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Identification and Characterization of the 5' UTR of the *Drosophila virilis* Insulin Receptor mRNA

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Abstract

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Nutrient signaling pathways are vital for the proper growth and maintenance of an organism. Detecting nutrient availability is important under both high and low nutrient conditions in order to determine the most advantageous and minimally sustainable cellular activity levels. One of the primary nutrient sensing systems in metazoans, found to be conserved between *D. melanogaster* and humans, is the insulin receptor pathway. Studies of model organisms, including the fruit fly, *Drosophila melanogaster* have provided a better understanding of the insulin signaling pathway at the cellular level. Previous studies in the fruit fly have shown internal ribosomal entry site (IRES) activity in the 5' untranslated region (UTR) of the *Drosophila melanogaster* insulin receptor (INR) transcript providing a novel level of control(Marr et al., 2007). In order to better understand the characteristics required for IRES activity, the 5' UTR of the INR in the distantly related species, *Drosophila virilis*, was cloned for analysis and comparison to the *D. melanogaster* UTR. Interestingly, we found the *virilis* INR transcript shares characteristics often found in IRES containing transcripts.

Introduction

Nutrient signaling pathways are vital for the proper development and maintenance of eukaryotic cells. Detecting nutrient availability is important under both high and low nutrient conditions in order to determine the most advantageous or minimally sustainable cellular activity levels. One such nutrient signaling pathway involves the detection of insulin by insulin receptors and the cellular responses which ensue.

In humans, high levels of blood glucose cause the beta cells of the pancreas to secrete the hormone insulin which elicits responses in the body to increase the uptake of glucose (Widmaier et al. 623). When this system fails in the human body it takes on one of two forms. Either the insulin is not secreted in response to glucose levels as it should be (type I diabetes mellitus) or the cellular sensitivity to the insulin is lower than normal (type II diabetes mellitus), (Widmaier et al. 628). Understanding the mechanisms which underlie this decreased sensitivity to insulin would be beneficial for the effective treatment of type II diabetes in humans.

The insulin signaling pathway is conserved in metazoans (See figure 1). Insulin binds to the insulin receptor and a cascade of events occurs. Though some intermediates in this pathway vary, the overall effect of insulin is the regulation of growth or

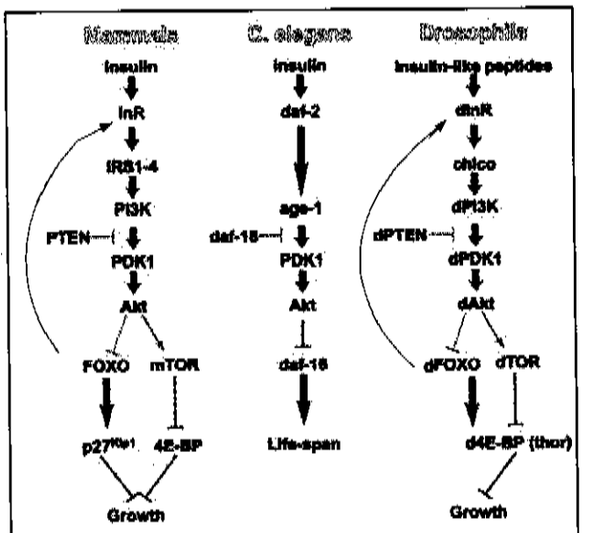

Figure 1. Insulin signaling pathway in mammals, *C.elegans*, and Drosophila

life span which is limited by finite numbers of cell divisions. This pathway conservation allows the information gained from studies in model organisms to be applied in humans. In order to gain a better understanding of the insulin signaling pathway at the cellular level, the fruit fly *Drosophila melanogaster* has been used as a model organism. Studies in *D. melanogaster* show that a mechanism for growth regulation mediated by the insulin receptor is conserved from humans to insects (Brogiolo et al., 2001).

In *D. melanogaster*, insulin-like peptides bind to the insulin receptor in high nutrient conditions. This begins a signaling cascade resulting in the inactivation of the translation inhibitor 4E-BP, and the transcription factor FOXO (See figure 2). This allows the cell to efficiently continue cap-dependent translation of mRNA into proteins(Marr et al., 2007).

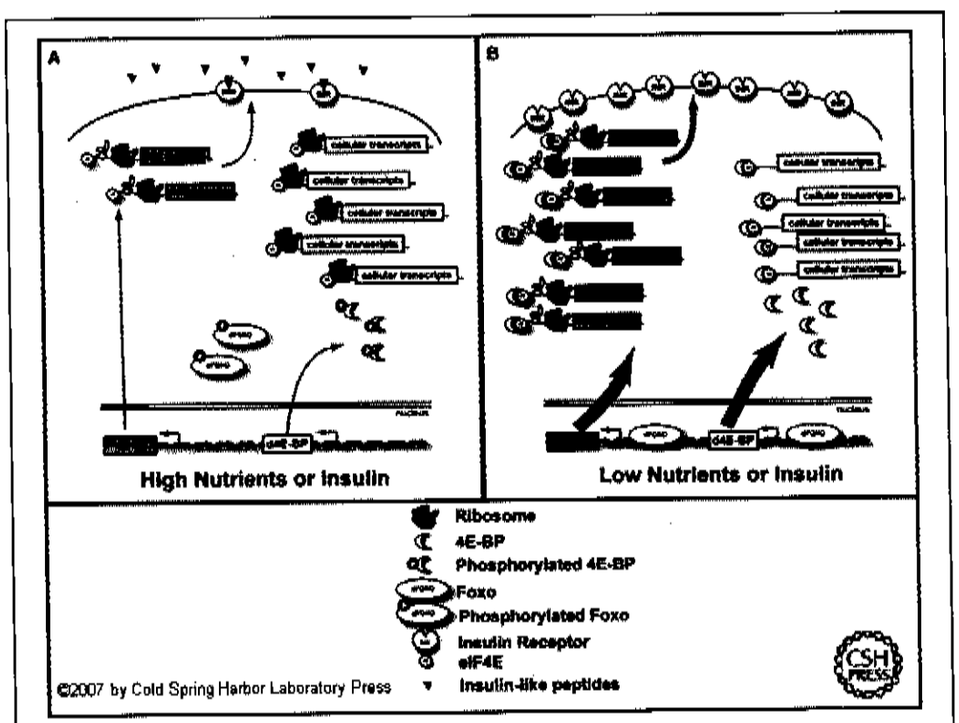

Figure 2. An overview of the INR signaling cascade events in *D. melanogaster*

However, under starvation conditions when nutrients are low, the cell responds by modulating certain cellular functions like protein synthesis. Without insulin to bind to the insulin receptor, FOXO and 4E-BP remain in their active states. Active 4E-BP is able to bind eIF4E which prevents the ribosome from forming a translation initiation complex (Miron et al., 2001). The binding of 4E-BP to eIF4E inhibits cap-dependent translation of mRNA into proteins. As the production of protein is limited within the cell, cellular growth is constrained as well.

Under cell starvation conditions, active FOXO is free to bind and stimulate the transcription of its target genes including the *Drosophila* insulin receptor (INR) within the nucleus (Marr et al., 2007). With a higher level of INR transcripts, the cell is poised to become extremely sensitive to nutrient signaling via insulin binding, but only if the INR transcripts can be translated into protein. When nutrients are low, and FOXO upregulates the transcription of INR, 4E-BP is simultaneously working to suppress cap-dependent translation in the cell. Therefore, the cell must somehow overcome the cap-dependent translation suppression if the INR is to be translated to sensitize the cell to insulin. An internal ribosomal entry site (IRES), has been proposed as the mechanism by which the INR transcripts are translated into protein (Marr et al., 2007). An IRES allows the ribosome needed for translation is able to bind directly to the transcript, bypassing the inhibitory 4E-BP eIF4E complex used to suppress cap-dependent translation under low nutrient conditions. Despite the inhibition of cap-dependent translation of the INR transcript via 4E-BP cap-binding, INR can still be translated via IRES activity. Since the insulin receptor pathway has been shown to be highly conserved it is important to know if the IRES function is also conserved.

