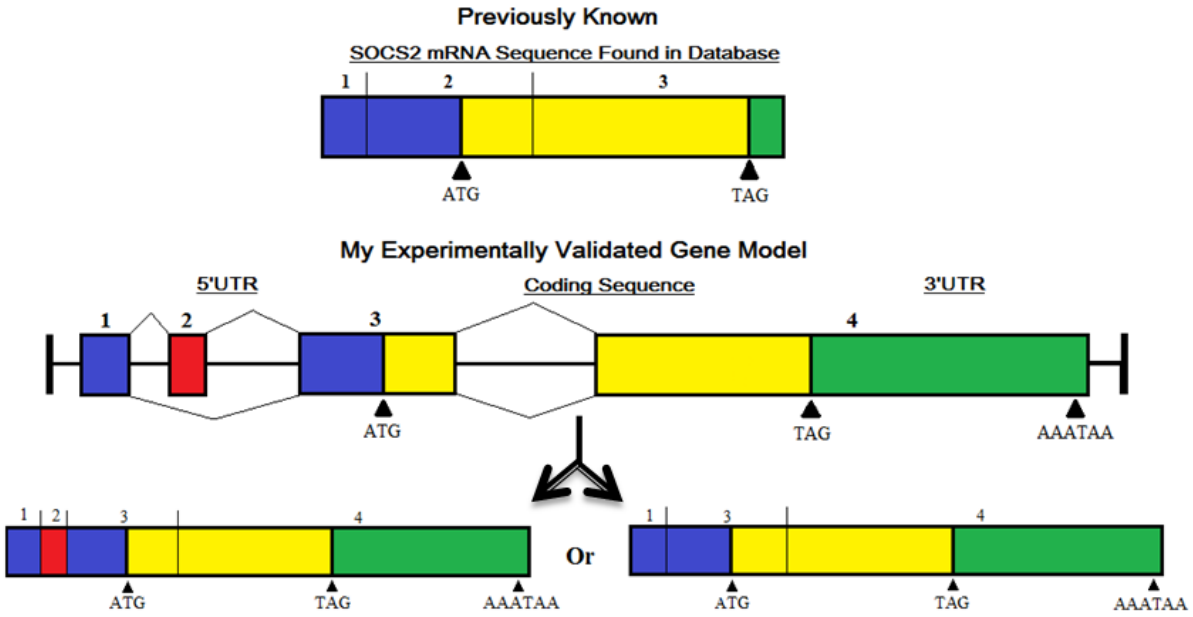

Figure 4. Two splice forms of the 5'UTR of SOCS2 were found. *All splice forms contained Exons 1, 3, and 4. Exon 2 was found to be not required but sometimes included.*

In situ Hybridization Correlated Expression of *SOCS2* that Contains Exon 2 to Regeneration

In situ hybridization was performed on retina sections 12 days after optic nerve crush to determine if the expression of the form of *SOCS2* that includes Exon 2 plays a role in optic nerve regeneration. This procedure revealed that the expression of Exon 2 increased during regeneration (Figure 5B) by using antisense cRNA probes. Total *SOCS2* expression detected by an antisense probe for the coding domain in the retina increased during optic nerve regeneration (Figure 5A), as expected from previous unpublished studies by the lab. *In situ* hybridization with a sense probe from Exon 2 showed no hybridization signal in either the regenerating or non-regenerating eyes (Figure 5C).

