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Analysis of *drongo*, a new *Drosophila* zinc
finger gene expressed during oogenesis and
neurogenesis.

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Declaration

I hereby declare that the work presented in this thesis is my own work unless otherwise stated in the text or figure legends. This work has not been submitted to any other institution or for any previous degree. All information sources are acknowledged.

Jane Pritchard

Abstract

This thesis is an investigation into the function of the *drongo*, a novel gene with a zinc finger motif, originally isolated via enhancer trapping from its expression in the embryonic neuroectoderm. Drongo has previously been shown to be expressed during oogenesis, neurogenesis and eye development.

In this project, sequencing of a Drongo cDNA clone, shows homology to human nucleoporin protein hRIP; and a lesser extent to other proteins including the mammalian ARF-1 GTPase activating protein (ARF-1 GAP), a protein involved in vesicular transport across the cell; and family of yeast zinc finger genes GTS1/GCS1/GLO3, members of which have also recently been shown to have GAP activity.

Overexpression of *drongo* during early oogenesis results in egg chambers with supernumerary nurse cells, probably as a result of a delay in follicle cell migration; a phenotype similar to that of *braniac* mutants. Overexpression during late oogenesis causes a mislocalisation of Oskar, producing embryos which lacked denticle belts and which often had posterior defects, suggesting that ectopic expression of the gene at different times in development can have different consequences.

A mutagenesis screen carried out generated a number of potential mutant alleles, although none as yet have identified a mutation in the *drongo* gene. The use of an peptide antibody to the protein on S2 cells and western blots has identified a possible localisation of the Drongo protein to the endoplasmic reticulum, suggesting a role in vesicle transport. Drongo has also been shown to be developmentally expressed and the gene appears to encode two proteins which may or may not be functionally distinct. The role of Drongo as a possible ARF GAP, are discussed.

Abbreviations.

The abbreviations used in this thesis are listed below in alphabetical order. Several classes of abbreviations have been omitted from this list these include (a) chemical formulae, (b) names of cell lines, (c) strains of animals or bacteria, accepted gene or phenotype designations, names of plasmids, and transcription factors and (d) restriction enzymes. -

A - adenine (base)

ADP - Adenosine diphosphate

ARF - ADP ribosylation factor

ast - asteroid

b - base

β -Gal - beta galactosidase

BAP - Bacterial alkaline phosphatase

bHLH - basic helix loop helix

bp - basepair

BRE - Bruno response element

BSA - Bovine serum albumin

C - cytosine (base)

CAT - chloramphenicol acetyltransferase

cDNA - complementary DNA

CNS - central nervous system

Con A - concanavalin A

CyO - Curly of Oster

DAB - 3,3' diaminobenzidine

dATP - 2'-deoxyadenosine 5'- triphosphate

DER - *Drosophila* EGF receptor
Dh33 - *Drosophila hydei* 33 (embryonic cell line)
Dig - digoxigenin
DMSO - dimethylsulphoxide
DNA - 2'-deoxyribonucleic acid
DNase - 2' deoxyribonuclease
DSHB - developmental studies hybridoma bank
EDTA - ethylenediaminetetraacetic acid
EGF - epidermal growth factor
ELISA - enzyme linked immunoadsorbant assay
EMS - ethyl methanesulphonate
env - envelope gene (HIV-1)
EST - expressed sequence tags
et al. - *et alia*, and others
FB/CS - fetal bovine/calf serum
FG - Phenylalanine-glutamine
FITC - fluorescein isothiocyanate
g - force of gravity
G - guanine (base)
GAP - GTPase activating protein
GC - golgi complex
GMC - Ganglion mother cell
GST - glutathione-S-transferase
GTP - Guaninetriphosphate
h or hrs - hours
HEPES - N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphoic acid]
His - histidine
HIV-1 - human immunodeficiency virus type 1
HRP - horse radish peroxidase
IgG - Immunoglobulin type G

IPTG - isopropyl β D-thiogalactopyranoside
Kb - kilobase
kDa - kiloDalton
KLH - Keyhole limpet haemocyanin
l - litre
LB - Luria Bertani broth
lbin² - pounds per inch
M - Molar
MBP - maltose binding protein
 μ Ci - microcurie
MCS - multiple cloning site
 μ g - microgramme
 μ l - microlitre
ml- millilitre
mM - millimolar
mRNA - messenger RNA
MTOC - microtubule organising centre
mV - millivolts
NES - nuclear export sequence
NGS - Normal goat serum
NLS - nuclear localisation signal
NPC - nuclear pore complex
O.D. - optical density
ONPG - o-nitrophenyl- β -D-galactopyranoside
OPD - o-phenylenediamine
ORF - open reading frame
PAGE - polyacrylamide gel electrophoresis
PBS - phosphate buffered saline
PBT - phosphate buffered saline with the addition of 0.1% Triton X-100
PCR - polymerase chain reaction

PHS - post heat shock
pmole - picomole
PMSF - phenylmethylsulphonylfluoride
PNS - peripheral nervous system
RER - Rough endoplasmic reticulum
RNA - ribonucleic acid
RNase - ribonuclease
rpm - revolutions per minute
RRE - Rev response element
RT-PCR - Reverse transcriptase PCR
RTK - receptor tyrosine kinase
S2 - Schneider 2 (*Drosophila melanogaster* embryonic cell line)
SA - Splice acceptor
SD - Splice donor
SOP - sense organ precursor
SV40 - simian virus 40
T - thymine (base)
Tat - trans activator gene (HIV-1)
TBE - electrophoresis buffer containing Tris, boric acid and EDTA
TEMED - N, N, N', N', - Tetramethylethylenediamine
TGF - transforming growth factor
TLC - thin layer chromatography
UAS - Upstream activating sequence
UTR - Untranslated region
UV - Ultra violet
WCS - White Canton S

For Mum, Dad and Jonathan.

Chapter 1:

Introduction.

1.1 Finding a role for *drongo* in *Drosophila*.

Identification and characterisation of new genes involved in the development of a multicellular organism is a rapidly growing subject in modern day biology. The use of genetic and molecular techniques in a variety of model animal systems has uncovered a number of genes and signalling pathways utilised throughout development. The discovery that many of these genes possess homologues involved in the development of many higher animals suggests that these model systems can be used to extrapolate information about the dynamic development of humans.

One of the most well established model systems in use today is that of the fruit fly, *Drosophila melanogaster* the organism utilised in this study.

Many of the genes identified in *Drosophila* have been found to mediate a function in multiple systems throughout development, suggesting the concept of 'signalling cassettes' (Ruohola *et al.*, 1991) in which the same signalling mechanisms are utilised in different developmental contexts with different consequences.

When this project was initiated, the overall aim was to characterise a new *Drosophila* gene called *drongo*. Previous work (Harris, 1995) involved the isolation and initial characterisation of this gene which offered clues to the gene's possible function. From the initial data, this project aimed to determine the role of *drongo* at the cellular level and in the development of the fly.

My aim in this introduction is to give an overview of the previous work on the *drongo* gene leading to a brief introduction of the recent developments in two of the processes where *drongo* is expressed, oogenesis and neurogenesis. The latter part of the introduction will then focus on an overview of nucleocytoplasmic transport and its relevance to this project, the introduction will finish with the aims of this study.

1.2 Previous analysis of *drongo*.

1.2.1 Isolation of *drongo*: Enhancer trapping.

The *drongo* gene was isolated and cloned using the method of enhancer trapping. The classical approach to identify developmental genes is to perform large

scale mutagenesis screens such as those performed by Nüsslein-Volhard and Wieschaus (1980) and Nüsslein-Volhard *et al.* (1984). Whilst these screens are still in use, the enhancer trap technique, first demonstrated by O’Kane and Gehring (1987) has proved useful as an additional way to identify genes, particularly where there is a partial redundancy of function, or when the mutant phenotype is too subtle to screen for easily.

The enhancer-trap technique (figure 1.1) utilises a modified *Drosophila* transposon, or P-element, which contains a β -galactosidase (*lacZ*) reporter gene fused to a weak promoter. A P-element is a specialised DNA structure in *Drosophila* which is able to move or ‘transpose’ from one place in the genome to another. There are many P-elements dispersed throughout the genome of many wild type strains and they contain inverted terminal repeats and a source of transposase, which are important for efficient transposition (For a review of P-elements, isolation and techniques, see: <http://www.wisc.edu/genetics/CATG/engels/Pelements/Pt.html>).

P-element enhancer constructs transformed into the *Drosophila* genome lead to transcription from the weak P-element promoter which can only be initiated upon insertion near an endogenous enhancer (figure 1.1). Expression of the *lac Z* gene often reflects all (or at least part) of the temporal and spatial expression of an endogenous gene, upon which the enhancer primarily acts. Genes are subsequently cloned on their expression pattern rather than via a mutant phenotype (Wilson *et al.*, 1989).

Enhancer-traps contain sequences which allow flanking genomic DNA from the insertion point to be cloned by plasmid rescue exploiting Bluescript sequences at the 3’ end of the enhancer trap. Plasmid rescue (Wilson *et al.*, 1989) is a technique whereby the genomic DNA from transformed flies can be digested, recircularised and transformed into *E.coli* cells. The flanking genomic DNA can then be analysed.

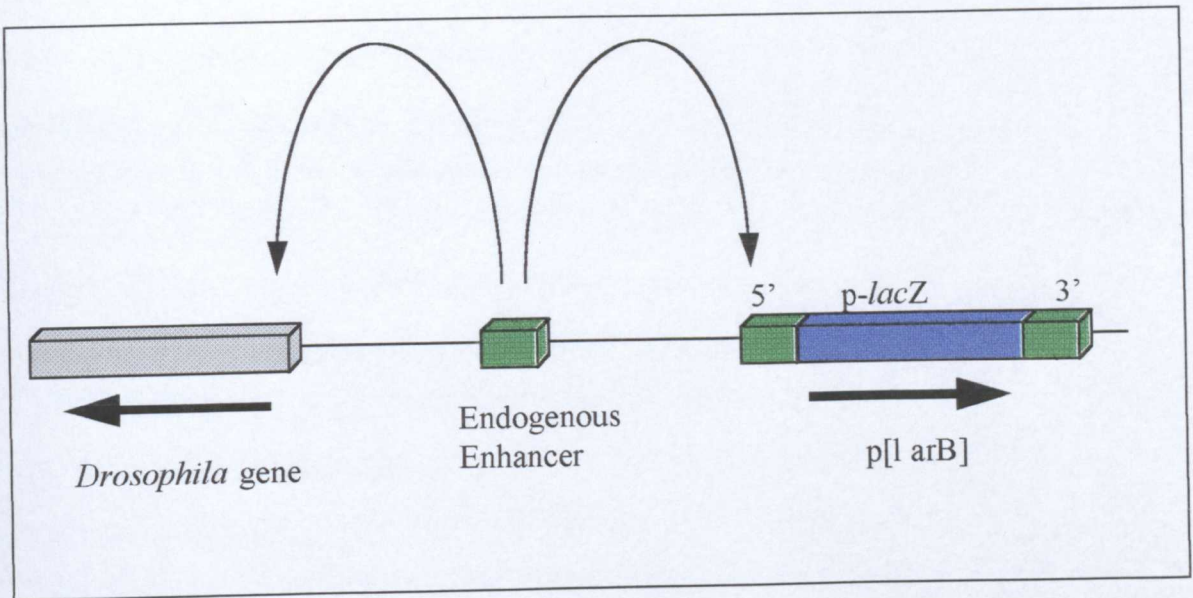


Figure 1.1

Enhancer Trapping

The basic enhancer-trap principle where an endogenous gene can be isolated via analysis of the expression of the *E. coli lac Z* gene. An enhancer trap is a modified P-element, which contains a weak promoter and a *lac Z* reporter gene, can be transformed into the genome. The endogenous enhancer which normally acts on a nearby gene can also influence expression of the enhancer-trap. The *lac Z* expression pattern often resembles that of the endogenous gene controlled by the same enhancer. In addition, the enhancer-trap contains sequences which allow flanking DNA to be cloned (Diagram from Wilson *et al.*, 1989).

The enhancer trap B75.1M2 (a p[larB] line) was recovered in a screen for embryonic *lacZ* expression patterns (Harris, 1995; Wilson *et al.*, 1989). It was primarily of interest after *lacZ* expression was observed in the neurogenic ectoderm, the region of ectoderm patterned to become nervous system in the embryo (see section 1.3). *lacZ* expression was also seen during oogenesis and eye development. The B75.1M2 insert was mapped cytologically to 21D on the second chromosome and DNA flanking the enhancer trap was cloned (plasmid rescue for the enhancer trap line was carried out by Harris, 1995). This clone was then hybridised to the genomic walk used to isolate the *ninaA* gene (Schneuwly *et al.*, 1989), situated between the breakpoints of two deficiencies in the area Df(2L)*ast4* and Df(2L)*ast6* (deletions in the DNA created by gamma ray irradiation of the second chromosome) (Roberts *et al.*, 1985). Data from previous work initially suggested that there were four essential genes between the deficiency breakpoints (Roberts *et al.*, 1985). Stocks containing mutations of these genes are no longer available.

Four transcribed regions were primarily identified near the B75.1M2 insert by reverse northern analysis (DeLorenzi *et al.*, 1988) Fragments from the transcribed regions were used to screen a 4 to 8 hour old embryonic cDNA library and one transcript, designated *drongo*, was expressed in a pattern similar, although not, identical to that of the B75.1M2 reporter construct (Harris, 1995).

The *drongo* transcript was expressed during embryogenesis, initially as a set of ectodermal stripes (figure 1.2A) and then later expression was seen in ventral ectoderm (figure 1.2B; and see section 1.3), expression also was observed in neuroblasts delaminating from this region (figure 1.2C). During later stages of neurogenesis (see section 1.3), expression was seen in what appeared to be a peripheral nervous system (PNS) precursor cell and precursors of the longitudinal glia (figure 1.2D). At stage 11 expression was observed in two clusters, probably the progeny of single neuroblasts. By stage 15 expression was confined mainly to the PNS and the longitudinal glia (figure 1.2 E and F).

Expression of the *drongo* transcript was also observed during oogenesis (see section 1.4). Using a DNA probe expression was seen in the germarium, probably in the precursors of the follicle cells and in the oocyte, which both express *drongo*

Figure 1.2

Expression of *drongo* during development

In situ hybridisation with the *drongo* cDNA.

A-F expression of *drongo* during embryogenesis.

(A). At cellularisation of the embryo, *drongo* was expressed in six stripes across the trunk of the embryo

(B) Upon germband extension, *drongo* expression was observed in the ventral ectoderm (arrow)

(C) *drongo* is then expressed in delaminating neuroblasts at stage 8 and 9

(D) During stage 10 expression was seen in a single cell per hemisegment these are probably PNS precursor cells.

(E and F). During early stage 11 *drongo* is expressed in the progeny of a single neuroblast and was confined to the PNS and the longitudinal glia (lg)

G-I expression during oogenesis.

(G) *drongo* was expressed in the germarium, although the cells were not identified. expression was seen in the follicle cells (open arrow) from stage 2 to stage 7 expression was also seen in the oocyte (small black arrow).

(H) From stage 9 *drongo* was localised to the anterior of the oocyte and nurse cell (large black arrow) expression of *drongo* continued until stage 12

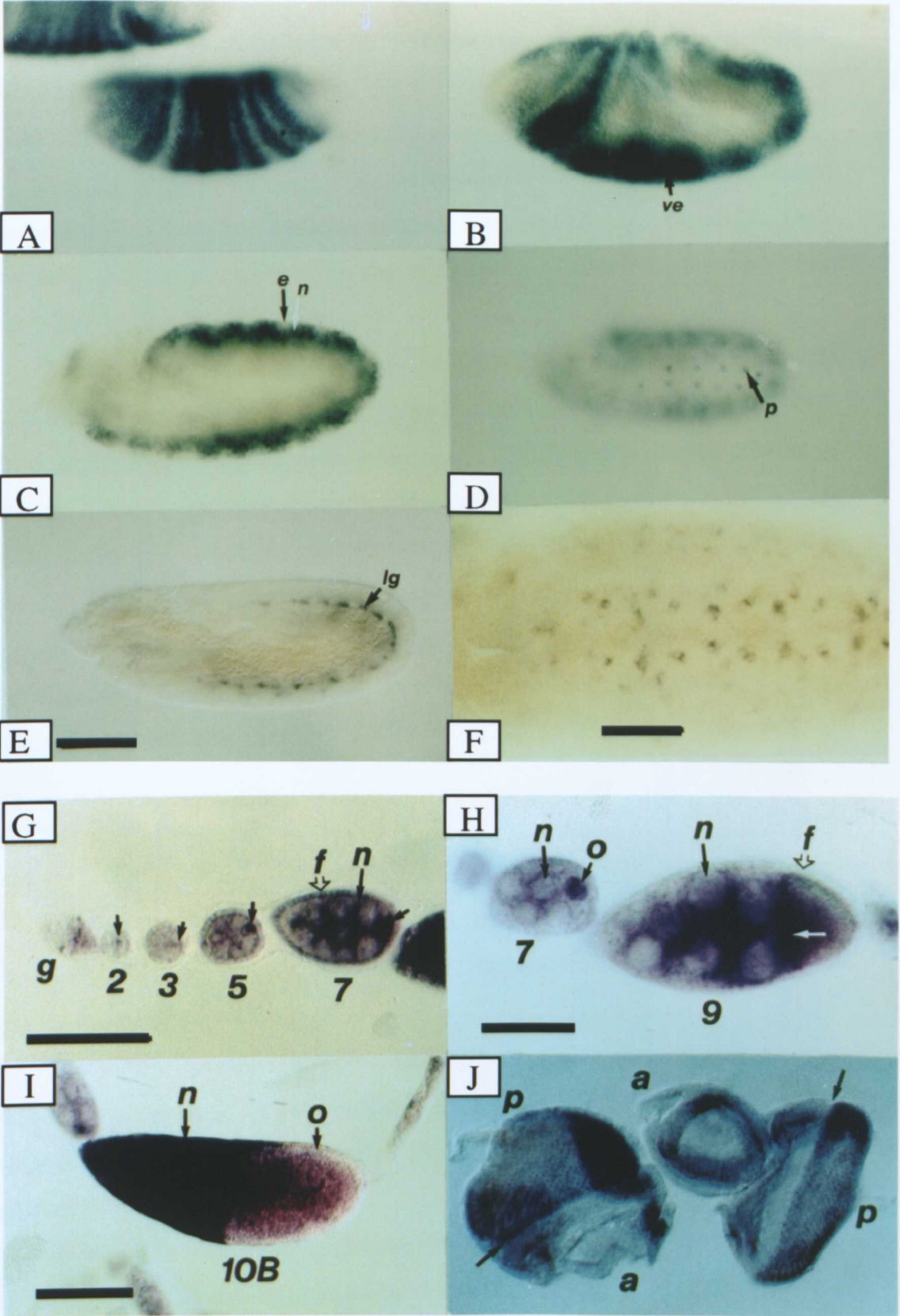
(I) Expression of the gene continued until nurse cell dumping into the oocyte occurred.

J expression of *drongo* in the eye imaginal disc

Expression of *drongo* was seen immediately behind the morphogenetic furrow (arrow) and was concentrated in a number of cells in each ommatidium. Expression of *drongo* was also observed in other groups of cells in third instar discs which are probably CNS precursors.

Key: Ve: ventral ectoderm; e: ectoderm; n: neuroblast; p: PNS; lg: longitudinal glia; n: nurse cells; o: oocyte; f: follicle cells; a: anterior; p: posterior; g: germarium. Numbers refer to stage of egg chambers.

Figure from Harris, 1995.



subsequently (figure 1.2 G). Expression was seen in the follicle cells from stage 2 to about stage 7 of oogenesis and in one of the cystocytes (probably the oocyte) until about stage 9, with weak expression in the nurse cells (figure 1.2 H). At stage 9, expression increased in the nurse cells and decreased in the oocyte until transfer of the nurse cell contents occurred at stage 12 (figure 1.2 I).

drongo expression was also analysed during eye development. Expression of *drongo* in the third instar eye-antennal imaginal disc began behind the morphogenic furrow and there appeared to be expression in a number of cells in each ommatidium (figure 1.2 J).

The *drongo* cDNA was transcribed from right to left on the chromosome and the B75.1M2 insertion was found 82bp into the transcription unit (Harris, 1995). northern analysis detected two major transcripts of 3.7kb and 3.2kb, the smaller of which was abundant only in 0-2 hour old embryos and adult females, indicating the 3.2kb transcript was probably a maternally expressed mRNA. Sequencing of *drongo* revealed a cDNA of 3495bp, which was close to the size of the 3.7kb transcript (the transcript which predominated at the stages of embryogenesis used to make the cDNA library). It is not clear whether these transcripts were the result of alternate splicing, initiation or termination.

1.2.2 Characterisation of *drongo*.

Following analysis of the expression pattern, investigations were carried out to determine the type of protein encoded by the *drongo* gene in an attempt to identify a function.

The cDNA contained an open reading frame (ORF) that encoded a protein containing a single zinc finger motif and other short motifs including numerous repeats of the dipeptide phenylalanine glycine (FG) and Opa (poly-glutamine) repeat sequences (figure 1.3; Harris, 1995).

In vitro translation of the protein in both plant and animal systems, identified two possible translational start sites, producing proteins of 84kDa and 61kDa. A series of deletions from the 5' end of the cDNA transcribed and translated *in vitro*,

Figure 1.3:

drongo cDNA and predicted protein sequence.

The DNA sequence is in lower case. Protein translated from ATT (605) or from GTG (613) is in upper case, and the protein translated from ATG (958) is in upper case and bold. Numbers on the left refer to DNA sequence and on the right to protein sequence. The position of the B75.1M2 insertion is indicated by a triangle with an arrow showing the direction of transcription of the *lac Z* gene. The putative polyadenylation signal is boxed and the final (a) indicates the position of the poly A tail.

In the protein sequence, the green box indicates the position of the zinc finger motif, grey boxes show phenylalanine-glycine (FG) repeats and pink boxes indicate positions of the polyglutamine (Poly Q, Opa) repeats.

cDNA sequence from Harris (1995) with corrections to the 3 ' end done in this work (section 2.3.1).

1 Ggttggttaggcgagcgggtcggaaatttgcgtcgaaattgtgttgagtcgagtcgcgctatnttgtttcagtgcgctgt
80 gctggttggtttcgctccgtttcgcttcgcttttcactcgtcttcgaatccgcaactgcaacaatttcgcaa
160 cactgtcgaaatttcaattgaaatctgaaatctgtgtggaattgcagcggcactcgggtgtaaatagattgaaattgaagc
240 gaaattctgcgagtcgaagaagtgaagtaacaataacacgggcaatcggaagtggttttcgataaatgcaccgg
320 acacacacactgtgagtggtgcaaaagtgagtggtgagtggtgcgtcgaaggaggaaggtaagccaaaaattgcg
400 taaagaaaaggaaaggaaagccttagaagaggggaagggaattacgtcagtttcgcattcccgataaaaaattttgatc
480 cctaaacgatcctgatagccagccattacgatagggcaaaaaacgataacagcggaaatttactgtaatttggagcagaaa
M Q F V Y A Y N S S A P 12
560 gtaaacgataccaattcccgaagaattcaaaaaatcaggcaagattcagttcgtgtagcgttacacagctcccgcgccc
H A A S S Y R T A V A P F A P Y A A E R S T H H H P L 39
640 cagcgcgctcctcctaccgcaaccgctgcccccttgcgctcagcggccgagcgtccaccaccaccaccact
P P P E E R V P S D T E Q L A G S D E D R A Q S H E A 66
720 cccgcccccgaggagcgggtgcccctcggacaccgagcagctgagctgacccgagcggatcgggctcagagcccaag
E A E E S E E E R A G S G P S V R P L I A A A G A T 92
800 cggaaagcggaggaatccgaggaggagcgcgagcagtgacggaccctccgtagcggcgtcatcgcagcagcaggcaaca
T T T T S T S V V P E A V Q R A A A K G A T E T A T M 119
880 acaacgacaacgtcagctcagtagtaccggaagcggtaacaaagggcggcggcaagggagcagacagagcggcagcaat
A V V R K K Q D D K Y L L A L R E L V V S G T G S N R 146
960 ggcggtggtgcgcaagaacaggacgacaagtacgtgctgcggaactagtggtcagcggagcgggagcgaatc
Q C F D C G Q K G P T Y V N M T I G S F V C T R C S 172
1040 ggcagtgcttcgactgcggccaaaaggaccaacctacgtgaacatgacctcggatcctttgtctgcaccgctgctcc
G V L R G L T P P H R V K S I S M A T F T Q D E I D F 198
1120 gvtcctcgtcgtgacagccacatcgatggccacattcaccacagcagatggccacattcaccacagcagatggccac
L R S H G N E L C A K T W L G L W D P K R A V H Q Q E 225
1200 cctgcgagccatggcaacgagctgtgtgccaagacctggtgggattgtgggatccgaagcgggctgtgcaccagcagg
Q R E L M M D K Y E R K R Y Y L E P A S P L K S L A 251
1280 agcagcgcgaactgatgatggacaagtatgagcggaaagcagataactcctggagccagctccttaagctcgtggcc
N A V N L K S S A P A T N H T Q N G H H N G Y A S I H 278
1360 aatgcggtcaacctgaagtctgtctcggcgagcaaccacactcagaatggccaccacaatgggtatgccagcatcca
L T P P A A Q R T S A N G L Q K V A N S S S N S S G K 305
1440 ttgacctcgtcgtgaccagccctcggcgaatgtagcagagtgtagcagagtgccacattcaccacagcagatggccac
T S S S I S R P H Y N H Q N N S Q N N N H D A F G L 331
1520 agacatcctcgtcagtaggccaattataatcaccagaacaacagccaaaacaacatcagatgctttggcctg
G A T L S S L N S A G S T S T G A L S D T S S C A S N 358
1600 ggtggcgattgagcagcctgacaagcgggttcacatccaccggagctccttcgacaccagcagttggccagcaa
G F G A D C D F V A D F G S A N I F D A T S A R S T G 385
1680 tggcttcggtgcgagctcagctttgtggctgactttggctcggccaacatttgcagccacatcggcgcggtccacag
S P A V S S V S S V G S S N G Y A K V Q P I R A A H 411
1760 gatcgcggcggtgctgctccgtgctcctcagtggttcagcaatggctacgccaaggtgcagccatccggcgagctcat
L Q Q Q Q Q L Q Q Q Q H Q Q Q L L N G N G H Q G T E N 438
1840 ctccagcagcaacagcagtcgagcagcagcagcagcagcagcagcagcagcagcagcagcagcagcagcagcagcagc
F A D F H A P I Y N A V G A P L Q N G H T T S P P K 465
1920 ctttgcgacttcgatcagctcccatctacaatcagtggtgctcccttcgagaacggccatacagcagctccgcca
G G N A A M A P S E D R Y A A L K D L D E Q L R E L 491
2000 agggcggaaatgcagcagtcgcccagtgaggatcagctcctccttaaggtatcgcagcagcagcagcagcagcagcagc
K A S E S A T E T P T P T S G N V Q A T D A F G G A L 518
2080 aaggccagcaaaagcggcagagagcggccaccgacggcaatggtcagccacagatgctttggtggccct
N N N P N P F K G Q Q Q Q Q L S S H V V N P F Q Q Q Q Q 545
2160 caacaacaatccaaatcccttcaaggccagcaacagcagcagcagcagcagcagcagcagcagcagcagcagcagc
Q Q Q Q H Q Q N L Y G Q L T L I P N A Y G S S S Q Q Q Q 571
2240 aacagc
M G H H L L Q Q Q Q Q Q Q Q S F F N F N N N G F A I S 598
2320 atggggccacatcctcctcagc
Q G L P N G C G F G S M Q P A P V M A N N P F A A S G 625
2400 gcaggtctgcccacggctcggcctcggcagcagcagcagcagcagcagcagcagcagcagcagcagcagcagcagcagc
A M N T N N P F L
2480 cgccatgaaccaacaatccattcttatgagactcaaccaggagaatccgctcggccactggcagagcagcagcagcagcagc
2560 gcgaacaagagc
2640 tgcagccggagagc
2720 atggcggaggcggactgggctgatatattctttacatggaactcactcactaacgtttacggatagcagatatttgt
2800 atttgtttgcccagactctatacttactaagcggccatgaacacttccactaacatagctactaatcctcat
2880 cctagtgaggatgcagttggtccagactcctgtttttttatctatcctcgtacttgtctttgtcccatttagca
2960 ctttcgttgggataagaactttgtcagttattgattgtgtggccttaataagattataaaactaaattatttagcgtac
3040 gactatacatatacggatagcagattcagacagcttagtacagatagatatacatatacagctttttagccta
3120 atgaattgcttctgtttccattgctaatactcgtcttttcggtgctaatttatacactagtagcagcagatcggcc

3200 gtgcagatagattgctcagctcgcgagtcaagcctcttttggtgcacccacggcagacatttgtacataactgtctga
3280 ttgtaagcctcgtgtaatacctccattaacaccactccccaccacccatccatcgaacccgaatccatgactcaattc
3360 actgtcacatgtccatgccatgccagataagaagcaagaattttccatttttagcattaacgtgtcaaacattatcga
3440 agccttaaagtttatttaaaaactacgaaatttcaataaaaacaataagaacgctatc (a)

suggested that the smaller product was initiated from the first AUG (position 959) whilst the larger product was possibly initiated from a non-AUG codon (either 613 (GUG) or 605 (AUU)). It was not clear whether the same codons were also utilised *in vivo* as recorded for other eukaryotic proteins (Acland *et al.*, 1990; Imataka *et al.*, 1997) or were an *in vitro* artifact (Kozak, 1989).

Homology searches with Drongo (figure 1.4) of protein sequence databases (using FASTA and BLAST search tools) initially revealed similarity (about 30-40% over the most highly conserved 50 amino acids) to a family of zinc finger proteins (Figure 1.4). Members of this family include the yeast GCS1/GTS1/GLO3 family, mutations of which suggest these proteins have a role in controlling cell cycle progression (Ireland *et al.*, 1994; Mitsui *et al.*, 1994). The gene was hence initially named *Drosophila* neural GT^S1-like OR^F, or *drongo*. Other more distantly related homologues were a mammalian and a *Drosophila* GTPase-activating protein (GAP) which act on the small GTPase ARF1 (Cukierman *et al.*, 1995) and which may regulate vesicle traffic from the Golgi complex. ARF-1(ADP ribosylation factor-1) is a monomeric GTP binding protein which plays a crucial role in the assembly and disassembly of coatomer and clathrin coated vesicle coats. ARF-1 requires a GTP exchange factor (GEF) for activation and vesicle coating and a GTPase activating protein (ARF1-GAP) to hydrolyse GTP and allow vesicle release (Vitale *et al.*, 1998).

More recent searches however have identified substantial similarity (77% identity over 73 amino acids) to a human protein hRIP/Rab (Rev interacting protein/Rev activation domain binding protein), which has been shown to bind the HIV-1 Rev protein and implicated in transport of HIV RNA from the nucleus to the cytoplasm (section 1.5) (Bogerd *et al.*, 1995; Fritz *et al.*, 1995). Nuclear export of intron containing HIV-1 transcripts is critically dependent on Rev function, hRIP is thought to bind to Rev and enhance its function. Homology with hRIP/Rab was observed around the zinc finger and also included other short motifs such as a number of the FG repeats and a sequence DRYAALKDLD (found once in the *drongo* cDNA, a similar sequence was found twice in the hRIP cDNA) (Figure 1.4).

Figure 1.4.

Homology of Drongo with other proteins.

The Drongo protein sequence aligned with the yeast zinc finger proteins GTS1, GCS1 and Glo3; the human nucleoporin protein hRIP and the rat and *Drosophila* ARF1 GAPs. Sequences were aligned and similarity assessed using the University of Wisconsin GCG sequence analysis software. For determining similarity, amino acids were grouped as follows (L, I, V, M, F, W, P, C, G, A) (D, E) (K, R, H) (S, T, N, Q, Y). Sequence similarity is shown if 5 or more have amino acids from the same group at that position. Sequence identity is shown if sequence similarity criteria are met and at least 6 of the sequences are identical.

Dark shading indicates a region containing similarity at least 6 sequences, light shading indicates sequence similarity. Numbers indicate positions of amino acids from the predicted start of translation; for *drongo* this was taken to be ATG (958) and dashes indicate no amino acids in a particular position. Amino acid sequences of rat and *Drosophila* ARF1-GAPs; yeast genes GTS1, GCS1, Glo3; and hRIP were taken from Genbank .

In view of the interesting nature of this gene and the exciting prospect of identifying a function for *drongo* in *Drosophila*, initial attempts were made to produce a mutation in the *drongo* gene from which a mutant phenotype could be analysed. The P-element, B75.1M2, had inserted into the 5' end of the *drongo* gene (as sequencing of the flanking DNA started at + 82 of the *drongo* cDNA and extended upstream of this). The insert was located on a CyO balancer chromosome and none of the chromosomes recovered were lethal over the deficiency Df(2L)*ast4*. It is possible to mobilise a P-element by using an additional defective P-element transposon that expresses high levels of a transposase enzyme, but cannot move itself. The enzyme can thus work in *trans* and mobilise the enhancer trap element. Attempts were made to produce “jump-start” males, containing both the B75.1M2 insert and the source of transposase ($\Delta 2-3$).

These jump-start flies were recovered at very low frequency (3 out of 3,000 screened) however and northern analysis showed that *drongo* was still expressed in these flies (*Pers. Comm.* K. Moffat).

This was the starting point of my study. I would now like to review some of the present work in neurogenesis and oogenesis and introduce the area of nuclear transport.

1.3 Neurogenesis : Genetic and Cellular aspects.

Development of the central nervous system (CNS) in *Drosophila* is a multistep process whereby a two dimensional ectodermal sheet is transformed into an organised three dimensional array of different types of neurons and glia (Hartenstein, 1993). Due to the growing interest in this field and the recent expansion of genes identified in *Drosophila*, this organism represents a good model system in which to study this highly regulated process.

This review of recent literature will concentrate on early neurogenesis as it is most relevant to this project. Axon guidance and brain development during larval and pupal life have been reviewed extensively elsewhere (Theranos *et al.*, 1995).

1.3.1 Embryonic development.

After fertilisation of the egg, a *Drosophila* embryo passes through 17 characteristic stages, which last approximately 18-24 hours (Campos-Ortega and Hartenstein, 1985; Hartenstein, 1993). Briefly, the main landmarks points are as follows: Stage 1 syncytial embryos form a cellular blastoderm at around stage 5. Neurogenesis is initiated at stage 9 (according to Campos-Ortega and Hartenstein, 1985), shortly after gastrulation (the process in which the germ layers are formed in the embryo, this occurs around stage 6-7). The nervous system continues to develop throughout embryogenesis until germ band retraction at stage 12. Dorsal closure and head involution occur at around stage 14 (Campos-Ortega and Hartenstein, 1985).

The CNS is derived from the ventral neurogenic ectoderm and this process occurs in three stages (figure 1.5). In the first step, all cells within the neuroectodermal portion of the embryo primarily acquire a neural fate (figure 1.5; stage 5-8). Only about 25% of these cells develop into neural progenitors or neuroblasts (nb). They do this in a sequential manner by segregation in three “waves” (SI-SIII) from the ectoderm to create three separate groups of neuroblasts forming a layer situated between the ectoderm and the mesoderm (figure 1.5; stage 9). In the third step the neuroblasts undergo a number of divisions to form intermediate precursors called ganglion mother cells (GMC; figure 1.5; stage 11), which will then give rise to sibling postmitotic neurons and glia (Hartenstein, 1993; figure 1.5; stage 13-17).

1.3.2 Nervous system development

1.3.2.1 Formation of the ventral neuroectoderm.

Early cell ablation experiments in the grasshopper, as well as cell transplantation experiments in *Drosophila*, indicated that cell-cell interactions are

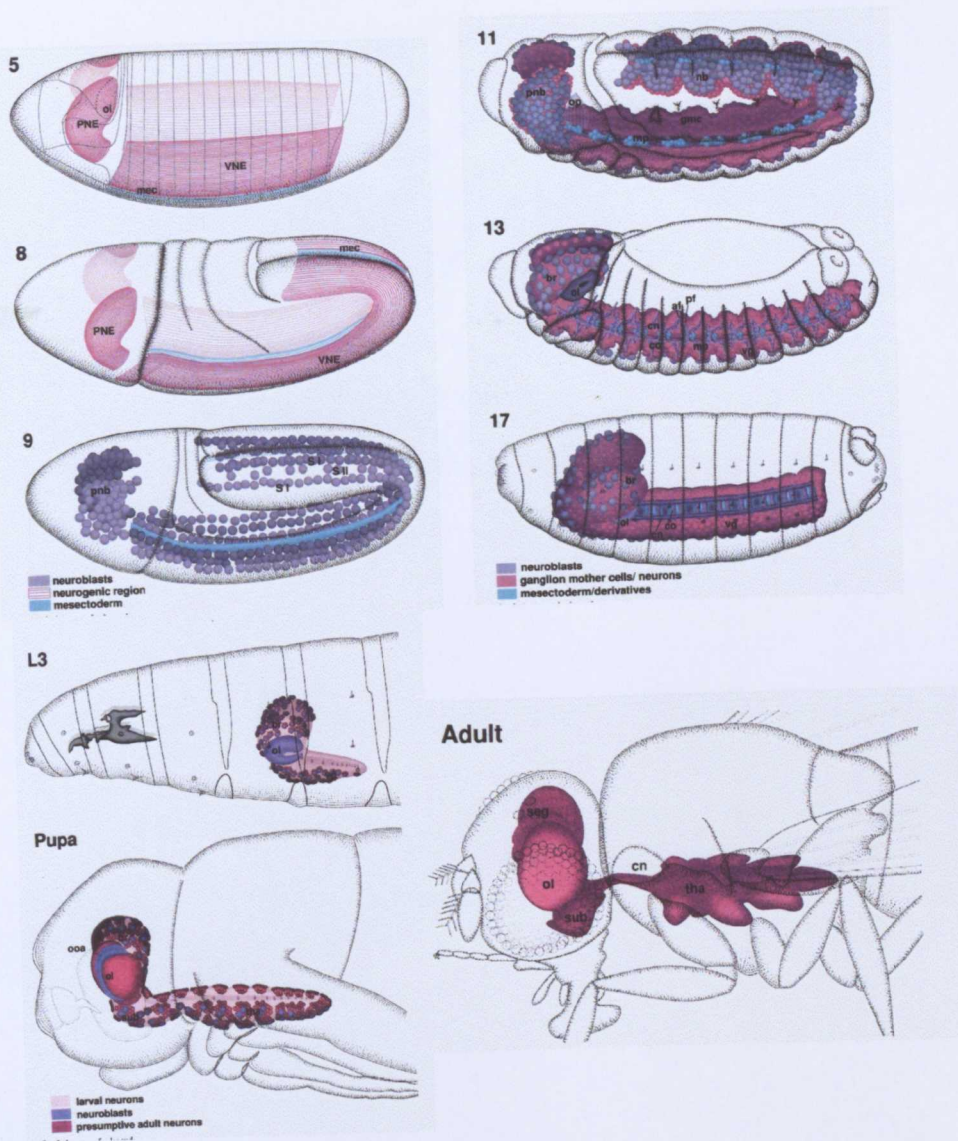


Figure 1.5.
 Diagrams of the developing *Drosophila* CNS throughout development.

The neuroblasts are the precursors of the nervous system derived from the neurogenic ectoderm. This is apparent in 2 regions : the ventral neurogenic (VNE) and the procephalic neurogenic region (PNE) – Stages 5 and 8. The mesectoderm (mec) is a single row of cells that gives rise to some neuronal precursors. The optic lobe anlage (ol) develops differently to the rest of the PNE. The first 2 waves of neuroblast delamination, starting at stage 9, are shown forming 3 rows of neuroblasts (S1 and S2). Procephalic neuroblasts also delaminate in a cluster (pnb). The neuroblasts (nb) divide asymmetrically to produce ganglion mother cells (gmc) that then produce the neurons. The mitosis taking place in stage 9 to 11 produce a nerve cord that is the length of the germ band extended embryo. This then retracts at stage 13, condensing until the end of embryogenesis producing a compact and complex nervous system, with features such as connectives (cn), commissures (co) and fascicles (af/pf) in the ventral ganglia (vg). The optic lobe precursors (op) form a vesicle (ol) attached to the basal surfaces of the brain hemispheres (br).

The morphogenetic changes can be seen as the CNS develops further in the third larval instar (L3) and pupa. Neuroblast divisions produce new neurons whilst cell death trims away the elements of the CNS that were used in the larva but are not required for the adult. The CNS resulting differs greatly from the larval form with a distinct head region (with supraesophageal ganglion (seg), subesophageal ganglion (sub) and optic lobes (ol)), connected to the large thoracic ganglia (tha) located in the thorax by a connective (cn).

From Hartenstein (1993).

important in the decision between neural and non-neural cell fates (Doe and Goodman, 1985; Techau and Campos-Ortega, 1987).

In the prospective neuroectoderm of the embryo, a gradient of the transcription factor Dorsal activates genes (such as *decapentaplegic* (*dpp*), *short gastrulation* (*sog*) *snail* (*sna*), and *twist* (*twi*)) which promote dorsoventral differentiation (figure 1.6a; Doe and Skeath, 1990). The dorsoventral side of the neuroectoderm is then subdivided into three domains: lateral, medial and intermediate (figure 1.6b). This dorsoventral subdivision is apparent at stage 5 (3 hours of development) when expression of genes such as *muscle specific homeodomain* (*msh*) and *ventral nervous system defective* (*vnd*) are expressed in the lateral and medial column, respectively, and *escargot* (*esg*) is expressed in both the medial and lateral columns (reviewed in Yagi *et al.*, 1998) (figure 1.6b).

Recent experiments, using transplantation and molecular markers for downstream genes in the EGF (epidermal growth factor) receptor signalling pathway, have shown a role for the *Drosophila* EGF receptor (DER) in promoting the formation, patterning and individual fate of early neuroblasts along the D/V axis (Udolph *et al.*, 1998).

DER mutants show defects in formation of intermediate and medial column neuroblast formation, although lateral neuroblasts form normally (Udolph *et al.*, 1998; Skeath, 1998). There thus appears to be DER-dependent and DER-independent regulatory pathways (Udolph *et al.*, 1998). *vnd*, encoding a homeobox protein, may be a good candidate for a DER independent ventral patterning gene. Along the dorsoventral axis, ventral cells express *vnd* (*Vnd* is thought to interact with DER and counteract DER mediated repression of *esg* and *scout* (*sc*)); expression of *intermediate neuroblasts defective* (*ind*) is required for intermediate column development, and the dorsal cells express *msh* (Chu *et al.*, 1998; Weiss *et al.*, 1998; McDonald *et al.*, 1998). These expression patterns are tightly regulated to establish the diversification of cells along the dorsoventral axis.

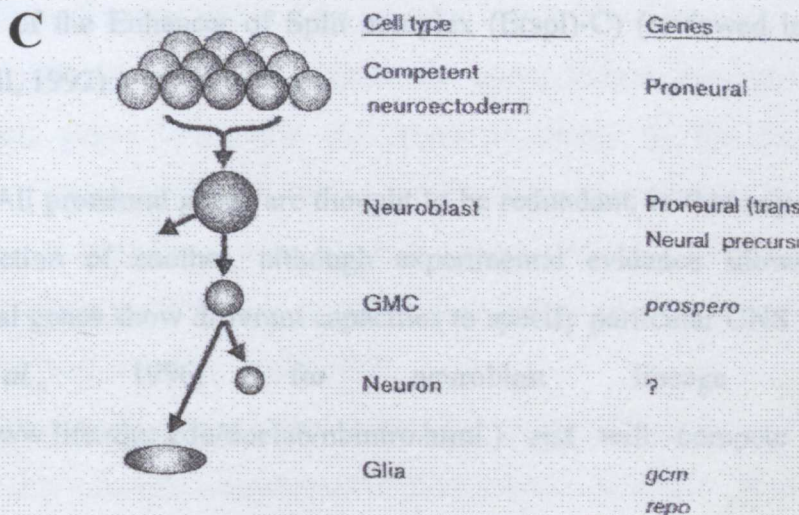
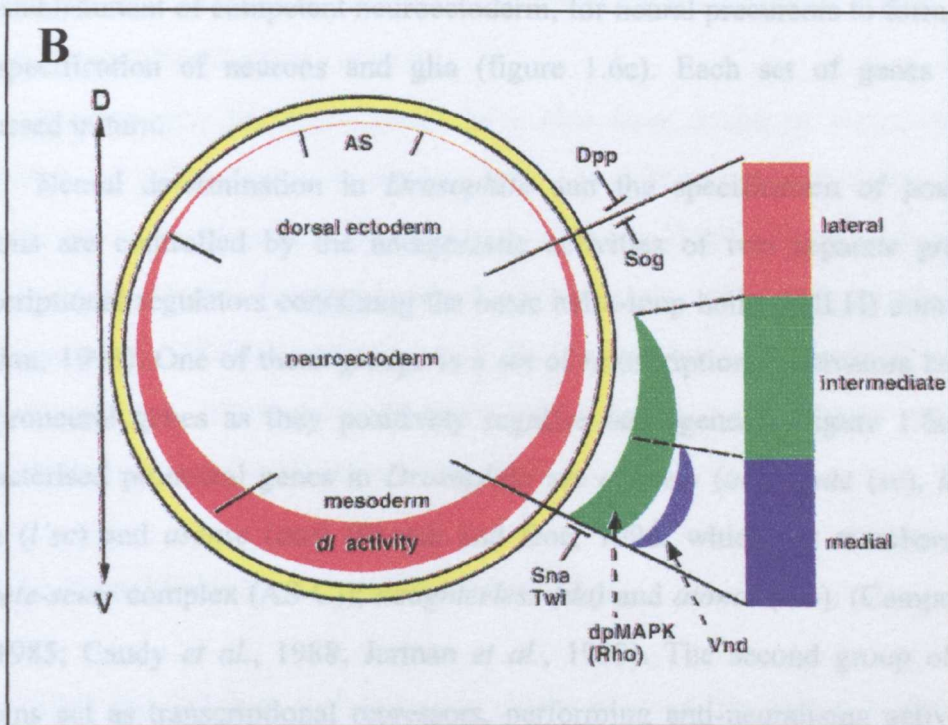
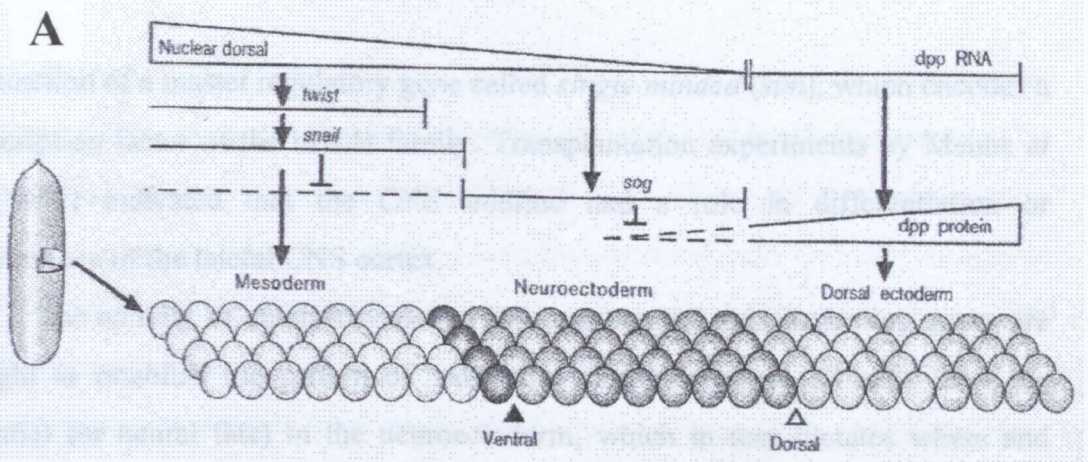
The CNS can be separated into two ventral halves connected by a special set of cells found at the CNS midline. The *Drosophila* midline comprises of a set of about 20 cells per neuromere. The development of the midline cells is dependent of

Figure 1.6

Formation of the neuroectoderm and development of neurons and glia

- A:** Establishment of the neuroectoderm. A hemisegment of the embryo becomes competent to produce neural precursors and a nuclear gradient of dorsal activates genes such as *twist (twi)*, *snail (sna)*, *short gastrulation (sog)* and *decapentaplegic (dpp)* which promote dorsal-ventral (D/V) differentiation.
- B:** Subdivision of the neuroectoderm along the dorsventral axis. In the stage 5 embryo, genes are expressed (see A) that promote D/V differentiation, which subdivides the neuroectoderm into lateral, intermediate and medial section. *rhomboid (rho)* determines the activation domain of the Drosophila EGF receptor (DER) and MAPK. Figure from Udolph *et al.* (1998).
- C:** Development of neuroblasts, Ganglion mother cells (GMCs) and neurons. Neuroblast formation requires the expression of one or more proneural genes which encode basic helix-loop-helix (bHLH) factors. All neuroblasts then divide asymmetrically to bud off smaller GMCs. GMC-specific localisation and expression is observed from genes such as *prospero* and *numb*. Although there is no known specific master regulatory gene for neuronal identity, a large number of genes are thought to be involved in neuronal cell type acquisition. Genes such as *glia cell missing (gcm)* and *reversed potential (repo)* can specify glial cell development. The genes expressed in each cell type are listed on the right. Ectoderm: grey; dark grey: neuroectoderm (and neuroblast, GMC, neuron and glia). (Figures A and C from Doe and Skeath, 1996)

Key: AS:aminoserosa; v: ventral; d: dorsal.



the function of a master regulatory gene called *single minded (sim)*, which encodes a transcription factor of the bHLH family. Transplantation experiments by Menne *et al.* (1997) indicated that the CNS midline had a role in differentiation or maintenance of the lateral CNS cortex.

The activity of anterior/posterior and dorsoventral-axis patterning genes are thought to establish the pattern of 'proneural clusters' (where all cells have the potential for neural fate) in the neuroectoderm, which in turn dictates where and when neuroblasts will form (Doe and Skeath, 1996). Specific genes are required for the establishment of competent neuroectoderm, for neural precursors to form and for the specification of neurons and glia (figure 1.6c). Each set of genes will be discussed in turn.

Neural determination in *Drosophila* and the specification of postmitotic neurons are controlled by the antagonistic activities of two separate groups of transcriptional regulators containing the basic helix-loop helix (bHLH) domain (Jan and Jan, 1993). One of these groups is a set of transcriptional activators known as the proneural genes as they positively regulate neurogenesis (figure 1.6c). Well characterised proneural genes in *Drosophila* are *achaete (ac)*, *scute (sc)*, *lethal of scute (l'sc)* and *asense (ase)* (Skeath and Doe, 1996; which are members of the *achaete-scute* complex (AS-C)); *daughterless (da)* and *atonal (ato)*. (Campuzano *et al.*, 1985; Caudy *et al.*, 1988; Jarman *et al.*, 1993). The second group of bHLH proteins act as transcriptional repressors, performing anti-neuralising activities by negatively regulating the proneural genes. This group includes Hairy and the related proteins of the Enhancer of Split complex (E(spl)-C) (reviewed in Campuzo and Modolell, 1992).

All proneural genes are thought to be redundant, in that any one can replace the function of another, although experimental evidence shows that different proneural genes show different capacities to specify particular CNS lineages (Parras *et al.*, 1996; for neuroblast lineage maps see <http://www.life.uiuc.edu/doelab/nbintro.html>.) and will compete for the same targets.

Proneural genes also function in the PNS for sense organ specification. *Drosophila* has a relatively simple PNS and much of the epidermis is innervated by two major subtypes of sense organ. The external sense organs are mechanoreceptors and chemoreceptors that are usually associated with external cuticular structures such as bristles, whilst the chordotonal structures are internal proprioceptors and vibration sensors whose dendrites are associated with internal scolopale structures. Both subtypes are derived from single ectodermal precursor cells called the sense organ precursors (SOPs). In the case of the PNS, the proneural genes have a more specific role, for example, *ato* expression in the SOPs specifies chordotonal organs, whereas *sc* specifies the sense organs .

Interestingly, proneural genes have also been found in many vertebrate organisms, including *cash4*, an *achaete-scute (AS-C)* homologue in chick. This has been shown to function as a proneural gene during chick neurogenesis and also displays a proneural function when heterologously expressed in *Drosophila* (Henrique *et al.*, 1997). Homologues of *AS-C* genes have also been identified in other vertebrates. These include *xash3* and *mash1*, in *Xenopus* and mouse respectively (reviewed in Henrique *et al.*, 1997).

1.3.2.2 Lateral Inhibition and neuroblast formation.

Only one cell from each proneural cluster of about five neuroectodermal cells will develop into a neuroblast/SOP by cell interactions mediated by the ligands Delta (Dl) or Serrate (Ser) and the receptor Notch (N) (Fehon *et al.*, 1990; Fleming *et al.*, 1997; Lendahl 1998). Proneural genes induce the expression of these neurogenic genes throughout the proneural cluster by the process of lateral inhibition. Lateral inhibition enables a cell to become committed to a neural fate by inhibiting its neighbours from becoming neural. All cells initially express both N and Dl and slight differences in the level of either will establish either a neural or ectodermal fate (Fleming *et al.*, 1997).

N and *Dl* encode transmembrane proteins at the cell surface. The extracellular domain of *N* contains a number of EGF-like repeats to which *Dl* and *Ser* have been

shown to bind. Intracellularly, N contains six ankyrin-like repeats which bind to Deltex (Dx), a positive regulator of N signalling (Fleming *et al.*, 1997).

In the absence of N or D1, there is a five fold increase in the formation of neuroblasts (Fleming *et al.*, 1997; Skeath and Carrol, 1992). In the majority of cases in the CNS and PNS, N function requires downstream nuclear proteins Mastermind (Mam), Suppressor of Hairless (Su(H)), Neuralised, and E(spl) (Reviewed in Artavanis-Tsakonas *et al.*, 1995). D1 and Ser are thought to be functionally interchangeable ligands which act in *trans* or *cis* with the N receptor (Jacobsen *et al.*, 1998).

The mechanism of N activation and subsequent activity has been come under much analysis recently and there are currently two theories as to how N acts *in vivo*.

In the first theory, three cleavage events of the N protein occur. The first being ligand dependent and results in an active N receptor at the cell surface. The second induces a cleavage in the extracellular domain of the N receptor (Lecoutois and Schweisguth, 1998). The third cleavage (which is thought to be ligand independent) results in production of a soluble N intracellular domain (NICD) which subsequently moves to the nucleus and binds to Su(H) (Kopan and Turner, 1996; Lecoutois and Schweisguth, 1998; Lewis, 1998; Rooke *et al.*, 1996). Su(H) in turn activates targets such as the *E(spl)-C* genes. *E(spl)-C* genes act as transcriptional repressors by repressing the *AS-C* complex of genes (Campuzano and Modolell, 1992).

The ADAM metalloproteinase Kuzbanian (Kuz), was thought to be involved in the third cleavage producing the NICD. Subsequent studies show, however, that Kuz may not be the principal protease. Mutations in *kuz* have less severe phenotypes than *N* mutations, suggesting other proteases are involved. It may also be involved in cleavage of the D1 protein, suggesting an alternative theory that D1 may act as a soluble ligand. (Qi *et al.*, 1999; Lewis, 1998; Nye, 1997).

As part of a feedback loop, Hairless (H) and Fringe (Fng) modulate N signalling. H binds to Su(H) antagonising transduction of the N signal whereas Fng is thought to antagonise the function of the Ser ligand (Fleming *et al.*, 1997; Panin *et*

al., 1997) and possibly the transmission of the DI signal (Panin *et al.*, 1997; Jacobsen *et al.*, 1998).

The second model suggests that N is associated with Su(H) in the cytoplasm of the cell. Ligand binding to N releases Su(H) from the cytoplasmic tail of N. Su(H) alone then enters the nucleus and acts as a transcription factor which subsequently activates the *E(spl)-C* (Bailey and Posakony, 1995).

Researchers have been unable to directly demonstrate localisation of N protein in the nucleus by immunostaining, although other evidence demonstrates N NICD activity (and therefore localisation) in the nucleus. This evidence also suggests that only minimal amounts of N are required for an effect (Struhl and Adachi, 1998; Weinmaster, 1998).

Lateral inhibition via the N pathway thus singles out neuroblasts from the neuroectoderm, these then move medially and divide or 'delaminate'.

1.3.2.3 Neuroblast division and development.

Once the neuroblasts have been specified, the next step is the repeated asymmetric division of the neuroblasts to bud off ganglion mother cells (GMCs). Within each neuroblast, the Inscuteable protein co-ordinates the mitotic spindle orientation with asymmetric protein and RNA localisation so that when a neuroblast divides a set of gene products (figure 1.7 A and B) asymmetrically segregate into the GMC (Knoblich *et al.*, 1999; Jan and Jan, 1998). These gene products include Numb, Miranda, Stauf (Stau), Prospero (Pros) proteins and *pros* RNA (Broadus *et al.*, 1998). Stau is an RNA binding domain which has been shown to localise *pros* (Broadus *et al.*, 1998). Pros is a transcription factor necessary to establish GMC-specific gene expression, as a wide crescent to the basal cortex of mitotic neuroblasts. Pros protein segregates exclusively into the GMC which buds off from the basal side of the neuroblast, where upon Pros translocates into the nucleus establishing differential gene expression between sibling cells (Ikeshima-Kataoka *et al.*, 1997; figure 1.7) Recent evidence suggests that Pros undergoes a cycle of phosphorylation and dephosphorylation as a requirement for (or a consequence of)

Figure 1.7

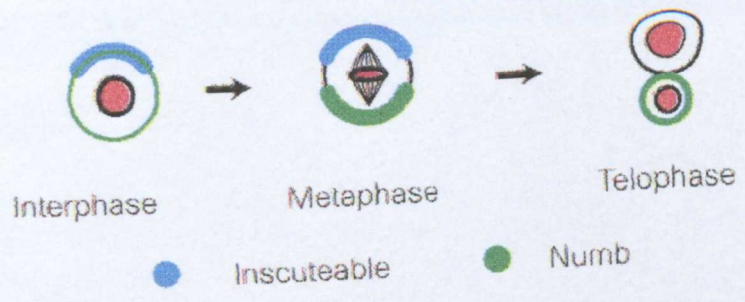
Asymmetric cell division in the CNS

A Two daughter cells are produced from the asymmetric division of a neural precursor. The inscuteable protein forms a crescent at one side of the neural precursor cell during interphase. Inscuteable is then required for the Numb protein to form a crescent at the opposite side of the cell during metaphase. After division one of the daughter cells inherits Numb and determines a cell fate different from its sibling.

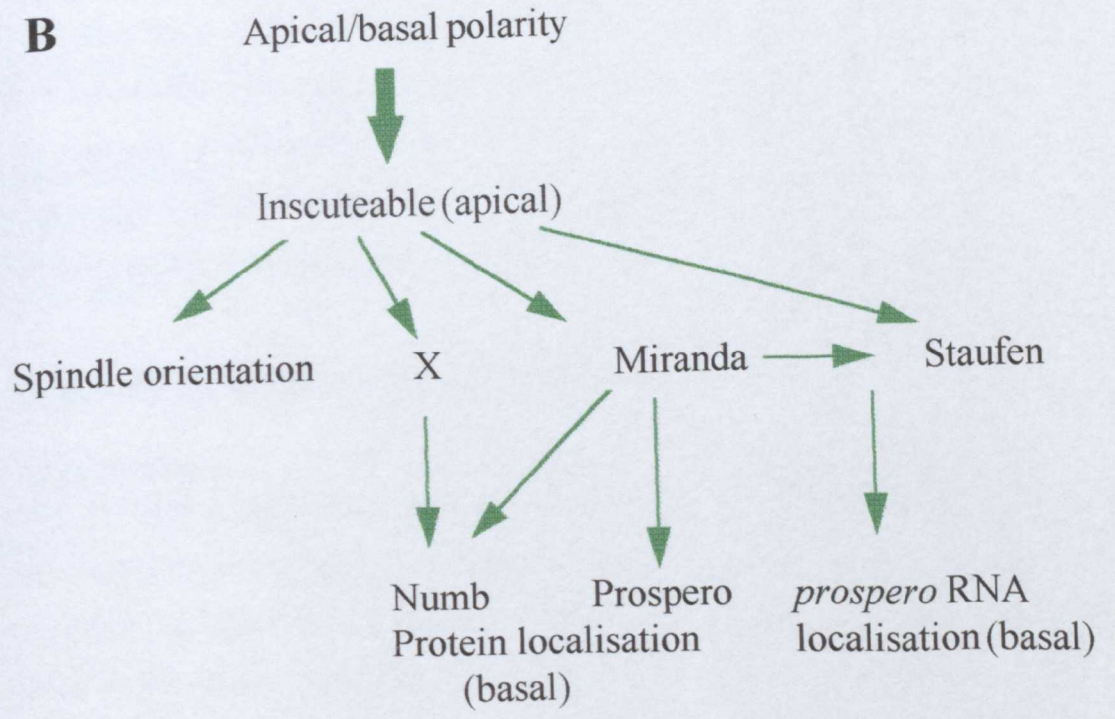
B The apical basal polarity laid down during embryogenesis directs the apical localisation of Inscuteable seen in A. This is necessary for spindle orientation along the apical axis and the asymmetric localisation of Numb. Prospero protein and RNA are localised by Miranda and Staufén to the basal side of the neuroblast.

Diagrams from Jan and Jan, 1998

A



B



localisation to the cortex and subsequent localisation to the nucleus (Srinivasan *et al.*, 1998).

Miranda co-localises with Pros protein and acts to partition both Pros protein and RNA. Miranda has also been shown to localise Stau and Pros to the basal cortex of dividing epithelial cells (Matsuzaki *et al.*, 1998). Miranda contains a coiled-coil structure and two leucine zipper motifs. Mutational analysis suggests that the ability of Miranda to bind Pros may be regulated by PKC sites in the C-terminus of the protein (Ikeshima-Kataoka *et al.*, 1997). Mutations in any of these genes result in incorrect neuronal determination and defects in axon tract formation later in neural development. In *stau* and *pros* deficient embryos, Miranda localisation is normal. It is thought that Miranda is localised via cytoskeletal elements as its localisation has been shown to be actin dependent (Matsuzaki *et al.*, 1998).

The last step of neurogenesis involves the division of the GMCs to produce a pair of post mitotic neurons (or glia), where the sibling neurons, in most cases, differ in gene expression and axon projections (Theranos *et al.*, 1995).

In summary, neural specification in *Drosophila* is a progressive process. Proneural genes are expressed in clusters of ectodermal cells and confer these cells with the competence to become neural precursors. Interactions between the cells via neurogenic genes ensure that only one cell from each cluster becomes a neural cell, which subsequently expresses neural-specific genes conferring neural identity.

1.4 Oogenesis in *Drosophila*: The formation of the egg.

Expression of the *drongo* gene has also been identified and analysed during oogenesis, the process in which the *Drosophila* egg is produced and develops in the female ovary. As I have further analysed the possible functions of *drongo* during oogenesis in this study, I will give a brief overview of the recent advances in this field.

This section concentrates on the genetic and cellular interactions that occur in the adult ovary, particularly during the early stages of oogenesis, rather than the

hormonal control or the structure of the ovary which is described elsewhere (King, 1970).

1.4.1 Egg chamber formation.

Each *Drosophila* ovary contains an average of 16 long thin structures called ovarioles (Spradling, 1993). Each ovariole consists of two main parts, the germarium, where the precursor of the mature oocyte, the egg chamber is produced; and the vitellarium, where each egg chamber passes through 14 characteristic developmental stages to form a mature egg (figure 1.8a).

In *Drosophila*, female gametes develop within small clusters or cysts of germline derived cells. Oogenesis begins in the most anterior structure of the ovary, the germarium. The germarium consists of 4 regions, 1, 2a, 2b, and 3 in which characteristic events at the start of oogenesis occur (figure 1.8b) (Spradling, 1993).

In region 1, a germline stem cell divides to produce a cystoblast and a daughter stem cell, which will proceed into another division (Spradling, 1993; figure 1.8b)). The cystoblast then undergoes four incomplete mitoses to produce a set of 16 cystocytes which are connected by cytoplasmic structures called ring canals. Within each set of 16 cystocytes, one of the cystocytes with the most ring canals (figure 1.9) will maintain meiotic arrest, become an oocyte and the chromatin in its nucleus will later condense to form a karyosome (a sphere of tightly packed chromatin). The other fifteen cystocytes will halt mitosis, enter meiosis and form supportive structures called nurse cells (Gonzalez-Reyes *et al.*, 1997). These nurse cells utilise the ring canals to supply nutrients to the oocyte, whose nucleus is transcriptionally inactive (Spradling, 1993). Mutants in the *encore* (*enc*) gene, show a disruption in these germ line divisions and egg chambers subsequently contain supernumerary nurse cells. In a germarium from an *enc* mutant mother, the stem cell undergoes an extra division in the germ line to produce a final number of 32 rather than 16 cystocytes (Hawkins *et al.*, 1996).