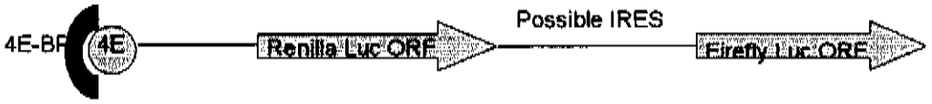

Figure 3. A schematic of the dual luciferase reporter assay

In *Drosophila melanogaster*, three 5' UTRs for INR, each originating from a different promoter, were isolated (Marr et al., 2007). These 5' UTRs isolated in *D. melanogaster* were tested for IRES activity with a translation assay which utilizes two different luciferase reporter proteins. The section of a transcript being examined for IRES activity is inserted into the reporter between the open reading frame (ORF) for renilla luciferase and the ORF for firefly luciferase (Grentzmann et al., 1998). The translation of renilla luciferase will be subject to the control of the 4E-BP binding cap-dependent translation discussed earlier (See figure 3).

Comparison of the ratio of firefly to renilla luciferase quantifies IRES activity located within the region of interest. Due to the increase of firefly luciferase activity with the 5' UTRs isolated from *D. melanogaster*, it has been proposed that these regions upstream of the initiator codon of the INR contain internal ribosomal entry sites (Marr et al., 2007). Comparison of the 5' INR UTR in a divergent species of fly, *Drosophila virilis*, may provide insight into the role of IRES activity in this pathway and cellular IRES's in general.

Results

Because there is already evidence of IRES activity in *D. melanogaster*, testing for conservation of IRES activity in a related yet distant species of *Drosophila* would provide more information about the mechanism than would testing for IRES's in closely related *Drosophila* species with very similar genomic sequences. However, the comparison species must also have a sequenced genome with which we can compare *D. melanogaster* homologs (such as INR) and also for the purpose of designing primers. For these two reasons, *Drosophila virilis* was chosen as its last shared ancestor with *D. melanogaster* diverged approximately 40 million years ago and its genome has been sequenced ("*Syntenic relationships*."; "*Evolution of genes*," (2007). To first verify the production of the INR transcript in the D. virilis tissue cultire cell line, WRDV-1, a PCR reaction designed to amplify a highly conserved region, proposed to encode the INR protein, was carried out (See figure 4). A cDNA reverse transcription followed by PCR confirmed that the INR transcript existed in the cell line and that WRDV-1 cells could be used for amplification of the 5' UTR of the INR.

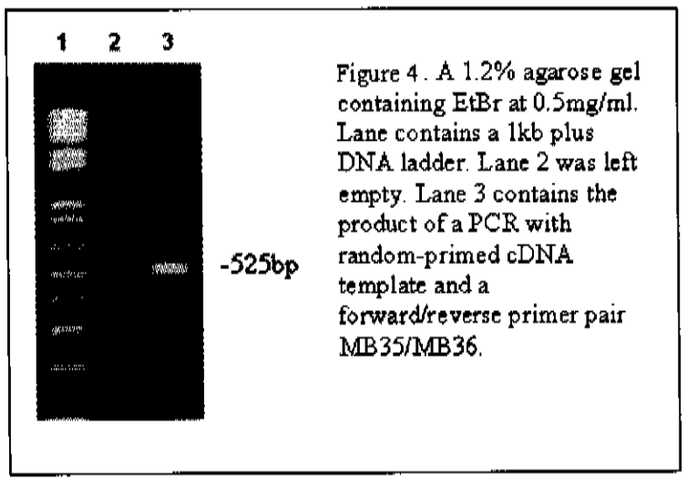

Figure 4. A 1.2% agarose gel containing EtBr at 0.5mg/ml. Lane contains a 1kb plus DNA ladder. Lane 2 was left empty. Lane 3 contains the product of a PCR with random-primed cDNA template and a forward/reverse primer pair MB35/MB36.

Cloning this 5' UTR of the INR transcript requires the use of a method known as RACE, or rapid amplification of complementary DNA ends (See figure 5). The procedure results in the production of DNA complementary to the desired transcript and two adjacent primers on one side of the transcript coupled with two nonspecific primers designed to target the other side for use in pairs during multiple PCR reactions (Ohara et al., 1989). Complementary DNA can be synthesized using the first of three primers specific to the 3' end of the transcript with the aid of reverse transcriptase. The 3' end of the cDNA is then c-tailed in a process during which terminal transferase is used to add cytosine residues to the end of the cDNA. Following the addition of the cytosine residues to the 3' end of the cDNA, two rounds of PCR are carried out with nested primers for increased specificity. The final product of 5' RACE contains the sequence for the 5' end of the transcript through the last gene specific primer used.

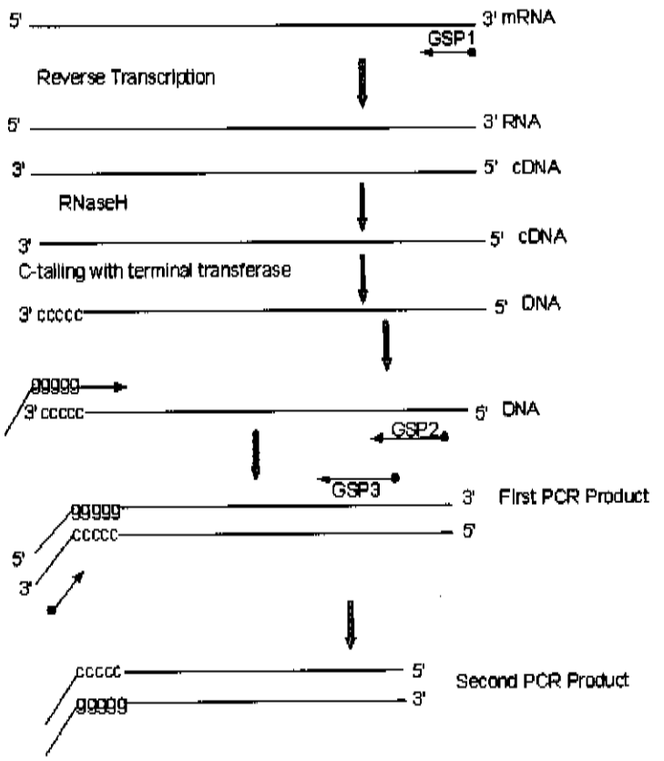

Figure 5. An overview of 5' RACE

5' RACE methods were first performed using gene specific primers designed over 1kb downstream of the proposed AUG of the INR. The high sequence conservation in this protein coding region across drosophila species gave the best evidence that we were designing primers to amplify regions of the INR transcript. 5' RACE products obtained using the first three gene specific primers designed revealed an intron within a region formerly believed to encode insulin receptor protein (See figure 6).

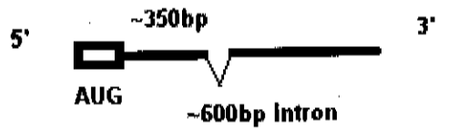

Figure 6. An intron discovered within the Coding region of the D. virilis INR

These initial 5' RACE products appeared to be incomplete. Sequencing data revealed multiple 5' ends and smearing of PCR products on agarose gels indicated that variable product lengths were present. Degraded mRNAs were being amplified and yielding products of varying length. Full-length transcripts were selected for by chemically modifying and capturing the 5' cap structures on complete mRNAs hybridized with strands of DNA following cDNA synthesis (Carninci et al., 1996). Selecting for full-length transcripts optimized the procedure by ensuring that only transcripts containing the entire 5' end of the transcript were used as template for cDNA synthesis.

Figure 7a. A 1.2% agarose containing EtBr at 0.5mg/ml. Lane contains a 1kb plus ladder. Lanes 2 and 3 sontain the firs and second PCR products of a 5' RACE carried out with full-length cDNA.