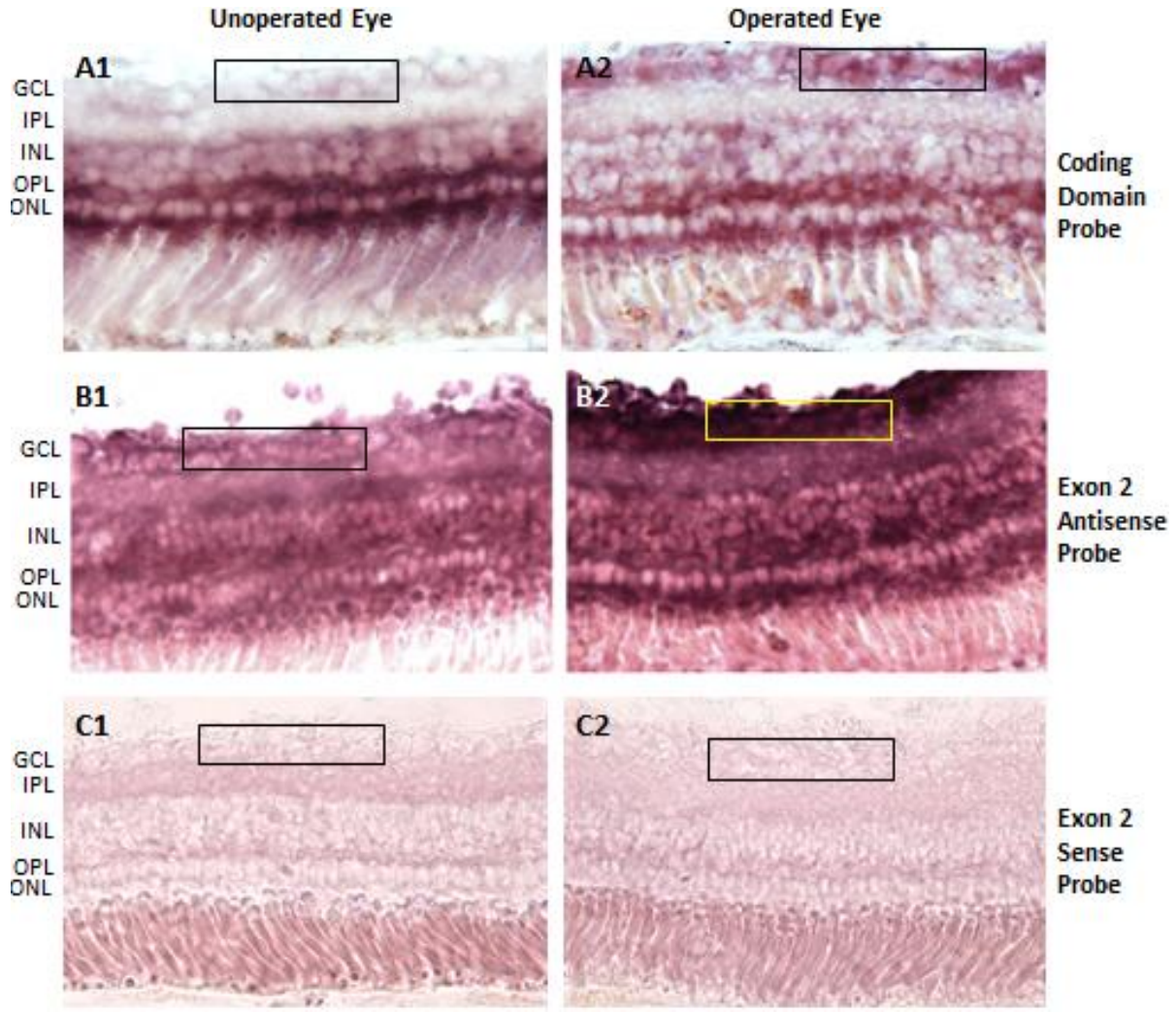

Figure 5. Expression of *SOCS2* containing Exon 2 increases in the ganglion cell layer (GCL) during regeneration. *An antisense probe targeting Exon 2 of* SOCS2 *mRNA (B1, B2), as well as one targeting the coding domain (A1, A2), show increased staining in retinal ganglion cells of the injured eye (A2, B2) relative to those of the uninjured, contralateral eye within the same section (A1, B1). A sense probe targeting Exon 2 of* SOCS2 *mRNA (C1, C2) shows no distinct staining or differences between the two eyes.*

A correlation between the elevated expression of Exon 2 form of *SOCS2* and the peak of axon outgrowth at 12 days during regeneration suggested that the two different splice forms of *SOCS2* were worthy of further examination. *In situ* hybridization could not be used to study the splice form that lacked Exon 2, since there is no region of the mRNA that is unique to this form.

PCR and qPCR at 7 Days Indicated Upregulation of the Exon 2 Form of *SOCS2*

To study the relative expression of the two forms of *SOCS2* mRNA, PCR was performed on cDNA isolated from eye during regeneration using primers that flanked Exon 2 (Table 1). Thus, both forms would be visible within the resultant PCR product and would migrate differently on an agarose/TBE gel. The PCR products from the cDNAs obtained from the operated eye, unoperated eye, and brain from *X. laevis* juvenile frogs 7 days after optic nerve crush can be seen in Figure 6.

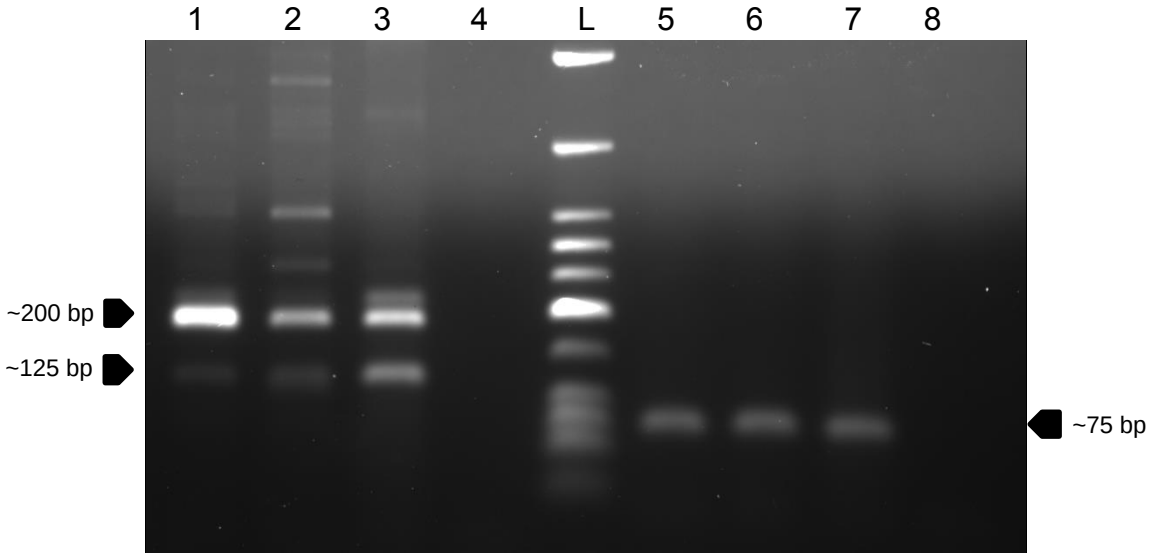

Figure 6. PCR suggested that the Exon 2 form of *SOCS2* is upregulated during optic nerve regeneration while No Exon 2 expression remains the same. *The Low Molecular Weight Ladder (New England Biolabs) was used in lane L. The No Exon 2 primers showed that the operated eye (1), unoperated eye (2), and brain (3) lanes all shared a ~200 bp band and a ~125 bp band.* GAPDH *primers revealed a band at ~75 bp that was shared by the operated eye (5), unoperated eye (6), and brain (7). No contamination was detected in the no template controls (4,8).*

The PCR data from Figure 6 using the "No Exon 2" primers showed the Exon 2 form (~200 bp) and No Exon 2 forms (~125 bp) of the *SOCS2* 5'UTR. The band representing the Exon 2 form in operated eye was clearly brighter than any other band at that size. The No Exon 2 form appeared to be slightly brighter in the unoperated eye than in the operated eye, but this may be due to primer competition between the two PCR products. *GAPDH* appears equivalent among

all the lanes (5, 6, and 7), except for the no template lane (8), which was expected. This shows that all tissues have approximately the same abundance of *GAPDH*, which was used as the endogenous gene for qPCR normalization.

qPCR of technical triplicates normalized to the average *GAPDH* C_T value can be seen in Appendix I Table 2. The normalized C_T values for operated eye and unoperated eye were graphed in Figure 7. The normalized C_T values in unoperated versus operated eye for both the Exon 2 and No Exon 2 forms were then tested for statistical significance within the technical replicates using a two-tailed, homoscedastic t-test. When comparing unoperated eye and operated eye, Exon 2 had a significance of $p = 0.02$ and No Exon 2 was not statistically significant between the operated and unoperated eyes ($p > 0.05$).