Figure 1.8

Oogenesis in *Drosophila*

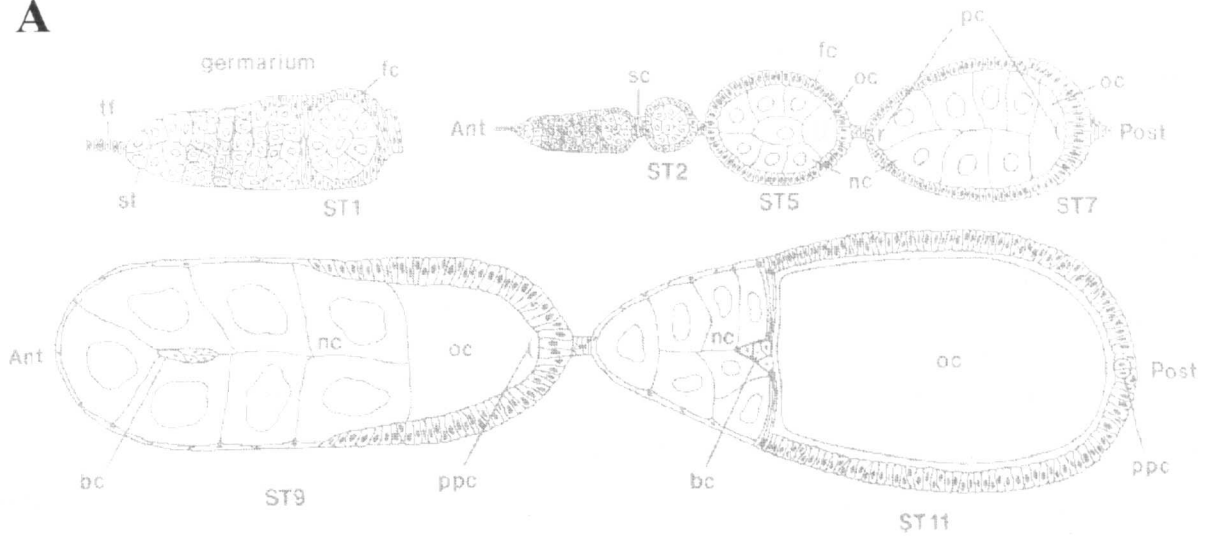
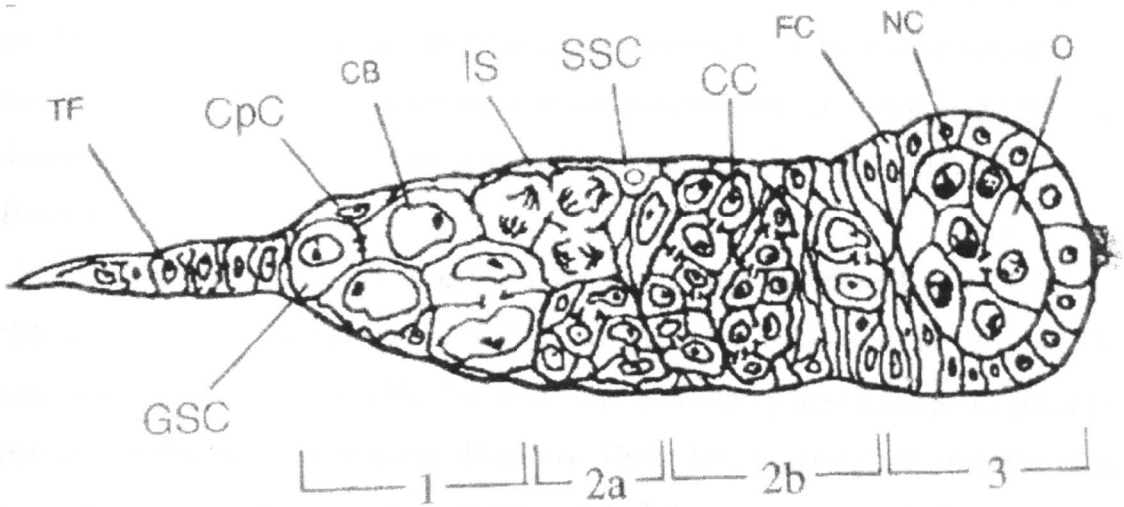
A: Schematic of a wild type ovariole.

Oogenesis begins in the anterior tip of the germarium (see part B), where a 16 cell germline cyst is produced. This is surrounded by a layer of follicle cells to form a stage one egg chamber (ST1) at which point the oocyte and nurse cells are defined. This passes through 14 characteristic stages (Stage 2, 5, 7, 9 and 11 are shown) to form a mature egg.

Key: tf: terminal filament; fc: follicle cells; st: stem cells; sc: stalk cells; oc: oocyte; nc: nurse cells; bc: border cells. Anterior (Ant) is left, posterior (post) is right. Figure from Peifer *et al.*, 1993.

B: Schematic of the germarium.

The germarium is divided into three regions. In region 1 a germline stem cell (GSC) divides asymmetrically to produce a cystoblast (CB) which further divides to form a 16 cell interconnected cyst. The stem cells are located basal to the terminal filament. In region 2a, differentiation of the 16 cystocytes (CC) into 15 nurse cells (NC) and one oocyte (O) occurs by transport of certain RNAs and proteins. The cyst is enveloped in region 3 by a layer of somatic follicle cells (FC) (these are the progeny of the somatic stem cells (SSC) to form a stage one egg chamber. Figure from Deng and Lin, 1997.

A**B**

1.4.1.1 Oocyte Specification.

The way in which the oocyte is determined is highly controversial, although recent detailed examination of this process offers us a number of theories on how the oocyte may be specified in a set of cystocytes. The ‘induction model’ (Rübsam *et al.*, 1998), suggests that oocyte specification occurs after cyst mitoses are complete. In this model, both cells with four ring canals (figure 1.9A) become ‘pro-oocytes’ but only one is chosen by expression of specific genes, to become the oocyte; the other reverts to a nurse cell fate (González-Reyes *et al.*, 1997; Rübsam *et al.*, 1998).

Mutations in a group of genes called the *spindle* (*spn*) genes cause a delay in determination of the pro-oocyte (González-Reyes *et al.*, 1997). The five *spn* genes, *spn-A*, *spn-B*, *spn-C*, *spn-D* and *spn-E*, were originally identified in a screen for maternal effect mutants as the females lay ventralised eggs. The function of the *spn* genes was found to be required in the germline (González-Reyes *et al.*, 1997). Spn B and C interact with Okra (a homologue of the yeast Rad52 DNA repair enzyme) and are thought to be involved in meiotic checkpoints in the 16 cell cysts (Ghabrial *et al.*, 1998). A null mutant in any of these genes does not block the choice between the two pro-oocytes, however, suggesting that there is an initial asymmetry and that the determination of the oocyte is just delayed by a mutation in these genes (González-Reyes *et al.*, 1997).

A second mechanism suggested by de Cuevas and Spradling (1997), the ‘Mosaic model’ by Rübsam *et al.* (1998), suggests the cystocytes are asymmetric and the oocyte is specified at the first cystoblast division by the creation of a polarity that is maintained in subsequent divisions. Their data suggests the presence of a cytoplasmic structure called a spectrosome which begins as a spherical structure in the oocyte and branches out through the ring canals to every cell in the cyst (figure 1.9a) forming a fusome. Fusomes consist of the membrane skeletal proteins α -spectrin (Lee *et al.*, 1997), β -spectrin, ankyrin and the adducin-like molecule Hu-li tai shao (Hts) (Yue and Spradling, 1992, Lin *et al.*, 1994). Mutants in any of these

Figure 1.9

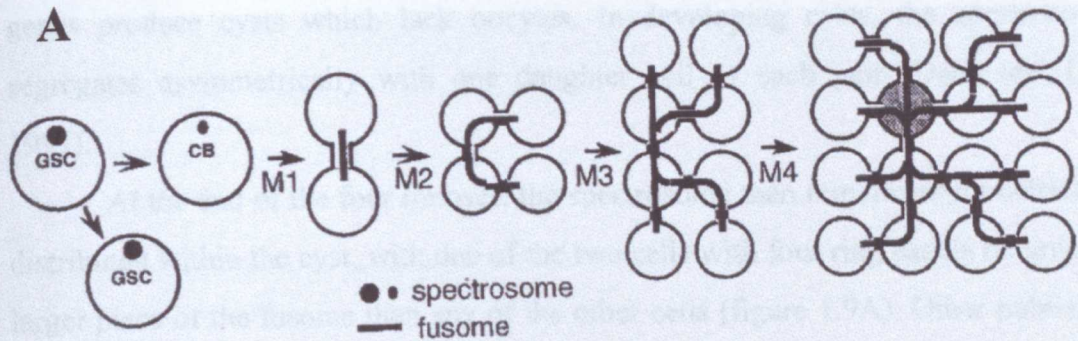
Oocyte determination and polarity

A: The fusome and polarisation of a microtubule network.

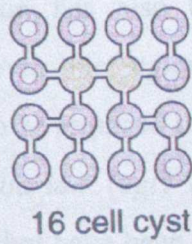
The fusome and its putative precursor, the spectrosome have been implicated in asymmetric germ cell division in the germarium. The spherical spectrosome has been observed in germ line stem cells, first observed in the oocyte, it develops into the large cytoplasmic fusome structure connecting individual cystocytes through ring canals. Fusomes and spectroosomes contain membrane skeletal proteins, like α and β spectrin, and are thought to be essential for proper cyst formation and oocyte determination. It is hypothesised that the fusome may act as a polarised cue to form a microtubule network for intracyst transport. Figure from Deng and Lin, 1997.

B: Polarisation and establishment of the A/P and D/V axes.

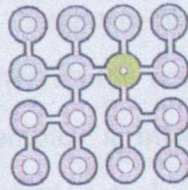
The A/P and the D/V axes become polarised due to a series of asymmetrical steps during oogenesis. (1) One cell in the 16 cell cyst is selected to become an oocyte (yellow shading). (2) By way of a microtubule network in the cyst, the oocyte moves to a posterior position. (3) A/P polarity is established when the oocyte nucleus signals via the gurken/EGFR pathway (white arrows) to establish the posterior follicle cells (red) which then directs movement of the oocyte nucleus to the anterior dorsal side of the oocyte. Gurken signals (white arrows) from the oocyte then induce dorsal follicle cell fate (green), polarising the D/V axis (4). Figure from González-Reyes *et al.*, 1997.



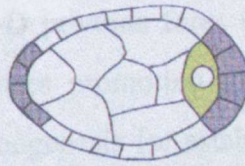
B



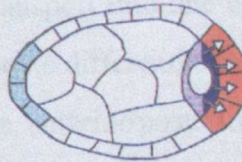
(1) selection of the oocyte



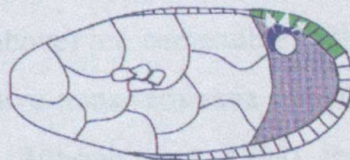
(2) Posterior positioning of the oocyte



(3) Polarisation of the AP axis of the follicle cell layer



(4) Polarisation of the DV axis of the follicle cell layer



genes produce cysts which lack oocytes. In developing cysts, the spectrosome segregates asymmetrically with one daughter cell of each pair (Deng and Lin, 1997).

At the end of the four mitoses, the spectrosome then remains asymmetrically distributed within the cyst, with one of the two cells with four ring canals retaining a larger piece of the fusome than any of the other cells (figure 1.9A). Other published data supports this theory of oocyte specification (de Cuevas *et al.*, 1997, Deng and Lin, 1997; Lin and Spradling, 1997).

A third model for oocyte specification has also been proposed. After formation of the 16 cell cyst when the cyst moves into region 2a of the germarium (figure 1.8 B), a number of genes are thought to be required in the oocyte in order to maintain its fate. *Bicaudal-D (Bic-D)*, *egalitarian (egl)* *cappucino (Cup)* and *Ovarian RNA binding protein (Orb)* are genes that have been shown to be expressed specifically in the oocyte at early stages (reviewed in Gonzalez-Reyes *et al.*, 1997; Wharton and Struhl, 1989). *Bic-D* and *egl* are thought to play a central role in the determination of oocyte and for the formation within the oocyte of an microtubule organising centre (MTOC). The MTOC assembles a microtubule network that extends to the other 15 cells and this polarised cytoskeleton later directs the accumulation of specific transcripts in the oocyte (reviewed in Mach and Lehman, 1997; Swan and Suter, 1996). *Egl* and *Bic-D* proteins form a complex which is thought to be required for transport of factors promoting oocyte differentiation (Mach and Lehman, 1997) and then later in oogenesis for establishing the anterior-posterior and dorsoventral axes of the embryo (section 1.4.2). This has been referred to as the 'transport' model where polarised transport into one of two central cluster cells in region 2 is thought to be crucial for oocyte differentiation.

Although it is well accepted that the anterior/posterior asymmetry in the oocyte is established by formation of a MTOC at its posterior pole neither the mosaic model (where one cell, the oocyte, possess factors different to the other 15 cells) or the transport model (described above) are compatible with mutants of the gene *egghead (egh)*, *egh* encodes a putative transmembrane protein, thought to be involved in germ cell-follicle interaction (Rübsam *et al.*, 1998). Presumptive nurse

cells can be seen to differentiate into an oocyte in certain *egh* alleles with no effect on spectrosome formation or on branching within each cyst. This suggests that neither model of oocyte specification is yet complete.

1.4.1.2 Enclosure of the 16 cell cyst.

At the same time the oocyte is beginning to differentiate, a layer of somatic follicle cells surrounds the germline cyst in region 2b of the germarium (Figure 1.8b; King 1970). The coordination of this migration depends upon communication between the germline and the somatic cells as well as between the somatic cells themselves. Genetic analysis has identified a number of genes which are involved in the development of the follicular epithelium. Two neurogenic genes, *brainiac* (*brn*) and *egh* are required in the germline (Goode *et al.*, 1992 and 1996; Rübsum *et al.*, 1998). *brn* acts in the germline to signal to the DER (encoded by the *torpedo* (*top*) locus) and this signal is required to establish and maintain a continuous follicle cell monolayer around each oocyte/nurse cell complex (Goode *et al.*, 1992). *Brn* also functions in the establishment of dorsal/ventral polarity by interacting with the TGF α homologue, Gurken (*Grk*), in the germline and with the DER in the somatic cells (Goode *et al.*, 1992, 1996). These functions are separable genetically. The product of the *egh* gene is also required for follicle cell migration and in *egh* mutant ovaries, pre-follicular cells do not build a continuous epithelium around each cystocyte cluster. Both *egh* and *brn* are not redundant and show an enhanced phenotype when double mutant with *top* alleles. This indicates a common role for both genes in DER signalling in the follicle cells which is required for correct egg chamber formation to occur (Rübsum *et al.*, 1998).

The proneural gene *da*, and the neurogenic genes *N*, *Dl* and *mastermind* (*mam*) are also required in the somatic cells for the enclosure of the germline cyst (Ruohola *et al.*, 1991). *N*, *Dl* and *Da* are all expressed in the prefollicular cells including the stalk cells, the collection of 4-6 cells which interleaf between each egg chamber in order to separate it from the previous one, indeed the *N/Dl* mutant displays defects in stalk cell formation (Xu *et al.*, 1992). It is thought that *N*, *Dl* and

da maintain the somatic cells in an uncommitted state and they are induced by signals such as the *hedgehog* (*hh*) gene or a signal from the germline cells, such as *brn* or *egh* (Ruohola *et al.*, 1991).

Another recently cloned coiled-coil protein, Toucan (Tuc), is also required in the germline and has been shown to be part of the signalling pathway controlling somatic cell migration (Grammont *et al.*, 1997). *tuc* has been genetically shown to interact with *da*, *N* and *Dl* in the formation of egg chambers. A P-element insertion mutant of *tuc* shows a decrease in the number of cells adopting the stalk cell fate and a delay in migration of follicle cells towards the oocyte later in oogenesis, the exact opposite of the phenotype observed in *brn* and *egh* mutant ovaries. One model proposed by Grammont *et al.* (1997) is that Tuc negatively regulates the activities of Brn and Egh proteins, although no dominant interaction has been shown between *tuc* and *brn* mutants.

The 16 cell cyst is surrounded by a layer of about 80 somatic follicle cells (Spradling, 1993). These begin to divide until there are approximately 1000 follicle cells, the cells then cease divisions and become polyploid. Subsequently, the oocyte associated cells (or stretch cells), at the posterior, then become columnar while the more anterior cells become squamous (Spradling, 1993; Lee and Montell, 1997).

1.4.1.3 Positioning of the oocyte.

As the cyst moves through the germarium into region 3 (figure 1.8B) to become a stage one egg chamber, the oocyte moves to achieve a posterior position, this is maintained throughout oogenesis (figure 1.9B). Positioning of the oocyte requires the function of the *spn* genes (section 1.4.1.1) as the oocyte in *spn* mutants can often be located at random positions in the cyst. The *egh* gene product is also required for correct positioning of the oocyte (Rübsam *et al.*, 1998). Cell adhesion is also important for oocyte positioning as mutations in *armadillo* (*arm*), an invertebrate homologue of the adhesive junction components plakoglobin and β -catenin, result in defects including disruption of the posterior positioning of the oocyte. Experiments suggests that certain mutations in *arm* affect follicle cell-germ

cell adhesion, resulting in a block in oogenesis producing small eggs which are never laid (Peifer *et al.*, 1993).

Consistent with this theory of cell adhesion positioning the oocyte, mosaic analysis has shown that the homophilic adhesion molecule, DE-cadherin, is required in both germline and somatic cells for correct oocyte localisation (Godt and Tepass, 1998). The oocyte selectively attaches itself to a set of DE-cadherin expressing follicle cells in a cell sorting process which depend on differential adhesion mediated by DE-cadherin (Godt and Tepass, 1998).

1.4.2 Egg Chamber development: Establishment of the Anterior/Posterior and Dorsal/Ventral axes.

Once the stage one egg chamber has been formed in the germarium. The egg chamber then undergoes further development in the vitellarium of the ovary. The two main body axes of the embryo, anteroposterior (A/P) and dorsoventral (D/V), are determined prior to fertilisation by the localisation of three main mRNAs, *oskar* (*osk*), *bicoid* (*bcd*), and *grk* to discrete localisation within the oocyte (reviewed in Morris and Lehmann, 1999). These are localised via a set of intercellular signals passed between the oocyte and follicle cells.

As it is well accepted as having a central role to play during oogenesis, the *DER* signalling pathway, can be seen as a focal point on which the two axes are set up. Sapir *et al.* (1998) suggest five different phases of *DER* activation which establishes the A/P and D/V axes through egg chamber development.

(A) Phase One of *DER* activation has a role in the early stages of oogenesis (section 1.4.1.2)

(B) Phase two occurs between stage 5-7 triggered by *Grk*, which is likely to be a ligand for the *DER* (Goode *et al.*, 1996; Neuman-Silberberg and Schüpbach, 1993 and 1996; Wasserman and Freeman, 1998; Twombly *et al.*, 1996). *grk* transcript is expressed at a position between the oocyte nucleus (positioned near the oocyte posterior) and the posterior of the oocyte (figure 1.9 B). *DER*, however, is expressed in all follicle cells but only activated in the posterior in a *grk* dependent

manner where it is responsible for the induction of posterior follicle cells, which then become distinct from the default anterior state maintained in all other follicle cells. The posterior follicle cells then signal back to the oocyte, where a reorganisation of a microtubule/microfilament system of the oocyte then ensues (Theurkauf *et al.*, 1992). This signal requires the activity of the *mago nashi* (*mago*) gene. *Mago* mutants display defects in the organisation of the microtubule cytoskeleton in the oocyte, although Grk localisation at the posterior is normal, and therefore fail to form the two body axes and cannot assemble pole plasm (Newmark *et al.*, 1997).

After repolarisation of the microtubule network in the oocyte the localisation of mRNAs, for example *bcd* and *osk*, along the A/P axes occurs (Grunet and St. Johnston, 1996; González-Reyes *et al.*, 1995; Roth *et al.*, 1995; Berlef *et al.*, 1988; Lehman and Nusslein-Volhard, 1986). *Osk* is an RNA binding protein involved in a signalling pathway which ultimately activates Nanos (Nos), a protein implicated in pole cell formation (Grunet and St. Johnston, 1996). The *vasa* (*vas*) locus, which encodes for a protein belonging to a family of a DEAD box RNA helicases (Tomancack *et al.*, 1997; Hay *et al.*, 1988) is thought to be involved in pole plasm assembly by regulating translation of *osk* and *nos* mRNAs (Lehman and Nusslein-Volhard, 1991). *osk* translation is also thought to be controlled by the Bruno (Bru) and Aubergine (Aub) proteins. Bruno response elements (BRE) have been identified in the 3'UTR of the *osk* transcript and are thought to provide a means of repressing *osk* translation until the transcript has been correctly localised (Webster *et al.*, 1997). Localisation of mRNAs into the oocyte is promoted by cis acting sequences in the 3'untranslated region (UTR) (Kim-Ha *et al.*, 1995; St Johnston, 1995) and in a number of cases such as *Bcd* and *K10*, it has been shown that RNA secondary structures present on the 3'UTR account for its action (St. Johnston, 1995). More recent work, however, indicates that the 5' coding sequence and not the 3'UTR, at least in some mRNAs, is involved in this microtubule-dependent transport and distribution of the RNA (Capri *et al.*, 1997). *Vas* has also recently been found to regulate *grk* translation during early oogenesis affecting A/P patterning (Styhler *et al.*, 1998). Interestingly, at least one BRE has been identified in the *vas* transcript,

that has been shown to bind Bru (Kim-Ha *et al.*, 1995). Vas and Bru may co-operate in the translational regulation of *grk* mRNA. The oocyte microtubular network also directs the migration of the oocyte nucleus to the anterior/dorsal cortex of the oocyte which is completed by stage 8-9 (figure 1.9 B).

(C) In the third cycle of DER activation, during stage 10 of oogenesis, *grk* expression at the oocyte nucleus this time induces a dorsal follicle cell fate (figure 1.9 B; Neuman-Silberberg, 1996). Levels of *grk* protein regulating D/V patterning are thought to be regulated by the Squid and K10 proteins (Tomancak *et al.*, 1998).

(D) In the fourth phase, these dorsal follicle cells then express *rhomboid* (*rho*) which appears essential for normal patterning of these cells (Ruohola-Baker *et al.*, 1993; Sapir *et al.*, 1998). Mutants of the *spitz* group (which include *spitz* and *star*) confirm the requirement of these genes in the follicle cells to generate a dorsalising signal. Rho is thought to process/activate the Spitz and the Argos proteins, then alters this signalling profile.

Cells which assume the default ventral state generate a ventralising signal mediated by the products of the *pipe* (*pip*), *windbeutel* (*wbl*) and *nudel* (*nd*) genes (Ray and Schüpbach, 1996) in restricting the domain in which Spätzle (Spz) is cleaved, in order to produce an active ligand of Toll (Tl). The current understanding of how the D/V pathway is established is shown in figure 1.10.

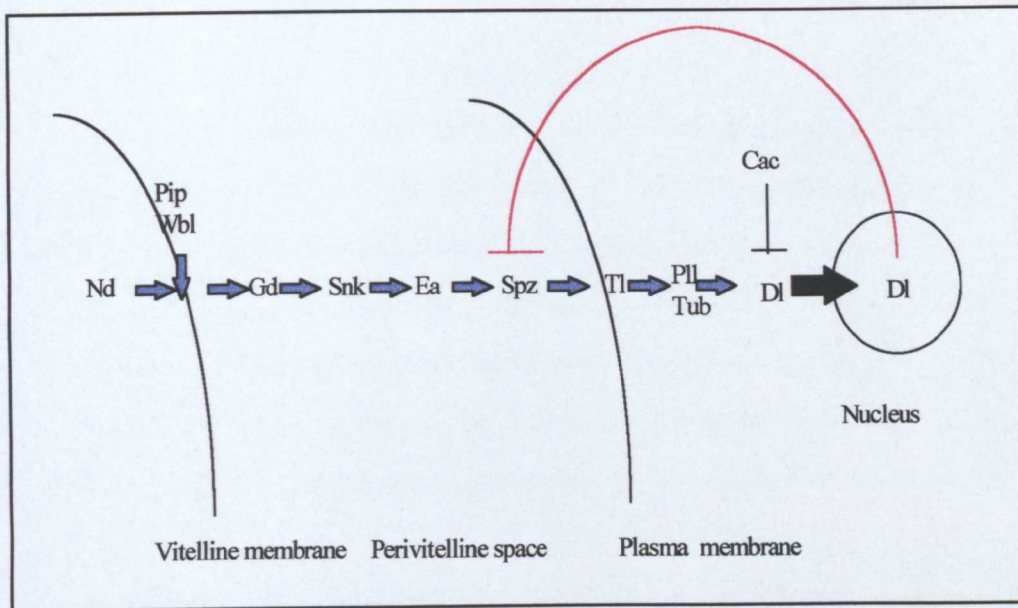


Figure 1.10

Dorsoventral patterning in the embryo.

The central component of the dorsoventral pathway is the transmembrane protein Toll (Tl). Toll is expressed throughout the embryo but only activated at the ventral side. Activated Toll triggers the formation of a nuclear concentration gradient of the NF- κ B/Rel-like transcription factor Dorsal (Df). Dorsal regulates a number of zygotic genes which specify cell fates along the D/V axis.

Activated Toll ligand is produced by cleavage from the protease Spätzle (Spz). Spz is activated by a cascade of proteases including Easter (Ea), Snake (Snk) and Gastrulation defective (Gd). Gastrulation defective has been shown to function upstream of Snake and Easter and may provide a link between clues in the follicle cells and the activation of these proteases. The Nudel protein (Nd) undergoes autoactivation and proteolytically cleaves Gd. The other two somatic components, Pipe (Pip) and Windbeutel (Wbl) act in parallel with Nudel in the cascade.

Data for this figure is taken from Ray and Schüpbach, 1996.

(E) At stage 10B, a cytoplasmic streaming occurs which disrupts the microtubular network. The final phase of DER activation occurs at stage 11-14. Once *rho* is induced, DER activation no longer requires accessibility to *grk* and the cells expressing *rho* from the cells of the dorsal appendages which continue to activate the DER pathway until dorsal appendage morphogenesis is complete.

Thus, there appears to be temporal separation between early Grk-mediated activation of the pathway and subsequent Rho-mediated activation (Sapir *et al.*, 1998).

In summary, an asymmetry established at the beginning of oogenesis defines the oocyte from a set of 16 germline cells in the germarium, the oocyte then accumulates specific factors necessary for its development. Enclosure of the cyst with a layer of somatic follicle cells (induced by interaction of germline factors such as Brn and Grk in the somatic cells) and positioning of the oocyte by cell adhesion molecules at the posterior, creates a stage one egg chamber. This progresses through the vitellarium, where the anterior/posterior and dorsoventral axes of the embryo are established mainly by components of the DER signalling pathway directing localisation of transcripts at the respective poles.

1.4.3 Signalling Cassettes

Two way cell-cell communication is a common theme in both neurogenesis and oogenesis, two systems in which cells must interact in a co-ordinate manner to produce different fates. This suggests the idea of signalling cassettes, where the same genes/signalling pathways can exist in different systems and have quite different results. For example, Notch is expressed both during oogenesis and neurogenesis, and functions as regulator of cell fate in both systems. A mutation can have a deleterious effect on neurogenesis, resulting in the conversion of too many cells to the neuroblast fate. An egg chamber from a *N* female, displays defects in stalk cell formation, suggesting *N/Dl* has a role in maintaining somatic cells in an

uncommitted state and they are later induced by signals such as *hedgehog* to differentiate. N therefore has different consequences in both developmental system but a common theme of controlling cell fate.

The proneural gene *daughterless* has also demonstrated a function during neurogenesis and oogenesis as well as many other systems (e.g. eye development) and can have different consequences in each (Bouzada *et al.*, 1996).

1.5 Transport across the nuclear membrane.

Homology searches using the Drongo amino acid sequence identified a number of homologues within different species. One of these proteins, the human hRIP/Rab, showed high degree of identity to the Drongo protein. hRIP/Rab has previously been implicated in the transport of molecules across the nuclear membrane and as a possible co-factor of the HIV-1 Rev regulatory protein. This indicates a potential role for the *drongo* gene. The co-factors of Rev have not yet been identified in human or in *Drosophila* where Rev has been shown to function (Ivey Hoyle and Rosenberg, 1990). The homology between hRIP and Drongo provides an appropriate reason to analyse whether Drongo is involved in interactions with Rev in *Drosophila* and in transport across the nuclear membrane.

The next section, therefore, is a review of the latest ideas in the field of nuclear transport. As this field covers an extensive amount of experimental work and because the homologues of Drongo are thought to be involved in RNA export from the nucleus, I will concentrate on this particular area. There will also be a short discussion about protein export from the nucleus as this is tightly linked with RNA export.

1.5.1 Nuclear transport: The problem.

In eukaryotic cells replication and transcription take place in the nucleus of the cell and translation takes place in the cytoplasm. This means proteins needed for nuclear functions must be imported back following translation and RNA/protein

complexes must also be shuttled out into the cytoplasm. The nuclear envelope, however, acts as a barrier to this transport. The envelope consists of an inner membrane component which is adjacent to the filamentous nuclear lamina structure and an outer membrane component continuous with the endoplasmic reticulum. Molecules of less than 90Å in diameter can cross the envelope by passive diffusion, but often molecules up to the size of 250Å have to enter the nucleus (Heese-peck and Räikhel, 1998).

Kinetic experiments have shown that macromolecular exchange occurs via nuclear pore complexes (NPCs). NPCs are large elaborate structures, composed of more than 100 different proteins and have an estimated molecular mass of about 125 million kDa. Extending from the outer surface are cytoplasmic filaments and from the inner surface, the nuclear basket. (Figure 1.11). The nuclear pore has previously been difficult to preserve for electron microscopy studies so 3D structures have been proposed from gold and dye labelling experiments (Heese-Peck and Raikhel, 1998; Feldherr and Akin, 1997; Grote *et al.*, 1995). More recently, field emission in lens scanning electron microscopy (FEISEM) has been used to complement and expand the current understanding of nuclear pore formation (Gant *et al.*, 1998). The role of the NPC in nuclear transport has subsequently been proposed.

Studies in *Xenopus* oocytes (Feldherr and Akin, 1997) have shown macromolecules enter the NPC via signal mediated transport through a 'central transporter' which contains a 'gate' that restricts passive diffusion. The opening of the NPC translocation channel involves a large conformational change to expand to a diameter greater than 25nm. Signal mediated transport through this central transporter involves several steps (1) initial binding of the transport substrate (cargo) to the cytoplasmic receptor on the pore, (2) association with the pore complex, and (3) translocation through the pore.

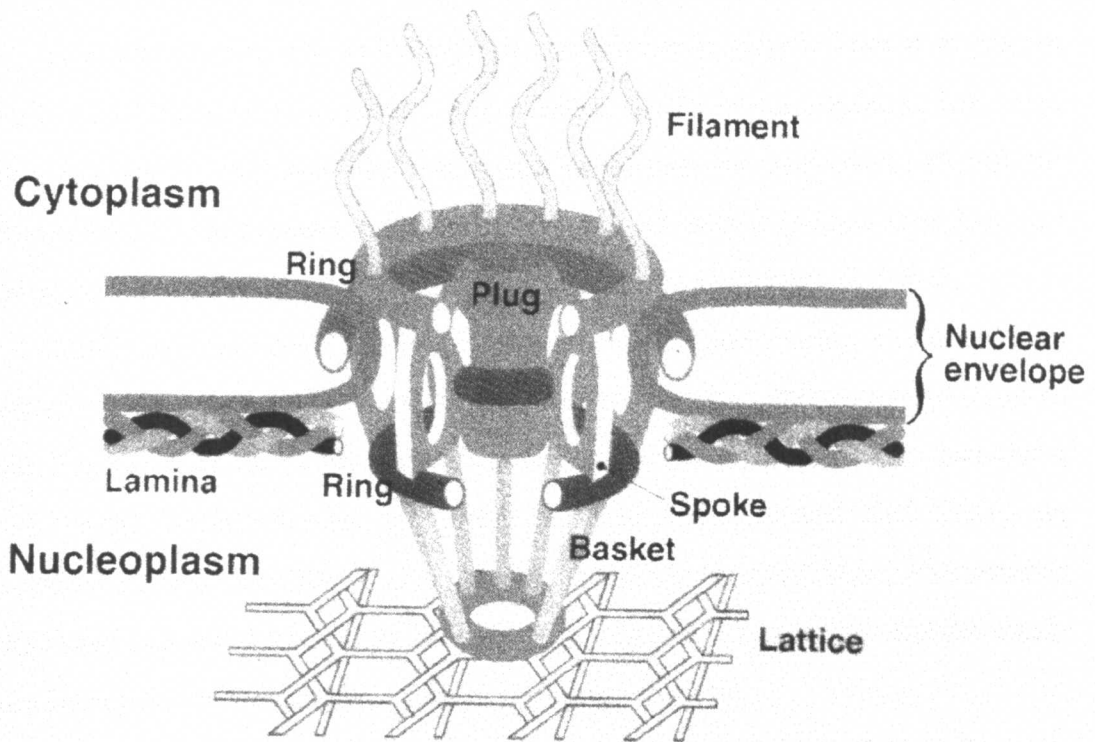


Figure 1.10
The nuclear pore complex.

The membrane spanning part of the nuclear pore complex consists of symmetrical arrangement of spoke structures. These form a ring which contains a central plug through which active transport occurs. Rings are a set of annular structures present on the cytoplasmic and nuclear side of the pore. The cytoplasmic ring extends 8 cytoplasmic filaments and the nuclear ring supports 8 fibres joined by a terminal ring, forming the nuclear basket. Although many studies has been carried out on this structure, it remains to be seen which aspects of the NPC are functionally important. Figure taken from Ohno *et al.*, 1998.

1.5.2 Model systems for nuclear transport.

In comparison to our knowledge on nuclear import, less is known at present about the mechanisms of export of macromolecules from the nucleus. The main approaches which have identified the components and the mechanism of nuclear transport are the retroviruses (often with the use of microinjection into *Xenopus* oocytes), and the yeast *Saccharomyces cerevisiae* (Cole and Hammel, 1998).

Retroviral replication requires the nuclear export and cytoplasmic translation of both incompletely spliced and fully spliced forms of the viral transcript (Cullen, 1998). For example the human immunodeficiency virus type 1 (HIV-1) encodes a set of “early” transcripts, the regulatory proteins Rev, Tat and Nef. These are encoded by fully spliced 2kb RNAs. The Rev protein is required then for expression of the “late” unspliced transcripts encoding the Gag and Pol proteins, and the singly spliced transcript encoding the structural glycoprotein Env.

The cellular machinery involved in transcription and translation, however repress the export of RNAs from the nucleus until they have been properly spliced by splicing factors residing in the nucleus, which recognise and define intronic sequences. The Rev protein has been shown to activate export of the viral mRNAs by either creating an alternative pathway or affecting the cellular splicing machinery. Recent experiments have disclosed many of the factors to which Rev binds and the possible mechanisms of how Rev functions (Pollard and Malim, 1998). These data in addition to studies of yeast mutants defective in transport of RNAs/import of proteins have suggested a number of models for nuclear transport, I will now discuss this work and the models put forward.

Rev was found to be a sequence specific nuclear RNA export factor following overexpression experiments in *Xenopus* oocytes (Fischer *et al.*, 1995). The sequence specificity is mediated by a *cis*-acting viral RNA stem-loop structure called the Rev Response Element (RRE), located in the *env* gene (Heaphy *et al.*, 1991; Jan and Belasco, 1996). Mutational studies suggest that Rev interacts via an arginine rich binding domain which also acts as an nuclear localisation signal (NLS). Rev is then thought to recruit other Rev molecules resulting in the formation of a

ribonucleoprotein complex (Pollard and Malim, 1998; Malim and Cullen, 1993). Further experiments revealed that Rev also contains a leucine rich nuclear export sequence (NES) (Fischer *et al.*, 1995) that can induce the efficient export of a linked substrate. The Rev NES is a prototype of leucine rich NESs, many of which have recently been observed in other viral and cellular proteins (Tiley *et al.*, 1991). Efforts to isolate a cellular cofactor that may mediate Rev activity by observing interactions of proteins in the yeast two hybrid assay, led to the identification of a human zinc finger protein hRIP/Rab (Bogerd *et al.*, 1995; Fritz *et al.*, 1995). RIP/Rab bears the hallmarks of a nucleoporin protein, a member of the class of proteins that make up the nuclear pore complex. The majority of nucleoporins contain a number of characteristic repeat sequences, GLFG and FXFG (where X is any amino acid) (Heese-Peck and Raikhel, 1998). Studies have shown these FG repeats are essential for the function of many nucleoporins and the repeats are likely to be involved in protein-protein interactions (Heese-Peck and Raikhel, 1998; Kasper *et al.*, 1999; Zolotukhin and Felber, 1999)

Overexpression of RIP/Rab can enhance (albeit modestly) Rev function *in vitro* (Fritz *et al.*, 1995). Two findings, however, shed doubt that RIP/Rab is the direct target for the Rev NES. Firstly, several other proteins have been subsequently found to interact with Rev NES with the same specificity as RIP/Rab (Fritz and Green, 1996; Stutz *et al.*, 1995) thus the interaction with RIP/Rab is not unique. Secondly, it proved difficult to observe an interaction between purified recombinant Rev and RIP proteins *in vitro*.

These findings suggested that the interaction between Rev and the nucleoporin may exist but are probably indirect and linked by other proteins. Two NES receptors have recently been identified. Crm1p/Exportin (chromosomal region maintenance protein), a highly conserved protein in yeast and human cells (Neville *et al.*, 1997; Stade *et al.*, 1997; Fornerod *et al.*, 1997) and CAS1 (reviewed in Ohno *et al.*, 1998). These proteins belong to the importin β group of nucleoplasmic transport factors, that are known to bind to nucleoporins. Crm1p mediates the export of proteins such as Rev and PKI (protein kinase A inhibitor) that contain leucine rich

NESs and CAS1 exports importin α , whose NES remains to be identified (Nakiely and Dreyfuss, 1997).

The activity of importin β , Crm1p and related proteins is thought to be regulated by the small monomeric GTPase Ran (Ras related nuclear protein), and in particular the GTP bound form of Ran (Dahlberg and Lund 1998; Moore and Blobel, 1993, 1994). The soluble transport factor p10/NFT2 has also been found to be required for import and also binds to Ran-GDP (Moore and Blobel, 1994; Paschal and Gerace, 1995). There is a high concentration of Ran-GTP inside the nucleus due to the cytoplasmic compartmentalisation of the Ran GTPase activating protein (Ran-GAP) and it's cofactor RanGAP binding protein 1 (RanBP1), conversely there is little RAN-GDP in the nucleus due to the nuclear compartmentalisation of Ran specific guanine nucleotide exchange factor (RCC1) (Mattaj and Englmeier, 1998; Shannon Moore, 1998). Receptor-cargo association is thought to be dictated by Ran during import of proteins and export of RNA (figure 1.12)

Although Ran does hydrolyse GTP via Ran GAP, it is thought contrary to earlier studies, that GTP hydrolysis is not required for import *per se*. It is more likely that the energy is used for recycling import components (Schwoebel *et al.*, 1998; Englmeier *et al.*, 1998; Nakiely and Dreyfuss, 1997).

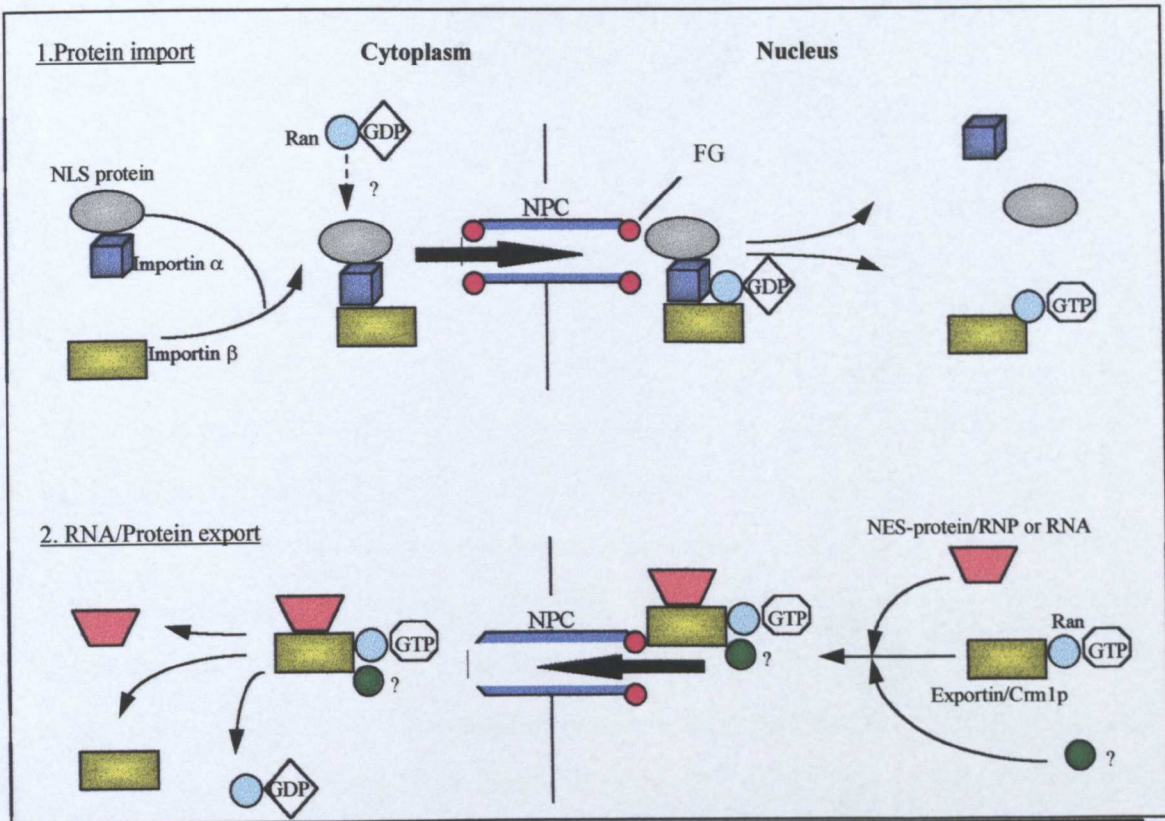


Figure 1.12.

A model for nucleocytoplasmic transport.

Import and export are controlled by the small GTPase Ran.

1: Import complexes form when Ran is in a GDP bound form (most of the Ran in the cytoplasm), cargo associates with importin, the complexes bind to importin β and is shuttled through the nuclear pore by sequential interaction of importin β (a family of transport factors of which the yeast Crm1p is a member) with several nucleoporins. After translocation, Ran converts GDP to GTP, the complex is released from the pore and dissociates.

2: Export receptor-cargo complexes are formed in the nucleus via association with Ran-GTP. A typical example of the cargo may be the HIV-1 Rev protein and associated RRE-RNA. This is then transported through the pore and is thought to be dissociated by hydrolysis of GTP, releasing the cargo into the cytoplasm and allowing the receptor to shuttle back to the nucleus. Signals for RNA export are thought to reside within the associated proteins.

Diagram based on one from Cole and Hammel (1998).

Key: NLS: nuclear localisation sequence; NES: nuclear export sequence; NPC: nuclear pore complex; FG: FG repeat containing nucleoporins.

The overall mechanism of Rev action (and thus the export of many NES-containing proteins) is thought to be as follows. In step one, after translation of Rev and import into the nucleus, multiple Rev molecules assemble onto the RRE and recruit several Ran-GTP bound Crm1p molecules to the RRE. In step two, Crm1p targets the ribonucleoprotein complex to the nuclear pore by interaction with the nucleoporins (probably including RIP/Rab). Translocation is thought to occur by the sequential interaction of Crm1p with several nucleoporins. Once the complex enters the cytoplasm, Ran GTP is hydrolysed to Ran-GDP by Ran GAP/RanBP1, releasing Crm1p from the Rev NES (step three). In step four, the HIV-1 incompletely spliced mRNA is then available for translation, while Crm1p and Rev are recycled back to the nucleus (reviewed by Cullen, 1998).

Import complexes are formed when Ran is absent or primarily in the GDP bound form (i.e. cytoplasmically located). The cargo binds to an importin α molecule (also known as Karyopherin α), this complex then binds to importin β (also known as Karyopherin β) and is shuttled through the nuclear pore via receptor interactions involving Ran. After translocation the complexes are dissociated by the conversion of Ran-GDP to Ran-GTP. Importin β is then recycled back to the cytoplasm by association with Ran GTP (Cole and Hammel, 1998).

Experimental evidence suggests there are 4 distinct pathways for the export of mRNA, tRNA, large ribosomal RNA, and 5S RNA/ U-rich small nuclear RNA (UsnRNA). Overexpression of Rev in *Xenopus* oocytes inhibits 5S RNA and small nuclear RNA (snRNA) export but not mRNA export (Fischer *et al.*, 1994) suggesting that the nuclear export pathway accessed by Rev is distinct from the cellular mRNA export pathway. Indeed, Fornerod *et al.* (1997) showed that a specific inhibitor of Crm1p, the drug leptomycin β , inhibits Rev but not mRNA export in eukaryotic cells.

In summary, the nuclear pore complex can be considered to represent the transporter which allows bidirectional traffic to occur between the nucleus and the cytoplasm. Both import and export involves the importin proteins (e.g Crm1p in yeast), the transport cargo (e.g mRNA/Rev protein) and the small GTPase Ran. Models have been compiled from studies such as the HIV-1 Rev dependent transport of unspliced and partially spliced viral mRNA transcripts and studies of mRNA export and protein import in the yeast *S. Cerevisiae*. These studies have defined many of the transport mechanisms involved in these systems yet there are still many protein factors to be discovered.

1.6 Aims of the study.

1) To overexpress the gene in a number of developmental systems and analyse any abnormal phenotype.

drongo has been shown to be expressed during neurogenesis, oogenesis and early eye development. At the onset of this work, I did not have a mutant of the *drongo* gene and so I was unable to analyse the phenotype. In the absence of a mutant, I therefore intended to produce a phenotype from ectopic expression of the gene and suggest a possible role for *drongo*.

2) To analyse the effect of Drongo on Rev activity and assay the involvement of the Drongo protein in nuclear transport.

As well as establishing a mutant phenotype it would be interesting to study the role of the protein in the cell directly.

Drongo shows extensive sequence similarity at the amino acid level to the hRIP protein, a putative co-factor of the HIV-1 Rev protein. As further analysis of the role of the gene it would be interesting to observe if Drongo could function, in a similar way to hRIP, with Rev to transport an unspliced reporter construct from the nucleus. It would also be interesting to observe which regions of the gene, if any, are involved in the function of Drongo in the cell.

3) To induce mutations in the gene and study the mutant phenotype.

One of the best ways to deduce the wild type function of the *drongo* gene is to analyse the mutant phenotype. This would be performed with a mutagenesis screen of the second chromosome with the possibility of identifying a mutant *drongo* allele(s).

4) Expression of the Drongo protein in *E.coli* and production of an antibody to the Drongo protein.

Expression of the Drongo protein in a prokaryotic system may enable production of enough pure protein to produce an antibody to Drongo. It would also allow analysis of the properties of the protein such as size and solubility. After analysis of the expressed protein, a pure sample of protein would be used to raise antibodies to the Drongo protein. An antibody could be used to look at the localisation of the protein during development and its localisation in the cell. This could offer valuable information about the function of the protein.

Chapter 2

Materials and Methods

2.1 Maintenance of fly stocks.

2.1.1 Preparation of food.

2.1.1.1 Standard Fly food.

Fly stocks were maintained at 18°C or 25°C in bottles or vials of standard food. Standard food was prepared as in Ashburner (1989).

All fine chemicals used for fly food or otherwise were obtained from Gibco BRL unless otherwise stated.

2.1.1.2 Grape juice agar plates.

Grape juice agar plates, used for egg collections, were prepared according to Roberts (1986). Plates were stored in the refrigerator. Before use, plates were warmed in the microwave for 3 seconds and a small amount of yeast paste was placed on each plate.

2.1.2 Fly stocks used.

| Stock | Details | Assigned chromosome. | Used for | Source |
|------------------|--|---------------------------------------|--|------------------|
| WCS | White Canton S (essentially wild type stock) | ----- | Overexpression experiments and mutagenesis | laboratory stock |
| hs-drongo 14M3 | <i>drongo</i> cDNA in hs (CaSpeR). | 3 rd . Balanced over TM6B. | Overexpression of <i>drongo</i> | laboratory stock |
| hs-drongo line 1 | <i>drongo</i> cDNA in hs (CaSpeR) | 1 st . balanced over FM7. | Overexpression of <i>drongo</i> | laboratory stock |
| hs-drongoR | <i>drongo</i> cDNA in hs (CaSpeR) | 2 nd . Balanced over CyO | Overexpression of <i>drongo</i> | laboratory stock |
| UAS- | <i>drongo</i> cDNA in | 3 rd . Balanced | Overexpression | laboratory stock |

| | | | | |
|--------------------|---|---------------------------------------|---------------------------------|--------------------------|
| drongo2M5 | pUASt | over TM6B. | of <i>drongo</i> | |
| UAS-drongo2F1 | <i>drongo</i> cDNA in pUASt | 1 st . balanced over FM7. | Overexpression of <i>drongo</i> | laboratory stock |
| UAS-drongoΔS13M6 | deleted <i>drongo</i> cDNA in pUASt | 3 rd . Balanced over TM6B. | Overexpression of <i>drongo</i> | laboratory stock |
| UAS-drongoΔS35M3 | deleted <i>drongo</i> cDNA in pUASt | 2 nd , balanced over CyO. | Overexpression of <i>drongo</i> | laboratory stock |
| P{GAL4-twi.G} | GAL4 line expressed in twist pattern | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| P{ GAL4-ninaE.GMR} | GAL4 line expressed behind morphogenetic furrow in the eye. | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| P{ GAL4-dpp.blk1} | GAL4 line expressed in a decapentaplegic pattern. | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| P{GAL4-arm.S} | GAL4 line expressed in a armadillo pattern | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| P{GawB}h[1J3] GAL4 | GAL4 line expressed in a hairy pattern. | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| P{GawB}69B GAL 4 | GAL4 line expressed in the embryonic epiderm. | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| P{ GawB}ptc-GAL4 | GAL4 line expressed in a patched pattern. | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| P GAL4-twi.2xPE} | GAL4 line expressed in a twist pattern. | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| P{GAL4-ve.NEE} | GAL4 line expressed in the | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |

| | | | | |
|--|---|-------|------------------------------------|-----------------------------|
| | embryonic neural ectoderm | | | |
| P{GawB}md509 /CyO; MKRS/TM2 | GAL4 line expressed in a caudal pattern | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| GAL4 P{GawB}bi | GAL4 line expressed in a bicaudal pattern. | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| P{ GAL4- Hsp70.PB}31- 1/T(2;3)B3 | GAL4 line expressed in the embryonic CNS and PNS. | ----- | Overexpression of <i>drongo</i> | Bloomington stock Centre |
| Df(2L) <i>ast4</i> | Contains deficiency on 21D Breakpoints: 21D1- 21D2:21E2 | ----- | Screening EMS mutants. | Bloomington stock Centre |
| Df(2L) <i>ast6</i> | Contains deficiency on 21D Breakpoints: 21E1- 21E2:21E2- 21E3 | ----- | Screening EMS mutants. | Bloomington stock Centre |
| Df(2L) <i>S2</i> | Contains deficiency on 21D Breakpoints:21C 3-21C4:22A2- 22A3 | ----- | Screening EMS mutants. | Bloomington stock Centre |
| Df(2L) <i>ast2</i> | Contains deficiency on 21D Breakpoints:21D 1-21D2:22B2- 22B3. | ----- | Screening EMS mutants. | Bloomington stock Centre |

Table 2.1

Fly stocks used in this study.

2.2 DNA manipulations.

2.2.1 Agarose gels.

Agarose gels used for DNA analysis were prepared using 1X TBE (10.8g tris, 5.5g boric acid, 4mls of 0.5M EDTA per 1l water) adding 0.8% agarose. Samples were mixed with DNA loading buffer (Sambrook *et al.*, 1989). The gel was then run at between 50-200mV until the DNA fragments had migrated far enough to be separated and 1kb ladder (Gibco BRL) was used to observe size separation. Ethidium bromide (0.5µg/ml) was added in order to visualise the DNA on a U.V. light box.

2.2.2 L.B. Agar Plates and L.B. cultures.

Bacterial cells were plated out on LB (Luria Bertani broth) agar plates. Per litre of LB agar, 10g bacto-tryptone, 5g of bacto-yeast extract, 10g NaCl, 10g of bacto-agar, 1l of water were mixed and the solution was autoclaved at 12 lbin² for 20 minutes. LB (same ingredients without the agar) was used as media for bacteria cells. Ampicillin/chloramphenicol was used at a final concentration of 50 µg/ml.

2.2.3 Large and Small scale preparation of plasmid DNA.

Plasmid DNA to be purified was excised from an agarose gel and cleaned with either a GeneClean kit (BIO 101) or a DNA extraction kit (Qiagen).

DNA minipreps were performed using Qiagen Qiaprep plasmid minipreparation kit. Large scale preparations were performed using Qiagen Qiaprep Plasmid Maxipreparation kit, according to manufacturer's instructions.

2.2.4 Restriction digestions.

DNA was routinely cut with restriction enzymes according to the manufacturer's instructions. Generally DNA was cut in a volume between 10 and 100 μ l reaction mix containing 1x reaction buffer (the relevant reaction buffer used for a particular enzyme) and 1/10th the volume of restriction enzyme. All digests were performed at 37°C for between 2 hours and overnight except for *Sma* I (25°C). Restriction enzymes and buffers were supplied by Gibco BRL or Amersham.

Restriction digests were terminated by separation on an agarose gel (2.2.1) and then cleaned via gel extraction kit (Qiagen), according the manufacturer's instructions.

2.2.5 Ligation of DNA fragments.

Ligations were carried out using T4 DNA ligase (Gibco BRL) according to manufacturer's instructions. A ratio of 1:3 vector to insert was used in ligation reactions. Approximately 250ng of vector DNA was used. For blunt ended ligation samples were incubated at room temperature for 4 hours and for sticky ended ligation, samples were incubated for 2 days at 4°C.

Vector cut with a single enzyme was de-phosphorylated with Bacterial alkaline phosphatase (BAP: Amersham life sciences). For de-phosphorylation reactions 1 μ l of enzyme was used and the reaction was placed at 37°C for 1 hour.

DNA was quantitated by spectrophotometry (using a Ultraspec III machine (Pharmacia Biotech)) and 5 μ l was run on an agarose gel.

2.2.6 Transformation of *E.coli* cells with DNA.

| Strain | Genotype | Source. |
|--------------|--|--|
| DH5 α | <i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> | Laboratory stock. |
| BL21 (DE3) | <i>hsdS gal</i> (λ <i>Its857 ind1 Sam7 nin5</i> <i>lacUV5-T7 gene 1</i>) | <i>Pers.Comm.</i> Lauren Porter. |
| XA90 | Δ <i>lacZ</i> Pr08 <i>argEam ara⁻ nalA thi⁻ rif^R</i> F' <i>lacI^q lacZY⁺ proAB⁺</i> | <i>Pers.Comm</i> Stuart Allen |

Table 2.2:

E.coli strains used

Bacteria were transformed according to this section. DH5 α , BL21 and XA90 cells were used for protein expression experiments (section 2.9.1). DH5 α cells were used for all other cloning experiments unless otherwise stated.

DH5 α cells were transformed with DNA unless otherwise stated. All cells used for transformations were made competent using the calcium chloride method (Sambrook *et al.*, 1989). Competent cells were stored at -70°C. Glycerol stocks were prepared of all transformed cultures used, i.e. 0.5 mls of 50% glycerol were added to 0.5 mls of culture and the cells were then frozen rapidly on dry ice and then stored at -70°C. Taken from Sambrook *et al.* (1989) and personal communication with Stuart Allen.

2.3 Double stranded DNA sequencing.

2.3.1 Preparation of DNA for sequencing.

Double stranded plasmid DNA to be sequenced was prepared using a Quiagen Maxi-prep kit, according to the manufacturer's instructions.

DNA was either sequenced on an automated sequencer (Perkin Elmer ABI PRISM 377 DNA sequencer) or by hand using a Sequenase Version 2.0 kit (Amersham life science), following manufacturer's instructions.

5-10 μ g of DNA template were used for each reaction in 20 μ l of water. DNA was denatured by adding 250 μ l of 5M NaOH, 2 μ l of 0.5M EDTA, 248 μ l of distilled water and incubated at room temperature for 5 minutes. DNA was precipitated by adding 3 μ l of 3M NaAc, 8 μ l of 1M Tris-HCl pH 7.0, 75 μ l of ethanol and placed on dry ice for 30 minutes and the template pellet was resuspended in 7 μ l of water. 10 μ Ci of S³⁵ dATP and 1pmole/ μ l of primers were used per reaction and the reaction carried out according to the manufacturer's instructions.

The following primers were used for sequencing the *drongo* deletion constructs:

For pGEX-*drongo* ΔS (see section 2.9.1):

Primer # 857: 5' TCATCGCAGCAGCAGGA 3'

and for UAS-*drongo* ΔF (see section 2.6.4):

Primer # 1493: 5' CCAACTCGTCGAGTAAC 3'

For sequencing the 3' end of *drongo* cDNA for correct sequence.

Bottom strand:

Primer # 1b: 5' GATAGCGTTCTTATTTATTT 3'

Primer # 2b: 5' GTCATGCATTCGGGGTTC 3'

Primer # 3b 5' GGCCACACAATCATTAAGT 3'

Primer# 4b 5' CGAGCAGGTAAGTTCGTC 3'

Top Strand:

Primer #1a: 5' CGATGGCCACATTCACAC 3'

Primer # 2a: 5' GCTCCCATCTACAATGCAG 3'

Primer # 3a: 5' GGATCGATATGCCGCT 3'

Primer # 4a: 5' CATACCAAATGCCTACGG 3'

Primers used for sequencing pTk34 cDNA (section 2.7.4):

SV40 promoter: 5' CCTGATTGGCAGAACTACAC 3'

SV40 promoter and splice site 1: CTCGGCCTCTGAGCTATTCC 3'

CAT gene; 5' GACGGTGACCTGGTGATATG 3'

5' AGCGGAGGCTAG AAGGAGAG3'

5' GACGGTGAGCTGGTGATATG3'

5' AAGGCGACAAGGTGCTGATG 3'

splice site 2

and 3: 5' TCCATGTTCGGCAGAATGCTT 3'

Env gene

(and RRE): 5' GGCAGGAAGAAGCGGAGACA 3'

5' CATGTGGCAGGAAGTAGGAA 3'

5' GAGCAGCAGGAAGCACTATG 3'

splice site 4: 5' ATAGTAGGAGGCTTGG 3'

Primers were manufactured by Genosys or *pers. comm.* Stephen Harris.

2.3.2 Sequencing gels.

0.4 mm thick sequencing gels were poured and used in a 40cm sequencing tank.

6% acrylamide gels were prepared (9:1 ratio of acrylamide:NN-methylbisacrylamide; 1x TBE; 47% Urea (w/v)). To polymerise the reaction, 70µl of TEMED and 800µl of 10% ammonium sulphate were added. Gels were run in 1 x TBE at 1000-1500 volts, fixed in 10% (v/v) methanol: 10% acetic acid and vacuum dried at 80°C before being autoradiographed.

2.4 Whole mount *in situ* hybridisation

2.4.1 Preparation of Dig labelled RNA probes.

Probes to be used for *in situ* hybridisations were prepared by a modified version of the protocol for the synthesis of Digoxigenin labelled probes using a Dig RNA labelling kit. (Boehringer Mannheim), according to manufacturer's instructions.

Templates used for Dig labelling transcriptions were prepared by linearising with restriction enzymes for 1-2 hours at 37°C. For probes used for *in situ* hybridisation see table 2.3

| Clone name. | Cloning Details. | Digested with | Transcription details. | Probe . |
|-----------------------|---|-----------------|---------------------------------|---------------------------|
| pBS- <i>drongo</i> | <i>drongo</i> ORF cloned into pBluescript via <i>Eco</i> RI sites. | <i>Xho</i> I | Transcribed with T7 polymerase | antisense <i>drongo</i> . |
| pBS- <i>drongo</i> | <i>drongo</i> cDNA cloned into pBluescript via <i>Eco</i> RI sites. | <i>Xba</i> I | Transcribed with T3 polymerase | sense <i>drongo</i> . |
| PBN40- <i>oskar</i> . | <i>Oskar</i> cDNA cloned into pBN40 | <i>Hind</i> III | Transcribed with T7 polymerase | antisense <i>oskar</i> . |
| PBN40- <i>oskar</i> . | <i>Oskar</i> cDNA cloned into pBN40 | <i>Not</i> I | Transcribed with SP6 polymerase | sense <i>oskar</i> . |

Table 2.3

Probes produced for *in situ* hybridisations.

RNA probes were acid hydrolysed (section 2.4.2) before use. Antisense *drongo* and sense *drongo* were used for *in situ* hybridisation to ovaries (section 2.4.4) and embryos (section 2.4.3), antisense and sense *oskar* were used for *in situ* to ovaries.

After linearisation, 2µl of each reaction was run on an agarose gel to check for complete digestion, the DNA was extracted and cleaned with phenol/chloroform, the upper phase was precipitated with ethanol as described by Sambrook *et al.*, (1989) and resuspended in 20µl of dH₂O. RNA was transcribed as described in the manufacturer's instructions.

The probe was precipitated by adding 80µl of distilled, deionised water, 20µl of 4M LiCl and 360µl of 100% ethanol. The probe was incubated on dry ice for 15 minutes and centrifuged in a bench top centrifuge at 13,000 rpm. The RNA probe was then resuspended in 100µl of H₂O and used as a 100 x stock. Before use RNA probes were acid hydrolysed according to the method in section 2.4.2. The labelling of the probes was also validated by dot blot testing, making dilutions of each probe from 1/10 to 1/10000 and dotting 2.5µl onto nylon membrane (Hybond N, Amersham). Filters were then dried and fixed DNA side down on a U.V. transilluminator for 3 minutes. Subsequent washing, antibody incubation and colour reaction steps are essentially as described in the manufacturer's instructions.

2.4.2 Acid hydrolysis of probes.

100µl of 0.2M NaHCO₃ were added to the precipitated RNA probe were added and the probe was heated to 60°C for t minutes as calculated by the formula below:

$$t \text{ (minutes)} = \frac{L_o - L_f}{(0.11)(L_o)(L_f)}$$

Where L_o= original length of the probe in kb

and L_f= desired length in kb (Usually between 0.3- 0.15 kb)

The probe was then precipitated with 20µl of 5% acetic acid, 20µl of 3M NaAc, 500µl 100% ethanol and incubated on dry ice for 15 minutes and then centrifuged at 13,000 rpm for 15 minutes in a microfuge.

Immediately before adding the probe it was heated to 95⁰C for 3 minutes in a heating block and allowed to cool on ice for 5 minutes.

2.4.3 *In situ* hybridisation of RNA probes to wholemount *Drosophila* embryos.

2.4.3.1 Embryo Collection and pretreatment.

Embryos were collected, dechorionated and devitellinsed as described in Roberts (1986).

2.4.3.2 In situ hybridisation.

For *in situ* hybridisation to embryos the protocol is that described in Tautz and Pfeifle (1989) with the following additions. The embryos were incubated in anti-Dig antibody (Dig-RNA kit, Boehringer Mannheim) at 1/2000 (diluted in PBT) for 2 hours at room temperature.

The antibody conjugate was then removed and the embryos were washed for 2 minutes in PBT, 10 minutes in PBT, 3 x 20 minutes in PBT.

2.4.4 *In situ* hybridisations of RNA probes to wholemount *Drosophila* ovaries.

In situ hybridisations to wholemount ovaries were carried out using essentially the same method as for embryos . The ovaries were dissected out of young females in 1x Robbs buffer (55mM NaAc; 40mM Kac; 100mM sucrose; 1.2mM MgCl₂ ;1mM CaCl₂; 100mM HEPES (pH 7.4) and then fixed in 4% formaldehyde; 0.1% D.M.S.O. in PBT for 1 hour.

The ovaries were then dehydrated through an ethanol series (50%, 70%, 90%, 90%, 100%, 100%) then transferred to methanol. Ovaries were then rehydrated in the same manner as embryos and following this step, the steps up to mounting were identical to *in situ* hybridisations to embryos except that the ovaries were incubated in Proteinase K (Boehringer Mannheim; 14.4µg/ml) for 1 hour at room temperature and the ovaries washed overnight in PBT after addition of the anti-Dig antibody. The ovaries were not

dehydrated before mounting but mounted in gelvitol (90% (v/v) glycerol; 50mM Tris-HCl (pH 7.5); 1%(w/v) propyl trihydroxy benzoate) under a supported coverslip.

2.5 Whole mount antibody staining.

2.5.1 Antibody staining to *Drosophila* embryos.

| Primary. (dilution used) | Source. | Tissue used in staining. | Secondary. (Dilution used) | Detection method. |
|---|---|-----------------------------|---------------------------------|----------------------|
| Mouse Even-Skipped (1/250) | Develomental Studies Hybridoma Bank (Described in Doe <i>et al.</i> , 1988) | Embryos. | HRP- Goat anti mouse. (1/250) | DAB staining. |
| Mouse anti-fasciclin III (1/10) | DSHB (described in Goode <i>et al.</i> , 1996) | Ovaries | HRP- Goat anti mouse. (1/250) | DAB staining. |
| Rabbit anti-Drongo (section 2.10.1/2) (1/10-1/250). | Made in laboratory. | Ovaries/ Embryos. | HRP- Goat anti-rabbit. (1/250) | DAB staining. |
| Mouse anti-Engrailed. (1/100) | DSHB (described in DiNardo <i>et al.</i> , 1985) | Embryos. | HRP- Goat anti mouse. (1/250) | DAB staining. |
| BP102 (mouse anti-CNS axons) (1/250) | DSHB | Embryos. | HRP- Goat anti mouse. (1/250) | DAB staining. |
| Rat anti-Gurken. (1/500) | <i>Pers.comm</i> with Trudi Schupbach. (Described in Neuman-Silberberg and Schupbach, 1996) | Ovaries | Rhodmine-Goat anti-rat. (1/250) | N/A. |
| Rabbit anti-Oskar. (1/2500) | <i>Pers.comm.</i> Clare Hudson. | Ovaries. | HRP-Goat anti-Rabbit. (1/250) | DAB staining. |

Table 2.4

Antibodies used for antibody staining to ovaries and embryos.

