Investigating the mechanism of translational stimulation by Deleted in Azoospermia-like

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Declaration

I declare

(a) That this thesis has been composed by myself, and

(b) That the work presented here is my own, unless where otherwise acknowledged, and

(c) That the work has not been submitted for any other degree or professional qualification

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December 2007

Abstract

The proper expression of a gene to a protein is a complicated process with many steps. One of the major steps is translation, the process of decoding a messenger RNA signal and the building of a protein from its component parts. The control of translation is one of the major steps for the overall control of gene expression and its dysregulation is associated with a wide variety of human diseases including neurological, metabolic and reproductive disorders. Dazl family proteins are germ cell restricted RNA binding proteins that contain a motif characteristic of this family, the DAZ domain. Whilst humans encode all three family members DAZ, DAZL and BOULE, flies only possess the *boule* gene. The members of this family have an essential conserved role in gametogenesis in a wide variety of organisms from worm to man with loss of function resulting in phenotypes ranging from male or female infertility or both. However, little is known about the molecular role of these proteins in germ cell development.

A previous study within the laboratory showed that several vertebrate Dazl family members can stimulate translation of a reporter gene in *Xenopus laevis* oocytes, suggesting a conserved role in mRNA specific translational control. This is consistent with studies in invertebrates. It was proposed that Dazl proteins fulfil this function through an interaction with a translation initiation factor, poly(A) binding protein, PABP. The aim of this thesis was to further refine this model of action. The work presented here investigates several fundamental questions regarding the mechanism of Dazl-mediated stimulation. First, it investigated the step of translation initiation that Dazl acts upon and explored the initiation factors that may be required. Second, it addressed in more detail the requirements for an interaction between Dazl and the poly(A) binding protein, PABP. Third, it examined the potential role of another factor, DAZ associated protein 1, DAZAP1, in Dazl-mediated stimulation. The role of multi-protein complexes containing Dazl bound to the 3'UTR that localise, repress and stimulate translation of specific mRNAs at defined times during gametogenesis are discussed.

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Well, after the best part of five years this whole thing is finally coming to an end; it has been good in parts and worse in others. In equal amounts I'm happy that I've got here but I'm also very glad it's over. Anyway, enough of the philosophising and onto the real reason anybody would even bother to pick up this irrelevant tome. As ever my eventual success is not solely down to my own efforts and there are many people to be thanked for helping me along the way.

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List of abbreviations

A	adenine
aa	amino acids
ADP	adenosine diphosphate
Ala	alanine
AMV	avian myeloblastosis virus
APS	ammonium persulphate
ARE	AU-rich elements
ARS	auto-regulatory sequence
AZF	azoospermia factor region
ATP	adenosine triphosphate
A-site	aminoacyl site
BRE	Bruno responsive elements
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CoIPe	Co-immunoprecipitation with endogenous proteins
CoIPo	Co-immunoprecipitation with over expressed proteins assays.
CPE	cytoplasmic polyadenylation elements
CSFV	classical swine fever virus
C-terminal	carboxy-terminal
C. elegans	*Caenorhabditis elegans*
Da	Daltons
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dH_20	distilled water
dpc	days post coitum
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
Drosophila	*Drosophila melanogaster*
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
eALAS	erythroid 5-aminolevulinate synthase
ECL	enhanced chemiluminescence
EDTA	ethylenediaminotetra-acetic acid
eEF	eukaryotic elongation factor
EF	elongation factor
eIF	eukaryotic initiation factors
EMCV	encephalomyocarditis virus
EMSA	electro-mobility shift assays

eRF	eukaryotic release factor
E. coli	*Escherichia coli*
G	guanine
GDP	guanine diphosphate
GEF	guanine-nucleotide exchange factor
GST	glutathione-S-transferase
GTP	guanine triphosphate
His	histidine
HRP	horse-radish peroxidase
IF	initiation factor
IH	immunohistochemistry
IRE	iron responsive element
IRES	internal ribosome entry site
ITAF	IRES *trans*-acting factors
kDa	kiloDaltons
LAR	luciferase assay reagent
LB	Luria Bertani
leu	leucine
luc	luciferase
M	molar
Met-tRNAi	initiator methionyl-transfer RNA
MFC	multifactor complex
min	minute
miR	microRNA
miRNP	microRNA containing ribonucleoprotein particles
mRNA	messenger RNA
NI	not injected
NMR	nuclear magnetic resonance
NRE	nanos response elements
N-terminal	amino-terminal
ODC	ornithene decarboxylase
OE	over expressing
ORF	open reading frame
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PBS(T)	phosphate buffered saline (with Tween-20)
PCR	polymerase chain reaction
PEG	poly ethyl glycol
PGC	primordial germ cell
post-TC	post-termination complexes
PV	poliovirus
P-site	peptidyl tRNA binding site
QRT-PCR	quantitative RT-PCR
RNase	ribonuclease

RNasin	ribonuclease inhibitor
rpm	revolutions per minute
RRM	RNA recognition motif
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
Ser	serine
S. cerevisiae	*Saccharomyces cerevisiae*
T	thymidine
TBS(T)	tris buffered saline (with Tween-20)
TCA	trichloroacetic acid
TEMED	N, N, N', N-tetramethylethylene diamine
TNT	transcription and translation
tRNA	transfer RNA
trp	tryptophan
U	units
uAUG	upstream AUG
uORF	upstream ORF
Xenopus	*Xenopus laevis*
Y2H	Yeast two hybrid
β-gal	β-galactosidase
3D	3 dimensional
3'UTR	3' untranslated region
5'UTR	5' untranslated region

Chapter 1: Introduction

1.1 Gene expression, translational control and disease

Gene expression is the process in which a gene is deciphered into a functional gene product, usually a protein. This process is a complex multi-step system and needs to be tightly controlled for proper health and development. The first stage in gene expression is transcription, where the gene is copied into a pre-messenger RNA (mRNA). The mRNA then undergoes post-transcriptional processing including splicing, the addition of a 5' cap and a 3' poly(A) tail. The next stage in gene expression is the localisation of the now mature mRNA from the nucleus where it is transcribed to the cytoplasm where it is subjected to the final stage in gene expression, translation. Translation is the process by which the mRNA is 'read' by the cellular protein factories, the ribosomes, and the gene's final protein product is produced. Some proteins then undergo post-translational modifications to fulfil their function or to regulate their activity. The mRNA template is ultimately destroyed. Each step within this process is highly regulated to ensure that genes are expressed in the correct place, in the correct form and at the correct time in order to properly perform their functions. One of the major levels of gene expression control occurs at translation (reviewed in (Day and Tuite, 1998))

Dysregulation of translation is an important source of genetic diseases and can also play a critical role in many infectious disease processes such as viral infection (Thompson and Sarnow, 2000). Causative mutations have been isolated from the protein synthetic machinery and its upstream regulators (Scheper *et. al.*, 2007), but also in the control elements embedded in specific mRNAs and the *trans*-acting factors that mediate mRNA specific control (Scheper *et. al.*, 2007).

1.2 Gametogenesis

One of the physiological processes that translational control plays a key role in is gametogenesis, and mutations in a variety of genes linked to translation lead to infertility, for example members of the Dazl family of proteins (Reynolds and Cooke,

2005; Yen, 2004). Gametogenesis is the process of making the haploid gametes, the sperm and the egg, from diploid primordial germ cells. In order to make haploid cells from diploid the cells must undergo both mitosis and meiosis and periods of differentiation and growth or maturation. Entry though different stages of gametogenesis is often controlled translationally. At certain points during this process DNA is undergoing complex mitotic and meiotic processes and the packaging of DNA makes it unavailable for transcription. Thus changes in the pattern of protein synthesis can only be achieved by the activation, repression or destruction of pre-existing mRNAs (Hake and Richter, 1997).

1.2.1 The stages of gametogenesis

Gametogenesis begins with specialist cells, named germatogonia, that act as stem cells for the production of gametes; these are known as oogonia in females and spermatogonia in males. The germatogonia undergo both mitosis and meiosis; some reproduce mitotically continually replenishing the supply of germatogonia, whereas others first divide mitotically before entering a pathway of differentiation and meiosis that will end with the production of gametes.

1.2.1.1 Oogenesis

In females the process is known as oogenesis and starts with the transformation of oogonia to primary oocytes in a process that takes place either prenatally or shortly after birth. The next step is the process by which a diploid primary oocyte is transformed to a haploid ootid and is called ootidogenesis. This process begins prenatally and arrests at prophase I of meiosis. The arrested primary oocytes then stay in this state until the beginning of menstruation when oocytes reactivate. When reactivated the primary oocyte completes meiosis I and divides asymmetrically to form one haploid secondary oocyte and one polar body with the majority of the cytoplasm being retained in the oocyte. Immediately post meiosis I, the secondary oocyte initiates meiosis II. This meiotic division is also halted, this time at metaphase II. This arrest lasts until

fertilisation when meiosis II is completed (a second polar body is also extruded at this point) and the newly formed ootid undergoes final differentiation into a mature ovum. Oogenesis is reviewed in (Hilscher, 1991).

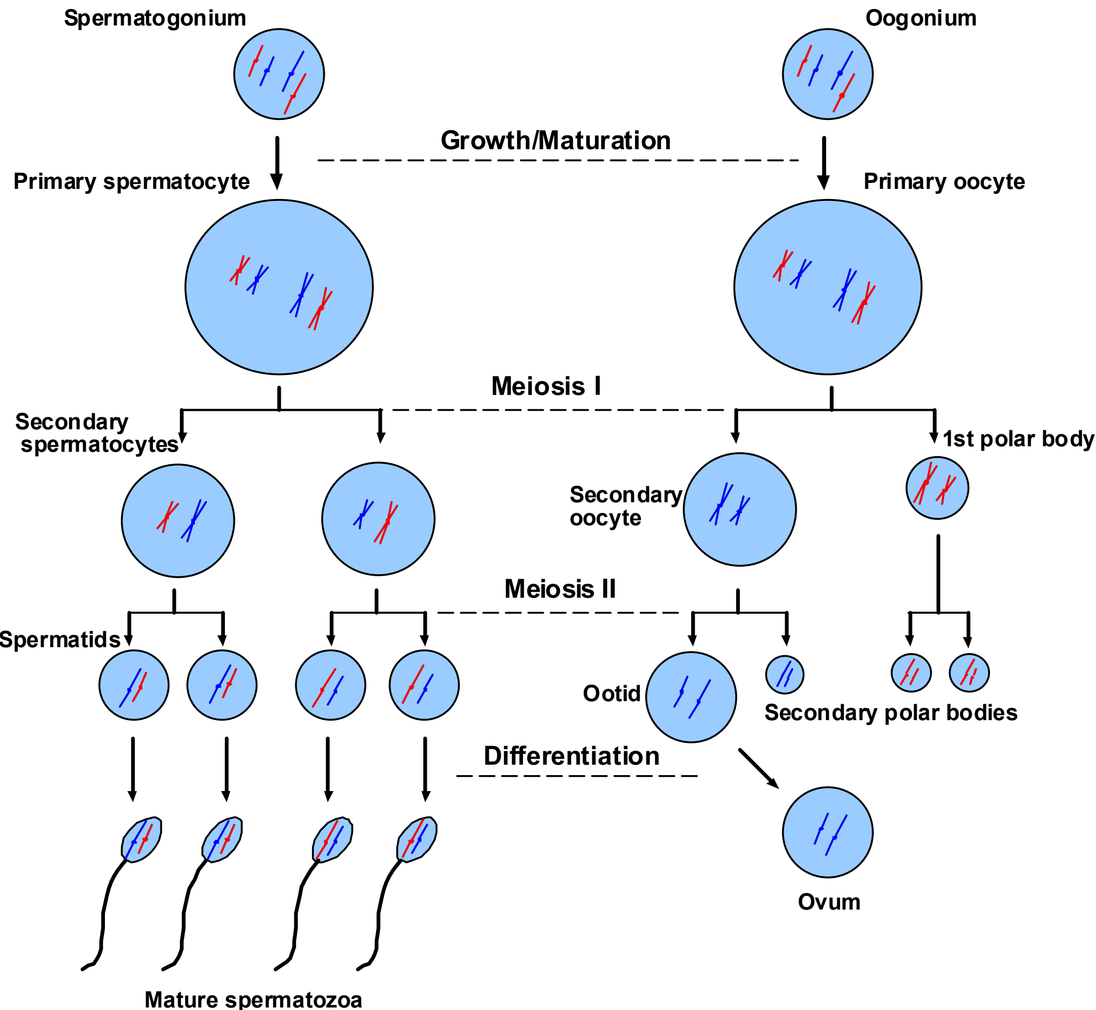


Figure 1.1: Gametogenesis
Spermatogonia and oogonia develop into gametes following a similar pathway. Spermatogonia (males) and oogonia (females) first undergo a period of growth and maturation to form diploid primary spermatocytes (male) or primary oocytes (female) before entering into meiosis I. After meiosis I is complete primary spermatocytes divide into two haploid secondary spermatocytes. Primary oocytes divide asymmetrically into one haploid secondary oocyte and the 1st polar body. Secondary spermatocytes undergo meiosis II and each forms two spermatids. Secondary oocytes undergo meiosis II and again divide asymmetrically to form an ootid and a second polar body. The final stage of gametogenesis is differentiation where spermatids develop into mature spermatozoa and the ootid develops into a mature ovum. (Diagram adapted from www.bio.miami.edu/dana/104/gametogenesis.jpg)

1.2.1.1 Spermatogenesis

In males the process of gametogenesis is known as spermatogenesis and begins when a diploid spermatogonium divides mitotically to produce two diploid cells called primary spermatocytes. Each primary spermatocyte duplicates its DNA and subsequently undergoes meiosis I to produce two haploid secondary spermatocytes. Secondary spermatocytes then rapidly enter meiosis II and divide to produce haploid cells known as spermatids. Spermatids are sometimes referred to as 'round spermatids' because of their shape when viewed on histological sections. The final stage is known as spermiogenesis and is the process of differentiation of the spermatids to mature spermatozoa. The spermatids elongate and grow a tail, and develop a thickened mid-piece where the mitochondria gather. Spermatid DNA also undergoes packaging, becoming highly condensed. The DNA is packaged firstly with specific nuclear basic proteins, which are subsequently replaced with protamines during spermatid elongation. The resultant tightly packed chromatin is transcriptionally inactive (Kierszenbaum and Tres, 1978). Spermatogenesis is reviewed in (Grootegoed *et. al.*, 2000).

1.2.1 The timing of gametogenesis

Although male and female gametogenesis share common features including a mitotic proliferative stage, entry into meiosis, and a post meiotic differentiation they differ greatly in the timing of these events, and indeed the stage of development at which these events take place. For example, female germ cells enter meiosis during fetal development whereas; in contrast, male germ cells enter meiosis postnatally. Also, in males gametogenesis completes the entire process once started whereas in the female the oocyte is arrested in the diplotene stage of meiotic prophase I, where it can remain for months or years. Following a growth period, the oocyte resumes meiosis, only to arrest at a second point, metaphase II. Finally the oocyte completes meiosis upon fertilization. The timing of gametogenesis is reviewed in (Rajesh and Pittman, 2006).

1.3 Translation

The translation of mRNA into protein within the cytoplasm is a complex process involving a large number of proteins, protein complexes, ribosomal subunits and tRNAs and can be divided into three steps: Initiation; which is the most complex of these phases and during which the majority of control takes place. Elongation; during which mRNAs are decoded by the ribosome to make the polypeptide chain encoded by the gene. And finally, termination; when completed peptides and translational machinery are released. Each of these steps can be regulated during global or mRNA specific control of translation, in which either the majority of cellular mRNAs or individual/subsets of mRNAs can be up or down regulated.

1.3.1 Initiation

The majority of translation initiation is dependent on the presence of the 5' m^7Gppp cap structure that is present on the majority of eukaryotic mRNAs. The second primary determinant of translational efficiency is the poly(A) tail present on all but one cellular mRNAs. The other methods of initiation are both cap-independent, the first is similar to the cap-dependent pathway but does not require the cap structure. The second is through the use of internal ribosome entry sites (IRESs).

1.3.1.1 Cap-dependent translation initiation

Cap-dependent translation initiation can be divided into two RNA independent steps and four mRNA dependent steps. The RNA independent steps are the formation of the ternary complex and the 43S pre-initiation complex. The mRNA dependent steps of initiation can be divided into four basic steps (see figure 1.2). First the binding of a protein complex at the 5' cap of the mRNA. Second, the recruitment of the 43S pre-initiation complex to the mRNA at or near the 5' end. Third, the scanning of the small ribosomal subunit through the 5'UTR to the initiation codon. The fourth and final

mRNA dependent step is the joining of the large ribosomal subunit at the initiation codon, the release of initiation factors and the start of elongation. All of these steps involve the binding of many accessory protein factors, called eukaryotic initiation factors (eIFs).