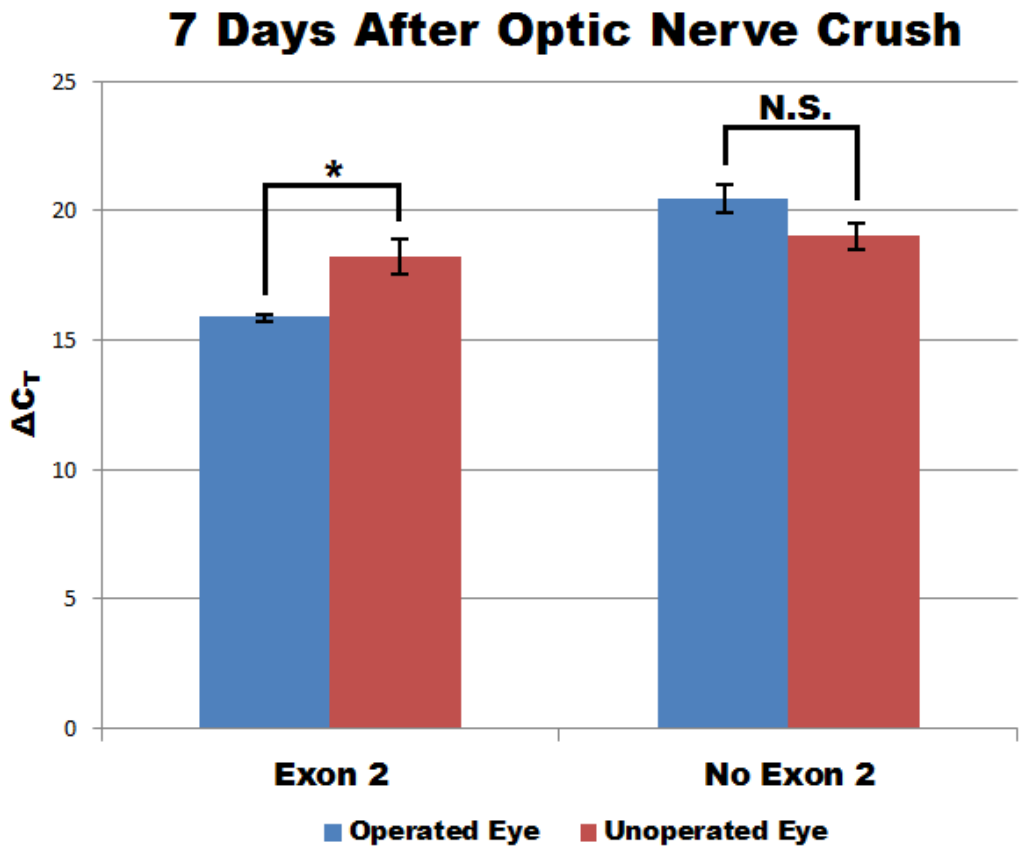

Figure 7. Graphical representation of normalized C_T values in operated eye and unoperated eye. *The average and normalized C_T value was plotted against the type of cDNA and detector. A lower C_T indicates greater expression levels of mRNA. There was a significant difference in Exon 2 expression during optic nerve regeneration according to the Student's t-test performed. No Exon 2 expression levels were not significant between operated and unoperated eyes. (* $p<0.05$, N.S. = not significant). Error bars indicate +/- SE, n = 3 replicates, 4 frogs per group.*

There was a difference of 2.32 cycles between the average normalized C_T values for Exon 2 in operated eye vs. unoperated eye. This represents a 5.01 fold increase in expression of Exon 2 mRNA in the operated eye. These technical replicates suggest that Exon 2 increases during optic nerve regeneration whereas the No Exon 2 form does not change its expression levels significantly at this time during regeneration.

PCR and qPCR at 3 Days Indicated Upregulation of the No Exon 2 Form of *SOCS2*

PCR products using cDNAs obtained from the operated eye, unoperated eye, and brain from *X. laevis* juveniles 3 days after optic nerve crush can be seen in Figure 8.

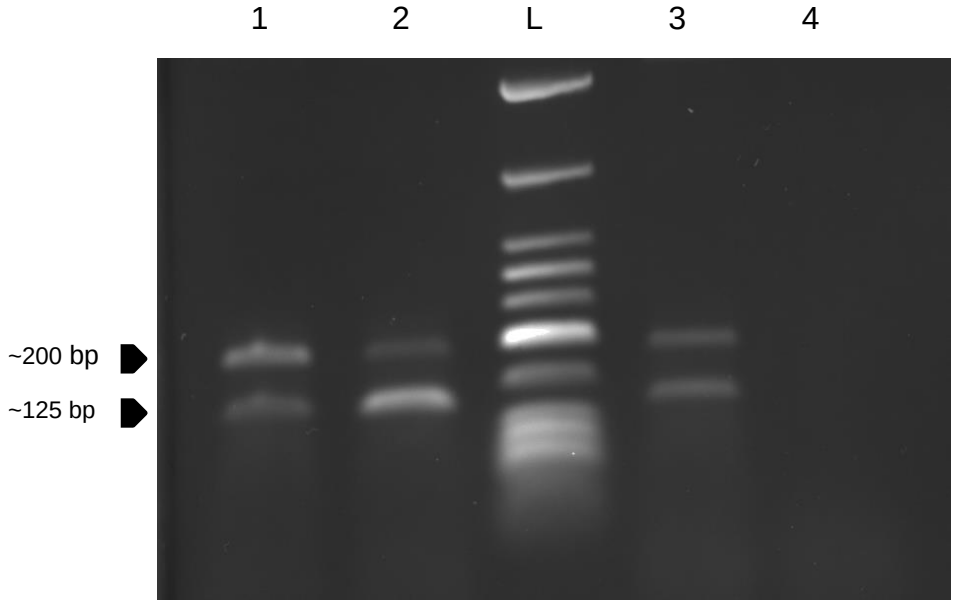

Figure 8. PCR suggested that the Exon 2 form of *SOCS2* is downregulated during optic nerve regeneration while the No Exon 2 form is upregulated. *The Low Molecular Weight Ladder (New England Biolabs) was used in lane L. The No Exon 2 primers showed that the unoperated eye (1), operated eye (2), and brain (3) lanes all shared a ~200 bp band and ~125 bp band. No contamination was detected in the no template control (4).*

