EFFECTIVE TRANSLATION OF THE SECOND CISTRON IN TWO DROSOPHILA DICISTRONIC TRANSCRIPTS IS DETERMINED BY THE ABSENCE OF IN-FRAME AUG CODONS IN THE FIRST CISTRON.

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The novel dicistronic transcript encoded by the Drosophila melanogaster stoned gene was recognised as being unusual in that the protein encoded by the first open reading frame, stoned-A (STNA), contains no internal methionine residues in a protein of 93kDa. The dicistronic nature of the stoned locus and the lack of methionine residues in STNA is conserved across dipteran species. A second methionine-free cistron, encoding Snapin, was identified in Drosophila and also found to be dicistronic, the second ORF encoding a methyltransferase. We have replaced the methyltransferase cistron with GFP and used this dicistronic construct to show that the GFP cistron is translated in Drosophila S2 cells. The insertion of in-frame AUG codons into the snapin ORF attenuates the translation of GFP, and the level of attenuation correlates with the number of inserted AUGs. Increasing the efficiency of translation-initiation of the Snapin cistron also attenuates the translation of GFP. This indicates that failure to initiate translation at the first AUG allows ribosomes to scan through the Snapin ORF and to initiate translation of the second cistron, unless new AUG codons are inserted. These data are used to interpret the expression of the stoned locus and in particular, to explain the altered stoned protein levels in the stoned-temperature-sensitive mutant allele, which replaces a lysine with a methionine codon early in the first, stonedA, cistron.

Although not unknown in eukaryotes, true dicistronic mRNAs (as opposed to alternatively spliced mRNAs) are rare. Where they do occur, they seem to fall into two categories. The first is best exemplified by the situation in certain eukaryotic viruses where a single mRNA can produce several proteins (1, 2). In this case there exists internal ribosome entry sites (IRES's) in the mRNA, where the necessity of the m7G 5’ cap to initiate ribosome binding is obviated (3). Hence two or more translational products can be synthesised from a single mRNA. Cellular genes that do not contain the 5’ terminal cap but allow internal ribosome binding in the 5’ leader sequence have been identified (4), and a non-viral dicistronic mRNA, with proposed IRES’s between the cistrons, has been recognised as an alternative transcript from the Adh locus in Drosophila (5). While the 1st ORF encodes the ADH protein, the function of the protein produced from the 2nd ORF (Adhr) is unknown (5), and so the evolutionary rationale for arranging two genes in this way is not clear. A screen of the Drosophila genome data-bases for possible dicistronic mRNAs yielded a number of single transcripts encoding two ORFs. Many were like the Adh locus with at least the possibility that IRES sequences might be present. Among these loci, some gave rise to alternative spliced mRNA variants with one monocistronic and another dicistronic, still other dicistronic loci produced a single transcript.

A possible mechanism for translating IRES-deficient dicistronic transcripts, involves the scanning of the ribosome from the termination signal at the end of the first ORF and the reinitiation of translation at the beginning of the second ORF. The best studied of this type of mRNA is represented by the GCN4 gene of S. Cerevisiae (6). In this case four small (3-4 codons) ORFs (uORFs), upstream of the structural gene, translationally regulate the expression of the main ORF in response to amino acid starvation (7, 8). It is proposed that this occurs via the starvation-induced phosphorylation of the translation initiation factor eIF2, making it unavailable as a substrate for the GDP/GTP exchange factor eIF2B. After translating the first uORF, it is presumed that the absence of an active eIF2.GTP.Met-tRNAi complex allows the 40S ribosomal subunit to scan through uORFs 2-4 but at the same time gives the 40S ribosome subunit time to recharge with eIF2.GTP.Met-tRNAt to allow reinitiation of translation at the AUG of the main ORF (7, 8). Although this proposed model is incomplete, there is
ample genetic evidence that the status of the eIF2 and other members of the initiation complex, play a role in regulating the reinitiation of translation at the main ORF (9). A similar combination of small uORFs has been shown to regulate the translation of mammalian ATF4 in response to environmental stress (10). However it has been proposed that the ability to reinitiate translation downstream of uORFs is dependent on the small size of these ORFs and, were there to be a large uORF, reinitiation at the 2nd ORF AUG would be considerably attenuated, if indeed it would happen at all (11, 12).

A third possible mechanism for translation of the second ORF in dicistronic mRNAs is “context-dependent leaky scanning” (13) where the AUG of the first ORF is in a sub-optimal configuration and so a population of ribosomes fail to initiate at this AUG and continue scanning the mRNA to initiate translation at the AUG of the second ORF. However there are major constraints on this method of translating downstream ORFs (13).

There are a few eukaryotic examples where the mechanism of translation of the second ORF is still in doubt. One of these is the stoned (stn) locus from Drosophila melanogaster (14). The stoned locus encodes a single transcript of 8.4kb, and when cDNAs corresponding to this transcript were isolated, it was observed that they included two tandemly arranged ORFs of 2.5 and 3.7 kb (known as stnA and stnB) with a 55bp intercistronic region (ICR) (14). Both ORFs have been shown to be translated in vivo (14). Structurally the stoned proteins (STNA and STNB) were novel, although subsequently genes corresponding to STNB homologues have been identified in mammals (15, 16) and C.elegans. There are no equivalents of the STNA gene upstream of the STNB cistrons in either C. elegans or mammalian STNB ORFs. One notable feature of the D. melanogaster STNA protein is its lack of internal methionine residues. In a protein of 95kDa, this is unusual. Here we report the comparison of the stoned locus from a number of insect species and the identification of another D. melanogaster dicistronic locus where the first ORF contains no in-frame AUG codons. We use this second locus to investigate the effect of in-frame AUG codons on the translation of a second ORF and conclude that the absence of in-frame AUG codons in the first ORF is essential for efficient translation of downstream ORFs. This data is then used to interpret the phenotype associated with a mutation at the stoned locus that inserts a methionine in place of a lysine at position 35 in the stnA ORF.

METHODS AND MATERIALS

Generating the Snapin::GFP dicistronic constructs.