Only after selecting for full-length transcripts prior to performing 5' RACE, did we obtain product containing the 5' end of the INR transcript (See figure 7a). The sequence data resulting from this 5' RACE product allowed for the design of a reverse primer for amplification of the 5' UTR but was not itself a complete UTR because the last primer used did not include the 3' end of the UTR. The 5' RACE performed with full-length cDNA also revealed an intron 26,664bp in length (See figure 7b). With a known

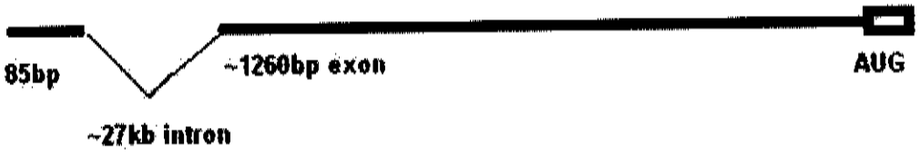

Figure 7b. A schematic of an intron discovered in the 5' UTR of the D. virlilis INR relative to the full length of the 5' UTR

sequence at the 5' end of the UTR, forward and reverse primers were designed to amplify the entire 5' UTR. Cloning and sequencing of 5' RACE products had only previously yielded portions of the 5' UTR. Performing this PCR confirmed that the entire predicted 5' UTR existed as a continuous portion of the INR transcript (See figure 8a). A PCR reaction with these primers was carried out using cDNA made with a gene specific primer was carried out. The product yielded a band of the expected size for the full length 5'

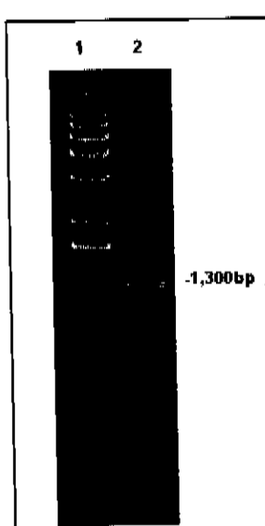

Figure 8a. A 1.2% agarose gel containing EtBr at 0.5mg/ml. Lane 1 contains a 1kb plus DNA ladder. Lane 2 contains the product of a PCR performed using primers designed to amplify the full length of the predicted 5' UTR of INR in *D.*

UTR of the *D. virilis* INR according to combined sequence and genomic data. The PCR program used was designed to select for longer transcripts so it is possible that the bias in the reaction accounts for the band strength differences in product. Multiple PCR product bands may be caused by alternative splicing of *D. virilis* INR transcripts within the amplified region or by nonspecific primer annealing and polymerization. The PCR product band around 1,300bp was gel purified and cloned for sequencing, confirming that the predicted sequence for the 5' UTR of INR existed as a region of transcript in the cells (See figure 8b).

5' UGUGCUGCCGCUCUCUCGCUCGCCUCACCAACAUCAACAUAAUCAACAACAACAACAGCAACAACAACUACGAAGAG
GCACCCUAGUUUCGACCAGCCGAGUAAAAACAACAACAACAACAACAACGACGACAACAAAAAGCAUUCCAACUGACGA
AGAUAAAUACAAAACACUACAAGUCUGAGAACACAACUGGUGUAUGUGUGUGUGUAUGUGUGAGGAAGAGAGAGCGAGA
GAGAGAAAGAGAGGGCUAGCGAGCGUGCAAGCGUGCAAGCGAGAGCUGCAAUGAUCACUUAGUGGGCCGGCGCCCCUAA
UUAAAUAAUUAAUAAUAAAAACAAUUUUUUUUUAAUUUAAUUAAAAUAUCAACAAAGCAAGAAAAACAAUGGAAUCAACA
AUAAUAACAUUUUUCAAUGGAUAUGUUUACUUACGUAUUCCUGAGGAAAAGCUGCCAAUGCCAAUAGCAAACACAAAUC
AAAGCGAUAGUGCAUGUUUGACCCUUACGAGAACAACAACAACAACAACAAAGCGAAAAAGAAGAAUAUCUAUGCU
CUUUGAUAGUAAACCGAUUCCGAGUAAACACAAUUGUGAAUUGCGCGAAACGCUAGCUCCCGGCAACAUGAUGCAGAAA
UCCAGUUGCAGUGCAACAGCAACAAAAACAAAAACAAAAACAUUAACAACUGAUAAGGUAUUAGAAACAACAAAAACAA
GGCCAGCAUCAUUGUCAACAACAACAACAACAACUAGAGCAACAACAACGCAUGGAAACAUUUGUGUUGUAUGCCGACA
AGUGUUCGCAACAACAACAUGUUCCUGCAUUCAGACAGCAACAAGCAGCAGUUCCUAUGCAGACAGCAGCAGCUGUAGC
AGCCAGCGCAGAAGCAAAAACAGAAACAGUAGCAGUAACAGUAACAGUAACAGCGGCGCAACAUCCACCACAU
UCCCCAGCAAACUGUCGUCGGCGCAACAUUGCCGCUGUCUGCAACAACAGCAGCAACAUCUGCAGCACCAGCAACAUGU
UGGCAGCGUCGCGCAUAUAUGUUGCUGUGCAAAUAGUAGUAGUAGUCGAAGAAAUAGUAGUAGUAGUAGUAGUAAU
AGUACAAAUGACAAUAACCAAAAAGUAGCAGCAACAACAGCCAAUUGCCACUGCAACAGCAACAACAAAAACAACAAUA
UUAAAAGCCACAUCCAUAAAAAGCAACGACUGCAGCAACACUUUAACAUUUACAGCAAUUGCUUAAGCCUUUUGGCCAC
ACUGCAAUUGUUAUUUCUGUUUGUGCAAAA3'

Figure 8b. Sequence of the 5' UTR identified in the *D. virilis* INR.

The sequence for the 5'UTR of INR in *D. virilis* shares some similarities with the longest of the 5' UTRs in *D. melanogaster*. In *D. melanogaster*, the longest and most abundant 5' UTR of INR is 1,118bp in length and contains 12 AUGs upstream of the initiator codon. The D. virilis 5' UTR of INR contains 17 or 18 AUGs upstream of the two most likely initiator codons. Aligning the 5' UTRs of INR from *D. virilis* with the longest 5' UTR of INR in *D. melanogaster* reveals a 22.5% conservation of base pair sequences overall (See figure 9a) as well as regions containing over 50% conservation (See figures 9b-9d).

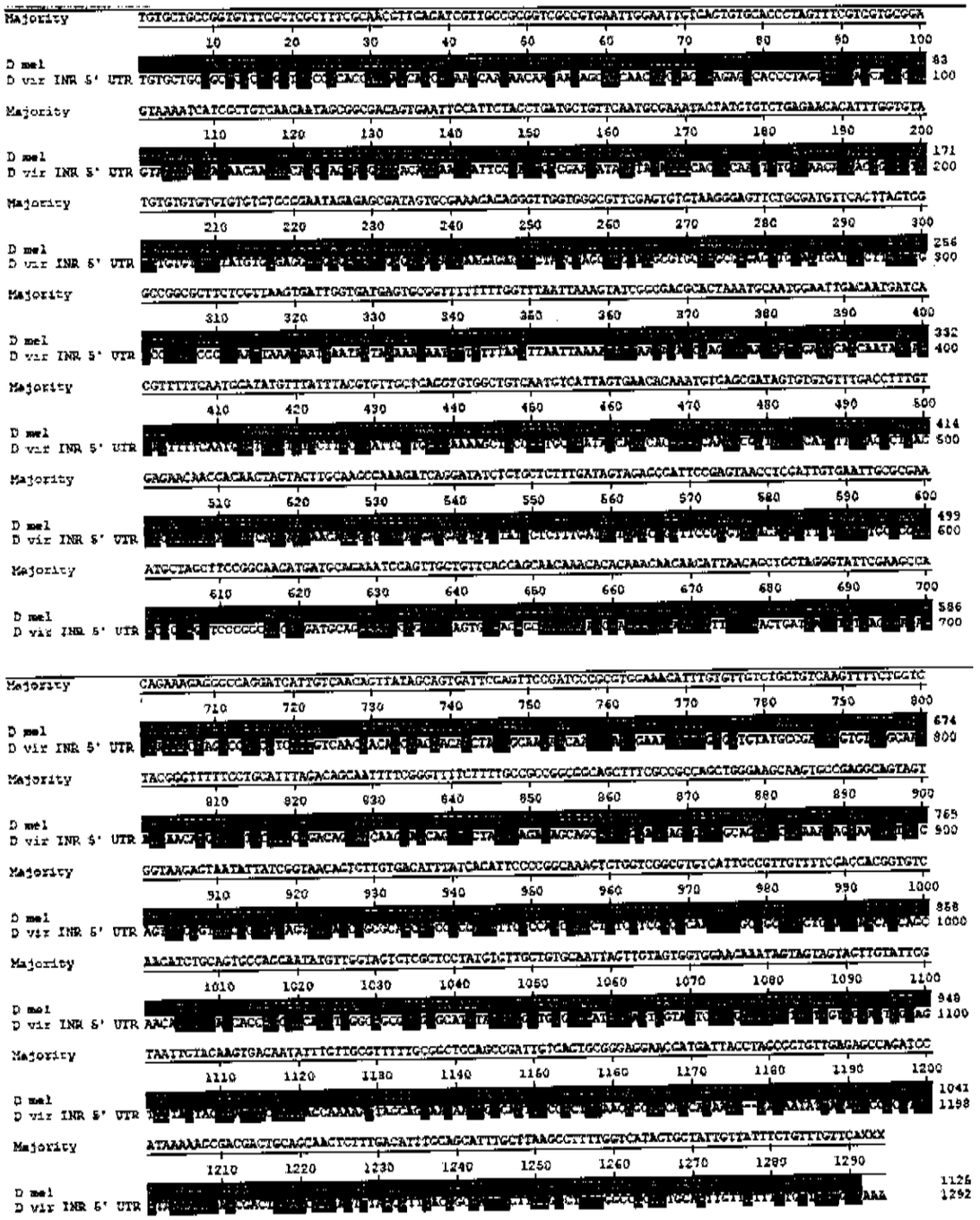

Figure 9a. An alignment of the longest 5'UTR of INR known in *D. melanogaster* and the 5' UTR of INR from *D. virilis*.