RNA independent steps of initiation

Step 1: Ternary complex formation

The first step in translation initiation is the assembly of what is known as the ternary complex. This complex consists of the initiator methionyl-transfer RNA (Met-tRNAi), eIF2 and GTP. The purpose of this complex is to bring the Met-tRNAi to the P-site of the ribosome and thus position the first amino acid of the polypeptide chain in place. eIF2 consists of three subunits, α, β and γ that all have separate functions (see below). GTP binding to eIF2 is necessary to keep the complex assembled and is hydrolysed to GDP during step 6 (see below). This hydrolysis reaction is catalysed by the GTPase activity of eIF2 and also requires the GTPase activating protein eIF5. eIF2 is then released from the ribosome in a complex with GDP and is recycled into eIF2-GTP for the next round of initiation by the action of a guanine nucleotide exchange factor named eIF2B.

eIF2α is responsible for the interaction with eIF2B protein and is a key target in translational regulation (Krishnamoorthy *et. al.*, 2001). When this domain is phosphorylated eIF2-GDP can no longer act as a substrate for eIF2B and acts instead as a competitive inhibitor, thus reducing the amount of free eIF2B, which is limiting within cells and consequentially the amount of available eIF2-GTP for translation. The structure of eIF2γ has been solved by X-ray crystallography and contains characteristics of a GTP binding domain (Schmitt *et. al.*, 2002). However eIF2γ needs another initiation factor, eIF5, to have functional GTPase activity. eIF5 is a GTPase activating protein specific for eIF2 and supplies an "arginine finger" to the catalytic site of eIF2γ (Das *et. al.*, 2001). The eIF2β subunit is responsible for a mutually exclusive interaction with either eIF5 or eIF2B (Asano *et. al.*, 1999). eIF2β also has been shown to have RNA

binding activity and it has been suggested that this enables eIF2 in also function in initiator AUG recognition (see below) (Laurino *et. al.*, 1999).

Step 2: 43S pre-initiation complex formation

The next step is the assembly of the 43S pre-initiation complex that contains the ternary complex, the 40S small ribosomal subunit plus the initiation factors eIF1, eIF1A, eIF3 and eIF5. Initiation factor eIF1A binds eIF3 and eIF5 and is said to be essential for binding the ternary complex to the 43S complex (Olsen *et. al.*, 2003). eIF3, which contains at least 10 subunits, binds to the 40S subunit directly in an interaction that is enhanced by eIF2 and the presence of eIF3 enhances 40S binding of eIF1 and eIF1A. Furthermore, eIF1 and eIF1A cooperate with one another in binding the ribosomal subunit (Majumdar *et. al.*, 2003).

In yeast it has been shown that eIF1, eIF2, eIF3 and the ternary complex can pre-assemble independent of the ribosome and is referred to as the multifactor complex (MFC) (Asano *et. al.*, 2000). In this complex eIF3 contacts eIF1, eIF2, and eIF5 directly and also the 40S ribosomal subunit, giving it a central role in 43S complex assembly (Valasek *et. al.*, 2004).

RNA dependent steps of initiation

Step 1: Cap binding complex assembly

The initial mRNA dependant step of translation is the assembly of a multi-protein complex at the cap known as the eIF4F complex (figure 1.2, step 1). In animal cells the eIF4F complex contains the proto-oncogene eIF4E that directly interacts with the cap, a scaffolding initiation factor called eIF4G and an RNA-dependant helicase named eIF4A. Another initiation factor, eIF4B, is closely associated with the complex (Mathews *et. al.*, 2007).

The eIF4E protein binds the methylated guanosine cap with high affinity and binds non-methylated precursors with a much lower affinity (Morino *et. al.*, 1996). It is this

interaction that is thought to be responsible for the assembly of the eIF4F complex on the mRNA (though some complex assembly can occur in the absence of the cap, see section 1.3.1.2 below) and is thus responsible for the directionality of ribosome entry onto the mRNA. The 3D structure of eIF4E bound to the cap has been solved by X-ray crystallography for mouse (Marcotrigiano *et. al.*, 1997) and by NMR for yeast (Matsuo *et. al.*, 1997). From this data the structure of eIF4E is said to be resemble a cupped hand with the 'cup' of the hand forming a slot for the binding of the cap structure and a contiguous region for mRNA binding.

eIF4G is the central core of eIF4F and is thought to be the 'scaffolding' protein that both dramatically increases affinity of the complex for cap binding (Haghighat and Sonenberg, 1997), by increasing the affinity of eIF4E for the cap by an unknown mechanism , and holds the rest of the complex together. It can be divided into three areas with the N-terminal third (amino acids (aa) 1-634) containing binding sites to eIF4E (Mader *et. al.*, 1995) and also to the poly(A) binding protein (PABP) (Imataka *et. al.*, 1998). The central third (aa 635-1039) binds to eIF4A and eIF3 (Imataka and Sonenberg, 1997) and also has RNA binding activity (Pestova *et. al.*, 1996b). The C-terminal portion (aa 1040-1560) contains a second eIF4A binding site (Imataka and Sonenberg, 1997) and is bound by the Mnk1 kinases (Pyronnet *et. al.*, 1999), which phosphorylate eIF4E.

eIF4A possesses RNA-dependent ATPase activity and is the founder member of the DEAD box family of RNA-helicases (Oguro *et. al.*, 2003). Its activity is stimulated by eIF4B and eIF4A appears more active when part of the eIF4F complex (Mathews *et. al.*, 2007). When this protein is bound to the eIF4F complex via its interaction with eIF4G it is thought that this helicase activity unwinds secondary RNA structure in the 5'UTR in an ATP dependent manner, thus enabling the 43S complex to bind the mRNA. eIF4A also has mRNA binding activity and is thought to be involved in further mRNA unwinding after 43S joining during scanning (Abramson *et. al.*, 1987). Another factor named eIF4H can also stimulate eIF4A activity and both eIF4B and eIF4H have been

proposed to allow eIF4A to unwind longer, more stable RNA structures and to increase the rate and range of this unwinding (Rogers *et. al.*, 1999).

Step 2: Joining of the 43S complex to the mRNA

The assembly of the cap-binding complex and the unwinding of secondary RNA structures in the 5'UTR facilitates the joining of the 43S complex to the mRNA (figure 1.2, step 2). The 43S complex is recruited to the mRNA in part by the association between eIF4G and eIF3 (Imataka and Sonenberg, 1997). Additional interactions between initiation factors may also help stabilise this interaction, for example the C-terminal domain of eIF5 can bind simultaneously to eIF4G and eIF3 and it has been proposed that this aids their binding to each other (Asano *et. al.*, 2001). eIF4B has been proposed to aid 43S recruitment via its RNA recognition motif (RRM), which has been suggested to bind simultaneously to ribosomal 18S RNA and a mRNA thus bridging the ribosome to the mRNA (Methot *et. al.*, 1996a). eIF4B also can bind eIF3, again aiding the recruitment of the 43S complex to the mRNA (Methot *et. al.*, 1996b).

Step 3: Scanning and AUG recognition

After the 43S pre-initiation complex has bound close to the 5' cap it "moves" through the 5'UTR to the start codon in an ill-defined process known as scanning. A linear movement of the 43S complex along the 5'UTR until an AUG codon is found is the most common view of scanning, but as elements of eIF4F such as eIF4G and eIF4A are required for scanning it is unsure if the complex moves along the mRNA away from the cap, or if the RNA is drawn toward the cap thus looping out the 5'UTR. It has also been suggested that scanning in some systems may be locally bidirectional and able to move both in a 3' and 5' direction (Berthelot *et. al.*, 2004). The movement of the 43S complex along the mRNA determines the tendency of most known RNAs to initiate translation at the start codon closest to the mRNA 5' end, the 'first-AUG' rule (Kozak, 1995). However in mammals the first-AUG rule is moderated by other factors. The site must be at least 8 nucleotides from the cap (Pestova and Kolupaeva, 2002) and generally more than 20, the sequence surrounding the AUG is also important with the strongest sequence corresponding to the 'Kozak' consensus: GCC(**A/G**)CCAUG**G** (the most

important residues aside from the AUG are shown in bold, with the +4 G being of even more important than the -3 purine) (Kozak, 1987b). The initiation factors eIF1 and eIF1A are also important for scanning and AUG recognition. A model has been suggested where 43S complexes exist in two different forms, a ‘closed’ scanning incompetent form in the absence of eIF1 and an ‘open’ scanning competent form in the presence of eIF1 (Pestova and Kolupaeva, 2002). eIF1 also prevents premature GTP hydrolysis by eIF2 before AUG recognition (Unbehaun *et. al.*, 2004). It has also been suggested that eIF1 and eIF5 inhibit eIF2-GTP hydrolysis at non-AUG codons (Valasek *et. al.*, 2004). As initiation of protein synthesis in cell-free systems is dependent on ATP hydrolysis, it is assumed that the scanning process itself is either directly or indirectly ATP driven (Kozak, 1980).

Step 4: 60S joining and factor release

Once scanning is complete and the 43S complex has been properly positioned at the start codon, the final step in translation initiation occurs; the joining of the large 60S ribosomal subunit to make the active 80S ribosome. For this step to take place initiation factors must be released. The first action in this process is the hydrolysis of the ternary complex bound GTP, catalysed by the GTPase activity of eIF2, which is activated by eIF5 as discussed earlier. This causes the release of eIF2 and all other initiation factors are also assumed to release at this point.

Until recently this single GTP hydrolysis was thought to be sufficient for 60S joining but the activity of a second GTPase initiation factor, eIF5B, is also required for 80S formation (Lee *et. al.*, 2002). eIF5B is already complexed with GTP and eIF5B-GTP stimulates 60S joining but GTP hydrolysis only occurs after the joining event that then causes the release of eIF5B-GDP (Lee *et. al.*, 2002). eIF5B interacts with eIF1A and this reaction is thought to enable eIF5B to localise to the 43S complex bound mRNA (Olsen *et. al.*, 2003).

After this second subunit joining step and initiation factor release occurs the ribosome is complete and in place and translation enters the next stage, elongation.

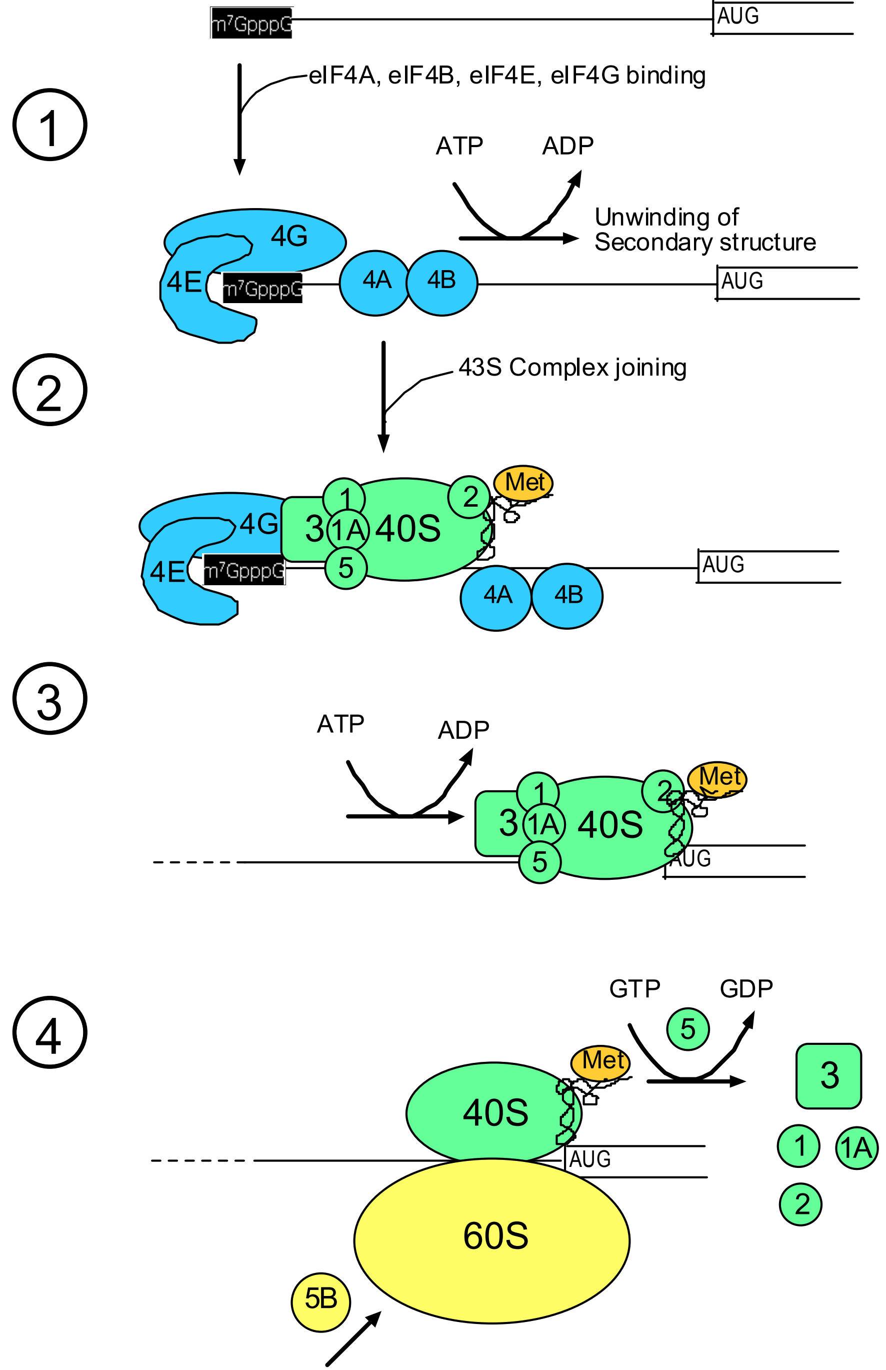


Figure 1.2: mRNA dependent steps of cap-dependent translation initiation.
This model is consistent with the majority of data available. For simplicity not all initiation factors have been shown. The sizes of the factors are not to scale, not all interactions nor the orientation of the factors within complexes is depicted.
Definitions: eIF= eukaryotic initiation factor, 1= eIF1, 1A= eIF1A, 2= eIF2, 3= eIF3, 4A= eIF4A, 4B= eIF4B, 4E= eIF4E, 4G= eIF4G, 5= eIF5, 5B= eIF5B, Met= Methionine tRNA, m7GpppG= 5' mRNA cap structure, 40S= Small ribosomal subunit, 43S= Pre-initiation complex, 60S= Large ribosomal subunit.

(1) The eIF4F cap-binding complex binds to the mRNA via an interaction between eIF4E and the m^7GpppG cap. There is also a possibility that the observed RNA binding activity of eIF4G may play a role in this process. The binding of the eIF4F complex facilities the ATP dependant unwinding of any secondary structure under the action of the RNA helicase activity of eIF4A. This activity of eIF4A is enhanced by eIF4B.
(2) Unwinding allows the 43S pre-initiation complex to join the mRNA via interactions between eIF4G in the cap binding complex and eIF3 in the 43S complex.
The 43S pre-initiation complex consists of 40S ribosomal subunit, the ternary complex (Met-tRNA, eIF2 and GTP) and various initiation factors (eIFs 1, 1A, 3 and 5). This complex assembles before joining the mRNA but the exact order of and nature of the formation of this complex is currently unclear.
(3) The 43S complex then moves through the 5'UTR to the start codon via ill-defined, but ATP-dependent, processes known as scanning.
(4) Scanning ends when the anticodon of the Met-tRNA is engaged to a start codon. GTP is hydrolysed by eIF5 and all initiation factors dissociate. eIF5B catalyses the joining of the 60S ribosomal subunit.

1.3.1.2 Alternative mechanisms of translation initiation

IRESs - viral and cellular

Some cellular and viral mRNAs utilise an alternative method of initiation using an element known as an internal ribosome entry site, or IRES. This method of initiation was first identified in picornaviruses and viral IRESs normally consist of multiple stem-loops or pseudoknot structures that can promote cap-independent initiation at a site that is internal to the mRNA, away from the cap structure (Schneider and Mohr, 2003). As in 'normal' initiation many accessory protein factors are required, however, in general the cap-binding protein eIF-4E is not required (Vagner *et. al.*, 2001). Whilst some IRESs require the majority of the canonical initiation factors, other IRESs require only a subset and in the most extreme cases none of the eIFs are required (Spahn *et. al.*, 2004) and the ribosome is recruited to the IRES directly in an event reminiscent of prokaryotic translation. In addition to eIFs many IRESs require other accessory proteins known as IRES *trans*-acting factors, or ITAFs, for their function, with a variety of different proteins being utilised by individual IRES elements. The tertiary structure of IRESs is in general thought to be very important for their function and in some cases it is suggested the ITAFs may act to stabilise these structures (Vagner *et. al.*, 2001).