The Snapin ORF was isolated by PCR from Drosophila melanogaster genomic DNA using the oligonucleotides 5′GAATTCTGCAATGGATTCGG3′ and 5′TCATGACGAACAGTAATTG3′. These oligonucleotides included the ATGs that initiate the Snapin and methyl transferase cistrons and create a 5′ EcoRI site and a 3′ BspHI site. This fragment was then cloned into EcoRI and Ncol cut pALX-190 (a gift from Alex Andrianopolous, University of Melbourne and consisting of the GFP ORF cloned into the Bluescript (Stratagene) vector) to generate pALX-190:AW. The dicistronic fragment was then removed from pALX190 using EcoRI and XbaI and cloned into the Drosophila expression vector pAc5.1/V5-HisA (Invitrogen) to produce plasmid pAcD1. All further manipulations were carried out in pALX-190:AW and then cloned into pAc5.1. The Snapin was tagged with a myc epitope by inserting annealed oligonucleotides 5′AGACAGAAACTTATTTCTGAAGAAGATCTTGAGC 3′ and 5′CAAGATCTTCTTTGGAGAAATTAAGTTTCTTCTTGCA3′ into PstI cut pALX-190:AW and subcloned into pAc 5.1 to produce pAcD2. This places the myc tag at position +150bp in the Snapin ORF. An in-frame ATG codon was inserted along with the myc tag using the same site and annealed oligonucleotides 5′ATGGAACAGAAACTTATTTCTGAAGAAGATCTTGAGC 3′ and 5′CAAGATCTTCTTTGGAGAAATTAAGTTTCTTCTTGCA3′ to produce pAcD3. The pAcD4 clone containing an inserted ATG at position 338, was produced from pAcD2 by inserting annealed oligonucleotides 5′GTACGATGAAGCTTATGGCC3′ and 5′GTACGATGAAGCTTATGGCC3′ to produce pAcD4. The pAcD5 clone containing both ATG150 and ATG338 was constructed by inserting the same two oligonucleotides into the BstEII site of pAcD3. A third ATG codon was inserted again at the BstEII site of pAcD3 using annealed oligonucleotides 5′GCAGGTCTGAGGAGGGCCATAGGCATGCTTATGGGC 3′ and 5′GTACGATGAAGCTTATGGCC3′ to produce pAcD6. The GFP in all of these constructs was then myc-tagged by cutting the pAcD2-D6 constructs with BspEI and XbaI and inserting annealed oligonucleotides: 5′CCGGTCCAGGAAACAGAAAACCTATTTCTTCTGAGC 3′
5’CCGTCTCGAGGAACAGAAACTTATTTCT  
G A A G A A G A T C T T A 3 ’  
and  
5’CTAGTAAGATCTTA3’ and  
5’CTAGTAAGATCTTA3’ thus creating a novel XhoI site at the 3’ end of the GFP ORF. To create the “optimised” version of pAcD2 and pAcD3, these two constructs were used as templates for PCR reactions using oligonucleotides:  
5’GAATTCAAAATGGATTCGGACAGC3’ and  
5’GGATCCTCTTCAGAAATAAGT3’. This replaces the normal 5’ TGCAATGG 3’ Snapin translation initiation site with 5’CAAAATGG 3’.

The PCR fragment was then cut with EcoRI and BamHI and used to replace the EcoRI (1) - Bgl II (190) fragment in pAcD2 and pAcD3 to produce pAcD2(Opt) and pAcD3(Opt) respectively. The composition of all constructs was confirmed by sequencing. A monocistronic GFP in pAc5.1 was prepared by removing the EcoRI/XbaI fragment from pALX-190 and inserting it directly into pAc5.1 to produce pAcM1.

The stnA::GFP fusion constructs were created by PCR from either wild-type or stn-ts genomic DNA. The constructs that included the small upstream ORF were generated using oligonucleotides  
5’GTGGTACCAACTATTTGCAGACC 3’ and  
5’GGATGCTGCAGGATGATCC 3’, creating a 5’KpnI and a 3’PstI site. These fragments were cloned into pAc5.1 DNA followed by the insertion of the PstI-XbaI fragment from pALX-190, containing GFP in-frame. The version of STNA without the small uORF was constructed by using sequential rounds of PCR with wild-type genomic DNA. First a PCR fragment was generated using oligonucleotides  
5’GACCAATCTCGGTGCCTCCGC 3’ and the same 3’ oligonucleotide described above. This fragment was then used as a template with  
5’GACCAATCTCGGTACCTCCGC 3’ and the same 3’ oligonucleotide. This again generated a PCR fragment with 5’KpnI and 3’PstI sites. The remainder of the construction was as described above.

All DNA for transfection was prepared using Qiagen Midi-prep DNA purification system according to the manufacturer’s protocol.

**Transfections**

*D. melanogaster* S2 cells were grown in GIBCO Schneider’s medium supplemented with 10% fetal calf serum, 100units/ml penicillin and 100 µg/ml streptomycin at 22°C. For transfection, cells (40-60% confluent) were washed in fresh medium and

Effectene (10µl) and Enhancer (8µl) (Qiagen) and DNA (1µg) was added to give a final volume of 2.6ml of culture as per the manufacturer’s instructions www.qiagen.com/transfectiontools/cell_list/default.asp. All cell cultures were incubated with the Effectene/DNA for 72 hours, unless otherwise stated, before being assayed for GFP fluorescence. Because the transfection efficiency varied between cultures, the lysates were initially subjected to Western blotting and the blots used to quantify the levels of Snapin or STNA. Blots were then re-run with loadings that approximately equalised the amounts of Snapin/STNA. Only the ratios of proteins within a transfection are used for comparative analysis.

**Immunohistochemistry.**

Cells were fixed using Histochoice (Astral Scientific). Rabbit anti-GFP antibodies (Chemicon) and monoclonal anti-c-Myc (Sigma-Aldrich) were used at 1/50 dilution, and secondary antibodies Alexa594 conjugated anti-rabbit and Alexa488 conjugated anti-mouse (Molecular Probes) were used at 1/250 dilution. Images were captured using an Olympus inverted microscope with an Olympus CCD camera. Comparative GFP fluorescing images were taken at the same aperture and exposure time with the lowest fluorescent transfection being used as the baseline for generating a clear image of individual cells. Negative images were generated using Adobe Photoshop.