For antibody staining to embryos, embryos were pretreated (section 2.4.3.1) and antibody stained according to this section. Ovaries were antibody stained according to section 2.5.2.

(DSHB: Developmental Studies Hybridoma Bank).

Antibodies were used to localise proteins in embryos, most antibody staining was carried out according to the method below except for Even-skipped antibody staining which was carried out according to the method from Doe *et al.* (1988).

Embryos were collected and pretreated as in method 2.4.3.1. Embryos were incubated in blocking solution (10% BSA with 3% normal goat serum (Vector) for 30 minutes and then in primary antibody (see table 3) overnight at 4°C. Embryos were then washed in PBT (PBS + 0.1% Triton X-100) for 3 x 3 minutes and 3 x 20 minutes. Embryos were incubated in secondary antibody diluted in PBS (see table 3) for 4 hours at room temperature.

After subsequent washes for 3 x 3 minutes and then at 3 x 20 minutes in PBT, embryos were incubated in ABC solution (16µl of solution A and 16µl of solution B from Vectastain kit, Vector laboratories) for 30 minutes. Embryos were washed in PBT for 3 x 20 minutes and incubated in DAB reagent (10ml 0.1M Tris-HCl pH 7.5, 0.1mls DAB at 50mg/ml, 12µl of H₂O₂) until colour developed. Embryos were then washed, dehydrated, cleared in Histoclear and mounted in Canada Balsam (as in method 2.4.3).

2.5.2 Antibody staining to ovaries.

Ovaries were dissected in Robbs buffer (as in section 2.4.4), and rinsed in PBS. Ovaries were then fixed for 1 hour in 4% formaldehyde containing 0.1% DMSO.

After rinsing 3x 20 minutes in PBT, ovaries were blocked for 15 minutes in 10% BSA + 3% normal goat serum. Subsequent steps were identical to antibody staining to embryos (section 2.5.1). Ovaries were mounted in gelvitol on a supported coverslip. For staining with Gurken antibody, the above method was used with the following exceptions, 2% formaldehyde was used for fixing.

2.6 Overexpression of transgenes in *Drosophila* tissues

2.6.1 Production of transgenic flies.

WCS embryos were collected in large laying cages (according to Roberts, 1986). Embryo injections were performed essentially as in Roberts (1986) collecting embryos at hourly intervals. 250ng/μl of helper plasmid (pπΔ2-3; Roberts *et al.*, 1986) was co-precipitated with the DNA to be injected. DNA was injected at 1μg/μl. After injection, embryos were placed at 18°C until hatching occurred and then the larvae were placed on standard fly food to develop to adulthood.

2.6.2 Mapping of insert in transgenic stocks.

Inserts were mapped by genetic crossing and then placed over a suitable chromosomal balancer (table 2.1).

2.6.3 Overexpression by heat shock and analysis of effects.

drongo cDNA was cloned into pP(CaSpeR-hs) via *EcoR* I restriction sites which placed the cDNA under the control of an inducible heat shock promoter (*hs-drongo*). The cDNA was also cloned into the same vector in a reverse direction. This construct was named *hs-drongoR*. Transgenic flies were produced by the embryo injection method described in section 2.6.1.

2.6.3.1 Overexpression during oogenesis.

Female adults were collected as virgins, conditioned with males for 3 days and then exposed to a 30 minute heat shock at 37° C. This was achieved by placing approximately 30 females in a food vial into a 37°C water bath. Heat shocks were repeated three times daily for three days and the ovaries were dissected one to three days post heat shock for analysis.

Ovaries were dissected in Robbs buffer, teased apart with fine forceps and fixed for 10 minutes in 3.7% formaldehyde in PBS. After washing in PBS the ovaries were incubated in 0.5 mls Hoechst # 33258 (Sigma: 1µg/ml in PBS) for 4 minutes. The ovaries were washed two times for 10 minutes in PBS and mounted in Gelvitol.

2.6.3.2 Overexpression during eye development.

Approximately 20 third instar larvae were placed into a small sealed petri dish and lowered into a 37°C water bath using a weight to hold the dish under water. The larvae were heat shocked for either 15, 30, 60 or 90 minutes and then placed into a food vial at 25°C until they emerged as adults. Adult eyes were then examined after eclosion.

2.6.3.3 Overexpression during embryogenesis.

Embryos were collected from grape juice agar plates (see section 2.1.1.2) in laying cages and placed into embryo collecting baskets. Embryos were submerged into a prewarmed 5% saline solution at 37°C for either 15, 30, 60 or 90 minutes and fixed and mounted at 7 hours of age for observation.

2.6.3.4 Overexpression during late oogenesis.

Analysis of heat shock effects during late oogenesis was performed by heat shocking female flies for 30 minutes at 37°C twice daily for three days. The eggs were collected from the females 2-18 hours after the final heat shock and analysis of the embryo cuticle was performed by hand removal of the chorionic and vitelline membrane as

described in Roberts (1986). Embryos were viewed using dark field view down a Zeiss Axioskop light microscope.

For all overexpression experiments WCS flies were subjected to the same heat shock conditions as *hs-drongo* and analysed identically as a control.

2.6.4 Overexpression of *drongo* by UAS-GAL4 system.

For restriction maps of vectors see appendix 10.1

drongo was overexpressed as a full length cDNA and as various deleted fragments. The *drongo* cDNA was cloned into pUAS_T vector, by linearising pUAS_T with *EcoR* I and excising *drongo* cDNA from pBS-*drongo* with *EcoR* I. The resulting fragments were ligated to produce the clone designated UAS-*drongo*.

The *drongo* cDNA fragment in pGEX-5X-1- *drongo* Δ S (prepared as described in section 2.9.1) was excised using *Not* I and *EcoR* I. pUAS_T was also digested with *Not* I and *EcoR* I. The two fragments were then ligated. The resulting construct was designated UAS-*drongo* Δ S.

UAS-*drongo* was also digested with *Fsp* I and nucleotides 1-970 and 1205-3493 were religated and this construct was designated UAS-*drongo* Δ F.

Transgenic flies were produced containing UAS-*drongo*, and flies were also produced which contained deletion constructs UAS-*drongo* Δ S and UAS-*drongo* Δ F as described in section 2.6.1.

2.7. Drosophila cell Culture

| Cell line | Details | Source/reference |
|-----------|--|---|
| Dh33 | <i>Drosophila hydei</i> embryonic cells. Grown as a monolayer culture. | Saunders <i>et al.</i> , 1989. <i>Pers. comm.</i> Susan Saunders, University of Sussex. |
| S2 cells | <i>Drosophila melanogaster</i> Oregon R embryonic cells. Female cells, 80% tetraploid. Grow partly in suspension . | <i>Pers.Comm.</i> Javier Vinos. MRC LMB, Cambridge. |

Table 2.5

Insect tissue culture cells lines used in this study

Cells were passaged 2.7.3 tranfected according to section section 2.7.5.

2.7.1 Preparation of cell culture media.

Drosophila Schneider's Insect Medium (Gibco) was used throughout the cell culture experiments. Before use of the media, 50 mls of sterile foetal bovine serum (Sigma), heat inactivated at 65°C for 30 minutes; 5 ml of penicillin-streptomycin solution (Sigma: 5,000 units penicillin; 5mg/ml of streptomycin); and 1ml of 200mM filter sterilised Glutamine were added to a 500 ml bottle of sterile media. All glassware for use with cell culture was sterilised at 180°C for 20 minutes before use and plastics cleaned with ethanol before use.

2.7.2 Freezing cell culture cells.

Before proceeding with cell culture work. Back up samples were frozen by the following method. The best results were obtained when cells were frozen slowly and thawed quickly.

Freezing medium (80% (v/v) media; 10% FCS, 20% DMSO) was prepared and chilled on ice. Cells were frozen at a concentration of 1.5×10^7 cells/ml. 0.5 ml aliquots of cells were added 1:1 with freezing media were placed into plastic ampules and chilled immediately as the DMSO was cytotoxic. The vials were then placed at -20°C for 1-2 hours and then transferred to -70°C overnight. The cells were then transferred into liquid nitrogen storage.

Cells were recovered after 1-2 weeks after freezing and tested for viability using the Trypan Blue exclusion test (Davis, 1994). Cells were thawed by gently thawing ampules by warming in the hand, the contents of the ampule was transferred to a 50ml tissue culture flask (Nunclon) flask containing 5ml of media. Cells were transferred after 2-3 days to wash out any cryoprotectant (DMSO).

2.7.3 Subculture of *Drosophila* cells.

DH33 cells were maintained as monolayer cultures. When cells reached a confluent monolayer (approximately every three days) they were passaged 1/5 dilution. Passaging was performed by knocking the side of the flask to release the cells into the media, the cell mixture was then poured into a sterile tube and diluted. 5mls of the diluted cell suspension was placed into 50cm^2 and 10ml into 200cm^2 flasks.

Schneider 2 cells were also maintained as monolayer cultures in *Drosophila* Schneider's medium at 25°C in Nunclon 200cm^2 vented flasks. Cells were grown until they were 80% confluent at which time they were 80-99% in suspension. The flask was then rocked gently to ensure a uniform cell density and the cells in suspension were subcultured at a 1/10 dilution into fresh media.

The cell density was measured using a haemocytometer (Hawksley Crystalite improved Nelbauer).

2.7.4 Cloning for tissue culture.

rev cDNA was excised from pGEMt, using *EcoR* I and *Xba* I restriction sites and subcloned into pUASt (also cut with *EcoR* I and *Xba* I). pTK34 contains a Rev responsive element (RRE) which drives expression of chloramphenicol acetyl transferase (CAT), pTK53 is an identical construct with a 2 nucleotide mutation at 7805-7806, which induces a conformational change in the RRE, disrupting Rev binding (pGEMt-*rev*, pTk34 and pTk53 constructs were kind gifts from Jon Karn at MRC, Cambridge).

pUASt, pTK34 and pTK53 were digested with *Not* I and *Xho* I (a 3kb fragment in each case included the *env* exon and the CAT gene and ligated to produce UAS-RRE and UAS-mRRE respectively.

2.7.5 Calcium Phosphate Transfection of *Drosophila* cells.

Cells were plated out at a density of $1-5 \times 10^6$ cells per 60mm dish (Nunclon) in 3mls of Schneider's *Drosophila* medium at least 6 hours prior to transfection. Cells were transfected using the calcium phosphate method as described in Kingston, 1997.

2.7.6 Preparation of cell lysates.

Media was removed from the dishes and 1ml of PBS was added to the transfected cells. Cells were scraped into microcentrifuge tubes using a Costar disposable scraper, pelleted by centrifugation for 2 minutes at 6,000 rpm in a microfuge (Sanyo MSE) and resuspended in 100 μ l of 0.25M Tris-HCl (pH7.8). Cells were lysed by three freeze/thaw cycles (30 seconds in liquid N₂ and 90 seconds at 37°C) and the debris was pelleted by centrifugation at 12,000 rpm for 2 minutes. Supernatants were transferred to fresh tubes and 30 μ l of each lysate was used for CAT assays as described in section 2.7.7 and 30 μ l was assayed for β -galactosidase activity as described in section 2.7.8. 10 μ l of the lysate was also used to measure the protein concentration using the Biorad protein assay according to manufacturer's instructions.

2.7.7 Assay for Chloramphenicol Acetyl Transferase (CAT) activity.

CAT activity was assayed as described in Ausubel (1996) with the following modifications. ¹⁴C chloramphenicol (ICN) was used at 25 μ Ci/ml and 1 μ l 10mM acetyl CoA. was used.

The samples were run on ascending thin layer chromatography (TLC) using 95% chloroform and 5% methanol. The plates were then dried and exposed to X-ray film overnight at room temperature. The CAT assays were then quantified by wrapping the plates in cling film and exposing them overnight to phosphoimager screen (Molecular Dynamics) according to the manufacturer's instructions. The ImageQuant programme was used to analyse the phosphoimaged samples, by measuring the volume of the 3-acetylchloramphenicol in each case compared to the background.

2.7.8 Assay for transfection efficiency (β -galactosidase activity) in tissue culture cells.

1 μ g of SV40 β Gal plasmid (expressing the enzyme β galactosidase) was co-transfected with the assay constructs and cell extracts were prepared as described in section 2.7.6 to measure β galactosidase activity.

β galactosidase activity in cell extracts was measured according to the protocol in Kingston (1997).

2.7.9 Extraction of proteins from tissue culture cells.

Proteins were extracted from *Drosophila* tissue culture cells were extracted as in method 2.7.6 and heated in 1x SDS loading buffer at 95 $^{\circ}$ C for 4 minutes and then subject to western analysis (2.11.5).

2.7.10 Immunofluorescence to tissue culture cells.

Antibody staining to S2 cells was carried out essentially as described in King and Prosse (1992), with the following modifications: cells were fixed in methanol at -20°C, primary antibody was added 1/50 (unless otherwise stated) in PBS +10% normal goat serum (NGS) for 2 hours and secondary antibody was added 1/100 for 30 minutes. Double labelling with Hoechst # 33258 (Sigma) was performed as follows: after the secondary antibody was added the cells were rinsed 5 times and then 1µg/ml of Hoechst # 33258 in PBS was added for 4 minutes, the cells were then rinsed 5 times and washed for 2 X 5 minutes and rinsed a further 5 times before mounting.

2.8 Ethylmethane sulphonate (EMS) mutagenesis of 21D region.

| Aberration | Class of aberration. | Breakpoints. | Mutagen/origin. |
|--------------------|----------------------|-------------------------|-----------------|
| Df(2L) <i>ast2</i> | deficiency | 21D1-21D2; 22B2-22B3 | gamma ray |
| Df(2L) <i>S2</i> | deficiency | 21C6-D1; 22A6-B1 | gamma ray |
| Df(2L) <i>ast4</i> | deficiency | 21D1-21D2; 21E2. | gamma ray |
| Df(2L) <i>ast6</i> | deficiency | 21E1-21E2; 21E2-21E3 | gamma ray |

Table 2.6

Deficiencies used in defining mutations in the 21D region.

Deficiency stocks and breakpoint information were obtained from the Bloomington stock centre. These stocks were used for screening EMS mutants according to section 2.8.2.

2.8.1 Isogenisation of wild type stocks

Isogenisation of a *white* Canton S (WCS) stock of flies was performed essentially as described in Greenspan (1997).

2.8.2 Mutagenesis of WCS stocks and subsequent crosses.

Male flies from isogenised WCS stock were fed 2.5mM EMS/sucrose solution according to EMS mutagenesis protocol of Roberts (1986). 100 males per bottle were then mated to *W*; *CyO/Sp* virgin females. At evenly spaced timepoints throughout the mutagenesis, crosses were also performed to assess the effectiveness of the EMS treatment by observing male lethals (see appendix 10.2A).

Single pair matings were performed by placing one male and one female in a vial. Large scale bottle matings were performed by adding approximately 20 females and 30 males together in a bottle. Non-complementation in any cross was taken as the absence of any *Cy*⁺ flies in over 100 progeny unless otherwise stated.

2.8.3 Analysis of progeny.

Mutants were placed in laying cages and a total of 200-300 eggs per allele were collected and kept at 18°C for up to 3 days . The eggs were scored for any lethality and any hatching larvae were then transferred to a food vial and then followed up to eclosion of the adult flies noting any lethality at any stage of development. Eggs which did not hatch in the time period allowed were first examined by cuticle preparation (as described in section 2.6.3.4), then subsequently by antibody analysis as described in method 2.5.1

2.9 Fusion Protein production and analysis.

2.9.1 Expression of fusion proteins in *E.coli*.

The *drongo* ORF was cloned into (1) pGEX-5X-1 expression vector (Pharmacia Biotech) using *EcoR* I restriction sites (appendix 10.1); into (2) pMAL-c2 expression vector (New England Biolabs) for expression of maltose binding protein (MBP)-fusion protein, using *EcoR* I restriction sites (appendix 10.1); and into (3) pQE32 vector (Qiagen) using *Sma* I and *EcoR* I sites for expression of a 6X his tagged -fusion protein (not shown)

As controls in determining the validity of induction and purification, pGEX-5X-1, pMal-p2 and pQE-32 vector alone were used alongside expression of GST-fusion proteins, MBP-fusion proteins and His-tagged fusion proteins respectively.

For expression of all fusion proteins, overnight cultures were diluted 1:100 into fresh media/antibiotic and grown at 37°C (unless otherwise stated) until O.D. $A_{600} = 0.6-0.8$. To induce expression of fusion proteins, IPTG was then added to the bacterial culture to a final concentration of 1mM (unless otherwise stated) and the culture was grown at 37°C for a further 4 hours. (To determine successful induction, various time points were taken throughout).

For GST-fusion proteins DH5 α was used initially as a bacterial strain, then protease deficient strains BL21 (DE3) and LE392 (Table 2.2).were used subsequently in order to minimise protein degradation.

Attempts were made to solubilise the fusion protein using the *E. coli* thioredoxin plasmid *pTtrx* or using heat shock chaperone plasmids *pTgroE* (these were a gift from Lauren Porter and described in Yasukawa *et al*, 1995). Both of these plasmids are also IPTG inducible (appendix 10.1H).

pGEX-5X-1 was also digested with *Sst* I and *Not* I, deleting amino acids 1650-3494, the remaining fragment was treated with S1 nuclease (from nested deletion kit, Pharmacia Biotech: used according to manufacturer's instructions) to form a deleted

Drongo fusion protein (pGEX-5X-1-*drongo* Δ S) in order to minimise steric hindrance of binding to the affinity matrix.

Cultures were then centrifuged at 3,000 rpm for 10 minutes at 4°C to pellet the cells and the cells were resuspended in an appropriate buffer for lysis.

2.9.2 Purification of fusion proteins by affinity binding.

Cells were either sonicated or lysed in a French pressure cell. For sonication, bacteria were sonicated in lysis buffer 1 (150mM NaCl, 1% triton X-100, 0.1% SDS, 50mM Tris-HCl pH 8.0, 1mM PMSF) using a Jencons sonicator using a 30 second pulse at 6 μ m repeated five times with 30 seconds break in between pulses.

The cell lysate was then centrifuged at 10,000 rpm for 20 minutes at 4°C and the supernatant transferred to a fresh test tube and the pellet was resuspended in lysis buffer 1. Samples were then analysed on an SDS-PAGE electrophoresis gel.

For French press, bacterial cells were resuspended in lysis buffer 2 (10mM Tris-HCl pH 8.0, 150mM NaCl, 1mM EDTA) with 1mM PMSF. Cells were then subject to French pressing twice at 1010 atm in a Aminco French pressure cell press and the cell lysate was centrifuged at 10,000 rpm for 20 minutes at 4°C.

The supernatant was transferred to a fresh test tube and the pellet was resuspended in 0.5% S.D.S.

After cell lysis the supernatant was used for a source of soluble fusion protein in the purification experiments. For elution of GST-fusion proteins, supernatants were incubated with 150 μ l of 50% slurry of Glutathione Sepharose 4B beads (prepared according to manufacturer's instructions (Pharmacia Biotech) per 10ml of culture on a rotator at 4°C for 1 hour-overnight. Purification was attempted according to the batch method. GST-Fusion proteins were eluted in 100mM Tris-HCl pH 9.5, 20mM reduced glutathione (Sigma). Elution was achieved by rocking gently at room temperature for 1-3 hours.

For isolation of MBP-fusion proteins, a similar method was used utilising an Amylose Resin (NEB). Elution was performed with 10mM maltose in 150mM tris-HCl pH 7.5.

All samples were analysed by SDS-PAGE to determine the purification of the fusion proteins.

2.9.3 Analysis of fusion protein on SDS-PAGE electrophoresis.

1x SDS-loading buffer (50mM Tris-HCl pH 6.8; 5% 2-mercaptoethanol; 2% SDS; 10% glycerol) was added to samples to be analysed. The samples were heated to 94°C for 4 minutes and 1µl of bromophenol blue dye was added to each sample.

SDS PAGE was performed according to Sambrook *et al.* (1989) using 12% acrylamide gels.

Prestained molecular weight protein marker were loaded onto the end wells of each gel. See Blue standards (Novex Novel experimental technology) or Broad range prestained protein marker (NEB) were used.

In order to visualise whole proteins, the gels were stained with 0.2% coomassie blue in 10% acetic acid 10% methanol, and destained after 1-2 hours with 10% methanol/10% acetic acid.

2.9.4 Concentration of protein solutions.

Protein solutions were often precipitated out of solution in order to produce a sample which was more concentrated.

Ammonium sulphate precipitation was carried out essentially as described in Harlow and Lane (1988) using 80% ammonium sulphate. For ammonium reneke precipitation, an equal volume of ammonium reneke (Sigma) was added and the solution of protein was incubated on ice for 10 minutes and then centrifuged for 10 minutes at 10,000 rpm at 4°C. The supernatant was discarded and the pellet was washed twice with 6:1 acetone :100mM HCl and then centrifuged for 3 minutes at 10,000 rpm at 4°C. The pellet was washed twice again with acetone and then vacuum dried and resuspended in 1X SDS loading buffer.

2.10. Immunisations and preparation of serum.

2.10.1 Production of synthetic peptide.

The following peptide was produced at the Department of Genetics in Cambridge and coupled to MBS activated Keyhole limpet haemocyanin (KLH) through the thiol group on the N-terminal cysteine. The efficiency was calculated using Ellmans reagent (Sigma) according to the manufacturer's instructions, which gives a colour reaction with the free cysteines before and after the coupling. 78% of 4mgs were coupled and the final concentration of the conjugate was 2mg/ml.

For immunisation procedures, 100µl of the peptide was used and mixed with 800µl of Freund's adjuvant (Morris, Guildhay Ltd.) and 400µl of PBS. This peptide was mixed to an emulsion between two syringes linked by a thin metal tube. Complete Freund's (prepared by adding 10 doses of intradermal BCG vaccine BP (Evans) to 20ml of Freund's) was used for the first injection and incomplete non ulcerative Freund's was used for subsequent injections.

Rabbits were injected subcutaneously every 4 weeks and bleeds taken 2 weeks after injection and analysed by ELISA (section 2.11.1) and by western analysis (section 2.11.5). The serum taken post immunisation was termed anti-Drongo peptide antibody.

2.10.2 Isolation of GST-fusion proteins from SDS-PAGE gels.

25µg of total bacterial extract was loaded onto a 12% SDS-PAGE gel and run as previously described. After Coomassie blue staining of the gel and localisation of the fusion proteins on the gel, the band was excised with a clean scapel and placed in an electroelution chamber (BioRad), where the protein was electroeluted for 6 hours at 18mA at 4°C and dialysed against PBS the fusion protein and used to immunise a rabbit as described in section 2.10.1. 200µg of protein was used for each vaccination. Before immunisation, SDS was removed by dialysis against PBS containing 1ml of DEAE-cellulose (Sigma) and 10% methanol. The serum taken post immunisation was termed anti FL (full length) Drongo antibody .

2.10.3 Preparation of rabbit serum.

Blood taken at each bleed was placed at room temperature for 1 hour, while a clot formed, the clots were detached from the sides using a spatula. The blood was then left overnight at 4°C. The serum was then taken and centrifuged at 12,000 for 5 minutes to pellet any red blood cells and then the supernatant was aliquotted and stored at -20°C. Pre immune serum was mixed with 50% glycerol and stored at -70°C.

2.11 Analysis of serum

2.11.1 ELISA (Enzyme Linked Immunoabsorbant Assay) for detecting antibodies in crude serum.

150µl of protein solution (purified protein or bacterial extract) at 1µg/ml, diluted in coating solution (50mM sodium carbonate pH 9.6) was placed into the wells of a 36 well dish (Nunclon), using a complete column of wells for each antibody dilution and reserving the first set of columns for blank (coating solution only). The plate was incubated overnight at 4°C. The coating solution was then removed from the wells and 2 x 160µl of blocking solution (0.5% w/v gelatine) was added to each well and the plate was incubated at 37°C for 30 minutes. Wells were then washed 3 x with PBS + 0.1% Tween-20 (PBS-Tw).

Serum was diluted (in the range : 1,1000-1,6400) in PBS and a control of pre immune serum was used at 1,1000. Each row of wells was filled each dilution of antibody solution (including the blank column) and incubated at 37°C for 30 minutes. The antibody solutions were then removed and the wells washed 5 x PBS-Tw.

Each well was then filled with secondary antibody (HRP conjugated goat anti-rabbit IgG) at 1,6000 (diluted in PBS) and incubated again at 37°C for 30 minutes.

The wells were washed 5 x with PBS-Tw and then the colour was developed with 150µl of substrate OPD (10mg OPD, 25ml dH₂O, 13ml 2.84% (w/v) Na₂HPO₄, 12ml 1.92% (w/v) citric acid, 20µl H₂O₂. and after a 30 minute incubation in the dark, the reaction was terminated with 100µl of 2M H₂SO₄. The reaction was quantitated by measuring the O.D. at A₄₉₀.

2.11.2 Protein A affinity purification of IgG from rabbit serum.

IgG fraction from anti-Drongo peptide antibody was purified using a 5 ml Protein A (Sigma) column. The protocol was essentially according to the manufacturer's instructions, adjusting the pH of the crude serum to pH 8.0 by adding 10mls of 1M Tris HCl (pH7.9), antibody was bound to the matrix and albumin was washed from the column with Tris HCl (pH 8.0) and elution was carried out with 100mM glycine pH 3.0 into 100µl of 1M Tris HCl pH 8.0. IgG containing fractions were analysed by reading absorbance at 280nm.

2.11.3 Subcellular Fractionation of *Drosophila* embryos.

Drosophila embryos were fractionated by the method according to Berrios *et al.* (1995).

1 x SDS loading buffer was added to samples from each fractionation step and the samples were boiled at 95°C for 4 minutes and loaded on a 15% SDS PAGE gel. *Drosophila* anti-lamin antibody (a generous gift from Paul Fisher, at Stony Brook, described in Gruenbaum *et al.*, 1988) was used as a control in subcellular fractionation experiments.

2.11.4. Protein extraction for western blotting.

Various *Drosophila* tissues were homogenised essentially as described in Govind *et al.* (1992) in homogenisation buffer (50mM Tris HCl (pH 7,5) 140mM NaCl, 5mM MgCl₂, 0.05% NP-40, 1mM PMSF, 10mg/ml pepstatin A, 10mM/ml aprotinin, 1mg/ml leupeptin) on ice. Homogenates were filtered and 20µg was loaded onto a SDS PAGE gel for western analysis.

2.11.5 Western Analysis.

Western analysis was performed essentially as described in Harlow and Lane (1989).

SDS-PAGE gels were run as described in section 2.9.3. All western blotting materials used were first soaked in 1x blot transfer buffer (10mM Tris-HCl, 76mM glycine, 0.0% SDS, 20% Analar grade methanol.) Proteins were transferred overnight at 25V with the buffer stirring continuously and the blots were blocked overnight in 5% Marvel in PBS-Tween-20 (0.1%:unless otherwise stated).

For the colour reaction, unless otherwise stated, filters were incubated in substrate (3,3' diaminobenzidine (DAB) tetrahydrochloride tablets (Sigma) at 0.5mg/ml) in Tris/NaCl buffer. 5 μ l of hydrogen peroxide was added for every 1mg of DAB. When the colour reaction was complete the blots were then rinsed well in PBS and dried on Whatman paper.

Chapter 3:

Overexpression of *drongo*

3.1 Introduction.

Previous *in situ* hybridisation analysis of *drongo* indicated expression of the gene at various developmental phases in *Drosophila* (Harris, 1995). *drongo* is expressed during development of the compound eye, early neurogenesis (section 1.3) and also as the ovary is developing in the female fly (section 1.4). In order to establish the function of *drongo*, one aim of this project was to investigate if overexpression of the *drongo* cDNA would have any deleterious effect in these tissues. It was hypothesised that, given the restricted expression pattern of the wild type gene, ectopic expression of *drongo* may produce defects which could be analysed later in the development of each phase, possibly suggesting a role for the Drongo protein in the wild type fly.

3.2 RESULTS: Overexpression via heat shock.

3.2.1 Cloning of *drongo* cDNA into a heat shock inducible vector.

In order to analyse the effects of overexpression of the *drongo* gene, the 2.9 kb *drongo* cDNA (nucleotides 584 - 3494) was cloned into the vector p(hsCaSpeR) (*pers. comm.* Marcus Allen). This cDNA fragment included both potential translational start sites in the cDNA (+605 and +958) (figure 1.3).

WCS embryos were injected with the clone P(hsCaSpeR-*drongo*) and three transgenic lines were produced (*pers. comm.* Marcus Allen). These *hs-drongo* flies are listed in table 2.1. The insert was mapped, crossed to produce a homozygous stock (table 2.1) and placed over a relevant balancer chromosome.

Preliminary experiments were performed by *in situ* hybridisation to embryos to determine if the *drongo* gene could be overexpressed. These embryos appeared to show staining all over the embryo (data not shown), demonstrating that *drongo* could

be overexpressed constitutively in *Drosophila* tissues of *hs-drongo* flies.

One line was also produced containing the *drongo* cDNA in reverse orientation and designated *hs-drongo R*, in an attempt to produce possible antisense effects (Table 2.1). Effects of overexpression of the gene were then analysed during oogenesis, eye development and embryogenesis (to determine if overexpression of *drongo* had any effect on the formation of the nervous system).

3.2.2 Ectopic expression of *drongo* during *Drosophila* oogenesis.

Overexpression of *drongo* was initially analysed in the ovaries due to the relative ease of identifying abnormal phenotypes in this organ without using specific markers or antibodies.

Preliminary experiments were performed where homozygous *hs-drongo* (line 1) females received a single heat shock at 37°C for 30 minutes. The ovaries from these females were then dissected one day post heat shock (PHS) (after the final heat shock). Ovaries were dissected and stained with Hoechst # 33258, a nuclear stain used previously for observing nuclei in somatic and germline cells of the ovaries (Swan and Suter, 1996).

No abnormal phenotypes were seen following analysis of these ovaries compared to WCS (essentially wild type) ovaries from females which had undergone an identical heat shock regime. It has previously been documented that the window of heat shock sensitivity is often minimal. Pulses of heat shock may therefore be more likely to produce an effect during a developmental process (Forbes *et al.*, 1996). Therefore one day old females were heat shocked twice a day for three days and then the ovaries were dissected one or three days PHS and analysed as before.

At the beginning of oogenesis in the wild type germarium (the most anterior structure of the ovary), a cystoblast divides to produce 16 cystocytes. One of these cystocytes becomes an oocyte and 15 become nurse cells (section 1.4). These are subsequently surrounded by a layer of follicle cells to form a stage one egg chamber (Spradling, 1993). When *drongo* was overexpressed there was an increase in nurse cell number in at least one egg chamber per ovary (figure 3.1). This was observed in 26%

Figure 3.1

The effect of overexpression of *drongo* on egg chambers during oogenesis.

A-F: Hoechst staining of *hs-drongo* and WCS egg chambers.

Hs-drongo and WCS females were heat shocked at 37°C for 30 minutes 3x a day for 3 days (section 2.6.3.1). Ovaries were then dissected from these flies one day post heat shock and stained with Hoescht # 33258 (section 2.6.3.1).

(A) WCS (essentially wild type) stage 7 egg chamber containing 15 nurse cells (nc) and 1 oocyte (O) surrounded by a layer of somatic follicle cells (fc).

(B) *hs-drongo* stage 8 egg chamber containing 30 nurse cells (arrow).

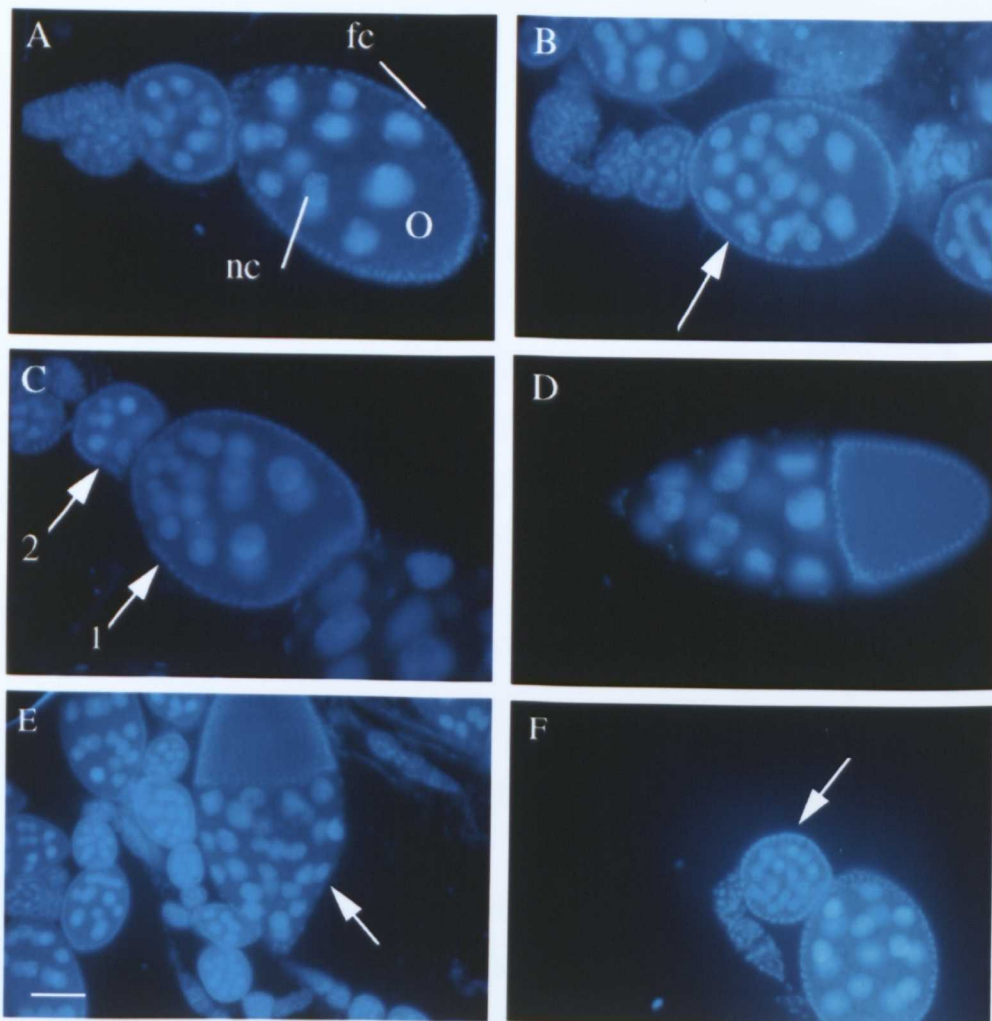
(C) *hs-drongo* stage 7 egg chamber containing 19 nurse cells (arrow 1). The more anterior egg chamber contains 11 nurse cells (arrow 2).

(D) WCS stage 10A egg chamber.

(E) *hs-drongo* stage 10A egg chamber containing 26 nurse cells (arrow). Anterior is downwards.

(F) *hs-drongo* stage 4 containing 24 nurse cells (arrow).

Anterior is to the left (unless otherwise stated). All egg chambers are staged according to Spradling 1993. Scale bar: 25µm.



of ovaries analysed one day post heat shock and 8% of *hs-drongo* ovaries analysed three days PHS (table 3.1).

| | No heat shock | Analysed one day PHS. | Analysed three days PHS. |
|------------------------|---------------|-----------------------|--------------------------|
| WCS | 0% of 77 | 0% of 85 | 0% of 104 |
| <i>hs-drongo</i> 14M3 | 0% of 62 | 26% of 80 | 8% of 75 |
| <i>hs-drongo</i> line1 | 0% of 50 | 26% of 58 | 8% of 60 |
| <i>hs-drongo</i> R | 0% of 91 | 0% of 82 | 0% of 90 |

Table 3.1.

Summary of defective egg chambers.

Females were heat shocked as in figure 3.1 and the ovaries were dissected and analysed as in figure 3.1.

Ovaries were analysed either minus heat shock; or heat shocked and dissected 1 or 3 days PHS. The percentages shown are the number of egg chambers containing more than 15 nurse cells as a percentage of the total number of ovaries analysed.

In affected egg chambers, between 19 (figure 3.1C) and 30 (figure 3.1B) nurse cells were observed, with an average of 26. This phenotype was not stage specific and it was observed at various stages as seen in figure 3.2.

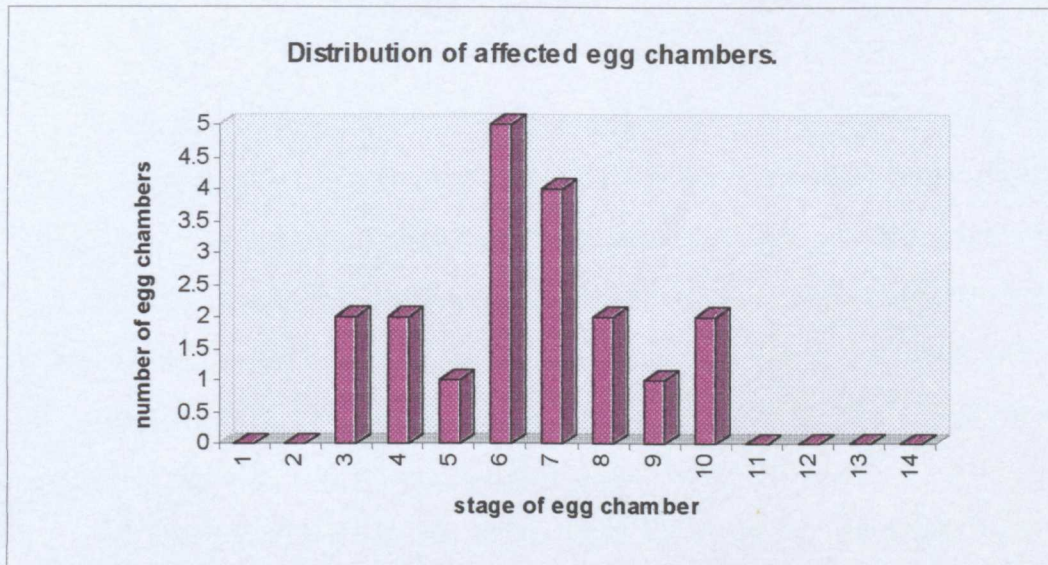


Figure 3.2.

Distribution of affected *hs-drongo* egg chambers.

The number of affected egg chambers from *hs-drongo* flies (heat shocked and treated as in figure 3.1). Egg chambers were staged according to Spradling (1993). Defective egg chambers prior to stage 3 and after stage 11 were not scored due to difficulty in counting the nurse cell number. The affect of overexpressing *drongo* during oogenesis is not stage specific.

Affected stages included stage 4 (figure 3.2 F), 7/8 (figure 3.1 B, C), and at stage 10A (figure 3.1 E). In most cases it was noted that there were adjacent egg chambers containing less than fifteen nurse cells (e.g. figure 3.1C, arrow 2) but the sum of the nurse cells in the two adjacent egg chambers was often equal to the number in two separate wild type egg chambers (i.e. 30). An increase in nurse cell number was also observed in ovaries from a second *hs-drongo* line (*hs-drongo14M3*), suggesting that the effect was not position dependent (table 3.1).

It was postulated that this defect observed following heat shock of *hs-drongo* females could be the result of two occurrences: either a cell division defect in the germline, or a defect in somatic follicle cell migration (section 1.4.1.2). The germ line divisions which occur in the germarium are known to be affected in mutants of *enc* (Hawkins *et al.*, 1996). Mutants have also been identified which disrupt the migration of the somatic follicle cells around the 16 cell cyst to form the stage one egg chamber. Previously documented analysis of known neurogenic mutants of *N* and *Dl* (Ruohola *et al.*, 1991) indicate that their gene products are involved in follicle cell development; another neurogenic gene *brn* (Goode *et al.*, 1996) is also required for prefollicular cells to migrate between egg chambers (section 1.4.1.2).

To determine if either a cell division defect or a follicle migration defect was the underlining cause of the phenotype, further analysis of the ovaries was performed. Firstly, egg chambers were observed in which a gradient in nurse cell size from the anterior to the posterior of the egg chamber was seen (figure 3.1 C). This suggested that some cystocytes originated earlier than others, thus the defect does not appear to be a cell division defect. Also the ratio of cystocytes between the supernumary egg chamber and the adjacent anterior egg chamber was not always 0:32, which may have indicated an extra cell division in the germline (figure 3.3).

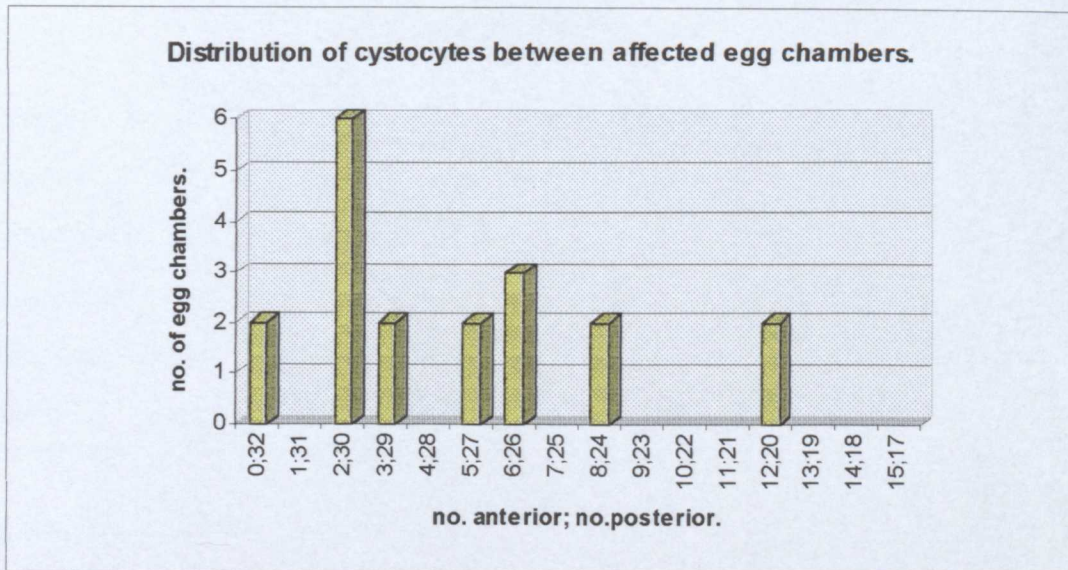


Figure 3.3

Distribution of cystocytes in affected egg chambers.

Ovaries were dissected and treated as in figure 3.1 and all affected egg chambers seen were scored. The ratio corresponds to the number of cystocytes (total = 32; 30 nurse cells and 2 oocytes between the two egg chambers) present in the anterior versus number in the adjacent posterior egg chamber.

Secondly, the timings of the division and encapsulation processes (according to Spradling, 1993) are correct for it to be a follicle cell migration defect but not a cell division defect. These timings are summarised in figure 3.4. Heat shocking the ovaries on day 1 of the regime (Extremes: heat shock for three days, dissected three days later) would result in the ovaries being analysed 6 days after the first heat shock.

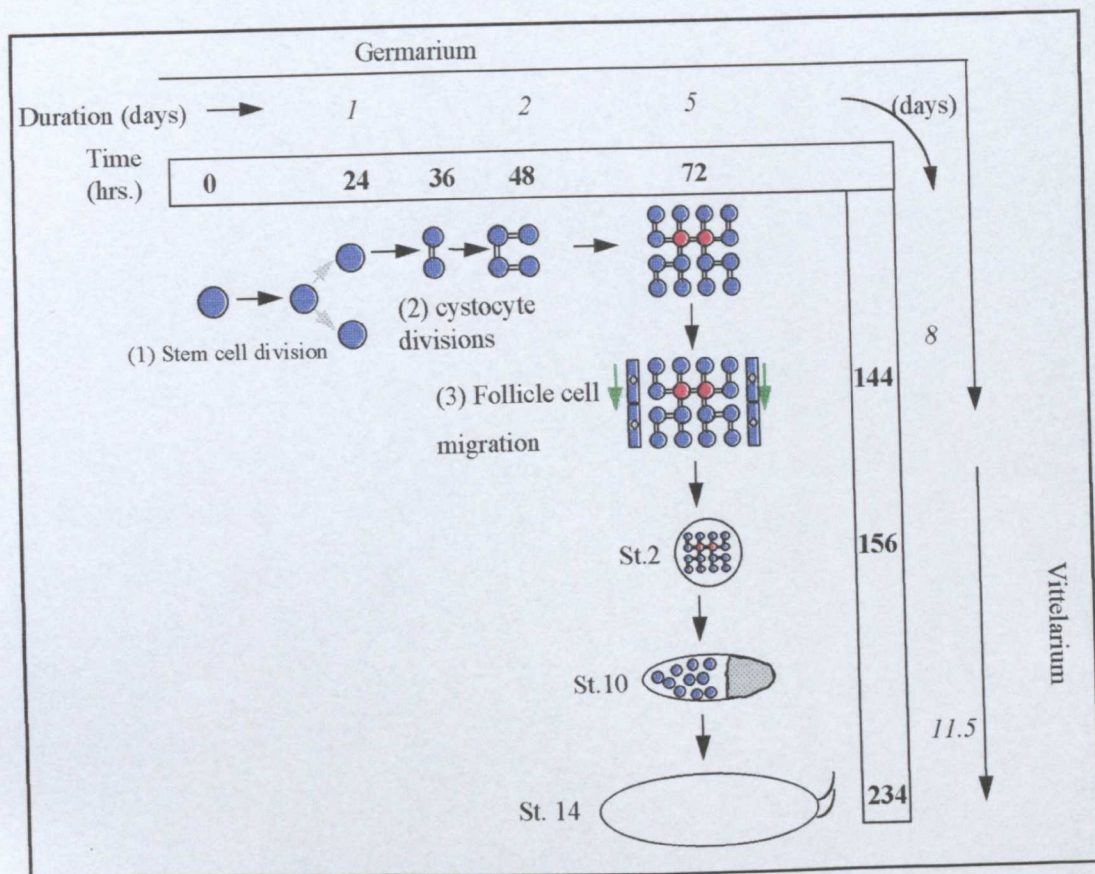


Figure 3.4

Summary of the duration of oogenesis.

Every 24 hours a stem cell divides to produce a cystoblast and a daughter stem cell. Taking stem cell division as time 0, the cystoblast then undergoes 4 divisions, each of 12 hours. It takes a further 3 days for follicle cell migration to occur, 12 hours to produce a stage 2 egg chamber and up to 78 hours for a stage 2 to become a stage 14 egg chamber. It is unlikely, therefore, even if the heat shock disrupted the final cystoblast division, that stage 8/9/10 egg chambers containing supernumary nurse cells would be observed. Timings taken from Spradling, 1993 and King, 1970.

If this heat shock was affecting a cystoblast division, even the final division, it is unlikely a defect would be seen in late stages such as stage 10A (which is 6-7 days after the final cystoblast division), this is clearly not the case. If defects at these stages are observed, the heat shock is more likely to affect a process that occurs after cell division but at least one-three days before stage 8-10A egg chambers are formed. This process may be follicle cell migration as this occurs in the correct time frame.

3.2.2.2 Further analysis of the germarium.

If follicle cell migration around the egg chamber was the process affected, it was postulated that a defect in the germarium (where the stage one egg chamber is formed) should be observed.

Previous studies have used the membrane protein, Fasciclin III as a marker for follicle cells in the germarium (Goode *et al.*, 1996). *hs-drongo* females were again heat shocked as before but this time dissected 6 hours post heat shock and stained with an anti Fasciclin III monoclonal antibody. In the wild type germarium, Fasciclin III protein is expressed in the follicle cells that have already migrated in the germarium, and appears as three thin bands of expression in the germarium as seen in previous work (Goode *et al.*, 1996, figure 3.5A). In *hs-drongo* ovaries, the Fasciclin III pattern was perturbed in 20% of 80 germaria. Only one, sometimes two, bands of expression were observed in the germarium from the ovaries of *hs-drongo* females (figure 3.5B), suggesting that ectopic expression of *drongo* resulted in a defect in migration of the follicle cells across the germarium over the 16 cell cyst. No germarial defects were seen in over 80 analyses of WCS flies and *hs-drongoR* flies that had experienced the same heat shock regime .

Previous *in situ* hybridisation analysis of *drongo* (Harris, 1995) had reported the gene to be expressed during early oogenesis. The analysis of *drongo* expression was therefore performed in the germarium to determine exactly where *drongo* is normally expressed as this had not previously been looked at in detail. *In situ* hybridisation showed expression of *drongo* in the follicle cells that had already migrated across the germarium (figure 3.5C), thus ectopic expression of *drongo* in the

Figure 3.5

The effect of overexpression on migration of follicle cells.

A-B Fasciclin expression in the germarium. **C-D** *in situ* hybridisation in the germarium

For analysis of Fasciclin III expression in the germarium, WCS and *hs-drongo* females were heat shocked 3 x a day at 37°C for three days for 30 minutes. Ovaries were then dissected 6 hours post heat shock and antibody stained with anti-Fasciclin III antibody (section 2.5.2).

For *in situ* hybridisation to ovaries from WCS females were dissected teased apart and *in situ* hybridisation was carried out according to section 2.4.4. Hybridisation was performed with a riboprobe made from the *drongo* cDNA (section 2.4.4).

(A) Wild type germarium/stage one egg chamber. Fasciclin III is expressed in three thin bands corresponding to each layer of follicle cells which have migrated across the germarium (arrows)

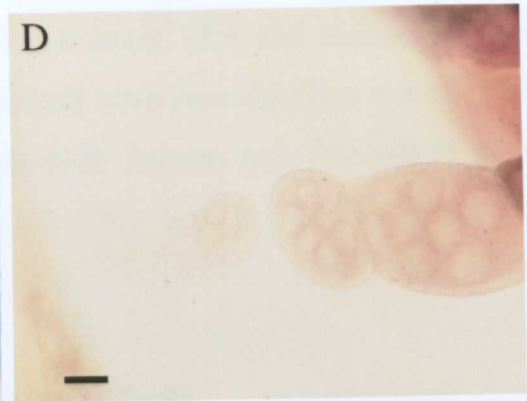
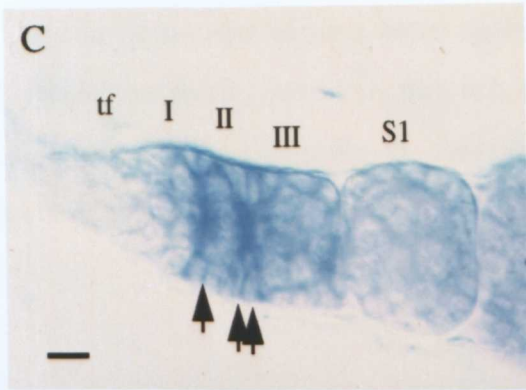
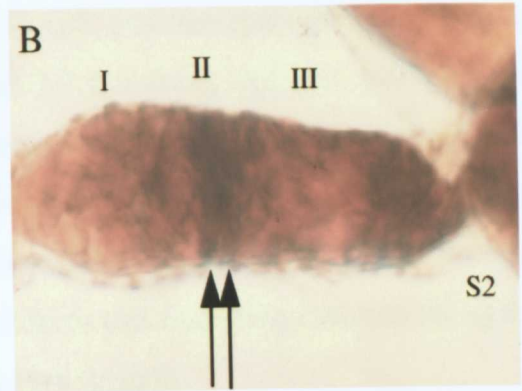
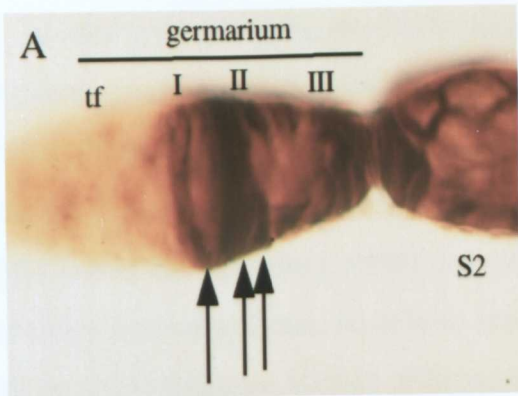
(B) *hs-drongo* germarium/stage one egg chamber, Fasciclin III is expressed in only two bands (arrows)

(C) *drongo* transcript is expressed in the migrated follicle cells in region II of the germarium.

(D) *In situ* hybridisation with sense riboprobe.

Anterior is to the left. Scale bars: 15µm A-C. 35µm in D.

Key: tf: terminal filament; I, II, III :stage I, II and II in the germarium; s:stage.



follicle cells in the pre-migration state may be responsible for the phenotype observed after heat shock.

In wild type germaria, the developing 16 cell germline cysts are spanned by a cytoskeletal structure called a fusome (Section 1.4.1.1; Deng and Lin, 1997; Lee *et al.*, 1997). It has been shown that alpha-spectrin, which is one of the components of the fusome, is required for cyst formation and oocyte differentiation in the germline cells (deCuevas and Spradling, 1996). I postulated that after overexpression of *drongo* in *hs-drongo* germaria, I may be able to see follicle cell migration between the cysts and hence across a fusome instead of between each fusome.

When *hs-drongo* ovaries were analysed 6 hours PHS and stained with an alpha-spectrin antibody, however, no such defects were detected (data not shown). This may be due to difficulty in observing each fusome and the follicle cells simultaneously.

3.2.3 Overexpression of *drongo* during eye development.

hs-drongo third instar larvae were used in analyses of overexpression effects of *drongo* during eye development, as *drongo* was shown previously to be expressed just behind the morphogenetic furrow by *in situ* hybridisation (Harris, 1995; figure 1.2J).

hs-drongo larvae were heat shocked at 37°C for either 15 minutes, 30 minutes, 45 minutes, 60 minutes or 90 minutes and then transferred to 25°C. After eclosion the adult eyes were analysed for any abnormalities (rough eyes may indicate a change in the structure of the eye).

Analysis of adult eyes after larval heat shocks (at 15, 30, 45 minutes) showed no effect on the formation of the eye and had no deleterious effect on any other body part upon gross morphological examination. Heat shocks for 90 minutes or more were lethal to the larvae. 76 *hs-drongo* third instar larvae were analysed for defects at each time point (15 minutes, 30 minutes, 45 minutes and 60 minutes) and 25 larvae were analysed for 90 minutes. 90 WCS third instar larvae, which were also subject to identical heat shocks and analysed in the same way, showed no defects in eye

development.

3.2.4 Overexpression during embryogenesis.

In order to analyse possible defects during neurogenesis (section 1.3), *hs-drongo* embryos were collected and heat shocked at stage 7, 3.5 hours into development (taking time 0 as fertilisation), as neurogenesis first begins in the embryo. 50 embryos at a time were heat shocked at 37°C for each time point (15, 30, 45, 60, 90 minutes), a total of 250 embryos were thus heat shocked.

Embryos were then analysed at stage 16/17, 13-17 hours of development, when the CNS was fully developed. Two antibodies were then used for analysis of the embryos, firstly BP102 (anti CNS axons: Carney *et al.*, 1997), a marker for central nervous system longitudinal connectives and axon commissures and secondly, anti-Even skipped, a more specific marker for a subset of neurons (Patel *et al.*, 1989). Analyses of embryos with both of these markers (data not shown), showed no obvious defects in nervous system development. 300 WCS embryos, also heat shocked as above and analysed with the same markers, showed no defects in CNS development. Heat shocks to embryos prior to 3.5 hours of development were detrimental to both *hs-drongo* and to WCS embryos, indicating sensitivity during early development to heat shock effects.

Heat shocks during embryogenesis seemed not to perturb any phase of embryonic development, suggesting overexpression of zygotic message had no effect. Overexpression of maternal message or a somatic message during oogenesis, however, may have an effect on embryo development.

One day old *hs-drongo* females were heat shocked at 37°C for 30 minutes, 3 x a day for 3 days and embryos were collected from the flies between 2-18 hours after the final heat shock. Cuticle preparations of 100 embryos from heat shocked *hs-drongo* females and embryos from heat shocked WCS females were analysed. In the wild type late embryo/1st instar larvae (figure 3.6 A), a pattern of segmentally repeated fine hairs or denticle belts can be observed on the ventral side of the embryos. One can also observe anterior structures such as the head skeleton and mouth parts and posterior structures such as the paired filzkörper, part of the embryo's

Figure 3.6

The effect of overexpression of *drongo* during late oogenesis on embryos

A-D: Cuticle preparations of late embryos.

hs-drongo females were heat shocked 3 x a day at 37°C for 3 days and fertilised eggs were collected 13-17 hours post heat shock. The embryos were cleared and cuticle preparations were performed (section 2.6.3.4).

E-F: Oskar antibody staining to ovaries .

hs-drongo females were heat shocked as above, their ovaries dissected 6 hours later and antibody stained with Oskar antibody (section 2.5.2).

(A) *hs-drongo* type A (essentially wild type morphology) first instar larva showing mouthparts (mt) as an anterior marker and the filzkörper (fz) as a posterior marker. The 11 denticle belts observed on the ventral side of the larva represent the 3 head (t1-3) and 8 abdominal segments (a1-8) present (ventral is facing).

(B) *hs-drongo* type B embryo. At least 3 denticle belts are absent (ventral is facing).

(C) *hs-drongo* type C embryo. Only half the wild type number of denticle belts are present (arrowheads), embryo is starting to show posterior defects (ventral is down).

(D) *hs-drongo* type D embryo, embryo appears to contain no abdominal segments, posterior defects are now obvious as the filzkörper is no longer paired (ventral is up).

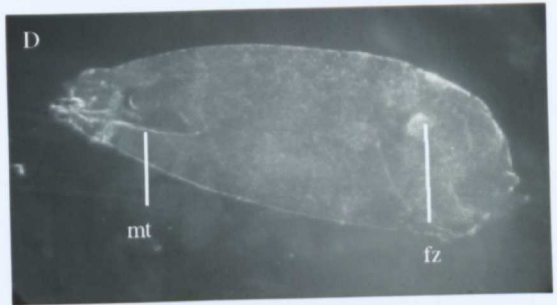
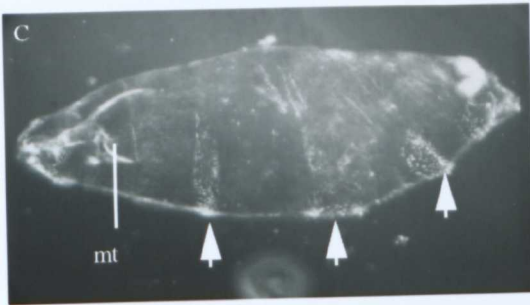
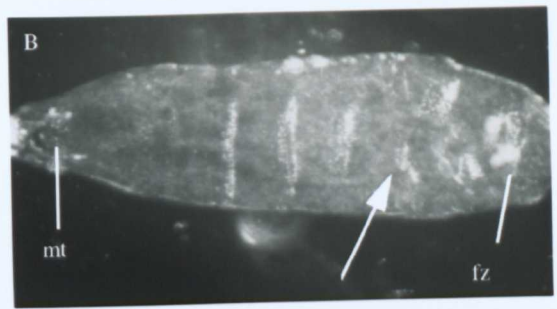
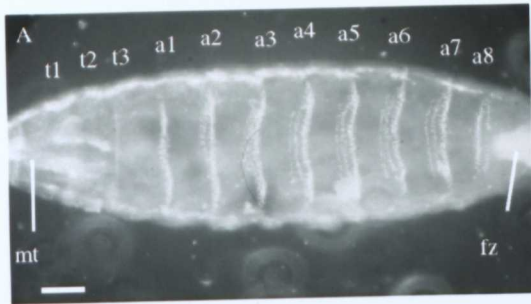
Abnormal embryos were seen 25% of the embryos analysed after overexpression of *drongo*.

Scale bar: 15µm. Anterior is to the left.

(E) Oskar expression in wild type stage 9 (S9) egg chamber, Oskar is located at the posterior tip of the oocyte (asterisk).

(F) Oskar expression in *hs-drongo* stage 9 egg chamber, Oskar is located at the dorsal and ventral sides of the oocyte (asterisks). This phenotype was observed in 20% of all ovaries observed after overexpression of *drongo*.

Scale bar: 35µm. Anterior is to the left.



breathing apparatus. The patterned denticle belts and the terminal structures provide easily identifiable markers where any perturbation in the embryos can be easily defined .

When embryos were observed from heat shocked *hs-drongo* females 25% of 100 embryos showed a variety of defects. The embryos often showed mostly segmental and posterior defects (figure 3.6 B-D; table 3.2). The majority of the embryos were classed into four types (A-D). Type A (which were essentially wild type) and then types B, C and D depending on severity of defect (table 3.2). Type B embryos displayed mild defects in cuticle formation. These embryos had one to three denticle belts missing. Type C embryos only had half the normal number of denticle belts and mild posterior defects (figure 3.6C); and type D embryos had a normal anterior but the posterior was obviously abnormal (figure 3.6D). Type D embryos often had no abdominal segments present.

| Conditions | Female | Class of Defect | | | |
|-------------|------------------|-----------------|---------|---------|---------|
| | | Type A: | Type B: | Type C: | Type D: |
| 37°C/3 days | WCS | 120 | 0 | 0 | 0 |
| 37°C/3 days | <i>hs-drongo</i> | 75 | 10 | 10 | 5 |
| 25°C/3 days | WCS | 104 | 0 | 0 | 0 |
| 25°C/3 days | <i>hs-drongo</i> | 110 | 0 | 0 | 0 |

Table 3.2

Classification of embryo defects.

Hs-drongo females were heat shocked at 37°C for 30 minutes 3x day for 3 days. Fertilised, embryos were collected and cuticle preparations were performed. Embryos were analysed and the cuticle defects were placed into four groups depending on severity of the phenotype (types A-D). **Type A:** essentially wild type morphology showing all 3 head and 8 abdominal denticle belts; **Type B:** Anterior and posterior structures normal but with one to three denticle belts absent; **Type C:** Anterior normal, posterior slightly abnormal but with half the normal number of denticle belts; **Type D:** Anterior normal, posterior abnormal with only a few denticle belts present. 25% of the embryos observed showed abnormalities (types B, C and D). A similar experiment was carried out where WCS and *hs-drongo* flies were incubated at 25°C instead of 37°C. Embryos were collected and the cuticle analysed as before to ensure no background mutation was causing the phenotype observed. Numbers represent embryos of each observed.

In over 120 analyses of cuticles prepared from WCS subject to an identical heat shock regime no abnormalities were seen in these embryos (table 3.2).

In order to ensure no background mutations were causing any effects, embryos from non-heat shocked WCS and *hs-drongo* females kept at 25°C were also collected and analysed by cuticle preparation (as before).

In type D embryos, other defects were observed in addition to the lack of denticle belts. Embryos where *drongo* had been overexpressed displayed a phenotype reminiscent of posterior group maternal mutants such as *osk* (Section 1.4.2; Ephrussi *et al.*, 1991; Gonzalez-Reyes *et al.*, 1995) and *aub* (Wilson *et al.*, 1996). Embryos from these mutants are unable to form a normal posterior due to defects in localisation or translation of posterior determinants. A similar phenotype is observed in type D embryos (figure 3.6D).

In view of this phenotype, subsequent experiments were carried out to determine the nature of this posterior perturbation. The anteroposterior axis is established during oogenesis when RNA determinants such as *osk* and *bcd* are localised (section 1.4.2; figure 1.10). An Osk antibody (*pers. Comm.* Clare Hudson; described in St Johnston *et al.*, 1991) was used to observe if any defect had occurred in the localisation or translation of the Osk protein. Any perturbation may indicate that overexpression of *drongo* has an effect on posterior formation in the embryo.

In the wild type ovary, Osk can be seen to localise to the posterior tip of the oocyte (figure 3.6 E) (Kim-Ha *et al.*, 1991). The mRNA is first localised and then translated in egg chambers after stage 9. Translation is usually dependent on localisation of the RNA at the posterior of the oocyte and translation is normally suppressed until this occurs (Kim-Ha *et al.*, 1995). In 20% of 73 *hs drongo* ovaries, Osk protein is observed on the dorsal and ventral sides of the oocyte (figure 3.6 F). In order to establish if the mRNA was correctly localised, *in situ* hybridisation was also performed with an *osk* RNA probe but localisation of the RNA appeared no different to the wild type (data not shown).

Overexpression of *drongo* appeared to affect posterior patterning during

oogenesis. Posterior patterning involves Grk function specifying the posterior follicle cells. These cells then signal back in order to localise mRNAs such as *osk* (St. Johnston *et al.*, 1996). I was interested in determining if any localisation of any of components of the posterior pathway were also affected.

Grk protein expression was therefore analysed in wild type and *hs-drongo* egg chambers. Like *osk*, *grk* mRNA is first localised and then the protein is translated in the specific region. An anti Grk antibody (Neuman-Silberberg *et al.*, 1993) was used in order to locate the protein in egg chambers. In wild type egg chambers the protein is located at the posterior tip of the oocyte up until stage 8. The protein then moves to a more central position at the anterior/dorsal side of the oocyte (Neuman-Silberberg *et al.*, 1993). In *hs-drongo* egg chambers the pattern of Grk expression does not deviate from wild type staining (data not shown) all through development of the ovary suggesting that overexpression of *drongo* does not have an effect on *grk* localisation or translation.