Viruses from many different families, including picornaviruses, flaviviruses and others, have been shown to use IRES mediated translation as a means to escape the shut-off of

host protein synthesis that typically occurs following viral infection (Schneider and Mohr, 2003). One mechanism of this shut off of translation is achieved by cleavage of eIF4G. Many different viruses employ this technique and one of the best characterised eIF4G proteases is the 2A protease of rhinovirus (Haghighat *et. al.*, 1996). The cleavage of eIF4G causes a reduction in cap-dependent translation but the cleaved eIF4G is still sufficient to support IRES mediated translation, so viral mRNAs are still expressed (Schneider and Mohr, 2003). Cellular IRESs are frequently less structurally complex than their viral counterparts and function during processes such as starvation when cap-dependent translation is down regulated (Vagner *et. al.*, 2001).

Non IRES cap-independent initiation

This mode of translation normally describes the ability of cellular mRNAs lacking the physiological cap or an IRES to initiate translation but can also occur on artificial mRNAs made with non-m^7GpppG caps. This form of initiation is generally inefficient but some viruses appear to initiate translation in this manner, for example the AMV virus (Gehrke *et. al.*, 1983). The mechanistic details of this process remain unclear but elements of the cap-binding complex appear to be required and it is proposed that they are still recruited to the 5'end of the mRNA, perhaps due to the RNA binding activity of several of the factors (Fletcher *et. al.*, 1990). The 43S complex is then recruited as normal via interactions with eIF3. Scanning, AUG recognition and 60S joining then proceeds as in 'standard' translation initiation.

Re-initiation

In most cases eukaryotic ribosomes only initiate translation once per mRNA, but in some cases re-initiation at a downstream AUG is possible when the 5' proximal AUG triplet is followed shortly by a terminator codon. In such cases ribosomes can reinitiate translation at the next AUG downstream (Kozak, 1984). The distance between the upstream cistron and the second AUG is important in eukaryotes with increasing gap increasing re-initiation with a 79 nucleotide gap after a 'minicodon' not affecting translational efficiency in one system tested (Kozak, 1987c). Re-initiation plays a vital

role in the translational control of a subset of cellular mRNAs, including GCN4, described in more detail in section 1.2.2.1.

Leaky scanning

Another mechanism by which initiation may occur at an internal rather than the 5' proximal AUG is known as 'leaky scanning', which can occur when the first AUG lies in an unfavourable context for initiation and is therefore passed over in favour of a later more distal AUG with a better context. This model has been developed by manipulating the context of the AUG in synthetic constructs (Kozak, 1986). Leaky scanning can be important for the correct translation of some biologically important genes, for example the ß-Site ß-amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1), which is linked to processing the proteins involved in Alzheimer's disease (Zhou and Song, 2006). Its mRNA contains a number of uAUGs that are skipped by leaky scanning and this combined with a re-initiation event results in weak expression of BACE1 under normal conditions. It is thought that alterations of the leaky scanning and re-initiation in *BACE1* gene expression could play an important role in Alzheimer's disease pathogenesis (Zhou and Song, 2006).

Shunting

As mentioned above, in most RNAs the small ribosomal subunit is thought to undergo a process called scanning whereby the ribosome moves through the 5'UTR to the AUG in an ill-defined process. However for a very limited number of mRNAs this mechanism is thought to be different with the ribosomal subunits bypassing, or shunting, segments of the 5'UTR as they move to the initiation codon. This effect has be observed in adenovirus (Yueh and Schneider, 2000) and cauliflower mosaic virus (Pooggin *et. al.*, 2001) and requires "take-off" and "landing"-pads although the mechanistic details of this process are yet to be determined.

Hopping

Another novel initiation mechanism is that of ribosome hopping. During translation of bacteriophage T4 topoisomerase subunit gene 60 mRNA the complete ribosome

disengages from the ORF at glycine codon 46 and re-engages at an identical codon after a 50 nucleotide gap, thus producing a single polypeptide from two separate ORFs (Weiss *et. al.*, 1990).

1.3.2 Elongation

Elongation is the step of translation in which the actual polypeptide synthesis takes place. This process begins with the positioning of the Met-tRNAi into the P-site of the ribosome during translational initiation. The next amino acid in the polypeptide chain, determined by the mRNA sequence is then delivered by its cognate tRNA into the empty aminoacyl 'A' site adjacent to the P-site. The aminoacyl-tRNA is complexed with GTP and eukaryotic elongation factor 1A (eEF1A). The base pairing between the mRNA codon and the tRNA's anticodon causes conformational changes in the ribosome and the hydrolysis of GTP and the release of the tRNA from eEF1A-GDP. Following hydrolysis of the GTP, eEF1A-GDP is released from the ribosome. This form cannot bind amino acyl-tRNA and is 'recycled' to the active GTP-bound form by eEF1B, a guanine nucleotide-exchange factor (GEF) for eEF1A. The release of the tRNA allows the aminoacyl-tRNA to form a peptide bond with the adjacent peptidyl-tRNA in a reaction catalysed by the ribosome's peptidyl transferase activity. During this reaction the growing polypeptide chain is passed from the first tRNA to the second, leaving the tRNA in the P-site deacylated. The next step is the translocation of the complex so that the deacylated tRNA moves to the exit 'E' site from the P-site, and the polypeptide bound tRNA in the A-site is moved to take the now vacant position in the P-site. The next codon of the mRNA is shifted to the now empty A-site ready to accept the next aminoacyl-tRNA. This translocation requires GTP hydrolysis and is mediated by eukaryotic elongation factor 2 (eEF2). Elongation continues until a termination codon (UAA, UAG or UGA) is recognised by the ribosome complex. Elongation is reviewed in (Mathews *et. al.*, 2007).

1.3.3 Termination

Termination of translation occurs when one of the three stop codons, UAA, UAG or UGA, reaches the A-site in the ribosome. When a stop codon enters the A-site of the ribosome, ribosome release factors are recruited to the mRNA/ribosome complex and mediate the hydrolysis of the peptide chain from the bound tRNA causing the dissociation of the newly synthesised peptide from the ribosome. In eukaryotes a single factor, eukaryotic release factor 1 (eRF1) recognises all three stop codons. The eukaryotic release factor 3 (eRF3), a GTPase, binds to eRF1 and stimulates its activity and together these factors induce hydrolysis of the ester bond of the P-site peptidyl-tRNA, thus releasing the finished polypeptide from the ribosome (Alkalaeva *et. al.*, 2006).

After peptide release the ribosome must be released from the mRNA in order to become available for further rounds of translation, a process that is known as 'ribosomal recycling' from post-termination complexes (post-TCs). In prokaryotes recycling of post-TCs requires an initiation factor, IF3, the EF-G elongation factor and a factor known as the ribosomal release factor (RRF). RRF and EF-G act to dissociate post-TCs into free 50S subunits and 30S subunits bound to both the mRNA and the deacylated P-site tRNA. IF3 then induces the release of tRNA from 30S subunits, which is followed by the spontaneous dissociation of the mRNA, thus leaving all the components of the post-TC free for re-use (Peske *et. al.*, 2005; Zavialov *et. al.*, 2005). There has been no equivalent RRF identified in eukaryotes and the mechanism of ribosomal recycling is currently unknown.

1.3.4 The closed loop model of translational initiation

Whilst initiation occurs at the 5' end of the mRNA, the poly (A) tail at the 3' end is also a primary determinant of translational efficiency like the 5'm7GpppG cap. The effects of the poly(A) tail are thought to be largely mediated by poly(A) binding protein (Kuhn

and Wahle, 2004; Sachs *et. al.*, 1987), which binds in an ordered manner to poly(A) tails (Baer and Kornberg, 1983). It has been shown that PABP binds to the poly(A) tail (Kuhn and Wahle, 2004) and also interacts with proteins that bind to the 5' cap region (Gallie, 1998), and it has been suggested that these interactions can cause the circularization of the mRNA (Wells *et. al.*, 1998) into a an end-to-end complex that will be discussed in more detail in section 1.5.2.1.

1.4 The control of translation

Translational regulation is important both in terms of genome diversity (Copeland, 2003) and for controlling temporal and/or spatial gene expression (Mignone *et. al.*, 2002). In the latter, effects can be either positive or negative and can act on an individual mRNAs or be more global in nature. Translational regulation of individual mRNAs is dependent on sequences within these mRNAs. In some cases they can be linked to changes in mRNA stability or localisation (Day and Tuite, 1998). Many different types of translational regulatory elements have been identified, with most located in the untranslated regions at the 3' and 5' ends (Wilkie *et. al.*, 2003). Most of these mechanisms work at the level of translational initiation and almost every step in initiation can be regulated (Gray and Wickens, 1998). However regulation of each of the other steps of translation have also been described, suggesting the evolution of multiple mechanisms of regulation where single mRNAs can be subject to multiple regulatory events due to the presence of different elements that function in response to different stimuli. Alternative initiation mechanisms also play an important role in regulating translation and several mRNAs have been shown to be controlled in this way (Gray and Wickens, 1998).

1.4.1 Global control of translation

There also exist systems that can regulate whole cell translation with overall levels changing in response to stimuli, for example stress (Dever, 2002). Subsets of mRNAs

important to reacting to changes in physiological conditions normally escape this global control due to elements with these mRNAs. Thus a complex network of global and mRNA specific controls allow the cell to respond to different conditions. Many of these global regulatory events operate via phosphorylation pathways (Raught and Gingras, 2007).

1.4.1.1 Global control of translation by eIF2

The pathway for translation initiation presented above started with eIF2-GTP and the production of this vital complex is one of the key limiting steps in translation. At the end of each round of translation eIF2-GDP must exchange GDP for GTP in order to be available for the next round. Nucleotide exchange of eIF2 involves eIF2B, the guanine-nucleotide exchange factor (GEF) for eIF2. eIF2B interacts with the α-domain of eIF2, (Krishnamoorthy *et. al.*, 2001). When eIF2 is phosphorylated on serine 51 the eIF2α domain translation is inhibited as eIF2-GDP can no longer act as a substrate for eIF2B and acts instead as a competitive inhibitor (Krishnamoorthy *et. al.*, 2001). Normally eIF2 is in excess to eIF2B, so if a small amount is phosphorylated eIF2 sequesters all the eIF2B and then there is a general loss of translational activity (Krishnamoorthy *et. al.*, 2001). This mechanism of translational control is usually the response to cellular stress. These stresses activate cellular kinases that then go on to phosphorylate eIF2α. For example yeast GC2 kinase can be activated in response to uncharged tRNAs accumulating due to amino acid starvation, infection by viruses can lead to activation of PKR and ER-stress leads to phosphorylation via PERK (Wek *et. al.*, 2006).

1.4.1.2 Global control of translation by 4E-BP

Regulatory sequestration proteins represent another type of global control mechanism; the eIF4E-binding protein (eIF4B-BP) is a regulatory protein that competes with eIF4G for binding to eIF4E and thus prevents formation of the eIF4F complex.

In humans there are three eIF4E binding proteins, eIF4E-BP1, eIF4E-BP2 (Pause *et. al.*, 1994a) and eIF4E-BP3 (Poulin *et. al.*, 1998). These binding proteins function by interacting with eIF4E leaving it unable to bind with eIF4G (Haghighat *et. al.*, 1995). This results in the eIF4F complex not being able to form and cap-dependent translation is thus reduced. The region of eIF4E that binds eIF4-BP is separate to the cap binding site (Matsuo *et. al.*, 1997) that means that eIF4E/eIF4EBP complexes can still bind the cap, but not translate due to an inability to bind eIF4G, leading to RNAs held in translationally inactive complexes (Proud and Denton, 1997).

Under stress conditions such as heat, shock or starvation eIF4E-BP1 becomes hypophosphorylated and binds to eIF4E whereas hyperphosphorylatred eIF4E-BP1 cannot bind eIF4E (Pause *et. al.*, 1994a). Interestingly not all stresses cause equal effects but may vary in their response to stimuli for example during heat shock (Scheper *et. al.*, 1997) or certain stages of adenovirus infection (Feigenblum and Schneider, 1996).

1.4.1.3 Global control by eIF4E phosphorylation

eIF4F complex binding is dependent on the affinity for the cap by the eIF4E protein, an affinity that has long been thought to be dependent on the phosphorylation state of that protein with increased phosphorylation on Ser-209 increasing affinity for the cap (Minich *et. al.*, 1994). However, mutation of Ser-209 to alanine does not affect the ability of eIF4E to mediate translation in an *in vitro* system (McKendrick *et. al.*, 2001) suggesting that eIF4E phosphorylation may not be as important. The importance of eIF4E phosphorylation as stimulatory to translation has been further challenged recently where it was proposed that phosphorylation instead reduces the affinity of eIF4E cap binding activity (Scheper and Proud, 2002).

1.4.1.4 eIF4G decoys

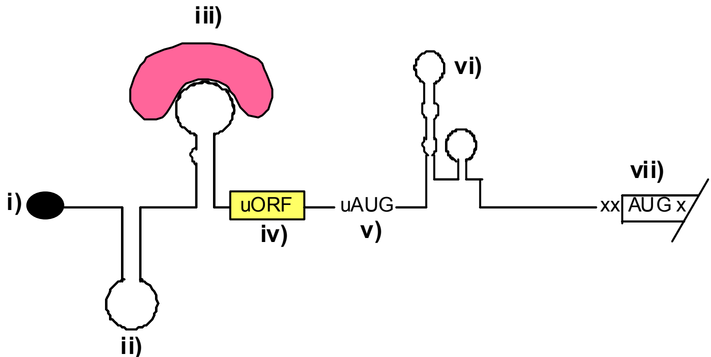
As mentioned above eIF4G is an important factor in translation initiation and its availability is important for translation to occur with levels of this protein being

regulated at both the level of synthesis and turnover (Morley *et. al.*, 1997). The ability of eIF4G to interact with initiation factors may be regulated by a protein called p97, DAP-5 or NAT1. This protein has 30% homology to the C-terminal two thirds of eIF4G (Morley *et. al.*, 1997), and appears to act as a decoy for eIF4G. It can interact with eIF3 and/or eIF4A *in vitro* (Imataka and Sonenberg, 1997) but lacks the ability to interact with eIF4E and is thought to suppress translation. High levels of expression of DAP-5 represses cell growth, but this effect was not mapped to the portion of the protein that binds eIF3 and eIF4A therefore is not clear whether this protein repress growth by titrating initiation factors away from ribosomes or by some other mechanism (Levy-Strumpf *et. al.*, 1997).

1.4.2 Specific control of translation

Specific regulation is conferred by *cis*-acting elements that are located most frequently within the 5' and 3' untranslated regions and often serve as binding sites for positive or negative *trans*-acting factors (Gebauer and Hentze, 2004; Gray and Wickens, 1998; Wilkie *et. al.*, 2003). These *trans*-acting factors can be RNA-binding proteins or miRNAs and multi-factor complexes are often required, especially for regulation mediated by the 3' end (Gray and Wickens, 1998). In the few examples that have been intensively studied to date, the regulation of these mRNAs appears to occur predominantly at the level of initiation (Gebauer and Hentze, 2004; Gray and Wickens, 1998). Examples of these different specific control sites are shown in figure 1.3.

A: 5' UTR Elements



B: 3' UTR Elements

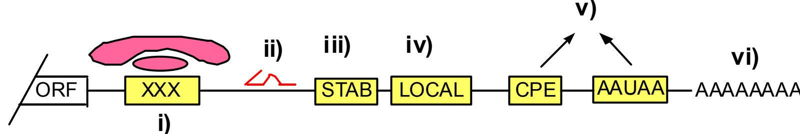


Figure 1.3: Translational regulatory elements located in the 5' and 3' UTRs.
A: 5'UTR Elements. i) the 5' cap, ii) stem-loop structures, iii) regulatory protein binding sites, iv) upstream open reading frames, v) upstream AUGs, vi) IRESs and Vii) AUG context
B: 3'UTR Elements. i) regulatory protein binding sites, ii) microRNA complementary sites, iii) stability elements iv) localisation elements, v) cytoplasmic polyadenylation elements and vii) the poly(A) tail.
(Figure adapted from (Wilkie *et. al.*, 2003).

1.4.2.1 5' translation control elements

Features within the 5' untranslated region that affect translation initiation include the 5' cap, leader length, secondary structure, regulatory protein binding sites, IRESs, upstream ORFs or AUGs, and initiation codon context (reviewed in (Gray and Wickens, 1998)).