The PCR data from Figure 8 using No Exon 2 primers showed the Exon 2 form (~200 bp) and No Exon 2 forms (~125 bp) of the *SOCS2* 5'UTR. The band for Exon 2 is dimmer in operated eye than in the unoperated eye and brain. The No Exon 2 form was brighter in the operated eye when compared to unoperated eye and brain. These results were exactly the opposite of the expression levels of the *SOCS2* 5'UTR splice forms seen at 7 days.

qPCR corroborated the PCR data. qPCR of technical triplicates normalized to the average GAPDH C_T value can be seen in Appendix I Table 3. The normalized C_T values for operated eye and unoperated eye were graphed in Figure 9. The normalized C_T values in unoperated versus operated for both the Exon 2 and No Exon 2 forms were then tested for statistical

significance within the technical replicates using a two-tailed, homoscedastic t-test. When comparing unoperated eye and operated eye, Exon 2 had a significance of p = 0.0006 and No Exon 2 had p = 0.0003.

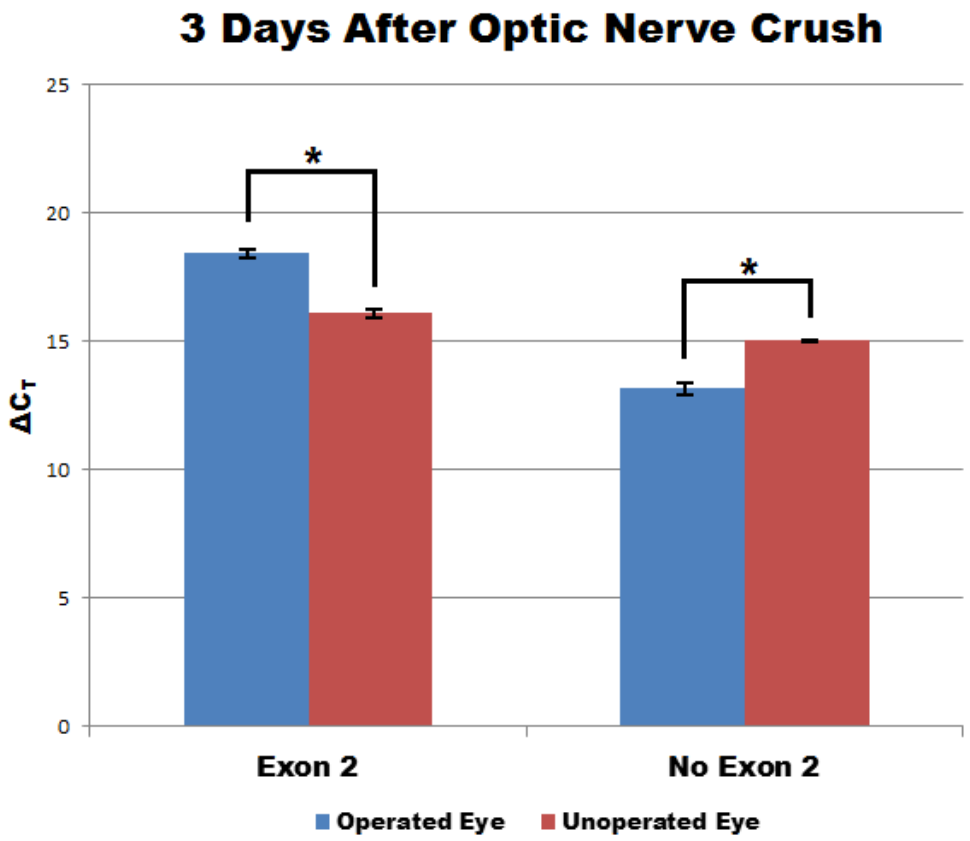

Figure 9. Graphical representation of normalized C_T values in operated eye and unoperated eye. *There was a significant difference in Exon 2 and No Exon 2 expression during optic nerve regeneration according to the Student's t-test performed. (* $p<0.05$, N.S. = not significant). Error bars indicate +/- SE, n = 3 replicates, 4 frogs per group.*

There was a difference of 2.33 cycles between the average normalized C_T values for Exon 2 in operated eye and unoperated eye. The difference between average normalized C_T for No Exon 2 was 1.86 cycles. There was a 5.01 fold increase in expression of No Exon 2 mRNA and a 3.6 fold decrease in the expression of the Exon 2 form in the operated eye relative to the unoperated eye. These data with 3 technical replicates suggest that at 3 days post optic nerve crush Exon 2 decreases during optic nerve regeneration whereas the No Exon 2 form increases in expression levels.

Development of an Assay for Validation of an IRES *in vivo*

One possibility for the regulatory role of the *SOCS2* 5'UTR is that the presence or lack of Exon 2 in the 5'UTR could be acting as an Internal Ribosome Entry Site (IRES). A common feature of IRESes is the presence of high secondary structures. The predicted secondary structures of the *SOCS2* 5'UTR using m-FOLD, an RNA structure prediction software, at 22°C with and without Exon 2 can be seen in Figure 10.