3.3 Overexpression of *drongo* using the yeast UAS/GAL 4 system.

3.3.1 Cloning of *drongo* into the pUAS_T vector.

drongo cDNA was also cloned into the pUAS_T vector (Brand and Perrimon, 1993; figure 3.7 A; appendix: figure 10.1) via *EcoR* I sites in order to overexpress the gene in a more defined manner in each developmental context. Ligation junctions were again sequenced along with GAL4 binding sites in pUAS_T (data not shown)

In order to examine whether a dominant negative effect could be produced, deletions of the *drongo* gene were produced and placed under the control of the UAS promoter (figure 3.7 B and C). Two deletion constructs were made, UAS-*drongo*ΔS, effectively making a deletion in the 3'end of the gene (figure 3.7 B); and UAS-*drongo*ΔF, where a deletion was produced in the zinc finger (figure 3.7 C). Three transgenic lines were produced by injection of embryos and designated UAS-*drongo*. Two transgenic lines were produced using UAS-*drongo* ΔS, however no transgenic lines were produced after injection of over 1000 eggs with the UAS-*drongo*ΔF. This construct may have been toxic to the embryos. Transgenic stocks were mapped, a

homozygous stock was produced, and placed over a relevant balancer chromosome (see table 2.1).

Figure 3.7

UAS-DRongo and deletion constructs and expression of GAL line P{GAL4-Hsp70.PB}31-1/T(2;3)B3

A-C: UAS-*drongo* deletions constructs. (For restriction maps and digestion checks see appendix 10.1).

D-E: Antibody staining with anti- β galactosidase on *Drosophila* stage 16 embryos from GAL4 line 31.1. Embryos were collected from GAL4 line P{GAL4-Hsp70.PB}31-1/T(2;3)B3 and antibody staining was performed (section 2.5.1).

(A) UAS-*drongo*: *drongo* cDNA cloned into pUAS_t. This clone was used to make the deletion clone in **(B)** and **(C)**

(B) UAS-*drongo* Δ S: a deletion in the OPA and FG repeats encoded at the 3' end of *drongo* (figure 1.3) was produced by digestion with *Sst*I. This clone was produced by digesting pGEX-5X-1-*drongo* DS (section 5.3.2) with *Eco*R I and pUAS_t with *Eco*R I and ligating the *drongo* fragment into pUAS_t.

(C) UAS-*drongo* Δ F was produced by digestion with *Fsp*I resulting in a deletion in the zinc finger.

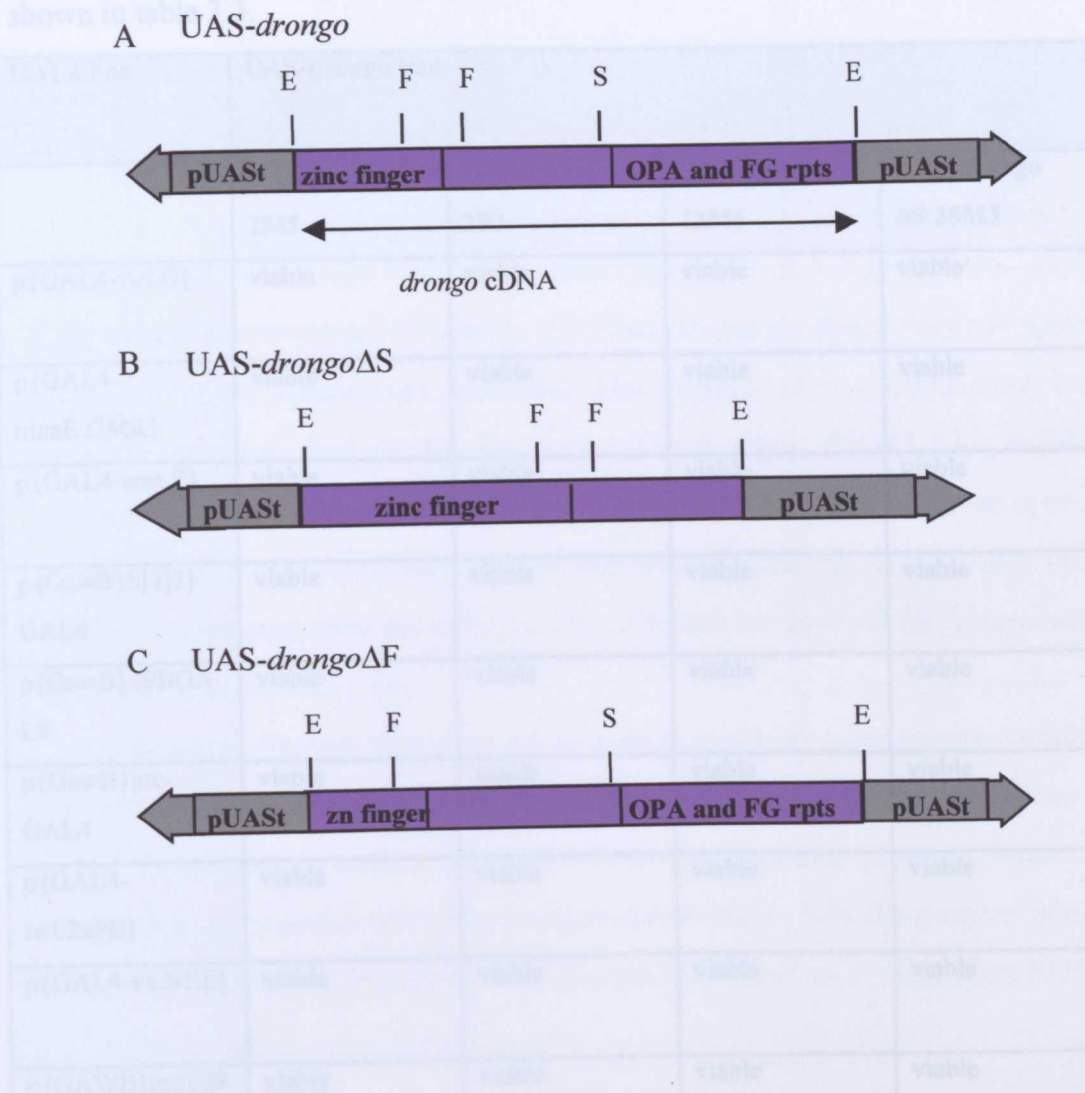
(D) Expression pattern of GAL4 line P{GAL4-Hsp70.PB}31-1/T(2;3)B3 pan neurally in the central nervous system (arrows). Embryos from GAL4 line P{GAL4-Hsp70.PB}31-1/T(2;3)B3 are crossed to embryos containing UAS-*lac* Z. β galactosidase protein expressed in the GAL pattern is detected by an anti- β galactosidase antibody (section 2.5.1).

(E) Expression pattern of GAL4 P{GAL4-Hsp70.PB}31-1/T(2;3)B3 in the peripheral nervous system (arrow).

Expression of UAS-*drongo* in the pattern of the GAL4 line produced lethality in first instar larvae.

3.3.2 Overexpression of *drongo* via UAS/GAL4.

UAS-drongo and *UAS-drongo Δ S* were crossed to a number of GAL4 lines as



Figures were crossed to various GAL4 lines. The absence of

3.3.2 Overexpression of *drongo* via UAS/GAL4.

UAS-*drongo* and UAS-*drongo*ΔS were crossed to a number of GAL4 lines as shown in table 3.3.

| GAL4 line | UAS- <i>drongo</i> line | | | |
|---------------------------------|---------------------------|---------------------------|-------------------------------|-------------------------------|
| | UAS- <i>drongo</i> 2M5 | UAS- <i>drongo</i> 2F1 | UAS- <i>drongo</i> ΔS 13M6 | UAS- <i>drongo</i> ΔS 35M3 |
| p{GAL4-twi.G} | viable | viable | viable | viable |
| p{GAL4-ninaE.GMR} | viable | viable | viable | viable |
| p{GAL4-arm.S} | viable | viable | viable | viable |
| p{GawB}h[1j3] GAL4 | viable | viable | viable | viable |
| p{GawB}69BGA L4 | viable | viable | viable | viable |
| p{GawB}ptc- GAL4 | viable | viable | viable | viable |
| p{GAL4-twi.2xPE} | viable | viable | viable | viable |
| p{GAL4-ve.NEE} | viable | viable | viable | viable |
| p{GAWB}md509 /CyO;MKRS/TM2 | viable | viable | viable | viable |
| GAL4 p{GawB}bi | viable | viable | viable | viable |
| p{GAL4.hsp70P} 31.1/T(2;3)B3 | lethal | lethal | viable | viable |

Table 3.3

Results of overexpression of *drongo* via GAL4 lines.

Flies were crossed as single pair matings, and the progeny scored. The absence of

marked flies in over 100 progeny was taken as an indication that driving UAS-*drongo* in the specific GAL4 pattern was lethal to the flies. In these crosses, 25% of the progeny would be expressing the GAL4 line and the UAS-*drongo* insert. The cross between UAS-*drongo* and GAL4 line P{GAL4.hsp70P}31.1/T(2;3)B was lethal for 25% of the progeny. These embryos hatched but died after the first larval instar stage.

When UAS-*drongo* was driven under GAL4 line 31.1 in embryos it was lethal in the progeny containing both inserts. All other crosses produced viable progeny (the progeny were all staged until eclosion). The progeny of the lethal cross hatched normally and then died at the first instar larval stage. P{GAL4-hsp70.PB}31-1 expresses in the embryonic CNS and PNS (figure 3.8D and E). The larvae upon gross morphological analysis are wild type in appearance (data not shown) and show no defects in movement were not affected. The affected larvae lived for 2 days, an extra day longer than the wild type.

It may be the case that these larvae cannot moult for some reason possible due to a hormonal excess or deficiency caused by overexpression of *drongo* or an effect on the peripheral nervous system inhibiting the moulting process. When UAS-*drongo* Δ S was crossed to the GAL4 lines available (Table 3.3), the progeny of each of the crosses was viable, including UAS-*drongo* Δ S crossed to P{GAL4-hsp70.PB}31-1.

3.4 DISCUSSION: Overexpression of *drongo*.

This chapter has described how *drongo* has been ectopically expressed in a number of developmental systems in *Drosophila*, including oogenesis, eye development and embryogenesis. Overexpression by heat shock results in egg chambers with supernumerary nurse cells (an average of 26 nurse cells in defective egg chambers). More detailed analysis of the germarium suggests a probable cause of the phenotype is a delay in follicle cell migration over the 16 cell cyst. In the wild type fly, this migration normally produces a stage one egg chamber of 15 nurse cells and 1 oocyte.

Mutations in many genes result in the production of supernumerary nurse cells. These include *brn* (Goode *et al.*, 1996), *N* (Ruohola *et al.*, 1994), and *encore* (Hawkins *et al.*, 1996). As mentioned previously, *enc* is thought to be involved in stem cell division in the germarium and *N* in determination of follicle cell fate. It appears that *drongo* overexpression has a consequence different to these and more similar to the defect seen in *brn* mutants. As *drongo* is expressed in migrated follicle cells in the germarium, it may act as a part of a signal to negatively control the next set of follicle cell migrations.

From sequence data it is unlikely that *Drongo* acts as a diffusible molecule. It is more likely to act as a transcription factor or function in the transport of molecules across the nuclear membrane. Both of these processes are important for ensuring communication between the germline and the follicle cells in order to form a stage one egg chamber.

Ectopic expression of *drongo* during late oogenesis results in embryos with cuticle defects and, in severe cases, posterior malformations. Upon further examination it appears a possible cause of this phenotype is the localisation of Osk protein in stage 9 egg chambers, although it does not appear that the localisation of the RNA is affected in these egg chambers. Overexpression of *drongo* appears to cause mislocalisation of Osk protein from the posterior tip to more dorsal and ventral positions in the oocyte of affected egg chambers. In the wild type egg chamber, Grk protein is expressed as a crescent around the germinal vesicle (oocyte nucleus)

(Neuman-Silberberg and Schüpbach, 1996) and acts to signal a set of follicle cells which then activate the *Drosophila* EGF receptor and adopt a posterior fate. N signalling is also known to be important for establishing the number of follicle cells which take a posterior fate. Osk is normally localised at the posterior by a microtubule network in the oocyte, which is polarised via a signal from the posterior follicle cells, factors such as *bruno* and *aubergine* normally regulating the translation of *osk* (Wilson, *et al.* 1996; Kim-Ha *et al.*, 1995). It is likely that *drongo* overexpression could be affecting one of these processes utilised to establish posterior identity in the embryo.

It is surprising that Grk protein localisation appears normal throughout oogenesis. Most mutations which disrupt the signal from the posterior follicle cell to the oocyte mislocalise posterior determinants. Mutations at the *mago nashi* locus (Newmark *et al.*, 1997) disrupt the oocyte nucleus localisation affect the second phase of *grk* localisation where dorsoventral pathway is established. This result may indicate that Grk mislocalisation may not be the cause of the abnormal embryos observed from *hs-drongo* females. Also the *drongo* gene is not specifically expressed in the posterior of wild type egg chambers (section 1.2.1), and is normally expressed in the follicle cells and the oocyte during early oogenesis and in the nurse cells during later stages, not indicative of a gene involved in the posterior pathway. The gene may however be ectopically expressed elsewhere in the egg chamber thus causing this effect. A mutant of *drongo* could be analysed for possible defects in oogenesis, and genetic interactions with posterior group genes could be analysed.

In order to define the role further, *drongo* was also overexpressed using the UAS/GAL4 system. When *drongo* was overexpressed its effect was lethal when driven by GAL4 line P{GAL4-hsp70.PB}31-1, which resulted in ectopic expression in the embryonic CNS/PNS. Expression of *drongo* has already been established in the CNS and PNS and so any defect caused by misexpression warranted further analysis. It was unclear however, why overexpression of *drongo* via this GAL4 line produced this effect.

When *drongo* is ectopically expressed by any of the other GAL4 lines which also express in the embryonic nervous systems (for example P{w^{+mC}=GAL4-ve.NEE}) there are no deleterious effects on development. Expression in these

lines was not established. I found that there were no reliable GAL4 lines available to use in which germline or somatic overexpression of *drongo* could be tested, so an effect on oogenesis could not be established using this system.

It is also interesting how a deleted form of the protein, when driven by GAL4 has no deleterious effect on development, suggesting perhaps that *drongo* may require some or all of the repeat sequences at the end of the gene, even to have an effect during overexpression. It was surprising that this deletion did not perhaps have a dominant negative effect by competing with the wild type protein and hindering its normal function.

drongo overexpression has produced interesting effects during early and late oogenesis and during CNS and PNS formation in the embryo. I was also able to analyse these phenotypes further. Although these analyses have offered clues to the possible roles of *drongo* in *Drosophila*, perhaps overexpression is not indicative of the true role of a protein in a developmental system. A more direct method is therefore required to assign a role to *drongo* at the cellular and molecular level.

Chapter 4:

**Analysis of *drongo* function in nuclear
transport:**

A Rev activity assay.

Chapter 4: RESULTS AND DISCUSSION: Analysis of *drongo* function in nuclear transport: A Rev activity assay.

4.1 Introduction

Analysis of the overexpression of *drongo* has shown that ectopic expression of the protein in *Drosophila* can produce an effect during various stages of development. It would be a strong statement, however, to define a role for the protein in any system just by observing an overexpression effect. Previous sequencing of the gene (section 1.2) reveals the highest amino acid identity (section 1.2.2) with hRIP/Rab, a human nucleoporin gene (Fritz *et al.*, 1995; Bogerd *et al.*, 1995). There is some evidence (Fritz *et al.*, 1995, Bogerd *et al.*, 1995; Stutz *et al.*, 1996) that hRIP may be a co-factor for the HIV-1 regulatory protein Rev. Rev is thought to be involved in activating the translation of viral structural proteins by enabling nuclear export of unspliced/partially spliced viral mRNA (section 1.5). Previous analyses suggest that Rev can function in *Drosophila* cells (Ivey Hoyle and Rosenberg, 1990) suggesting that a cellular co-factor exists in insect cells and that similar nuclear transport pathways to those in mammals may prevail in this organism.

It seemed logical therefore to observe if Drongo had any effect on Rev function in nuclear transport. Before this analysis, an assay was required to measure Rev binding and Rev activity.

A number of constructs were obtained or made in the laboratory and tested in an assay set up to measure Rev activity. The constructs did not function in *Drosophila* cells and therefore it was not possible to test if Drongo had an effect on Rev function in tissue culture. The following chapter is therefore a documentation of the constructs made, the cell lines used and how the assay was carried out, leading to a discussion on the problems of this approach.

4.2 Co-transfection of *Drosophila* cells.

Transfection of tissue culture cells offered a well established system and was used as an assay for a gene's function.

In order to observe possible interactions, previous studies have co-transfected more than one plasmid and evidence suggests that if cells are successfully transfected they will take up all plasmid DNA in the assay (*pers. comm.* Marcus Allen).

Two types of plasmids were used in this co-transfection analysis. An 'effector plasmid' has been utilised which expresses the gene(s) under test; and a 'reporter plasmid', where a reporter gene is fused to a promoter which is acted on by the protein expressed by the gene under test. A change in expression of the reporter gene may indicate an effect from the test gene or a possible interaction between the two, be it direct or indirect.

4.3. The Rev activity assay.

Previous studies have shown that HIV-1 Rev uses a conserved cellular pathway for the export of viral RNAs (Fritz and Green, 1996; Stutz and Rosbash, 1998; Neville *et al.*, 1997). This suggests that putative co-factors such as hRIP (Fritz *et al.*, 1995) may also show similarities in sequence between organisms. Fritz *et al.* (1995) indeed showed that the amount of hRIP was proportional to the activity of Rev in tissue culture cells, therefore a possible cofactor in *Drosophila* may show a similar effect. As mentioned earlier, Rev has also been previously shown to function in *Drosophila* cells (Ivey Hoyle and Rosenberg, 1990) suggesting that a co-factor exists in *Drosophila* cells. Due to the similarity with hRIP, Drongo was a likely candidate for this role in *Drosophila*. An alignment between Drongo and hRIP is shown in figure 4.1.

Figure 4.1

Homology between Drongo and hRIP.

Drongo shows substantial similarity to human Rev interacting protein (RIP). RIP has been shown to bind to HIV-1 regulatory protein Rev and implicated in the export of viral RNA from the nucleus. Homology was highest around the zinc finger in both protein at 77% over 73 amino acids but also include short motifs towards the C terminal of the protein.

Key: Drongo: Drongo amino acid sequence; hRIP: human RIP amino acid sequence.

Drongo : ---MAVVRKKQDDKYLLALRELVVSGTGSNRQCFDCGQKGPTYVN : 42
 hRIP : --MAASAKRKQEEKHLKMLRDM--TGLPRNRKCFDCDQRGPTYVN : 41

Drongo : MTIGSFVCTRCSGVLRGL----TPPHR-VKSISMATFTQDEIDF : 81
 hRIP : MTVGSFVCTSCSGSLRGL----NPPHR-VKSISMTTFTQOEIEF : 80

Drongo : LRSHGNELCAKTWLGLWDPKRAV-----HQEQRELMMDKYERKR : 121
 hRIP : LQKHGNEVCKQIWLGLFDRLSSAIPDFRDPQKVKEFLQEKYEKKR : 125

Drongo : YYLEPA-----SPLKSL---- : 133
 hRIP : WYVPPEQAKVVASVHASI----SGSSASSTSSTPEVKPLKSLG : 166

Drongo : -ANAVNLK---SSAPATNHTQNGHHN-----GYASIHHTPP : 165
 hRIP : SAPTLHLNKGTPSQSPVGRSQGQQQEKKQFDLLSDLGSDIFAAP : 211

Drongo : AAQRTSANGLOKQVANSSNSSGKTSSSISRPHYNHQN--NSQNNN : 208
 hRIP : APQSTATANFANFAHFNASHAAQNSA-----NADFAN : 242

Drongo : HDAFGLGGGLSSLNSAGSTSTGAL----SDTSSCASNGFGADCDF : 249
 hRIP : FDAFGQSSGSSNFGGFPTASHSPFPQPTTGGSAASVNFANFAHFDN : 287

Drongo : VADFGSANIFDATSARSTGSPAVSSVSSVGSNGYAKVQPIRAAH : 294
 hRIP : FPKSSADF--GTFNTSQSHQTASAVSKVSTNK-----AG : 320

Drongo : LQQQQLQQQQHQQQLLN-GNGHQGTENFADFHDHAPIYNAVGA : 338
 hRIP : LQTADKYAALANLDNIFSAQGGDQGSFGTTGKAPVGSVSVPS : 365

Drongo : QNGHTTSPPKGNAAMAPSEDRYAALKDLDEQLRELKASESATET : 383
 hRIP : QSS-----ASSDKYAALAEELDSVFSSAATSSNAYTS : 396

Drongo : PTPTSGNVQATDAFGGALNNP-----NPFKGQQQ : 413
 hRIP : TSNASSNVFGTVPVVASAQTQPASSVVPAPFGRTPTNPFVAAAG : 441

Drongo : QQLSSHVVNPFQHQHQONLYGQLTLIPNAYG----- : 448
hRIP : PSVAS-STNPFQTNARGATAATFGTASMSMPTGFGTPAPYSLPTS : 485

Drongo : -SSSQQMGHLLQQQQQQSFFNFNNNGF-AISQGLPNGCGFG : 491
hRIP : FSGSFQPAFPAQAAPFOQTAFSQPNAGAGFAAFGQTKPVVTPFG : 530

Drongo : SMQPAPVMANNPF---AASGAMNTN---NPFL : 517
hRIP : QVAAAGV-SSNPFMTGAPTGQFPTGSSTNPFL : 562

An assay has been developed which utilised the UAS/GAL4 system in *Drosophila* cells and attempts were made to measure Rev activity following measurement of expression from a CAT reporter gene. The assay used an effector plasmid containing an Adh-GAL4 construct which utilised the constitutive alcohol dehydrogenase (Adh) promoter fused to a GAL4 gene. Adh-GAL4 will then activate any plasmid that is driven by the UAS (upstream activating sequence) promoter in every cell transfected.

The principle plasmid used in this assay was the HIV-1 derived 'Rev responsive' reporter plasmid, pTK34 (Kind gift from John Karn) (figure 4.2). The reporter plasmid, carrying the bacterial chloramphenicol acetyltransferase gene (CAT; Gorman, 1983) inserted downstream of the HIV-1 *gag* (*gag* encodes a viral capsid protein) 5' splice site (see figure 4.2), transcribes sequences from the molecular clone pNL4-3 (nucleotides 5943-8888) under the control of the SV40 (Simian virus 40) early promoter and enhancer. Nucleotides 5943-8888 of pNL4-3 include the *env* exon, which contains the Rev responsive element (RRE). The RRE is a secondary structural loop in the RNA which is thought to bind Rev and be essential for it's function in prevention of RNA splicing during HIV infection (section 1.5). The CAT gene in this construct is therefore flanked by splice donor (SD) and splice acceptor (SA) sites (figure 4.1).

The construct was partially sequenced in this study to check the sequences highlighted (the SV40 promoter, the CAT gene, the *env* gene and location of the RRE and location of splice sites: appendix figure 10.1 I).

In the event of cellular splicing, CAT RNA produced from this construct can only enter the cytoplasm in the presence of the Rev protein (figure 4.3). In the absence of Rev, CAT will spliced out as an intron.

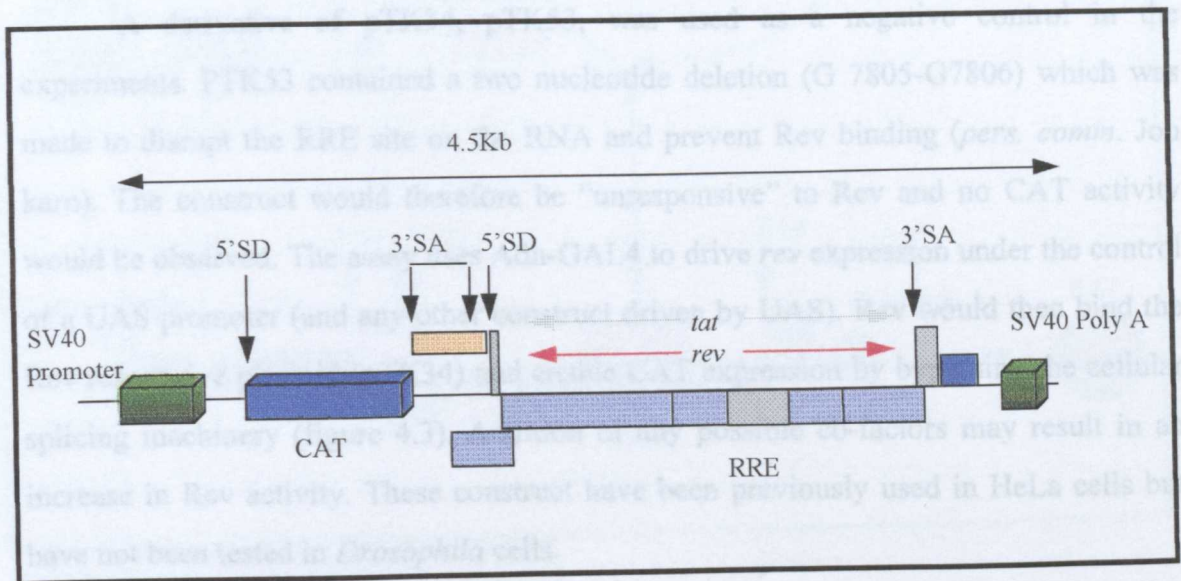


Figure 4.2

Structure of the Rev-responsive CAT reporter gene, pTK34.

The CAT gene is placed under the control of an SV40 promoter and flanked by a 5' splice site from the HIV-1 clone pNL4-3. pTK34 also contains the *tat*, *rev*, *vpu*, and *env* genes. The *env* gene contains the Rev responsive element (RRE) to which Rev is thought to bind and prevent or bypass RNA splicing. Endogenous *rev* function is inactivated by a frame shift mutation at the BamHI site in the second exon of *rev* (Nucleotide 8466). The SV40 polyadenylation signal was also placed downstream of the HIV-I sequence. A second plasmid, PTK53 is a plasmid derived from pTK34 which contains a two nucleotide deletion at the RRE site resulting in a disruption to Rev binding and was used as a negative control. (*pers.comm.* John Karn.)

Endogenous *rev* expression from pTK34 was prevented by a frame shift mutation in the clone.

A derivative of pTK34, pTK53, was used as a negative control in the experiments. PTK53 contained a two nucleotide deletion (G 7805-G7806) which was made to disrupt the RRE site on the RNA and prevent Rev binding (*pers. comm.* Jon karn). The construct would therefore be “unresponsive” to Rev and no CAT activity would be observed. The assay uses Adh-GAL4 to drive *rev* expression under the control of a UAS promoter (and any other construct driven by UAS). Rev would then bind the Rev responsive plasmid (pTK34) and enable CAT expression by bypassing the cellular splicing machinery (figure 4.3). Addition of any possible co-factors may result in an increase in Rev activity. These construct have been previously used in HeLa cells but have not been tested in *Drosophila* cells.

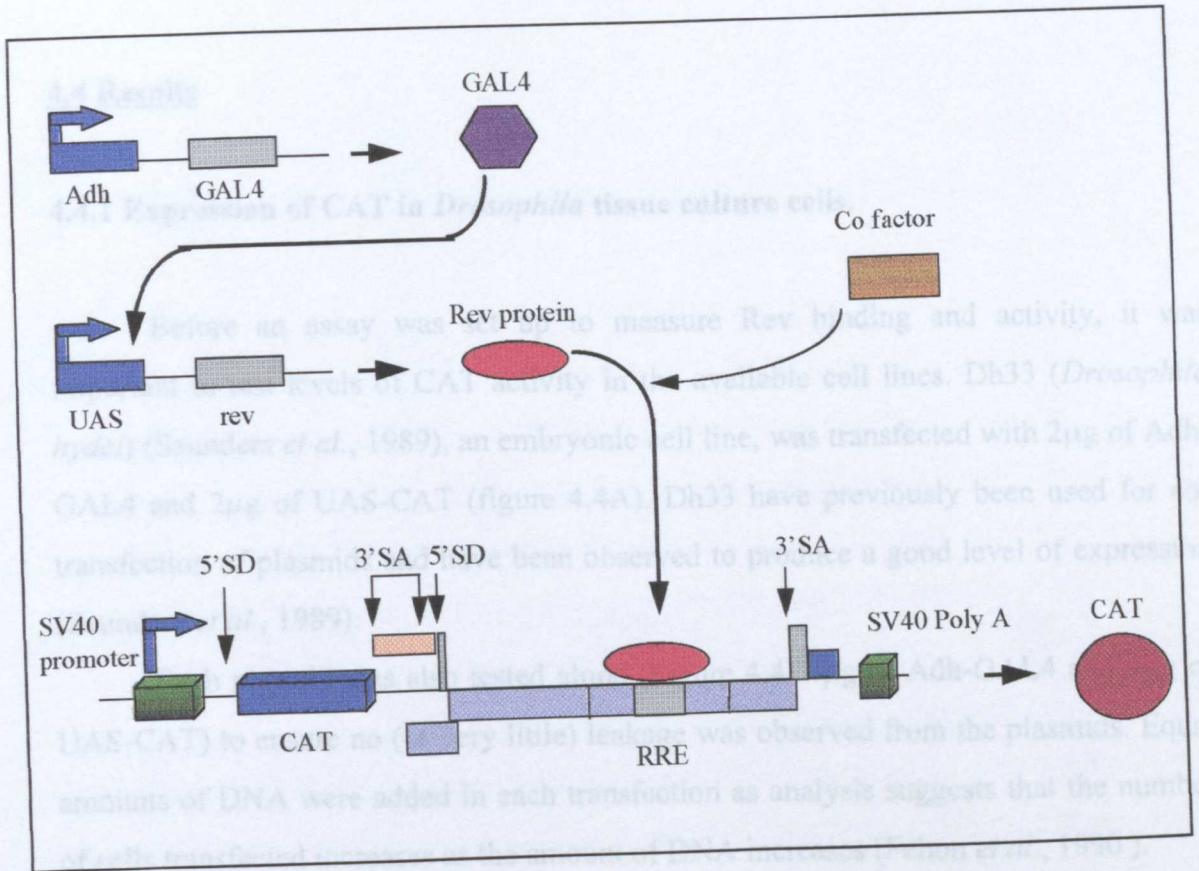


Figure 4.3:

The Rev activity assay:

Adh-GAL4 was used as a constitutive promoter to drive expression of GAL4 which drives *rev* expression under a UAS promoter. Rev binds to the RRE site on the Rev responsive plasmid, prevents splicing and enables expression of the *CAT* gene via the SV40 promoter, thereby allowing analysis of Rev activity indirectly through measurement of *CAT* activity. In the absence of Rev, splicing occurs and no/reduced *CAT* expression is seen.

4.4 Results

4.4.1 Expression of CAT in *Drosophila* tissue culture cells.

Before an assay was set up to measure Rev binding and activity, it was important to test levels of CAT activity in the available cell lines. Dh33 (*Drosophila hydei*) (Saunders *et al.*, 1989), an embryonic cell line, was transfected with 2 μ g of Adh-GAL4 and 2 μ g of UAS-CAT (figure 4.4A). Dh33 have previously been used for co-transfection of plasmids and have been observed to produce a good level of expression (Saunders *et al.*, 1989).

Each plasmid was also tested alone (Figure 4.4: 4 μ g of Adh-GAL4 and 4 μ g of UAS-CAT) to ensure no (or very little) leakage was observed from the plasmids. Equal amounts of DNA were added in each transfection as analysis suggests that the number of cells transfected increases as the amount of DNA increases (Fehon *et al.*, 1990).

Other controls for the experiment included adding no DNA to cells, using cells which had not been transfected at all (to check for any bacterial CAT activity) and no cells in the assay. After 3 days, cells were harvested and extracts of the cells were run on thin layer chromatography TLC plates (Data not shown). The protein concentration (figure 4.4 B) for each extract was also measured (section 2.7.9) and the TLC plates were quantitated (section 2.7.7) on the phosphoimager (figure 4.4 B). In order to check the transfection efficiency of each set of experiments, 10% of the total DNA amount of pSV β gal was cotransfected. pSV β gal (*Pers. Comm.* Phil Gardener) drives expression of *E.coli* β galactosidase under the control of an SV40 promoter, this was measured using a colourimetric assay (section 2.7.8). The efficiency of each transfection and the amount of protein used for each CAT assay was probably often different so it was important to normalise the data. The CAT activity was normalised by calculating the phosphoimager reading per units of β gal per μ g of protein present in assay (figure 4.4 B+C). As transfections were usually performed in duplicate (some later in triplicate) an average

Figure 4.4

Schematic, table and histogram of control transfections in Dh33 cells.

(A) Constructs used in the control transfections.

Dh33 cells were transfected with 4 μ g of Adh-GAL4 alone, 4 μ g of UAS-CAT alone or 2 μ g both constructs (section 2.7.5). 1 μ g of SV β gal (to assay for transfection efficiency) was also transfected into every set of cells used.

Negative controls were no DNA transfected (to check for the presence of any bacterial CAT protein), untransfected (untreated) cells, and no cells in the CAT assays.

(B) Table showing results from the transfections.

Cells were harvested 3 days after transfections, extracts were made and assayed for CAT activity and run on TLC plates (section 2.7.6 and 2.7.7). The TLC plates (data not shown) were used to obtain a phosphoimager reading for each sample (section 2.7.7).

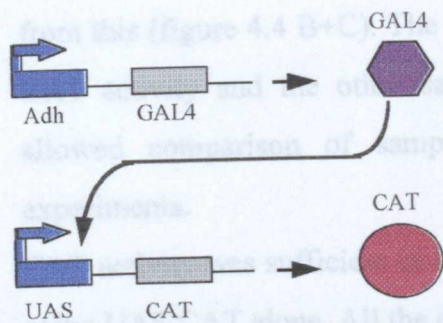
CAT activity was calculated by normalising the phosphoimager reading (2.7.9) against amount of protein in each sample (section 2.7.9) and transfection efficiency (β galactosidase units). The average reading was calculated per μ g of protein/units of β Gal. the positive control was taken as 100% CAT activity and the other samples were a percentage of this figure.

(C) The data from (B) was plotted as a histogram.

This shows that although the CAT reporter constructs displays a little leakage, Adh-GAL4 is able to drive UAS-CAT in Dh33 cells and that all of the other negative controls showed no CAT activity.

Abbreviations: Adh: alcohol dehydrogenase; CAT: Chloramphenicol acetyl tranferase; AG: Adh-GAL4; UC: UAS-CAT; β GAL: β galactosidase.

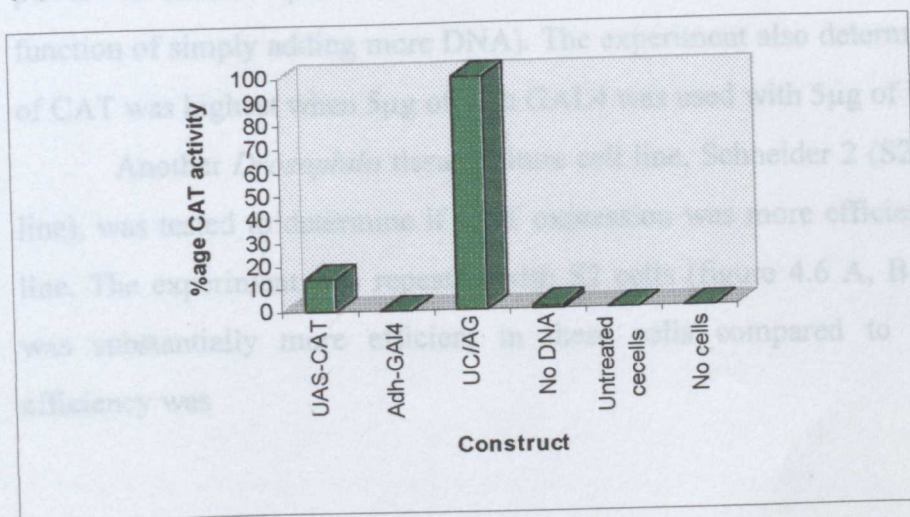
(A)



(B)

| Construct | Reading | β GAL activity | Protein in assay (μ g) | Reading / μ g of protein | β GAL activity / μ g of protein | Units CAT / units β GAL / μ g of protein | Average Units CAT / units β GAL | % CAT activity |
|-----------|---------|----------------------|-----------------------------|------------------------------|---|--|---------------------------------------|----------------|
| | X | Y | Z | (X/Z) A | (Y/Z) B | (A/B) C | | |
| UAS-CAT | 447387 | 186 | 2.98 | 150130 | 62.4 | 2405.3 | 3881.6 | 14.3 |
| UAS-CAT | 1071584 | 200 | 2.8 | 382709 | 71.4 | 5357.9 | | |
| Adh-GAL4 | 3713 | 399 | 3.8 | 978 | 105 | 9.3 | 10 | 0.04 |
| Adh-GAL4 | 2168 | 203 | 2.6 | 834 | 78.8 | 10.7 | | |
| UC/AG | 1189504 | 269 | 3.3 | 3604558 | 81.5 | 4421.9 | 27200.2 | 100 |
| UC/AG | 6313030 | 117 | 3.4 | 1856774 | 34.4 | 53957.5 | | |
| No DNA | 28940 | 82 | 3.09 | 9366 | 26.5 | 352.9 | 355.3 | 1.3 |
| No DNA | 38987 | 109 | 3.2 | 12183 | 34.1 | 357.7 | | |
| Untreated | 27252 | 0.00 | 4.62 | 5898 | 0.00 | 0.00 | 0.00 | 0.00 |
| No cells | 33324 | 0.00 | 0.00 | | 0.00 | 0.00 | 0.00 | 0.00 |

(C)



reading/units β gal/ μ g protein was taken and the percentage CAT activity was calculated from this (figure 4.4 B+C). The positive control in each experiment was taken as 100% CAT activity and the other samples were calculated as a percentage of this. This allowed comparison of samples within the same experiment but not between experiments.

CAT activity was sufficient in Dh33 cells, although a basal level of CAT was produced using UAS CAT alone. All the other controls were negative.

4.4.2 Optimising the expression of CAT.

I wanted to select the best cell line in which to measure CAT activity and also to determine the amount of a UAS-driven construct that gives the highest measurable level of CAT activity from the reporter construct. Dh33 cells were transfected with increasing amounts of UAS-CAT (UC) (figure 4.5: 2 μ g UAS-CAT; 3 μ g UAS-CAT; or 5 μ g UAS-CAT) with 5 μ g of Adh-GAL4 (AG) in each case). A reverse experiment was performed whereby Dh33 cells were transiently transfected with increasing amounts of Adh-GAL4 (AG) (Figure 4.5: 2 μ g Adh-GAL4; 3 μ g Adh-GAL4; or 5 μ g of UAS-CAT (UC) for each case. As negative controls, extracts from cells transfected with 5 μ g of UAS-CAT and with 5 μ g of Adh-GAL4 were also loaded on the TLC plate (data not shown).

The data (figure 4.5 B and C) showed that the increase of CAT activity was relatively (although not exactly) proportional to the addition of more construct (adding pUAS_t to ensure equal amounts of DNA were transfected ensured this was not a function of simply adding more DNA). The experiment also determined that expression of CAT was highest when 5 μ g of Adh GAL4 was used with 5 μ g of UAS-CAT.

Another *Drosophila* tissue culture cell line, Schneider 2 (S2: also an embryonic line), was tested to determine if CAT expression was more efficient in a different cell line. The experiment was repeated with S2 cells (figure 4.6 A, B and C). Expression was substantially more efficient in these cells compared to Dh33. Transfection efficiency was

Figure 4.5

Schematic, table and histogram of increasing amounts of Adh-GAL4 and UAS-CAT in Dh33 cells.

(A) Constructs used in the transfections.

Dh33 cells were transfected with 5 μ g of UC alone or 5 μ g of AG alone or with 2, 3 or 4 μ g of UC with 5 μ g of AG in each transfection. Cells were also transfected with either 2, 3 or 4 μ g of AG with 5 μ g of UC in each transfection. Cells were transfected as in figure 4.4.

(B) Table showing results from the transfections.

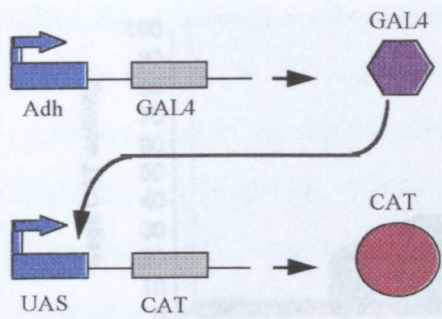
Cells were harvested and assayed for CAT activity, β GAL activity and protein concentration as in figure 4.4.

(C) The data from the table was plotted as a histogram.

There appeared to be a general increase in CAT activity with the increase in the amount of each construct. The data also suggests that 5 μ g of each construct shows the highest CAT activity in this cell line. Key: 1: 5 μ g UC; 2: 5 μ g AG; 3: 2 μ g UC/5 μ g AG; 4 3 μ g UC/ 5 μ g AG; 5: 2 μ g AG/ 5 μ g UC; 6: 3 μ g AG/ 5 μ g UC; 7: 5 μ g UC; 5 μ g AG.

Abbreviations: as in figure 4.4.

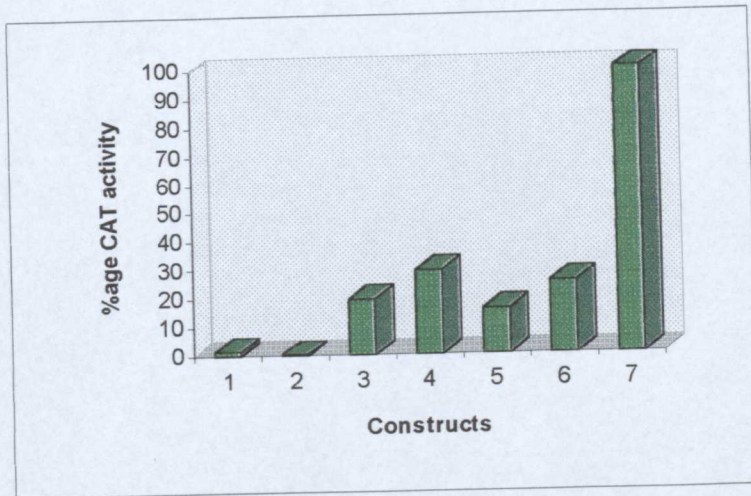
(A)



(B)

| Construct | Reading | β GAL activity | Protein in assay (μ g) | Reading/ μ g of protein | β GAL activity/ μ g of protein | Units CAT/ units β GAL/ μ g of protein | Average Units CAT/ units β GAL | % CAT activity |
|-------------------------------|----------|----------------------|-----------------------------|--------------------------------|---|--|---|----------------|
| | X | Y | Z | (X/Z) A | (Y/Z) B | (A/B) C | | |
| 5 μ g UC | 558391 | 304 | 4.1 | 136192.9 | 74.2 | 1822.0 | 2570.9 | 1.3 |
| 5 μ g UC | 986022 | 297 | 2.4 | 410842.5 | 123.8 | 3319.9 | | |
| 5 μ g AG | 6701 | 265 | 2.3 | 2913.5 | 115.2 | 25.3 | 25.5 | 0.01 |
| 5 μ g AG | 7792 | 304 | 1.2 | 6493.3 | 253.3 | 25.6 | | |
| 2 μ g UC/ 5 μ g AG | 8967330 | 152 | 3.1 | 2892687.1 | 49.0 | 58995.6 | 39825.6 | 19.5 |
| 2 μ g UC/ 5 μ g AG | 6403219 | 310 | 3.5 | 1829491.1 | 88.6 | 20655.5 | | |
| 3 μ g UC/ 5 μ g AG | 9870123 | 132 | 4.2 | 2350029.3 | 31.4 | 74773.7 | 59817.2 | 29.3 |
| 3 μ g UC/ 5 μ g AG | 7940347 | 177 | 3.2 | 2481358.4 | 55.3 | 44860.7 | | |
| 2 μ g AG/ 5 μ g UC | 9903621 | 325 | 2.2 | 4501645.9 | 147.7 | 30472.7 | 31338.8 | 15.4 |
| 2 μ g AG/ 5 μ g UC | 10949620 | 340 | 2.5 | 4379848 | 136 | 32204.8 | | |
| 3 μ g AG/ 5 μ g UC | 12950351 | 269 | 3.5 | 3700100.3 | 76.9 | 48142.6 | 50632.7 | 24.8 |
| 3 μ g AG/ 5 μ g UC | 13068197 | 246 | 3.0 | 4356065.7 | 82 | 53122.8 | | |
| 5 μ g UC/ 5 μ g AG | 88950351 | 351 | 3.0 | 29650117 | 117 | 253419.8 | 203890.2 | 100 |
| 5 μ g UC/ 5 μ g AG | 17905831 | 116 | 3.2 | 5595572.2 | 36.3 | 154360.6 | | |

(C)



measured by assaying β galactosidase activity (figure 4.6 B) for each experiment. The S2 cells were thus used for all further experiments. This cell line also had the added advantage of being a *Drosophila melanogaster* cell line so that any results may be directly related to the organism of study. Although UAS-CAT was not to be the construct used in the final assay, this was an important test to determine the amount of UAS-driven construct in this particular cell line, a general conclusion from this experiment was 5 μ g of a UAS-driven construct was sufficient to produce CAT expression (either directly via UAS-CAT or indirectly via UAS-Rev).

4.4.3 Expression of Rev Responsive CAT constructs.

The second stage of the assay was to assess whether the Rev activity assay (figure 4.3) works efficiently and Rev responsive CAT constructs could be expressed in S2 cells.

Before the assay was carried out, the pTK34 plasmid was partially sequenced in order to verify that there was no mutations present which affect expression of the construct. This sequence is shown in appendix 10.1 I (the relevant sequences are highlighted). There appeared to be no mutations in the construct that may affect the assay.

As a preliminary experiment, S2 cells were transiently transfected with 0.2-5 μ g of pTK34 (the rev responsive CAT reporter construct shown in figure 4.2) or 0.2-5 μ g of pTK53 (a derivative of pTK34 containing a deletion in the RRE site) (data not shown) to ensure that no basal activity (or at least low enough to be insignificant) could be observed as this may have confused further experiments.

With up to 5 μ g of each plasmid no leakage could be observed (data not shown), suggesting that export and translation of the plasmid RNA was repressed in the absence of Rev. In order to test if expression of pTK34 could be observed in the presence of Rev, 5 μ g of Adh-GAL4, 5 μ g of UAS-Rev and 5 μ g pTK34 were used to transfect S2

Figure 4.6

Table and histogram of increasing amounts of Adh-GAL4 and UAS-CAT in S2 cells.

(A) Table showing the results from the transfections.

The experiment from figure 4.5 was repeated with S2 cells. Cells were transfected, harvested and assayed as in figure 4.4

(B) The data from the table was plotted as a histogram.

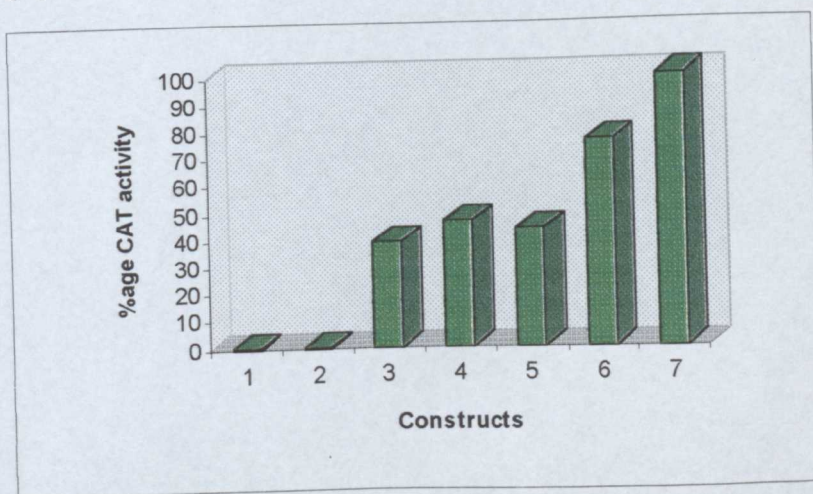
There appeared to be an increase in CAT activity with an increase in the amount of each construct added. The overall CAT activities in this cell line appear higher than in Dh33 cells. Key: 1: 5µg UC; 2: 5µg AG; 3: 2µg UC/5µg AG; 4 3µg UC/ 5µg AG; 5: 2µg AG/ 5µg UC; 6: 3µg AG/ 5µg UC; 7: 5µg UC; 5µg AG.

Abbreviations: as in figure 4.4.

(A)

| Construct | Reading X | β GAL activity Y | Protein in assay (μ g) Z | Reading/ μ g of protein (X/Z) A | β GAL activity/ μ g of protein (Y/Z) B | Units CAT/ units β GAL/ μ g of protein (A/B) C | Average Units CAT/ units β GAL | % CAT activity |
|-------------------------------|--------------|---------------------------|----------------------------------|--|---|--|---|----------------|
| 2 μ g UC/ 5 μ g AG | 37809471 | 187 | 4.1 | 9221822.2 | 45.6 | 202232.9 | 170490.1 | 39.2 |
| 2 μ g UC/ 5 μ g AG | 29678045 | 214 | 2.3 | 12903498 | 93.0 | 138747.3 | | |
| 5 μ g UC | 347189 | 195 | 2.7 | 128588.5 | 72.2 | 1785.9 | 1545.9 | 0.36 |
| 5 μ g UC | 220960 | 170 | 1.8 | 122755.6 | 94.4 | 1305.9 | | |
| 3 μ g UC/ 5 μ g AG | 26093021 | 150 | 1.7 | 15348836 | 88.2 | 174023.1 | 203083.3 | 46.6 |
| 3 μ g UC/ 5 μ g AG | 30902941 | 133 | 2.6 | 11885747 | 51.2 | 232143.5 | | |
| 2 μ g AG/ 5 μ g UC | 28679567 | 167 | 4.0 | 7169891.8 | 41.8 | 171528.5 | 189653.1 | 43.6 |
| 2 μ g AG/ 5 μ g UC | 41378906 | 199 | 3.5 | 11822545 | 56.9 | 207777.6 | | |
| 5 μ g AG | 6697 | 107 | 3.2 | 2092.8 | 33.4 | 62.7 | 53.4 | 0.01 |
| 5 μ g AG | 5073 | 115 | 1.7 | 2984.1 | 67.6 | 44.1 | | |
| 3 μ g AG/ 5 μ g UC | 44089671 | 179 | 1.7 | 25935101 | 105.3 | 246297.3 | 332857.2 | 76.4 |
| 3 μ g AG/ 5 μ g UC | 39089674 | 93 | 4.0 | 9772418.5 | 23.3 | 419417.1 | | |
| 5 μ g AG/ 5 μ g UC | 48890856 | 100 | 2.8 | 17461020 | 35.7 | 489104.2 | 435307.1 | 100 |
| 5 μ g AG/ 5 μ g UC | 40089067 | 105 | 3.7 | 10834883 | 28.4 | 381509.9 | | |

(B)



cells (Figure 4.7 A). 5µg of each plasmid was used for transfection (unless otherwise stated) using pUAS_t to make up the DNA amount to 20µg for each experiment. As a negative control the experiment was repeated using the defective plasmid, pTK54, in order to ensure that any mutation in the RRE would prevent CAT expression (presumably by preventing Rev binding and export of the construct RNA from the nucleus). However, when CAT activity was assayed, there was no significant activity observed from pTK34 (figure 4.7 B and C). In order to control the experiment, each plasmid was transfected alone (Figure 4.7 B and C: Adh-GAL4 (AG); UAS-Rev (UR); pTK34 (TK34) or pTK53 (TK53)) and then with each of the other plasmids in turn (Adh-GAL4 and pTK34; Adh-GAL4 and pTK53; UAS-Rev (UR) and pTK34; UAS-Rev and pTK53; or UAS-Rev and Adh-GAL4). When each of the cell extracts were analysed by TLC (data not shown), no CAT activity was observed (figure 4.7 B and C).

There may be a number of reasons why no CAT activity was observed using this construct. One explanation may be the control of expression of the plasmid. Expression of CAT is controlled by the SV40 promoter/enhancer and it is essential that this promoter works efficiently in this particular system. Some recent work (Ratzui *et al.*, 1998; Mehlen *et al.*, 1995; Angelichio *et al.*, 1991) suggest that the SV40 promoter does not promote efficient transcription of plasmids transfected into *Drosophila* cells, although there are other experiments which dispute this idea (Pfeifer *et al.*, 1997).

4.4.4 Expression of the Rev responsive plasmids under UAS control.

In order to alleviate the possible problem of the SV40 promoter not working efficiently in this cell line, the construct was placed under the control of the UAS promoter. It is known from this experiment and others that the UAS-promoter works highly efficiently when transfected in *Drosophila* tissue culture cells (Toba *et al.*, 1999; Phelps and Brand, 1998). pTK43 and pTK53 (in order to perform a negative control also under UAS control) were digested with *AvrII* and *XhoI* and a 4kb fragment was ligated into pUAS_t (digested with *XbaI* and *XhoI*). The resulting UAS-RRE (the

Figure 4.7.

Schematic, table and histogram of transfections of S2 cells with pTK34.

(A) Constructs used in the transfections

For control experiments: 5 μ g of Adh-GAL4, UAS-Rev and pTK34 (Rev responsive construct under the control of the SV40 promoter/enhancer) and pTK53 (derivative of pTK34 with a mutation in the RRE-Rev binding site) were all transfected alone, or with each other construct in a pair.

In the final assays 5 μ g of UAS-REV, Adh-GAL4 and pTK34/pTK53 were all transfected into the same cells.

Transfections were carried out as in figure 4.4.

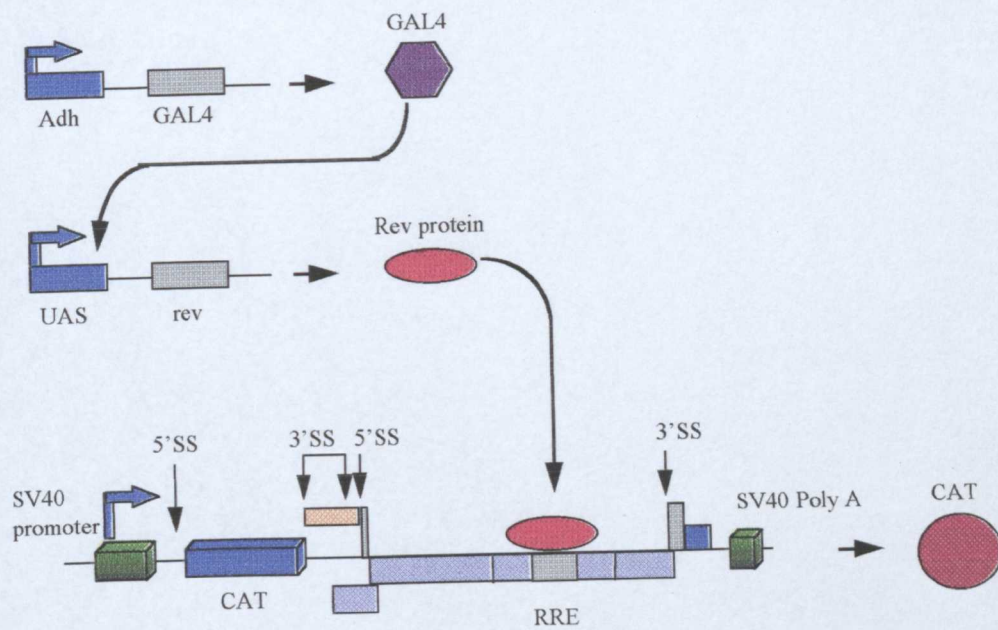
(B) Table of the results from the transfections.

Cells were harvested, and assayed as in figure 4.4. The constructs did not appear to produce any measurable CAT activity in the final assays.

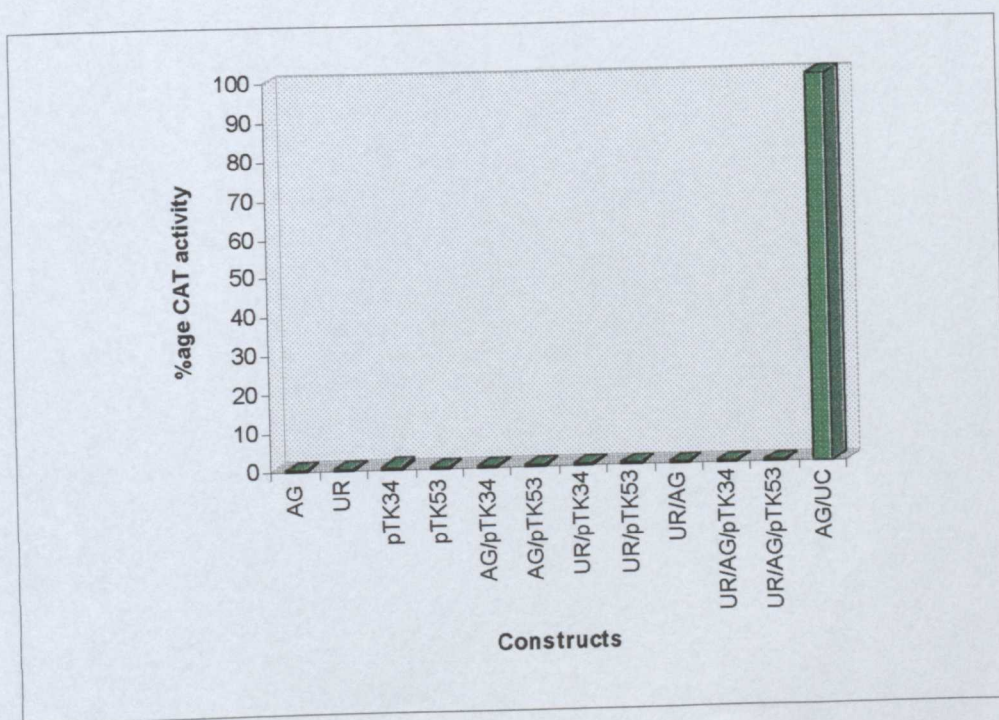
(C) The data from (B) was plotted as a histogram.

Abbreviations: (as in figure 4.4 with the following additions) UR: UAS-Rev; SV: Simian Virus promoter/enhancer-driven construct.

(A)



(B)



(C)

| Construct | Reading | β GAL activity | Protein in assay (μ g) | Reading/ μ g of protein | β GAL activity/ μ g of protein | Units CAT/ units β GAL/ μ g of protein | Average Units CAT/ units β GAL | % CAT activity |
|--|----------|----------------------|-----------------------------|-----------------------------|--|--|---|----------------|
| | X | Y | Z | (X/Z) A | (Y/Z) B | (A/B) C | | |
| 5 μ g AG | 4586 | 406 | 2.6 | 1763.8 | 156.2 | 11.29 | 11.9 | 0.04 |
| 5 μ g AG | 8956 | 705 | 4.0 | 2239 | 176.3 | 12.7 | | |
| 5 μ g UR | 5565 | 499 | 2.5 | 2226 | 199.6 | 11.2 | 8.5 | 0.03 |
| 5 μ g UR | 2310 | 396 | 1.6 | 1443.75 | 247.5 | 5.8 | | |
| 5 μ g TK34 | 80931 | 406 | 4.0 | 20232.8 | 101.5 | 199.3 | 191 | 0.60 |
| 5 μ g TK34 | 67810 | 371 | 3.6 | 18836.1 | 103.1 | 182.7 | | |
| 5 μ g TK34 | 3420 | 544 | 3.6 | 950 | 151.1 | 6.3 | 13.9 | 0.37 |
| 5 μ g TK54 | 6453 | 300 | 2.5 | 2581.2 | 120 | 21.5 | | |
| 5 μ g AG/ 5 μ g TK34 | 2318 | 271 | 3.8 | 610 | 71.3 | 8.5 | 8.9 | 0.03 |
| 5 μ g AG/ 5 μ g TK34 | 4528 | 483 | 2.9 | 1561.4 | 166.6 | 9.4 | | |
| 5 μ g AG/ 5 μ g TK53 | 6009 | 299 | 3.0 | 2003 | 99.6 | 20.1 | 11.5 | 0.04 |
| 5 μ g AG/ 5 μ g TK53 | 1345 | 473 | 2.6 | 517.3 | 181.9 | 2.8 | | |
| 5 μ g UR/ 5 μ g TK34 | 1571 | 991 | 2.9 | 541.7 | 341.7 | 1.6 | 4.2 | 0.013 |
| 5 μ g UR/ 5 μ g TK34 | 6051 | 693 | 2.6 | 2327.3 | 266.5 | 8.7 | | |
| 5 μ g UR/ 5 μ g TK53 | 4633 | 274 | 3.3 | 1403.9 | 83.0 | 16.9 | 13.6 | 0.04 |
| 5 μ g UR/ 5 μ g TK53 | 5097 | 500 | 4.0 | 1274.3 | 125 | 10.2 | | |
| 5 μ g UR/ 5 μ g AG | 3622 | 588 | 2.7 | 1341.5 | 217.8 | 6.2 | 7.9 | 0.025 |
| 5 μ g UR/ 5 μ g AG | 3821 | 400 | 4.7 | 812.9 | 85.1 | 9.6 | | |
| 5 μ g UR/ 5 μ g AG/ 5 μ g TK34 | 3008 | 555 | 3.2 | 940 | 85.1 | 11.0 | 24 | 0.75 |
| 5 μ g UR/ 5 μ g AG/ 5 μ g TK34 | 3652 | 394 | 3.8 | 961.1 | 103.7 | 9.3 | | |
| 5 μ g UR/ 5 μ g AG/ 5 μ g TK34 | 2045 | 550 | 2.7 | 757.4 | 203.7 | 3.7 | | |
| 5 μ g UR/ 5 μ g AG/ 5 μ g TK53 | 3000 | 794 | 4.9 | 612.2 | 162.0 | 3.7 | 5.6 | 0.018 |
| 5 μ g UR/ 5 μ g AG/ 5 μ g TK53 | 3721 | 495 | 4.4 | 845.7 | 112.5 | 7.5 | | |
| 5 μ g AG/ 5 μ g UC | 20584912 | 863 | 2.7 | 7624041.2 | 319.6 | 23854.9 | 31895.9 | 100 |
| 5 μ g AG/ 5 μ g UC | 19804739 | 496 | 3.0 | 6601579.7 | 165.3 | 39936.9 | | |

fragment from pTK43 under the control of the UAS promoter) and UAS-mRRE (the fragment from pTK53 under the control of the UAS promoter). 5µg of Adh-GAL4, 5µg of UAS-Rev and 5µg of UAS-RRE were used to transfect S2 cells (figure 4.8A). A identical experiment was set up using 5µg of UAS-mRRE as a negative control.

As controls, each plasmid alone was used for transfection (figure 4.8 B and C: Adh-GAL4; UAS-Rev; UAS-RRE; or UAS-mRRE) and then with each other plasmid in turn (Adh-GAL4 and UAS-RRE; Adh-GAL4 and UAS-mRRE; UAS-Rev (UR) and UAS-RRE; UAS-Rev and UASmRRE; or UAS-Rev and Adh-GAL4) as for the previous experiment.

In both this and the previous experiment (4.4.3) 5µg of Adh-GAL4 and 5µg of UAS-CAT were used to transfect cells and the extract loaded onto each TLC plate as a positive control for the CAT assays (data not shown). No significant amount of CAT activity was observed in extracts from either of the Rev responsive plasmids, UAS-RRE or UAS-mRRE (figure 4.8B and C). This suggests that either there was a problem with expression of the plasmids other than promoter control or for some reason Rev was not expressed or did not bind to the RRE in UAS-RRE/UAS-mRRE.

4.5 Discussion

A co-transfection assay in *Drosophila* tissue culture cells has been utilised and has shown that GAL4 can activate a standard CAT reporter plasmid under UAS control, attempts have been made to assess whether a Rev responsive plasmid can be used to determine if Drongo had a function in tissue culture cells in enhancing Rev activity. Two sets of Rev responsive plasmids were used, one under the control of the SV40 promoter and the other under the control of the UAS promoter. Both sets of plasmids showed no Rev responsive CAT activity, and so the influence of the Drongo protein on Rev activity could not be assessed.

Figure 4.8:

Schematic, table and histogram of tranfection of S2 cells with UAS-RRE and UAS-mRRE.

(A) Constructs used in the assays.

For control experiments: 5 μ g UAS-RRE (a subclone of pTK34 with the Rev responsive fragment under the control of the UAS promoter), UAS-mRRE (a subclone of pTK53 under the control of the UAS promoter), Adh-GAL4 or UAS-Rev were transfected alone or with 5 μ g of each other plasmid.

In the final assays, 5 μ g UAS-RRE, UAS-mRRE, Adh-GAL4 and UAS-Rev were all transfected into the same cells.

Cells were transfected as in figure 4.4.

The experiment was a repeat of figure 4.8 but using the constructs under UAS control.

(B) The data from (C) was plotted as a histogram.