<u>5' cap</u>

The 5' cap is a primary determinant of translational efficiency and must be methylated to stimulate translation via eIF-4E recruitment, though an unmethylated cap still increases mRNA stability by resisting decapping and protecting the mRNA from 5' to 3' exonucleases (Grudzien *et. al.*, 2006).

Secondary structure

Stable secondary structure within the 5'UTR is associated with decreased translation efficiency. Relatively weak secondary structures (-30 kcal/mol) are capable of inhibiting translation when located close to the cap structure whilst more stable structures (-61 kcal/mol) are required to prevent efficient initiation from downstream sites (Kozak, 1989a). Stem-loops located close to the cap physically prevent the binding of the 43S complex whereas those in a cap-distal position prevent scanning of the 43S complex (reviewed in (Day and Tuite, 1998)). Ornithene decarboxylase (ODC) is an example of a gene that is regulated in this way. ODC mRNA translation is impeded by 140 nucleotide GC-rich region that is predicted to form a very stable stem loop structure in the 5'UTR close to the cap structure (Gray and Hentze, 1994b). If the sequence is mutated in such a way that the loop is destabilised, then the translational efficiency of the mRNA is increased (Gray and Hentze, 1994b). It has also been established that if the inhibitory region was inverted translation of ODC mRNA was still poor, indicating that the structure itself is inhibitory rather than serving as a binding site for a repressor protein (Gray and Hentze, 1994b).

Not all secondary structures are inhibitory: Secondary structures positioned downstream from the initiation codon can enhance its recognition. If a stem loop structure is present 14 nucleotides downstream from the initiation codon this forces pausing of the 43S complex at the AUG with the initiation centre directly above the initiator codon thus aiding recognition (Kozak, 1990).

RNA binding proteins

Stem-loop structures are frequently the binding sites for regulatory proteins, which can repress translation in a manner similar to secondary structures, but in these cases the repression is often highly regulated by controlling the binding of the repressor protein.

A well characterised example of a stem-loop that acts as a binding site for a regulatory protein is the iron responsive element (IRE) that is found in the 5'UTR of a number of mRNAs including ferritin and erythroid 5-aminolevulinate synthase (eALAS). The IRE

is the binding site for iron regulatory proteins 1 and 2 (IRP-1 and IRP-2) and its mode of action is dependent on its position in the 5'UTR. If the IRE is more cap-proximal then 43S complex joining is blocked (Gray and Hentze, 1994a). If the IRE is moved to a more cap-distal position then 43S joining can occur but there is still some action of bound IRPs with a delay in scanning being detected (Paraskeva *et. al.*, 1999). However it appears that the stalled 43S complexes can overcome the effect of the IRE by displacing IRP in an unknown mechanism, though the action of helicases disrupting the RNA structure of the IRE is suggested (Paraskeva *et. al.*, 1999).

Not all regulatory protein-binding sites within the 5'UTR are structured (as above) and not all lead to repression as exemplified by the ITAFs that interact with IRES elements. An example of a different type of protein binding site is that of the adenine-rich auto-regulatory sequence (ARS) in the 5'UTR of the PABP protein mRNA. This binding site is cap-distal, does not have a stable secondary structure and binds a multi protein complex of PABP and two other proteins, IMP1, an orthologue of chickenzip-codebinding polypeptide, and UNR, a protein that can bind PABP (Patel *et. al.*, 2005). This complex bound to the ARS represses translation of the PABP mRNA by interfering with ribosome scanning and thus auto regulates the expression of PABP (Bag, 2001).

<u>Upstream ORF</u>

Some transcripts have open reading frames upstream of the main open reading frames (ORFs) that are known as upstream ORFs (uORFs). uORFs in general cause a decrease in translation from the main open reading frame by sequestering ribosomes from the main open reading frame but are often involved in intricate patterns of control. Translation of the main ORF in transcripts containing a uORF is dependent on re-initiation or leaky scanning (see above).

Although most uORFs are inhibitory, on some genes they can be responsible for the activation of translation, as in the case of the yeast gene *GCN4*. GCN4 is a transcription factor that activates the genes in yeast that are responsible for amino acid biosynthesis. The *GCN4* mRNA contains four short uORFs (uORF 1, 2, 3 and 4) upstream of the

initiation codon of the main ORF. In nutrient rich conditions, these uORFs restrict the flow of scanning ribosomes to the *GCN4* AUG and thus the protein is produced at low levels. The translation of uORF4 is especially inhibitory as a GC-rich region adjacent to its stop codon causes full disassembly of the ribosome. Following translation of the first uORF, the 60S ribosomal subunit is thought to dissociate, but the 40S subunit remains bound and has the potential to reinitiate translation further down the mRNA, but this requires additional ternary complexes. When ternary complexes are plentiful, the ribosome can re-initiate efficiently and the probability of translation of uORF4 increases, and thus translation of GCN4 decreases. However, under starvation conditions, when eIF2 is phosphorylated and ternary complex formation is thus inhibited (see section 1.2.1.1) the efficiency of re-initiation is reduced and the translation of uORF4 decreases, allowing more 40S subunits to reach the *GCN4* AUG and thus initiate translation of the GCN4 protein and activation of the amino acid biosynthetic pathways occurs (GCN4 translational control is reviewed in (Hinnebusch, 1997)).

In some cases the peptide encoded by the uORF can be involved in the regulation of the main ORF as in the case of S-adenosylmethionine decarboxylase (AdoMet) mRNA in response to polyamine levels. The uORF is predicted to produce a short peptide and if the sequence of the uORF is altered so the peptide sequence is changed then translation of the main ORF is increased, indicating that the uORF peptide regulates the translation of the main ORF (Hill and Morris, 1993).

Upstream AUGs

Some mRNAs have upstream AUGs (uAUGs) (or other initiation codons, see viii below) 5' of the main ORF that unlike uORFs do not have in frame termination codons. As the standard initiation pathway will end when they reach the uORF any initiation of the downstream AUG will be the result of leaky scanning (see section above). The presence of a uAUG is usually strongly inhibitory, with the inhibition being stronger the better the context of the AUG is (Kozak, 1987b). Because uAUGs are not necessarily in frame with the main ORF and because there are no termination codons, uAUGs do not give the same options for re-initiation as uORFs so are generally more inhibitory. These

are frequently found in mRNAs that encode cytokines, growth factors and transcription factors and are proposed to maintain low levels of expression (Kozak, 1991).

In addition to controlling translation from the main open reading frame, multiple start codons can also be utilised to produce more than one protein from a single mRNA. An example of this type of translational control is the liver-enriched transcriptional activator protein (LAP) and the liver-enriched transcriptional inhibitor protein (LIP) (Descombes and Schibler, 1991). LAP is an activator of transcription and LIP, which is produced by leaky scanning on the same mRNA, lacks the transcriptional activator domain and therefore antagonises the action of LAP. Changes in the ratio of the two proteins coincide with their function of LAP in liver differentiation (Descombes and Schibler, 1991).

Cellular IRESs

As mentioned earlier, an IRES enables an mRNA to initiate translation in a cap-independent manner. In addition to viruses some cellular mRNAs also have IRESs. Because of the IRES the mRNA is subject to different regulation when compared to other mRNAs and can allow translation of a specific mRNA even when general cap-dependent translation is impaired (Schneider and Mohr, 2003).

For example the human FGF-2 gene, which encodes a growth factor, contains an IRES that can initiate translation even when general translation is inhibited due to sequestering of eIF4E (Vagner *et. al.*, 1995). This allows FGF-2 to be translated under conditions such as cell growth arrest or stress when normal translation is inhibited. Production of this growth factor could result in the reactivation of translation, stimulation of cell proliferation or differentiation and has been suggested to play a key roll in wound healing (Vagner *et. al.*, 1995).

Length of UTR

The length of the 5’UTR can affect translational efficiency with short UTRs (approximately less than 20 nucleotides) leading to ‘leaky scanning’, meaning that 40S

ribosomal subunits do not efficiently recognise the first AUG due to its proximity to the cap-structure and continue to scan to downstream AUGs (Kozak, 1991). Conversely increasing the length of the 5'UTR can increase translational efficiency if the sequences are unstructured by increasing the number of 40S ribosomal subunits that can bind to an mRNA at a time (Dever, 2002), however most 5'UTRs are between 20-100 nucleotides (Kozak, 1987a). The upper limit is thought to be in part because an increase in length beyond this is more likely to encompass secondary structures, uAUGs or uORFs (Kozak, 1987a).

Start site context

Start site context influences the efficiency of translation by affecting the ability of the small ribosomal subunit to recognise the initiation codon (Kozak, 1987b). Consequently, context not only plays a role in recognising the initiator codon (AUG or non-AUG) at the start of the open reading frame but also at upstream uORFs and AUGs and this contributes to amount of inhibition mediated by these elements. Non-AUG codons, such as GUG or CUG can sometimes serves as initiation codons but even the most efficient of these (GUG) has only 3-5% the activity of AUG (Kozak, 1989b).

1.4.2.2 3' translation control elements

Elements that affect translation within the 3' untranslated region include the poly(A) tail, microRNA complimentary sequences, regulatory protein binding sites, RNA localisation elements and stability elements.

Poly(A) tail and changes in poly(A) tail length

Like the 5'cap, the poly(A) tail is also a primary determinant of translation efficiency. Messenger RNAs emerge from the nucleus with a poly(A) tail of approximately 250 nucleotides and after translocation to the cytoplasm, the poly(A) tail becomes progressively shorter over time finally signalling the mRNA for degradation (Sheiness *et. al.*, 1975). However, poly(A) tail length can also be subject to tight regulation and mRNAs can undergo both regulated deadenylation and polyadenylation within the

cytoplasm (Richter, 1999). The length of the poly(A) tail is thought to be linked to translation such that polyadenylation leads to translational activation and deadenylation with translational silencing (Wickens *et. al.*, 1997). The main effector protein for the poly(A) tail is the poly(A) binding protein, PABP, which will be discussed in detail below in section 1.5. This control system by poly(A) tail length has been best characterised during oogenesis and oocyte maturation and early development in mice, flies and *Xenopus*.

The signals in the 3'UTR that promote cytoplasmic polyadenylation and deadenylation have perhaps been most intensively studied in *Xenopus* oocytes, but both the elements and the *trans*-acting factors appear to be conserved between species. The signals for cytoplasmic polyadenylation are bipartite; the first part consists a highly conserved AAUAAA sequence that is also required for nuclear polyadenylation (Mendez and Richter, 2001). The second element is a U-rich element that is known as a cytoplasmic polyadenylation element (CPE) and includes diverse sequences but a consensus is UUUUUAU (Mendez and Richter, 2001). In addition to directing active polyadenylation the CPE, can promote deadenylation (Kim and Richter, 2006) and can also recruit a translation-repressive complex (de Moor and Richter, 1999). The activities of CPEs are dependent on a RNA-binding protein called CPE-binding protein (CPEB). Repressed mRNAs have multiple CPEs that bind CPEB that have been suggested to recruit a repressor protein named Maskin that binds to eIF4E and prevents its binding eIF4E, thus preventing translation initiation (Stebbins-Boaz *et. al.*, 1999). The switch to polyadenylation is achieved by the phosphorylation of CPEB on serine 174, by the serine/threonine kinase Aurora A (Sarkissian *et. al.*, 2004), which leads to activation of a CPEB-associated poly(A) polymerase complex that contains the cleavage and specificity factor (CPSF) that binds to the germline development deficient (GLD2) poly(A) polymerase (Barnard *et. al.*, 2004). This polymerase then elongates the poly(A) tail that then binds PABP, which interacts with eIF4G and helps displace Maskin from the repressive Maskin-cap complex, resulting in translational activation.

This system of regulating polyadenylation and translation is especially important in oocyte development, spermatogenesis, early embryogenesis, and some areas of neurons where the majority of gene expression is regulated at the mRNA level (Richter, 1999). Unlike most cell types there is no decay of unadenylated mRNAs in *Xenopus* oocytes through oocyte development and early embryogenesis until the mid-blastula transition (Mendez and Richter, 2001). Messages can be adenylated and deadenylated as needed to modulate translation. It is known that adenylation state is the primary determination of gene expression on most (but not all, see below) mRNAs in oocytes. One of the main activation points of mRNAs with CPEs is oocyte maturation when oocytes re-enter meiosis following fertilisation. Not all mRNAs in oocytes contain CPEs and housekeeping genes lacking CPEs are adenylated and active until oocyte maturation. Following oocyte maturation these mRNAs undergo default deadenylation by a maturation-activated deadenylase that is released from the nucleus upon germinal vesicle breakdown (Wickens, 1990). Therefore overall at oocyte maturation, some poly(A) tails get longer, activating their mRNAs, and some poly(A) tails get shorter, deactivating their mRNAs.

MicroRNAs as translational repressors

MicroRNAs (miRs) are small (approximately 22 nucleotides) regulatory RNAs that inhibit the translation of the mRNAs to which they bind (Pillai *et. al.*, 2007). These micro-RNAs are anti-sense but do not form perfect duplexes with their target sites, which are often found in the 3'UTR of mRNAs (Pillai *et. al.*, 2007). miRs are thought to bind to their target mRNAs as part of a large RNA/protein particles known as 'microRNA containing ribonucleoprotein particles' or miRNPs (Pillai *et. al.*, 2007). MicroRNAs inhibit protein synthesis as miRNPs containing Argonaute family proteins but the mechanisms of regulation remain highly controversial. A number of studies have reported that these miRNP complexes block the initiation phase by a mechanism that requires a functional cap (Humphreys *et. al.*, 2005). Whilst other studies have implicated the elongation phase (Olsen and Ambros, 1999), the observation that miRNPs migrate in heavy pseudo-polysomal like complexes (Thermann and Hentze, 2007) may underline some of the disparity of opinion.

A novel class of small non-coding mRNAs have been identified in the mouse male germline that have been termed piwi-interacting RNAs (piRNAs) (Aravin *et. al.*, 2006) (Girard *et. al.*, 2006). piRNAs are longer than miRs and are typically around 30 nucleotides in length (Aravin *et. al.*, 2006) (Girard *et. al.*, 2006). piRNAs are associated with MIWI, a spermatogenesis-specific PIWI subfamily member of the Argonaute protein family, and depend on MIWI for their biogenesis and/or stability (Grivna *et. al.*, 2006). A subpopulation of these piRNAs associate with polysomes, suggesting a potential role in translational regulation (Grivna *et. al.*, 2006).

Protein complexes

Binding sites for regulatory proteins are also present within the 3'UTR. Unlike their 5' counterparts these sequences are normally unstructured and are thought to recruit protein complexes rather than single factors, which are often sufficient to achieve regulation within the 5'UTR. This suggests that regulation within the 3'UTR may be mechanistically more complex, perhaps due in part because of its relative distance from the site of initiation. Proteins bound to the 3'UTR can be translational repressors, translational activators, stability factors or a combination.

An example of a repressor complex is the control of *Drosophila hunchback* mRNA by a complex containing the Nanos protein. *hunchback* encodes a transcription factor that is required to be expressed differentially across early *Drosophila* embryos to establish the anterior/posterior axis. To ensure proper axis establishment Hunchback protein must only be expressed in the posterior of the embryo however, maternal *hunchback* mRNA is present uniformly throughout the embryo and its translation would result in too high a concentration of Hunchback protein in the anterior of the embryo. Because of this translation of non-posterior localised *hunchback* mRNA has to be inhibited. *hunchback* mRNA is repressed via the Nanos protein binding sites known as nanos response elements (NREs) in the 3'UTR. Interestingly, Nanos does not bind to the NRE directly, instead an adaptor protein, Pumilio, does so and then recruits Nanos to the NRE and thus represses translation (Wickens *et. al.*, 2002). Maternal *nanos* mRNA is localised to the

embryonic posterior, thus establishing a gradient of Nanos protein from posterior to anterior, to ensure Hunchback is only expressed in the anterior. The second cofactor is Brain Tumour, which is recruited jointly via interactions with Pumilio and Nanos (Edwards *et. al.*, 2003).

Another well-characterised translational repression system is that of 15- lipoxygenase (LOX) in erythroid precursor cells, where control of gene expression often occurs at the level of translation as the nucleus is lost during the course of erythroid differentiation. The function of LOX, which attacks intact phospholipids, is to participate in the breakdown of the internal membranes, such as those of mitochondria, during reticulocyte maturation. The mRNA encoding LOX is synthesized in the early stages of erythropoiesis, but is only activated for translation in peripheral reticulocytes when it is required. Until then its expression controlled by multiple CU-rich elements, known as DICE, in the 3'UTR that are bound by and repressed by a complex of two proteins, hnRNP K and hRNP E1 (Ostareck *et. al.*, 1997). This 3'UTR bound complex represses translation by preventing the 60S subunit from joining the mRNA (Ostareck *et. al.*, 2001).