**Fly stocks and crosses.**

All *Drosophila* stocks were maintained at 22°C in a 12 hour light/dark cycle. Flies for the preparation of head extracts were all 4 days post-eclosion. The “wild-type” used for all the studies was the white-eyed *w*1118 strain obtained from the Bloomington stock center. The *stoned* alleles *stn*-α, *stn*-c and *stn*-13-120 have been described previously (17, 18). All of the *stoned* strains had the white eyed mutation crossed into them. The *stn*-13-120 allele is embryonic lethal and is maintained as a heterozygote balanced over FM7. The *stn*-c/*stn*-13-120 heterozygotes were generated by crossing *stn*-13-120/FM7 females with *stn*-c males.

**Western blots.**

Harvested S2 cells were lysed by boiling directly in SDS sample buffer, and loaded on to 15% SDS gels. For *Drosophila* extracts, flies were lightly anaesthetised by placing on ice. Their heads were then removed using a scalpel and rapidly homogenised in SDS sample buffer, boiled, and centrifuged at 5000g for 10 minutes
and loaded on to 7% SDS gels. The sample buffer for fly heads contained no reducing agent.
Western blots were stained with 0.2% Ponceau S (Sigma-Aldrich) in 3% TCA and the stained blots scanned to ensure equivalent loading of material. The blots were then probed with either anti-STNA antibodies (14) at 1/20,000, anti-STNB antibodies (19) at 1/5000, anti-myC (Sigma-Aldrich) at 1/500, or anti-GFP (Chemicon) at 1/500. Secondary antibodies, anti-mouse or anti-rabbit, labelled with HRP were used, and the blots were developed using ECL (Pharmacia Biotech). Quantitation of the blots was performed by scanning the Ponceau stained blots and the ECL films, and using MacBas v2.1 (Fuji Photo) to determine the pixel intensity of the area of the band.

Identification of stoned homologous sequences in other organisms.
The 3’ nucleotide sequence of the Drosophila melanogaster stnA ORF was used to BLAST against the NCBI trace archives of various species. Sequences detected in this way were then translated in all three reading frames and compared to the carboxy-terminal amino acid sequence of STNA and the amino-terminal sequence of STNB from D. melanogaster. Both of these amino acid sequences appear highly conserved across species. The intercistronic sequences were then extracted. Where possible, 5’ extensions of translation products from the contigs identified in these BLAST searches were then compared to the D. melanogaster STNA sequence to obtain amino acid sequence alignments. In this way the presumptive amino acid sequence of the STNA proteins were identified. Where it was possible to identify a complete STNA ORF, all but the Apis melifora STNA amino acid sequence were found to be free of internal in-frame AUG codons.

RESULTS.
Structural comparisons of two dicistronic transcripts in Drosophila and Anopheles species.
In the recently released Anopheles gambiae and Drosophila species genomes, we have identified equivalent genomic arrangements of STNA- and STNB-encoding ORFs. Although the sequences that flank the intercistronic regions (ICRs) are highly conserved, the ICRs themselves are entirely divergent (Fig. 1A), and range in length from 15bp (D. simulans) to 101bp (D. pseudoobscura). It would appear that the sequence of the ICR per se, is of little consequence. However, in all of the cases where the complete stnA ORF can be identified, no internal in-frame AUG codons are present. This is despite the fact that an amino acid comparison of the STNA proteins from D. melanogaster and A. gambiae show only 37% identity (Fig. 1B), and in evolutionary terms, it might be expected that there would be random mutations that substituted methionine for other residues that would be of little consequence to the overall structure and function of the STNA protein. The D. melanogaster STNA protein contains 30 leucine and 28 isoleucine residues that, with a single base change, could produce a methionine codon and a relatively conservative amino acid substitution. Hence, in a protein of some 850 amino acids, the likelihood that the absence of in-frame AUG codons evolved by chance seems low. The absence of internal methionine codons in the STNA ORF appears to be a highly selected property of STNA in these species. In contrast, the STNB proteins from D. melanogaster and A. gambiae show some five amino acid residues, in conserved regions, that are substitutions of leucine/isoleucine for methionine. This indicates that, over all, there is no evolutionary prejudice against methionine substitutions at the stoned locus.

In screening 100 proteins from various eukaryotic sources and of random size that were held in a local database of synaptic proteins, the average methionine content was found to be 2.5% with the range being from 1.2-5.5%. This average is consistent with expectations (20). However one protein was found that contained no internal methionine residues. Drosophila Snapin is an evolutionarily highly conserved protein, consisting of 134 amino acid residues, but containing no internal methionines. The mammalian Snapin (21) does contain internal methionines but at the lower end of the range of methionine content in the randomly selected proteins. This lack of methionines in D. melanogaster Snapin might therefore be a chance event. However, when the D. melanogaster Snapin transcript was investigated, it too was found to be dicistronic (Flybase CG32951), with the 2nd ORF containing a highly conserved methyl-transferase protein (MTr) (Flybase CG9960). The ICR between Snapin and the MTr is 21 base pairs long and there is no evidence of an IRES sequence (Fig. 1A).
The sequence flanking the Snapin/MTr ICR has no nucleotide sequence identity, nor nucleotide content similarity, to those surrounding the STNA/STNB ICR. It might be concluded that despite the similarity in ICR-adjacent sequences in the stoned genes, they are unlikely to play a role in translation-initiation at the 2nd
ORF. In contrast to the conserved adjacent location of the stoned ORFs, the Snapin and MTr genes in *A. gambiae* are found at very separate locations on chromosome 3. The *A. gambiae* Snapin protein also contains 3 internal methionine residues, showing that the absence of methionine residues is not a structural or functional requirement of this protein in insects.