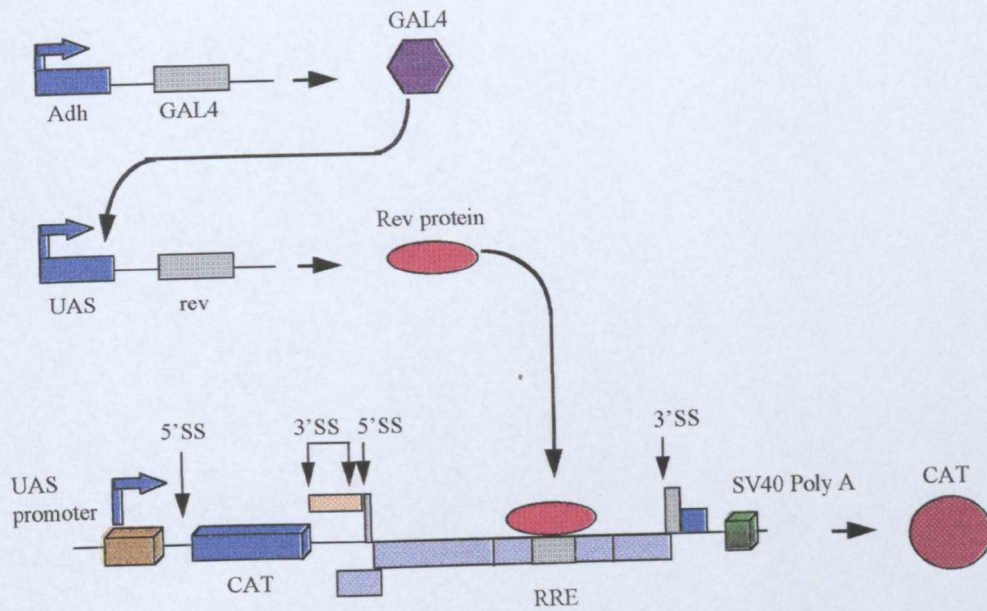
Cells were harvested and assayed as in figure 4.4.

(B) Table of results from the CAT assays.

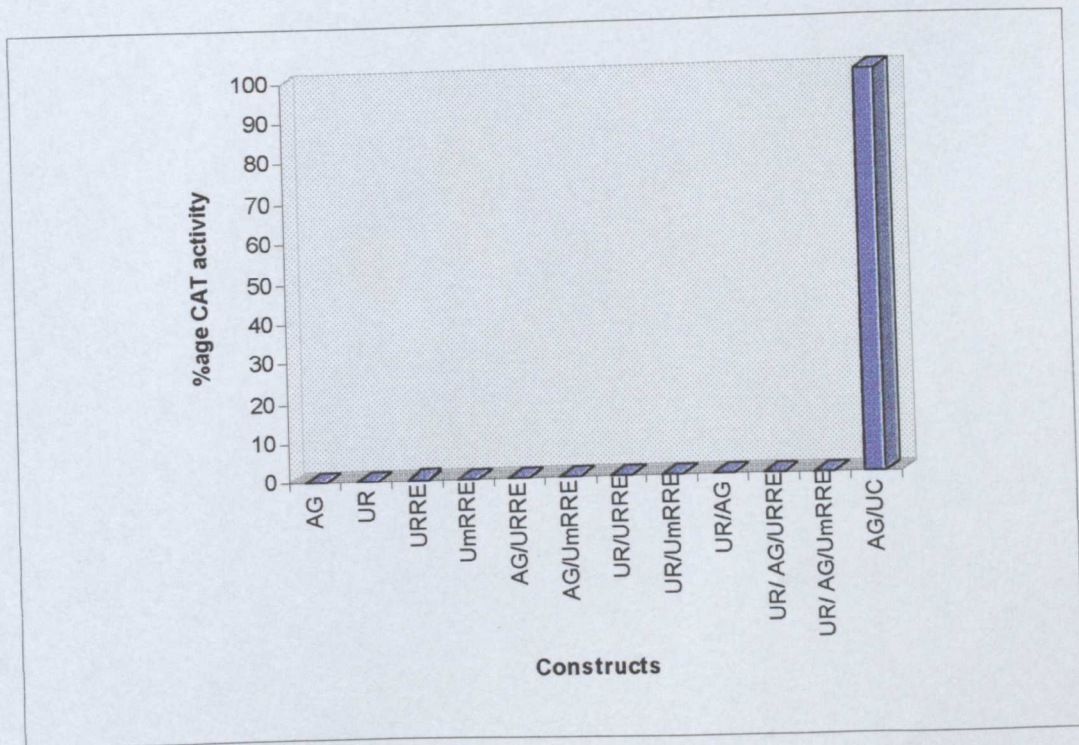
The constructs in the final assays do not appear to produce any measurable CAT activity in S2 cells.

Abbreviations: as in figure 4.4.

(A)



(B)



(C)

| Construct | Reading | β GAL activity | Protein in assay (μ g) | Reading / μ g of protein | β GAL activity/ μ g of protein | Units CAT/ units β GAL/ μ g of protein | Average Units CAT/ units β GAL | % CAT activity |
|------------------|--------------|----------------------|-----------------------------|------------------------------|---|--|---|----------------|
| | X | Y | Z | (X/Z) A | (Y/Z) B | (A/B) C | | |
| AG | 3078 | 180 | 4.9 | 628.2 | 36.7 | 17.1 | 29.95 | 0.05 |
| AG | 4967 | 116 | 3.6 | 1379.7 | 32.2 | 42.8 | | |
| UR | 1057 | 70 | 5.8 | 182.2 | 12.1 | 15.1 | 17.9 | 0.28 |
| UR | 4078 | 197 | 2.0 | 2039 | 98.5 | 20.7 | | |
| URRE | 6079 | 20 | 2.7 | 2251.5 | 7.4 | 303.9 | 170.9 | 0.26 |
| URRE | 7045 | 186 | 1.7 | 4144.1 | 109.4 | 37.9 | | |
| UmRRE | 4068 | 500 | 5.9 | 689.5 | 84.7 | 8.1 | 11.1 | 0.02 |
| UmRRE | 2298 | 164 | 3.7 | 621.1 | 44.3 | 14.0 | | |
| AG/ URRE | 2067 | 112 | 5.4 | 382.8 | 20.7 | 18.5 | 64.8 | 0.09 |
| AG/ URRE | 3330 | 30 | 7.8 | 426.9 | 3.8 | 111 | | |
| AG/ UmRRE | 4035 | 109 | 5.0 | 807 | 21.8 | 37.0 | 23.5 | 0.03 |
| AG/ UmRRE | 2057 | 205 | 4.3 | 478.4 | 47.7 | 10.0 | | |
| UR/ URRE | 6079 | 113 | 6.9 | 881.0 | 16.4 | 53.7 | 50.4 | 0.08 |
| UR/ URRE | 9022 | 164 | 6.5 | 1388 | 25.2 | 55.0 | | |
| UR/ UmRRE | 3065 | 185 | 2.6 | 1178.8 | 71.2 | 16.6 | 11.6 | 0.02 |
| UR/ UmRRE | 2007 | 300 | 3.0 | 669 | 100 | 6.69 | | |
| UR/ AG | 3076 | 278 | 4.8 | 640.8 | 57.9 | 11.1 | 28.6 | 0.04 |
| UR/ AG | 3958 | 123 | 2.9 | 1364.8 | 42.4 | 32.2 | | |
| UR/ AG/ URRE | 9784 | 206 | 5.6 | 1747.1 | 36.8 | 47.5 | 52.4 | 0.08 |
| UR/ AG/ URRE | 10897 | 135 | 5.8 | 1878.8 | 23.3 | 80.7 | | |
| UR/ AG/ URRE | 3960 | 137 | 3.9 | 1015.4 | 35.1 | 28.9 | | |
| UR/ AG/ UmRRE | 1108 | 145 | 7.0 | 158.3 | 20.7 | 7.6 | 28.7 | 0.04 |
| UR/ AG/ UmRRE | 5947 | 263 | 5.7 | 1043.3 | 46.1 | 22.6 | | |
| UR/ AG/ UmRRE | 7957 | 142 | 8.7 | 914.6 | 16.3 | 56.1 | | |
| AG/ UC | 1098057 6 | 165 | 3.0 | 3660192 | 55 | 66548.9 | 64876.3 | 100 |
| AG/UC | 8605000 3 | 200 | 6.5 | 1323846 2 | 30.8 | 42982.0 | | |
| AG/UC | 1107890 6 | 130 | 4.7 | 2357214 | 27.7 | 85097.9 | | |

4.5.1. Assessment of the Rev activity assay.

These experiments were carried out in order to enable us to use a reporter system in which a change in activity of the reporter gene could be measured, this was achieved successfully.

The second objective of the assay was to test the Rev responsive plamids in order to ascertain whether we could indirectly measure Rev activity via measuring CAT activity. This was not possible using the constructs available and the constructs produced in the laboratory.

The reason this did not work is unclear. There are several possible reasons why the assay could not be carried out using either pTK34 or UAS-RRE in this study:

(A) There has been several lines of evidence that the SV40 promoter driving pTK34 may not work very efficiently in *Drosophila* cells although other research disputes this. The plasmid used for checking the efficiency of transfection is also under the control of the SV40 plasmid and this appears to transcribe efficiently in this system.

(B) A mutation in any of the sequences required for the assay to work in this system may produce a non functional transcript. However, the *rev* gene and pTK34 were sequenced and the relevant sequences were analysed including the SV40 promoter, the CAT gene, the RRE and the splice sites. None of these sequences appeared to contain any mutations/deletions.

(C) The Rev protein may not be expressed or may be expressed at a level insufficient to promote RRE binding, this was not been verified and would need to be if the experiment is repeated.

(D) Expression was also not possible using UAS-RRE. The ligation junctions after production of this clone were also sequenced (data not shown) and this also appeared to contain no mutations.

(E) Poor codon usage (McPherson, 1988) may present another factor which puts constraints on the assay. We have no indication of the codon usage. This has not been tested but could be measured by the method of Grantham *et al* (1980).

Previous experiments did not find it necessary to include the addition of a co-factor in order to assay Rev function in *Drosophila* (Ivey Hoyle and Rosenberg, 1990), so this is not likely to be a limiting factor in this study (although it is likely that different Rev clones were used in our study and in others), it remains a possibility that Rev requires a basal level of an endogenous co-factor in the cells in which it functions.

Other co-transfection assays reported have used successfully different types of reporter systems, such as the metallothionein (Mt) assay for measuring Rev activity (Ivey-Hoyle and Rosenberg, 1990). Cells stably transfected with the reporter gene under the control of the Mt promoter will express protein upon metal induction of the promoter. Although the plasmids used in this study have previously been reported to work efficiently in human cells it seemed that expression and subsequent measurement of Rev activity was not possible.

The ultimate objective of the assay was to determine if the Drongo protein could affect Rev activity *in vitro*, it was not possible to test this in this assay and so the result of this is not yet known. It would be interesting to analyse different inducible vectors in this particular line and observe any effects of Drongo in this way. It is known that a co-factor is required for Rev *in vivo* and the identification of hRIP in human suggests that a conserved co-factor(s) may be will be found in *Drosophila*. No other molecule has yet been identified in *Drosophila* that fulfils this role, we have a case therefore to analyse whether the Drongo has an analogous function to hRIP.

4.5.2. Analysing Drongo function *in vivo*.

From this study, it has not yet been possible to observe if Drongo has a function *in vitro*, via tissue culture experiments. Prior to attempts to try a different set of vectors in the cell line established in this study, it would be wise to assess as much as possible if

a function can be assigned to Drongo *in vivo*. This could be performed by analyses of any mutations of the gene, this being one of the principal aims of the study.

Production of an antibody to the Drongo may also establish the location of the protein in the cell. The use of tissue culture studies to may then be re-examined with a revised view on the function of the Drongo protein.

Chapter 5:

EMS Mutagenesis

Chapter 5: RESULTS AND DISCUSSION: EMS Mutagenesis.

5.1 Introduction.

One of the most informative ways of determining the function of a gene in *Drosophila* is to isolate a mutation of the gene and analyse the mutant phenotype. Initial attempts to produce deletions of the *drongo* gene by excision of the P-element were unsuccessful (section 1.2.2) therefore a mutagenesis screen of the region was carried out in an attempt to produce lethal alleles of *drongo*.

This screen was initiated after a review of Roberts *et al.* (1985) in which EMS mutagenesis was carried out on the second chromosome, in the region 21C6-D1-22A6-22B1, an interval which encompasses the *drongo* gene and its neighbours. This work offered us a starting point from which to proceed with the mutagenesis as we could obtain the deficiencies (the *asteroid* deficiencies) they produced. Roberts *et al.* (1985) established four mutable regions (complementation groups) which may correspond to four transcribed regions, one of which may have been *drongo*. Unfortunately these stocks were lost and so it was not possible for us to test them in our study.

5.2 Analysis of the 21D region.

When this screen was initiated, we had obtained some information on the genetics of the 21D region on the second chromosome (figure 5.1) from a number of sources (Harris, 1995 and *pers. comm.* Edwin Chan). The original enhancer trap (B75.1M2) screen (Harris, 1995) and hybridisation of B75.1M2 genomic clones to the *ninaA* genomic walk (Schneuwly *et al.*, 1989) (section 1.2) placed B75.1M2 between the left breakpoints of deficiencies Df(2L)*ast4* and Df(2L)*ast6* (Roberts *et al.*, 1985) (figure 5.1). Reverse northern blots identified 3 transcripts. The first gene isolated was *drongo*. The second gene, *arouser*, encoded a putative eps8 homologue; and the third, *kracken*, encoded a putative atropine diesterase

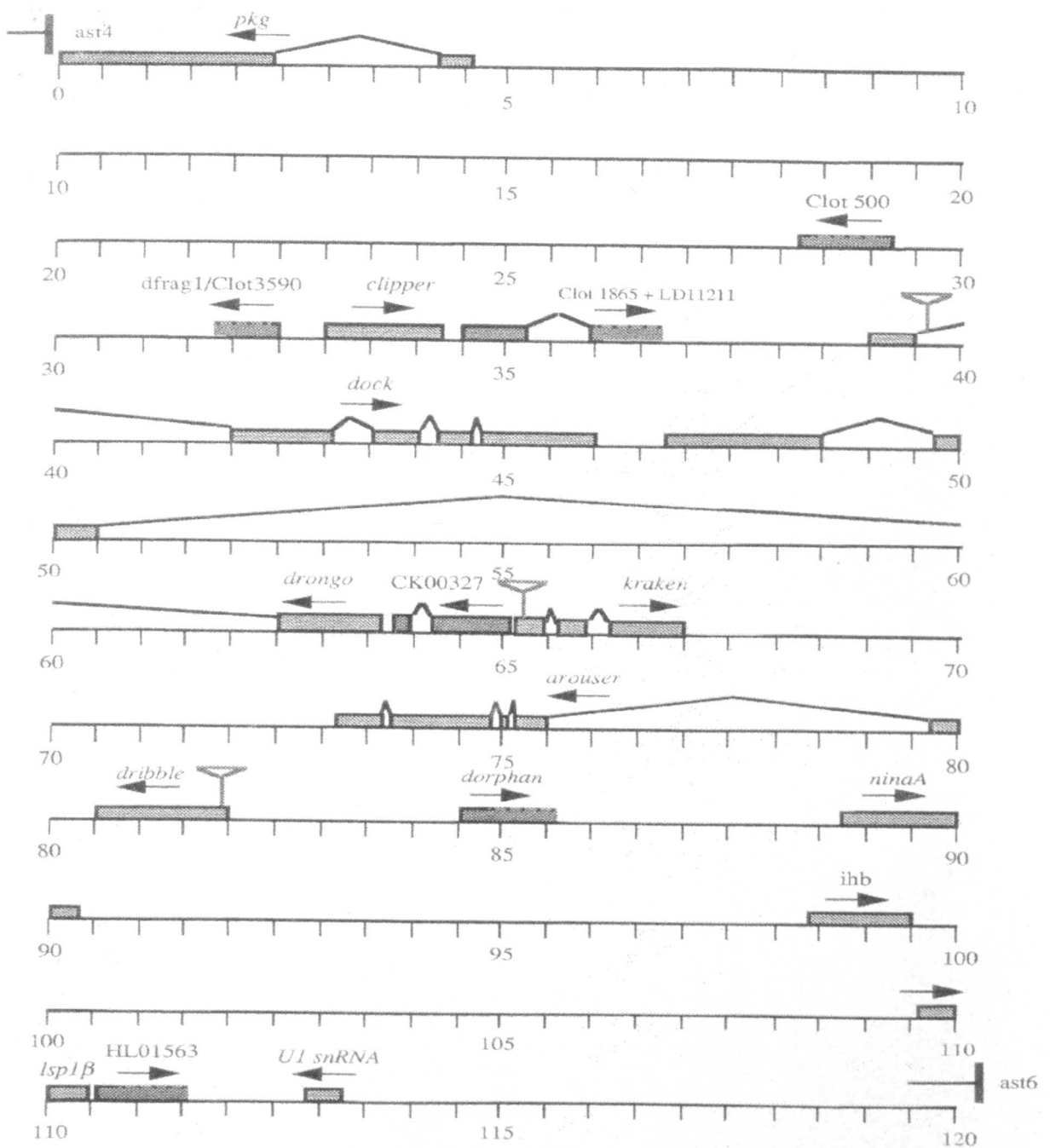
Figure 5.1

Molecular map of the 21D region.

drongo is situated between the breakpoints of two deficiencies, Df(2L)*ast4* and Df(2L)*ast6*. Localisation of the enhancer trap B75.1M2 identified two other genes in the region, *arouser* and *kracken*. Further sequencing of the surrounding area isolated nearby transcripts, *dribble* and *dorphan*. EST sequences from cDNA libraries arranged into clots (homologous EST sequences) were also positioned on the map (clot 500, clot3590, clot 1865)

Key: —| = Deficiency breakpoints; CK, HL and LD are cDNA libraries (see <http://www.fruitfly.org>). \wedge = represent intronic region. Numbers represent bases in the genomic DNA. Shaded boxes represent transcribed regions. Arrows indicate direction of transcription.

∇ = P-element enhancer trap. pkg= Plakaglobin; dock: dreadlocks; lsp1 β : larval serum protein β . Map compiled by Edwin Chan.



homologue (Harris *et al.*, in press). Plasmid rescue (section 1.2.1) and cytological mapping of these cDNAs placed them in relative positions to each other on the walk near to known transcripts. These transcripts include *dreadlocks* (*dock*), a gene required in axon guidance of photoreceptor cells in the eye (Garrity *et al.*, 1996) (situated about 750 bp to the left of *drongo*); *ninaA* (Schneuwly *et al.*, 1989) (which is approximately 25kb to the right of *drongo*); and *clipper*, an endonuclease (Bai and Tolia, 1996). Further sequencing around the region (Edwin Chan *pers. comm.*) identified two other regions of transcribed DNA, named *dribble* and *dorphan*. *dribble* maps to approximately 18 kb to the right of *drongo* and *dorphan* maps to approximately 2.5 kb to the right of *dribble*. The whole of this region (120kb) of the map has now been sequenced (BDGP: contig pkg21D (<http://www.fruitfly.org/sequence/drosophila-regions.html>)).

Localisations of lethal P-element deletions in *dock* (l(2)05423) and *dribble* (l(2)k06708) are also shown in figure 5.1. EP elements (Rorth, 1996) have also been mapped to the region (figure 5.1). EP elements are insertion lines which carry a regulatable promoter for misexpression of the endogenous genes at the site of insertion. Many of these elements have been mapped by *in situ* hybridisation to polytene chromosomes and, in many, the flanking genomic DNA has been sequenced (BDGP, Berkeley Fly database).

Further analysis with Expressed Sequence Tags (ESTs) have also identified other possible transcripts in the region. An EST is the sequence of the 5' or 3' end of a cDNA used to identify expressed genes in the genome and these are available on EST databases (<http://www.fruitfly.org/EST/index.html#ESTs>). cDNA clones from each library have been compared to other clones in the library using nucleotide sequence searches using a BLAST tool and clones homologous to each other are grouped as "clots". The position of known clots in the 21D region is shown on figure 5.1.

The molecular map obtained was sufficient to consider initiating a mutagenesis screen as we could use this information to screen the mutants and identify mutant alleles, primarily in *drongo* and secondarily in any other genes in the region.

5.3 Isogenisation of the original stock.

The mutagenesis screen consisted of finding new lethal mutations in homozygous lines and so it was important the WCS strain to be mutagenised did not contain any lethal mutations already in the stock. Spontaneous mutations may occur in any culture, some of these may be recessive and present in a heterozygous state, and thus would usually go unnoticed. A spontaneous mutation frequency of 1% of flies in a stock subsequently used for mutagenesis would lead to the majority of 'new' variants being pre-existing mutations (Greenspan, 1997) and a new lethal phenotype may be incorrectly assumed.

Isogenisation meant starting a new stock from a single chromosome and using the stock before it acquired any natural lethal mutations. The isogenisation scheme used in this study was carried out as shown in figure 5.2 (see section 2.8.1).

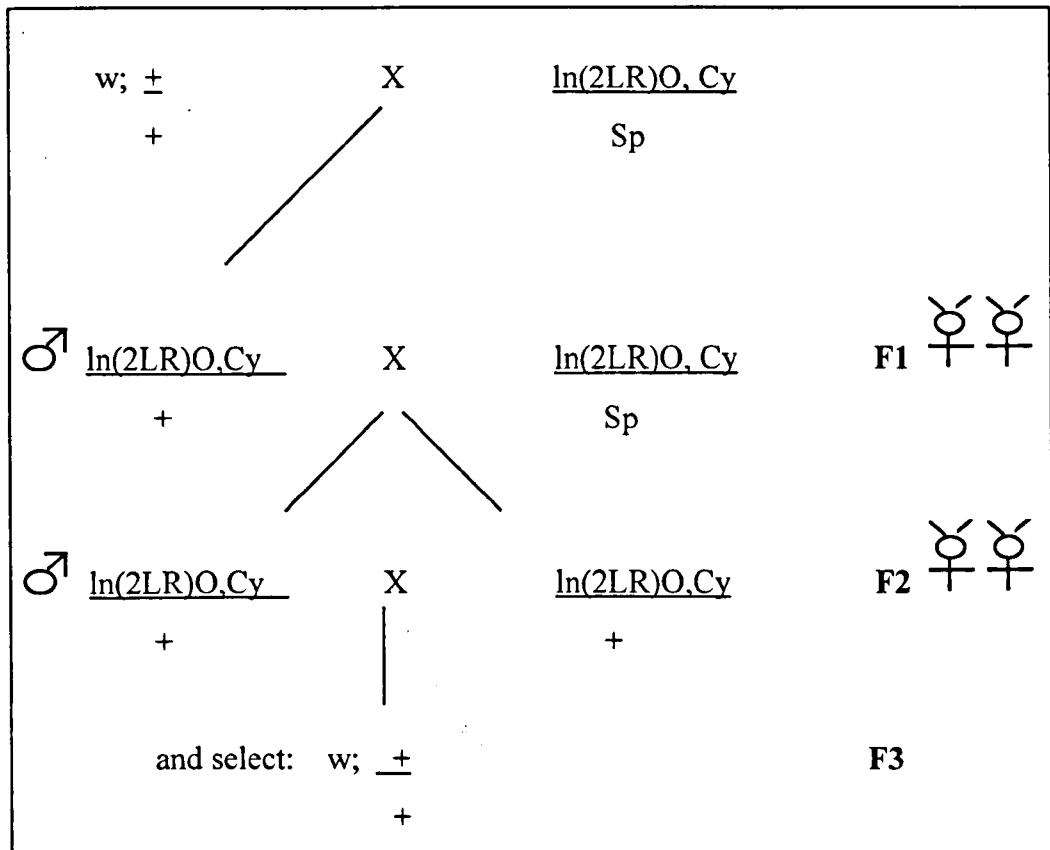


Figure 5.2

Crossing scheme for isogenisation of the second chromosome.

The crossing scheme was performed as in Greenspan (1997). At F1 a single w; +/+ chromosome was propagated by selecting CyO males (more than one vial was set up as some may be lethals). All of the progeny in the F2 generation, carry the wild type (+) chromosome. This chromosome cannot recombine with its homologue, the balancer *ln(2LR)O,Cy*. F2 siblings were then crossed together.

The first of these crosses was designated bottles 4D and 3A (the numbering system is explained in the text). By F3 the stock was isogenic. See section 2.8.1.

5.4 RESULTS

5.4.1 EMS mutagenesis.

The mutagenesis was performed using EMS (section 2.8.2). This is an alkylating agent which binds to one strand of the DNA double helix, usually producing point mutations and sometimes small deletions. Flies were mutagenised with 25 mM of EMS (this should induce 4.3×10^6 alkylations/sperm as reviewed in Ashburner, 1989). Males to be mutagenised were briefly starved for 12 hours before treatment to increase the frequency of recovered mutations. The mutagenesis was performed in bottles using 100 males in each. Bottles were organised into a series for ease of labelling the origin of the mutants. Bottles were labelled according to the isogenisation stock from which they originated, and isogenisation stocks used were 4D and 3A . Bottles were labelled 4DA-4DZ; 4EA-4EQ and 3AA-3AZ; 3BA-3BN and used in alternate order for example, 4DA, 3AA, 4DB, 3AB. Males were mutagenised, crossed to virgin females (figure 5.3a) and the progeny were used for further analysis.

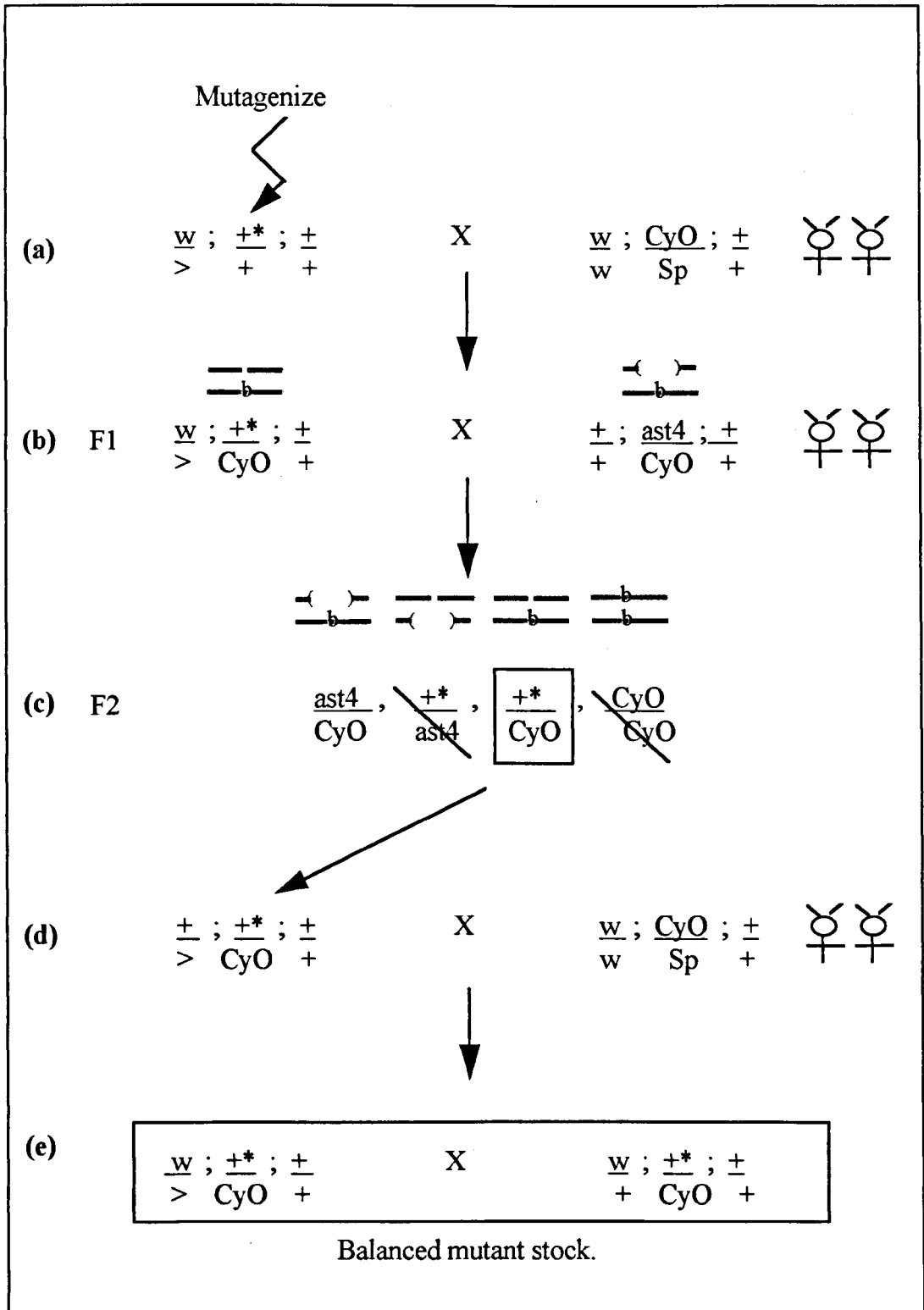


Figure 5.3

Crossing scheme for mutagenesis and screening of mutants over Df(2L)ast4.

(a) Males from the isogenised stock (section 5.3) were mutagenised with 25mM EMS (section 2.8.2) and crossed to virgins of a balanced stock (w; CyO/Sp)



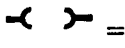
(b) The mutations balanced over CyO were then crossed to Df(2L)ast4.

(c) In cases of non-complementation crosses were set up as in (D)

(d) Mutations balanced over CyO were used to backcross to w; CyO/Sp virgins.

(e) The progeny were balanced over CyO and used to generate a stock.

7120 mutant males were isolated by this screen. 55 mutations did not complement Df(2L)ast4 and thus were in the region of interest.

Key:  = balancer chromosome,  = EMS induced mutation,  = Deficiency (Df(2L)ast4). For progeny numbers see section 10.2 B.

5.4.2 Analysis of the mutants.

Male progeny from the above cross were then analysed for any lethal mutations in the 21D region.

5.4.1 Deficiency analysis of the mutants.

Males were screened over a deficiency (for deficiency breakpoints see table 2.6) which uncovers the *drongo* locus, Df(2L)ast4 (figure 5.3b), in order to select any mutations in the 21D region (figure 5.1). Mutants in the region (lethal over Df(2L)ast4; figure 5.3c) were recovered at a rate of about 1/150 male progeny screened. 7120 chromosomes were screened over Df(2L)ast4 and 55 mutants did not complement Df(2L)ast4. The progeny of the deficiency cross, balanced over CyO (figure 5.3d) were then used to generate a stock (figure 5.3e). A white eye phenotype was introduced to this stock (using allele w¹¹⁸) in order to attempt

subsequent rescue of any mutant alleles by using cloned transgenes marked with w^+ . Stocks were then used for further analysis.

Mutant stocks were then tested for complementation over another deficiency, $Df(2L)ast6$ (figure 5.4), the breakpoints of which map further downstream of *drongo* (table 2. 6 and figure 5.1). Non-complementation was taken as the absence of Cy^+ flies in over 100 progeny. Mapping the breakpoints of $Df(2L)ast4$ and $Df(2L)ast6$ had determined that they overlap further downstream of the 21D region (Roberts *et al.*, 1985). Complementation testing over both deficiencies would therefore attenuate the number of mutants down to those in the 21D region (figure 5.1).

Mutants which complemented $Df(2L)ast6$ (and thus were not in the $Df(2L)ast6$ region (figure 5.4) were then chosen for further analysis. Of the 55 mutants lethal over $Df(2L)ast4$, 47 mutants were viable over $Df(2L)ast6$. These were therefore in the region of interest. Mutants which did not complement $Df(2L)ast6$ were kept as stocks but not used further in this study.

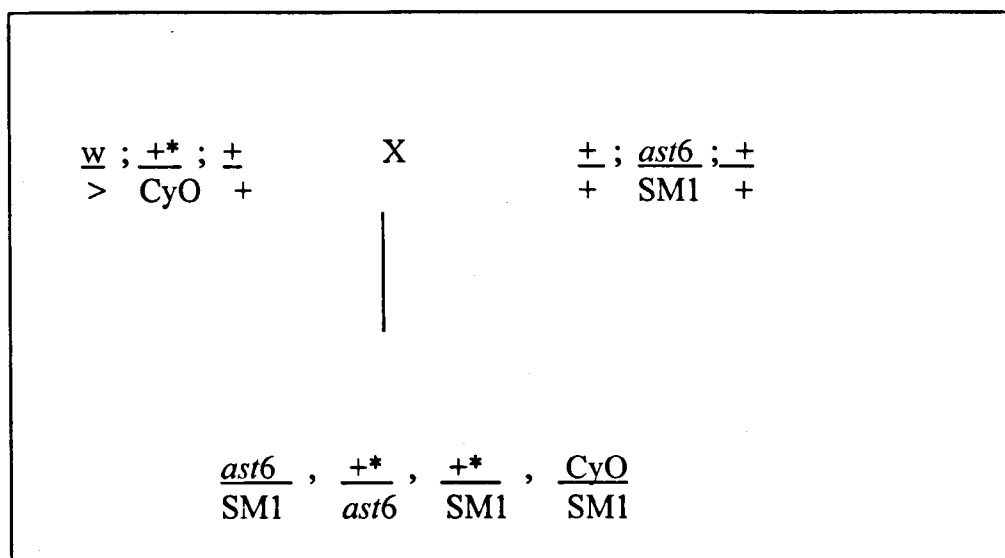


Figure 5.4

Crossing scheme for complementation testing over $Df(2L)ast6$.

Males from mutant stocks which were lethal over $Df(2L)ast4$ were crossed to $Df(2L)ast6$, in a test for complementation. Non-complementation was taken as the

absence of CyO⁺ flies in over 100 progeny. Mutants were crossed to Df(2L)*ast6* virgin females in single pair matings. Df(2L)*ast6* was balanced over SM1 in order to assess the lethality of the mutation. Alleles which were lethal over Df(2L)*ast4* and viable over Df(2L)*ast6* were used for further analysis. Any alleles which were lethal over Df(2L)*ast6* were kept as stocks but not analysed any further. Using this scheme, 47 mutants did not complement Df(2L)*ast4* but complemented Df(2L)*ast6* and thus were in the region of interest.

5.4.4 Complementation analysis.

5.4.4.1 Classification of mutants into lethal complementation groups.

Selected mutant stocks were then organised into allelic groups by lethal complementation analysis. This was performed in order to determine the number of lethally mutable regions and thus the number of essential transcripts in the genomic area. Mutant stocks were crossed as single pair matings (section 2.8 2), taking virgins of the first mutant obtained (4DA-122) and crossing them to males of all of the other mutant stocks (figure 5.5). In this way, the first complementation group was established by grouping alleles which did not complement 4DA-122 (figure 5.5). Group two was determined by taking the next mutant which was not in group one (thus this mutant complemented 4DA-122) and crossing it to all the other mutants which did not fit into this group. This was continued in the same manner until all of the mutants had been grouped (For progeny numbers see section 10.2 C). This scheme determined that there were at least six complementation groups with at least two mutants in each group (Table 5.1).

Crossing the mutants in each group *inter se* showed that there appeared to be some mutants which existed in more than one group (3AC-184, 4DI-1893 and 4EC-3906) and some in more than two groups (4DS-2678, 4DS-2715 and 4DS-2716) (Table 5.1). 4DS-2678, 4DS-2715 and 4DS-2716 may have contained deletions as a consequence of the mutagenesis.

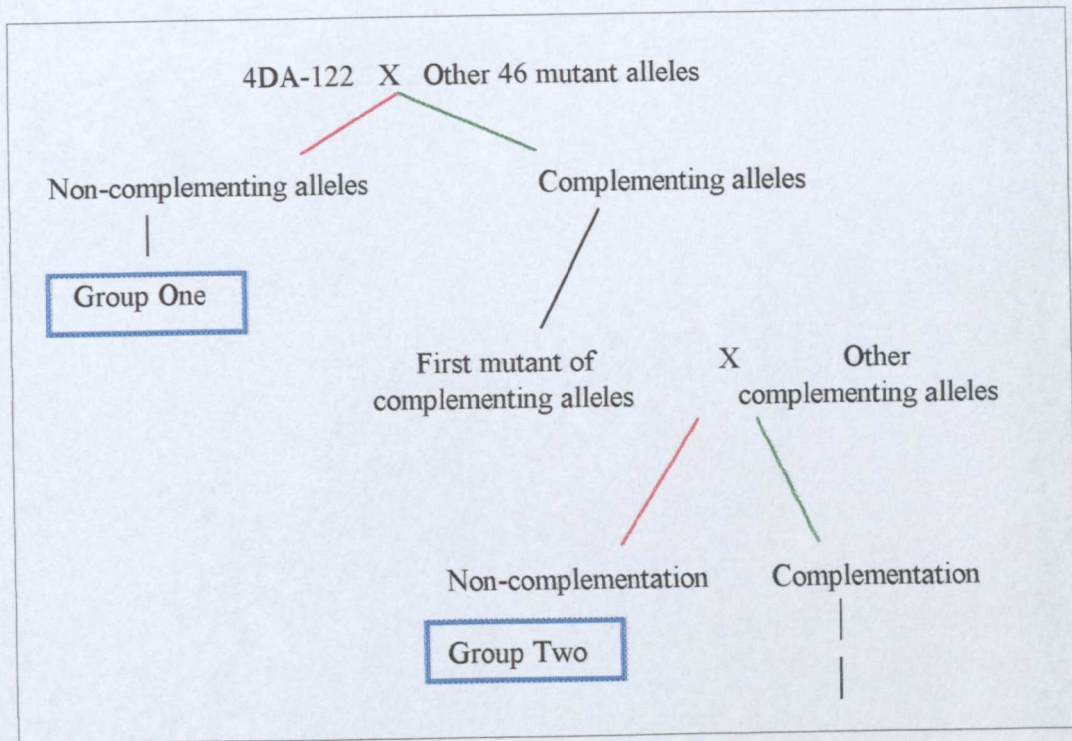


Figure 5.5

Flow diagram indicating how lethal complementation analysis was performed.

Virgin females of the first mutant (4DA-122) were crossed to males of all of the other mutant stocks, any which did not complement 4DA-122 were placed into group one. The next mutant (in order of those obtained) was then crossed to all of the other mutants which complemented 4DA-122, thus any which showed non-complementation were placed into group two. In this way 6 complementation groups were established and all of the mutants were placed into at least one group.

| One | Two | Three | Four | Five | Six |
|-------------------------|-------------------------|------------------|-------------------------|-------------------------|------------------|
| 4DA-122 (HL) | 3AC-184 (HL) | 4DG-1524 (HV) | 3AC-184 (HL) | 4DL-1801 (HV) | 4DS-2715 |
| 4DD-418 (HV) | 4DG-777 (HL) | 3AL-1872 (HV) | 4DD-201 (HV) | 4DI-1893 (HL) | 4DS-2716 |
| 4DH-1251 (HV) | 4DI-1893 (HL) | | 4DD-288 (HV) | 4DL-1980 (HV) | 4DS-2678 |
| 4DI-1367 (HV) | 4DS-2705 (HV) | | 4DS-2678 | 4DN-2014 (HL) | 4EB-3455 (HL) |
| 4DS-2678 (HL) | 4DS-2678 | | 4DS-2715 | 4DS-2678 | 3BC-3677 (HL) |
| 4DS-2715 (HL) | 4DS-2715 | | 4DS-2716 | 4DS-2715 | 4EC-3906 (HL) |
| 4DS-2716 (HL) | 4DS-2716 | | 3AU-2858 (HL) | 4DS-2716 | 4EF-4391 (HL) |
| 3AZ-3013 (HL) | 3BL-5823 (HL) | | 4DY-3071 (HL) | 3BC-3629 (HL) | 3BE-4401 (HL) |
| 3BK-5333 (HL) | 3BL-6666 (HL) | | 3AY-3216 (HL) | 3BC-3677 (HL) | 4EC-4540 (HL) |
| 4EQ-6548 (HL) | | | 4DY-3291 (HL) | 4EC-3742 (HL) | |
| | | | 4EC-3781 (HV) | 3BF-4311 (HL) | |
| | | | 4EC-3906 (HL) | 3BE-4331 (HL) | |
| | | | 3BE-4178 (HL) | 3BK-5476 (HL) | |
| | | | 4EH-4705 (HV) | 3BK-5587 (HL) | |
| | | | 4EK-4975 (HL) | 3BN-6246 (HV) | |
| | | | 4EK-5076 (HL) | | |
| | | | 4EP-6332 (HL) | | |
| | | | 4ER-6893 (HL) | | |

Table 5.1

EMS complementation groups.

Six groups were established by lethal complementation crossing (as performed in figure 5.5). Those in plain type are probably point mutations. Those in bold are in more than one group and so possibly have more than one point mutation or contain a small deletion. (HV= homozygous viable (these mutants were hemizygous lethal over the deficiency), HL=homozygous lethal). (For full details of progeny numbers see appendix 10.2C)

5.4.4.2 Complementation of mutants over P-element deletions.

Once the complementation groups were established, the task was set to specify if any of the groups were *drongo* alleles. This was facilitated by production of a number of P-element deletions/insertions in the region (*pers. comm.* Edwin Chan; Table 5.2). Unfortunately, none of the deletions uncovered the *drongo* locus alone, which made assignment of a *drongo* group more abstruse. However a number of the EMS alleles thought to be deletions (4DS-2678, 4DS-2715 and 4DS-2716) were shown to be in five of the six complementation groups (Table 5.1) and so there is a strong possibility these uncovered the *drongo* gene. The P-element deletions were also used in an attempt to assign the complementation groups to known transcripts in 21D region (figure 5.1). Table 5.2 shows the results of these complementation crosses (*pers. comm.* Edwin Chan) These were performed in single pair matings to virgins of the P-element deletion/insertion stocks.

Details of the P-element insertions and deletions are as follows (*Pers. Comm.* Edwin Chan): lethal 4723 is a P element insertion into the *Dreadlocks* (*Dock*) allele; 5428 is a lethal Pelement insertion into *dribble* (*dbe*). C16 is an precise excision uncovering *arouser* and *dbe* and 32/16 uncovers *dbe*. There are also two Pelement excisions uncovering *dbe*, D120 extends leftwards from *dbe* and D125 extends rightwards.

The molecular breakpoints of all the deletions and insertions have not yet been molecularly defined.

Table 5.2

Complementation analysis with P-element deletions/insertions.

Males from mutant stocks in each lethal complementation group were crossed (as single pair matings) to virgins of each of the P-element lethals. Non-complementation was taken as the absence of CyO^+ flies in over 100 progeny and shown as a red box. 4DS-2678, 4DS-2715 and 4DS-2716 appear to be deletions (see appendix 10.2 for progeny numbers). Insertion 4723 is a lethal insertion into the *dock* gene. No mutants thought to be point mutations showed non-complementation over 4723 or in 5428, suggesting no alleles were in *dock* alone. A number of alleles from group two did not complement C16 (a deletion in *arouser* and *dribble*) but not D120 (a deletion which extends leftwards from *dribble*), suggesting putative *arouser* alleles. Three mutants from group six and a number from group four did not complement D125 (an excision which extends outwards from *dribble* into *kraken*) or D120. These data suggest that groups one, three and five were candidates for *drongo* mutations. For progeny numbers see appendix 10.2D.

All of the mutations affecting single complementation groups complemented *dock* (4723) and *dribble* (32/16). This suggested that none of the groups we found were alleles of either of these two genes. The imprecise excision C16 fails to complement two alleles in different groups, 4DI-1893 and 3EC-3906. These alleles do not overlap. Additionally C16 does not complement any other alleles in these two groups, suggesting another group may exist which may also include the *dribble* gene (does not complement C16). 3AC-184, however does not fit easily into this model as it affects alleles in both groups two and four.

These results may place group two as a possible *arouser* group, as all complement C16 but not D120 (uncovers *dribble*). Group six may contain alleles of *kraken*, they are lethal over D120 (extends leftwards from *dribble*) and D125,

but viable over C16. The excision D125 extends outwards from *dribble*, and does not complement 4EC-3906 (groups six and four). The results of some of the complementation suggest that groups one, two and five were candidates for *drongo*. However given the tentative nature of these results, groups three, four and six were not ruled out.

5.4.5.1 Phenotypic analysis of each group.

Two homozygous lethal stocks were chosen from each group, in order to determine the earliest lethal phase of the alleles in each group and thus determine a scheme for rescuing possible *drongo* mutants. Alleles considered to be most likely contain point mutations were selected. Flies were crossed in three ways in order to determine the earliest lethal phases (table 5.3). Firstly, the alleles were crossed to produce homozygous progeny which were subsequently scored for stage of lethality. Secondly each mutant was then crossed to Df(2L)*ast4* stock to produce progeny with the mutation over the deficiency. This was performed to ensure no mutations in a different region to the one under study on the chromosome were producing lethality thus confusing the results. Thirdly, a second potential complication may also arise from crossing the mutants to a deficiency. The original chromosome from which the *asteroid* deficiencies (Roberts *et al.*, 1985) were made could not be isogenised and so may also contain 'background' mutations. Therefore finally the first mutant chosen from each group was crossed with the second mutant chosen from that group and the progeny scored (section 2.8.3). The results are shown in Table 5.3. For progeny numbers see section 10.2 E.

| <i>Group No.</i> | <i>Allele.</i> | <i>Cross analysed.</i> | <i>Earliest Lethal Phase of progeny</i> |
|------------------|----------------|---|---|
| One | 4DA-122 | 4DA-122/4DA-122 4DA-122/ <i>ast4</i> 4DA-122/3BK-5333 | Larval (1st instar) Larval (1st instar) Larval (1st instar) |
| | 3BK-5333 | 3BK-5333/3BK-5333 3BK-5333/ <i>ast4</i> 3BK-5333/4DA-122 | Larval (3rd instar) Larval (3rd instar) Larval (3rd instar) |
| Two | 3BL-6666 | 3BL-6666/3BL-6666 3BL-6666/ <i>ast4</i> 3BL-6666/3BL-5823 | Embryonic Embryonic Embryonic |
| | 3BL-5823 | 3BL-5823/3BL-5823 3BL-5823/ <i>ast4</i> 3BL-5823/3BL-6666 | Embryonic Embryonic Embryonic |
| Three | 4DG-1524 | 4DG-1524/4DG-1524 4DG-1524/ <i>ast4</i> 4DG-1524/3AL-1872 | NT Larval (1st instar) Larval (1st instar) |
| | 3AL-1872 | 3AL-1872/3AL-1872 3AL-1872/ <i>ast4</i> 3AL-1872/4DG-1524 | NT Embryonic Embryonic |
| Four | 3AY-3216 | 3AY-3216/3AY-3216 3AY-3216/ <i>ast4</i> 3AY-3216/4EP-6322 | Embryonic Embryonic Embryonic |
| | 4EP-6322 | 4EP-6322/4EP-6322 4EP-6322/ <i>ast4</i> 4EP-6322/3AY-3216 | Embryonic Embryonic Embryonic |
| Five | 3BK-5588 | 3BK-5588/3BK-5588 3BK-5588/ <i>ast4</i> 3BK-5588/3BK-5476 | Larval (3rd instar) Larval (3rd instar) Larval (3rd instar) |
| | 3BK-5476 | 3BK-5476/3BK-5476 3BK-5476/ <i>ast4</i> 3BK-5476/3BK-5588 | Larval (3rd instar) Larval (3rd instar) Larval (3rd instar) |
| Six | 4EF-4391 | 4EF-4391/4EF-4391 4EF-4391/ <i>ast4</i> 4EF-4391/4EC-4540 | Embryonic Embryonic Embryonic |
| | 4EC-4540 | 4EC-4540/4EC-4540 4EC-4540/ <i>ast4</i> 4EC-4540/4EF-4391 | Larval Larval Larval |

Table 5.3

First lethal phase of alleles from each complementation group.

Two representative mutant alleles were chosen from each group to analyse when the earliest lethal phase of the mutation arose. Siblings from each vial were crossed and scored, noting that 1/4 of the progeny would be CyO/CyO and embryonic lethal. For progeny numbers see section 10. 2E

5.4.5.2 Rescue of potential *drongo* alleles.

Arrangement of the mutants into complementation groups and complementation testing over the P-element excisions offered some intimation of which groups to analyse in order to find an alleles which may contain a *drongo* mutation. I decided to perform a rescue experiment using the *hs-drongo* flies produced earlier in this study (section 3.2). Rescue of a mutant phenotype with expression of the wild type gene can offer strong evidence for a role of the gene in development. I wanted to observe whether we could rescue any of the mutant alleles isolated in this screen by expression of *drongo*. Rescue may indicate that we had isolated one or more alleles of the gene. Determination of the earliest lethal phase gave some indication of when to perform the heat shocks, if these were necessary. Rescue of mutant flies was performed as in figure 5.6. Heat shocks were performed before the earliest lethal phase of each alleles studied. Results from these crosses are shown in table 5.4. Rescue was not achieved using this crossing scheme, neither was rescue successful after heat shocks treatment (for progeny numbers see sections 10.2 F and G).

A. Generation of stock no. 1: Rescue of each mutant allele as a homozygote.

(1) $\frac{+}{>}; \frac{+*}{\text{CyO}}; \frac{+}{+}$ X $w; \frac{\text{CyO}}{\text{Sp}}; \frac{\text{TM6B}}{\text{rf10}}$

$\frac{+}{+}; \frac{+*}{\text{CyO}}; \frac{\text{TM6B}}{+}$

(2) $\frac{+}{>}; \frac{+}{+}; \frac{\text{HSD}}{\text{TM6B}}$ X $w; \frac{\text{CyO}}{\text{Sp}}; \frac{+}{+}$

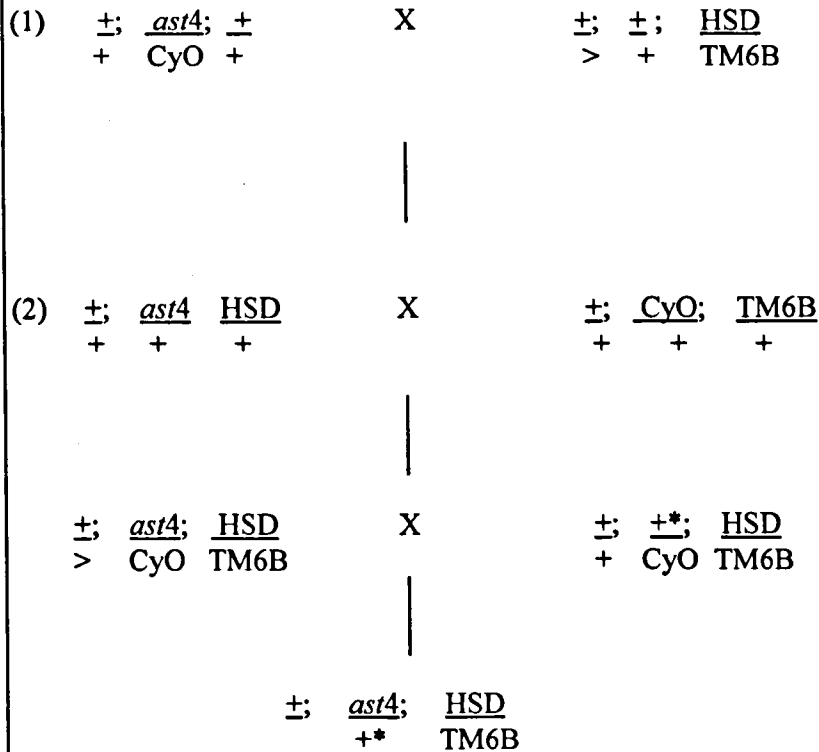
(2a) $\frac{+}{>}; \frac{\text{CyO}}{+}; \frac{\text{HSD}}{+}$ X $\frac{+}{+}; \frac{\text{Sp}}{+}; \frac{\text{TM6B}}{+}$

$\frac{+}{+}; \frac{\text{CyO}}{\text{Sp}}; \frac{\text{HSD}}{\text{TM6B}}$

(3) $\frac{+}{>}; \frac{+*}{\text{CyO}}; \frac{\text{TM6B}}{+}$ X $\frac{+}{+}; \frac{\text{CyO}}{\text{Sp}}; \frac{\text{HSD}}{\text{TM6B}}$

| | | |
|--|---|--|
| $\frac{+}{>}; \frac{+*}{\text{CyO}}; \frac{\text{HSD}}{\text{TM6B}}$ | X | $\frac{+}{+}; \frac{+*}{\text{CyO}}; \frac{\text{HSD}}{\text{TM6B}}$ |
|--|---|--|

B. Rescue of mutant alleles over Df(2L)ast4.



C. Rescue of each mutant allele over one other in the same complementation group.

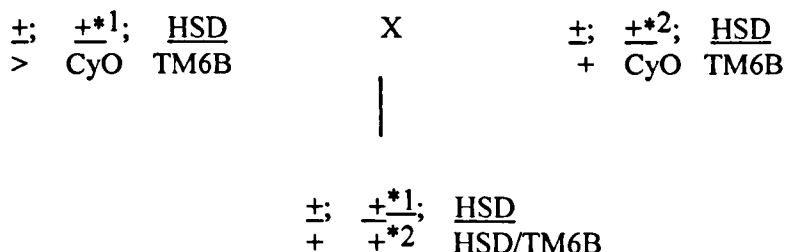


Figure 5.6

Rescue of mutant alleles with *hs-drongo*.

A: Rescue of each mutant allele in a homozygous state. (1) Males from each mutant stock balanced over CyO were crossed to a third chromosome balancer stock. (2) *Hs-drongo* males, balanced over TM6B were then crossed to a second chromosome balanced stock. In order to produce flies which were balanced on the second and the third chromosome. (3) The progeny of cross 1 was then crossed to the progeny of cross 2a producing siblings with both the mutation balanced over CyO and *hs-drongo* balanced over TM6B. When males and females of stock was crossed all of the resulting progeny would contain a balancer except for flies containing both *hs-drongo* and mutation in a homozygous state.

B: (1) Males from *Df(2L)ast4* were crossed to the balanced *hs-drongo* stock. (2) The progeny of cross 1 was then crossed to each other to produce the progeny some of which contained the *ast4* deficiency and *hs-drongo* in the same fly, the progeny of which all contain a balancer except those which carry *hs-drongo* in the homozygous state and the deficiency over the mutation.

C: The crossing scheme in A was repeated for the other allele chosen from each group. Males from one mutant stock were then crossed to females of the other, to produce flies containing *hs-drongo* in the homozygous state and both mutations in a transheterozygous state.

| | | | Rescue conditions | | | | | | |
|--------------|----------|-----------------------|-------------------|------------|--------|-------------|---------|---------|--|
| | | | No heat shock | Heat shock | | | | | |
| | | | | Embryo | | | Larval | | |
| Group number | Allele | Cross | | 30 min. | 1 hour | 3 x 30 min. | 30 min. | 1 hour. | |
| One | 4DA-122 | 4DA-122/4DA-122 | X | nt | nt | nt | X | X | |
| | | 4DA-122/ <i>ast4</i> | X | nt | nt | nt | X | X | |
| | | 4DA-122/3BK-5333 | X | nt | nt | nt | X | X | |
| | 3BK-5333 | 3BK-5333/3BK-5333 | X | nt | nt | nt | X | X | |
| | | 3BK-5333/ <i>ast4</i> | X | nt | nt | nt | X | X | |
| | | 3BK-5333/4DA-122 | X | nt | nt | nt | X | X | |
| Two | 3BL-6666 | 3BL-6666/3BL-6666 | X | X | X | X | nt | nt | |
| | | 3BL-6666/ <i>ast4</i> | X | X | X | X | nt | nt | |
| | | 3BL-6666/5823 | X | X | X | X | nt | nt | |
| | 3BL-5823 | 3BL-5823/3BL-5823 | X | X | X | X | nt | nt | |
| | | 3BL-5823/ <i>ast4</i> | X | X | X | X | nt | nt | |
| | | 3BL-5823/3BL-6666 | X | X | X | X | nt | nt | |
| Three | 4DG-1524 | 4DG-1524/4DG-1524 | N/A | N/A | N/A | N/A | N/A | N/A | |
| | | 4DG-1524/ <i>ast4</i> | X | nt | nt | nt | X | X | |
| | | 4DG-1524/3AL-1872 | X | X | X | X | X | X | |
| | 3AL-1872 | 3AL-1872/3AL-1872 | N/A | N/A | N/A | N/A | N/A | N/A | |
| | | 3AL-1872/ <i>ast4</i> | X | X | X | X | nt | nt | |
| | | 3AL-1872/4DG-1524 | X | X | X | X | X | X | |
| Four | 3AY-3216 | 3AY-3216/3AY-3216 | X | X | X | X | nt | nt | |
| | | 3AY-3216/ <i>ast4</i> | X | X | X | X | nt | nt | |
| | | 3AY-3216/4EP-6322 | X | X | X | X | nt | nt | |
| | 4EP-6322 | 4EP-6322/4EP-6322 | X | X | X | X | nt | nt | |
| | | 4EP-6322/ <i>ast4</i> | X | X | X | X | nt | nt | |
| | | 4EP-6322/3AY-3216 | X | X | X | X | nt | nt | |
| Five | 3BK-5588 | 3BK-5588/3BK-5588 | X | nt | nt | nt | X | X | |
| | | 3BK-5588/ <i>ast4</i> | X | nt | nt | nt | X | X | |
| | | 3BK-5588/3BK-5476 | X | nt | nt | nt | X | X | |
| | 3BK-5476 | 3BK-5476/3BK-5476 | X | nt | nt | nt | X | X | |
| | | 3BK-5476/ <i>ast4</i> | X | nt | nt | nt | X | X | |
| | | 3BK-5476/3BK-5588 | X | nt | nt | nt | X | X | |
| Six | 4EF-4391 | 4EF-4391/4EF-4391 | X | nt | nt | nt | X | X | |
| | | 4EF-4391/ <i>ast4</i> | X | X | X | X | X | X | |
| | | 4EF-4391/4EC-4540 | X | X | X | X | X | X | |
| | 4EC-4540 | 4EC-4540/4EC-4540 | X | nt | nt | nt | X | X | |
| | | 4EC-4540/ <i>ast4</i> | X | nt | nt | nt | X | X | |
| | | 4EC-4540/4EF-4391 | X | X | X | X | X | X | |

Table 5.4

Rescue of potential *drongo* alleles with *hs-drongo*.

Flies containing the mutant allele and *hs-drongo* insert were produced as in figure 9 and the progeny from these crosses were scored. A lethal phenotype (i.e. no rescue) was recorded as a red cross (✗), Group two alleles were homozygous viable and so could not be tested for rescue in the homozygous state (N/A). Progeny were scored primarily without heat shock and then heat shock regimes were applied. Progeny which had previously presented a lethal phenotype at embryonic stages (table 5.3) were heat shocked at this stage (groups 2,4 3 and 6) and those which presented a lethal phenotype at larval stage given a heat shock at this stage (groups 1 and 5). Rescue was not possible with the crossing scheme shown in figure 5.6 or when the mutants were subject to heat shock.

It was found that neither the basal level or overexpression of the *drongo* transcript in these flies at the heat shock time points chosen was enough to rescue mutants from any of the six groups.

5.5 Discussion

A mutagenesis screen was performed using the alkylating agent EMS, at 21D on the second chromosome. This is the region where *drongo* has been previously mapped (Harris, 1995). Mutant chromosomes were tested for non-complementation over the *Df(2L)ast4* deficiency, which covered the region of interest. Over 7,000 chromosomes were screened and 55 mutants were obtained. 47 of these mutants were in the region of interest (they did not complement *Df(2L)ast4* but they complemented *Df(2L)ast6*). It was then determined if mutants were homozygous lethal or homozygous viable. Mutants which were viable as

homozygotes but lethal when heterozygous with the deficiency may be hypomorphic alleles, or haplo-insufficient lethals. Previous studies suggest that this occurs relatively frequently in mutagenesis screens (reviewed in Ashburner, 1989).

These mutants were then placed into six lethal complementation groups by allelic analysis. Analysis of each of the complementation groups was performed by complementation crosses within each group and also against a number of P-element deletions/insertions in order to determine if any of the groups may contain *drongo* alleles. These data were used in conjunction with molecular information about the region to assign complementation groups to identified transcripts. For this study I was particularly interested in isolating a mutation at the *drongo* locus. From these crosses it appeared a number of point mutations and possibly a number of deletions had been isolated.

It is thought that base pair substitutions and chromosomal aberrations (deletions) may be a consequence of excision-repair mechanism in the DNA following depurination of the DNA after EMS treatment. Previous studies show that the relative proportions of aberrations to point mutations after EMS mutagenesis depends upon the extent to which the breeding protocol allows sperm storage in the female (Ashburner, 1989). For unstored sperm the number of chromosomal aberrations in one study was shown to 3.7% of the total number of mutants. In this study there was estimated to be 6.3% deletions to point mutations. I suggest that mutations that appeared to be in two groups probably contain two point mutations rather than a small deletion.

The complementation data appeared a little confusing as some of the mutations did not complement the excisions expected. Thus excisions may be more complicated than the simple deletions they seem to be. These deletions may also contain other unknown local rearrangements/ deletions in the region.

In light of previous studies (Roberts *et al.*, 1985), this study has provided evidence that although we may have not saturated the region with mutagenesis, all the mutants we have recovered fit into at least one of 6 groups, each group having more than one member. Data from Southern blotting experiments around the region, suggests that there may be at least 16 transcripts (*pers. comm.* Edwin Chan). It is not known whether it is possible to obtain lethals of any of these

transcripts. It is also not known whether all the loci are equally mutable. The data from Roberts *et al.* (1985) suggest that there are only four complementation groups in the region but this only tested 4,000 chromosomes in a region of 120kb uncovered by Df(2L)*ast4* or Df(2L)S2. We also noted that some of the complementation groups in the study of Roberts *et al.* (1995) contain only one allele.

This study obtained a mutant in the region (lethal over Df(2L)*ast4*) approximately 1 in 130 chromosomes screened, this was a lower rate to the one obtained by Roberts *et al.* (1985) (1 in 75 chromosomes screened) this was probably because I screened over one deficiency whereas Roberts *et al.* (1985) screened over two, and thus had a higher chance of finding a mutant of interest.

The EMS mutagenesis method used in general provides an effective, low toxicity approach for recovering mutants. The method of administering EMS used in this study is not without its drawbacks, however. It is difficult to administer an accurate dose of the mutagen by feeding EMS to the male flies. The uptake of the mutagen may occur by contact or vapour from the source. Non-palatibility is also a source of artifactual results, that is, less mutants are recovered than expected (Ashburner, 1989). The data suggested, however, that the mutants appeared to be evenly spread across the total number of flies screened and the complementation groups all contained more than one mutant in the region of interest suggesting that we had isolated mutant alleles of at least some of the genes in the 21D region.

As the complementation results on the whole appeared very tentative, and the analysis could not unequivocally define *drongo* mutant alleles, two representatives from each group were chosen to perform rescue with *hs-drongo*. The earliest lethal phase of the two members was determined to offer an idea of when the mutations were lethal and so a starting point for rescue experiments.

Attempted rescue with *hs-drongo* by a variety of heat shock regimes of all of the groups was unsuccessful and no *drongo* mutant was identified. This does not necessarily indicate that no *drongo* mutant alleles have been isolated. It may indicate that the heat shock regimes were incorrect or that overexpression of the cDNA was not enough to rescue a *drongo* mutation. Construction of the genomic fragment including regions of the promoter that may not be in the cDNA may be

necessary for rescue. There was not time however to produce this construct, make transgenic flies and perform the rescue but it would be one of an interesting number of future experiments.

The timings of the heat shocks may also not have been correct to rescue the mutation. The earliest lethal phase of each complementation group measured in this study only reflects the time at which the cumulative abnormalities resulting from the mutation become too severe for development to proceed any further and may not be the ideal guide to the function of the wild type allele during development (Ashburner, 1989). Previous studies have also shown that the penetrance of a lethal mutation is influenced by genetic and environmental variation and should not be taken to be an absolute property of a particular mutant (Ashburner, 1989). Another reason for the absence of rescue could be the level of expression produced by the *hs-drongo* transcript, although this may be enough to produce an overexpression effect (section 3.2.2), it may not be enough to rescue a mutation in the gene.

Finally upon observation from northern analysis of the gene (Harris, 1995) there appears to be two transcripts produced from *drongo*, which may occur via alternate splicing or alternate transcriptional start sites. There may be the possibility that the *hs-drongo* construct may produce only one of these transcripts, and perhaps one transcript is less active than the other, preventing rescue of a mutation in this way. Again, this may be overcome by overexpression of the genomic fragment of *drongo*.

In summary, a *drongo* mutant was not identified using this mutagenesis screen. Further analysis may be required to reach this goal by other means. In the absence of a mutant, another method of studying the function of a gene may be to observe the properties of the protein encoded by the gene of interest.

Chapter 6:

Expression of the Drongo protein.

Chapter 6: Expression of Drongo protein.

6.1 Introduction.

Analysis of the predicted amino acid sequence encoded by the *drongo* gene (section 1.2.2), suggested that Drongo contains a putative zinc finger motif at its N-terminus and numerous repeat sequences at the C-terminal end of the protein. *In vitro* translation of the gene also suggested that there may be two potential start sites at + 605 and + 958 (Harris, 1995), it was not known which of these are utilised.

Overexpression of the gene had been shown to cause an effect during oogenesis and embryogenesis (chapter 3). In order to analyse the function of *drongo* further, it was beneficial to purify the full length protein encoded by the gene and to use the protein for generation of an antibody. An antibody to the protein could be used to observe the expression and localisation of the protein *in vitro* and *in vivo*.

A number of different bacterial purification systems were utilised in this chapter, although it was not possible to isolate a sufficient amount of full length or partial length protein. I will describe and discuss the approaches used and consider alternative ways of generating an antibody to the protein.

6.2 Choosing an expression system: affinity purification.

There are a number of different expression systems utilised in *E.coli* to express and purify eukaryotic proteins. The requirements of the system to express and purify Drongo were as follows: firstly, to be able to produce a minimum amount of protein to enable subsequent purification of the protein. Secondly, the purification would be aided by production of soluble protein. Finally, a single step purification of the protein under non-denaturing conditions would be an advantage. Affinity purification systems have been used to purify numerous

proteins in *E.coli* and have been shown to fit all of these requirements for other proteins.