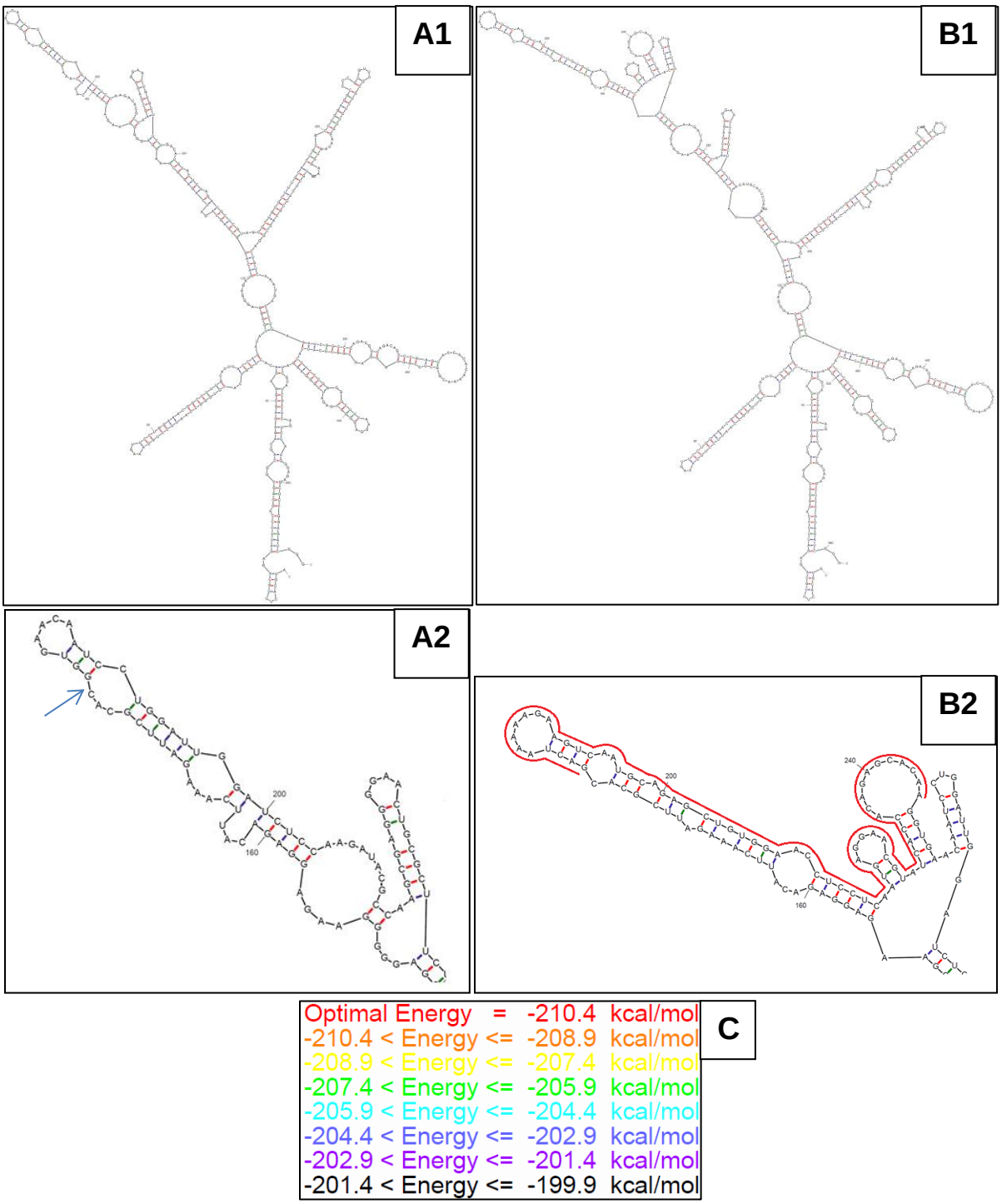

Figure 10. Inclusion of Exon 2 in the 5'UTR adds a stable secondary structure not found when Exon 2 is absent. *The 5'UTR when Exon 2 is absent (A1) is similar to the 5'UTR that includes Exon 2 (B1) except in the area of the exon junction (A2, arrow). The addition of Exon 2 (B2, red) increases the overall stability of this structure, with many bonds in the secondary structure having the optimal energy (C).*

The predicted secondary structure of the *SOCS2* 5'UTR is suggestive that the UTR may be functioning as an IRES, which can be tested by inserting the putative IRES into a bicistronic reporter plasmid (Figure 11). Four constructs were created, differing only in the putative IRES sequence inserted between green lantern Green Fluorescent Protein (glGFP) and td-Tomato. For full plasmids maps for each bicistronic reporter construct, refer to Appendix II.

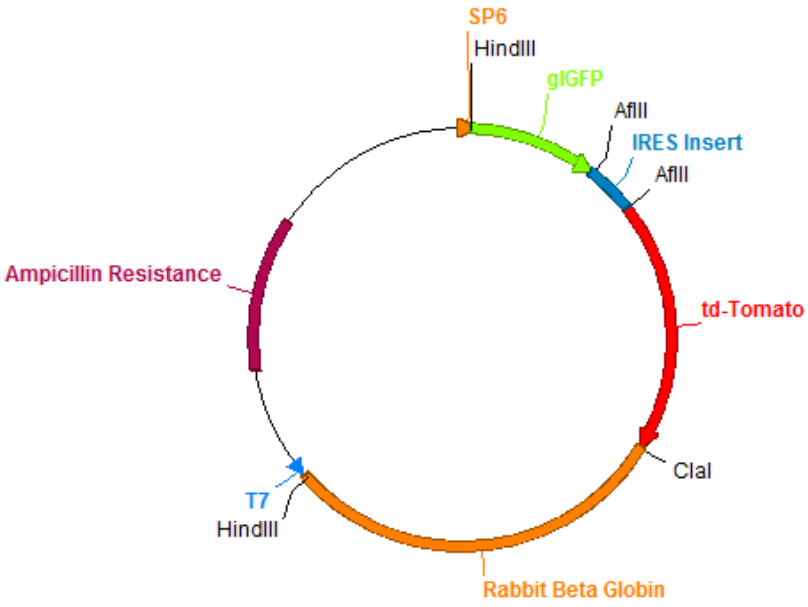

Figure 11. All bicistronic reporter plasmids were created with the same general sequence and organization. *A pGEM-3z (Promega) vector was manipulated to test for the presence of an IRES. A positive control was created by inserting a commercially validated IRES (derived from pIRES2-dsRed2). The plasmid that lacked an IRES sequence between the two fluorescent protein coding sequences was generated and used as a negative control. The experimental plasmids contained the complete* SOCS2 *5'UTR, either including or excluding Exon 2.*

The control plasmids that included the pIRES2-dsRed2 IRES sequence and the no IRES insert were linearized and *in vitro* transcribed into synthetic mRNAs. These mRNAs were injected into two-cell stage *X. laevis* embryos. At stages 37/38 in development, the embryos were imaged for green and red fluorescence indicating IRES activity or lack of IRES activity thereof (Figure 12).