During the final stage of spermatogenesis, where nuclear restructuring of the nucleus is taking place in the differentiating spermatid, the expression of a number of important proteins are controlled at the level of translation. Translational control is important during this stage as transcription terminates during mid-spermiogenesis (Kierszenbaum and Tres, 1978). During the restructuring of the nucleus the histone proteins that package the DNA are replaced with smaller packaging proteins, first with the transition proteins and later with protamines. The 3'UTRs of the mRNAs that encode the protamine 1 and 2 proteins contain *cis* acting RNA elements that regulate their temporal expression in the spermatogenic pathway by repressing the mRNAs until a change in phosphorylation releases the inhibitor (Hecht, 1998; Kwon and Hecht, 1993). These elements are amongst those bound by the Testis Brain-RNA-binding protein (TB-RBP), which can bind to conserved sequence elements in a large number of translationally regulated testicular proteins (Hecht, 1998).

Localisation elements

3'UTRs often contain both translation repression and mRNA localisation elements that are often in close proximity. For some RNAs translational control is coupled to mRNA localisation to achieve proper temporal and spatial expression. An example of this system is the *Drosophila oskar* mRNA, which is silenced during transport to its site of action in the oocyte (Gunkel *et. al.*, 1998). The localisation and translation of *oskar* mRNA is thought to be mediated by a RNA binding protein named Staufen, which has distinct domains that mediate localisation and translation separately (Micklem *et. al.*, 2000). It has been demonstrated by investigation of a *Drosophila kinesin heavy chain* mutant that the Staufen-*oskar* mRNA complex is localised by the Kinesin motor protein, as in the mutant the complex is not transported as normal (Brendza *et. al.*, 2000). During localisation this mRNA is repressed by a protein named Bruno that binds to sequences in the 3'UTR that are required for this translational repression, named Bruno responsive elements (BREs) (Castagnetti *et. al.*, 2000). Loss of Bruno-mediated repression is not sufficient for full Oskar activation and the functions of several proteins that are located in the posterior of the oocyte contribute to its full activation (Wilson *et. al.*, 1996).

Stability elements

Rapid turnover of an mRNA will affect the amount available for translation and thus gene expression. There are a number of sequence elements that contribute to the control of mRNA stability by either stimulating or inhibiting degradation. AU-rich elements (AREs) are sequence elements found in the 3'UTR of a number of mRNAs that are rich in adenosine and uridine bases and are known to target mRNAs for rapid degradation. AREs are bound by ARE-binding proteins (ARE-BPs) of which there are many examples, with AUF1, HuR and Triestetrapolin being the most extensively studied (Gonzalez *et. al.*, 2007). Some of these proteins promote mRNA stabilisation whilst others prompt mRNA turnover by recruiting components of the degradation machinery (Gonzalez *et. al.*, 2007). The mechanism of ARE-dependent degradation is an initial deadenylation event followed by decapping and degradation by exonucleases. The principal degradation pathway in mammalian cells is by a large multi-protein complex

called the exosome that contains 3'-5’ exonucleases rather than 5’ to 3’ via Xrn1 homologues (Barreau *et. al.*, 2005).

In addition to a reduction in protein synthesis as a result of mRNA turnover, AREs can also repress the translation of the mRNAs that contain them (Barreau *et. al.*, 2005). The inhibitory effects of ARE-BPs on translation are thought to be mediated by the deadenylation of the mRNA leading to a loss of PABP and thus a destabilisation of the end to end complex (Barreau *et. al.*, 2005).

1.4.2.3 Regulation of translation by both the 5’ and 3’UTR

Some translational control systems operate via both the 5’ and 3’UTR. The *Drosophila male-specific-lethal* 2 (*msl-2*) gene products are vital for the hyper-transcription of the single male X chromosome and the dosage compensation needed to balance gene expression (Bashaw and Baker, 1997). In females dosage compensation does not occur due to a lack of MSL-2 that is caused by the inhibition of the msl-2 mRNA by the female-specific RNA-binding protein Sex-lethal (SXL). The SXL protein partially inhibits gene expression by affecting splicing but also affects translation directly with the protein binding sites in the 5’ and 3’UTR. SXL binding the 3’UTR inhibits the recruitment of the 43S subunit to the mRNA (Beckmann *et. al.*, 2005) in an association with another RNA binding factor, UNR (upstream of N-ras) (Duncan *et. al.*, 2006). 43S complexes escaping this block and binding to the 5’UTR are further challenged by SXL bound to the 5’UTR where it interferes with the scanning process thus further inhibiting translation initiation in a so called “fail-safe” mechanism (Beckmann *et. al.*, 2005).

1.5 The poly(A) binding protein family

As mentioned in section 1.3.4 the poly(A) tail is a primary determinant of translation efficiency with its length thought to be linked to translation such that polyadenylation leads to translational activation and deadenylation leads to translational silencing. The main effector protein for the poly(A) tail is the poly(A) binding protein, (PABP) a protein defined by its ability to bind to poly(A) tails.

In *Xenopus laevis* oocytes, the poly(A)-tail can be functionally replaced by tethering either PABPC1 or ePABP to unadenylated reporter mRNAs (Gray *et. al.*, 2000) showing that the poly(A) tail primarily provides a scaffold for recruiting these proteins. In oocytes, PABPs are also thought to protect mRNAs from deadenylation (Voeltz and Steitz, 1998), and over expression of *Xenopus* PABPC1 during maturation delays the deadenylation that would normally be silenced (Wormington *et. al.*, 1996).

1.5.1 The PABP family

There are six cytoplasmic PABPs, four of which (PABPC1, tPABP, PABP4, ePABP) share a similar structure, a further cytoplasmic PABP (PABP5) has a distinct structure, and the final cytoplasmic PABP (ePABP2) shares a very different structure with the one nuclear PABP (PABPN1). The general structure of the PABP family members are represented in figure 1.4.

PABP1	RRM1	RRM2	RRM3	RRM4	P-rich	PABPC
tPABP	RRM1	RRM2	RRM3	RRM4	P-rich	PABPC
PABP4	RRM1	RRM2	RRM3	RRM4	P-rich	PABPC
ePABP	RRM1	RRM2	RRM3	RRM4	P-rich	PABPC
PABP5	RRM1	RRM2	RRM3	RRM4		
PABPN1		RRM				
ePABP2		RRM				

Figure 1.4: General structure of PABP family members
PABP1 contains four non-identical RNA recognition motifs (RRMs) linked by an unstructured proline-rich region (P-rich) to a globular C-terminal domain (PABPC). tPABP, PABP4 and ePABP maintain a similar structure to PABP1. By contrast, PABP5 does not contain either the proline-rich linker or the PABPC domain. PABPN1 and ePABP2 contain only one RRM and have a long acidic N-terminus and a shorter arginine-rich C-terminus.
(Figure adapted from (Gorgoni and Gray, 2004)).

Nuclear PABP and ePABP2

Nuclear PABP has a structure (predicted by amino acid sequence) that is very different to that of most of the cytoplasmic PABPs. It only has one RNA recognition motif (RRM) and an arginine-rich carboxy-terminal domain (Kuhn and Wahle, 2004). Nuclear PABP functions in the polyadenylation of mRNAs in the nucleus. It binds to nascent poly(A) tracts of 11-14 adenosines (Meyer *et. al.*, 2002) and along with CPSF, it forms a complex with poly(A) polymerase (PAP) and stimulates PAP to switch from distributive to processive, continuous synthesis (Bienroth *et. al.*, 1993). PABPN1 monomers continue to bind the poly(A) tail until it reaches its full length of approximately 250 nucleotides (Keller *et. al.*, 2000). This binding is accompanied by the formation of a 21nm spherical particle that is thought to consist of the growing poly(A) tail and bound sequential molecules of PABPN1 (Keller *et. al.*, 2000). These particles have been suggested to act as a ‘molecular ruler’ that dictates the final length of the poly(A) tail; the particles encompass a stable complex until the tail reaches

approximately 250 nucleotides at which point the whole adenylation complex destabilises and PAP can no longer add any more nucleotides (Keller *et. al.*, 2000).

A second PABP that shares the structure of PABPN1 was identified in *Xenopus*, and was named ePABP2. Unlike PABPN1 that is expressed ubiquitously in the nucleus, this protein is predominantly expressed during early embryogenesis and the oocyte (Good *et. al.*, 2004) and is cytoplasmic. It was suggested that ePABP2 may play a similar role to PABPN1 but in cytoplasmic, rather than nuclear, polyadenylation.

PABPC1

The primary cytoplasmic PABP is called PABPC1, though when the term “PABP” is used it normally refers to this protein (it is also referred to as PAB, PAB1 and PABP1). The poly(A) binding protein (PABP1) is a 68kDa protein characterised by its ability to bind to the poly(A) tails of mRNAs. PABP binds specifically to poly(A), requiring a minimum of 12 residues to bind but covering about 25 nucleotides when bound to a long section of poly(A) (Gorgoni and Gray, 2004). It contains four N-terminal RNA recognition motifs (RRMs) separated by short linker sequences. PABP1 also contains a C-terminal region that does not bind RNA and is thought to largely mediate protein-protein interactions (Kozlov *et. al.*, 2001). This C-terminal domain (PABC) is composed of 5 α-helices and is linked to the RRMs by a proline- and glutamine- rich linker region (Gorgoni and Gray, 2004). The PABC domain together with the linker region is sometimes referred to as the PABP C-terminal region (PABPCt).

PABPC1 is often regarded as being ubiquitously expressed but experimental data suggests that though it is widely expressed there may be differences in expression levels between different tissues. Northern blots suggest that human PABPC1 is expressed highly in heart, lung, liver (Yang *et. al.*, 1995), testis and ovary (Feral *et. al.*, 2001). Compared to other tissues it is less highly expressed in brain, skeletal muscle (Yang *et. al.*, 1995), and colon (Feral *et. al.*, 2001). A northern blot of mouse PABP1 showed low levels of expression in muscle, liver and brain (Kleene *et. al.*, 1994).

Traditionally antibodies to PABP have not discriminated between the family members and have likely been cross reactive to multiple PABPs, making expression analysis at the protein level unreliable with regards to which PABP is expressed where. However a current project seeks to address this issue but is still in the initial stages (Ross Anderson and Hannah Burgess, unpublished).

tPABP

A testis specific PABP (tPABP, also known as PABP3) has also been identified, originally in mouse (Kleene *et. al.*, 1994) but subsequently in human (Feral *et. al.*, 2001). The gene coding for this protein is thought to have derived from a retrotransposition of the PABPC1 gene (Kleene *et. al.*, 1998). The gene is intronless and codes for a protein with high homology (92.5%) to PABPC1 (Feral *et. al.*, 2001).

Unlike PABP1 that is widely expressed, northern blot analysis has shown tPABP to be restricted to testis in humans (Feral *et. al.*, 2001), though the experiment only queried eight tissues. RT-PCR followed by southern blotting has suggested a similar result in mice (Kleene *et. al.*, 1998). tPABP was also shown to be localised to round spermatids in human adult testis sections (Kleene *et. al.*, 1998). Northern blots of fractionated testis cells suggested that tPABP RNAs are present in spermatocytes and round spermatids but are absent in elongating spermatids in mouse testis (Kleene *et. al.*, 1994).

This gene's distinct expression profile from PABPC1 may indicate that the protein has either functional differences or a different biological role. The subcellular localisation seen in the testis would imply a direct function in spermatogenesis.

PABP4

PABP4, (also known as inducible PABP and PABPC4) was originally described as an mRNA upregulated in activated T-cells and as a protein expressed on the surface of activated platelets (Yang *et. al.*, 1995). PABPC4 also shares homology with PABPC1 but is more divergent sequence than tPABP; the human proteins share 89% identity in the RRMs (Sladic *et. al.*, 2004) and 45% in the PABPC domain (Okochi *et. al.*, 2005).

The expression pattern of PABPC4 awaits to be determined at the protein level but it appears to be more widely expressed than tPABP by northern blotting (Yang *et. al.*, 1995).

ePABP

ePABP was initially identified in *Xenopus* (Voeltz *et. al.*, 2001) and also appears to be present in mammals (Seli *et. al.*, 2005; Wilkie *et. al.*, 2005). ePABP has been shown to be highly expressed in *Xenopus* laevis oogenesis and embryogenesis (Cosson *et. al.*, 2002) and in ovary and testis in mouse with levels well exceeding that of PABPC1(Seli *et. al.*, 2005; Wilkie *et. al.*, 2005). As the dominant PABP in oogenesis this PABP may play a significant role in this process. ePABP appears to have the same general structure as PABPC1 but is the most divergent of the similar cytoplasmic PABPs (PABPC1, tPABP, PABPC4 and ePABP) and shows most divergence in RRM3 and the proline rich linker region (Voeltz *et. al.*, 2001).

PABP5

PABP5 is encoded by a gene located on the X chromosome identified in humans and mice and like tPABP appears to be mammalian specific. It differs from the first three PABPs mentioned here in that is lacks the PABPCt domain. A low level of expression has been noted in various human tissues at the RNA level and a higher level of expression seen in the ovary (Blanco *et. al.*, 2001). It has not been investigated further and its function is currently unknown.

1.5.2 PABP functions

Not all the PABPs have an identified function; PABPC1 has been shown to function in mRNA translation and stability in the cytoplasm. Of the other cytoplasmic PABPs only ePABP has also been shown to function in translation (Wilkie *et. al.*, 2005), tPABP, iPABP, PABP5 and ePABP2 have all yet to be examined, though ePABP, ePABP2, tPABP, iPABP have all been shown to bind to poly(A).

1.5.2.1 The function of PABP in translation

The poly(A) tail is a major determinant of translational efficacy and PABP is the effector molecule for the poly(A) tail (Kuhn and Wahle, 2004) but the exact mechanism of PABP stimulation of translation is unclear.

In yeast based cell-free experimental systems a complex of PABP and poly(A) binds to eIF4G, which interacts in turn with eIF4E bound to the cap (Tarun and Sachs, 1995). Atomic force microscopy showed that such a complex formed from purified proteins could be visualised as a ring structure with 3' and 5' binding portions interacting and the RNA forming a loop between them (Wells *et. al.*, 1998) and also that these components could stimulate the translation of a reporter RNA *in vitro* (Tarun and Sachs, 1995). A model for translational stimulation via PABP, in which PABP circularises mRNAs it binds to and promotes stable 48S complex formation thus enhancing translation, was formulated on the basis of these results. This model of action was enhanced by the discovery that the PABP-eIF4G interaction enhances the binding affinity of PABPC1 for poly(A) (Le *et. al.*, 1997) as measured by electro mobility shift assay. The PABP-eIF4G interaction also increases eIF4E affinity for the cap as shown by using a rabbit reticulocyte lysate *in vitro* system to examine the potential of cap analogue to displace eIF4E from a reporter in the presence and absence of PABP (Borman *et. al.*, 2000).

In vitro coimmunoprecipitation assays have shown that PABP also interacts with a protein called the PAB-interacting protein (PAIP1) (Craig *et. al.*, 1998). Subsequently, Paip1 interacts with eIF4A as shown by further coimmunoprecipitations and was shown to stimulate translation of a reporter mRNA in cultured mammalian cells (Craig *et. al.*, 1998). The interaction with eIF4A was proposed to stimulate translation possibly by stimulating the RNA unwinding activity of eIF4A in the 5'UTR of an mRNA and thus enabling more efficient ribosome joining (Craig *et. al.*, 1998). eIF4A is known to be important for translation of all mRNAs, as dominant-negative mutants of eIF4A repress translation in a rabbit reticulocyte translation system (Pause *et. al.*, 1994b). PABP also interacts with eIF4B as demonstrated by Far Western blotting of proteins purified from

wheat germ extracts (Le *et. al.*, 1997) and *in vitro* coimmunoprecipitations (Bushell *et. al.*, 2001). The interaction of PABP with eIF4G had a positive effect on the ATPase and RNA helicase activity of a complex of eIF4A, eIF4B and eIF4G as shown by an ATPase and helicase assay respectively using purified proteins (Bi and Goss, 2000). On the basis of these results it was suggested that PABP may enhance the rate of RNA unwinding in translational initiation thus enhancing translation (Bi and Goss, 2000).