**Both ORFs are translated in a Snapin/GFP dicistronic construct in Drosophila S2 cells.** In an initial series of experiments we constructed a dicistronic gene that included the complete Snapin ORF, the intercistronic region and initiating AUG of the 2nd ORF, but with the MTr cistron replaced by GFP. For this investigation, Snapin was chosen over STNA as both Snapin and GFP have similar molecular weights and would run in the same region on SDS-PAGE. This reduces the effect of differential transfer efficiency due to size when quantifying data on Western blots. The Snapin/GFP construct was then transfected into *Drosophila* S2 cells. As compared to a monocistronic GFP, there was a reduction in the fluorescence level in cells transfected with the dicistronic construct (data not shown), however the transfected cells still showed strong fluorescence, indicating that the GFP cistron was efficiently translated in those cells, with maximum fluorescence obtained at 72 hours post-transfection. To be able to determine the production of the Snapin protein in S2 cells, Snapin was tagged with a myc epitope inserted at the PstI site at nucleotide 150 in the Snapin ORF. The tagging had no effect on the level of fluorescence in cells transfected with this construct. Using double labelling of transfected cells, we concluded that the expression of GFP was restricted to those cells that were also expressing Snapin (Fig. 2A). However it was also apparent that while GFP was cytosolic, the Snapin protein appeared to localize to the plasma membrane (Fig. 2A). This necessitated the use of whole cell lysates rather than just cytosolic extracts in subsequent Western blot experiments.

We then tagged the GFP protein with the myc epitope (see Fig. 2B and Experimental Procedures). This allowed the use of the same anti-myc antibody to identify both GFP and Snapin on blots and to compare their stoichiometry. Western blots of cultures transfected with this construct showed that both GFP and Snapin were present and could be identified using the anti-myc antibody (Fig. 2C). A time course of expression of Snapin and GFP showed that GFP, as a function of total cell protein, was maximal at 72 hours post-transfection (Fig. 2D), and subsequently reduces as the cells continue to grow and the plasmid is diluted out or degraded. During this time course the expression levels of Snapin and GFP were also measured (Fig. 2E). The ratio of GFP to Snapin remained constant throughout the first 96 hours post-transfection, but at 168 hours there is a decrease in Snapin levels, indicating that it is less stable than GFP in S2 cells over extended periods. It is interesting that the stoichiometry of Snapin to GFP was always in favor of GFP and that the ratio of GFP/Snapin did not vary significantly over the first 96 hours (Fig. 2E). It seems unlikely, therefore, that the ratio in favor of GFP at the early time points, is due entirely to the preferential degradation of Snapin.

**Addition of in-frame AUG codons to the Snapin ORF attenuate translation of a second ORF.** We then introduced a series of in-frame AUG codons into the Snapin ORF. These constructs were transfected into S2 cells. In all cases, the introduction of in-frame AUG codons attenuated the GFP signal both in terms of fluorescence (Fig. 3A) and the GFP::myc signal on Western blots (Fig. 3B). Although there was considerable variation in transfection efficiency, we found that for any given construct the ratio of GFP/Snapin was invariant. The effect of a single AUG insertion on the level of GFP as compared to Snapin, was determined either by its position in the ORF or, more likely, by the sequence context surrounding the AUG (Fig. 3D). Comparing wild-type with AUG150 and AUG338 the ratio of GFP to Snapin was 1.77 in the AUG-free construct, dropped to 1.1 in AUG150 but to 0.25 in AUG338 (Fig. 3C). The ratio dropped further to 0.055 when AUG130 and AUG338 were combined, and the addition of a third AUG at position 342 reduced the GFP signal even further (Fig. 3C). Obviously the presence of in-frame AUG codons in the Snapin ORF has a significant effect on the translation efficiency of the GFP ORF.

We also observed an extra Snapin protein species on the blots (arrow in Fig. 3B). This protein derives from the Snapin ORF as it reacts with the anti-myc antibodies but not with anti-GFP antibodies, and is present in constructs where the GFP is not myc-tagged (data not shown). Indeed its molecular weight corresponded to that expected from a truncation derived from initiation of translation at AUG150. The level of expression of this truncated protein as a proportion of the Snapin is quite...
variable (10-45% of full length Snapin levels) although it is always less or equal to the reduction in the GFP/Snapin ratio associated with AUG. This variability may indicate a lack of stability of the truncated protein as compared with the full length Snapin. Although the amino acids surrounding the myc tags are different, which might produce differences in the affinity of the anti-myc antibodies, this data does suggest that translation initiation at the 2nd (GFP) ORF is independent of the prior translation and termination of the 1st (Snapin) ORF. Termination/reinitiation would be expected to produce either equal quantities of Snapin and GFP, or less GFP than Snapin, whereas the data (Fig. 2E) suggests that more GFP is produced than Snapin. Translation of the GFP ORF is therefore unlikely to be due to reinitiation.

“Optimising” the Snapin initiating AUG attenuates GFP translation.
The stoichiometry of the two proteins in the AUG-free construct and presence of the truncated Snapin protein in AUG suggested that the translation of the 2nd ORF occurs through context-dependent leaky scanning where the scanning ribosome fails to initiate translation at the start of the Snapin ORF. If the efficiency of translation initiation of Snapin were increased, then it might be expected that translation of the GFP ORF might be accordingly decreased. The sequence surrounding the initiating AUG in Snapin is far from the consensus sequence for Drosophila initiation (Fig. 4A) (22). We therefore “optimised” the sequence preceding the Snapin initiating AUG to bring it closer to the consensus sequence (Fig. 4A) and placed this in the dicistronic constructs. Consistent with the leaky scanning model, this optimised sequence reduced the stoichiometry of GFP to Snapin in both the AUG-free construct as well as those where in-frame AUGs have been inserted in the Snapin ORF (Fig. 4B). This indicates that the level of translation initiation at the 2nd ORF is related to the inefficiency of translation initiation at the 1st ORF.

Lack of evidence for an Internal Ribosome Entry Site in the stoned transcript.
Having identified a possible mechanism for the translation of dicistronic transcripts in D. melanogaster, we sought to extrapolate this to the stoned locus. A major difference between stnA and Snapin is that the stnA ORF is 2553bp, while the Snapin ORF is considerably smaller (405bp). Although the inter-cistronic region of the stoned transcript is small and not conserved, it is feasible that an IRES does exist at this locus. The stn mutation is homozygous lethal allele that consists of an insertion of a ‘doc’ element in the stnA cistron, and appears to produce a very much larger than normal transcript (14). If stoned contains an IRES then it might be expected that the translation of the stnB ORF would be unaffected by the stn mutation. Another stoned mutation, stnC, is viable and arises from a base pair mutation in stnB that produces a UGA stop codon at position 415 in the STNB amino acid sequence (19). This truncates the STNB protein but still allows for a small amount of read-through producing >10% of the wild-type levels of STNB (19). We created stnC stn heterozygous flies and reasoned that if there were an IRES controlling STNB translation independently of STNA, then the stn allele would contribute a large amount of STNB protein and the levels in these fly heads would be at least 50% of the normal level. In fact the extremely low level of STNB in the stnC/stnC heterozygotes (Fig. 5A) can be accounted for by the low levels of read-through of the stnC mutation, indicating that the stn allele exhibits a polar effect on STNB expression and does not contribute to STNB levels in the heterozygote. While indirect, this data suggests that there is no IRES in stoned. Although it might be argued that the ‘doc’ insertion in STNA disrupts the IRES in the stn mutant, the data to follow confirms the absence of an IRES in stoned.