6.3 RESULTS

6.3.1 Production of Glutathione-S-Transferase (GST) -fusion proteins.

The glutathione-S-transferase (GST)-fusion system (Smith and Johnson, 1988) was the primary system chosen as it has previously been shown to fulfil the above requirements for a number of different eukaryotic proteins (Toye *et al.*, 1990, Kaelin *et al.*, 1991).

An inducible pGEX vector (Promega) (Appendix 10.1) was used containing a multiple cloning site (MCS) which enabled production of a fusion protein when transformed into *E.coli*. The expression of this protein was promoted by induction of an otherwise repressed *tac* promoter with IPTG. The fusion protein was then bound to a matrix of glutathione-sepharose beads, washed with buffer and eluted with reduced glutathione. The pure protein of interest can then be separated from GST by cleavage with endoproteases factor X or thrombin (Smith and Johnson, 1988).

6.3.1.1 Cloning of drongo into pGEX-5X-1.

The *drongo* cDNA was cloned into the pGEX-5X-1 vector (Smith and Johnson, 1988) via *EcoRI* sites (section 2.9.1). DH5 α cells were then transformed with this construct (section 2.2.6). Prior to any expression analysis, the plasmid DNA from these cells was prepared (section 2.2.3) and restriction digests were performed (section 2.2.4) in order to ensure authenticity of the plasmid (appendix 10.1).

6.3.1.2 Expression of GST-Drongo fusion proteins.

DH5 α cells transformed with pGEX-5X-1/*drongo* were cultured to an A₆₀₀ of 0.6, then induced with 1mM IPTG (section 2.9.1). An induction timecourse was carried out by taking samples over 0.5-4 hours after induction. Samples were subsequently analysed by SDS-PAGE (Figure 6.1A; section 2.9.3). The cDNA fragment used for expression contained both predicted translational start sites. Translation was predicted to begin at the first ATG at position 604 in the cDNA, producing a protein of approximately 84kDa.

Induction of the *tac* promoter with IPTG in cells transformed with pGEX-5X-1-*drongo* resulted in the synthesis of a 110kDa protein (figure 6.1A), 84kDa Drongo protein plus 25kDa GST. The size of Drongo in the fusion protein was in agreement with the predicted size from the cDNA. By estimation of protein amount from the SDS PAGE gels, 500 μ g -1.5mg of Drongo-fusion protein were produced from 500mls of culture. As a control, DH5 α cells were also transformed with pGEX-5X-1 vector alone. These cells were cultured and induced in an identical way to pGEX-5X-1-*drongo* (figure 6.1A).

The Drongo fusion protein was, however, insoluble and most of the protein was present in the pellet of cells lysed by sonication (section 2.9.2) and subjected to centrifugation (figure 6.1A). In an attempt to determine if any of the GST-Drongo fusion protein was soluble and could be purified, the supernatant was used to attempt purification, by incubation with glutathione-sepharose beads (section 2.9.2). Any unbound material was collected, the beads were washed and then any bound material was eluted with reduced glutathione (figure 6.1A). The absence of any protein in the eluate, however, suggested that the purification was unsuccessful or that there was not enough GST-fusion protein in the soluble fraction. (figure 6.1A). After an identical cell lysis and purification with pGEX-5x-1 transformed and induced cells, the GST protein was observed in the eluate suggesting that there were no problems with the purification system (figure 6.1A). It has previously been documented that many eukaryotic proteins, especially full length proteins, are produced in *E.coli* as insoluble aggregates (inclusion bodies). The formation of inclusion bodies may be caused by the production of "inappropriate" protein-protein interactions due to the lack of proper protein folding. Previous studies co-expressing fusion proteins with the

Figure 6.1

SDS-PAGE analysis of the full length GST-Drongo fusion protein.

A: Expression and affinity purification of GST-Drongo fusion protein.

DH5 α cells transformed with pGEX-5X-1 or pGEX-5X-1-*drongo* (section 2.2.6) were cultured to A₆₀₀ of 0.5, induced with 1mM IPTG and grown for a further 4 hours taking timed samples at **0.5h**, **2h** and **4h** (section 2.9.1). Total lysate from cells induced with pGEX-5X-1-*drongo* was prepared and centrifuged to separate the soluble (**s/n**) and insoluble (**pt**) fractions. The soluble fraction was then incubated with glutathione beads and any unbound material removed (**un**). The beads were then washed and bound material was eluted from the beads with reduced glutathione (**e**¹). Sonication, centrifugation and purification was repeated with cells transformed with pGEX-5X-1 alone and the eluant also analysed (**e**²). (section 2.9.2)

Samples were analysed on 10% SDS PAGE (section 2.9.3) and stained with Coomassie blue.

Cells transformed with pGEX-5X-1-*drongo* expressed a 110kDa band (84kDa Drongo protein and 26kDa GST: **arrow a**). After cell lysis the 110kDa protein was present mostly in the insoluble fraction (**pt**). When purification was attempted most of the protein was observed in the unbound fraction (**un**) and none could be purified in the eluate (**e**¹). As a test of the purification method, pGEX-5X-1 alone was induced. A 26kDa band was expressed (**arrow b**) which could be purified on glutathione sephrose (**e**²).

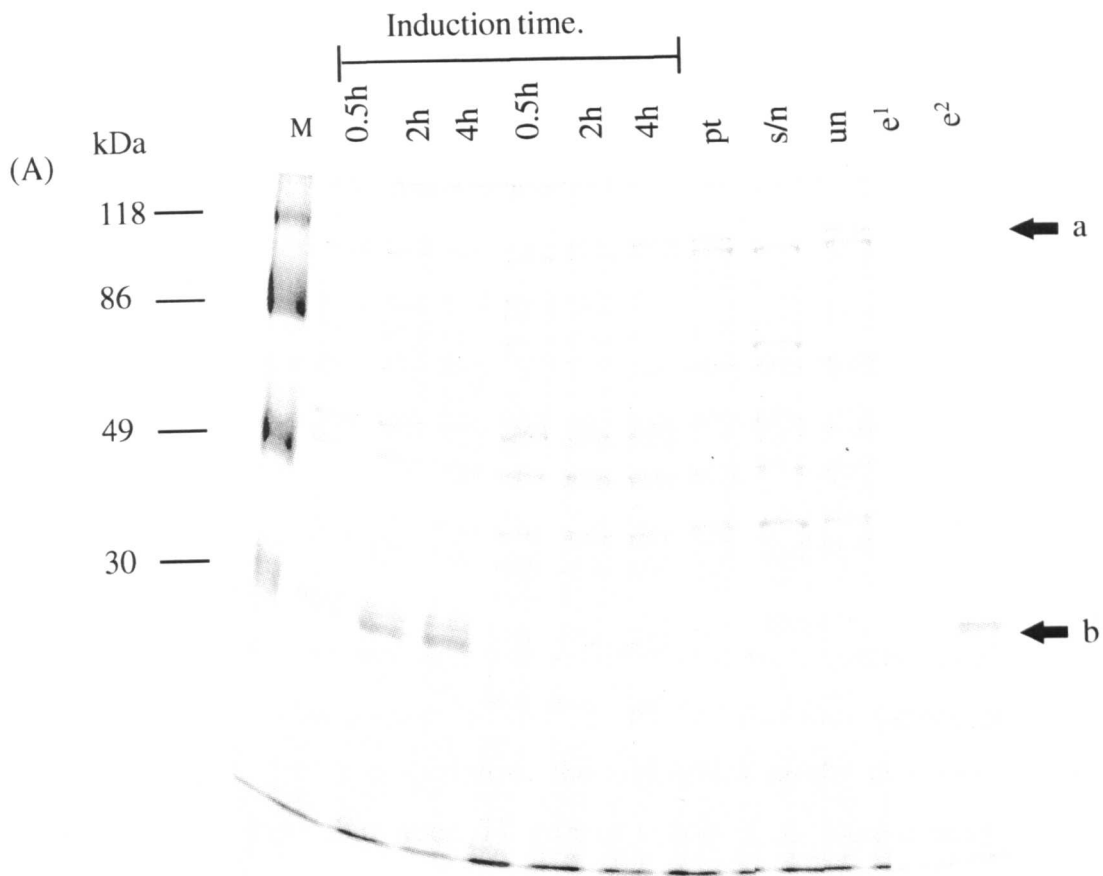
M= molecular weight markers.

B: Co-expression of GST-Drongo/ GroESL and GST-Drongo/Thioredoxin.

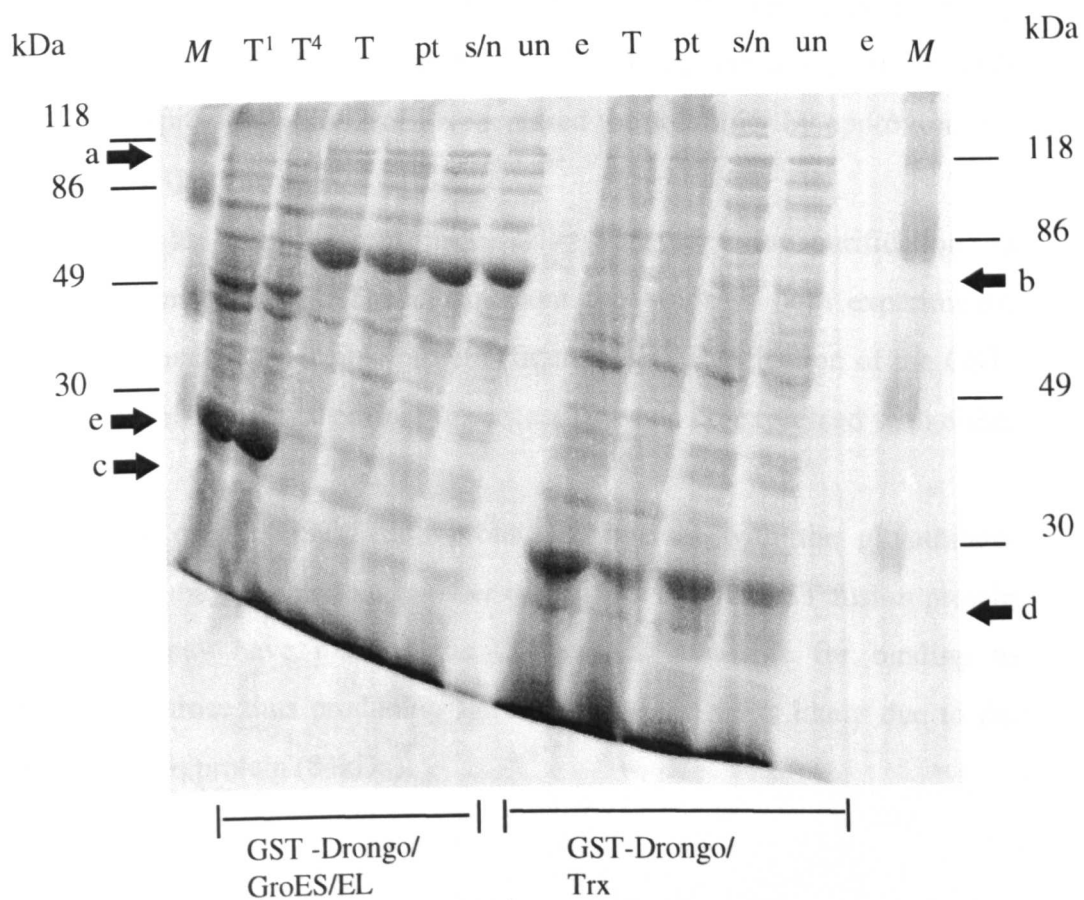
DH5 α cells transformed with pGEX-5X-1-*drongo*/T-groE and cells transformed with pGEX-5X-1-*drongo*/pT-Trx were grown up, induced (For GST alone (**arrow e**, timed samples taken at 1 hour and 4 hours: **T**¹ **T**⁴). Cells were lysed and purification was attempted as in A except GST alone was not tested. Samples were analysed as in A.

Cells transformed with pGEX-5X-1-*drongo* and pT-groE expressed a 110kDa GST-Drongo fusion protein (**arrow a**), a 60kDa GroEL (**arrow b**), and a 14kDa GroES protein band (**arrow c**). Co-expression with GroESL resulted in an increase in the GST-Drongo fusion band in the soluble fraction (**s/n**), although none of the fusion protein could be purified (**e**) and the majority was observed in the unbound (**un**) fraction. Co-expression of the Drongo-GST fusion protein was with Trx (15kDa band: **arrow d**) also resulted in an increase in the Drongo GST fusion protein in the soluble fraction (**s/n**), but again none was observed in the eluate (**e**) after incubation with glutathione-sepharose beads.

M= molecular weight markers.



(B)



E.coli chaperone, GroESL can increase the solubility significantly (Yasukawa *et al.*, 1995).

A second parameter that may affect the solubility of eukaryotic proteins could be the difference in redox potential between *E.coli* cells and eukaryotic cells. It has been suggested that *E.coli* cells have a relatively oxidative environment compared to mammalian cells. This may induce the formation of abnormal intramolecular disulfide bonds that aggregate the protein (Yasukawa *et al.*, 1995). Co-expression with the reducing agent thioredoxin has been shown to increase solubility in some cases (Yasukawa *et al.*, 1995). Increasing the solubility of the GST-Drongo fusion protein may enable successful purification via glutathione-sepharose.

In light of these previous studies, DH5 α cells were co-transformed with pGEX-5X-1-*drongo* and either pTgroE or pT-Trx plasmids (appendix 10.1: Yasukawa *et al.*, 1995). Induction of these plasmids results in expression of GroESL or Trx from the strong T7 promoter. Inductions were carried out as described, cells were lysed and separated into soluble supernatant and insoluble pellet fractions (figure 6.1B).

Co-expression of GST-Drongo with GroESL and Thioredoxin increased the solubility of Drongo substantially. Co-expression with thioredoxin increased the amount of protein in the soluble fraction by at approximately 60% (figure 6.1B) and co-expression with GroESL increased the solubility by approximately 50% (figure 6.1B).

The soluble protein from these cells were used for purification on glutathione-sepharose beads. The eluant from the beads in both experiments, however, contained no detectable protein (figure 6.1B). Purification of the GST-Drongo fusion protein was therefore unsuccessful when co-expressed with either Trx or GroESL.

Inability of the GST-fusion protein to interact with the glutathione-sepharose beads may be due to a number of reasons. As the GST fusion protein folds, Drongo may have masked the GST site(s) available for binding to glutathione-sepharose thus producing steric hindrance. This is likely due to the size of the Drongo protein (84kDa).

In an attempt to reduce the steric hindrance of the fusion protein and enable binding to the glutathione-sepharose beads for purification, a deletion was produced in the Drongo protein by digestion of the plasmid pGEX-5X-1-*drongo* with *Sst*I (section 2.9.1), leaving 1Kb cDNA (coding for mostly N-terminal amino acids) fragment in pGEX-5X-1 which was subsequently ligated. DH5 α cells were again transformed with this deleted construct.

Induction of the deleted protein produced a 80kDa band on an SDS PAGE gel (figure 6.2A) and at least 50% of the protein was soluble (figure 6.2B) However when purification was attempted with this deleted GST-fusion protein, two bands were observed in the eluant running at approximately 27kDa and 30kDa (figure 6.2B), as if two degradation products had been purified. Isolation of these proteins on glutathione-sepharose beads would have required the presence of the GST protein, which is 25kDa. The purified products therefore did not contain an adequate amount of the Drongo protein fused to GST to warrant any further investigation. In order to overcome this degradation problem, pGEX-5X-1-Drongo and pGEX-5X-1-Drongo Δ S were used to transform a protease deficient strain of *E.coli*, BL21(DE3). Expression was improved in these cell lines (figure 6.3A), and degradation was not observed. GST-Drongo Δ S was, however, unable to bind to the glutathione-sepharose beads and purification was unsuccessful (figure 6.3A).

6.3.2 Production of Maltose binding protein (MBP) -fusion proteins.

The GST-fusion system produced a good level of expression of a Drongo-fusion and after modification of conditions, produced an adequate amount of soluble fusion protein, however purification of the GST-fusion product or a deleted derivative (GST-Drongo Δ S) was not possible.

The second expression system utilised was the MBP-fusion protein expression approach (Maina *et al.*, 1988). This was similar to the GST-fusion system in that it utilised affinity purification of a fusion protein and a linked support, but the two differed in two main ways. The MBP-fusion protein is

Figure 6.2

SDS-PAGE analysis of the GST-Drongo Δ S fusion protein.

A: Expression of GST-Drongo Δ S fusion protein.

DH5 α cells transformed with pGEX-5X-1-*drongo* Δ S and were grown up and induced as in figure 6.1A. Samples were analysed as in figure 6.1A.

GST-Drongo Δ S, the deleted form of Drongo was expressed as a 75kDa band on SDS-PAGE gel (**arrow a**). In order to test induction, GST-Drongo was also induced (**arrow b**).

M=molecular weight markers.

B: Purification of GST-Drongo Δ S.

Total lysates (**T¹** and **T⁴**) from DH5 α cells expressing pGEX-5X-1-*drongo* Δ S were centrifuged and separated into soluble (**s/n**) and insoluble (**pt**) fractions. The soluble fraction was incubated with glutathione-sepharose and bound material was eluted (**e**). Samples were analysed as in figure 6.1A.

The majority of the GST-Drongo Δ S protein (**arrow a**) was observed in the soluble fraction (**s/n**) although some was seen in the insoluble fraction (**pt**). Upon purification two bands of approximately 27kDa and 29kDa (**arrow b**) were observed in the eluate (**e**). However if both contained the 26kDa GST protein, the size of the additional Drongo protein did not suffice any further analysis.

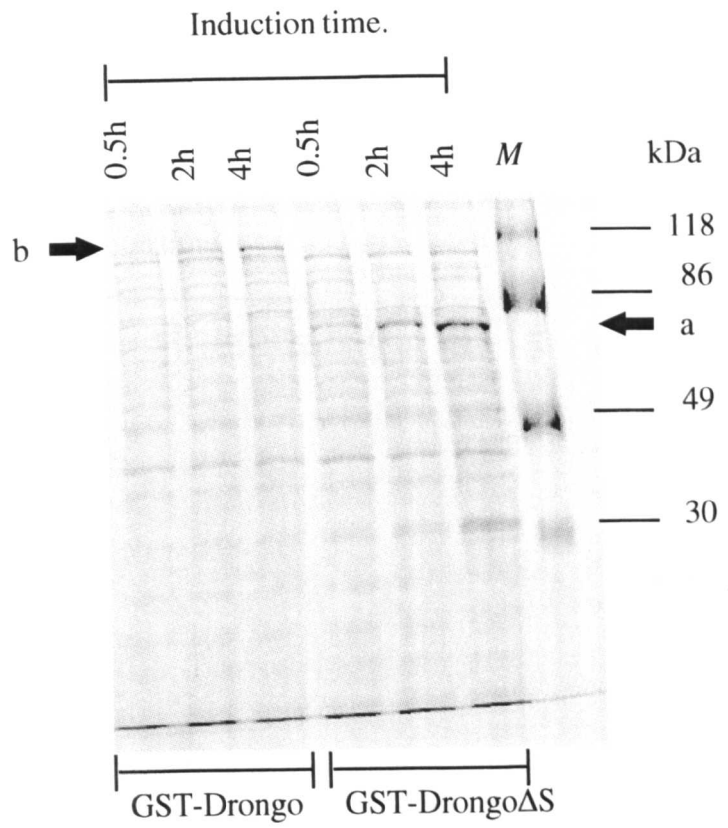
C: Expression of GST-Drongo Δ S in BL21 cells.

BL21 cells transformed with pGEX-5X-1 and pGEX-5X-1-*drongo* were grown up, induced (**0.5h**, **1h**, **4h**), sonicated and separated into soluble (**s/n**) and insoluble pellet (**pt**) fractions as in figure 6.1A. The soluble fraction was incubated with glutathione-sepharose, unbound proteins (**un**) were washed through, and any proteins bound were eluted with reduced glutathione (**e**). Samples were analysed as in figure 6.1A.

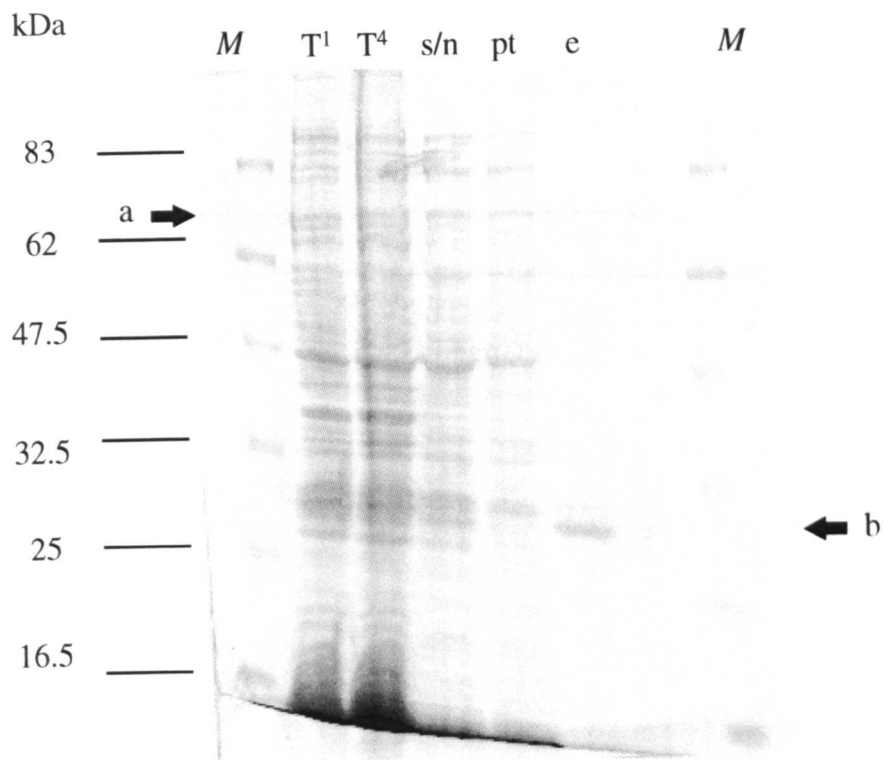
GST-Drongo Δ S was expressed as a 75kDa band in BL21 cells. The protein was mostly soluble (**s/n**) but could not be purified after incubation with glutathione sepharose (**e**) and was observed in the unbound (**un**) fraction.

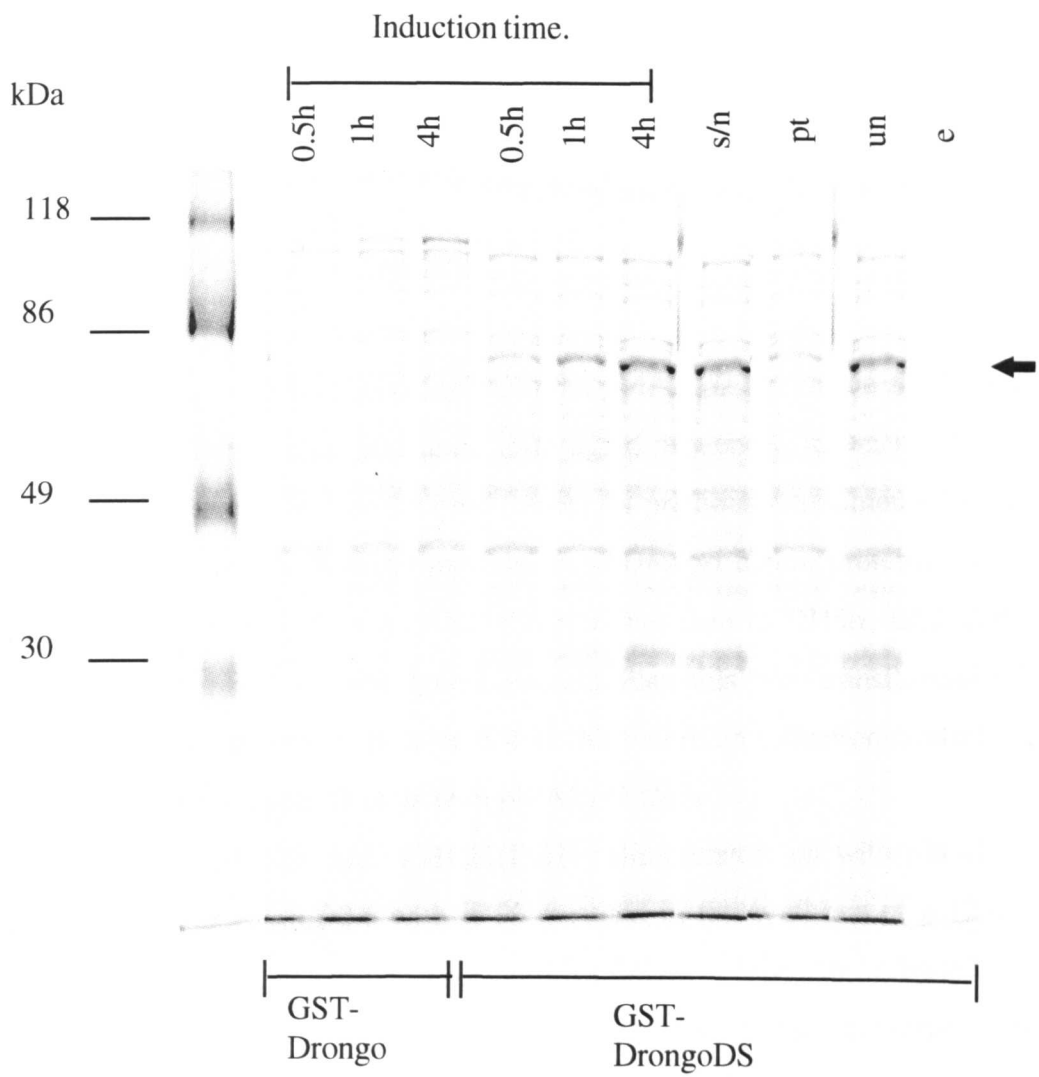
M= molecular weight markers; *h* = hours.

(A)



(B)





exported to the periplasm of the cell in which it is expressed. This aids release of the fusion protein upon cell lysis and it is therefore more likely to be soluble. The MBP also utilises a maltose resin through which it binds the fusion protein. One can then elute the purified fusion protein with free amylose (and cleave in the same way as GST-fusion proteins). The MBP-fusion system has been utilised for purification of a number of proteins, particularly large fusion proteins (Maina *et al.*, 1988; Tear *et al.*, 1996).

6.3.2.1 Cloning of *drongo* into pMAL-p2.

The *drongo* cDNA was cloned into the pMAL-p2 vector via *EcoRI* sites (section 2.9.1). The ligation junctions were again sequenced to ensure that the protein was in the correct translational frame. As previous experiments (section 5.4.2) suggested that the level of expression of Drongo fusion proteins may be improved by expressing them in BL21(DE3) rather than in DH5 α , BL21(DE3) cells were transformed with the ligated plasmid. Plasmids from transformed cells were checked as previously (section 5.4.1). As before an induction control was set up by transforming BL21 cells with pMAL-p2 alone .

Induction of a culture of BL21(DE3) cells transformed with pMAL-p2-*drongo* grown to A_{600} of 0.6 with IPTG resulted in the synthesis of a 124kDa fusion protein (84 kDa Drongo plus 40 kDa MBP) which was released in a soluble form after cell lysis by sonication (data not shown). However when purification an amylose resin was attempted (section 2.9.2) no protein were observed in the eluant (data not shown). Again it appeared that steric hindrance may be the rationale for unsuccessful purification.

6.3.3 Production of Drongo-6 x His tagged fusion protein.

Both GST-fusion and MBP-fusion systems did not facilitate successful purification of Drongo. Therefore a third affinity purification system was employed. The 6 x His tag system again involved induction of Drongo fusion

protein via IPTG and single step purification via an affinity resin, in this case, a Ni-NTA nickel resin (Chen *et al.*, 1994)

6.3.3.1 Cloning of *drongo* into pQE-32.

The *drongo* cDNA was cloned into pQE-32 (appendix 10.1) via *Sma*I and *Xma*I sites (section 2.9.1). XA90 cells were transformed with the ligated plasmid (section 2.2.6). XA90 cells contain the pREP4 plasmid which acts to repress the T5 promoter until the cells are induced with IPTG. Transformed cells were again induced over a time course with IPTG and samples of the induction were analysed on SDS-PAGE (data not shown). However induction of the 6 x His tag-Drongo fusion protein (predicted size approximately 84kDa) was not observed and induction was unsuccessful (data not shown). Induction procedures were revised so that the fusion protein was induced at different temperatures, 37°C and 30°C. However induction at these different temperatures was still unsuccessful.

6.3.4 Other methods of purification.

6.3.4.1 Purification of GST Drongo fusion protein on an SDS-PAGE gel filtration column.

Following unsuccessful attempts to purify Drongo by affinity purification methods, the supernatant from induced GST-Drongo transformed cells after lysis was further utilised. The Mini-Prep Cell gel purification system was used. The supernatant was run through an 8% SDS-PAGE column gel (section 2.9.3) and fractions were collected at 0.2ml/minute. Although this technique resulted in excellent separation of the Drongo-fusion protein, the yield of protein from the column was poor (data not shown) and could not be used for further analysis of the protein or immunisations.

6.3.4.2 Purification of GST-Drongo on an anti-GST/protein A column.

As mentioned previously one theory for the unsuccessful binding and elution of the fusion protein to the glutathione-sepharose beads, was that of steric hindrance. In order to resolve this problem a different strategy was tried. A polyclonal anti-GST antibody would recognise more than one epitope on the GST protein, therefore if only a percentage of the active site(s) were masked by Drongo in the fusion protein; immunoaffinity purification with the antibody may be possible. The GST antibody was linked to a solid support for ease of purification, in this case protein A beads. Protein A has been previously been shown to bind IgG type antibody (Harlow and Lane, 1988). The supernatant was then passed through the beads and eluted using a low pH buffer (section 2.9.2). Binding of the fusion protein to the anti-GST/Protein A beads was not observed however (data not shown), showing that more sites may be masked by Drongo than originally conceived.

6.4 Discussion.

With a view to studying the interactions of the protein and to observe the expression and localisation of the protein by subsequently producing an antibody, the Drongo protein was expressed in *E.coli* via a fusion protein and purification was attempted.

Drongo was expressed successfully as a GST-fusion protein and, after modifications of the expression and cell lysis, the protein was 50% soluble following cell lysis but could not be purified by binding and elution from glutathione-sepharose beads. This technique has been successfully used for other proteins, however most of these tend to be smaller proteins, for example, GST-fusions to MOMP (Major outer membrane protein) of *Chlamydia trachomatis*, 32kDa (Toye *et al.*, 1990), and a series of 25-50kDa deleted forms of the retinoblastoma protein (pRB) produced as a fusion protein with GST (Kaelin *et al.*, 1991). A different pGEX vector (pGEX-2T) was used on both of these

studies but the expression cell lysis and purification method were similar to this study.

A soluble MBP fusion protein was also expressed but could not be affinity purified. From this data it appeared that the Drongo protein was masking the binding sites of GST to the glutathione-sepharose beads.

Previous studies have shown that if inclusion bodies are formed by expressed fusion proteins, high concentrations of chaotropic agents in the presence of sulphhydryl reducing agents can be used to unfold the chains, which can then be refolded (Schein, 1990). The drawback to this is that many partially folded proteins are excellent protease substrates. Thus a technique such as the 6 x his-tag method which can be performed in the presence of a detergent may have been alternative method of purifying the Drongo protein. The 6x His tag method of purification (Chen *et al.*, 1994) also utilised a smaller linker in the fusion protein to overcome the problem of steric interference, however induction of a 6 x His-tagged fusion protein was not observed for this protein and so purification could not be attempted.

Although affinity purification via fusion proteins was unsuccessful in these three cases, the expression of the protein confirmed the predicted size of the protein calculated from translation of the cDNA from the first predicted start site (AUG) at +604 in the cDNA of 84kDa. As affinity purification experiments yielded substantial amounts of soluble protein a number of other experiments were attempted. Purification was attempted with an anti-GST/protein A column, in order to resolve the problem of steric hindrance, binding and elution were again unsuccessful.

There are many alternative ways of producing a pure protein sample although in light of the unsuccessful attempts in this study using an N-terminal fusion protein, a fusion to the C-terminus may produce better results. The Drongo protein can be expressed in *E.coli* successfully but it proved difficult to purify the protein. This chapter has provided some information on the protein size in a prokaryotic expression system and its relative stability and solubility, but has not proved useful in providing a large amount of pure sample for generation of antibodies.

Production of an anti-Drono antibody for observing the cellular localisation and the interactions of the Drono protein could be attempted by synthesis of a peptide and subsequent immunisations.

Another method of analysing the *in vivo/in vitro* location of a protein is to produce a tagged version of the protein, for example using a GFP (green fluorescent protein) fusion. This non-invasive technique has been used to observe proteins and label cells by genetically marking with a reporter gene encoding GFP, which emits green light at a certain wavelength (e.g Chalfie *et al.*, 1994). GFP has been expressed in various organisms including *E.coli*, *Drosophila*, plant and mammalian cells (Chalfie *et al.*, 1994; Brand, 1995; Yeh *et al.*, 1995; Haseloff and Amos, 1995; Pines, 1995). This technique has also under gone many modifications in order to improve sensitivity in detection by, for example, producing nuclear directed GFP to define the signal (Shiga *et al.*, 1996).

Chapter 7:

**Production of peptide antibodies to the
Drongo protein.**

Chapter 7: RESULTS AND DISCUSSION: Production of peptide antibodies against the Drongo protein.

7.1 Introduction

Generation of an antibody to a specific protein and immunological analysis *in vitro* and *in vivo* can offer information about the role of a protein.

In order to further analyse the role of *drongo* during development, I wished to determine whether the Drongo protein was regulated developmentally in the fly. Furthermore an analysis of the expression domains of the protein could be carried out to determine whether or not they were similar to the mRNA expression domains determined by wholemount *in situ* hybridisation (Harris, 1995). An antibody to Drongo would also enable analysis of the subcellular localisation of the protein and offer indications of its role in the cell and therefore the possible types of protein Drongo may interact with.

Previous attempts made to purify the full length Drongo protein via affinity isolation with a view to raising an antibody to the whole of the Drongo protein were unsuccessful (chapter 6).

7.2 RESULTS: Production of an anti-Drongo peptide antibody.

7.2.1 Selection of a peptide sequence for Drongo antisera production.

A pure sample of the Drongo protein had not been isolated using the expression systems described in chapter 6, therefore a peptide antibody was produced. A 20 amino acid peptide from the Drongo sequence was chosen (figure 7.1). The peptide stretch located from amino acid 310 to 329 was chosen for two reasons. Firstly the peptide showed high antigenicity (figure 7.1A) on peptide plots, therefore making it more likely to raise a response in rabbits than other regions of the protein. It also displayed low hydrophobicity and high surface probability (figure 7.1B and C) and therefore was more likely to be exposed.

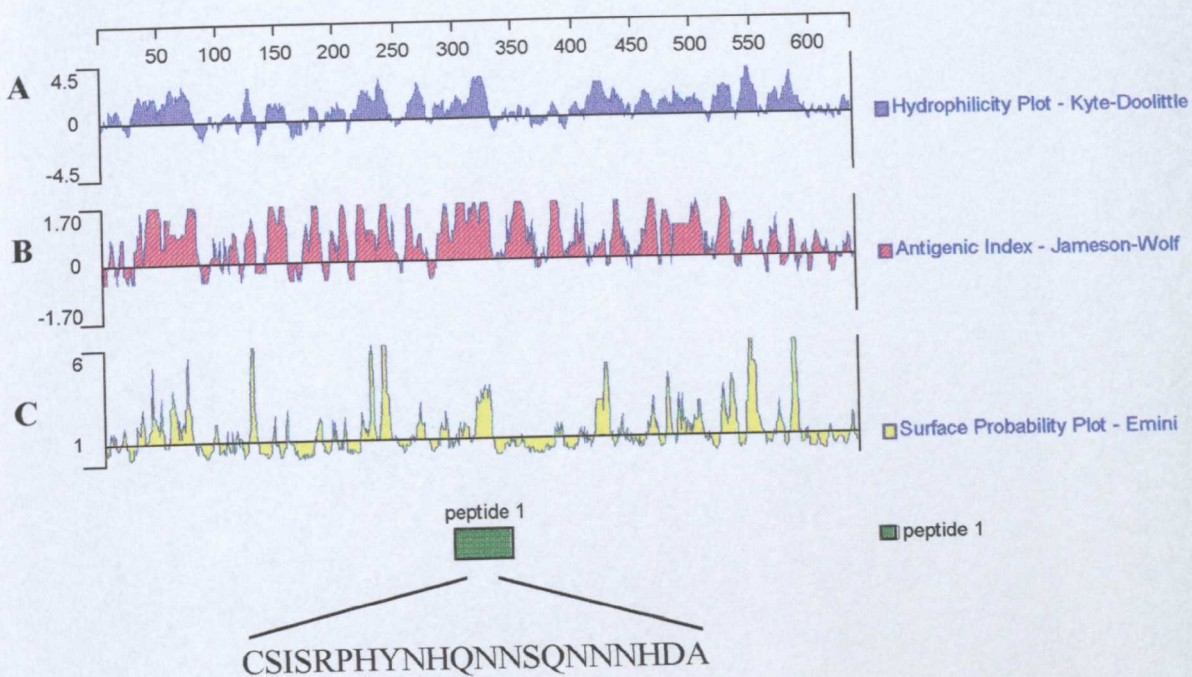


Figure 7.1:

Peptide plots of Drongo showing location of the peptide chosen to generate antibody.

The *drongo* cDNA sequence was translated and analysed for hydrophathy, surface characteristics and antigenicity in order to select a suitable peptide (Green box and sequence under plots) to generate an antibody.

A: Hydrophathy values according to the Kyte-Doolittle index (Kyte *et al*, 1982) were assigned for all amino acids, where positive is hydrophilic and negative is hydrophobic, and the average is plotted at the midpoint of the window.

B: The Jameson-Wolf (Jameson and Wolf, 1988) antigenic index predicts potential antigenic determinants using composite values from hydrophilicity, surface probability and secondary structure.

C: The surface probability (Emini *et al*, 1985) predicts the probability that a particular region lies on the surface of the protein. Numbers along the top represent amino acid numbers.

■ = 20 amino acids.

The second reason the peptide was chosen was due to its position within the protein. The peptide was not situated in the zinc finger and did not contain any of the FG or Opa repeats. We wanted the antibody raised from this peptide to be specific to Drongo and not cross react with any other proteins which contained homology to either the zinc finger or the repeat sequences in the protein located at the C-terminal end (section 1.1: figure 1.2).

The peptide was then coupled to Keyhole Limpet haemocyanin (KLH) (section 2.10.1), a highly antigenic carrier molecule, in order to raise an immediate response upon immunisation.

7.2.2 Production of the antiserum.

A rabbit was immunised with the KLH-linked peptide (section 2.10.1) every 4 weeks. Two weeks after each injection, 2ml bleeds were taken (section 2.10.3) and the serum was tested by western blotting (section 2.11.5) and ELISA (section 2.11.1).

Four injections were given to the rabbit as the immunogenic response was enhanced after each boost. The serum was titered by ELISA (data not shown). The final bleed was tested on western blots (figure 7.2 A) containing extracts from DH5 α cells expressing GST-Drongo fusion protein (section 5.3). The anti-Drongo antiserum recognised a 110 kDa band in the lanes containing GST-Drongo fusion protein but not in the lanes containing extracts from cells expressing GST alone. The antibody appeared to be specific to the Drongo protein as the pre immune serum (figure 7.2 B) and secondary antibody only (figure 7.2 C) did not recognise any non specific proteins in the extracts.

7.3 Characterisation of the antiserum.

7.3.1 Analysis of the developmental expression of Drongo.

In order to decrease background detection, the IgG antibodies from anti-Drongo antiserum were first purified on a protein A column (section 2.11.2) (data

Figure 7.2:

Western blot analysis with antiserum raised against the Drongo peptide.

A-C, Western blots with unpurified serum. **D** Coomassie stain.

DH5 α cells transformed with pGEX-5X-1*drongo* or pGEX-5X-1 alone (GST) were grown to an O.D of $A_{600} = 0.6$, induced with IPTG and cultured for a further 4 hours (0h-4h). Samples were lysed in SDS loading buffer (section 2.10.2) and electrophoresed on 10% SDS-PAGE gels (Section 2.10.2). 20 μ g of total protein was loaded per lane.

Proteins were transferred onto nitrocellulose membrane (2.11.5) and the blot was probed with either:

(A) Pre immune serum. No response was seen with the pre immune serum from the rabbit.

(B) anti-drongo peptide antiserum

The anti-Drongo antiserum (the 4th bleed from the rabbit) recognises a 110kDa band on the western blot in extracts of cells expressing GST-Drongo fusion protein (0h-4h) but not in extracts from cells expressing GST only.

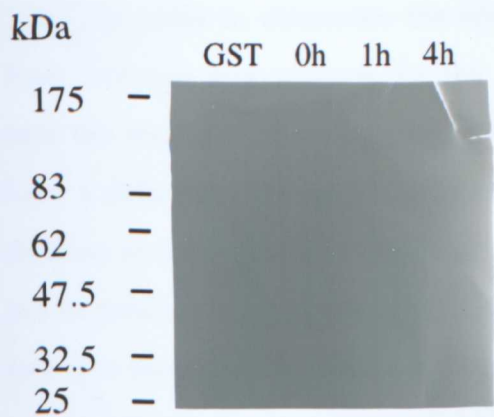
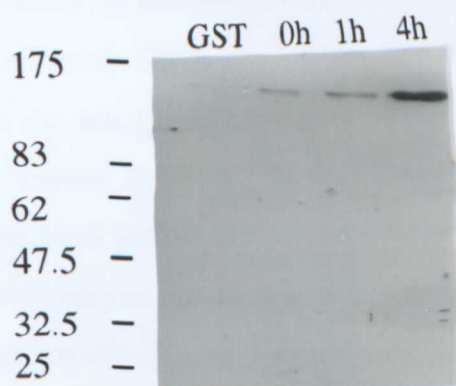
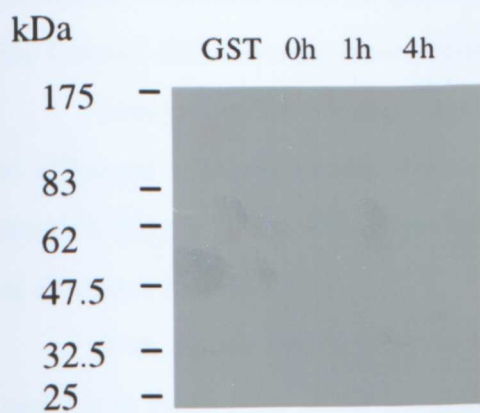
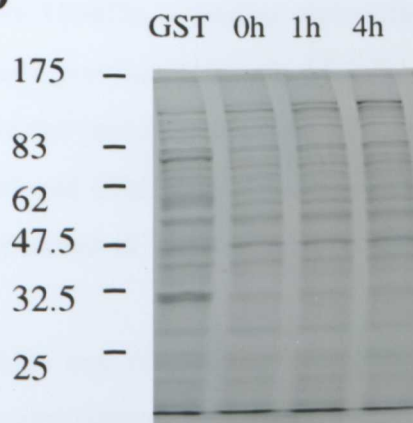
(C) secondary antibody alone (HRP-conjugated Goat anti-Rabbit).

No response was seen with the secondary antibody alone in either cells expressing GST or GST-Drongo.

(D) Coomassie Stain of the samples from (A).

Protein amounts were calibrated on a Coomassie Blue stained gel with the samples from 1A. Protein concentrations were also determined using the BioRad protein assay.

This figure shows that the peptide antiserum appeared specific to Drongo and response was not due to non-specific binding of the secondary antibody or from antibodies already present in the rabbit before immunisation to any bacterial proteins. The 110 kDa band seen was also the size expected of the GST-Drongo fusion protein.

A**B****C****D**

not shown). This IgG fraction will be referred to as the anti-Drongo peptide antibody.

The anti-Drongo peptide antibody was used to observe the developmental profile of the protein by western blotting (section 2.11.5). The western blot shown in figure 7.3 contained homogenised WCS embryos, larvae, male and female adults (section 2.11.4) and was probed using the anti-Drongo peptide antibody.

In order to determine the specificity of the antiserum, proteins extracted from embryos homozygous for the deficiency *Df(2L)ast4* were also transferred onto the western blot and probed with the anti-Drongo antibody. These embryos have a deficiency on the second chromosome (section 2.8) that corresponds to a deletion in the region where *drongo* has been previously mapped. Crosses carried out to produce homozygous *Df(2L)ast4* embryos are shown in figure 7.3B. These embryos should not produce any Drongo protein. The antiserum does not recognise any proteins in this lane (figure 7.3A).

As an additional control, bacterial extracts from a timed induction of GST-Drongo fusion protein were also on the western blot (figure 7.3), the antibody recognises a protein band of approximately 110kDa, a similar molecular weight to the induced GST-Drongo fusion protein seen previously (section 5.3.2).

Analysis of the western blot shows the presence of an 86 kDa protein band at all stages of development and an additional 60kDa band is seen in the embryo fraction (figure 7.3A). Other bands are observed at 73kDa (faint band) and 53kDa at all stages (figure 7.3A).

Two of the bands observed (86kDa and 60kDa) have similar molecular weights to those seen *in vitro* translation experiments (84kDa and 61kDa) (Harris, 1995). This western blot also indicates that the protein is developmentally expressed and the lower molecular weight protein is specifically observed in embryos at a higher abundance.

The presence of a number of additional bands in each fraction may indicate that degradation of the proteins during preparation had occurred. The extra bands were also not observed in the extracts from *Df(2L)ast4* embryos. This indicated that the additional bands are more likely to be Drongo products/degradation products rather than non-specific bands.

Figure 7.3.

Western analysis with purified antibody throughout development.

(A) Western blot of different developmental stages using IgG purified anti-Drongo peptide antibody.

Tissues from a WCS stock at different stages of development (embryos: lane 1; larvae: lane 2; male adults: lane 3; female adults: lane 4) were homogenised (section 2.11.4), filtered and separated by SDS PAGE (section 2.9.3).

15mg of protein (determined using the Bio Rad protein assay) was loaded into each lane. A western blot was performed with (as in figure 2) the anti-Drongo peptide antibody.

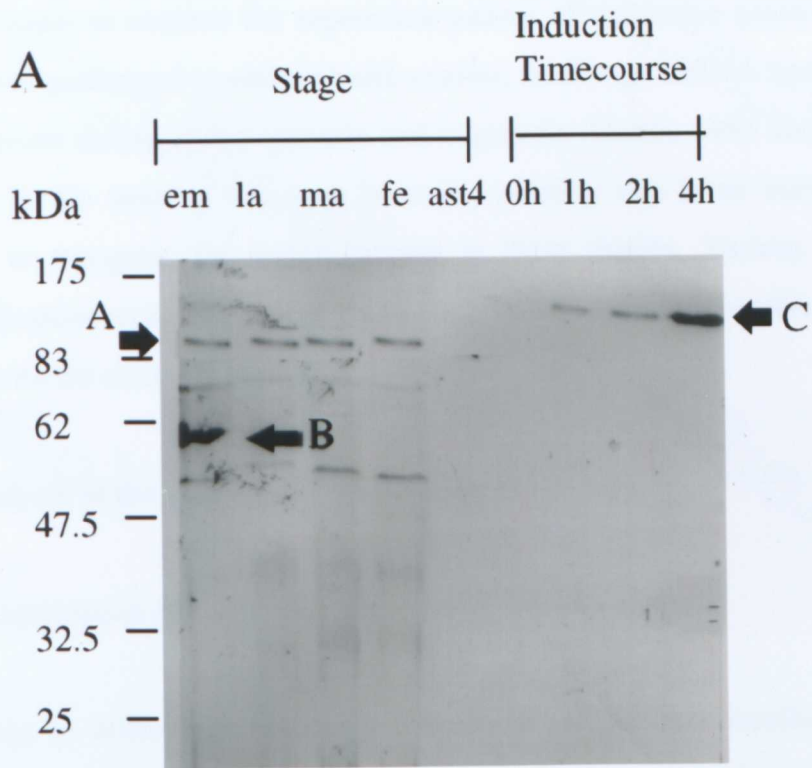
As a positive control, an induction timecourse (0h: lane 6; 1h: lane 7; 3h:lane 8; 4h: lane 9) was run of DH5 α cells expressing the GST-Drongo fusion protein (see section 5.x). As a negative control, embryos homozygous for Df(2L)ast4 (see cross adjacent to western blot) were also homogenised and loaded on the gel. These embryos contain a deletion in the 21 D region where *drongo* has previously been mapped and so should not produce any Drongo protein (ast 4: Lane 5).

The antiserum recognised proteins of approximately **86kDa** at all developmental stages (arrow A) and a **60kDa** band (arrow B) was also seen in embryos. Less intense bands at approximately **54kDa** and **73kDa** were also in all stages (em, la, ma, and fe). These may be degradation products. No bands were detectable in the lane containing Df(2L)ast4 embryos (ast4). The antibody also recognised a 110kDa band (arrow C) in the lanes containing the induced GST-Drongo fusion protein, which is similar in size to that seen during expression experiments (section 6.3.1.2).

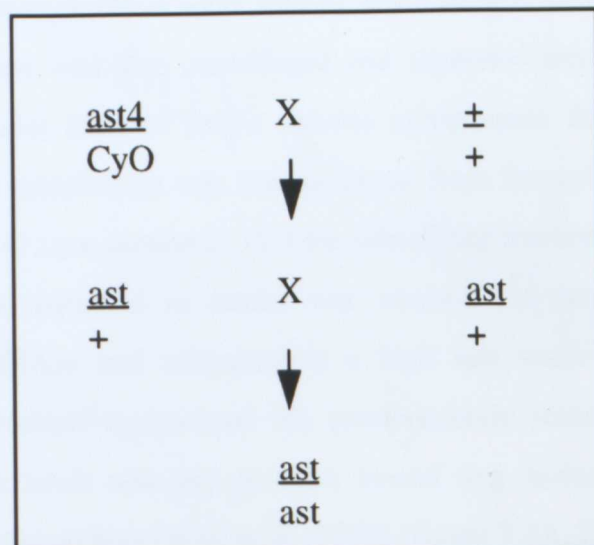
Key: em: embryos; la: larvae; ma: male adults; fe: female adults; h: hours post induction with IPTG; ast4: embryos homozygous for the deficiency Df(2L)ast4.

B) Crossing scheme to generate homozygous Df(2L)ast4 (ast4) embryos.

Virgin females from Df(2L)ast4 balanced over CyO were crossed with WCS males. Df(2L)ast4 +/- flies were collected (Straight wings, rough eyes) and siblings were crossed. Homozygous Df(2L)ast4 embryos were collected from this cross. embryos were checked to ensure that they had fertilised.



B



7.3.2 Analysis of the expression of the Drongo protein *in vivo*.

In order to further analyse the role of the *drongo* gene, we needed to know where the protein was expressed and its localisation in the cell. This has the potential to offer a clearer indication of the role of the gene than *in situ* hybridisation experiments.

In order to observe the expression pattern of the native protein, antibody staining was performed to embryos and ovaries, as *drongo* mRNA had previously been observed during embryogenesis and oogenesis (Harris, 1995 and this work: section 3.2). No staining was seen however, possibly due to an inability of the antibody to recognise the native protein in these tissues. Various fixing and permeabilisation techniques were attempted but we were still unable to observe staining with the antibody (data not shown).

7.3.3 Analysis of the subcellular localisation of Drongo.

7.3.3.1 Examination of subcellular fractions by western analysis.

Due to difficulties observing expression and cellular localisation of the Drongo protein in *Drosophila* tissues, the anti-Drongo antibody was used on a western blot containing subcellular fractions from homogenised embryos (figure 7.4). The homogenised embryos were filtered and a sample taken as whole cells. The remaining filtrate was then centrifuged and separated into a soluble and an insoluble crude nuclei fraction and a soluble cytoplasmic fraction. A fraction enriched in cellular membranes was also prepared from the cytoplasmic fraction (figure 7.4A, B and C ; see section 2. 11.3 for subcellular fractionation technique).

The fraction enriched in nuclei was washed and treated with 1µg/ml DNase, 1µg/ml RNase and subjected to a high salt wash (1M NaCl). This 'nuclear matrix' fraction represented the proteinaceous structure left after the removal of nucleic acids and any proteins bound (e.g histones) and has been described by other researchers (Wan *et al.*, 1999) (figure 7.4A, B and C).

Figure7.4:

Western blotting to determine the Subcellular localisation of Drongo.

(A,B) Western blots of subcellular fractions. **(C)** Coomassie stain.

Embryos were homogenised, filtered and fractionated as described in section 2.11.3.

Fractions were loaded onto a 10 % SDS-PAGE gel (5µg of total protein per lane), electrophoresed and transferred onto nitrocellulose membrane.

Two identical blots were produced and probed with:

(A) Affinity purified anti-*Drosophila* Lamin.

This antibody recognised protein of molecular weight of 75kDa in fractions enriched in whole cells, nuclei and nuclear matrix.

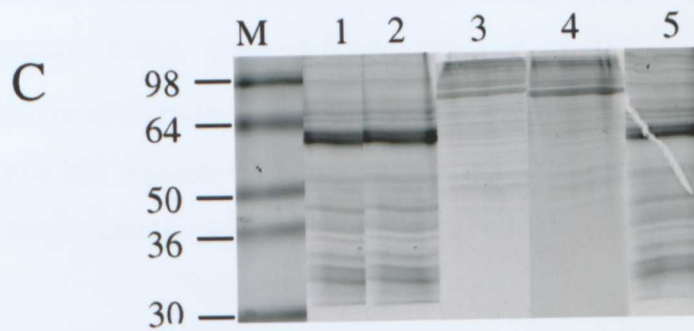
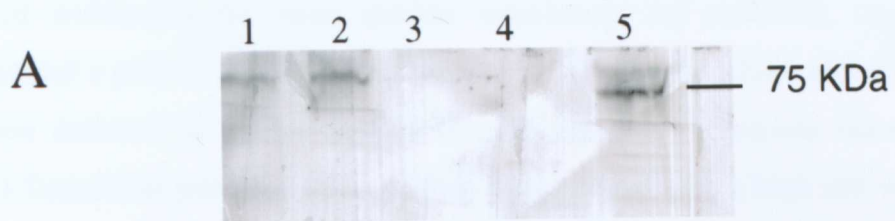
Protein markers on each side indicate that all bands were 75 kDa (markers not shown).

(B) IgG purified anti-Drongo peptide antibody.

The anti-Drongo protein recognised proteins of molecular weights 60kDa and 86kDa in the fractions enriched in whole cells, nuclei and nuclear matrix.

(C) Protein amounts per track were calibrated on a Coomassie Blue stained gel with the same samples. All lanes are from the same gel.

Key: M: molecular weight protein markers (See blue markers). Subcellular fractions: 1: embryo homogenate; 2: fraction enriched in nuclei; 3: fraction enriched in cytoplasm; 4: fraction enriched in membranes; 5: fraction enriched in nuclear matrix.



Prior to analysis of Drongo localisation in the cell, it was necessary to determine the contents of each of the fractions, therefore three identical SDS-PAGE gels were run; two were used to perform western blots and one was stained with Coomassie blue in order to quantify the protein loading.

The first western blot was probed using an anti-*Drosophila* lamin antibody (figure 7.4A) (a kind gift from Paul Fisher: Lin and Fisher, 1990). This antibody has been shown to recognise lamin L_o, an 75kDa intermediate filament protein located underneath the inner nuclear membrane. As predicted, the antibody recognised a protein band of approximately 75kDa in the whole cell fraction, the fraction enriched in nuclei and the fraction enriched in nuclear matrix (figure 7.4A). Lamin has previously been shown to be present after a high salt wash of the nuclei (Lin and Fisher, 1990).

Although this does not show that the fractions are pure it does suggest that the majority of the nuclei are contained in the 'nuclear' fraction (figure 7.4A, B and C) and are absent from the cytoplasmic fraction. It also shows that the nuclear matrices are still intact after the salt wash and still contain components of the inner and outer nuclear membranes. Although a number of antibodies to *Drosophila* cytoplasmic/membrane proteins were tested, none of them gave a good enough response on the western blot in order to validate the identity/contents of the other fractions.

The second western blot was then probed using the anti-Drongo peptide antibody. Two bands of 86kDa and 60kDa were observed in the whole cell fraction in the fraction enriched in nuclei and also in the fraction enriched with nuclear matrix (figure 7.4B). The antibody did not recognise any proteins in the cytoplasmic or membrane fractions .

From the western data, it appeared that Drongo was probably localised to the fraction enriched in nuclei and was not cytoplasmically localised.

7.3.3.2 Analysis of the subcellular localisation on tissue culture cells.

To verify the data observed on the western blot of subcellular fractions derived from embryos (section 7.3.3.1), the anti-Drongo antibody was then used on

S2 cells (section 2.7.9, also used in chapter 4), a *Drosophila melanogaster* embryonic primary cell line. The hypothesis from the western blot data was that Drongo may be observed in or around the nucleus, perhaps on the nuclear envelope. This may support the idea that Drongo is an hRIP/Rab homologue which has also been shown to be localised around the nuclear envelope in a punctate pattern (Fritz *et al.*, 1985).

In order to check that the anti-Drongo peptide antibody specifically recognised the Drongo protein in S2 cells, the pre immune serum and secondary antibody alone were used alongside the peptide antibody in cell staining experiments. Whilst there was no specific expression in the cells treated with pre immune serum (figure 7.5A) nor secondary antibody alone (figure 7.5 B), the peptide antibody recognised the Drongo protein in a perinuclear pattern which extended out into the cytoplasm (figure 7.5C). The staining appeared to be excluded from the nucleus.

In order to characterise the location of this staining further, S2 cells were double stained with anti-Drongo peptide antibody and Hoechst #33258 (section 2.7.9; figure 7.6C) (section 3.2.2). The anti-Drongo peptide antibody and the Hoechst stain did not co-localise (figure 7.6C), and there appeared to be a gap between where Drongo localised around the nucleus and the Hoechst stain suggesting that Drongo did not localise to the nuclear membrane. However, when cells were also double labelled with anti-*Drosophila* lamin, an antibody to a cytoskeletal protein known to be localised to the inner nuclear membrane (figure 7.6E see section 7.3.3.1; Lin and Fisher, 1990) and Hoechst (figure 7.6F), this gap was still present (figure 7.6G) and is probably a artifact caused by nuclei shrinkage after methanol fixing. Double labelling experiments with the anti-lamin antibody and the anti-Drongo peptide antibody would have been difficult to perform as both antibodies were raised in rabbit.

In summary staining S2 cells with anti-Drongo antibody, a nuclear marker (Hoechst # 33258) and a inner nuclear membrane marker (anti-*Drosophila* lamin antibody) demonstrated that Drongo was excluded from the nucleus. These experiments showed that Drongo was localised in close proximity to the nucleus, and the staining pattern extended out into the cytoplasm.

Figure 7.5.

Immunostaining of S2 cells with anti-Drongo peptide antibody and analysis of its subcellular localisation.

(A-C) Analysis of Drongo localisation in S2 cells.

S2 cells (a *Drosophila melanogaster* embryonic primary cell line) were plated out, fixed and washed as described in section 2.7.9. Cells were then immunostained (as described in section 2.7.9) and viewed under the microscope using the FITC filter.

Cells were stained with:

(A) Pre immune serum

(B) Secondary antibody alone (FITC-conjugated goat anti-rabbit).

Both pre-immune serum and secondary antibody showed low level non-specific staining.

(C) Anti-Drongo peptide antibody (with goat anti-rabbit secondary)

Drongo appeared to be localised in a punctate pattern around the nucleus (arrow) which often extended out into the cytoplasm (arrowheads).

Scale bar: A-C = 10µm.

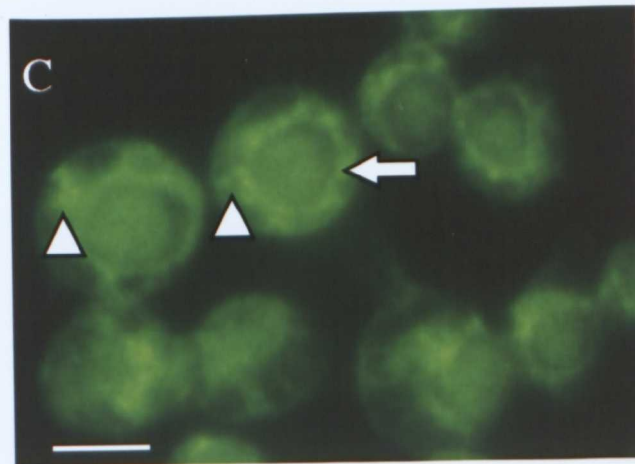
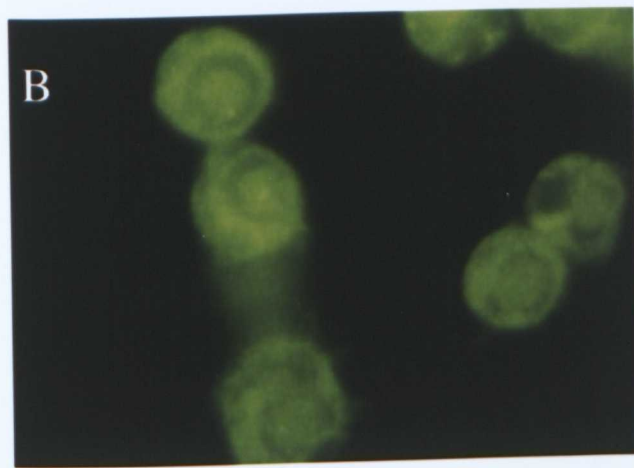
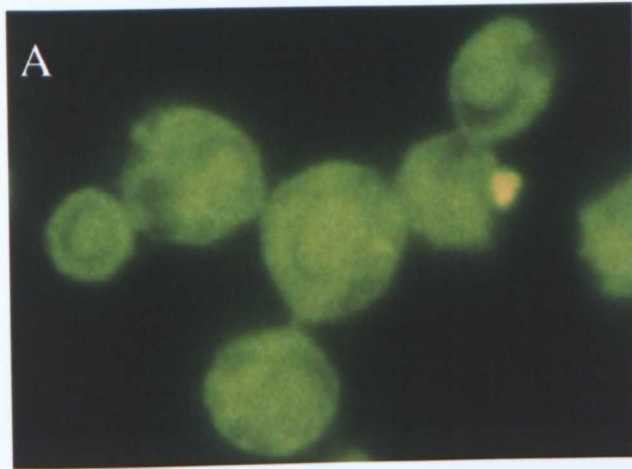


Figure 7.6.

Further characterisation of Drongo localisation.

(A-C) Co-localisation analysis of Drongo with Hoechst (section 2.7.9).

(D-F) Co-localisation of lamin and Hoechst.

In order to characterise this staining cells were treated with:

(A) Anti-Drongo peptide antibody,

(B) Hoechst # 33258, and

(C) Double stained with anti-Drongo peptide antibody and Hoechst # 33258.

Drongo and Hoechst did not co-localise and Drongo appeared to be present as a ring outside the nucleus (arrow a). There appears to be a gap between the two staining patterns (arrow b).