Though the *in vitro* evidence for PABP's role as a translational activator gives some information, its role in living cells is less clear. It has been suggested that yeast PAB is important for translation *in vivo*: Yeast *pab1* mutations show synthetic lethal interactions with eIF4E mutations indicating that they interact *in vivo* (Tarun *et. al.*, 1997). Other evidence for PABP's role *in vivo* again came from yeast with depletion of PAB in *S. cerevisiae* by promoter inactivation that resulted in the inhibition of translation initiation (Sachs and Davis, 1989).

Tethered function assays in *Xenopus* oocytes also provide direct support for a role of PABP as a stimulator of translation. PABP tethered to the 3'UTR of a reporter mRNA stimulates translation of that reporter in *cis* and can do so without the presence of a poly(A) tail (Gray *et. al.*, 2000). Tethered function assays also reveal that multiple and separate parts of the *Xenopus* PABP protein can stimulate translation with RRMs 1-4, 1-2 and 3-4 all being able to stimulate translation as well as the full length protein (Gray *et. al.*, 2000). This experiment implies that the interaction of PABP with eIF4G is not the only mechanism by which PABP stimulates translation as RRMs 3-4 are unable to bind eIF4G but still stimulate the reporter. This is consistent with reports that PABP can stimulate translation through multiple mechanisms (Kahvejian *et. al.*, 2005).

PABP has not just been implicated in stimulating 40S joining; effects of PABP binding on 60S ribosomal subunit joining have also been reported (Sachs and Davis, 1989). A yeast genetics approach showed that the absence of eIF5B or a defect in eIF5, proteins involved in 60S joining, specifically reduced the translation of a polyadenylated RNA, suggesting that poly(A), and therefore PABP may promote a role in 60S joining

(Searfoss *et. al.*, 2001). It was further suggested that PABP may act in this manner by inhibiting two putative RNA helicases (genes *SKI2* and *SLH1*) that otherwise inhibit eIF5 and eIF5B (Searfoss *et. al.*, 2001). A role for PABP in 60S joining is supported by experiments in a mammalian cell-free system where it was demonstrated by sucrose gradient analysis that depletion of PABPC1 leads to reduced 80S complex formation in addition to reduced 43S complex formation (Munroe and Jacobson, 1990), though mechanisms of this action remain unclear. A second *in vitro* system using PABP depleted mammalian cell extracts showed both 48S and 80S complex formation was inhibited by the depletion of PABP and identified stronger inhibition (approximately twofold) of 80S complex formation and argued that PABP plays a direct role in 60S subunit joining (Kahvejian *et. al.*, 2005).

PABP also interacts with a eukaryotic release factor, eRF3, as shown by yeast-two hybrid assays and *in vitro* GST coimmunoprecipitations (Hoshino *et. al.*, 1999). It was suggested that PABP may link translational termination to translational initiation, thus promoting ribosomal recycling (Uchida *et. al.*, 2002) but no exact mechanism has been proposed.

1.6 The Dazl family

Dazl is a member of a gene family of RNA binding proteins called the DAZ family. DAZ was first described as a candidate factor underlying spermatogenesis defects in infertile men (Reijo *et. al.*, 1995; Reynolds and Cooke, 2005). It is located on a region of the Y chromosome deleted in roughly 10-15% of men who fail to produce sperm (azoospermia) in the ejaculate; and is referred to as the azoospermia factor region, or AZF (Vogt *et. al.*, 1996). Subsequent studies have shown that this region contains several candidate genes that could be the cause of the genetic defect (Foresta *et. al.*, 2001). Two RNA binding proteins were identified from this region; the first was named RBMY (RNA-binding motif) (Ma *et. al.*, 1993) and is believed to play a role in splicing, based on finding that it co-localises with splicing factors as assessed by co-

immunohistochemistry on human testis sections (Elliott *et. al.*, 1998). The second protein was identified in a study examining microdeletions of the AZF region in patients suffering from spermatogenic failure (Reijo *et. al.*, 1995). This gene was named Deleted in Azoospermia (DAZ). Both DAZ and RBMY are present in multiple copies within this region (Chai *et. al.*, 1997; Yen *et. al.*, 1997).

1.6.1 Evolutionary distribution

Analysis of the DAZ sequence has identified a family of related proteins that has three members, DAZ, Dazl and Boule, known collectively as the Dazl family of proteins. These proteins are found in many different species across evolution ranging from invertebrates to humans but not all species have the same complement of family members (Haag, 2001; Xu *et. al.*, 2001). DAZ is restricted to humans and Old World monkeys (Shan *et. al.*, 1996). An autosomal homologue of DAZ is found on chromosome 3 in humans called Dazl (DAZ-Like). This gene is also found in other vertebrates, including the popular laboratory models mice, *Xenopus laevis* and zebrafish (Cooke *et. al.*, 1996; Houston *et. al.*, 1998; Maegawa *et. al.*, 1999). An ancestral autosomal homologue, called Boule, has also been identified, originally in *Drosophila* (and is the only DAZ family member found in *Drosophila*) (Eberhart *et. al.*, 1996). Boule has also been shown to exist in mouse, human and worm, it is also predicted to exist in zebrafish and *Xenopus laevis* (Karashima *et. al.*, 2000; Xu *et. al.*, 2001). It has been proposed that Boule is the original representative of the family and that DAZL arose from Boule in an ancestor of vertebrates (Haag, 2001). The DAZ gene is thought to have originated from a duplication-transposition of the DAZL gene to the Y-chromosome of an ancestor of today's Old World monkeys (Foresta *et. al.*, 2001). Repeated amplification of the DAZ gene has lead to most men having four DAZ genes, though about 2% of the male population have only two DAZ genes due to a 1.6-Mb deletion (Repping *et. al.*, 2003). The multiple copies of the DAZ genes are not all identical and they vary in both the number of RNA Recognition Motifs (RRMs) and DAZ repeats (Saxena *et. al.*, 2000; Yen *et. al.*, 1996). Western blotting of human testis

extracts with a DAZ-specific antibody has only detected one band, suggesting that not all of the human DAZ genes are translated (Reijo *et. al.*, 2000; Slee *et. al.*, 1999).

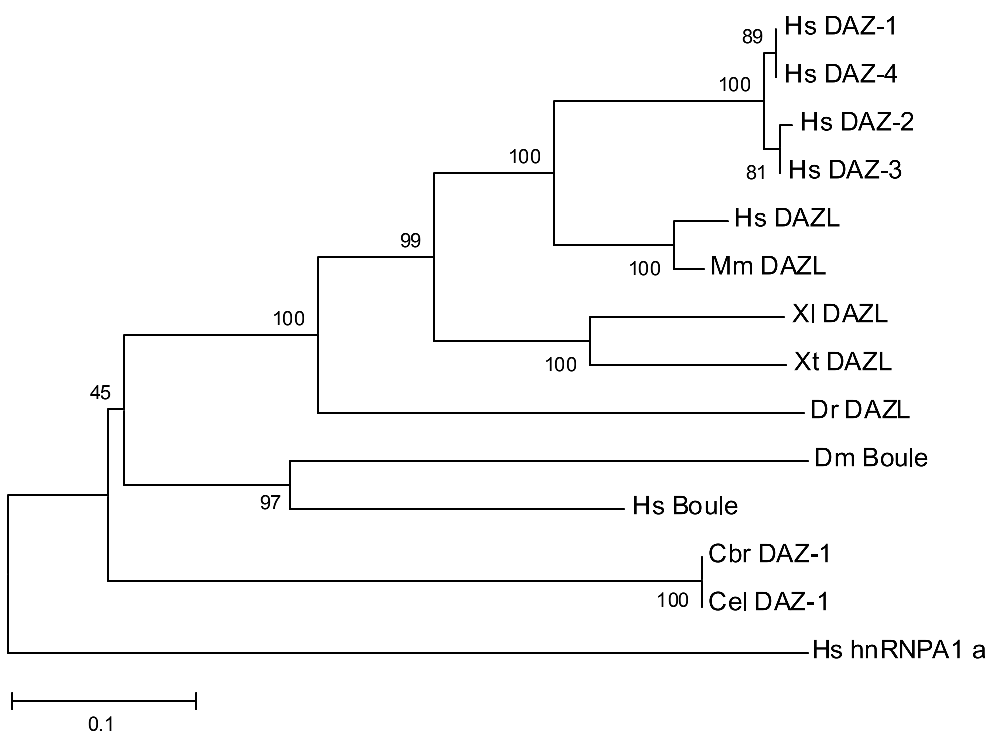


Figure 1.5: Phylogenetic tree based on the protein structure of Dazl family genes. Hs = *Homo sapiens*. Mm = *Mus muscularis*. Xl = *Xenopus laevis*. Xt = *Xenopus tropicalis*. Dr = *Danio rerio*. Ce = *Caenorhabditis elegans*. Dm = *Drosophila melanogaster*. Tree routed to human hnRNPA1.

1.6.2 Dazl family protein structure

The structure of Dazl family proteins has not been solved but they contain two recognisable motifs, an RNA recognition motif and a DAZ motif. RRM motifs are found in many RNA-binding proteins including those involved in mRNA splicing, translation, localisation and stability. Solution of the structure by NMR of a number of RRMs bound to RNA have illustrated how the face of the protein that does not interact with RNA can, in a number of investigated cases, be utilised for protein-protein interactions to mediate effects on RNA utilisation. For example RRMs 1-2 of PABP have been shown, when bound to RNA, to form a globular structure composed of anti-

parallel β-sheets backed by two α-helices (Deo *et. al.*, 1999). This structure allows one face of the domain to bind RNA, leaving the other face free for protein-protein interactions (Deo *et. al.*, 1999). Whilst Dazl and Boule only contain a single RRM, some copies of human DAZ contain two RRMs (Moore *et. al.*, 2004). However, as mentioned above, not all the human DAZ genes appear translated (Reijo *et. al.*, 2000; Slee *et. al.*, 1999), leaving the relevance of DAZ proteins with multiple RRMs unclear. A number of studies have demonstrated that Dazl proteins are capable of binding RNA and have sought to identify the RNA binding specificity of Dazl proteins (see RNA targets). The DAZ motif, which appears unique to this family, consists of repeats of a 24 amino acid sequence rich in glutamine, proline and tyrosine and is believed to mediate protein-protein interactions and polysome association (Tsui *et. al.*, 2000b). The number of DAZ repeats varies between different family members, DAZ proteins can contain between 8-24 copies of the repeat (Foresta *et. al.*, 2001) although DAZL and Boule only possess a single repeat. Homology between Boule and DAZL/DAZ is essentially limited to the RRM (78% similar) and the DAZ repeat (50% similar). DAZL and DAZ are similar through most of their lengths apart from the difference in the number of DAZ repeats; DAZL also has an extended C-terminal region after its DAZ repeat that is not present in the DAZ protein.

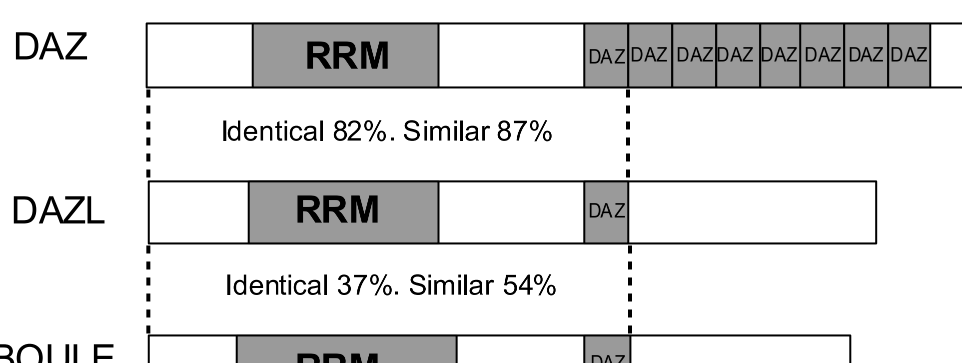


Figure 1.6: Protein structure of Dazl family.
The structures are based on the amino acid sequence of the human proteins. DAZ contains one RNA recognition motif (RRM) and 8 DAZ repeats (DAZ). DAZL is overall 50% identical and 57% similar to DAZ, but is much more conserved up to the end of its first DAZ repeat (82% identical, 87% similar) thereafter the two proteins begin to differ with DAZL lacking the multiple DAZ repeats that DAZ contains but having a extended C-terminal region that is not present in the DAZ protein. BOULE has the same general structure as DAZL with one DAZ repeat and the extended C-terminal region. However the sequence of BOULE is much more divergent from DAZL with an overall 30% identity and 44% similarity. Again the area containing the RRM and DAZ repeat is the most conserved with a 37% identity and 54% similarity.

1.6.3 Expression pattern of Dazl family members

The expression patterns of the different Dazl family members are not all the same. Often their expression patterns overlap but are not identical suggesting the possibility that the different proteins may have separate functions in gametogenesis and may interact with different protein partners or mRNA targets in doing so. This section will discuss what is known about the expression pattern of Dazl family genes in different species.

Invertebrates; *Drosophila* and *C. elegans*

As mentioned above invertebrates only possess one Dazl family gene, which is homologous to human *BOULE*, which confusingly in *C. elegans* is called *daz-1*. *daz-1* has been shown to be expressed in the gonad of *C. elegans* by whole mount in situ worms hybridisation (Karashima *et. al.*, 2000). *daz-1*started expression in the distal mitotic region of the gonad, began to accumulate in the meiotic transition zone, and

reached the maximum level in the proximal pachytene region (Karashima *et. al.*, 2000). In *Drosophila* Boule protein is expressed in testis as shown by Western blot and immunofluorescent staining, with expression being observed in pre-meiotic and meiotic spermatocytes and post-meiotic spermatids, (Cheng *et. al.*, 1998). An isoform of Boule has also been found expressed in the nervous system of *Drosophila* using immunohistochemistry (Joiner and Wu, 2004), which represents a possible role for a Dazl family member outside of the germline.

Xenopus and zebrafish

Xenopus laevis Dazl (Xdazl) RNA expression was first identified by northern blot in adult testis and ovary (Houston *et. al.*, 1998). In situ hybridisations found Xdazl RNA to be localised to the vegetal cortex of oocytes, exclusively in the germ plasm of early embryos and to be expressed in the spermatogonia and spermatocytes of *Xenopus* testis (Houston *et. al.*, 1998). The presence of Xdazl protein expression in the ovary and testis was later confirmed by Western blotting (Mita and Yamashita, 2000). Xdazl expression was further characterized and was shown to be present in all stages of male and female germ cells except mature spermatozoa (Mita and Yamashita, 2000). In embryogenesis Xdazl protein is not localised to any specific region in early-stage embryos (in contrast to the strict localization of its mRNA in the germ plasm) and disappears after gastrulation before reappearing in the primordial germ cells situated at the genital ridge (Mita and Yamashita, 2000).

Zebrafish Dazl (zDazl) mRNA has been shown to be expressed in the gonads of both sexes by northern blot, in ovary it was localised in the region of oocytes that becomes the germ cells using in situ hybridisations (Maegawa *et. al.*, 1999). Further experiments of the same type revealed that the *zDazl* gene is expressed in spermatogonia and spermatocytes but not in secondary spermatocytes, spermatids or mature sperm (Maegawa *et. al.*, 2002).

Mouse

Mouse Dazl (mDazl) transcript was detected predominantly in the testis, but also at lower levels in ovaries (Reijo *et. al.*, 1996). In testes of wildtype mice, Dazl transcription is detectable 1 day after birth, then increases steadily as spermatogonial stem cells appear before it plateaus as the first wave of spermatogenic cells enters meiosis and then is sustained thereafter (Reijo *et. al.*, 1996). Immunostaining with an anti-mouse Dazl antibody on sectioned testis showed that mDazl protein is expressed in the germ cells with spermatogonia and early and late spermatocytes identified as positive for Dazl (Ruggiu *et. al.*, 1997). Dazl protein was also identified in germ cells in the ovary by the same method (Ruggiu *et. al.*, 1997).

In contrast to the work of Riejo in 1996, a further study detected Dazl transcripts before birth (Seligman and Page, 1998). Northern blotting of developing gonads in mouse detected Dazl transcripts at 12.5 days post coitum (dpc) in male and female gonads, during which period the only germ cells present are primordial germ cells (Seligman and Page, 1998). Dazl transcript was also detected by RT-PCR in testis and ovary RNA samples isolated from 14.5 dpc mouse embryos (Brekhman *et. al.*, 2000).

Both the *Boule* transcript and the Boule protein were found to be confined to the testis, however its cellular distribution is different to that of other family members, with Boule not being expressed in the mitotic germ cells but instead being restricted to the meiotic cells (Xu *et. al.*, 2001).