The efficiency of translation of stonedB is altered in the stoned-temperature–sensitive mutant in vivo.
The evidence from the Snapin experiments suggest that the absence of in-frame AUG codons in the stnA cistron would allow ribosomes that fail to initiate at stnA to scan through and initiate translation at the second, stnB cistron. The stoned-temperature–sensitive, stn mutant carries an A to T mutation in stnA, and substitutes an AUG codon for an AAG lysine codon at amino acid position 35 (23, and see Fig. 1B). This novel AUG lies within the sequence “GACCTAatgG” and might be expected to produce a relatively high frequency of translation initiation. We therefore asked whether the stnA mutation might alter the expression of the stnB cistron. Western blots of whole extracts from wild-type and stnA fly heads were probed with anti-STNB and anti-STNA antibodies. The level of STNB protein in stnA fly heads is approximately 30% of that found in wild-type flies (Fig. 5B). This is consistent with our
previous report that showed an equivalent reduction in STNB levels in fractionated stn\textsuperscript{a} head extracts as compared with wild-type (19). We then asked if this mutation also altered the levels of the STNA protein. STNA exists in two forms on SDS-PAGE, one form shows the expected mobility and corresponds to a molecular weight of 95kDa. A second molecular form shows aberrant mobility on SDS-PAGE with a molecular weight estimate of 145kDa. Both forms are increased by approximately 40\% in stn\textsuperscript{a} head extracts (Fig. 5B). The nature of the Western blots precludes a direct comparison between experiments. However, although the absolute values of STNB and STNA vary from blot to blot, we find a consistent 5-fold higher STNB/STNA ratio in wild-type compared to stn\textsuperscript{a} heads.

To confirm that the difference in the level of STNB in the stn\textsuperscript{a} flies is due to a cis-acting rather than trans-acting effect, reciprocal crosses were performed using stn\textsuperscript{a} and wild-type flies. The males from the reciprocal crosses were either stn\textsuperscript{a} or wild-type and the females from both crosses were heterozygous. Again there is a reduction in STNB levels which segregated with the stn\textsuperscript{a} mutation, while the heterozygous females showed a level of STNB intermediate between wild-type and stn\textsuperscript{a} (Fig. 5C). This implies that the reduction in STNB is inherent in the stn\textsuperscript{a} transcript and is not due to some unlinked trans-acting factor. Heterozygous stn\textsuperscript{a} flies also show a level of STNA intermediate between wild-type and stn\textsuperscript{a} (Fig. 5C). This data indicates that translation of the stnB cistron is not independent of stnA translation, reinforcing the stn\textsuperscript{13,126} results and confirming the absence of an IRES in stoned. In all of the Western blots of stn\textsuperscript{a} flies, we never observed a smaller, truncated form of STNA that might arise from the initiation of translation at the novel AUG codon present in the stn\textsuperscript{a} flies. However there was a consistent increase in the level of STNA that might correspond to the added initiation of translation from the novel AUG codon and there did appear to be a slight mobility shift in the STNA proteins in stn\textsuperscript{a}. There are several possible reasons for the inability to observe both the full-length form of STNA and a truncated form in stn\textsuperscript{a}. It may be that the novel AUG is not in a favourable context for translation initiation. However it might also be that the truncated form is unstable and subject to degradation, or it could be due to the fact that the fragment of STNA that would be missing in the truncated form is lysine-rich (Fig. 1B). Its absence would make the protein overall much more negatively charged, and it is already highly acidic (pKi 4.36). Highly acidic proteins are known to show anomalous mobility on SDS-PAGE (24) and it is possible that a mobility shift caused by a reduction in size is counteracted by its increase in acidity. These possibilities were further investigated.

Expression of a truncated form of STNA does occur in transfection assays.

To determine if the methionine for lysine substitution in stn\textsuperscript{a} flies does indeed alter the translation initiation of STNA, we constructed STNA::GFP fusions for S2 cell transfection. The D. melanogaster stoned transcript contains a small upstream ORF, encoding eight amino acids, that extends from –19bp to +5bp. This uORF is conserved in other Drosophila species, and while differing in its termination codon and encoding just a tripeptide, is also present in A. gambiae (Fig. 6A). Because translation of this uORF may regulate translation initiation of the stnA ORF via a reinitiation shunt (13), we included this sequence. The stnA sequence from –41 to +226bp, with and without the stn\textsuperscript{a} AUG at position +103bp, were therefore fused in-frame with GFP, which itself contains an initiating AUG (Fig. 6B). Cells transfected with these constructs were harvested, processed for Western blotting and probed with anti-STNA antibodies. The presence of the stn\textsuperscript{a} AUG\textsuperscript{103} in the constructs produced a truncated protein of 31kDa as well as the expected full length (35kDa) STNA::GFP fusion (Fig. 6C). This truncated protein is absent from the cells transfected with the wild-type fusion construct. Perhaps surprisingly, the level of the truncated form of STNA in the stn\textsuperscript{a} AUG\textsuperscript{103} construct is approximately twice that of the full-length protein. When the equivalent fractions were probed with anti-GFP antibodies, again the wild-type construct exhibited one (35kDa), while the stn\textsuperscript{a} AUG\textsuperscript{103} construct two (35 and 31kDa) larger molecular weight protein species (Fig. 6D), equivalent to those identified with anti-STNA antibodies (Fig. 6C). However novel bands were observed that corresponded in molecular weight to the native GFP protein (27kDa) in both the wild-type and AUG\textsuperscript{103} constructs (Fig. 6C). This suggests that ribosomes failing to initiate translation at the stnA-initiating AUG, or at stn\textsuperscript{a} AUG\textsuperscript{130}, can effectively scan through and initiate translation at the GFP-initiating AUG. These data suggest that the stoned locus is exhibiting leaky scanning, both in situ and in
transfection assays, in a manner comparable to the Snapin dicistronic system. We also determined if the presence of the small uORF had any effect on the levels of translation initiation at the STNA AUG. A wild-type STNA::GFP fusion construct that removed the small uORF by starting at −15bp, was transfected into S2 cells. The Western blot of these cells along with samples from transfections that did contain the small uORF were probed with anti-GFP antibodies (Fig. 6E). It is clear that the absence of the small uORF does increases the levels of initiation at the STNA AUG as compared with the initiation at the GFP AUG. In fact the ratio of the STNA-fusion/GFP increases from 0.1 in the presence of the small uORF to 0.56 in its absence. This suggests that the small uORF could play a role in the regulation of translation-initiation of STNA.