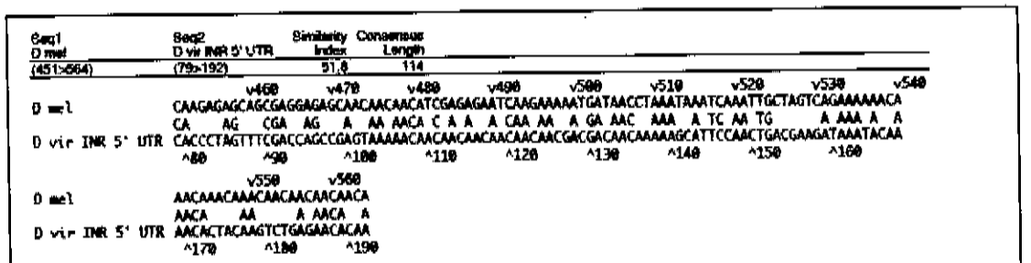

Figure 9b. An alignment of highly similar regions of the *D. melanogaster* and *D. virilis* 5' UTRs of INR sharing 51.8% sequence conservation.

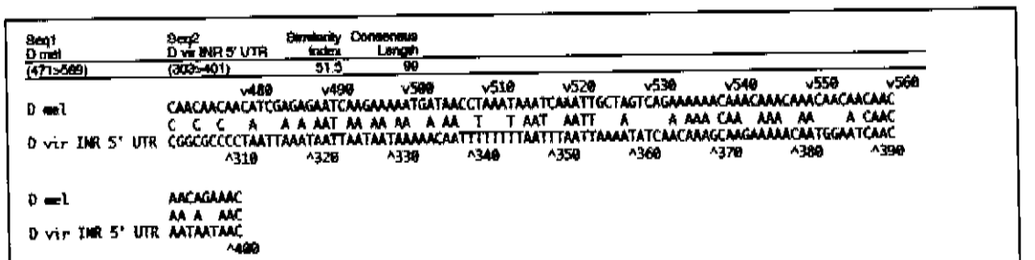

Figure 9c. An alignment of highly similar regions of the *D. melanogaster* and *D. virilis* 5' UTRs of INR sharing 51.5% sequence conservation.

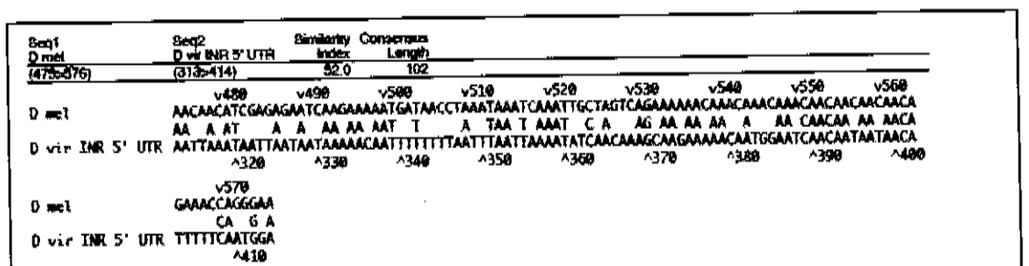

Figure 9d. An alignment of highly similar regions of the *D. melanogaster* and *D. virilis* 5' UTRs of INR sharing 52.0% sequence conservation.

Discussion

Performing 5' RACE with gene specific primers nested within the INR ORF led to the discovery of an intron within the translated region of the INR transcript (Fig. 6). Unexpectedly, the procedure carried out with the intention of characterizing the 5' UTR

helped to characterize the protein coding sequence of the *D. virilis* INR.

Cloning the 5' UTR of INR in *D. virilis* did reveal shared sequence traits with those identified and shown to exhibit IRES activity in *D. melanogaster*. The *D. virilis* 5' UTR of INR is longer than 1kb, like the longest of the 5' UTRs in *D. melanogaster* (Fig.8a). It also contains a 26,664bp intron, placing the promoter for the 5' UTR approximately 28,000bp upstream from the proposed start of the INR ORF (Figs. 7a, 7b). The longest 5' INR UTR in *D. melanogaster* also contains large introns and initiates from a promoter approximately 38,000bp upstream from the beginning of the INR ORF(Marr et al., 2007). The *D. virilis* 5' UTR of INR contains 17 to 18 AUGs upstream of the initiator codon, depending on which of the proposed ORF start sites is chosen (Fig. 8b). All three of the 5' INR UTRs found to have IRES activity in *D. melanogaster* contain multiple AUGs upstream of the translational start site (Marr et al., 2007). Although we have not yet determined whether or not the 5' UTR of INR in *D. virilis* contains an IRES, these similarities to the IRES-containing transcripts in *D. melanogaster* support the hypothesis that the IRES activity will be conserved.

After the presence of the transcript was confirmed (Fig.4), cap-trapping methods which selected for full-length transcripts were ultimately the only manner by which the 5' end of the INR transcript was finally cloned and subsequently sequenced (Fig. 7a). Prior to selection for full-length mRNAs, 5' RACE products ran as smears on agarose gels indicating multiple lengths of product. Library cloning and sequencing of these products revealed that numerous 5' ends were being amplified. By oxidizing and chemically modifying the diol groups present on the 5' caps of complete mRNAs, we were able to select for only full-length transcripts hybridized with DNA following reverse

transcription. Ultimately, the PCR product achieved via 5' RACE with full-length cDNA provided the sequence data necessary for the design of a reverse primer for more direct amplification of the 5' INR UTR. The PCR carried out with these primers at the 5' and 3' ends of the 5' UTR confirmed the presence of the full-length 1,300bp fragment which had previously only been sequenced in fragments (Fig. 8a). The PCR product was gel purified to clone only the 1,300bp band, but it is possible that the other product bands correspond to alternatively spliced 5' UTRs within the region. Three distinct 5' INR UTRs were identified in *D. melanogaster*, providing support for the possibility that multiple 5' UTRs might also exist in *D. virilis* (Marr et al., 2007).

Provided the 5' INR UTR contains and IRES, the sequence data acquired for the 5' INR UTR may offer insight into the elements required for IRES activity specifically as it pertains to the INR pathway. Furthermore, as more IRES's are identified across species, this UTR may provide information regarding IRES functionality in general. Cloning of the full-length 5' UTR into a dual luciferase assay will allow for the determination of IRES activity in future experiments. The sequences of the 5' UTR of INR in *D. virilis* and the longest 5' UTR of INR in *D. melanogaster* are 22.5% conserved (Fig. 9a) which would provide an interesting comparison of IRES character if the activity is conserved. Both IRES's would be functioning in the same pathway but are not identical. Also in the future, FOXO could be mutated to resist phosphorylation by Akt and studying the effect of this mutant FOXO on INR transcription levels would provide insight into the conservation of the pathway between *D. melanogaster* and *D. virilis*. Deletion mutants of the 5' UTR could also serve to identify regions of the transcript required to maintain IRES activity. Conversely, if the 5' INR UTR in *D. virilis* does not appear to have IRES

activity, then it would be interesting to identify the genomic changes requires to evolve the IRES activity observed in *D. melanogaster* and compare these novel transcript characteristics with the *D. virilis* 5' UTR. The characterization of the 5' INR UTR in *D. virilis* allows for the comparison of this function between two highly diverged species with unique genomic sequences.

Materials and Methods

1. Confirmation of INR transcription in WRDV-1 cells

cDNA was synthesized from total RNA isolated from WRDV-1 cells and used as template in a PCR reaction designed to amplify a region of the INR transcript proposed to encode protein.