The cells were also stained with:

(D) Anti-lamin antibody,

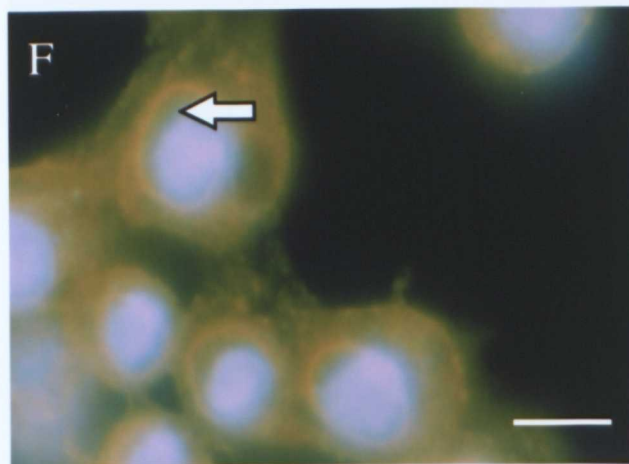
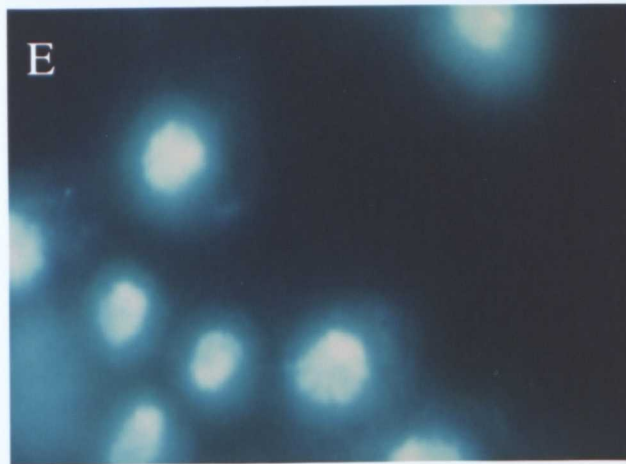
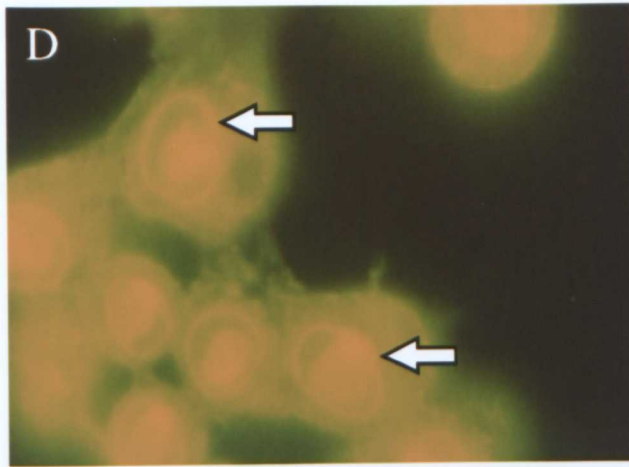
(E) Hoechst # 33258, and

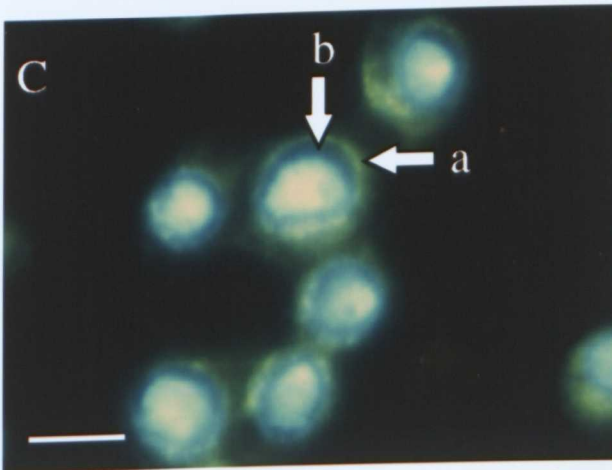
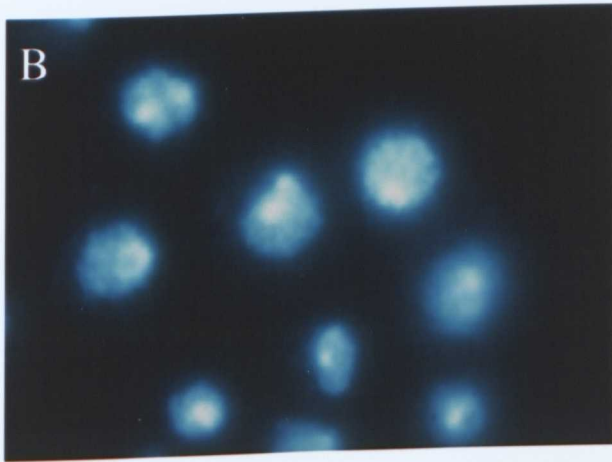
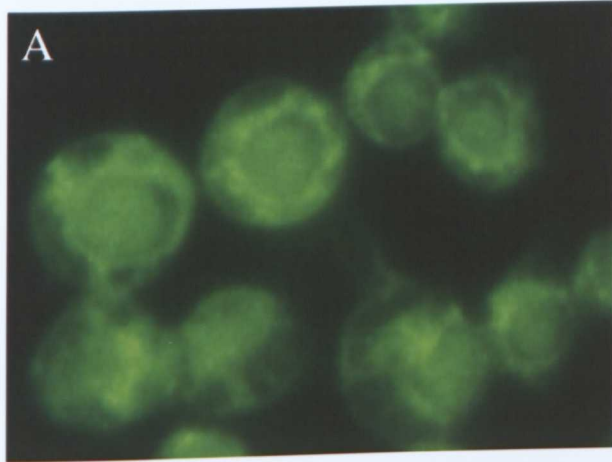
(F) Anti-lamin and Hoechst # 33258.

Anti-lamin localises as a tight ring around the nucleus (**D**: arrows), (there is also a high level of background staining).

This demonstrated that there was also a gap between the inner nuclear membrane in the nucleoplasm (**F**: arrow), probably as an artifact of the pretreatment of the cells.

Scale bar: A-F = 10 μ m





The Drongo staining pattern appeared to be similar to that observed when mammalian cells were stained with markers of the rough endoplasmic reticulum (RER) or Golgi complex (GC). In mammalian cells the RER is a membranous structure continuous with the nuclear envelope. The GC is a set of stacked membranes located further out into the cytoplasm (Skoufias *et al.*, 1990). Communication between the RER and the GC is via numerous vesicles which transport proteins and lipids between the two (figure 7.7)

In *Drosophila*, however, previous analysis of the GC (Thomopoulos *et al.*, 1992) and identification of proteins localised to the GC, such as the protein Commisureless (Tear *et al.*, 1996) suggest that in *Drosophila* cells, rather than the peri-centriolar localisation observed in mammalian cells, the Golgi complex exists as vesiculated cisternae located in between the membranous RER (Thomopoulos *et al.*, 1992; Ripoche *et al.*, 1994) (figure 7.7).

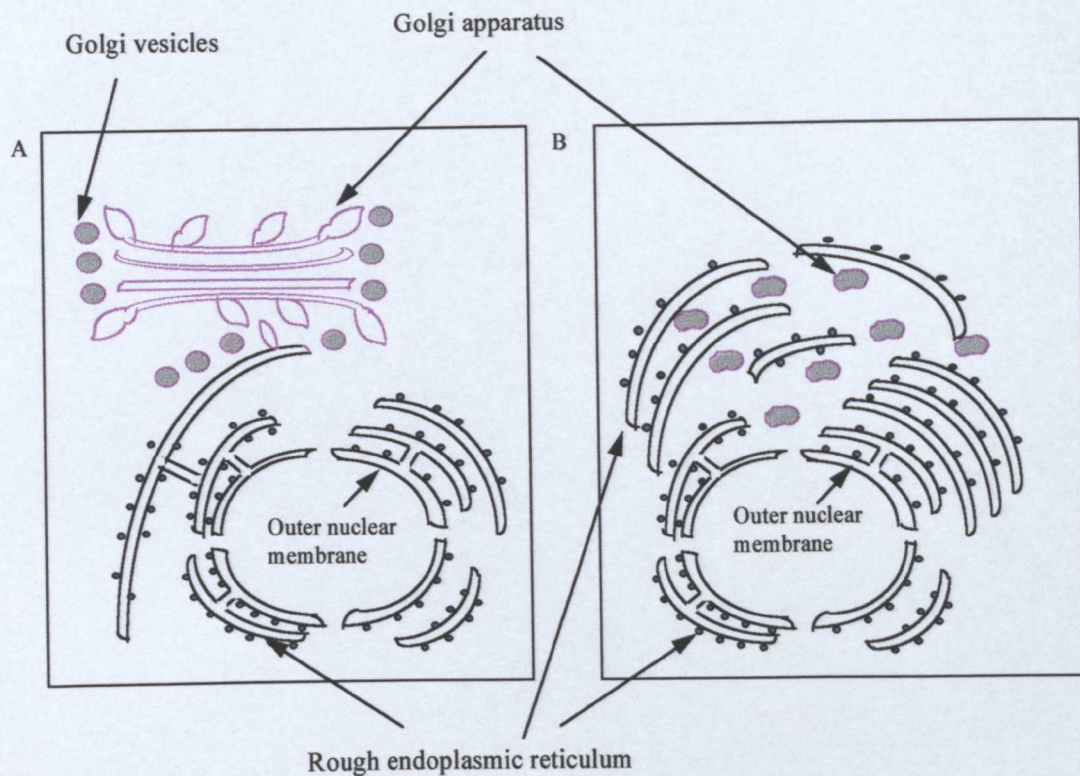


Figure 7.7.

Comparison of the RER and the Golgi complex in mammalian and *Drosophila* cells.

Schematic of the secretory apparatus in (A) a typical mammalian cell and (B) in a typical *Drosophila* cell. In a mammalian cell, the RER is continuous with the nuclear membrane and a single Golgi apparatus forms a set of stacked cisternae in a pericentriolar region. Cycles of vesicle budding and fusion permit communication between the RER and the Golgi. In *Drosophila*, the RER still appears to be continuous with the nuclear envelope but extends further out into the cytoplasm. The Golgi complex consists mainly of vesiculated cisternae located throughout the cytoplasm (schematics derived from diagrams in Thomopoulos et al., 1992 and Skoufias et al., 1990).

Figure 7.8:

Effects of Colchicine on Drongo localisation.

A-C Immunostained untreated cells. **D-I** colchicine treated immunostained cells. S2 cells were plated out, and fixed as in figure 7.5 (section 2.7.9) or plated out and treated incubated with 0.2 μ g/ml of Colchicine for 4 hours then fixed as before (figure 7.5: section 2.7.9).

A-C Immunostained untreated cells (- colchicine)

A: Untreated cells immunostained with anti-Drongo peptide antibody.

B: Cells stained with Concanavalin A (Con A)

C: Cells double labelled with Con A and anti-Drongo peptide antibody.

Drongo displays a similar localisation to Con A around the nucleus and emanating into the cytoplasm, and upon double labelling shows partial co-localisation with Con A, but the extent of the staining is slightly different for Con A and Drongo. Drongo is localised more tightly around the nucleus (**A** and **C** arrows and Con A extends further into the cytoplasm (**B** arrow and **C** arrowhead)

D-I Colchicine treated cells (+ colchicine)

D and **G:** Cells treated with colchicine and immunostained with anti-Drongo peptide antibody.

E and **H** Cells stained with Con A.

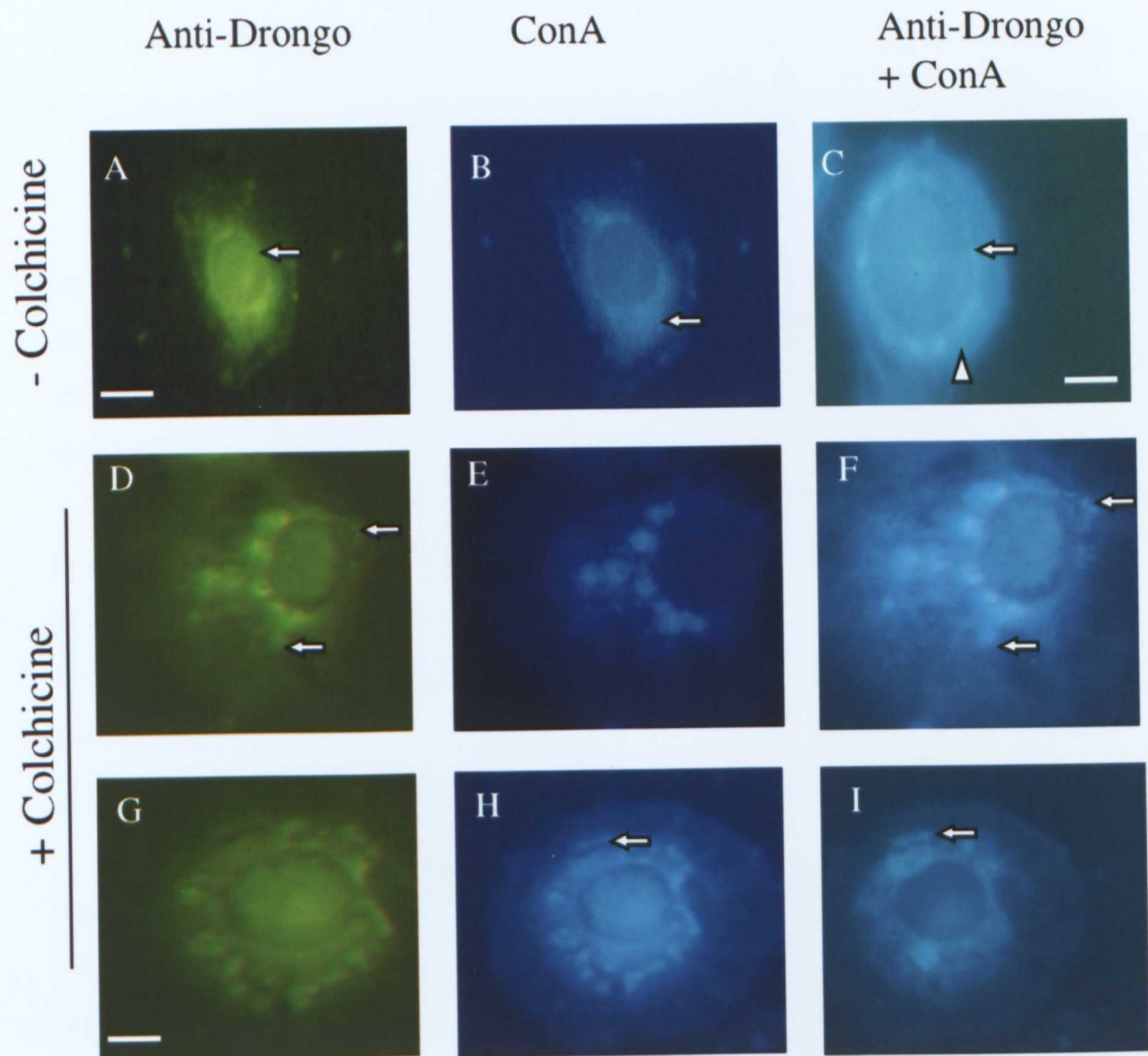
F and **I:** Cells double labelled with anti-Drongo antibody and Con A.

Upon treatment with colchicine, Drongo and Con A staining is dispersed into vesicles around the nucleus and into the cytoplasm. Upon double labelling, Drongo and Con A appear to stain some of the same vesicles but again the extent of the staining into the cytoplasm is different to Con A staining. Drongo has a slightly different localisation pattern to Con A (**D** arrows and **F** arrows compare to **E**; and **H** arrows and **I** arrows compare to **G**).

Scale bars: A+B =5 μ m

C = 3.5 μ m

D-I =3 μ m



The Commissureless (COMM) protein, which is known to be involved in axon guidance in the *Drosophila* CNS (Tear *et al.*, 1996), has been shown to localise to vesicular structures distributed in the cytoplasm of COMM expressing S2 cells. COMM co-localises with the *Drosophila* β -COP homologue as small vesicles in the cytoplasm. A myc-tagged version of COMM also co-localises with Mab 23C (a monoclonal antibody raised to a Golgi associated fragment in Monkey cells) in COS cells but displays a different localisation in mammalian cells, surrounding the nucleus as a single Golgi complex. (Tear *et al.*, 1996)

The staining pattern seen in S2 cells with the anti-Drongo peptide antibody appeared significantly different to that observed with the COMM antibody. This may suggest that Drongo is not localised to the Golgi complex. However it may be localised to the rough endoplasmic reticulum (RER) or to another structure in a similar location in the cell.

Indeed the location of the antibody staining appears similar to that of the Porcupine (Porc) protein, a putative multi-transmembrane protein involved in *wingless* signalling (Kadowaki *et al.*, 1996). The 59kDa Porcupine protein co-localises with the ER luminal protein BiP in Kc cells (a *Drosophila* embryonic line) cells in a perinuclear pattern which extends out into the cytoplasm (Kadowaki *et al.*, 1996)

To test the hypothesis that Drongo may be located in the RER, Concanavalin A (Con A), a marker used in previous studies for the endoplasmic reticulum (Parkkinen *et al.*, 1997) was used. Con A is a lectin molecule which binds to glycosylated mannose residues in the RER. It can also bind to similar residues in the Golgi and plasma membrane.

Cells were stained with anti-Drongo peptide antibody (figure 7.8A) and with Con A (figure 7.8B). Both Drongo and Con A appear to be localised around the nucleus and extend into the cytoplasm, but upon double labelling (figure 7.8 C) and comparison of the two, the extent of the staining is different. Drongo shows tight localisation around the nucleus and extends into the cytoplasm (figure 7.8A

and figure 7.8C), whilst Con A shows more cytoplasmic staining (figure 7.8B and figure 7.8C).

Drongo co-localised with the Con A pattern partially, the extents of the staining patterns covering different parts of the cell. This warranted further investigation. I therefore attempted to disrupt the RER and the Golgi apparatus to observe if Drongo staining could be disturbed.

Both the RER and the Golgi show sensitivity to the microtubule disrupting drug, colchicine. Upon treatment of mammalian cells with colchicine, the endoplasmic reticulum is seen to collapse onto the nuclear envelope and the Golgi has been observed to dissipate into vesicles throughout the cytoplasm (Thyberg and Moskalewski, 1985).

Upon treatment of S2 cells with colchicine (figure 7.8D-I) cells were immunostained with the anti-Drongo peptide antibody (Figure 7.8D and G) and stained with Con A (figure 7.8E and H). When observed, Drongo and Con A staining appeared to be affected by colchicine treatment with both becoming localised to vesicles surrounding the nucleus and emanating from the cytoplasm. Double labelling of the cells (figure 7.8F and I) revealed that the majority of the Drongo staining co-localised with that of Con A but the extent of the staining was slightly different. Drongo was localised to different regions of the cell to those seen with the Con A staining (figure 7.8D arrows and figure 7.8F compared to figure 7.8E and figure 7.8H and figure 7.8I compared to figure 7.8G).

These data suggest that the organelle(s) in which Drongo is localised is sensitive to colchicine treatment and so is maintained in the cell by microtubules. The data also demonstrates that the organelle to which Drongo is localised can be stained with Con A, a marker which localises to the RER, the Golgi and the plasma membrane.

7.3.3.3 Is Drongo localised to the RER?

The cell staining experiments raised the possibility that Drongo was localised to the RER in S2 cells. Previous western data (section 6.3.3.1), however,

suggested that Drongo was localised to a fraction enriched in nuclei and a fraction enriched in nuclear matrices. As the RER is continuous with the nuclear envelope, some or all of the RER may have still been attached to the nucleus and even the nuclear matrix when the fractions were made as no detergent was used in these steps. Other studies which utilised subcellular fractionation to localise proteins, suggest that treatment of a 'crude' nuclear fraction with detergent may solubilise the RER (Stuart Allen *pers. comm.*). The nuclear fraction made in section 7.3.3.1 was therefore treated with 0.1% Triton X-100 ranging between 10 and 40 times the volume of the fraction (as described in section 2.11.3). These were incubated at 4°C for 20 minutes and the fractions then centrifuged for 10 minutes at 6,000rpm and separated into soluble and insoluble fractions. These fractions were then analysed by western blotting (figure 7.9) using the anti-Drongo peptide antibody. Firstly, in order to ensure that nuclear proteins were not removed by addition of Triton X-100 to the fraction, the experiment was performed using anti-Lamin antibody on the western blot. Lamin was present as a 75 kDa band in the pellets from all treated fractions but was not released in any of the supernatants (figure 7.9A) after treatment with Triton X-100, suggesting that the nuclei in the fraction were largely undamaged by the detergent treatment.

Using anti-Drongo peptide antibody on the western (figure 7.9B) showed that the amount of Drongo protein decreased in the pellet as more Triton X-100 was added (figure 7.9B: compare 40x volume to 10x volume). The amount of protein in the supernatant also increased when the nuclear fraction was incubated in 40x the volume of Triton X-100 (figure 7.9B: compare 40x vol to 10 x vol). These data suggest that the treatment with detergent releases the fraction in which Drongo is contained, but does not appear to disrupt any nuclear proteins, stipulating a location for Drongo in an organelle in close proximity to the nucleus, such as the RER.

7.4 Discussion.

In order to analyse the expression profile of Drongo to investigate the subcellular location of the protein, an antibody was raised to a synthetic peptide.

Figure 7.9

Western analysis of the nuclear fraction after treatment with detergent.

(A,B) Western blots of nuclei treated with varying amounts of Triton X-100.

The fraction enriched in nuclei from figure 3 was incubated with 10x, 20x, 30x, 40x the volume (of fraction) with lysis buffer (section 2.11.3) containing 0.2% Triton X-100.

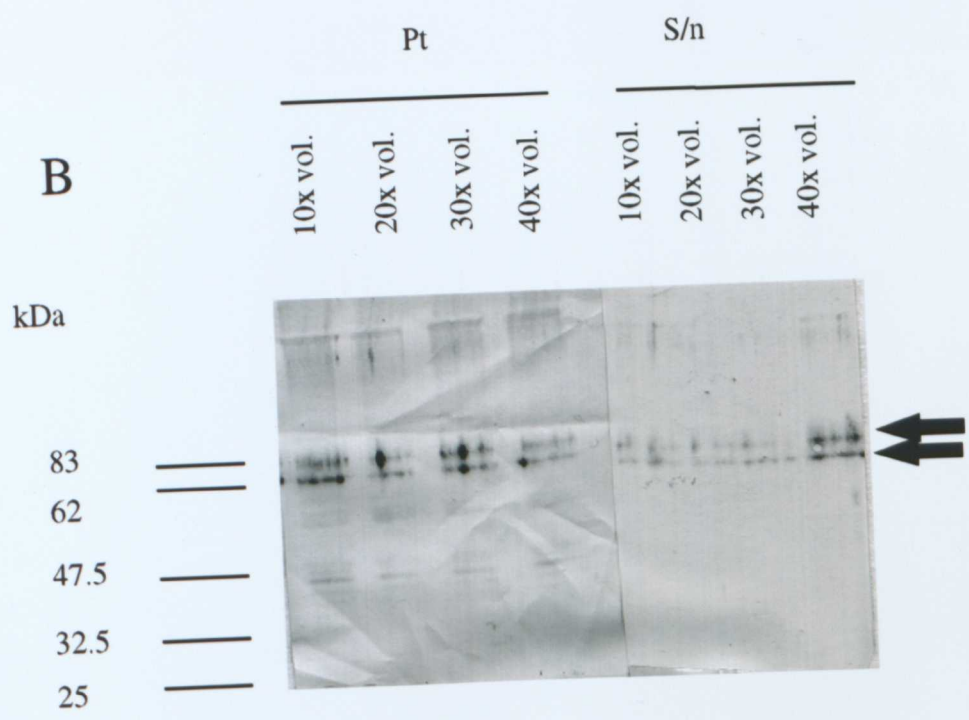
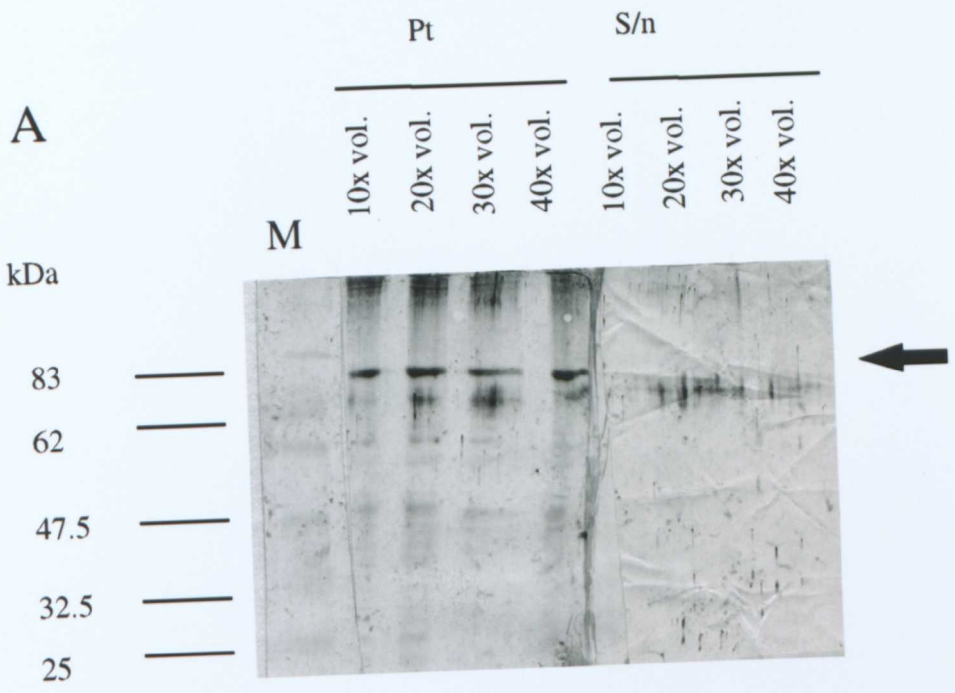
The samples were then centrifuged and separated into pellet (pt) and supernatant (s/n) fractions and run on a 10% SDS-PAGE gel. The proteins were then transferred to nitrocellulose and probed with:

(A) Anti-lamin antibody

(B) Anti-Drongo peptide antibody.

There appears to be increase in the amount of Drongo protein (**B: arrows**) in the supernatant as more of the lysis buffer is added (compare lane 4 to lanes 1-3) and a slight decrease in the amount pelleted (compare lane 8 with lanes 5-7).

There is no decrease in the amount of nuclear lamin upon treatment with lysis buffer (**A: arrow**).



The peptide was chosen as it displayed high predicted scores both hydrophilicity and antigenicity. An antiserum to the full length Drongo protein was also produced but this antiserum showed non-specific binding and so was not used further (data not shown). An IgG purified fraction of the antibody used for western analysis specifically recognised Drongo and showed that Drongo was developmentally expressed. A number of proteins were observed including proteins with molecular weights of approximately 86 kDa and 60 kDa. These proteins appear similar in size to those seen by previous *in vitro* translation experiments (Harris, 1995). Interestingly, the 60 kDa band appears to be more abundant than the 86 kDa protein. The presence of these two proteins *in vivo* may indicate a number possibilities. The first is that there are two possible translation start sites, (assuming that the other bands observed are degradation products). This idea was first suggested by Harris (1995) following deletion experiments and *in vitro* translation, where the two proteins seen were initiated from two different translational start sites, one being a non-AUG start codon (Harris, 1995). Previous reports, however, suggest that non-AUG codons are highly inefficient *in vivo* and are only utilised in exceptional cases (Kozak, 1989). The second possibility is that alternative splicing occurs. This seems unlikely as no splice sites have been found within the genomic sequence that may generate proteins of this size.

The antibody did not recognise the native protein in whole mount embryos and ovaries, so western analysis was used to determine the subcellular localisation of the protein. Due to the high identity of Drongo to the hRIP/Rab protein, it was postulated that Drongo may be localised to the nucleus/nuclear matrix. Indeed observations with the anti-Drongo peptide antibody on western blots of fractionated embryos localised Drongo to a fraction enriched in nuclei and nuclear matrix, primarily suggesting a nuclear function. An anti-lamin antibody was used to validate the fractions. Assuming that all nuclei remained whole (and were not themselves fractionated) using this antibody showed that the fractions enriched in cytoplasmic constituents and membranes contained no detectable levels of the particular lamin protein recognised by the antibody. We may therefore also postulate that the cytoplasmic and membrane fractions do not contain nuclear proteins.

Observation of the antibody staining pattern in S2 cells, however, and comparison to the nuclear localised Hoescht #33258 and anti-lamin antibody appears to exclude Drongo from the nucleus. Drongo is localised as a punctate pattern around the nuclear membrane and emanating out into the cytoplasm. In comparison to the localisation of the hRIP/Rab protein (Fritz *et al.*, 1995; Bogerd *et al.*, 1995) Drongo showed a similar punctate localisation around the nucleus but hRIP was more tightly localised when observed in S2 cells and did not show the cytoplasmic staining (Fritz *et al.*, 1995). The antibody to Rab showed that the protein was primarily located in the nucleus and nucleolus, but this analysis used CV1, a primate cell line (Bogerd *et al.*, 1995).

There is evidence that Drongo may localise to the endoplasmic reticulum in these cells. As in mammalian cells the RER can be observed as a reticular perinuclear structure in *Drosophila* cells, Drongo partially co-localises with a ER/Golgi marker Con A, consistent with a localisation to the ER. Drongo shows a pattern different to that of *Drosophila* Golgi specific proteins (e.g COMM) and similar to that of *Drosophila* proteins located to the RER (e.g Porcupine).

Western data after treating the fractions with detergent (0.1% Triton X-100) supports the theory that Drongo is localised to the RER, but although the experiment was controlled by using a nuclear antibody, it would be interesting to observe if an RER specific antibody would also show an increase in RER specific proteins as more detergent was added to the nuclear preparation.

There are no specific domains in the predicted Drongo protein sequence that suggest it is a resident ER protein, such as the KDEL sequence. For example, a similar sequence (KEEL) is present in the Windbeutel (Wind) protein a homologue of the rat endoplasmic reticulum protein Erp29 (Konzolaki and Schüpbach, 1998). Wind also contains a thioredoxin-like catalytic motif (Cys-X-X-Cys) a domain known to mediate the function of a family of ER-resident proteins such as Protein Disulphide Isomerase (PDI). Neither does Drongo contain any transmembrane domains or signal sequences suggestive of either a receptor/transport channel (e.g Porcupine; Kadowaki *et al.*, 1996 and COMM; Tear *et al.*, 1996) or a secreted protein.

Although we have some evidence that Drongo may be localised to the RER in S2 cells, we were not able to use an ER specific antibody for co-localisation experiments due to the lack of availability of ER specific antibodies in *Drosophila*. The ER antibodies available were raised in rabbit or the antibodies were monoclonal to the protein raised and would not have recognised the homologous protein in *Drosophila*. This problem appears to be widespread in the *Drosophila* field and a lack of ER markers has prompted other researchers to take different approaches to localisation experiments. For example, in the absence of a high affinity antibody specific to the Porcupine protein, a HA-tagged version of the protein was expressed in Kc cells (Kadowaki *et al.*, 1996) and localised via an antibody to the tag. Indeed a similar experiment was performed for localisation of COMM protein in S2 and Cos cells. This may be performed with the Drongo protein in order for a more detailed analysis of its localisation. In conclusion therefore observations of the subcellular localisation of the Drongo protein may offer implications firstly about its role in the cell and secondly about the dynamics of the endoplasmic reticulum in *Drosophila* cells. The organelle(s) labelled by Drongo appeared to include the endoplasmic reticulum. A caveat to this is that upon treatment with colchicine, the organelles were not affected in the way expected. This may be because the endoplasmic reticulum in *Drosophila* cells may be stabilised in a different way to that of mammalian cells.

Although *Drosophila* is not as well established as the yeast *S. cerevesiae* for studies in vesicle transport, it has been proved useful as an additional model for studying the ER (Konzolaki and Scüpbach, 1988; Wickham *et al.*, 1999) As I have discussed, the secretory apparatus is quite different to that in a mammalian cell. Any markers of the ER and Golgi in *Drosophila* must therefore be of some benefit in aiding our understanding of the structure of this apparatus and the communication between the ER and the Golgi. Drongo may represent a *Drosophila* marker for the endoplasmic reticulum, markers of which are rare at present. Further analysis with Drongo may also offer information on the compartments of the ER in *Drosophila* cells.

Chapter 8:

General discussion.

Chapter 8. General Discussion.

8.1 Introduction.

This thesis incorporates the results of an analysis into the functions of a *Drosophila* gene, *drongo*. The results of the experimental work (chapters 3-7) have been discussed and analysed at the end of each chapter. The thesis was divided into the following analyses: effects of overexpression of the gene via heat shock and UAS-GAL4 mis-expression; the analysis of the role of the gene in *Drosophila* tissue culture cells; generation and analysis of an antibody to the Drongo protein and mutational analysis of the gene and its chromosomal region.

This discussion will outline the experimental work overall, its advantages and limitations, and determine how much further we have come in understanding a role for the *drongo* gene.

8.2 The role of *drongo* during development.

8.2.1 Does *drongo* have a role during oogenesis and /or neurogenesis?

drongo is expressed during both early and late oogenesis and also during early neurogenesis (Harris, 1995) so both stages of development were obvious places to look for a role for the protein.

Overexpression experiments (chapter 3) showed that mis-expression of the *drongo* gene behind a heat shock promoter resulted in defects during early and late oogenesis.

drongo overexpression during early oogenesis resulted in the delayed migration of the follicle cells across the germarium to produce supernumary nurse cells in affected cysts. During late oogenesis, overexpression of *drongo* resulted in the production of abnormal embryos with denticle belts missing. Many of these embryos had posterior defects of varying degrees of severity. It was shown through

antibody staining that this was likely to be due to the mislocalisation of the posterior determinant *oskar*.

The cause of the overexpression defect during early oogenesis may be different to that seen during late oogenesis. Both early and late stages of oogenesis are involved in establishing the polarity of the egg (Grunert and St Johnston, 1996).

The polarisation of the two major body axes in *Drosophila* depends on a number of stages during oogenesis, the position of the fusome/spectrosome is thought to determine the oocyte (deCuevas and Spradling, 1997; Lin and spradling, 1994); the position of the oocyte determines the posterior of the egg chamber (Rüsbam *et al.*, 1998; Sapir *et al.*, 1998), and the position of the oocyte nucleus determines the dorsal side (Sapir *et al.*, 1998; Tomancak *et al.*, 1997). These cytological asymmetries result in the polarisation of the cytoskeleton which directs the localisation of specific mRNAs to subsequently set up axis formation (St Johnston, 1995; Gonzalez-Reyes *et al.*, 1995; Roth *et al.*, 1995).

From analysis it appeared that the polarity of the egg chamber is not affected by *drongo* mis-expression during early oogenesis but may be affected during late oogenesis. *drongo* may directly or indirectly act to inhibit the domain of *oskar* localisation; overexpression of the gene may expand this domain, producing the abnormal embryo. If *drongo* has a role during early oogenesis it may act as a part of a negative signal normally preventing migration of follicle cells until a 16 cell cyst has been formed.

Experiments were also carried out using the UAS-GAL4 system in an attempt to mis-express the gene in a more defined manner. Overexpression of *drongo* in the CNS via GAL4 line AB31 lead to lethality at the first larval instar stage, although I have not characterised this further.

It is unclear at present therefore whether *drongo* has a role during neurogenesis. If we analyse where *drongo* has been shown to be expressed, we can observe common signalling pathways such as the EGF receptor signalling, Notch/Delta and Wingless signalling. The role of the EGF receptor has been established during oogenesis (Sapir *et al.*, 1998), early neurogenesis (Udolph *et al.*, 1998; Skeath, 1998), and also during early eye development (another system where *drongo* is expressed). Notch/Delta and Wingless signalling have also been shown to

function in these systems with different consequences in each (Cooper *et al.*, 1999; Gonzalez-Reyes *et al.*, 1998; Zhang *et al.*, 1998; Forbes *et al.*, 1996; Bhat *et al.*, 1998). It is too early in this analysis, however, to confer a role in any signalling pathway at present. It should also be noted that production of a defect by overexpression is not necessarily an indication that the gene has a role in these systems.

8.2.2 How might the protein act?

Analyses using a specific anti-Drongo peptide antibody have shown that Drongo is developmentally regulated, and a number of different proteins are recognised by the antibody on western blots including two main proteins of approximately 60kDa (being abundant in embryos only) and a larger band of approximately 86kDa. These proteins are similar in size compared to those seen in *in vitro* translations, suggesting the concept of two translational start sites.

The nucleotide sequence surrounding the initiator codon(s) in the *drongo* cDNA is GGCAAGAAUUCAG (at 604) or CAGUUCGUGUAC (at 613). These are similar (although not identical) to the consensus sequence for translation from non-AUG codons (Imataka *et al.*, 1997). The rule states that purines are favourable at positions -3 and +4 (the first nucleotide being defined as +1). Kozak (1989) also suggests that adenines at the +5 position, a G at +4 and an A at +5 are important for efficient translation.

Many regulatory genes (Hann *et al.*, 1988 and Ackland *et al.*, 1990) have non-AUG initiators in addition to downstream in-frame AUG codons so that translation from the non-AUG codon generates amino terminally extended proteins. Some of these proteins show intracellular localisations different to their shorter counterparts (Ackland *et al.*, 1990). Post translational processing of a full length protein or alternative termination sites would also remain a possibility. It may be that different forms of the protein are involved at different stages of development.

Do any other genes that are involved in oogenesis and neurogenesis show a similar modifications in the proteins expressed? The Brainiac protein, for example, contains two potential N- and O-linked glycosylation sites which aid its function as a

secreted protein, signalling to the follicular epithelium (Goode *et al.*, 1996). An example of a gene where more than one protein is produced is that of the *Drosophila maternal nuclear kinase (Dmnk)* gene. Two proteins are also seen on western blots using antibodies to the protein (Oishi *et al.*, 1998). The long (Dmnk-L: 56KDa) and short (Dmnk-S: 54KDa) forms of Dmnk are thought to be produced by alternative splicing of the transcript. The two forms appear to be functionally distinct and although it is not possible to distinguish between the forms on whole mount tissues. *Dmnk-S* mRNA is abundant in ovaries and *Dmnk-L* mRNA is rather constant throughout embryogenesis. Although no developmental role as yet has been assigned to the gene, the restricted expression pattern of Dmnk proteins during oogenesis and in the posterior pole plasm/germ line cell lineages is characteristic of a maternal effect posterior group gene and suggests a role for Dmnk in pole cell formation (Oishi *et al.*, 1998).

If two translational start sites are used for the Drongo protein, there do not seem to be any sites that are likely to be post translationally modified between the two codons used, so it is unlikely that the two proteins are functionally distinct although this would require further analysis.

8.3 Is Drongo a hRIP orthologue?

A running theme throughout this thesis is the idea that Drongo may act as a hRIP orthologue. Sequencing of the gene (Harris, 1995 and this work) and comparisons of the predicted amino acid sequence to others in the databases (using Blast search tool) also determined a number of other homologues (see chapter 1). These included the yeast family of Zinc finger proteins GTS1/GCS1/Glo3 and the mammalian and *Drosophila* ARF-1 GAP. The human Rev interacting protein hRIP, however, showed the highest amino acid identity and so it seemed logical to study this protein and to determine if Drongo had a similar role in nuclear transport in the cell.

A number of analyses carried out in this thesis have offered information on whether Drongo acts in a similar way to hRIP. Western analysis suggested Drongo may be localised in the nucleus, a similar location to that of hRIP/Rab, however

upon further analysis in tissue culture cells, a more likely location of the protein was the rough endoplasmic reticulum and it became clear that the subcellular fractions produced were not as homogenous as originally thought.

Alternatively Drongo may not be exclusively localised to the RER and may be found on the nuclear membrane. Indeed, western analysis showed that Triton X-100 treatment of nuclei does not remove all of the protein from the nuclear fraction. GTP binding proteins of the Rab family are thought to translocate through membranes and have been found to accumulate in the cytoplasm and the nucleus of cells in culture (Perez *et al.*, 1994), although no nuclear transport role has yet been attributed to Rab.

We were not able to determine if Drongo could enhance Rev function *in vitro* by using a Rev activity assay in tissue culture, therefore we could not confirm or rule out an interactions with Rev and a role in nuclear transport. The majority of the Drongo protein appears to be localised to the rough endoplasmic reticulum, however the protein may well interact with Rev with additional functions in the RER.

Interestingly Drongo also displays amino acid homology to GTPase activating protein (GAP), which regulates the small GTP binding protein ARF1 (ADP-ribosylation factor) in mammals (Section 1.1; Huber *et al.*, 1998). ARF1 is required for both anterograde vesicle transport from the ER to Golgi and retrograde transport from the Golgi to the ER. Retrograde transport retrieves escaped material or recycles transport components for continued anterograde transport.

In an active GTP bound state, ARF recruits vesicle protein such as COPI (for coatamer coated vesicles) and AP1-1 and AP-3 (for clathrin coated vesicles; Kim *et al.*, 1997) to the membrane. A number of ARF1 GAPs have recently been cloned in yeast (Zhang *et al.*, 1998; Poon *et al.*, 1999), in mammals (Randazzo, 1997) and in *Drosophila* (Frolov, 1997).

Recently both Gcs1 and Glo3, also Drongo homologues identified from the yeast *S. cerevisiae*, have been reported to have GAP activity by *in vivo* and *in vitro* assays (Poon *et al.*, 1996; Poon *et al.*, 1999). Gcs1 and Glo3 form an essential pair of overlapping ARF-GAPs required for retrograde transport (from the Golgi to the ER) (Poon *et al.*, 1999).

Interestingly, the GAP activity domain has also been studied in a number of GTPase activating proteins. For the ARF1-GAP protein, ARD1 (Vitale *et al.*, 1998) for example, the putative amino terminal GAP domain contains a single zinc finger and two essential arginines located near the zinc finger. A N-terminal zinc finger may also be found in the *drongo* gene sequence (figure 1.1) although whether any of the arginine present in the sequence are in the correct place and are essential is yet to be determined.

There is also a sequence found in ARD1-GAP domain which is similar to a consenses sequence found in the Rho/Rac GAPs, KXXXXXXLPXPL (where X is any amino acid), although in ARD1, only the first lysine and the last two amino acids of the sequence, proline and leucine (underlined) appear essential for GAP activity (Vitale *et al.*, 1998). A similar sequence to this can be located in the Drongo protein sequence at lys¹¹⁹ to leu¹²⁹ (KRYYLEPASPL). In view of this sequence similarity, especially as many of these sequences have been shown in other proteins to be required for GTP hydrolysis, and given the localisation of the Drongo protein it would be interesting to assay Drongo for ARF1-GAP activity in a similar method to those described (Cuckierman *et al.*, 1995; Poon *et al.*, 1999), although a pure sample of the protein would be required for this procedure.

An EMS mutagenesis screen (Chapter 4) was also carried out in this study in an attempt to produce a mutation in the *drongo* gene. A *drongo* mutant was not identified, however, this may indicate that the region was not saturated by mutagenesis, although this is unlikely as more than one allele was found in each complementation group (section 5.4.4.1). Of course, the gene may not be lethally mutable and other phenotypes such as male/female sterility have not been analysed. In view of the possible evidence that Drongo may function as an ARF-GAP and that many previous ARF-GAPs have been found to be functionally redundant (e.g. Gcs1 and Glo3 as discussed above), it is likely that *drongo* also may show redundancy (and may not be lethally mutable) but be able to produce an effect after overexpression. It is also interesting to note that a null mutant of *Gap69C*, ARF-GAP cloned from *Drosophila* has not yet been identified (Frolov, 1997).

Functioning as an ARF1-GAP may explain the reason that Drongo has an effect after overexpression in the central nervous system via GAL4 line 31.1. If

vesicular transport is affected in the neurons it may well be lethal. There are two problems with this theory, however, an effect on vesicular transport might produce an effect earlier in the embryo (not larval as was reported in this study); also a deletion in the C-terminal of the protein does not have any deleterious effect when overexpressed via GAL4 31.1 and yet this protein would still contain an intact N-terminal (and so putative ARF GAP) domain.

8.5 How do we determine a more defined role for Drongo in *Drosophila*.

Further attempts to isolate a mutant have been carried out (*Pers. Comm.* Edwin Chan) by X-ray mutagenesis and P-element localisation. Recently, two P-element inserts have been identified by PCR (*Pers. Comm.* Edwin Chan) and are thought to be located within the *drongo* gene, both of these inserts are lethal, although neither are in the coding region. Analysis of these inserts with the anti-Drongo peptide antibody suggests that the insertion may result in a reduced amount of protein expressed by the gene. Further studies with these inserts may yield some exciting information about the role of the *drongo* gene.

ER markers recently obtained (a kind gift from David Roberts, University of Oxford) used on tissue culture cells with confocal analysis may also elucidate the localisation of the Drongo protein further.

Another method of defining the function of a gene in *Drosophila* is that of double stranded RNA gene interference (dsRNA-i). This technique has been established in *C. elegans* to produce specific genetic interference in the worm (Montgomery *et al.*, 1998) and has been used recently in the fly to study the function of many genes (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999). Studies have shown that the introduction of dsRNA corresponding to either a portion or the entire coding region of a gene can interfere with the function of the endogenous gene to give a phenotype similar to the known genetic mutation. Surprisingly results from studies suggest that the dsRNA may be acting catalytically after injection into embryonic cells (Misquitta and Paterson, 1999). As no mutant

has yet been identified in *drongo* it would be interesting to apply this technique in future analyses.

8.6 Concluding remarks

The analysis of the *drongo* gene has provided interesting information about possible roles for the gene in *Drosophila*, further study of the *drongo* gene and its protein may reveal exciting information about oogenesis and neurogenesis as well as offering more clues about the dynamics of the rough endoplasmic reticulum the *Drosophila* cell.

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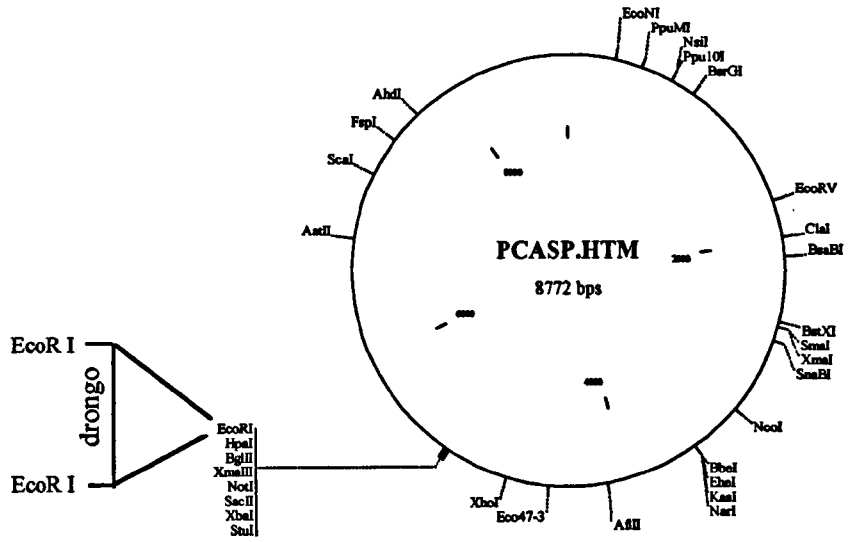
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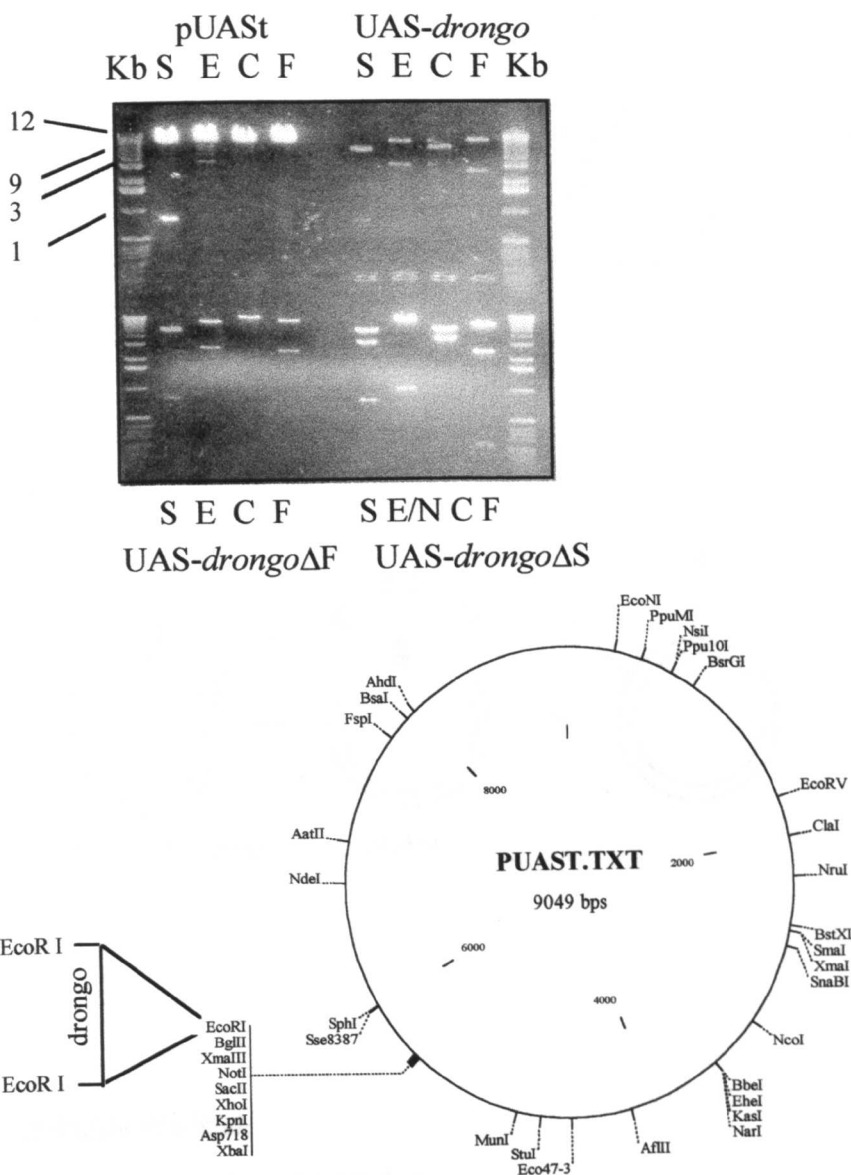
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10.1 Restriction maps and digestion checks



A) pCaSpeR-*drongo*.

drongo cDNA was cloned into the pCaSpeR vector using *EcoR* I digestion sites.



B) UAS-drongo and deletion constructs.

1) Agarose gel to show successful cloning.

2) Plasmid map and insert site of *drongo* cDNA.

drongo was cut from pBluescript with *EcoR* I and cloned into pUAST. UAS-*drongo* was also digested with *Fsp* I and religated (UAS-*drongo*ΔF). PGEX-5X-1-*drongo* ΔS (10.2 a) was digested with *EcoR* I and *Not* I and cloned into pUAST (UAS-*drongo*ΔS).

In order to validate the vector, cutting pUAST with *Sal* I releases a 0.9 kb fragment; cutting with *EcoR* I, *Cla* I and *Fsp* I all linearises the vector to give a 9 kb band.

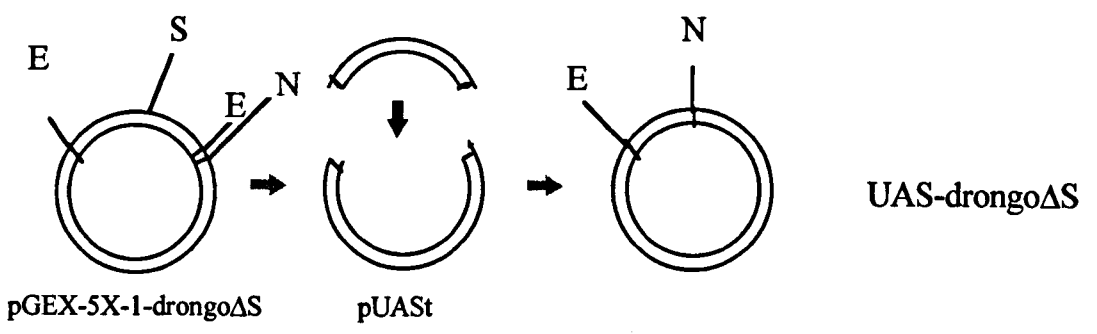
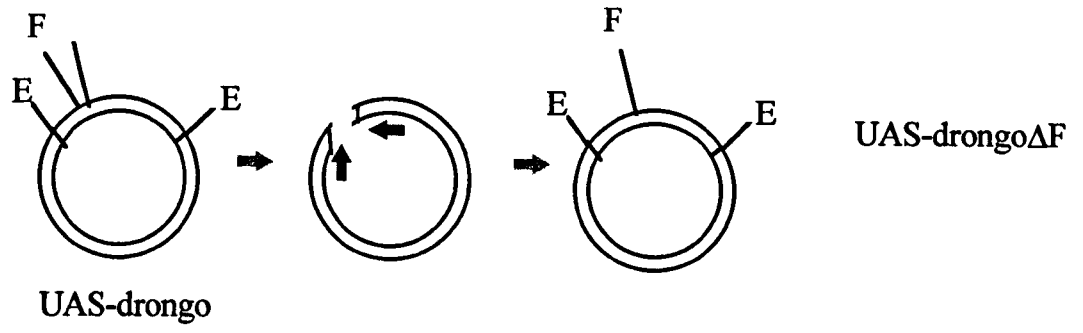
Cutting UAS-*drongo* with *Sal* I gives a 0.9 kb fragment, and two 6 kb bands so the fragment has inserted in the correct orientation. Cutting with *EcoR* I excises the 3.5 kb *drongo* insert in addition to the 9 kb pUAST fragment.

Cutting with *Fsp* I gives fragments of 2.8 kb and 6.2 kb if the insert is in the correct orientation. Cutting with *Cla* I gives a 3.5 kb and a 9 kb fragment showing that the insert is in the correct orientation.

Cutting UAS-*drongo* ΔF with *Sal* I, *EcoR* I, *Cla* I and

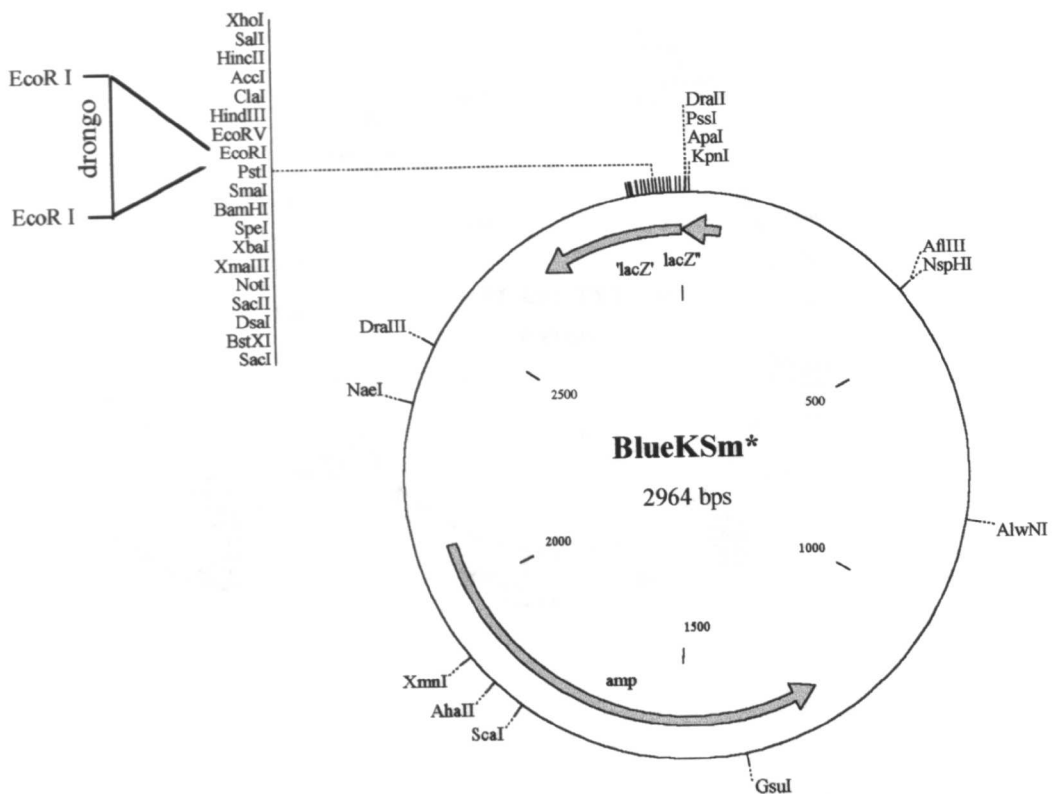
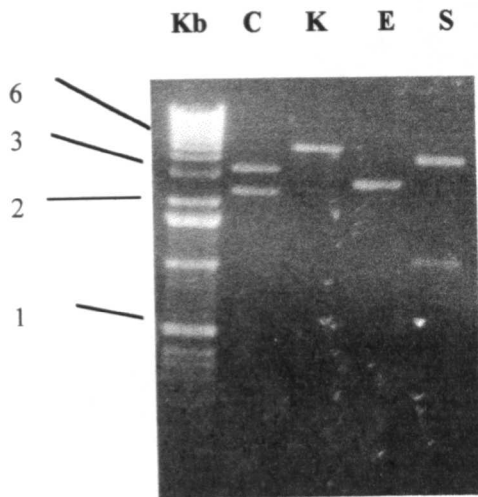
Fsp I result in a similar pattern to the restriction digests of UAS-*drongo*, but the lower molecular weight bands are slightly smaller. Cutting UAS-*drongo* ΔF demonstrates that the *Fsp* I site has reformed after cloning.

Digestion of UAS-*drongo* ΔS with *EcoR* I and *Not* I gives a fragment of 1.1 kb showing the 3' end of the *drongo* fragment has been deleted. Digestion with *Cla* I gives a 5 kb and a 6 kb fragment showing a deletion has been made.



C) UAS-drongo deletions.

See B for details of deletions of UAS-drongo.



D) pBS-drongo

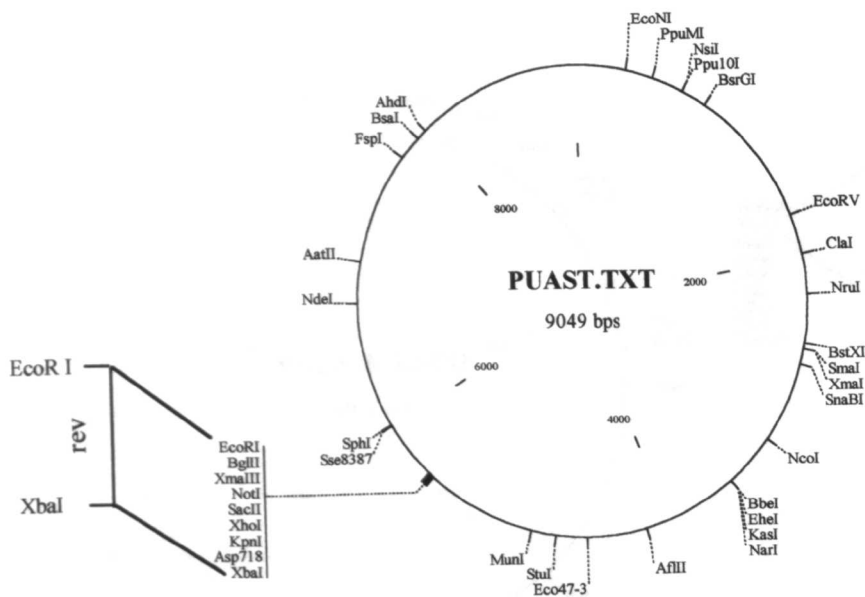
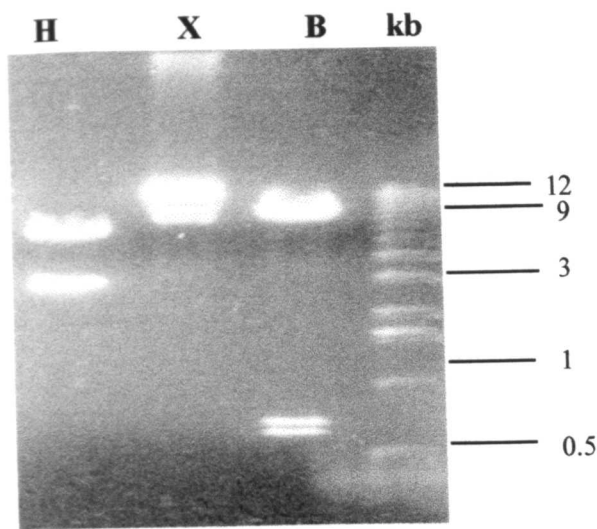
1) restriction digest of pBS-*drongo*.

2) Plasmid map of pBS-*drongo*.

Digestion of pBS-*drongo* with *Kpn* I linearises the construct, demonstrating pBluescript is present.

Digestion with *Eco*RI excises the 3.5kb *drongo* cDNA fragment and results in the production of 2 x3kb fragments demonstrating the presence of the *drongo* cDNA in pBluescript.

Digestion with *Sst* I results in a 1.1kb fragment demonstrating the *drongo* cDNA is in the correct orientation in the vector.



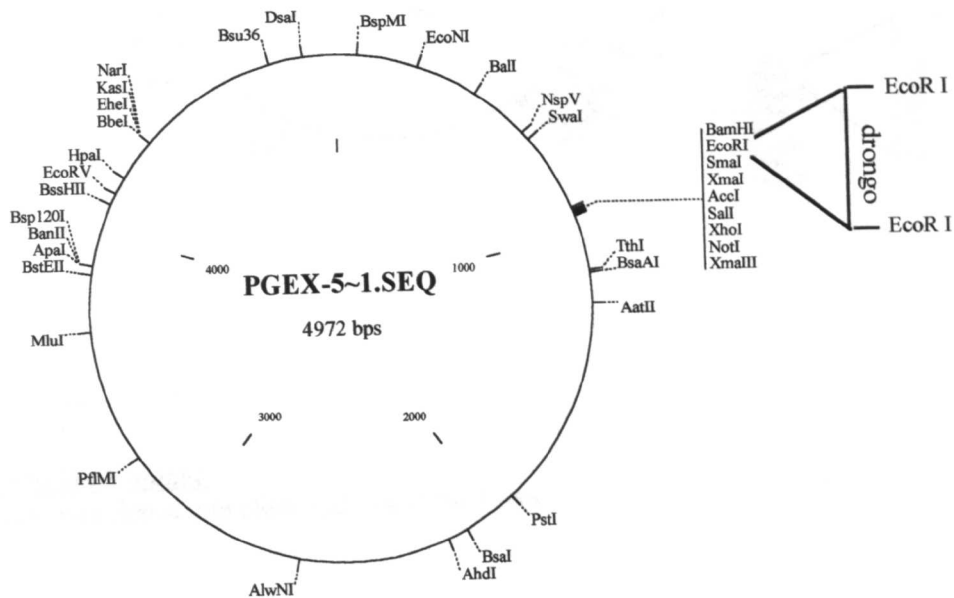
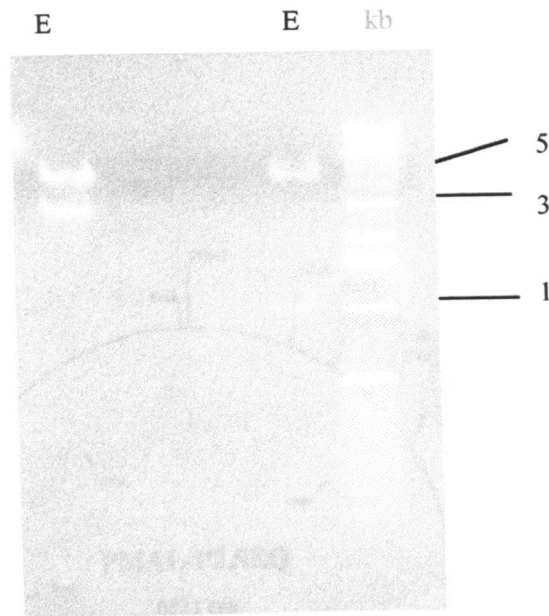
E) UAS-Rev

1) Agarose gel showing digestions of UAS-*rev*

2) Restriction map

Digestion with *Hind*III gives 6kb and 3kb vector fragments. *rev* has been excised in a 350bp fragment. *Xho*I (which does not cut in pUASt but cut in the original plasmid, pGEMt) demonstrates that Rev has been cloned into pUASt. *Bam*H I digestion results two bands at approximately 750bp and a 7.5kb band demonstrating the presence of the pUASt and an additional *Bam*H I site in the *rev* cDNA.

Digestion of UAS-*rev* with *Sal* I results in fragments of 0.8kb, 3kb and 6kb demonstrating that pUASt is present and demonstrating a *Sal* I site in *rev*.

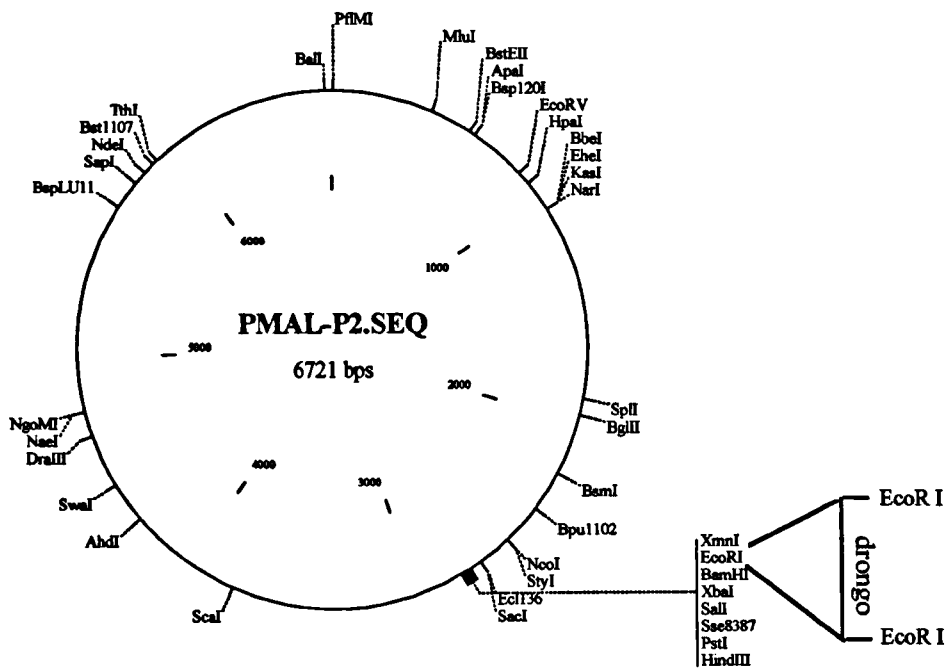


F) pGEX-5X-1 *drongo* and pGEX-5X-1 *drongo* Δ S.

1) Agarose gel of restriction digests.