**DISCUSSION.**

In the case of GCN4 the first of the small uORFs is read and yet the ribosome can apparently terminate and then re-initiate translation at a second ORF (7). In theory this termination/reinitiation mechanism might account for the translation of the downstream ORF in dicistronic transcripts. However the data we present for the stoichiometry of Snapin and GFP in a dicistronic context would argue that a termination-reinitiation mechanism is not operating here. If the initiation of translation of the GFP ORF were dependent on the translation and termination of the Snapin ORF then the levels of GFP would be, at most, equal to that of Snapin. With the caveat that there may be some difference in the ability of the anti-myc antibodies to bind to the two myc-tags, it is difficult to see how termination-reinitiation could bring about a higher level of GFP as compared to Snapin. This leaves the probability that there is a high level of “leaky scanning” of the ribosome through the Snapin ORF and these scanning ribosomes are then capable of initiating translation at the GFP ORF. The “leaky scanning” model is supported by the presence of the truncated Snapin protein in the constructs containing an AUG codon inserted before the myc tag. To obtain such a truncated protein the ribosome must have failed to initiate translation at the normal translation start site and scanned through to use the inserted AUG for translation-initiation. However, we cannot exclude the possibility that the insertion of a methionine residue before the myc tag makes the protein more sensitive to in vivo proteolysis, and the truncated protein is a proteolysed product of the full length Snapin.

This scanning through the initiating AUG of the Snapin ORF then explains the effects of the inserted AUG codons on the reduction in GFP levels. The internal AUG codons act as translation initiation sites for scanning ribosomes, thus reducing the number of scanning ribosomes that will reach the GFP ORF. This is the mechanism used by the rice tungro bacilliform virus to create three protein products from a single pre-genomic mRNA (25). The effect of each inserted AUG codon will be context dependent with the sequence surrounding the AUG being critical for initiation-site selection by the ribosome. Hence the difference between the effects on GFP expression of AUG150 as compared to AUG338 is most probably due to the context in which the AUG has been placed rather than its position in the ORF with regards to the normal termination codon. However, it is possible that an AUG codon close to the termination of the first ORF and so producing a small uORF, could act like the small uORFs in GCN4, allowing for reinitiation and having little effect on the downstream reading of the second ORF. Two pieces of information relate to this possibility. Firstly in the *Apis mellifora stnA* ORF there is an in-frame AUG codon two codons upstream from the ICR. While we cannot say that this has no effect on the expression of STNB, it seems unlikely that such a substitution would be tolerated if it did have a major effect. The second point arises from the presence of out-of-frame AUG codons in both the Snapin [1] and STNA [3] ORFs. If translation were to initiate at any of these out-of-frame AUG codons only short peptides would be produced, with the largest being 18 amino acids but most of them being 6-8 amino acids in length. A scanning ribosome that initiates at these AUG codons would terminate well before the ICR, and might be able to continue scanning to the AUG of the second ORF and reinitiate. However all of the out-of-frame AUGs might also be in a poor context for translation initiation. While it is not without precedence that a leaky scanning ribosome might traverse a mRNA the size of the Snapin ORF, in fact distances of up to 890nts have been reported for scanning ribosomes in some viral mRNAs (25, 26), in the case of the *stoned* mRNA the scanning ribosome has to cover 2,500nts before reaching the STNB ORF. It is possible that the presence of small out-of-frame ORFs in *stnA* act as “staging posts” for the scanning ribosome, maintaining its association with the mRNA and allowing it to traverse this distance.
Given that the scanning mechanism occurs both in Snapin and STNA, the selection for the absence of internal methionines in the STNA protein may have two components. Obviously the presence of in-frame AUG codons will exhibit a polar effect on STNB expression as is shown to be the case for the stn\(^{st}\) mutant. However there is also the possibility that such methionine insertion mutations would produce truncated STNA products. Large scale production of such truncations could have dominant negative effects, as they would be missing the N-terminal component of the STNA protein. We believe that this might well be the case for the stn\(^{st}\) mutation. Although there does appear to be a polar effect of the stn\(^{st}\) mutant AUG on the efficiency of translation of STNB, the decrease is to 30% of the normal level. By way of contrast, the stn\(^{c}\) mutation reduces the levels of full length STNB to <10% of the normal levels (19), yet the stn\(^{st}\) mutation has been shown to complement all of the phenotypes associated with the stn\(^{c}\) mutation and vice versa (18). It seems highly unlikely therefore, that the stn\(^{st}\) phenotype derives from a reduction in STNB. However the stn\(^{st}\) mutation does result in the N-terminal truncation of the STNA protein when expressed in S2 cells, and the levels of the truncated protein are two fold higher than the wild-type protein. If this is mirrored in situ, then the stn\(^{st}\) mutant would be expected to increase STNA levels. The data in Fig. 5B confirm this. This increased level of STNA would also consist of a considerable amount of truncated form of STNA, presumably with altered “activity”. Unlike much of STNA, the N-terminal region of the protein is highly conserved across species (Fig. 1B) and might therefore be expected to harbor an important function. Its loss in >60% of the STNA products may be the sole reason for the stn\(^{st}\) phenotype.