1.1 RNA Isolation

Total RNA was isolated from WRDV-1 cells using the RNeasy® Mini Kit (Qiagen, California, USA) as per protocol.

1.2 cDNA Synthesis

cDNA was synthesized from total RNA using random priming methods. A mixture of 1.0µl of Oligo(dT) primer at 40µM, 1.0µl of random hexamers at 0.3mg/ml, 5.0µl of total RNA (at 928µg/ml) and 8.5µl ddH20 was incubated at 70°C for 10 minutes. The mixture was then placed at 0°C for the addition of 2.µl 10X RT Buffer (NEB, Massachusetts, USA), 1.0µl of 10mM dNTP mix, 5.0µl ddH2O and 1.0 µl MMLV RT. This reaction was then incubated at 25°C for 20 minutes, 42°C for 50 minutes, and 70°C for 15 minutes.

1.3 RNase Treatment and Column Purification

The cDNA reaction was then treated with RNAses A and H at 37°C for 30

minutes. 500µl PB (5M guanidine hydrochloride, 30% isopropanol) were added to the mixture and passed over a QIAprep® spin miniprep (Qiagen, California, USA) purification column. The column was washed twice with 650 µl PE (80% ethanol, 10mM Tris pH 7.5) and centrifuged for 1 minute to dry the column. The cDNA was eluted in 30µl 10mM Tris pH 8.0

1.4 PCR for INR

A forward and reverse primers MB35 and MB36 were used in a 100µl PCR reaction to amplify a region of the genome encoding the insulin receptor protein. The PCR program used included a 1 minute incubation at 95°C followed by 30 cycles of an incubation at 95°C for 15 seconds, 55°C for 30 seconds, and at 72°C for 30 seconds. 15 µl of PCR products were analyzed using 1.2% agarose gels containing EtBr at 0.5mg/ml.

2. 5' RACE within the INR coding region

A 5' RACE using three gene specific primers (MB12, MB13, and MB14) was performed. RNA used to synthesize the cDNA was isolated from cells transfected with *D. virlis* FOXO (See appendix). The product was then cloned and sequenced.

2.1 cDNA synthesis

5µg of total RNA collected from cells transfected with pKN157-2 (see appendix) were used in a cDNA synthesis utilizing a modified protocol with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, California, USA). After mixing mRNA, annealing buffer, and a gene specific primer, MB12, as per protocol, the sample was incubated at 90°C for 1 minute, followed by a temperature gradient of 0.1°C/s to 74°C and incubated for ten minutes. Following this incubation was a temperature gradient of 0.1°C/s to 55°C when the 2X reaction mix and enzyme mix was

added as outlined by the Invitrogen protocol. A 1 hour incubation was then carried out at 55°C in place of the 50 minute incubation at 50°C recommended in the protocol.

2.2 RNase Treatment and Column Purification

The cDNA reaction was then treated with RNAses A and H at 37°C for 30 minutes. 500µl PB (5M guanidine hydrochloride, 30% isopropanol) were added to the mixture before being passed over, washed, and dried on a QIAprep® spin miniprep (Qiagen, California, USA) purification column. The cDNA was eluted in 30µl 10mM Tris pH 8.0

2.3 c-tailing of cDNA

A reaction mixture containing 2.5 µl 10X NEB buffer #4 (NEB, Massachusetts, USA), 1.0 µl 2.5mM CoCl2, 2.5 µl 2mM dCTPs, and 18.0µl of the column-purified cap-trapped cDNA mixture was incubated at 94°C for 2 minutes and then placed at 0°C for the addition of 1.0 µl terminal deoxynucleotidyl transferase (TdT). The 25µl reaction was incubated at 37°C for 10 minutes followed by 10 minutes at 65°C.

2.4 First PCR

A 50µl PCR reaction containing 5.0µl 10X ThermoPol buffer, 1.0µl 10mM dNTPs, 34.5µl ddH20, 5.0µl of the c-tailed cDNA reaction mixture, 2.0µl of a 5' RACE Anchor primer at 10µM, 2.0µl of a gene specific primer, MB13, at 10 µM and 0.5µl of a 1:100 Pfu:Taq mixture. The reaction was incubated at 94°C for 2 minutes, followed by ten cycles of an incubation at 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 3 minutes. 31 additional cycles were carried out with incubation at 94°C for 15 seconds, 55°C for 30 seconds, and 72°C incubations which began with a length of 3 minutes for the first cycle followed by subsequent incubations with the addition of 10 seconds per

cycle.

2.5 Second PCR

A 50µl PCR reaction containing 5.0µl 10X Thermopol buffer, 1.0µl 10mM dNTPs, 34.5µl ddH20, 5.0µl of a 1:100 dilution of the first PCR product in TE 10-1 buffer pH 8.0, 2.0µl of an abridged universal amplification primer (AUAP) at 10µM, 2.0µl of a gene specific primer, MB14, at 10µM and 0.5µl of 1:100 Pfu:Taq mixture. . The reaction was incubated at 94°C for 2 minutes, followed by ten cycles of an incubation at 94°C for 15 seconds, 70°C for 30 seconds, and 72°C for 3 minutes. 31 additional cycles were carried out with incubation at 94°C for 15 seconds, 70°C for 30 seconds, and 72°C incubations which began with a length of 3 minutes for the first cycle followed by subsequent incubations with the addition of 10 seconds per cycle.

3. *Cloning 5' UTR of* D. virilis *INR*

The full length 5' UTR of the *D. virilis* INR was accomplished using 5' RACE methods to characterize the 5' end of the transcript followed by a PCR reaction with forward and reverse primers flanking the entire 5' UTR.

3.1 RNA extraction

RNA from *Drosophila virilis* WRDV-1 cells was isolated using a modified invitrogen TRIzol® Reagent protocol. Following lysis with TRIzol® Reagent, as well as the addition of chloroform, the mixture was not incubated. During the isopropanol precipitation, 0.4ml of isopropanol were used per 1 ml of TRIzol® Reagent used for the initial homogenization. The mixture was not incubated with isopropanol and was centrifuged for 30 minutes in place of the 10 minutes outlined by the invitrogen protocol. The ethanol wash was carried out with 0.5ml 80% ethanol per 1ml of TRIzol® Reagent

used for the initial homogenization. The RNA was resuspended in RNAse-free water at room temperature.

3.2 mRNA Isolation

111μg total RNA dissolved in 500ul RNAse-free ddH20 for use in the protocol for small-scale mRNA isolation: PolyATract® Systems III and IV as outlined by the Promega Corporation with two procedural changes. During the elution step, each resuspension of the mixture in water was heated to 65°C for 1-2 minutes in order to facilitate the release of mRNA from the beads.

3.3 Characterization of the 5' end of the transcript

The 5' end of the *D. virilis* INR transcript was characterized using cap-trapped cDNA to carry out 5' RACE.

3.3a cDNA synthesis for cap-trapping

cDNA was synthesized from mRNA utilizing a modified protocol with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, California, USA). After mixing mRNA, annealing buffer, and a gene specific primer, MB14, as per protocol, the sample was incubated at 90°C for 1 minute, followed by a temperature gradient of 0.1°C/s to 74°C and incubated for ten minutes. Following this incubation was a temperature gradient of 0.1°C/s to 55°C when the 2X reaction mix and enzyme mix was added as outlined by the Invitrogen protocol. A 1 hour incubation was then carried out at 55°C in place of the 50 minute incubation at 50°C recommended in the protocol.

3.3b Biotinylation

For biotinylation of the diol groups of the RNA hybridized to the cDNA synthesized as outlined above, 80μl ddH2O, 4μl 0.5 M EDTA pH 8.0, and 8μl 5 M NaCl

were added to the cDNA synthesis reaction mixture prior to the addition of 100µl of phenol chloroform isoamyl alcohol (PCI) for the first PCI extraction. 80µl of the aqueous phase was removed and to this, 80µl of 1X phosphate buffered saline, 50mM Tris pH 7.5, and 80µl of PCI were added for a second PCI extraction. 320µl (2 volumes) of ethanol were added for an ethanol precipitation. The pellet was resuspended in 25µl of 66mM sodium acetate buffer pH 4.5, 5nM NaIO4 as outlined in the procedure for biotinylation of diol groups of RNA (Carninci et al., 1996). Changes to the biotynilation protocol include an oxidation on ice for 60 minutes in place of 45 minutes, a 3 hour and 10 minute incubation of cDNA/mRNA hybrid at -20°C in place of a 30 minute incubation, followed by a centrifugation at 15,000rpm for 30 minutes instead of 15minutes and an 80% ethanol wash in place of a 70% ethanol wash. After the overnight incubation at room temperature, the sample was precipitated as per protocol, but there was no incubation on ice before being pelleted by centrifugation and no ethanol washes were performed. Also, the pellet was not reswuspended in water, but in 16µl 10X Ambion RNAse structure buffer and 144µl ddH2O.