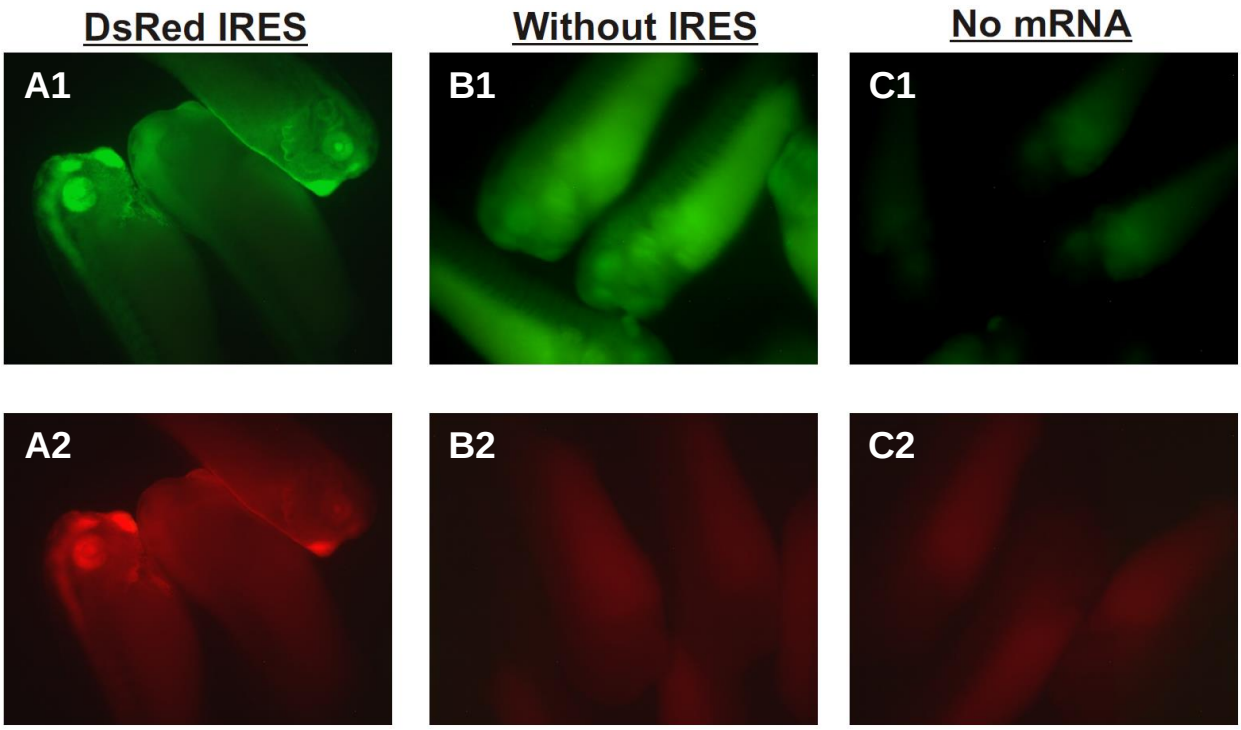

Figure 12. Bicistronic reporter constructs were functional in *X. laevis* embryos. *Absence of injected mRNA (C1, C2) showed only autofluorescence of the yolk. Injection of mRNA that had no putative IRES sequence in the AflII site resulted in expression of glGFP (B1), but no expression of td-Tomato (B2). Injection of mRNA that contained the commercial IRES (pIRES2) expressed both glGFP (A1) and td-Tomato (A2).*

The expression of these mRNAs with the appropriate fluorescence indicated that a method was created to test *in vivo* IRES activity. In Figure 12, the fluorescence of the mRNA that lacked an IRES was dim in expression of glGFP, and will need to be replicated for a more prominent image of fluorescence.

Discussion

I found that the mRNA sequence of *SOCS2* expressed in juvenile eye and embryos was different from that predicted from the NCBI database sequence. Using 3'RACE, I found an extended 3'UTR with a polyadenylation and termination sequence, strongly indicating that this longer form represents the true 3' end. It is likely that this longer form was missed, since the 3'UTR contains a string of A's that could result in mispriming by oligo-d(T) during the reverse transcriptase reaction. Indeed, only one of 20 clones represented the longer 3'UTR. However,

since the other 19 lack the termination and polyadenylation signal (AAAUAA) present in the longer form, it seems likely that this longer form represents the true 3'UTR.

The 5'UTR was revealed to be more nuanced, with two splice forms within the 5' UTR, but there were no changes to the coding domain. The addition of two alternatively spliced forms of the *SOCS2* 5'UTR provides fresh perspectives on how *SOCS2* could be regulated at the post-transcriptional level. 5'UTRs of mRNAs are often involved in translational regulation, functioning as response elements that bind proteins and miRNAs or as alternative sites of ribosomal entry to initiate translation internally within the mRNA instead of at the 5'-capped end. Thus, I have hypothesized that this 68 bp exon (called 'Exon 2' in this paper) could be involved in the post-transcriptional regulation of *SOCS2* expression, promoting increased expression of SOCS2 protein during the intermediate to late phases of optic nerve regeneration. Through *in situ* hybridization and qPCR, the expressions of the alternatively spliced forms were correlated with different stages of the early to intermediate phase of the regeneration process. I found that the form lacking Exon 2 was upregulated during the earliest phase of regeneration (3 days), while the splice form that included Exon 2 was downregulated. At the intermediate stage of optic nerve regeneration, upregulation of expression was only seen for the form of the 5'UTR that contained Exon 2. Thus, the two alternative splice forms were differentially expressed in the eye at different time points during regeneration.

These changes in expression could reflect a stress or injury response at early time points during regeneration when cap-dependent translation of many genes is suppressed. There are two possibilities, one where Exon 2 could be forming translation inhibitory structures such as hair-pin loops. The inhibitory structures could function as riboswitches or as microRNA binding sites. These would prevent ribosomes from progressing further down the mRNA transcript into

the coding domain. An alternative role Exon 2 could be playing is it could form secondary structures that would regulate the internal recruitment of ribosomes to promote translation of genes that could be required to cope with injury, when the cap dependent machinery is turned off. This would explain why one form is preferentially expressed early in regeneration.

An internal ribosome entry site (IRES) is one such possibility as it provides an alternative translation initiation mechanism. IRESes are sequences typically present on the 5'UTR of an mRNA that are capable of recruiting ribosomes to initiate translation in a cap-independent manner under the conditions of stress or injury. Since SOCS2 protein expression is regulated during injury and cellular stress, Exon 2 could be an IRES. Structural differences, taken together with their location in the 5'UTR, further suggest that Exon 2 may play a role in the differential expression of *SOCS2*. Secondary structure predictions made using m-FOLD indicate that Exon 2 forms a stem loop structure. Such structures are a common secondary structural characteristic found in IRESes, but there are no defining characteristics of an IRES. IRESes can be identified only through direct experiments. One such approach is to use a bicistronic reporter assay (as reviewed by Thompson, 2012). To test whether this might be a valid approach in *Xenopus*, I made and tested a bicistronic reporter construct. I showed that a commercially available IRES functions in *Xenopus* as expected, thus demonstrating the feasibility of this assay.