There is an interesting consequence of the scanning mechanism on the possible regulation of STNA/STNB levels. The levels of GFP in the stn\(A::GFP\) constructs would suggest that a major proportion of the ribosomes effectively scan through the STNA translation-initiation site, and this can be regulated by the presence of the small uORF. Any factor that increases the selection of the small uORF AUG for ribosome initiation, would automatically reduce the levels of STNA and increase the levels of STNB. By analogy with GCN4, this factor might be the phosphorylation of eIF2. On the other hand, any increase in STNA initiation would result in a decrease in the levels of STNB. The small uORF may therefore act as a reinitiation shunt (13). If such a regulatory mechanism were to exist, then a reasonable supposition for this dicistronic arrangement of the two ORFS might be that STNA and STNB are components of two parallel pathways achieving the same end point but through different mechanisms. Both of these proteins are found at synaptic terminals (27, 28) and from both amino acid sequence and mutant analyses, have been implicated in synaptic vesicle recycling (14, 18, 28, 29). There are presumed to be at least two mutually exclusive methods for recycling synaptic vesicles, clathrin-dependent recycling (30, 31) and “kiss and run” (32). If STNA and STNB were rate-limiting components of each of these two pathways then their regulation with respect to one another could alter the balance between these two mechanisms for synaptic vesicle recycling. Another possibility is that STNA and STNB act in opposition, where increased levels of STNA might inhibit a vesicle recycling pathway and STNB activate it. Exactly how the levels of the two proteins encoded by the stoned locus are regulated and whether the regulation of initiation of the small uORF or other extrinsic factors, play any part, awaits further investigation of these proteins, including a delineation of their role(s) in synaptic vesicle recycling.

**Footnotes and Acknowledgments:**

Abbreviations used are: IRES, internal ribosome entry site; ORF, open reading frame; uORF upstream open reading frame; GFP, green fluorescent protein; eIF, eukaryotic initiation factor; ICR, intercistronic region; nts, nucleotides.

‡AAW and AMP contributed equally to this work.

LE Kelly acknowledges support from the NH&MRC.
Figure Legends.

Figure 1: Comparison across species of the intercistronic sequences of dicistronic genes.

A. The intercistronic (ICR) sequences between STNA and STNB are shown for a number of *Drosophila* species as well as *Anopheles gambiae*. While there is similarity in size and sequence between *D. melanogaster*, *D. yakuba* and *D. erecta*, this breaks down entirely in the other three *Drosophila* species as well as in *A. gambiae*. There is also no similarity between the ICR from the *D. melanogaster* Snapin/methyl transferase dicistronic transcript and any of the STNB/STNA sequences.

B. Sequence comparison of the STNA protein from *Anopheles gambiae* and *Drosophila melanogaster*. Despite the obvious divergence of amino acid sequence between these two proteins, neither contains internal methionines. The lysine (35) that is mutated to methionine in the *Drosophila stn* mutant, is indicated in bold and underlined.

Figure 2: Both Snapin ORF and GFP are expressed from a dicistronic construct in S2 cells.

A Dual labelling of S2 cells transfected with a dicistronic myc-tagged Snapin/GFP construct. Cells were fixed and probed with both anti-GFP and anti-myc antibodies and imaged. All cells that are immuno-positive for GFP are also positive for the myc antigen. Whereas the distribution of GFP appears cytosolic, the Snapin appears to be associated with the periphery of the cell (arrow in the Snapin panel).

B Diagrammatic representation of the Snapin/GFP dicistronic construct indicating the position of the myc tags, the ICR and the inserted in-frame AUG codons. Note that the AUG^{150} insertion is before the myc tag and so any translation initiation that occurs from this AUG codon can be identified as being myc-positive.

C Time course of expression of Snapin and GFP in a transfection assay. Cells from a single transfection using the wild-type construct but where both Snapin and GFP were myc-tagged, were harvested at various times after transfection and the lysates subjected to Western blotting, the blots Ponceau stained, and then developed using anti-myc antibodies. Densitometric scans of the GFP, Snapin and total protein were quantified as described in Experimental Methods. The loading control represents the 50kDa region of the Ponceau-stained blot.

D Quantitation of the results shown in C. GFP synthesis, determined as the ratio of GFP to total protein, peaked at 72 hours, after which time the continued growth of the cells dilutes the GFP.

E Quantitative comparison shows that the relative levels of GFP and Snapin, as determined by the GFP/Snapin ratio, remains constant for the first 96 hours post-transfection, after which time the Snapin level is seen to decay relative to GFP.

Figure 3: Introduction of in-frame AUG codons in the Snapin ORF reduces GFP expression.

A Fields showing the relative GFP fluorescence in S2 cells transfected with the various dicistronic constructs (see Fig 2B) at 72 hours post transfection. The exposure time is identical for each field and the images have been converted into negatives using Adobe Photoshop.

B Western blots of extracts of cells transfected with the various constructs, harvested at 72 hours, and probed with anti-myc antibodies. The arrow head indicates the novel cross-reacting protein seen in the transfectants containing the constructs carrying AUG^{150}. Samples from the cell lysates were loaded to equalise the Snapin levels as closely as possible.

C Quantitation of the results shown in B. and expressed as the ratio of GFP to Snapin for each of the constructs shows the additive effect of AUG codons on GFP expression.

D Sequences surrounding the AUG codons inserted into the Snapin open reading frame.
Figure 4: Improving the translation initiation of Snapin reduces the translation of the GFP ORF.

A The “consensus” sequence for translation initiation in Drosophila as defined by Cavener (22), as compared with the native Snapin sequence and the “optimised” translation initiation sequence.

B Extracts of S2 cells transfected with either the wild-type and AUG150 constructs having the native sequence surrounding the AUG, or the equivalent constructs where the sequence around the initiating AUG had been “optimised”, were Western blotted and probed with anti-myc antibodies. Again samples from the cell lysates were loaded to try to equalise the Snapin levels. The arrow head indicates the presumptive N-terminally truncated form of Snapin. The histogram shows the ratio of GFP to Snapin for each of the transfected constructs and indicates that “optimising” the translation initiation of the Snapin ORF reduces the translation of GFP.

Figure 5: STNB and STNA levels in stoned mutants.