3.3c RNase Treatment

To the 160µl suspension containing the cDNA, 1.5µl of T1 RNase and 1.5µl of RNase A were added. This mixture was incubated at 37°C for a minimum of 30 minutes.

3.3d Capture and elution of capped RNA

Blocking of 100µl of Promega streptavidin MagneSphere® paramagnetic particles was performed by adding 80µl of yeast tRNA at 5mg/ml and incubating the mixture on ice for at least 1 hour. The beads were then washed using the magnetic stand for capture 3 times with 500µl of 2M NaCl, 50mM EDTA pH 8.0 containing1mg/ml

tRNA. At this point, the RNAse-treated cDNA was added to the blocked beads and incubated at room temperature for at least 1 hour with mixing. Beads were washed 4 times with 500µl 2M NaCl, 50mM EDTA pH 8.0 containing1mg/ml tRNA and once with 500µl RNase buffer (NEB, Massachusetts, USA). The beads were then resuspended in 100µl RNase H buffer before the addition of 1µl RNase H (NEB, Massachusetts, USA) and placed at 37°C for 30 minutes to 1 hour with mixing every 5 minutes.

The supernatant was removed and saved. 100µl 100mM NaOH, 5mM EDTA pH 8.0 were used to resuspend the beads which were then incubated at 65°C for 10 to 15 minutes. This supernatant was then pooled with the previous supernatant. 1 volume of 2M Tris pH 7.0 (220µl) was added to neutralize the supernatants.

3.3e Qiagen® column purification

2ml PB (5M guanidine hydrochloride, 30% isopropanol) were added to the supernatant pool which was then passed over, washed, and dried on a QIAprep® spin miniprep (Qiagen, California, USA) purification column as per protocol. The cDNA was eluted in 30µl 10mM Tris pH 8.5.

3.3f c-tailing of cap-trapped cDNA

A reaction mixture containing 2.5 µl 10X NEB buffer #4 (NEB, Massachusetts, USA), 1.0 µl 2.5mM CoCl2, 2.5 µl 2mM dCTPs, and 18.0µl of the column-purified cap-trapped cDNA mixture was incubated at 94°C for 2 minutes and then placed at 0°C for the addition of 1.0 µl terminal deoxynucleotidyl transferase (TdT). The 25µl reaction was incubated at 37°C for 30 minutes followed by 10 minutes at 65°C.

3.3g First PCR

A 50µl PCR reaction containing 5.0µl 10X ThermoPol buffer, 1.0µl 10mM dNTPs,

34.5µl ddH20, 5.0µl of the c-tailed cDNA reaction mixture, 2.0µl of a 5' RACE Anchor primer at 10µM, 2.0µl of a gene specific primer, MB231, at 10 µM and 0.5µl of a 1:100 Pfu:Taq mixture. The reaction was incubated at 94°C for 2 minutes, followed by ten cycles of an incubation at 94°C for 15 seconds, 61°C for 30 seconds, and 72°C for 3 minutes. 31 additional cycles were carried out with incubation at 94°C for 15 seconds, 61°C for 30 seconds, and 72°C incubations which began with a length of 3 minutes for the first cycle followed by subsequent incubations with the addition of 10 seconds per cycle.

3.3h Second PCR

A 50µl PCR reaction containing 5.0µl 10X Thermopol buffer, 1.0µl 10mM dNTPs, 34.5µl ddH20, 5.0µl of a 1:100 dilution of the first PCR product in TE 10-1 buffer pH 8.0, 2.0µl of an abridged universal amplification primer (AUAP) at 10µM, 2.0µl of a gene specific primer, MB261, at 10µM and 0.5µl of a 1:100 Pfu:Taq mixture. The reaction was incubated at 94°C for 2 minutes, followed by ten cycles of an incubation at 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 3 minutes. 31 additional cycles were carried out with incubation at 94°C for 15 seconds, 58°C for 30 seconds, and 72°C incubations which began with a length of 3 minutes for the first cycle followed by subsequent incubations with the addition of 10 seconds per cycle.

3.3i Agarose gel electrophoresis

15 µl of PCR products were analyzed using 1.2% agarose gels containing EtBr at 0.5mg/ml. EtBr was also added to the 1X TBE running buffer at 0.5 mg/ml.

3.3j Column purification of second PCR product

500µl PB (5M guanidine hydrochloride, 30% isopropanol) were added to 35µl of the

second PCR product and passed over a QIAprep® spin miniprep (Qiagen, California, USA) purification column. The column was washed twice with 650 µl PE (80% ethanol, 10mM Tris pH 7.5) and centrifuged for 1 minute to dry the column. The PCR product was eluted in 30µl 10mM TE 10-1 buffer pH 8.0.

3.3k Cloning second PCR product

The second PCR product was cloned using the TOPO®-TA cloning kit (Invitrogen, California, USA) and chemically competent DH5-α cells. Transformed cells were selected for using kanamycin in 2YT media agar plates and in 5ml LB overnight cultures. Plasmids were purified using QIAprep® spin miniprep (Qiagen, California, USA) purification methods. Following 20µl digests with EcoR1 to verify the presence of an insert into the cloning vector, selected plasmids were sequenced.

3.4 Cloning the full-length 5' UTR

The full-length 5' *D. virilis* 5' UTR was amplified and cloned using a PCR reaction with forward and reverse primers designed to amplify the entire UTR and add NcoI cut sites to the sequence. The cDNA used for template was synthesized using a gene specific primer.

3.4a cDNA synthesis

cDNA was synthesized from mRNA utilizing a modified protocol with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, California, USA) . After mixing mRNA, annealing buffer, and a gene specific primer MB231 as per protocol, the sample was then incubated at 90°C for 1 minute, followed by a temperature gradient of 0.1°C/s to 61°C and incubated for ten minutes. Following this incubation was a temperature gradient of 0.1°C/s to 55°C when the 2X reaction mix and enzyme mix were

added as outlined by the Invitrogen protocol. A 1 hour incubation was then carried out at 55°C in place of the 50 minute incubation at 50°C recommended in the protocol.

3.4b PCR for the full-length 5' UTR

A 50µl PCR reaction containing 5.0µl 10X ThermoPol buffer, 1.0µl 10mM dNTPs, 34.5µl ddH20, 5.0µl of the cDNA reaction outlined above, 2.0µl of a forward primer (MB287) at 10µM, 2.0µl of a reverse primer (MB288) at 10 µM and 0.5µl of a 1:100 Pfu:Taq mixture. The reaction was incubated at 94°C for 2 minutes, followed by ten cycles of an incubation at 94°C for 15 seconds, 61°C for 30 seconds, and 72°C for 3 minutes. 31 additional cycles were carried out with incubation at 94°C for 15 seconds, 61°C for 30 seconds, and 72°C incubations which began with a length of 3 minutes for the first cycle followed by subsequent incubations with the addition of 10 seconds per cycle.

3.4c Agarose gel purification of PCR product

35 µl of the PCR product described above was run on a 1.2% agarose gel containing EtBr at 0.5mg/ml. EtBr was also added to the 1X TBE running buffer at 0.5 mg/ml. A product band located at approximately 1,300bp was removed and gel purified using the Wizard® SV gel and PCR clean-up system (Promega, WI, USA) as per protocol. The product was eluted from the column in a volume of 30µl RNase-free water.

3.4d Cloning the gel-purified PCR product

The gel-purified PCR product was cloned using the TOPO®-TA cloning kit (Invitrogen, California, USA) and chemically competent DH5-α cells. Transformed cells were selected for using kanamycin in 2YT media agar plates and in 5ml LB overnight cultures. Plasmids were purified using QIAprep® spin miniprep (Qiagen, California,

USA) purification methods. Following 20µl digests with EcoR1 to verify the presence of an insert in the cloning vector, as well as 20µl digests with NcoI to confirm the addition of the restriction site via the primers used in the PCR (MB288 and MB287), selected plasmids were sequenced.