Human

In humans, Dazl transcript was detected by RT-PCR in testis and ovary RNA samples isolated from human foetuses at approximately 23 weeks gestation (Brekhman *et. al.*, 2000). RNA in-situ hybridisation of human embryonic ovary from the same time point found Dazl transcripts in oogonia, oocytes and granulosa of primordial follicles in ovary and in germ cells in human testis, also from foetuses at approximately 23 weeks gestation (Brekhman *et. al.*, 2000). Immunohistochemistry was used to show that

DAZL is expressed in embryos in germ cells of girls and in mature oocytes (Dorfman *et. al.*, 1999).

Dazl family proteins are expressed only in germ cells, in fact DAZ protein was investigated using an antibody for immunostaining and was found to be expressed in the spermatogonial stem cells, spermatocytes and prenatal primordial germ cells in humans but is absent from late spermatocytes or postmeiotic cells (Moore *et. al.*, 2004). DAZL is expressed in spermatogonia, primary and secondary spermatocytes, spermatids and postmeiotic cells, and also in foetal germ cells (Reijo *et. al.*, 2000; Ruggiu and Cooke, 2000; Ruggiu *et. al.*, 1997). DAZL transcript and protein has also been detected in mature human spermatozoa (Lin *et. al.*, 2002). BOULE protein is expressed in spermatocytes, persists through meiosis and decreases in early spermatids in humans, no expression is seen in spermatogonial stem cells, as determined by immunostaining of sectioned tissue (Luetjens *et. al.*, 2004; Xu *et. al.*, 2001).

1.6.4 Knock out phenotypes and effects in humans

It has been suggested by knock-out and knock-down phenotypes that the DAZ family have a common function in germ cell formation in a variety of species, although clear differences in the phenotypes exist. This section will examine the phenotypic effects of Dazl family disruption in model organisms and also the effects seen in humans.

Invertebrates: *Drosophila* and *C. elegans*

In *Drosophila*, disruption of *boule* results in a block at the first meiotic division during spermatogenesis (Eberhart *et. al.*, 1996). Interestingly, no effect on oogenesis has been reported in *Drosophila*, despite the fact that no other family members are present.

In *C. elegans* only the female line appears affected, loss of function mutants in daz-1 leads to an eventual arrest at pachytene during oogenesis (Karashima *et. al.*, 2000), and knock-down in *C. briggsae* prevents the sperm/oocyte switch in hermaphrodites (Otori *et. al.*, 2006).

Xenopus

Xdazl has been shown to be essential for primordial germ cell proliferation and migration as specific depletion of maternal Xdazl mRNA by injecting an antisense DNA oligonucleotide results in tadpoles lacking, or severely deficient in, primordial germ cells (PGCs) (Houston and King, 2000). Immunostaining of primordial germ cell markers in embryos revealed that in Xdazl depleted embryos, PGCs are lost at the late tailbud stage, at or near the time when they begin their dorsal migration to the site of the gonads (Houston and King, 2000).

Mouse

In mice knocking-out Dazl leads to loss of germ cells and the complete absence of gamete production in both sexes. In females, this germ cell loss has been characterised by looking at ovary sections; in the *Dazl* null mice the ovaries appear relatively normal at 15 dpc but by 19 dpc there is a marked deficiency of oocytes, and many of those remaining are degenerating and being absorbed (Ruggiu *et. al.*, 1997). It was also noted that in the absence of Dazl expression no follicles form in the mouse ovary (McNeilly *et. al.*, 2000). A later study investigated the ovary at 17.5 dpc and showed that germ cell loss had occurred at this point (Saunders *et. al.*, 2003).

In male *Dazl* null mice 19 dpc embryo, testis histology reveals that the ratio of somatic Sertoli cells to germ cells raises from the 4:1 ratio seen in wild type individuals to 8:1 (Ruggiu *et. al.*, 1997). This male germ cell loss occurs at a late prenatal stage and the postnatal testes have very low levels of germ cells and even these arrest early during the spermatogenic differentiation, before meiosis (Schrans-Stassen *et. al.*, 2001). Another study on the same mice strain discovered that some surviving germ cells could reach the leptotene stage of meiotic prophase I before arresting (Saunders *et. al.*, 2003). It was concluded that in the absence of Dazl germ cells can embark on functional differentiation but cannot progress through the meiotic prophase (Saunders *et. al.*, 2003). A later study investigated the influence of genetic background on the nature of the meiotic arrest seen in Dazl knockout mice (Lin and Page, 2005). Here the knockout was

bred onto a C57BL/6 background. The effect of this was loss of germ cells at embryonic stage e14.5, earlier than in previous studies, indicating that genetic background has a clear effect on Dazl knockout phenotype in mice.

It should be noted that, although the phenotypes observed in male and female mice occur at different times and stages during development, the timings of cell death in the ovary is at the point at which meiosis is starting and that this phenotype parallels the situation in the male. The difference in the time of onset of the phenotype reflects the difference in the timing of entry into meiosis in the male and female germ cells (see section 1.2.1).

Human

As mentioned earlier in humans azoospermia is associated with loss of the region of the Y chromosome that contains DAZ, the AZF (Tiepolo and Zuffardi, 1976). The AZF is commonly divided into three (Vogt *et. al.*, 1996), and the region containing DAZ, the AZFc region, has been found to be much more likely to be deleted in azoospermic men than the other regions with 71% missing AZFc compared with 13% missing AZFa and 31% missing AZFb (Ferlin *et. al.*, 1999). Though it must be considered that the sample size investigated was limited to 180 men and that although DAZ is a strong candidate for the AZFc azoospermia factor it has not been formally proven. In a separate study loss of DAZ gene was shown to be related to problems with spermatogenesis ranging from azoospermia to oligospermia (Reijo *et. al.*, 1995).

Since the initial observations that loss of *DAZ* causes problems with spermatogenesis the situation has become more complicated with the knowledge that the area of the Y chromosome that contains the AZF is prone to mutation, recombination and deletions (Repping *et. al.*, 2006). Therefore some of the variations in the phenotypes seen in deletions of the AZF regions could be explained by genetic variations in the locus deleted, with different haplotypes having different implications for an AZF deletion with different specific *DAZ* genes being present before or after the deletion. Deletion of the gene cluster containing *DAZ1* and *DAZ2* is responsible for severe oligospermia (Fernandes *et. al.*, 2002) whereas deletion of *DAZ2* alone does not appear to have any

affect on fertility (Fernandes *et. al.*, 2004). Deletion of both *DAZ3* and *DAZ4* is common and has deleterious effect on fertility (Fernandes *et. al.*, 2004). Different ethnic groups are also typified by different deletions or modifications in the AZF region. For example, partial duplication at AZFc on the Y chromosome is a risk factor for impaired spermatogenesis in Han Chinese in Taiwan (Fernandes *et. al.*, 2004) and certain Chinese men possess a Y chromosomal haplotype that increases the chance of a complete AZFc deletion (Zhang *et. al.*, 2007).

The evidence for links between the other Dazl family members and infertility in humans is less compelling. A lack of BOULE protein was detected in the testis biopsies with meiotic arrest by immunochemistry (Luetjens *et. al.*, 2004), but mutations in the BOULE gene were not found and the absence of the protein could have been due to the meiotic arrest rather than the cause. A genetic study investigating women with premature ovarian failure and infertile men has linked mutations in *DAZL* to these conditions (Tung *et. al.*, 2006), though no functional work was undertaken.

In all cases humans studies have been limited to observations of gene or protein loss and associated problems with fertility. Due to the difficulty of using humans as an experimental system no molecular evidence of causality has been presented, but taken together these results strongly support a critical and evolutionarily conserved role for these proteins in gametogenesis.

Dazl family redundancy

There is a partial but not complete redundancy within the Dazl family of proteins. Human DAZ (Slee *et. al.*, 1999) or DAZL (Vogel *et. al.*, 2002) can partially rescue the phenotype of a Dazl knock-out mice and *Xenopus* dazl (Xdazl) and human BOULE have both been shown to partially rescue the *Drosophila* BOULE phenotype (Houston *et. al.*, 1998) (Xu *et. al.*, 2003). However this redundancy is limited, for example a Dazl knockout mouse still has Boule but the gametogenesis failure phenotype is still apparent. However the degree of overlap in expression within the testis is yet to be determined. Thus this could be caused by a dosage effect or differential expression. An alternative

explanation is that the different family members have partially distinct functions or targets.

1.7 Dazl family function

Genetic studies have shown that the Dazl family proteins are important in gametogenesis but their exact molecular functions remain unclear. As a RRM has been identified in all the Dazl family proteins it has been suggested that at least one function of Dazl must be as an RNA binding protein. Their predominantly cytoplasmic localisation also suggests that their role is probably a cytoplasmic one. This was confirmed in elegant experiments in *Drosophila* where the nuclear localisation of Boule was disrupted without affecting entry into meiosis (Cheng *et. al.*, 1998)

The ability of Dazl proteins to bind RNA (Houston *et. al.*, 1998) and their presence within germ cells in a variety of species (see section 1.4.3) suggested a potential role in gene regulation in these cells. It has been suggested that Boule acts in the cytoplasm to regulate the stability or translation of messenger RNA encoding an essential meiotic factor (Cheng *et. al.*, 1998). In invertebrates the first genetic link to a role in translational regulation came from experiments in *Drosophila* where Boule was shown to be required for the translation of *twine* mRNA (Maines and Wasserman, 1999). However, these studies did not address whether this was a direct effect mediated by binding of Boule to this mRNA. More recently, *C. elegans* daz-1 was shown to bind FBF mRNAs and loss of function mutations lead to a modest reduction in the accumulation of these proteins (Otori *et. al.*, 2006). In vertebrates, Tsui et al initially demonstrated that mouse Dazl could be found on actively translating polysomes (Tsui *et. al.*, 2000b) from testis suggesting a likely role in mRNA translation or stability, a finding that was later confirmed for zebrafish dazl transfected into somatic cell lines (Maegawa *et. al.*, 2002).

1.7.1 Dazl family members as translational regulators

A direct role for a variety of vertebrate DAZL proteins in translation was shown by tethering these proteins to reporter mRNAs in *Xenopus* oocytes (Collier *et. al.*, 2005). It was demonstrated that DAZL family proteins can interact with PABP proteins providing a potential model for their roles as translational activators (Collier *et. al.*, 2005). A conserved interaction between DAZL proteins including Xdazl, mDazl, hDAZL, hDAZ and hBOULE and two poly(A)-binding proteins (PABP1 and ePABP) was detected by yeast-two hybrid analysis (Collier *et. al.*, 2005). Both of these PABP proteins are present in the *Xenopus* oocytes in which the DAZ proteins above were shown to stimulate translation in tethered assays making them attractive candidate factors. Directed yeast two-hybrid analysis demonstrated that Xdazl did not interact with a panel of other canonical translation initiation factors including subunits of eIF1, eIF1A, eIF2, eIF2B, eIF3, eIF4A, eIF4E, eIF4G, eIF4H, eIF5, eIF5A and eIF5B. Co-immunoprecipitation experiments using isolated stage VI oocytes or testis extracts showed RNA-independent interactions between endogenous Xdazl and PABP1 and ePABP, demonstrating a true protein-protein interaction. This interaction was shown to be mediated by the C-terminus of *Xenopus* PABP. This region of *Xenopus* PABPs does not show a strong capacity to stimulate translation in isolation, in contrast to other parts of the protein. Thus simultaneous interactions of PABP with DAZL and key factors required for its role in promoting initiation would not be precluded. The interaction with PABP was mediated by amino acids 99-166 of mouse Dazl. Deletion of this PABP binding domain was shown to completely abrogate the stimulatory effect of Dazl in the tethered function assay although these mutants were still bound to mRNA via the tether protein.

It was suggested that the DAZL family of proteins act as translational enhancers of specific mRNAs during gametogenesis (Collier *et. al.*, 2005). A mechanism for translational stimulation by Dazl was proposed following these results and a working model suggested. The model is as follows; Dazl binds to the 3'UTR of its target mRNAs, it then recruits molecules of PABP thorough direct protein-to-protein interactions (Collier *et. al.*, 2005). These molecules of PABP then stimulate translation

initiation through multiple interactions with translation initiation factors as demonstrated previously (Gray *et. al.*, 2000).

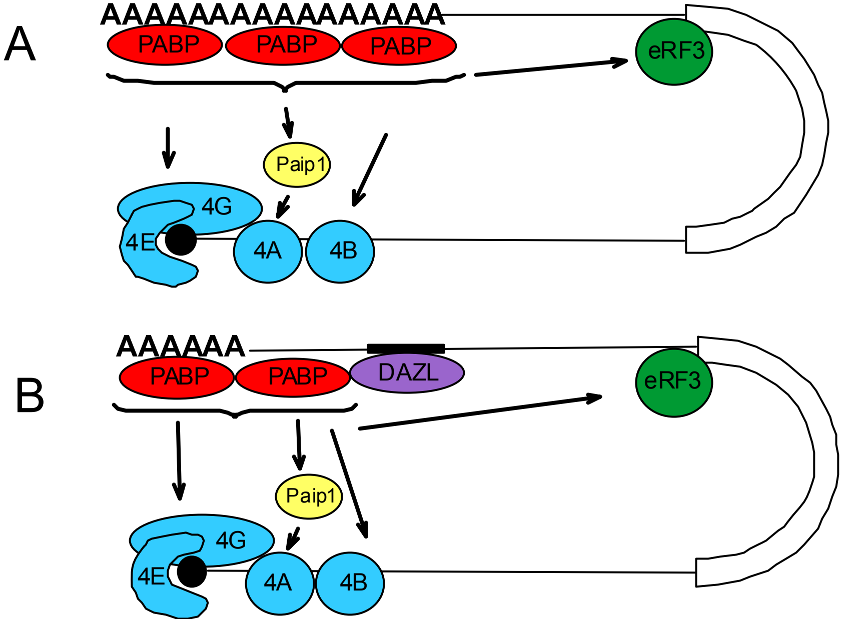


Figure 1.7: Models for the role of PABPs in DAZL-mediated stimulation of target mRNAs.
A: Molecules of PABP interact with other translation factors, circularising the mRNA and promoting translation.
B: Dazl family members directly recruit additional molecules of PABP to mRNAs with short poly(A) tails, increasing end-to -end complex formation, leading to enhanced ribosomal subunit recruitment (not shown). Multiple DAZL proteins may recruit multiple PABPs. This does not require changes in polyadenylation. Model adapted from Collier et al (Collier *et. al.*, 2005).

Figure 1.7 represents the model for translational stimulation by Dazl. Dazl binds to a sequence within the 3'UTR of the mRNA and thus brings extra molecules of PABP to the mRNA. These extra PABP molecules then interact with eIF4B, eIF4G and Paip1, and perhaps other factors, thus stimulating translation of the mRNA. The key concept of this working model is the ability of Dazl to bind to PABP and which results in translational stimulation.

The model proposed for Dazl action leads to the prediction that Dazl proteins should stimulate the translation of mRNAs with short poly(A) tails more than those with long poly(A) tails, because long poly(A) tails will already have multiple PABP molecules

bound (Collier *et. al.*, 2005). To test this prediction Collier et al utilised two reporters identical except for the fact that one had a poly(A) tail and the other did not. Mouse Dazl stimulated both of these reporters and the mRNA with the poly(A) tail gave the highest levels of translation. However it was the mRNA without the poly(A) tail that was stimulated to the greatest degree by mDazl.

During gametogenesis many stored mRNAs have relatively short poly(A) tails and are translationally inactive (Thompson *et. al.*, 2007). This phenomenon has been extensively studied in the oocytes of *Xenopus* and these changes in polyadenylation have been shown to be critical for oocyte maturation in both *Xenopus* and mouse (Gebauer *et. al.*, 1994; Sheets *et. al.*, 1994). Polyadenylation of these mRNAs in discrete waves during gametogenesis has been shown to lead to translational activation of many mRNAs, due to the recruitment of PABP (Belloc *et. al.*, 2008). However, not all activated mRNAs undergo polyadenylation and recruiting PABP via protein-protein interaction may provide an explanation for the activation of some of these mRNAs (Collier *et. al.*, 2005). Cytoplasmic polyadenylation has also been shown to have more extensive roles in gametogenesis, with CPEB knock-out mice showing defects at the pachytene stage of meiosis in males and females (Tay and Richter, 2001). Conditional knock-outs showed cytoplasmic polyadenylation is also important at the dictyate stage of oocyte growth and for oocyte-follicle signalling (Racki and Richter, 2006).

1.7.2 mRNA targets

The confirmation of the function of Dazl as a translational regulator was made more difficult by the lack of identified targets of Dazl, either *in vitro* or *in vivo*. This section will examine some potential targets of Dazl family proteins.