The data from my experiments suggest that the newly found splice forms of the *SOCS2* 5'UTR have distinctive roles in the regulation of *SOCS2* during optic nerve regeneration. The splice form that lacks Exon 2 is upregulated at only 3 days, an early timepoint in regeneration. At this early stage, debris from degenerating axons is being removed by macrophages and regeneration is just being initiated. By 7 and 12 days, expression of this form declines. During this time, regenerating axons are working toward the optic chiasm, which they reach around 10

days and the tectum is reached around 15 days (Ostberg and Norden, 1979). Full, comprehensive vision is restored several months after first sustaining damage (Szaro et al., 1985). The regenerating axons reach and cross the lesion site about 5 days after injury (Wilson et al., 1992). Although the alternative splice form of *SOCS2* that includes Exon 2 is downregulated at 3 days, it is upregulated at 7 days and 12 days, the intermediate and late stages of regeneration, respectively. At 7 days it is the predominant form of *SOCS2* mRNA present in the eye.

One possible explanation for these results is that during the early stages of regeneration, SOCS2 protein expression is low due to the higher expression of the No Exon 2 form, which lacks an IRES or other translation enhancing mechanism. At 3 days, SOCS2 protein is only beginning to be expressed (unpublished data), and this protein may be derived primarily from the Exon 2 containing mRNA. However, in the intermediate to late stages, the Exon 2 form of *SOCS2* increases and surpasses the No Exon 2 form, allowing for even more *SOCS2* translation. SOCS2, which peaks in protein expression at 12 days (unpublished data), could result in the degradation of SOCS3, which is known to inhibit optic nerve regeneration in mammals (Liu et al., 2015; Tannahill et al., 2005). In mammals, an absence of this translation enhancing mechanism in the 5'UTR may result in the failure of rise in *SOCS2* expression preventing the process of regeneration to occur.

The unusually long (43,484 bp) sequence of the intron within the coding domain of *SOCS2* was obtained from Xenbase GBrowse 7.2. Due to the length of the intron, the previous genome sequence of *X. laevis* was compared using GBrowse 6.0 (XenBase), where *SOCS2* is located on Scaffold 17487. The length of the intron using this older genome sequence was 21,775 bp. Both introns have extensive stretches of non-specified nucleotides (>9,500 bp). However, the

presence of such a large intron within a coding domain is unlikely. It is plausible that this region of the genome is difficult to sequence and the true length of this intron within *SOCS2* has not been elucidated. When PCR products from *SOCS2* cDNA using coding domain primers are examined with agarose/TBE, the length of the coding domain matches the database length. This means that the two exons of *SOCS2* within the genome are correct; their distance is the uncertain aspect. Therefore the accuracy of the intron data obtained from Xenbase can only be experimentally verified.

Less is known about the splice form that lacks Exon 2 due to no PCR or qPCR having been performed on cDNA from frogs 12 days after optic nerve crush. This experiment will need to be done in the future, along with biological replicates of the 7 and 3 days data. *In situ* hybridization will also need to be performed in 3 and 7 days post optic nerve crush retina sections to determine if these changes in expression of the Exon 2 form of SOCS2 are located in the retinal ganglion cell layer, as *SOCS2* expression can be seen throughout the retina (unpublished data). The bicistronic reporter assay must be performed with the *SOCS2* 5'UTR with and without Exon 2 in order to determine whether our IRES hypothesis is worth pursuing further. The control constructs containing an IRES or no IRES are positive indications that our method is reproducible, however we will need to reproduce our experiment to be sure. If these results are reproducible, then I have developed an assay to detect IRES activity that can be performed *in vivo*, which could arguably be more informative than an assay using cell lines. My thesis thus lays the foundation for future studies of the role of SOCS2 in successful CNS axon regeneration.

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APPENDIX I

Table 2. Normalized Ct Data for TaqMan qPCR at 7 Days after Optic Nerve Crush

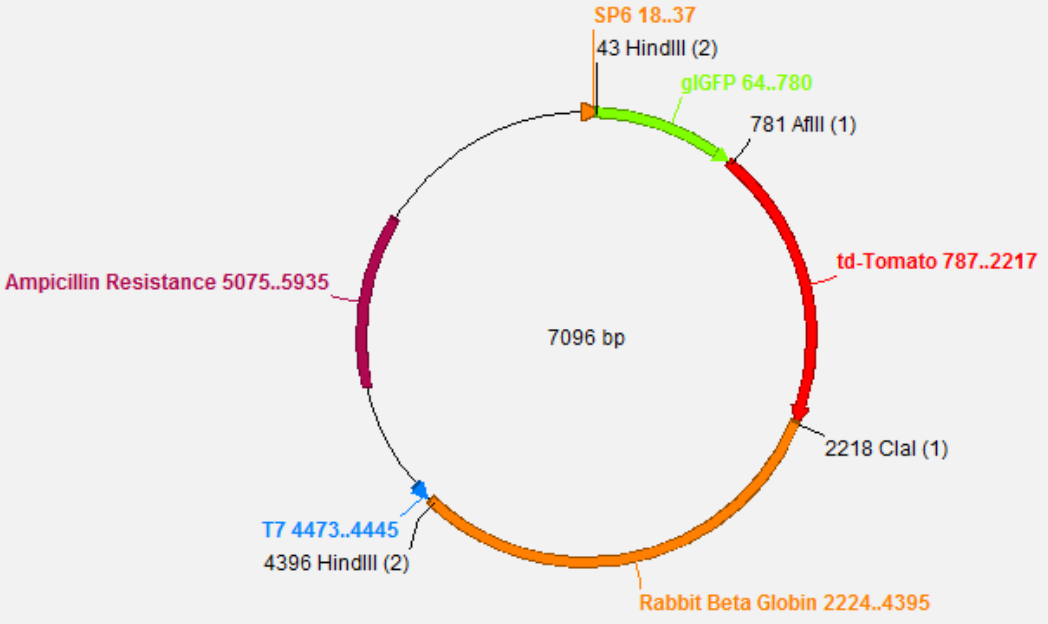
Detector	Sample	Normalized Ct Sample	Avg Normalized Ct	Avg Normalized St Dev
SOCS2 Exon 2	Unoperated Eye	19.30	18.23	1.12
		17.08		
		18.32		
	Operated Eye	15.83	15.91	0.13
		16.05		
		15.85		
	Brain	17.76	18.12	0.64
		17.74		
		18.86		
	No Template	N/A		
SOCS2 No Exon 2	Unoperated Eye	19.76	19.07	0.72
		18.32		
		19.12		
	Operated Eye	21.10	20.46	1.04
		19.26		
		21.01		
	Brain	16.48	16.92	0.39
		17.24		
		17.04		
	No Template	N/A		