3.4e Ligation of the 5' UTR into pMARL vector for assay

6µg of the pMARL-2F plasmid were digested with NcoI for removal of the 2F insert. Calf intestinal phosphatase (CIP) was added to the digest reaction and incubated at 37°C for at least one hour. Plasmid containing the 5' UTR band gel purified and cloned as described above was also digested with NcoI. The pMARL backbone as well as the 1,300bp 5' UTR insert were then gel purified on a 0.75% agarose gel using the Wizard® SV gel and PCR clean-up system (Promega, WI, USA) with a protocol modification. Both of the dissolved gel slices were passed over the same purification column prior to the washes and eluted together in 50µl ddH2O. 18µl of this purified elution were used in a 21µl T4 ligase reaction incubated at room temperature for at least one hour.

3.4f Cloning the assay plasmids

2µl of the ligation reaction described above were transformed into chemically competent cells provided in the TOPO®-TA cloning kit (Invitrogen, California, USA). Transformed cells were selected for using ampicillin in 2YT media agar plates and in 5ml LB overnight cultures. Plasmids were purified using QIAprep® spin miniprep (Qiagen, California, USA) purification methods. Plasmids were then digested with NheI for determination of insert directionality as well as insert presence.

Primer Name	Primer Sequence
MB35	5' CACGCACGATTGCTCCAGCAGTAGCGC 3'
MB36	5' GAGTCAGCTGGCGAACTGTACG 3'
MB43	5' GGGTACCACGACGGGTTCGCTCAGGACTGGCCC 3'
MB44	5' CCCCGGGATGCACCCAGGAGGGCGGCGAG 3'
MB14	5' CGATGATCGTACAGTTCGCCAGCTG 3'
Anchor primer	5' GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG 3'
MB231	5' ACGCCGAAGACAACAAATACAGCCAC 3'
AUAP	5' GGCCACGCGTCGACTAGTACG 3'
MB261	5'GCGAACACTTGTCGGCATACAAC
MB231	5'ACGCCGAAGACAACAAATACAGCCAC 3'
MB287	5' TGTGCTGCCGCTCTCTCGCTCGCCTCACCAA 3'
MB288	5' GTTTTTGCACAAACAGAAATAACAATTGCAGTGTGGC 3'
MB12	5' GTATAACGCTCAAATTGGGAAAGATTTGCG 3'
MB13	5' ACGATATCAGATTATGTACACG 3'

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Appendix

Cloning *D. virilis* FOXO

Results and Discussion

In hopes that a higher concentration of the targeted INR transcript would improve 5' RACE conditions, the transcription factor, FOXO was cloned for transfection of *D. virilis* cells. Forward and reverse primers were designed to amplify the FOXO homolog in *D. virilis* (See figure 10). Endogenous Hsp70 was found to be a major contaminant in the PCR reaction carried out to amplify FOXO. The lower band around 1,100bp visible in lane 2 was cloned,

Figure 10. A 1.2% agarose gel containing EtBr at 0.5mg/ml. Lane 1 contains a 1kb plus DNA ladder. Lane 2 contains the product of a PCR for FOXO carried out with primers MB43 and MB44.

5'ATGCACCCAGGAGGGCGGCGAGGTGACCACGCTGGGCTGTGAGTACTGGACGCGGGCGTTCAGATTTGCAGTCGCC
GTTTGTGTGGCCAGTTCCAAGCTGTTGTTGCTATTGTTGTTGTTGTTATTGTTGTTATTGTTGCTCATCAGCAAC
TGTTGCTGATGCTGTTGTTGTTGCTGTTGCTGTTGTTGTTGCTGCTGCAATTGCAGCTGAGCCTGCAACTGATTGAGC
TGAACACCTGCGCTGCTGTTGGAGGCGGCATTGCTGCTGCCGCTGTTTGTACTATTATTGGTTAAGATCACATTGTTG
GTATTCGTGTTGACCGCCGCGAGCGGTATATTAATGTCCAGCAAGCCGTCGTATCGCATCTCCTGCTGCAGCAGCTCC
TCGACGTTGCACTCGAGTCCACCCTGAAAGTCCTCCAGATTAAATTCGTCAATTTGCTCGCTGTTGAGCGCCTCCAGG
CATTGCCCCTCCAGACCTGAGGACAGCTGCTGCCTCTGCTGTTGCTGTTGCTGTTGTTGCTGTTGCTGATGCTGGACA
AGAAGTGCAGCATTATCCGCAGAGCCATCTAGCAGGACGTTGCTGTATGTGTTCAGGGAGTCGGGGCGCTGTGCAGGCAG
TTGGGATACGCAGGCGACATTGTGGTGGTAACTGAGTTGGGCGACAGGCCATCTCGCACTGGGGCGCTGTGCAGGCAG
CCGCAGTTGAGCGAGCGATGCAGCAGGCATTGGCCCTGCCCCTGTGGCTGTGGCTGCGGCTGCATTGCGGCATATCCT
GGTCCATTCAGGGAATAGCCCTGTGGAAGCTGCGGCTGCTGCGGCGGCTGATAGGGCGGCGGCGGCTGCGTCGGCAGG
CCAGATGCGGCGCTGAACCCTTGCTGCTGCAACATGTCGCTCTGGAGTTTCAGCTCTTCGGCCATGGAGCCGGCCAGC
TGGTCAAGCTGTTGGGCCTGTGCCTGCGTCATTGTCGTGTTCTGATAGTCAACCGAGAAGCCCCAGTCGGGCTCGAGA
TCCAATGCCCGTATGGGACTCAGACGGCCGCAGGAGCTGGCATTCGATGAGGCGCGCTGACGAAAATCGGGCGACAAT
TGAAAGCCGCCGCTGTGCAGCGGACTCTCTGGAAAGTGATCCAGCCCCTCGCTGACGCTGCTGCTCGGCGAGGGCGTG
GCATCATTGAGACCGACAACGCCGCCCTGGCGCAAAGCCTCGACACGCTTCTTGGCCCGTCCGCGGCGCTTCTCGTAG
CGCGATGTCTCCATGGAGGCGGCACGACGTCGCACCGATTTGCCAGGCTTAGCCTCCGGGTTGAGCATCCACCAGGAG
GACTTGCCGGTGCCCTCGTTCTGCACGCGCATAAACCGATTGTGCAGCGATAGATTGTGCCGAATGGAGTTCTTCCAG
CCAGCACTGCTATTCGAGTCGCCTTTATCCTTAAAGTAGGACACGTTTTGGACCATCCACTCGTAGATTTGGCTCAGC
GTTAGACGCTTGTCGGTGGCGGAGCCTATGGCGTGCGTGATGAGATCGGCATAGGACAGATTGCCCCATGCGTTGCGA
CGGGATGAGTTCTTTTTGGCCGCATTCGCATTCTGCATGGCCTGCTGTGGATCGCCGCTGGCCAGCTGCTGATTGCTA
GCCTTGGTACTGTCCAGCTCATCGACCGGCTCCACAAAGTTCTCAGGCCGTGGACATGGCCACGTGTTGGAGCGGGCG
CGCGTCTGCGGCTCGAAGCCGCCATCTGTTGGCAGCTCGCCGACCAGCTGATCCATATGCGCCCGTTGTCCGACCGAG
GCAAGTTGGGCCAGTCCTGAGCGAACCCGTC3'

Figure 11. Sequence data for D. virilis FOXO

sequenced and found to be closely related by sequence to Hsp70 sequences across several species of *Drosophila* genomes. The lower band around 1,100bp visible in lane 2 is PCR contamination encoding portions of Hsp70. The larger band located at approximately 1,800bp was cloned, sequenced and found to be the desired FOXO product (See figure 11). After cloning the PCR product, the sequence data acquired most closely matched other *Drosophila* genomic sequence data for Foxo proteins. The amino acid sequence for *D. virlis* FOXO is largely conserved with respect to *D. melanogaster* (See figure 12). Transfecting *D. virilis* cells with FOXO prior to collecting RNA for use in 5' RACE did not improve reaction conditions.