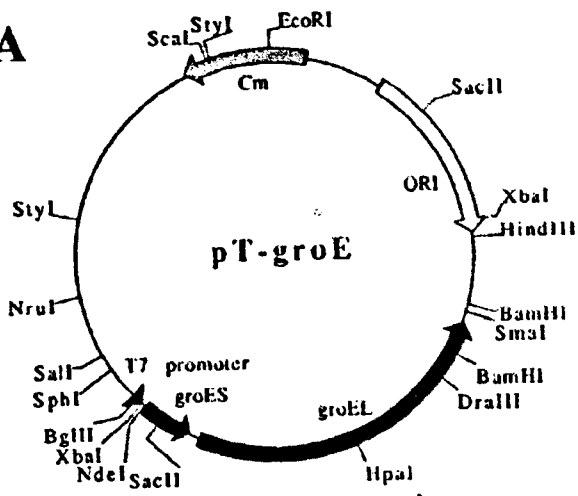
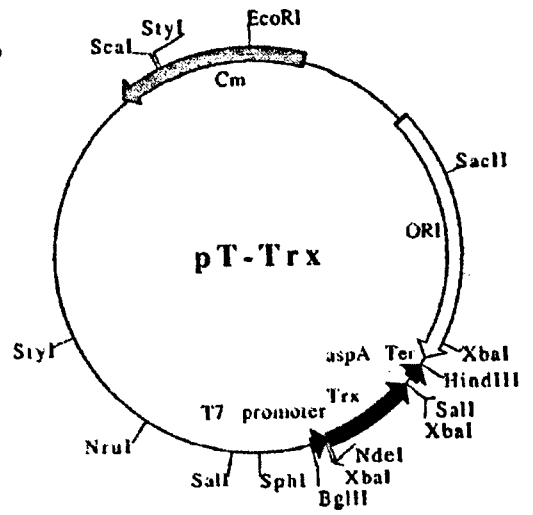
2) Plasmid map showing location of the *drongo* cDNA and restriction sites

pGEX-5X-1 *drongo* cut with *EcoR* I gives a 3.5kb *drongo* fragment and a 6kb vector band showing *drongo* has been excised from the vector.
pGEX-5X-1 *drongo* Δ S cut with *EcoR*I excises a deleted 1.1kb band (and a 6kjb vector band) from this construct, showing the succesful deletion of the 3'end of the *drongo* gene after digestion with *Sst* I.



G) pMAL-p2-*drongo*.

drongo was cloned into pMAL-p2 via *EcoR* I sites.

A**B**

10.1 I

Sequence of ptk34.

ptk34 was partially sequenced in this study to check the sequence highlighted.

The sequences checked were the SV40 promoter, all four splice sites. The CAT gene, the RRE site in the env gene and the SV40 poly A. No mutations were found in the sequence that would appear to affect the transcription or translation of the plasmid.

TTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGT 60

CTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGG 120

TGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGACTGAGAGTGCACCATAAC 180

TTNN 240

NN 300

NN 360

NN 420

NNNTGGAA 480

GGGCTAATTGGTCCAAAAAGACAAGAGATCCTTGATCTGTGGATCTACCACACACAA 540
SV40 PROMOTER
GGCTACTTCCTGATTGGCAGAACTACACACCAGGGCCAGGGATCAGATGATCCCTGTGG 600

AATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAA 660

AGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGG 720

AGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCTAACTCCG 780

CCCATCCCGCCCTAACTCCGCCAGTCCGCCATTCTCCGCCCATGGCTGACTAATT 840

TTTTTTATTTATGCAGAGGCCGAGGCCGCTCGGCCTCTGAGCTATTCCAGAAGTAGTGA 900

GGAGGCTTTTTGGAGGCTTAGGCTTTTGCAAAAGCTTGCCTTGAGTGTCAAAGTAGT 960

GTGTGCCCGTCTGTTGTGACTCTGGTAACTAGAGATCCCTCAGACCCTTTTAGTCAGT 1020

GTGGAAATCTCTAGCAGTGGCGCCCGAACAGGGACTTGAAAGCGAAAGTAAAGCCAGAG 1080

GAGATCTCTCGACGAGGACTCGGCTTGCTGAAGCGGCACGGCAAGAGGCGAGGGGCGG 1140
5' SPLICE SITE
CGACTGGTACTACGCCAAAATTTGACTAGCGGAGGCTAGAAGGAGAGAGGATCAGCT 1200

TGCGGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAATCACTGGATATACCA 1260

CCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGGAGCATTTTCAGTCAGTTGCTC 1320

AATGTACCTATAACCAGACCCTTCAGCTGGATATTACGGCCTTTTTAAAGACCCTAAAGA 1380

AAAATAAGCACAAGTTTTATCCGGCCTTATTACATCTTGCCCGCCTGATGAATGCTC 1440

ATCCGGAATTCGGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTCACC 1500
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 GGGTGAGTTTCACCAGTTTTGATTTAAACGTGGCCAATATGGACAACCTCTTCGCCCCCG 1740
 TTTTCACCATGGGCAAATATTATACGCAAGGCCACAAGGTGCTGATGCCGCTGGCGATTCC 1800
 AGGTTTCATCATGCCGCTCTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGAATPACAAC 1860
 AGTACTGCGATGAGTGGCAGGGCGGGCGTAATTTTTTAAGGCAGTTATTGGTGGCATC 1920
 CCCCATGGAAGATCTGGATCCTAGAACTAGTGGATCGATCCCCCATGACAAAAGCCTTA 1980
 3' SPLICE SITE rev
 GGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAGCTCATCAGAACAGTCAG 2040
 5' SPLICE SITE
 ACTCATCAAGCTTCTCTATCAAAGCAGTAAGTAGTACATGTAATGCAACCTATAATAGTA 2100
 GCAATAGTAGCATTAGTAGTAGCAATAATAATAGCAATAGTTGTGTGGTCCATAGTAATC 2160
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 env
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 CTGTGTACCCACAGACCCCAACCCACAAGAAGTAGTATTGGTAAATGTGACAGAAAATTT 2520
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 AACAGGACCATGTACAAATGTCAGCACAGTACAATGTACACATGGAATCAGGCCAGTAGT 3000

| | |
|---|------|
| ATCAACTCAACTGCTGTTAAATGGCAGTCTAGCAGAAGAAGATGTAGTAATTAGATCTGC | 3060 |
| CAATTTACAGACAATGCTAAAACCATAATAGTACAGCTGAACACATCTGTAGAAATTAA | 3120 |
| TTGTACAAGACCCAAACAATAACAAGAAAAAGTATCCGTATCCAGAGGGGACCAGGGAG | 3180 |
| AGCATTGTGTACAATAGGAAAAATAGGAAATATGAGACAAGCACATTGTAACATTAGTAG | 3240 |
| AGCAAAATGGAATGCCACTTTAAACAGATAGCTAGCAAATTAAGAGAACAATTTGGAAA | 3300 |
| TAATAAAACAATAATCTTTAAGCAATCCTCAGGAGGGGACCCAGAAATGTAACGCACAG | 3360 |
| TTTTAATTGTGGAGGGGAATTTTCTACTGTAATTCACACAACCTGTTAATAGTACTTG | 3420 |
| GTTTAATAGTACTTGGAGTACTGAAGGGTCAAATAACACTGAAGGAAGTGACACAATCAC | 3480 |
| ACTCCCATGCAGAATAAAACAATTTATAAACATGTGGCAGGAAGTAGGAAAAGCAATGTA | 3540 |
| TGCCCTCCCATCAGTGGACAAATTAGATGTTTCATCAAATATTACTGGGCTGCTATTAAC | 3600 |
| AAGAGATGGTGGTAATAACAACAATGGGTCCGAGATCTTCAGACCTGGAGGAGGCATAT | 3660 |
| GAGGGACAATTGGAGAAGTGAATTATATAAATATAAAGTAGTAAAAATGAACCATTAGG | 3720 |
| AGTAGCACCCACCAAGGCAAAGAGAAGAGTGGTGCAGAGAGAAAAAGAGCAGTGGGAAT | 3780 |
| AGGAGCTTTGTTCCCTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCTGCACGTCAAAT | 3840 |
| GACGCTGACGGTACAGGCCAGACAATTATTGCTGATATAGTGCAGCAGCAGAACAAATTT | 3900 |
| GCTGAGGGCTATTGAGGGCAACAGCATCTGTTGCAACTCACAGTCTGGGGCATCAAACA | 3960 |
| GCTCCAGGCAAGAATCCTGGCTGTGGAAAGATACTAAAGGATCAACAGCTCCTGGGGAT | 4020 |
| TTGGGGTTGCTCTGAAAACTCATTGCACCACTGCTGTGCCTTGGAAATGCTAGTTGGAG | 4080 |
| TAATAAATCTCTGGAACAGATTGGAATAACATGACCTGGATGGAGTGGGACAGAGAAAT | 4140 |
| TAACAATTACACAAGCTTAATACACTCCTTAATTGAAGAATCGAAAACCAGCAAGAAAA | 4200 |
| GAATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTAACAT | 4260 |
| AACAAATTGGCTGTGGTATATAAAATATTTCATAATGATAGTAGGAGGCTTGGTAGGTTT | 4320 |
| AGAATAGTTTTTGCTGTACTTTCTATAGTGAATAGAGTTAGGCAGGGATATTCACCATT | 4380 |
| ATCGTTTCAGACACCTCCCAATCCCGAGGGGACCCGACAGGCCCAAGGAATAGAAGA | 4440 |
| AGAAGGTGGAGAGAGAGACAGAGACAGATCCATTGATTAGTGAACGGATCGATCCTTAG | 4500 |
| CACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAGAGACT | 4560 |

RRE
→

RRE
←

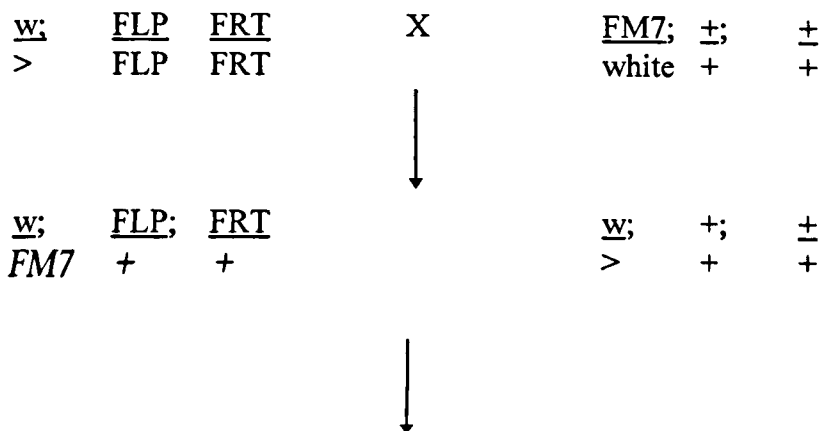
3' SPLICE SITE



| | |
|---|------|
| TACTCTTGATTCTAACGAGGATTGTGGAACCTCTGGGACGCAGGGGGTGGGAAGCCCTCA | 4620 |
| AATATTGGTGGAAATCTCCTACAGTATTGGAGTCAGGAACTAAAGAATAGTGCTGTAACT | 4680 |
| TGCTCAATGCCACAGCCATAGCAGTAGCTGAGGGGACAGATAGGGTTATAGAAGTATTAC | 4740 |
| AAGCAGCTTATAGAGCTATTCGCCACATACCTAGAAGAATAAGACAGGGCTTGGAAAGGA | 4800 |
| TTTTGCTATAAGATGGGTGGCAAGTGGTCAAAAAGTAGTGTGATTGGATGGCCTGCTGTA | 4860 |
| AGGGAAAGAATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGTATCTCGAGAT | 4920 |
| CCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACCTAGAATGCAGTGAAAA | 4980 |
| AAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGC | 5040 |
| SV40 POLY A | |
| AATAAA CAAGTTAACAAACAATTGCATTCAATTTTATGTTTCAGGTCAGGGGGAGGTGT | 5100 |
| GGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTATGGCTGATTATGATCCTGCCT | 5160 |
| CGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCAC | 5220 |
| AGCTTGCTGTGAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGT | 5280 |
| TGGCGGGTGTCCGGGCGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTATACTGG | 5340 |
| CTTAACATATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATA | 5400 |
| CCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACT | 5460 |
| GACTCGCTGCGCTCGGTGTTCCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTA | 5520 |
| ATACGGTTATCCACAGAATCAGCGGATAACGCAGGAAAGAACATGTGAGCAAAGGCCAG | 5580 |
| CAAAGGCCAGGAACCGTAAAAGGCCGCTTGCTGGCGTTTTTCCATAGGCTCCGCCCC | 5640 |
| CCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTA | 5700 |
| TAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCCTG | 5760 |
| CCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGC | 5820 |
| TCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTGTGCAC | 5880 |
| GAACCCCCGTTTCAGCCCAGCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAAC | 5940 |
| CCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCG | 6000 |
| AGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGA | 6060 |

10.2 Raw data from EMS screen

A) Crosses to assess the effectiveness of EMS treatment by looking for male lethals.



Ratio of FM7 males to normal from each single pairmating. If mutation is lethal will only get *white* males. Expect 30% lethals from X chromosome.

Results from control crosses (*pers. comm.* Marcus Allen)

| Cross number | Number of viables | Number of lethals | Total | Percentage lethal |
|--------------|-------------------|-------------------|-------|-------------------|
| 1 | 66 | 30 | 96 | 31.3% |
| 2 | 18 | 7 | 25 | 28.0% |
| 3 | 18 | 17 | 35 | 48.6% |
| 4 | 65 | 42 | 107 | 39.2% |
| 5 | 66 | 21 | 87 | 24.1% |

B) Collection of CyO males from bottles and screening of males over Df(2L)ast4.

| Bottle | No. of flies | No. sterile | No. of mutants | Bottle | No. of flies | No. sterile | No. of mutants |
|--------|--------------|-------------|----------------|--------|--------------|-------------|----------------|
| 4DA | 145 | 35 | 2 | 3AA | 0 | 0 | 0 |
| 4DB | 0 | 0 | 0 | 3AB | 0 | 0 | 0 |
| 4DC | 0 | 0 | 0 | 3AC | 263 | 65 | 1 |
| 4DD | 260 | 50 | 3 | 3AD | 0 | 0 | 0 |
| 4DE | 203 | 47 | 1 | 3AE | 0 | 0 | 0 |
| 4DF | 0 | 0 | 0 | 3AF | 76 | 20 | 2 |
| 4DG | 132 | 26 | 2 | 3AG | 85 | 26 | 0 |
| 4DH | 52 | 9 | 1 | 3AH | 79 | 19 | 0 |
| 4DI | 52 | 10 | 2 | 3AI | 70 | 20 | 0 |
| 4DJ | 39 | 8 | 0 | 3AJ | 58 | 16 | 0 |
| 4DK | 81 | 14 | 0 | 3AK | 92 | 18 | 1 |
| 4DL | 95 | 18 | 2 | 3AL | 41 | 8 | 1 |
| 4DM | 87 | 16 | 0 | 3AM | 53 | 14 | 0 |
| 4DN | 75 | 15 | 1 | 3AN | 77 | 22 | 0 |
| 4DO | 92 | 16 | 1 | 3AO | 69 | 13 | 0 |
| 4DP | 58 | 10 | 0 | 3AP | 81 | 11 | 0 |
| 4DQ | 42 | 10 | 0 | 3AQ | 38 | 8 | 0 |
| 4DR | 34 | 7 | 0 | 3AR | 27 | 5 | 0 |
| 4DS | 32 | 5 | 4 | 3AS | 17 | 3 | 0 |
| 4DT | 10 | 2 | 0 | 3AT | 42 | 8 | 0 |
| 4DU | 25 | 4 | 0 | 3AU | 37 | 9 | 0 |
| 4DV | 72 | 18 | 0 | 2AV | 38 | 6 | 0 |
| 4DW | 71 | 14 | 0 | 3AW | 21 | 4 | 0 |
| 4DX | 21 | 5 | 0 | 3AX | 89 | 17 | 0 |
| 4DY | 105 | 21 | 3 | 3AY | 75 | 15 | 2 |
| 4DZ | 84 | 13 | 0 | 3AZ | 92 | 14 | 1 |

| Bottle | No. of flies | No. sterile | No. of mutants | Bottle | No. of flies | No. sterile | No. of mutants |
|--------|--------------|-------------|----------------|--------|--------------|-------------|----------------|
| 4EA | 60 | 10 | 0 | 3BA | 75 | 11 | 0 |
| 4EB | 49 | 9 | 1 | 3BB | 60 | 7 | 0 |
| 4EC | 135 | 24 | 3 | 3BC | 84 | 15 | 3 |
| 4ED | 53 | 8 | 0 | 3BD | 40 | 7 | 1 |
| 4EE | 140 | 25 | 0 | 3BE | 98 | 16 | 2 |
| 4EF | 149 | 30 | 1 | 3BF | 41 | 7 | 1 |
| 4EG | 49 | 7 | 0 | 3BG | 80 | 15 | 0 |
| 4EH | 88 | 12 | 1 | 3BH | 109 | 15 | 0 |
| 4EI | 48 | 6 | 0 | 3BI | 42 | 8 | 0 |
| 4EJ | 12 | 2 | 0 | 3BJ | 75 | 14 | 0 |
| 4EK | 125 | 23 | 2 | 3BK | 151 | 38 | 3 |
| 4EL | 162 | 42 | 0 | 3BL | 128 | 31 | 2 |
| 4EM | 79 | 19 | 0 | 3BM | 102 | 14 | 0 |
| 4EN | 26 | 6 | 0 | 3BN | 41 | 13 | 1 |
| 4EO | 116 | 36 | 0 | 3BO | 67 | 18 | 0 |
| 4EP | 57 | 18 | 1 | 3BP | | | |
| 4EQ | 108 | 20 | 0 | 3BQ | | | |
| 4ER | 168 | 23 | 1 | 3BR | | | |

C) Complementation analysis of the EMS mutation lines (1): Organising the mutants into lethal complementation groups

| | <i>4DA-59</i> | <i>4DA-122</i> | <i>3AC-184</i> | <i>4DD-201</i> | <i>4DD-288</i> | <i>4DD-418</i> | <i>4DG-777</i> | <i>4DH-1251</i> |
|-----------------|---------------|----------------|----------------|----------------|----------------|----------------|----------------|-----------------|
| <i>4DA-59</i> | 21;36 | 20;51 | 21;32 | 22;31 | 20;32 | n/a | n/a | n/a |
| <i>4DA-122</i> | 27;42 | 0;120 | 22;41 | 23;41 | 21;41 | 0;85 | 21;40 | 0;98 |
| <i>3AC-184</i> | 13;50 | 21;55 | 0;92 | 0;89 | 0;82 | 32;40 | 0;57 | 22;56 |
| <i>4DD-201</i> | 25;70 | 18;20 | 0;48 | 25;50 | 0;72 | 37;66 | 34;67 | 25;37 |
| <i>4DD-288</i> | 18;33 | 12;59 | 0;71 | 0;73 | 7;13 | 35;64 | 36;51 | 35;55 |
| <i>4DD-418</i> | 2;5 | 0;59 | 17;37 | 17;24 | 34;58 | 18;39 | 36;64 | 0;97 |
| <i>4DG-777</i> | 15;27 | 17;52 | 0;85 | 18;27 | 13;46 | 21;34 | 0;106 | 1;7 |
| <i>4DH-1251</i> | 29;53 | 0;79 | 15;30 | 22;41 | 4;17 | 0;98 | 3;7 | 37;85 |
| <i>4DI-1367</i> | 37;48 | 0;105 | 41;80 | 23;55 | 15;36 | 0;87 | 9;19 | 0;100 |
| <i>4DG-1524</i> | 2;10 | 8;19 | 12;28 | 17;34 | 7;20 | 32;46 | 18;37 | 17;46 |
| <i>4DL-1801</i> | 29;33 | 17;67 | 19;37 | 30;42 | 12;25 | 35;67 | 26;48 | 23;51 |
| <i>3AL-1872</i> | 21;55 | 21;59 | 23;38 | 14;27 | 15;27 | 32;58 | 32;50 | 34;58 |
| <i>4DI-1893</i> | 7;18 | 11;25 | 0;112 | 15;31 | 12;32 | 11;22 | 0;68 | 15;46 |
| <i>4DL-1980</i> | 7;9 | 19;37 | 12;18 | 17;35 | 7;13 | 40;74 | 12;23 | 21;55 |
| <i>4DN-2014</i> | 19;22 | 21;38 | 27;41 | 27;58 | 21;39 | 48;35 | 35;60 | 9;29 |
| <i>4DS-2678</i> | 27;54 | 0;122 | 0;72 | 0;102 | 0;95 | 0;104 | 0;96 | 0;93 |
| <i>4DS-2705</i> | 32;43 | 22;47 | 0;85 | 27;61 | 9;13 | 12;19 | 0;103 | 9;30 |
| <i>4DS-2715</i> | 17;60 | 0;103 | 0;92 | 0;95 | 0;103 | 0;105 | 0;103 | 0;86 |
| <i>4DS-2716</i> | 12;52 | 0;104 | 0;78 | 0;96 | 0;85 | 0;68 | 0;104 | 0;95 |
| <i>3AU-2858</i> | 33;65 | 9;17 | 0;108 | 0;97 | 0;76 | 19;25 | 20;40 | 23;40 |
| <i>3AZ-3013</i> | 12;40 | 0;89 | 22;45 | 27;48 | 23;55 | 32;66 | 0;100 | 0;96 |
| <i>4DY-3071</i> | 14;50 | 14;26 | 0;97 | 30;64 | 0;96 | 13;30 | 21;47 | 18;24 |
| <i>3AY-3216</i> | 31;62 | 22;59 | 0;100 | 21;36 | 0;106 | 26;68 | 16;45 | 23;54 |
| <i>4DY-3291</i> | 11;51 | 18;34 | 0;85 | 22;42 | 0;97 | 32;67 | 22;36 | 34;58 |
| <i>4EB-3455</i> | 17;35 | 22;41 | 22;38 | 30;79 | 23;50 | 32;69 | 19;39 | 29;47 |
| <i>3BC-3629</i> | 10;21 | 28;30 | 19;39 | 1;5 | 2;12 | 40;70 | 24;51 | 2;17 |
| <i>3BC-3677</i> | 35;64 | 6;13 | 28;42 | 22;33 | 23;46 | 24;66 | 19;46 | 4;30 |
| <i>4EC-3742</i> | 17;46 | 12;42 | 35;51 | 17;28 | 32;48 | 11;54 | 26;58 | 25;47 |
| <i>4EC-3781</i> | 21;40 | 18;50 | 41;57 | 18;52 | 0;70 | 24;57 | 16;43 | 29;45 |
| <i>4EC-3906</i> | 5;17 | 19;42 | 0;106 | 32;50 | 0;98 | 23;98 | 30;59 | 2;36 |
| <i>3BE-4178</i> | 50;68 | 17;46 | 0;119 | 19;42 | 0;85 | 21;47 | 17;35 | 18;46 |
| <i>3BF-4311</i> | 30;41 | 19;27 | 17;47 | 20;30 | 21;30 | 17;34 | 28;55 | 22;58 |
| <i>3BE-4331</i> | 32;34 | 42;75 | 14;29 | 22;41 | 22;40 | 2;5 | 18;31 | 22;40 |
| <i>4EF-4391</i> | 40;61 | 32;66 | 15;31 | 12;33 | 6;13 | 17;37 | 34;58 | 16;32 |
| <i>3BE-4401</i> | 21;60 | 21;39 | 21;27 | 20;34 | 40;59 | 23;46 | 37;83 | 17;32 |
| <i>4EC-4540</i> | 16;51 | 18;72 | 27;65 | 26;55 | 21;20 | 2;8 | 32;40 | 21;34 |
| <i>4EH-4705</i> | 40;71 | 20;30 | 0;103 | 0;90 | 0;105 | 40;68 | 17;49 | 17;48 |
| <i>4EK-4975</i> | 39;63 | 13;29 | 0;94 | 0;65 | 0;100 | 18;37 | 18;30 | 24;48 |
| <i>4EK-5076</i> | 5;18 | 42;61 | 0;105 | 0;48 | 0;90 | 22;41 | 22;59 | 17;30 |
| <i>3BK-5333</i> | 30;59 | 0;85 | 21;35 | 28;46 | 21;23 | 0;86 | 17;23 | 0;97 |
| <i>3BK-5476</i> | 34;50 | 19;58 | 17;46 | 13;29 | 17;34 | 18;35 | 22;35 | 17;43 |
| <i>3BK-5587</i> | 21;38 | 20;48 | 31;50 | 27;30 | 35;55 | 22;35 | 14;22 | 26;43 |
| <i>3BL-5823</i> | 40;46 | 18;34 | 0;98 | 30;47 | 16;32 | 13;45 | 0;98 | 34;60 |
| <i>3BN-6246</i> | 20;23 | 17;37 | 19;43 | 21;45 | 17;46 | 34;67 | 30;56 | 23;53 |
| <i>4EP-6332</i> | 40;58 | 15;47 | 0;97 | 0;69 | 0;95 | 21;25 | 28;65 | 18;31 |
| <i>4EQ-6548</i> | 14;28 | 0;102 | 21;40 | 30;60 | 23;39 | 0;108 | 29;55 | 0;95 |

| | | | | | | | | |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|
| 3BP-6666 | 35;59 | 38;61 | 0;107 | 12;30 | 32;39 | 24;30 | 0;78 | 8;22 |
| 4ER-6893 | 21;38 | 21;25 | 0;96 | 0;78 | 0;104 | 30;40 | 21;28 | 23;50 |

| | 4DI-1367 | 4DG-1524 | 4DL-1801 | 3AL-1872 | 4DI-1893 | 4DL-1980 | 4DN-2014 | 4DS-2678 |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 4DA-59 | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| 4DA-122 | 0;95 | 18;32 | 29;57 | 19;40 | 10;17 | 22;49 | 21;45 | 0;68 |
| 3AC-184 | 22;48 | 17;48 | 27;65 | 39;70 | 0;90 | 12;30 | 22;37 | 0;90 |
| 4DD-201 | 12;33 | 20;46 | 27;61 | x | 17;38 | 16;50 | 35;69 | 0;70 |
| 4DD-288 | 20;47 | 18;53 | 13;46 | 17;20 | 18;40 | x | x | 0;98 |
| 4DD-418 | 0;67 | 17;45 | 10;30 | 27;69 | 23;58 | 12;47 | 6;16 | 0;94 |
| 4DG-777 | 16;45 | 27;56 | 22;60 | 42;79 | 0;100 | 10;31 | x | 0;55 |
| 4DH-1251 | 0;87 | 31;56 | 7;18 | 18;31 | 19;39 | 11;41 | 26;74 | 0;77 |
| 4DI-1367 | 32;68 | 18;61 | 121;35 | 23;59 | x | x | 26;46 | 0;67 |
| 4DG-1524 | 40;89 | 15;38 | 13;96 | 0;83 | 12;32 | 19;38 | x | 12;40 |
| 4DL-1801 | 40;74 | 38;60 | 22;71 | x | 0;106 | 0;119 | 0;97 | 0;78 |
| 3AL-1872 | 26;59 | 0;98 | 20;48 | 30;55 | x | x | 3;16 | 13;40 |
| 4DI-1893 | 34;57 | 12;50 | 0;100 | 17;40 | 0;100 | 1;12 | 0;103 | 0;105 |
| 4DL-1980 | 23;45 | 16;59 | 0;104 | 19;49 | 0;94 | 20;41 | x | 1;103 |
| 4DN-2014 | 34;59 | 5;15 | 0;96 | 11;22 | 0;92 | 0;109 | 0;101 | x |
| 4DS-2678 | 0;95 | 2;39 | 0;119 | x | 0;97 | 0;100 | 0;92 | 0;90 |
| 4DS-2705 | 25;47 | 29;50 | 17;35 | 10;13 | 0;97 | 31;50 | 18;20 | 0;105 |
| 4DS-2715 | 0;76 | 20;48 | x | x | x | x | x | x |
| 4DS-2716 | 0;87 | 18;67 | 0;90 | 19;39 | 0;75 | 0;97 | 0;89 | 0;99 |
| 3AU-2858 | 45;68 | 32;79 | 1;8 | 8;26 | 19;30 | 13;29 | 18;34 | 0;86 |
| 3AZ-3013 | 0;78 | 30;57 | 4;9 | 13;45 | 30;41 | 18;33 | 32;40 | 0;69 |
| 4DY-3071 | 17;46 | 32;61 | 36;70 | 11;58 | 39;68 | 17;26 | x | 0;93 |
| 3AY-3216 | 30;62 | 13;35 | 13;40 | 10;30 | 21;30 | 28;38 | 37;80 | 0;58 |
| 4DY-3291 | 18;45 | 8;40 | 20;48 | 11;37 | 23;40 | 11;51 | 25;63 | 0;95 |
| 4EB-3455 | 22;43 | 18;65 | 29;68 | 16;34 | 31;55 | 15;23 | 27;51 | 0;77 |
| 3BC-3629 | 19;56 | 32;48 | 0;115 | 25;50 | 0;72 | 0;106 | 0;98 | 0;58 |
| 3BC-3677 | 17;35 | 1;6 | 0;96 | 2;19 | 0;92 | x | 1;18 | 0;90 |
| 4EC-3742 | 8;30 | 37;50 | 0;95 | 27;64 | 0;102 | 0;57 | 0;90 | 0;105 |
| 4EC-3781 | 17;23 | 3;7 | x | 17;48 | 21;55 | 10;48 | 9;19 | 0;88 |
| 4EC-3906 | 12;40 | 27;47 | x | 19;39 | x | 12;47 | x | 0;85 |
| 3BE-4178 | 5;11 | 22;46 | 12;34 | 13;47 | 22;32 | x | 7;28 | x |
| 3BF-4311 | 20;45 | 20;39 | 0;57 | 11;27 | 0;101 | 0;94 | x | 0;27 |
| 3BE-4331 | 22;34 | 36;70 | 0;100 | x | 0;87 | 0;76 | x | 0;39 |
| 4EF-4391 | 12;40 | 18;31 | 30;68 | 22;37 | 21;50 | 23;47 | 5;30 | 0;90 |
| 3BE-4401 | 28;43 | 23;40 | 17;41 | 30;48 | 50;107 | 40;87 | 16;25 | x |
| 4EC-4540 | 35;55 | 17;37 | 36;52 | 16;38 | 22;41 | x | 14;23 | 0;99 |
| 4EH-4705 | 17;46 | 19;42 | 17;47 | 12;37 | 23;41 | 19;37 | x | 0;77 |
| 4EK-4975 | 34;46 | 18;68 | 23;54 | 13;67 | 20;39 | 30;69 | x | 0;69 |
| 4EK-5076 | 12;43 | 28;61 | 36;60 | 13;17 | 14;26 | x | x | 0;105 |
| 3BK-5333 | 0;89 | 25;80 | 17;48 | 10;21 | x | 30;68 | 23;53 | 0;96 |
| 3BK-5476 | 20;45 | 32;66 | 0;68 | 2;7 | 0;100 | 0;95 | 0;100 | 0;39 |
| 3BK-5587 | 23;34 | 31;69 | 0;96 | 9;17 | x | x | 0;94 | 0;68 |

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|----------|-------|-------|-------|-------|-------|-------|-------|-------|
| 3BL-5823 | 17;68 | 18;58 | x | 20;47 | 37;83 | 30;47 | 26;57 | x |
| 3BN-6246 | 12;45 | 13;47 | 0;99 | 23;54 | 0;104 | 0;93 | 0;95 | 0;109 |
| 4EP-6322 | 38;65 | 12;48 | 20;46 | 22;69 | 10;29 | x | 16;52 | 0;94 |
| 4EQ-6548 | 0;98 | 15;37 | 21;47 | 1;4 | x | 30;60 | 22;43 | x |
| 3BP-6666 | 6;15 | 42;74 | x | 10;31 | 30;75 | 12;40 | x | 0;56 |
| 4ER-6893 | 35;56 | 30;54 | x | 5;10 | x | 10;48 | 21;38 | x |

| | 4DS-2705 | 4DS-2715 | 4DS-2716 | 3AU-2858 | 3AZ-3013 | 4DY-3071 | 3AY-3216 | 4DY-3291 |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 4DA-59 | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| 4DA-122 | 21;50 | 0;96 | 0;94 | 7;16 | 0;68 | 9;17 | 19;35 | 6;16 |
| 3AC-184 | 0;72 | 0;103 | 0;89 | 0;97 | 15;32 | 0;105 | 0;95 | 0;66 |
| 4DD-201 | x | 0;84 | 0;106 | 0;107 | 18;40 | 0;118 | x | 0;99 |
| 4DD-288 | x | 0;72 | 0;94 | 0;99 | 31;98 | 0;110 | x | 0;106 |
| 4DD-418 | x | x | 0;96 | 7;40 | 0;90 | 12;30 | 20;44 | 22;40 |
| 4DG-777 | 0;114 | 0;89 | 0;103 | 6;30 | 37;80 | 12;37 | 23;53 | 19;44 |
| 4DH-1251 | 10;18 | 0;91 | 0;95 | x | x | 3;12 | 8;38 | 12;38 |
| 4DI-1367 | x | 0;82 | 0;120 | 19;40 | 13;26 | 16;46 | 9;20 | 50;68 |
| 4DG-1524 | 5;15 | 20;67 | 15;31 | 31;70 | 4;19 | 26;50 | x | 37;82 |
| 4DL-1801 | 7;19 | 0;98 | 0;113 | 40;86 | 13;62 | x | 12;34 | 40;86 |
| 3AL-1872 | 18;32 | 9;19 | 13;40 | 21;59 | 26;78 | 40;69 | 18;29 | x |
| 4DI-1893 | 0;105 | 0;92 | 0;95 | 34;50 | x | 2;4 | 21;46 | 17;34 |
| 4DL-1980 | x | 0;107 | 0;102 | 17;66 | 11;37 | 23;49 | 38;69 | x |
| 4DN-2014 | 22;55 | 0;98 | 0;119 | 23;58 | 13;47 | 15;28 | 20;37 | 10;40 |
| 4DS-2678 | 0;51 | 0;95 | 0;113 | 0;80 | 4;40 | 0;90 | x | 0;90 |
| 4DS-2705 | 26;60 | 0;58 | 0;73 | 12;50 | 20;36 | 13;40 | 19;44 | 30;66 |
| 4DS-2715 | 0;76 | 0;72 | 0;101 | x | x | x | x | x |
| 4DS-2716 | 0;87 | 0;97 | 0;83 | 0;98 | 0;79 | 0;89 | 0;98 | 0;78 |
| 3AU-2858 | 15;40 | 0;108 | 0;74 | 0;109 | 13;17 | 0;104 | 20;44 | 0;94 |
| 3AZ-3013 | 14;55 | 0;59 | 0;89 | x | 0;52 | 14;28 | 30;70 | 23;54 |
| 4DY-3071 | 7;13 | 0;78 | 0;66 | 0;69 | 10;32 | 0;100 | 21;48 | 10;35 |
| 3AY-3216 | 8;17 | 0;87 | 0;88 | 0;98 | x | 0;190 | 0;82 | 0;95 |
| 4DY-3291 | 9;17 | 0;112 | 0;75 | 0;67 | 15;36 | 0;72 | 19;31 | 0;83 |
| 4EB-3455 | 15;51 | x | 0;112 | 30;58 | 5;10 | 23;40 | 18;54 | 12;34 |
| 3BC-3629 | 22;55 | x | 0;103 | x | 4;26 | 15;34 | 21;40 | 50;107 |
| 3BC-3677 | 21;50 | x | 0;67 | 18;38 | 13;42 | 9;19 | x | 19;43 |
| 4EC-3742 | 22;55 | x | 0;85 | 12;33 | x | 23;41 | 17;20 | 21;48 |
| 4EC-3781 | 28;31 | x | 0;114 | 0;90 | 15;37 | 0;109 | 23;55 | 0;90 |
| 4EC-3906 | 28;37 | x | 0;58 | 0;50 | 31;60 | x | 18;31 | 0;67 |
| 3BE-4178 | 21;41 | x | 0;98 | 0;99 | x | 0;90 | 0;114 | 22;52 |
| 3BF-4311 | 35;70 | x | 0;85 | 23;45 | 15;36 | 17;38 | 30;65 | 21;37 |
| 3BE-4331 | 22;41 | x | 0;104 | 42;71 | 20;45 | 18;38 | 2;16 | 21;38 |
| 4EF-4391 | 17;31 | x | 0;121 | x | 8;16 | x | x | 23;66 |
| 3BE-4401 | x | x | 0;106 | 6;23 | 15;37 | 10;47 | 8;10 | x |
| 4EC-4540 | 22;38 | 0;98 | 0;90 | 45;67 | 8;16 | x | 22;72 | 8;17 |
| 4EH-4705 | 22;35 | 0;92 | 0;95 | x | 11;44 | 0;106 | 0;129 | 0;98 |
| 4EK-4975 | 21;55 | x | 0;105 | 0;97 | 19;44 | 0;90 | 23;41 | 0;78 |
| 4EK-5076 | 9;17 | 0;84 | 0;40 | 0;123 | x | 0;107 | 0;102 | x |
| 3BK-5333 | 13;28 | x | 0;96 | 4;30 | 0;120 | 30;69 | 19;42 | 16;39 |
| 3BK-5476 | 27;40 | x | 0;77 | x | 26;40 | 40;89 | 33;67 | 19;25 |

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|----------|-------|------|-------|-------|-------|-------|-------|-------|
| 3BK-5587 | 52;68 | x | 0;85 | 14;38 | 19;44 | 14;48 | 16;51 | 28;39 |
| 3BL-5523 | 0;106 | x | 0;100 | x | 15;35 | 24;33 | 17;47 | 19;46 |
| 3BN-6246 | 40;86 | x | 0;100 | 12;34 | 15;60 | 21;57 | 27;42 | 8;15 |
| 4EP-6322 | 23;57 | 0;94 | 0;105 | 0;85 | 37;79 | 0;105 | 0;99 | 0;107 |
| 4EQ-6548 | 34;85 | x | 0;38 | 22;42 | 0;119 | 27;62 | x | 20;45 |
| 3BP-6666 | 0;125 | x | 0;90 | 25;68 | x | 21;39 | 20;36 | x |
| 4ER-6893 | x | x | 0;108 | 0;66 | 24;68 | 1;70 | x | 0;103 |

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|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| | 4EB-3455 | 3BC-3629 | 3BC-3677 | 4EC-3742 | 4EC-3781 | 4EC-3906 | 3BE-4178 | 3BF-4311 |
| 4DA-59 | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| 4DA-122 | 10;32 | 21;30 | 21;39 | 41;62 | 12;34 | 23;46 | 20;56 | 12;30 |
| 3AC-184 | 19;30 | 30;68 | 30;69 | 30;43 | 0;92 | 0;95 | 0;50 | 22;43 |
| 4DD-201 | 42;50 | x | 6;18 | 21;46 | 0;95 | 19;32 | 0;78 | 21;50 |
| 4DD-288 | 32;50 | 10;40 | 40;92 | 19;44 | 0;105 | 0;78 | 0;78 | 5;34 |
| 4DD-418 | 15;37 | 12;40 | 3;20 | 20;44 | 18;36 | 23;46 | 26;45 | 4;27 |
| 4DG-777 | 8;16 | 20;38 | 15;38 | x | 5;20 | 24;47 | 34;57 | 9;28 |
| 4DH-1251 | 11;23 | 40;85 | 30;56 | 15;35 | 21;48 | x | 28;45 | 2;5 |
| 4DI-1367 | 27;40 | 7;20 | 20;49 | 13;38 | 15;28 | x | x | 29;47 |
| 4DG-1524 | 33;68 | x | x | 12;24 | 17;38 | 40;89 | 9;27 | 18;23 |
| 4DL-1801 | 38;50 | 0;72 | 0;91 | 0;93 | x | 18;52 | 16;28 | 0;94 |
| 3AL-1872 | x | 31;70 | 9;31 | 35;70 | 19;45 | 22;57 | 15;46 | 14;60 |
| 4DI-1893 | 17;34 | 0;90 | x | 0;92 | 8;17 | 19;42 | 0;103 | 0;77 |
| 4DL-1980 | 21;40 | 0;60 | x | 0;90 | 10;41 | 22;41 | 21;55 | 0;58 |
| 4DN-2014 | 15;35 | x | 0;105 | x | x | 12;33 | 9;29 | 0;96 |
| 4DS-2678 | 0;59 | 0;96 | 0;95 | 0;105 | 0;116 | 0;35 | 0;49 | 0;106 |
| 4DS-2705 | 15;27 | 21;50 | 9;30 | 10;28 | 19;35 | x | 3;16 | x |
| 4DS-2715 | x | x | x | x | x | x | x | x |
| 4DS-2716 | 0;97 | 0;86 | 0;77 | 0;67 | 0;89 | 0;56 | 0;30 | 0;95 |
| 3AU-2858 | 13;45 | 21;30 | 3;16 | 7;14 | x | 0;90 | 0;48 | 13;36 |
| 3AZ-3013 | 17;40 | 34;89 | 37;50 | 8;16 | 19;30 | x | | 19;25 |
| 4DY-3071 | 8;17 | 8;17 | x | 27;36 | 0;90 | 0;95 | 0;69 | 22;41 |
| 3AY-3216 | x | 34;69 | x | 15;36 | 0;76 | 0;67 | 0;100 | x |
| 4DY-3291 | 23;63 | x | 19;35 | 20;45 | 19;37 | 0;96 | 21;30 | 15;46 |
| 4EB-3455 | 0;71 | 0;72 | 23;66 | 0;72 | x | 1;125 | 19;30 | x |
| 3BC-3629 | x | 0;112 | 0;114 | 0;82 | 27;60 | 39;67 | 30;61 | 0;97 |
| 3BC-3677 | 0;90 | 0;90 | 0;48 | 0;95 | 8;17 | 0;103 | 15;28 | 0;95 |
| 4EC-3742 | 15;36 | 0;50 | 0;108 | 0;75 | 5;18 | 40;86 | 19;32 | 0;79 |
| 4EC-3781 | 18;38 | 53;105 | 3;16 | x | 15;41 | 0;90 | x | 10;17 |
| 4EC-3906 | 2;98 | 4;18 | 23;47 | 13;40 | 0;79 | 0;57 | 0;85 | 23;41 |
| 3BE-4178 | 11;51 | 14;30 | 35;49 | 15;47 | 0;90 | 0;78 | 0;82 | x |
| 3BF-4311 | 2;9 | 0;105 | 0;67 | 0;106 | 23;55 | 13;45 | 23;43 | 0;58 |
| 3BE-4331 | 22;52 | 0;78 | x | 0;47 | x | x | 5;36 | 0;68 |
| 4EF-4391 | 0;90 | 21;30 | 30;45 | 30;65 | x | 0;90 | 47;82 | 9;18 |
| 4EC-4540 | 0;79 | 38;60 | 31;55 | 40;87 | x | 1;79 | 17;25 | 17;42 |
| 3BE-4401 | 0;108 | x | 21;47 | x | x | 0;79 | 32;52 | 22;31 |
| 4EH-4705 | 18;42 | 40;89 | x | 15;37 | 0;67 | 0;90 | 0;94 | x |
| 4EK-4975 | 13;32 | 21;46 | 23;51 | 2;6 | 0;100 | 0;78 | 0;92 | x |
| 4EK-5076 | x | 39;71 | x | 8;17 | 0;89 | 0;73 | 0;66 | 20;32 |
| 3BK-5333 | 30;41 | 30;75 | 24;56 | 5;11 | 8;19 | 13;45 | 5;16 | x |

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|-----------------|-------|--------------|--------------|--------------|-------------|-------------|-------------|-------------|
| 3BK-5476 | 16;48 | x | 0;106 | 0;105 | x | x | 19;35 | 0;60 |
| 3BK-5587 | 15;32 | 0;90 | 0;89 | 0;95 | 14;29 | 36;73 | x | 0;96 |
| 3BL-5823 | 31;62 | 10;70 | x | 6;17 | x | 28;57 | 19;38 | 37;80 |
| 3BN-6246 | x | 0;109 | 0;105 | 0;113 | 15;36 | x | 30;56 | 0;95 |
| 4EP-6322 | 15;46 | 17;37 | 15;89 | 16;32 | 0;90 | 0;96 | 0;75 | 2;15 |
| 4EQ-6548 | 15;39 | 17;31 | 31;67 | 5;15 | 14;64 | 34;57 | x | 30;41 |
| 3BP-6876 | x | x | 20;46 | 23;54 | 10;19 | 30;67 | 40;87 | 20;41 |
| 4ER-6893 | 21;55 | 9;37 | 14;60 | 18;35 | 0;60 | 0;69 | 0;95 | 2;9 |

| | 3BE-4331 | 4EF-4391 | 3BE-4401 | 4EC-4540 | 4EH-4705 | 4EK-4975 | 4EK-5076 | 3BK-5333 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 4DA-59 | na/ | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| 4DA-122 | 8;16 | 23;56 | 13;50 | 11;24 | 30;66 | 21;44 | 21;45 | 0;70 |
| 3AC-184 | 11;44 | 13;62 | 18;37 | 8;20 | 0;105 | 0;100 | 0;100 | 10;21 |
| 4DD-201 | 10;25 | x | 10;13 | 9;21 | 0;100 | 0;100 | 0;109 | 2;6 |
| 4DD-288 | 15;28 | 2;27 | x | 4;12 | x | 0;94 | 0;100 | 13;17 |
| 4DD-418 | 23;66 | 1;6 | 22;43 | x | x | 30;53 | 9;18 | 0;108 |
| 4DG-777 | x | 37;58 | 18;46 | 19;39 | 14;65 | 30;41 | 23;45 | 14;25 |
| 4DH-1251 | 23;51 | 2;10 | 18;46 | 17;21 | 17;30 | 30;72 | 18;45 | 0;68 |
| 4DI-1367 | 16;51 | 50;78 | x | 27;58 | 18;51 | 17;48 | 31;40 | 0;129 |
| 4DG-1524 | 17;36 | 36;60 | 17;34 | 40;62 | 22;43 | 17;58 | 12;32 | 19;37 |
| 4DL-1801 | 0;86 | 5;13 | 20;45 | 4;7 | 1;10 | 36;49 | 12;36 | 2;14 |
| 3AL-1872 | x | 1;4 | 22;41 | 22;42 | 14;29 | x | 4;18 | x |
| 4DI-1893 | 0;97 | 2;9 | x | 5;16 | 5;10 | 20;36 | 10;13 | x |
| 4DL-1980 | 0;100 | x | x | 34;57 | 34;66 | 4;17 | 7;36 | 3;7 |
| 4DN-2014 | 0;100 | 10;17 | x | x | 30;41 | 21;54 | x | 10;31 |
| 4DS-2678 | 0;77 | 0;78 | 0;104 | 0;75 | 0;107 | 0;100 | 0;100 | 0;59 |
| 4DS-2705 | 19;28 | 9;16 | 23;46 | 6;18 | 21;53 | 21;40 | 7;42 | 18;37 |
| 4DS-2715 | x | 0;78 | 0;105 | x | x | 0;105 | x | x |
| 4DS-2716 | 0;103 | 0;68 | 0;100 | 0;68 | 0;77 | 0;47 | 0;105 | 0;112 |
| 3AU-2858 | 13;47 | 3;7 | 20;40 | 6;15 | 0;100 | 0;102 | 0;100 | 9;17 |
| 3AZ-3013 | 17;48 | 7;20 | 8;17 | 3;27 | x | 32;40 | x | 0;100 |
| 4DY-3071 | 30;41 | 13;46 | 30;42 | 10;36 | 0;100 | 0;100 | 3;11 | 23;45 |
| 3AY-3216 | 16;28 | 4;8 | 12;38 | 5;9 | 0;101 | 0;100 | 7;42 | 13;20 |
| 4DY-3291 | 17;29 | 27;34 | 30;65 | x | 0;105 | 0;104 | 5;27 | 4;8 |
| 4EB-3455 | x | 0;109 | 0;100 | 0;103 | 16;47 | 43;80 | 10;35 | x |
| 3BC-3629 | 0;100 | 2;4 | 20;47 | 30;65 | x | 22;58 | 18;34 | 6;16 |
| 3BC-3677 | 0;102 | 0;97 | 0;102 | 0;104 | 7;36 | 19;36 | 18;37 | 21;38 |
| 4EC-3742 | 0;109 | 31;60 | x | 6;19 | 21;28 | 31;60 | 20;44 | 6;10 |
| 4EC-3781 | x | 17;51 | x | 17;35 | 0;100 | 0;105 | 0;101 | 7;18 |
| 4EC-3906 | 22;45 | 0;98 | 0;102 | 2;96 | 0;100 | 0;100 | x | 3;11 |
| 3BE-4178 | 37;57 | 30;47 | 25;31 | 10;18 | 0;100 | 0;100 | 0;100 | 12;39 |
| 3BF-4311 | 0;100 | 15;50 | 3;15 | x | 41;57 | x | x | x |
| 3BE-4331 | 0;100 | 7;28 | 20;41 | 12;25 | 17;60 | 12;35 | 5;28 | 5;28 |
| 4EF-4391 | x | 0;104 | 0;104 | 0;100 | 8;16 | 11;44 | 10;22 | 11;26 |
| 3BE-4401 | x | 0;100 | 0;100 | 0;100 | 15;36 | 21;46 | x | 13;19 |
| 4EC-4540 | 15;34 | 0;120 | 0;100 | 0;79 | x | 11;58 | 10;18 | x |
| 4EH-4705 | x | x | 22;32 | 7;15 | 18;43 | 0;82 | 21;38 | x |
| 4EK-4975 | 6;17 | 6;15 | 25;61 | 32;67 | 0;105 | 0;120 | 13;30 | 6;15 |
| 4EK-5076 | 18;38 | 6;32 | 22;46 | x | 0;88 | 0;100 | 0;79 | 5;27 |

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|----------|-------|-------|-------|-------|-------|-------|-------|-------|
| 3BK-5333 | x | 7;22 | 17;23 | 31;56 | x | 16;38 | 8;26 | 0;98 |
| 3BK-5476 | 0;100 | 9;21 | 16;53 | 21;55 | x | 4;11 | 13;57 | 18;45 |
| 3BK-5587 | 0;100 | 8;26 | 30;41 | 28;42 | 23;55 | 12;27 | 15;46 | x |
| 3BL-5823 | 20;32 | 12;36 | 48;85 | 27;62 | 21;34 | 8;23 | x | 21;38 |
| 3BN-6246 | 0;100 | 10;13 | 28;41 | x | 21;35 | x | 6;15 | 18;34 |
| 4EP-6322 | 16;51 | 15;48 | x | x | 0;100 | 0;105 | 0;90 | 20;44 |
| 4EQ-6548 | 42;75 | 10;18 | x | 12;33 | x | 28;31 | 7;24 | 0;104 |
| 3BP-6666 | x | 22;40 | 18;51 | 23;56 | 22;36 | 7;20 | x | 8;21 |
| 4ER-6893 | 2;7 | 30;42 | 17;20 | x | 0;100 | x | 0;78 | 10;13 |

| | 3BK-5476 | 3BK-5587 | 3BL-5823 | 3BN-6246 | 4EP-6322 | 4EQ-6548 | 3BP-6666 | 4ER-6893 |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 4DA-59 | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| 4DA-122 | 12;50 | 23;65 | 23;45 | 19;35 | 20;37 | 0;100 | 23;54 | 23;50 |
| 3AC-184 | 15;37 | 45;70 | 0;69 | 8;15 | 0;70 | 23;36 | 0;100 | 0;103 |
| 4DD-201 | 2;14 | x | 23;45 | 15;31 | 0;121 | 34;58 | 3;6 | 0;90 |
| 4DD-288 | 14;27 | 21;45 | 3;7 | 17;34 | 0;85 | 7;18 | 22;47 | 0;100 |
| 4DD-418 | 9;18 | 11;25 | 23;48 | 21;46 | 30;34 | 0;101 | 7;20 | 6;13 |
| 4DG-777 | 20;43 | 15;46 | 0;106 | 15;38 | 15;31 | 9;17 | 0;98 | 4;17 |
| 4DH-1251 | 16;25 | 7;37 | 10;18 | x | 30;43 | 0;109 | 13;50 | 28;49 |
| 4DI-1367 | 9;57 | 10;31 | 15;37 | 18;54 | 14;28 | 0;90 | x | 17;62 |
| 4DG-1524 | 10;18 | x | 4;19 | 20;45 | 1;5 | 15;23 | 12;31 | 4;12 |
| 4DL-1801 | 0;96 | 0;95 | 5;16 | 0;84 | 30;79 | 14;37 | x | 9;16 |
| 3AL-1872 | 12;45 | 34;50 | x | 22;45 | 22;46 | 13;30 | 5;17 | x |
| 4DI-1893 | 0;108 | 0;94 | 0;56 | 0;98 | 15;23 | x | 0;94 | 18;34 |
| 4DL-1980 | 0;109 | x | 10;31 | 0;58 | 16;27 | 5;17 | 30;41 | x |
| 4DN-2014 | 0;100 | 0;98 | x | 0;97 | 17;20 | 19;39 | x | 22;42 |
| 4DS-2678 | 0;99 | 0;108 | 0;99 | 0;100 | 0;105 | 0;75 | 0;90 | 0;109 |
| 4DS-2705 | 30;58 | 13;20 | 0;95 | 17;35 | 12;25 | 9;16 | 0;96 | 17;28 |
| 4DS-2715 | x | x | x | x | x | x | x | x |
| 4DS-2716 | 0;58 | 0;89 | 0;65 | 0;79 | 0;47 | 0;105 | 0;104 | 0;60 |
| 3AU-2858 | 18;51 | 20;36 | 7;28 | x | 0;95 | 18;54 | 20;41 | 0;100 |
| 3AZ-3013 | x | 10;31 | 11;24 | 23;57 | 21;48 | 0;96 | 30;41 | 21;55 |
| 4DY-3071 | 9;38 | x | 13;62 | 14;62 | 0;81 | 18;53 | 5;16 | 0;100 |
| 3AY-3216 | 15;54 | 3;13 | 10;17 | 30;41 | 0;126 | 31;63 | x | 0;100 |
| 4DY-3291 | 3;10 | x | x | x | x | 7;25 | 22;48 | 0;102 |
| 4EB-3455 | x | 13;62 | x | 19;39 | 2;11 | 4;7 | 1;3 | 15;35 |
| 3BC-3629 | 0;100 | 0;68 | x | 0;67 | 9;18 | 17;31 | x | 18;38 |
| 3BC-3677 | 0;103 | 0;96 | 1;4 | 0;69 | 4;17 | 24;63 | x | x |
| 4EC-3742 | 0;100 | 0;48 | 20;36 | x | 20;45 | 22;59 | x | 15;29 |
| 4EC-3781 | 12;59 | 19;46 | 10;31 | 19;35 | 0;72 | 11;52 | 5;17 | 0;100 |
| 4EC-3906 | 2;5 | 8;16 | x | 6;17 | 0;87 | 28;40 | 40;61 | 10;42 |
| 3BE-4178 | 0;90 | 17;35 | 14;23 | 16;37 | x | 13;46 | 30;42 | 0;100 |
| 3BF-4311 | 0;89 | 0;96 | 7;20 | 0;58 | 30;45 | x | 8;19 | 23;66 |
| 3BE-4331 | 0;97 | 0;82 | 21;45 | 0;97 | 32;47 | x | 32;69 | 11;45 |
| 4EF-4391 | x | 10;18 | x | 19;36 | 18;61 | 16;27 | 17;34 | x |
| 3BE-4401 | x | 19;37 | 15;37 | 10;27 | 17;21 | 1;5 | 16;51 | 12;57 |
| 4EC-4540 | x | 16;47 | 31;62 | x | 16;51 | 30;75 | 2;37 | 11;44 |
| 4EH-4705 | 5;9 | 7;36 | 23;45 | x | 0;65 | 7;9 | 19;41 | 0;102 |

| | | | | | | | | |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|
| 4EK-4975 | | 20;36 | 2;13 | 22;33 | 0;98 | 20;47 | 16;28 | 0;105 |
| 4EK-5076 | 13;20 | 3;11 | x | 24;57 | 0;72 | 9;29 | 16;57 | 0;100 |
| 3BK-5333 | x | 3;11 | 13;25 | 17;35 | 13;67 | 0;92 | x | 15;39 |
| 3BK-5476 | 0;105 | 0;93 | x | 0;72 | x | 31;62 | 17;20 | 21;38 |
| 3BK-5587 | 0;98 | 0;95 | 8;26 | 0;81 | x | 12;41 | 23;41 | x |
| 3BL-5823 | 7;42 | 18;34 | 0;90 | x | x | x | 0;95 | 32;66 |
| 3BN-6246 | 0;68 | 32;65 | 12;36 | 0;104 | 16;51 | x | 22;41 | x |
| 4EP-6322 | 11;48 | 13;26 | 6;15 | 18;23 | 0;69 | 30;41 | 8;15 | 0;100 |
| 4EQ-6548 | x | x | 7;28 | 14;55 | x | 0;79 | 17;58 | 15;28 |
| 3BP-6666 | 17;52 | x | 0;95 | 21;58 | 23;56 | 13;31 | 0;100 | x |
| 4ER-6893 | x | x | 2;6 | x | 0;58 | 27;36 | x | 0;100 |

E) Raw data of progeny numbers in determining earliest lethal phase.

Mutant alleles were crossed to Df(2L)ast4. 25% of the progeny are homozygous for the balancer and be embryonic lethal.

| <u>Group number</u> | <u>Mutant allele</u> | <u>Dish number</u> | <u>Number of embryos</u> | <u>Number of larvae</u> | <u>Number of adults</u> | <u>Earliest lethal phase</u> |
|---------------------|----------------------|--------------------|--------------------------|-------------------------|-------------------------|------------------------------|
| 1 | 4DA-122 | 1 | 100 | 76 | 51 | Larval |
| | | 2 | 100 | 75 | 50 | |
| | | 3 | 100 | 74 | 51 | |
| 1 | 3BK-5333 | 1 | 100 | 70 | 50 | Larval |
| | | 2 | 100 | 75 | 48 | |
| | | 3 | 100 | 72 | 52 | |
| 2 | 3BL-6666 | 1 | 100 | 52 | 52 | Embryonic |
| | | 2 | 100 | 50 | 50 | |
| | | 3 | 100 | 51 | 51 | |
| 2 | 3BL-5823 | 1 | 100 | 50 | 49 | Embryonic |
| | | 2 | 100 | 52 | 51 | |
| | | 3 | 100 | 51 | 51 | |
| 3 | 4DG-1524 | 1 | 100 | 76 | 51 | Larval |
| | | 2 | 100 | 65 | 45 | |
| | | 3 | 100 | 74 | 50 | |
| 3 | 3AL-1872 | 1 | 103 | 52 | 52 | Embryonic |
| | | 2 | 88 | 50 | 50 | |
| | | 3 | 101 | 48 | 48 | |
| 4 | 3AY-3126 | 1 | 100 | 51 | 51 | Embryonic |
| | | 2 | 100 | 55 | 55 | |
| | | 3 | 100 | 60 | 58 | |
| 4 | 4EP-6322 | 1 | 100 | 51 | 50 | Embryonic |
| | | 2 | 115 | 62 | 62 | |
| | | 3 | 100 | 50 | 50 | |
| 5 | 3BK-5588 | 1 | 100 | 77 | 51 | Larval |
| | | 2 | 100 | 75 | 50 | |
| | | 3 | 100 | 72 | 47 | |
| 5 | 3BK-5476 | 1 | 100 | 76 | 50 | Larval |
| | | 2 | 100 | 75 | 53 | |
| | | 3 | 100 | 72 | 48 | |
| 6 | 4EF-4391 | 1 | 100 | 50 | 50 | Embryonic |
| | | 2 | 100 | 51 | 50 | |
| | | 3 | 100 | 50 | 49 | |
| 6 | 4EC-4540 | 1 | 100 | 87 | 87 | Larval |
| | | 2 | 100 | 79 | 79 | |
| | | 3 | 100 | 86 | 83 | |

F) Rescue experiment (1):**Table: A.**

| Group Number | Allele | Progeny (Cy⁺;Cy) |
|---------------------|---------------|--|
| One | 4DA-122 | 0; 203 |
| | 3BK-5333 | 0; 260 |
| Two | 3BL-6666 | 0; 281 |
| | 3BL-5823 | 0; 301 |
| Three | 4DG-1524 | / |
| | 3AL-1872 | / |
| Four | 3AY-3216 | 0; 140 |
| | 4EP-6322 | 0; 193 |
| Five | 3BK-5588 | 0; 250 |
| | 3BK-5476 | 0; 284 |
| Six | 4EF-4391 | 0; 310 |
| | 4EC-4540 | 0; 195 |

Table:B.

| Group Number | Allele | Progeny Cy⁺;Cy |
|---------------------|-------------------|--------------------------------------|
| One | 4DA-122/3BK-5333 | 0; 190 |
| Two | 3BL-6666/3BL-5823 | 0; 291 |
| Three | 4DG-1524/3AL-1872 | 0; 274 |
| Four | 3AY-3216/4EP-6322 | 0; 283 |
| Five | 3BK-5588/3BK-5476 | 0; 231 |
| Six | 4EF-4391/4EC-4540 | 0; 301 |

Table: C.

| Group Number | Allele | Progeny Cy⁺;Cy |
|---------------------|-------------------------|--------------------------------------|
| One | 4DA-122/ Df(2L)ast4 | 0; 300 |
| | 3BK-5333/ Df(2L)ast4 | 0; 295 |
| Two | 3BL-6666/ Df(2L)ast4 | 0; 201 |
| | 3BL-5823/ Df(2L)ast4 | 0; 246 |
| Three | 4DG-1524/ Df(2L)ast4 | 0; 150 |
| | 3AL-1872/ Df(2L)ast4 | 0; 316 |
| Four | 3AY-3216/ Df(2L)ast4 | 0; 254 |
| | 4EP-6322/ Df(2L)ast4 | 0; 234 |
| Five | 3BK-5588/ Df(2L)ast4 | 0; 312 |
| | 3BK-5476/ Df(2L)ast4 | 0; 275 |
| Six | 4EF-4391/ Df(2L)ast4 | 0; 263 |
| | 4EC-4540/ Df(2L)ast4 | 0; 239 |

Rescue experiment (2) with heat shocks.

Table A:

| | | <u>Heat shock</u> | | |
|---------------------|---------------|--------------------------|----------------------------------|------------------------------------|
| Group number | Allele | Stage | Duration | Progeny (Cy⁺;Cy) |
| One | 4DA-122 | Larval | 30 mins 1 hour | 0; 130 0; 150 |
| | 3BK-5333 | Larval | 30 mins 1 hour | 0; 136 0; 102 |
| Two | 3BL-6666 | Embryonic | 30 mins 1 hour 3 x 30 mins | 0; 134 0; 127 0; 107 |
| | 3BL-5823 | Embryonic | 30 mins 1 hour 3 x 30 mins | 0; 128 0; 123 0; 110 |
| Three | 4DG-1524 | / | / | / |
| | 3AL-1872 | / | / | / |
| Four | 3AY-3216 | Embryonic | 30 mins 1 hour | 0; 99 0; 104 |
| | 4EP-6322 | Embryonic | 30 mins 1 hour | 0; 93 0; 58 |
| Five | 3BK-5588 | Larval | 30 mins 1 hour | 0; 90 0; 76 |
| | 3BK-5476 | Larval | 30 mins 1 hour | 0; 102 0; 75 |
| Six | 4EF-4391 | Embryonic | 30 mins 1 hour | 0; 119 0; 103 |

Table B:

| | | <u>Heat shock</u> | | |
|--------------|-------------------------|-------------------|----------------------------------|-------------------------------|
| Group number | Allele | Stage | Duration | Progeny (Cy ⁺ ;Cy) |
| One | 4DA-122/ Df(2L)ast4 | Larval | 30 mins | 0; 120 |
| | | | 1 hour | 0; 145 |
| | 3BK-5333/ Df(2L)ast4 | Larval | 30 mins 1 hour | 0; 127 0; 152 |
| Two | 3BL-6666/ Df(2L)ast4 | Embryonic | 30 mins | 0; 101 |
| | | | 1 hour 3 x 30 mins | 0; 96 0; 85 |
| | 3BL-5823/ Df(2L)ast4 | Embryonic | 30 mins 1 hour 3 x 30 mis | 0; 68 0; 120 0; 94 |
| Three | 4DG-1524/ Df(2L)ast4 | Larval | 30 mins | 0; 118 |
| | | | 1 hour | 0; 86 |
| | 3AL-1872/ Df(2L)ast4 | Embryonic | 30 mins 1 hour 3 x 30 mins | 0; 76 0; 99 |
| Four | 3AY-3216/ Df(2L)ast4 | Embryonic | 30 mins | 0; 104 |
| | | | 1 hour | 0; 130 |
| | 4EP-6322/ Df(2L)ast4 | Embryonic | 30 mins 1 hour | 0; 94 0; 107 |
| Five | 3BK-5588/ Df(2L)ast4 | Larval | 30 mins | 0; 104 |
| | | | 1 hour | 0; 68 |
| | 3BK-5476/ Df(2L)ast4 | Larval | 30 mins 1 hour | 0; 197 0; 109 |
| Six | 4EF-4391/ Df(2L)ast4 | Embryonic | 30 mins | 0; 112 |
| | | | 1 hour | 0; 95 |
| | 4EC-4540/ Df(2L)ast4 | Larval | | |

Table C:

| | | <u>Heat shock</u> | | |
|---------------------|-----------------------|-------------------------|----------------------------------|------------------------------------|
| Group number | Allele | Stage | Duration | Progeny (Cy⁺;Cy) |
| One | 4DA-122/ 3BK-5333 | Larval | 30 mins 1 hour | 0; 130 0; 114 |
| Two | 3BL-6666/ 3BL-5823 | Embryonic | 30 mins 1 hour 3 x 30 mins | 0; 130 0; 97 0; 117 |
| Three | 4DG-1524/ 3AL-1872 | Embryonic Larval | 30 mins 1 hour 30 mins | 0; 112 0; 87 0; 102 |
| Four | 3AY-3216/ 4EP-6322 | Embryonic | 30 mins 1 hour | 0; 115 0; 126 |
| Five | 3BK-5588/ 3BK-5476 | Larval | 30 mins 1 hour | 0; 90 0; 140 |
| Six | 4EF-4391/ 4EC-4540 | Embryonic Larval | 30 mins 1 hour 1 hour | 0; 130 0; 68 0; 90 |