Table 3. Normalized Ct Data for TaqMan qPCR at 3 Days after Optic Nerve Crush

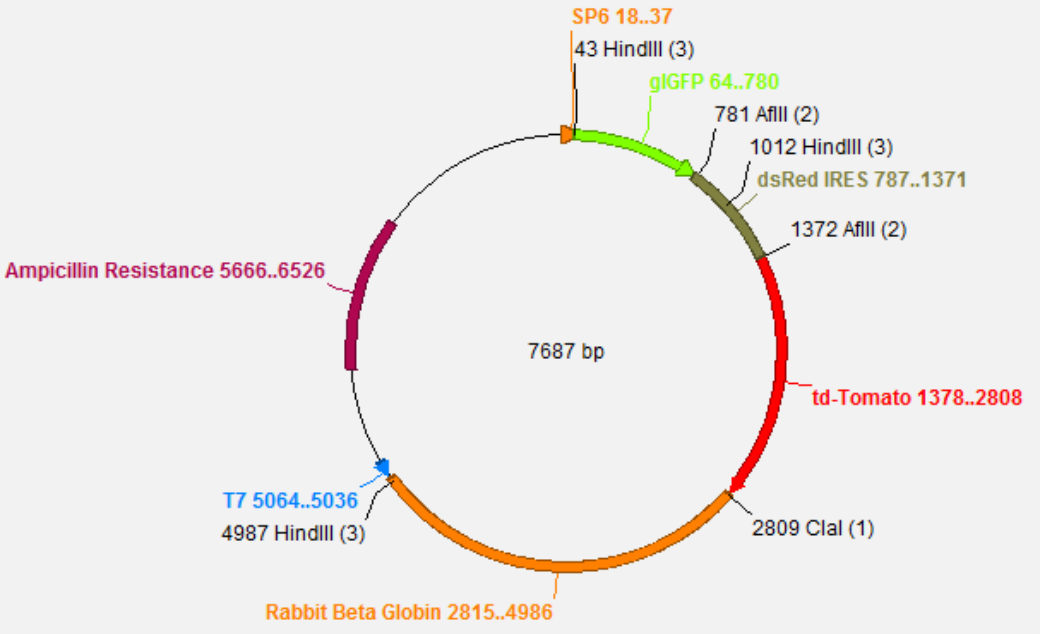
Detector	Sample	Normalized Ct Sample	Avg Normalized Ct	Avg Normalized St Dev
SOCS2 Exon 2	Unoperated Eye	16.41	16.09	0.30
		15.81		
		16.05		
	Operated Eye	18.48	18.42	0.28
		18.67		
		18.11		
	Brain	18.98	21.07	3.53
		19.07		
		25.14		
	No Template	44.13		
SOCS2 No Exon 2	Unoperated Eye	15.04	15.05	0.05
		15.10		
		15.01		
	Operated Eye	12.99	13.19	0.28
		13.51		
		13.08		
	Brain	15.28	14.89	0.37
		14.86		
		14.53		
	No Template	N/A		

APPENDIX II

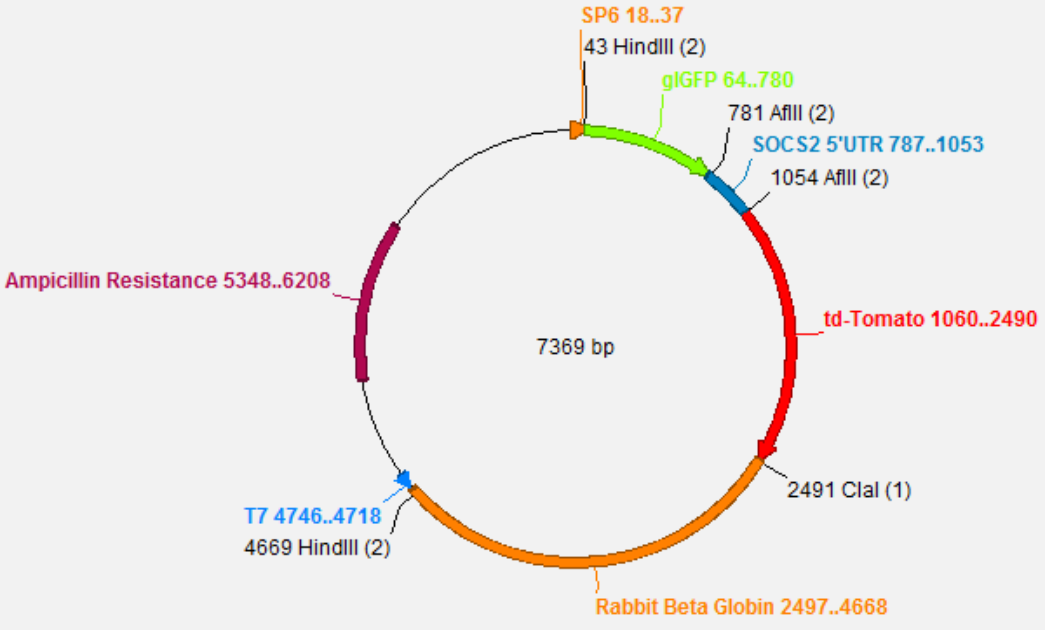
No IRES Insert



pIRES2-DsRed2 IRES



SOCS2 5'UTR without Exon 2



SOCS2 5'UTR with Exon 2