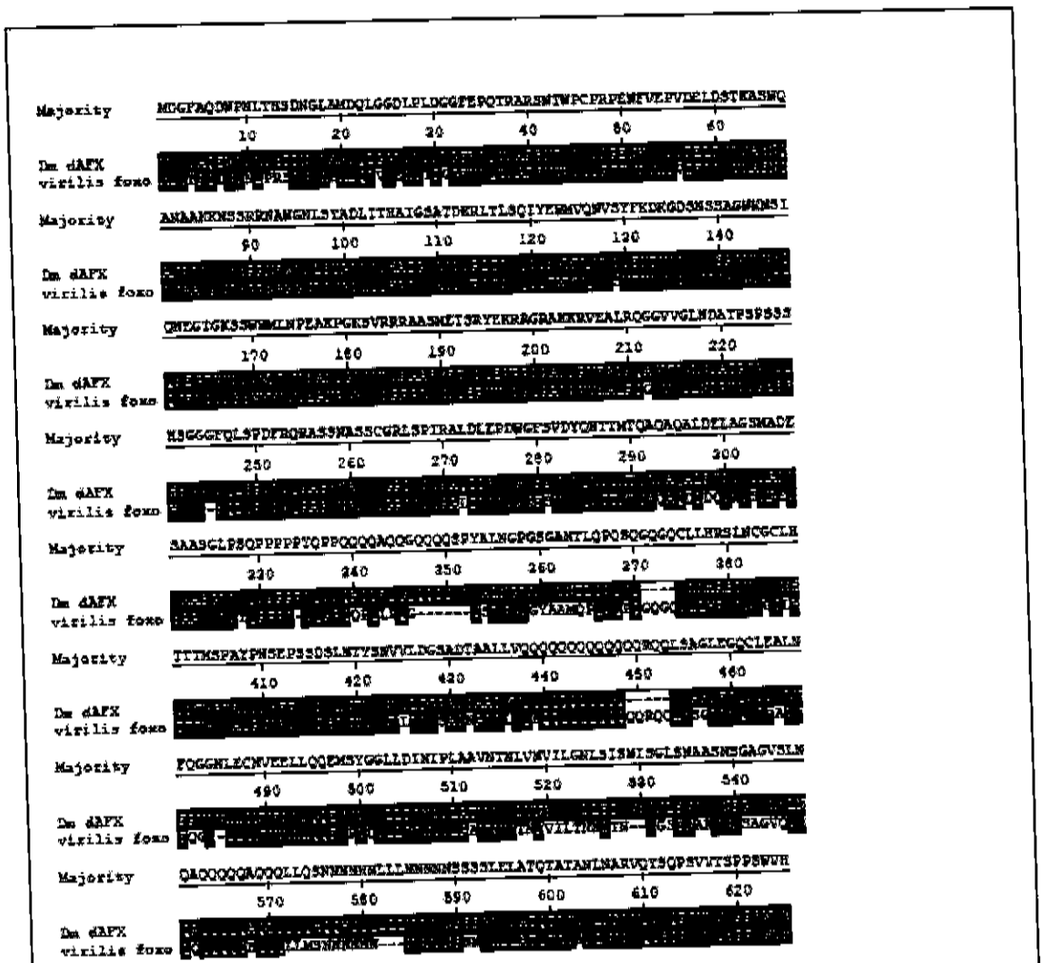

Figure 12. Amino acid sequence alignment of *D. virilis* and *D. melanogaster*

Materials and Methods

mRNA Isolation

332µg total RNA was brought to 500ul total volume in RNAse-free ddH20 for use in the protocol for small-scale mRNA isolation: PolyATract® Systems III and IV as outlined by the Promega Corporation with two procedural changes. During the elution step, each resuspension of the mixture in water was heated to 65°C for 1-2 minutes in order to facilitate the release of mRNA from the beads.

cDNA Synthesis

cDNA was synthesized from mRNA utilizing a modified protocol with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, California, USA). After mixing mRNA, random hexamers, and annealing buffer as per protocol, the sample was incubated at 90°C for 1 minute, followed by a temperature gradient of 0.1°C/s to 72°C and incubated for ten minutes. Following this incubation was a temperature gradient of 0.1°C/s to 55°C when the 2X reaction mix and enzyme mix was added as outlined by the Invitrogen protocol. A 1 hour incubation was then carried out at 55°C in place of the 50 minute incubation at 50°C recommended in the protocol.

RNase Treatment and Column Purification

The cDNA reaction was then treated with RNAses A and H at 37°C for 30 minutes. 500µl PB (5M guanidine hydrochloride, 30% isopropanol) were added to the mixture and passed over a QIAprep® spin miniprep (Qiagen, California, USA) purification column. The column was washed twice with 650 µl PE (80% ethanol, 10mM Tris pH 7.5) and centrifuged for 1 minute to dry the column. The cDNA was eluted in 30µl 10mM Tris pH 8.0

PCR for D. virilis FOXO

Using the cDNA described above as template, a PCR reaction with a forward primer, MB43, and reverse primer, MB44, was carried out with Pfu to amplify the D. virilis Foxo transcript. The PCR program used included a 1 minute incubation at 95°C followed by 30 cycles of an incubation at 95°C for 15 seconds and at 75°C for 5 minutes. 15 µl of the PCR product was analyzed using a 1.2% agarose gel containing EtBr at 0.5mg/ml.

Agarose gel purification of FOXO PCR product

85 µl of the PCR product described above was run on a 1.2% agarose gel containing EtBr at 0.5mg/ml. A product band located at approximately 1,800bp was removed and gel purified using the Wizard® SV gel and PCR clean-up system (Promega, WI, USA) as per protocol. The product was eluted from the column in a volume of 30µl RNase-free water.

Cloning FOXO

The gel-purified product was cloned using the TOPO®-Blunt cloning kit (Invitrogen, California, USA) and chemically competent DH5-α cells. Transformed colonies were identified by colony PCR with T3 and T7 primers to amplify the inserts in the TOPO® vector. Colonies containing the inserts were grown and selected for using kanamycin in 2YT media agar plates and in 5ml LB overnight cultures. Plasmids were purified using QIAprep® spin miniprep (Qiagen, California, USA) purification methods. Plasmids were digested with EcoR1 to verify the presence of an insert before being sequenced.

Preparation of purified plasmid containing FOXO

5µg of TOPO® vector containing the FOXO insert and 5µg of a control plasmid pFLAGMTF-f1 were digested with KpnI and XmaI at 37°C for a minimum of 30 minutes. Digests were run on a 1.2% agarose gel for purification. The pFLAGMTF backbone as well as the FOXO insert were gel purified using the Wizard® SV gel and PCR clean-up system (Promega, WI, USA) as per protocol. A 20µl T4 DNA ligase reaction containing equal volumes of gel-purified backbone and insert was then carried out at room temperature for at least 1 hour. 4µl of the ligation reaction described above were used to

transform 100µl of chemically competent DH5-α cells. Transformants were selected for using ampicillin in 2YT agar media plates and 5mL 2YT liquid cultures. Plasmids were purified using QIAprep® spin miniprep (Qiagen, California, USA) purification methods. Plasmids were digested with XmaI and KpnI to confirm the presence of both insert and backbone. A positive colony was then used to inoculate a 100ml 2YT culture with ampicillin. This culture was used to collect purified plasmid, pKN157-2 utilizing a QIAGEN® Plasmid Midi Kit as per protocol (Qiagen, California, USA).

3.2 Transfection of WRDV-1 Cells with FOXO

12 ml of WRDV-1 cells at $0.5x10^6$ cells/ml were added to 2 10cm plates and allowed to plate down for 30 minutes to 1 hour. One plate was transfected with 6µg of plasmid pKN157-2 containing the FOXO insert ligated into the pFLAGMTF backbone using the Effectene® Transfection Reagent as per protocol (Qiagen, California, USA). The second plate was transfected using 6µg of plasmid pFLAGMTF-f1 using the Effectene® Transfection Reagent as per protocol (Qiagen, California, USA).

RNA isolation

RNA from *Drosophila virilis* WRDV-1 cells transfected with pKN157-2 as described above was isolated using a modified invitrogen TRIzol® Reagent protocol. Following lysis with TRIzol® Reagent, and chloroform addition, the mixture was not incubated. During the isopropanol precipitation, 0.4ml of isopropanol were used per 1 ml of TRIzol® Reagent used for the initial homogenization. The mixture was not incubated with isopropanol and was centrifuged for 30 minutes in place of the 10 minutes outlined by the invitrogen protocol. The ethanol wash was carried out with 0.5ml 80% ethanol per

1ml of TRIzol® Reagent used for the initial homogenization. The RNA was resuspended in 100µl of RNAse-free water at room temperature.