A There is no evidence for an IRES at the stoned locus. Homogenates of six heads from wild-type (y1118), stnC and stnC/stn13120 heterozygous flies were run on SDS-PAGE, transferred to nitrocellulose and probed with anti-STNB antibodies. The levels of STNB in the stnC/stn13120 fly heads are no greater than those seen in the stnC heads, showing that there is unlikely to be an internal ribosome entry site in stoned. The loading control represents the 50kDa region of the Ponceau-stained blot.

B The levels of STNA and STNB were measured in stn fs and wild-type fly heads. Increasing numbers of heads from wild-type (y1118) and stn fs flies were homogenised and the extracts prepared for Western blotting. The blots were probed with anti-STNA and anti-STNB antibodies. Densitometric scans of the Western blots allowed the plotting of STNA and STNB levels against the number of heads used in the preparation of the extracts. This shows that the levels of STNA and STNB, as measured by densitometry, increases linearly with the number of heads and that while STNA levels appear 40% higher in the stn fs mutant, the STNB levels are reduced to ~30% of the wild-type level. The loading control represents the 50kDa region of the Ponceau-stained blot.

C Reciprocal crosses of y1118 females with w, stn fs males and y1118 males with w, stn fs females produce y1118 males and w, stn fs males and heterozygous females all of which contain the same autosome compliment. Four heads from each of the genotypes were run on SDS-PAGE Western blotted and probed with anti-STNA and anti-STNB antibodies. The blots were scanned to generate the data in the histogram. The increase in the level of STNA and the reduction in STNB are seen to segregate with the stn fs allele and are intermediate in the heterozygote indicating a cis-effect of the stn fs mutation on the levels of STNA/STNB. The loading control represents the 50kDa region of the Ponceau-stained blot.
Figure 6: Translation initiation of the STNA protein is inefficient and is regulated by a small upstream ORF

A Alignment of the small upstream ORF from two *Drosophila* species and *A. gambiae*. The beginning of the STNA ORF is capitalised and the small uORF is underlined. Despite the dissimilarity between the ICR sequences of *D. melanogaster* and *D. pseudoobscura* (see Fig. 1), the small uORF is highly conserved between these two species. *A. gambiae* also has a small uORF, but in this case the uORF does not extend into the STNA ORF.

B Diagrammatic representation of the STNA::GFP fusion construct showing the position of the STNA AUG, the AUG that is present in the *stnt* mutant (AUG130) and the AUG that starts the GFP ORF (AUGGFP). The shaded region indicates the position of the small upstream ORF that may regulate translation initiation at the STNA AUG.

C Western blots of lysates from S2 cells that were untransfected, transfected with the wild-type STNA::GFP fusion and with the STNA::GFP fusion carrying AUG130 from the *stnt* mutant. The blots have been probed with anti-STNA antibodies. The anti-STNA antibodies recognise a non-specific protein of 39kDa in the untransfected S2 cells, but in the transfected cells the 35kDa protein recognised by these antibodies corresponds to the expected size of the STNA::GFP fusion. Note the presence of the truncated protein at 31kDa in the lysate from the STNA::GFP fusion carrying AUG130.

D A blot identical to that in Fig. 5C probed with anti-GFP antibodies. As well as showing the same 35kDa and 31kDa proteins corresponding to the STNA::GFP and truncated STNA::GFP as seen in Fig. 4C, both transfectants also show a considerable signal corresponding to the size (27kDa) of unfused GFP.

E Transfectants carrying the wild-type STNA::GFP fusion with and without the small upstream ORF, were Western blotted and probed with anti-GFP antibodies. The absence of the small upstream ORF increases the STNA::GFP signal and correspondingly decreases the GFP signal as compared to the same material prepared from transfectants where the small upstream ORF is present.
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A. GFP and Snapin images with arrows indicating specific areas.

B. Schematic diagram showing the location of AUG150, AUG338, and ICR within the SNAPIN and GFP sequences.

C. Graph showing the ratio of GFP to total protein over time (24, 72, 96, 168 hours after transfection).

D. Graph showing the ratio of GFP to Snapin protein over time (24, 72, 96, 168 hours after transfection).

E. Graph showing the ratio of GFP to Snapin protein over time (24, 72, 96, 168 hours after transfection).
AUG$^{150}$ - CTGCAAatgG
AUG$^{338}$ - GTCACGatgA
AUG$^{342}$ - AAGCTTatgG
A

Consensus  - CAAAatgG
Snapin  - TGCAatgG
Snapin “optimised”  - CAAAatgG

B

![Graph showing GFP and Snapin expression levels]

Ratio GFP/Snapin

wild-type  wild-type opt  AUGopt  AUGopt
ccgatctcgatcgtccgcaat-tagataATGCTTAAG  D. pseudoobscura.
ccgatctcgatcgtccgcaat-tagataATGCTTAAG  D. melanogaster.
acgt-tcgatctcgatcgtccgcaat-tagataATGCTTAAG  A. gambiae.

A

\[
\begin{align*}
    &\text{ccgatctcgatcgtccgcaat-tagataATGCTTAAG} \\
    &\text{ccgatctcgatcgtccgcaat-tagataATGCTTAAG} \\
    &\text{acgt-tcgatctcgatcgtccgcaat-tagataATGCTTAAG}
\end{align*}
\]

B

\[
\begin{align*}
    &\text{AUG}^{\text{STNA}} \\
    &\text{AUG}^{\text{ORF}} \\
    &\text{GFP}
\end{align*}
\]

C

\[
\begin{align*}
    &\alpha-\text{STNA} \\
    &\text{untransfected} \\
    &\text{w.t. STNA} \\
    &\text{st}^f\text{ STNA} \\
    &35 \text{kDa} \\
    &31 \text{kDa}
\end{align*}
\]

D

\[
\begin{align*}
    &\alpha-\text{GFP} \\
    &\text{untransfected} \\
    &\text{w.t. STNA} \\
    &\text{st}^f\text{ STNA} \\
    &35 \text{kDa} \\
    &31 \text{kDa} \\
    &27 \text{kDa}
\end{align*}
\]

E

\[
\begin{align*}
    &\alpha-\text{GFP} \\
    &\text{+uORF} \\
    &\text{-uORF} \\
    &35 \text{kDa} \\
    &27 \text{kDa}
\end{align*}
\]
Effective translation of the second cistron in two drosophila dicistronic transcripts is determined by the absence of in-frame AUG codons in the first cistron

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