A potential target of *Drosophila* boule was identified in a genetic study based on the observation that the *twine* mutant had a similar phenotype to *boul*e mutants. *twine* encodes a meiosis specific Cdc25C phosphatase that activates cyclin B1 that is required for the G2/M transition of meiosis. In *boule* mutants the level of Twine protein, but not

the *twine* mRNA, is significantly reduced (Maines and Wasserman, 1999). Furthermore, expression of Twine from a spermatocyte specific transgene led to the partial rescue of the *boule* mutant (Maines and Wasserman, 1999). However a direct interaction between Boule protein and *twine* mRNA remains to be demonstrated.

Drosophila twine mRNA was shown to be able to recruit zDazl using reporter mRNAs containing the *twine* 3'UTR. In this cell-free assay zDazl was found to mildly stimulate this reporter dependent on a 'GUUC' sequence in this UTR (Maegawa *et. al.*, 2002; Maegawa *et. al.*, 1999). An interaction between CDC25A (the mammalian homologue of Twine) and BOULE has been suggested to be conserved in humans. A study showed that there was a correlation between a lack of BOULE and a lack of CDC25A by immunochemistry in human testis samples (Luetjens *et. al.*, 2004). However no causality was established so this interaction still remains to be convincing in any system.

In *C. elegans* DAZ-1 protein was shown by co-immunoprecipitation and electromobility shift assays to bind specifically to the mRNAs encoding the FBF proteins, which are translational regulators for the sperm/oocyte switch (Otori *et. al.*, 2006). The authors went on to present data that they claimed showed that the level of the FBF proteins was lowered in the *daz-1* mutant thus implying a direct translational effect, but the data was unconvincing.

Other potential targets of mouse Dazl have been identified from screens using immobilised recombinant GST-Dazl fusions that were incubated with mouse testis extracts to bind mRNAs that were then identified by differential display (Jiao *et. al.*, 2002). This method identified a number of mRNAs including Tpx-1, which is a testicular cell adhesion protein essential to the progression of spermatogenesis, GRSF-1, an mRNA binding protein involved in translational activation and TRF2, a TATA box binding protein involved in transcriptional regulation (Jiao *et. al.*, 2002). A similar screen was undertaken in human testis looking for mRNAs that could be bound by DAZL and one of its protein partners, PUM-2 (see below). Immobilised fusion proteins were washed with human testis mRNA extract before bound mRNAs were eluted,

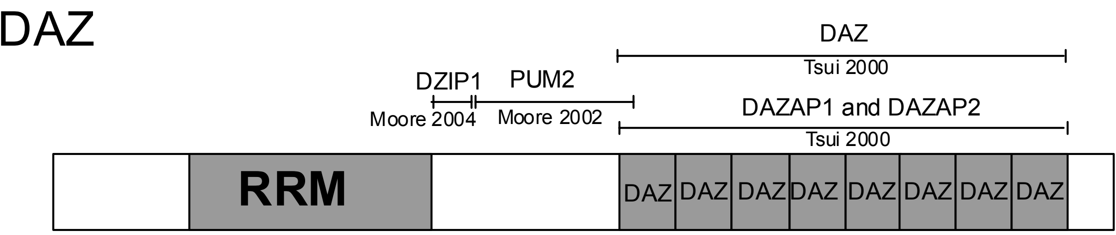
amplified by RT-PCR and sequenced (Fox *et. al.*, 2005). 61 possible targets were identified but only one was investigated in more detail, SDAD1, which is homologous to the *sda1* (*severe depolymerisation of actin*) gene in yeast. The value of this sort of screen remains to be seen as none of the interactions have been verified *in vivo*, though they produce plenty of possible avenues of investigation.

A recent screen using a combination of microarray and immunoprecipitation analysis has identified a number of mRNA targets of mouse Dazl (Maratou *et. al.*, 2004). Two of these targets have been explored in detail: The first of these targets is the mouse vasa homologue (*Mvh*) (Reynolds *et. al.*, 2005), a gene that is known to be essential for male gametogenesis. In particular, the *Mvh* knockout phenotype is a block at leptotene to zygotene of meiotic prophase I (Tanaka *et. al.*, 2000), a phenotype that corresponds to that of the Dazl knockout mouse (Saunders *et. al.*, 2003). The second characterised target is the synaptonemal complex protein 3 (Sycp3). This protein is essential for gametogenesis with a mouse knockout model showing a block in meiotic prophase that was similar to the Dazl knockout mouse. It was shown using *Xenopus* oocyte reporter assays that Dazl stimulates translation via both the *Mvh* and *Sycp3* 3'UTRs (Reynolds *et. al.*, 2007; Reynolds *et. al.*, 2005). It was also shown via immunofluorescence of testis sections that germ cells of Dazl null mice contain reduced levels of both proteins, indicating that Dazl mediated regulation of these mRNAs contributes to changes in their protein expression (Reynolds *et. al.*, 2007; Reynolds *et. al.*, 2005).

1.7.3 Dazl interacting proteins

The potential functions of Dazl family proteins in translation and some of the mRNA targets have been discussed. The importance of the interaction with PABP, was discussed in section 1.5.1, however Dazl family members are also known to interact with a number of proteins. This section will explore what is known about these partners.

The evidence for Dazl protein-protein interactions is summarised in figure 1.6 and table 1.1. Figure 1.8 shows the protein interactions that have been mapped to a specific area of a Dazl family member and table 1.1 details both mapped and unmapped interactions and also indicates the experimental methodologies employed to verify these interactions.



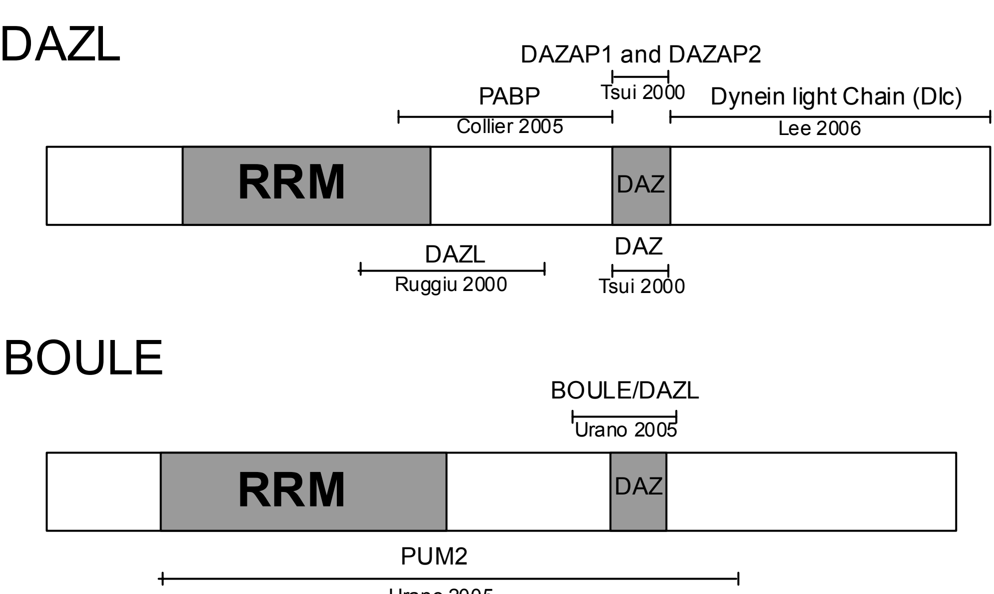


Figure 1.8: Known protein partners for the Dazl family proteins.
Proteins known to interact with Dazl family members are shown for Dazl, DAZ and BOULE. Note that this figure is not limited to one species, but instead maps all interactions across species. The area of the protein required to bind known protein partners for each of the Dazl family members are shown as horizontal lines.

Protein	Interacts with	Interaction Mapped?	Methods used	Reference
Hs_DAZ	Hs_DAZ	Yes	GST	(Tsui *et. al.*, 2000a)
	Hs_PUM2	Yes	Y2H, CoIPo, IH	(Moore *et. al.*, 2003)
	Hs_DZIP1	Yes	Y2H, CoIPo, IH	(Moore *et. al.*, 2004)
	Hs_DAZAP1	Yes	Y2H, GST	(Tsui *et. al.*, 2000a)
	Hs_DAZAP2	Yes	Y2H, GST	(Tsui *et. al.*, 2000a)
	Hs_DZIP2	No	CoIP	(Moore *et. al.*, 2004)
	Xl_PABP1	No	Y2H	(Collier *et. al.*, 2005)
Hs_DAZL	Hs_DAZ	Yes	GST	(Tsui *et. al.*, 2000a)
	Hs_DAZL	Yes	Y2H, GST	(Ruggiu and Cooke, 2000)
	Xl_PABP1	Yes	Y2H	(Collier *et. al.*, 2005)
	Hs_DAZAP1	Yes	Y2H, GST	(Tsui *et. al.*, 2000a)
	Hs_DAZAP2	Yes	Y2H, GST	(Tsui *et. al.*, 2000a)
Hs_BOULE	Hs_DAZL	Yes	Y2H	(Urano *et. al.*, 2005)
	Hs_BOULE	Yes	Y2H, CoIPo	(Urano *et. al.*, 2005)
	Hs_PUM2	Yes	Y2H, CoIPo	(Urano *et. al.*, 2005)
	Xl_PABP1	No	Y2H	(Collier *et. al.*, 2005)
Mm_Dazl	Xl_PABP1	Yes	Y2H	(Collier *et. al.*, 2005)
	Xl_ePABP	Yes	CoIPo	(Collier *et. al.*, 2005)
	Mm_Dlc	Yes	GST, CoIPo, CoIPe	(Lee *et. al.*, 2006)
Xl_Dazl	Xl_PABP1	No	Y2H	(Collier *et. al.*, 2005)
	Xl_ePABP	No	CoIPe	(Collier *et. al.*, 2005)
	Xl_PUM2	No	CoIPo	(Padmanabhan and Richter, 2006)
Ce_DAZ-1	Ce_CPE-3	No	CoIPo, IH	(Hasegawa *et. al.*, 2006)

Table 1.1: Dazl family member interacting proteins
Species specific protein interactions with Dazl family protein members and the methods by which these interactions were discovered are detailed.

Definitions: Y2H = Yeast two hybrid. CoIPe = Co-immunoprecipitation with endogenous proteins. CoIPo = Co-immunoprecipitation with over expressed proteins. GST = GST pulldown assays. IH = immunohistochemistry. Hs_DAZ = Human DAZ. Hs_DAZL = Human Dazl. Hs_BOL = human BOULE. Mm_dazl = Mouse Dazl. Xl_dazl = *Xenopus laevis* dazl. Ce_DAZ-1 = *C. elegans* DAZ-1.

DAZAP1 and DAZAP2

Using DAZ as bait in a two-hybrid screen two novel interacting proteins were identified (Tsui *et. al.*, 2000a). These proteins were named DAZ-associated protein 1 and 2 (DAZAP1 and DAZAP2). DAZAP1 is an RNA binding protein that shuttles from the nucleus to the cytoplasm during spermatogenesis (Dai *et. al.*, 2001), and has also been isolated as Prrp in *Xenopus* oocytes (Zhao *et. al.*, 2001), as a protein implicated in the localisation of mRNAs. This Dazl family interacting protein is the focus of chapter 5 of this thesis and will be described in more detail then.

Very little is known about DAZAP2, it lacks any recognisable functional domains and has been shown to be expressed ubiquitously at the RNA level by northern blot (Tsui *et. al.*, 2000a). This expression pattern makes it an unlikely partner of the germ cell specific Dazl family but so little is known about it that it is impossible to comment further on any possible functions.

Pumilio

Human DAZ was first identified to interact with human PUMILIO-2 (PUM-2) in a yeast-two hybrid screen (Moore *et. al.*, 2003). This interaction was shown to require amino acids 124-173 of DAZ (see figure 1.6). PUM-2 was shown to be expressed in human embryonic stem cells and germ cells and it has also been shown that it co-localised with DAZ and DAZL in germ cells (Moore *et. al.*, 2003). The ability to interact with PUM-2 was also established for human BOULE, as demonstrated by yeast-two hybrid and *in vitro* co-immunoprecipitation (Urano *et. al.*, 2005).

Overexpressed *Xenopus* Pumilio was also shown to interact with overexpressed tagged *Xenopus* Dazl from *Xenopus* oocytes by co-immunoprecipitation (Padmanabhan and

Richter, 2006). Pumilio was also shown to co-immunoprecipitation with *Xenopus* ePABP in the same system. It was suggested that Pumilio, Dazl and ePABP could all interact together and modulate the expression of a newly identified potential target mRNA named *RINGO/Spy* (Padmanabhan and Richter, 2006). However all the interactions were observed separately and it was not established if such a multi-protein complex is formed on the mRNA at any point.

Pumilio proteins have well characterised functions in germ cell line maintenance and differentiation and have been previously shown to control translation as part of a multi-protein complex (Wickens *et. al.*, 2002). They have typically been associated with translational repression of specific mRNAs. The function of the interaction between PUM-2 and Dazl has yet to be investigated. It is possible that it could act as a translational repressor of Dazl or it could be involved in aiding the binding of Dazl to an mRNA as it does for Nanos in *Drosophila* (Wickens *et. al.*, 2002).

DZIP

Another novel protein that interacts with DAZL is (Moore *et. al.*, 2004) DAZ-interacting protein or DZIP. This protein was identified in a yeast 2-hybrid screen (Moore, 2003) as one that interacted with human DAZ, and confirmed by *in vitro* co-immunoprecipitation (Moore *et. al.*, 2004), though the Western blot data was unconvincing. The DZIP gene is expressed predominantly in human embryonic stem cells and foetal and adult germ cells (Moore *et. al.*, 2004). Some immunohistochemical evidence has been produced suggesting that DZIP co-localises with both DAZ and DAZL in mouse testis but no functional characterisation has been undertaken. The lack of further information on DZIP is such that it will not be investigated further in this thesis, though there is potential for a possible function in translation with Dazl that may form the basis of future work.

Dynein light chain

The C-terminal portion of human Dazl has been shown to interact with the dynein light chain, a component of the dynein-dynactin motor complex, initially on the basis of *in*

vitro co-immunoprecipitations with over expressed proteins that were later verified by co-immunoprecipitations of endogenous proteins from testis extracts (Lee *et. al.*, 2006).

It was suggested that this could indicate that Dazl has a RNA localisation function and this hypothesis was investigated by immunohistochemical investigation of Dazl in cultured cells in the presence and absence of inhibitors of microtubules. It was discovered that Dazl was localised to specific areas of a cell, in a manner that was dependent on intact microtubules, suggesting that Dazl could indeed function in RNA localisation in concert with dynein (Lee *et. al.*, 2006). Such a function in RNA transport could be important for Dazl's overall function of ensuring that its target mRNAs are expressed in the correct place at the correct time for their potential roles in gametogenesis.

CPEB

In *C. elegans* DAZ-1 (BOULE homologue) was shown to be co-expressed with and interact with CPB-3 (shown by immunohistochemistry and co-immunoprecipitation respectively), a homologue of CPEB (Hasegawa *et. al.*, 2006). It was suggested that this may have relevance to DAZ-1's proposed function in gametogenesis in *C. elegans* but no function was determined experimentally (Hasegawa *et. al.*, 2006).

1.8 Thesis aims

The observation that multiple Dazl family members from a variety of species can stimulate the translation of specific mRNAs suggests that this conserved function is likely to be critical to their roles in oogenesis and spermatogenesis. Consequently, it is of interest to understand how they stimulate translation. As the functions of mRNA specific translational activators are poorly understood, few if any paradigms exist for the function of Dazl proteins. The observation that Dazl stimulates translation via recruitment of PABP still leaves many important questions unanswered. PABP proteins are thought to enhance translation by multiple mechanisms (Gray *et. al.*, 2000) (Kahvejian *et. al.*, 2005), in keeping with the finding that multiple domains of this protein (Gray *et. al.*, 2000) can stimulate translation and interact with a wide variety of basal translation factors (Gorgoni and Gray, 2004). It is unclear whether DAZL bound PABP maintains all its interactions with other translation factors and which of the steps of initiation DAZL stimulates. Moreover, this observation does not rule out the possibility that other Dazl-interacting factors may aid or co-operate with PABP to promote Dazl-mediated stimulation.

The results of this thesis address three separate but related aims pertaining to the molecular mechanism of Dazl-mediated translation:

i) To examine the stage and factors required for Dazl-mediated translational stimulation.
ii) To delineate the residues within DAZL that are important for its interaction with PABP.
iii) To investigate the role of DAZAP1 in DAZL-mediated regulation.

Chapter 2: Materials and Methods

2.1 Solutions and reagents

All chemicals and reagents used during this project were supplied by either Sigma or Roche unless detailed otherwise. All solutions used were as described in Molecular Cloning – A Laboratory Manual (Sambrook *et. al.*, 1989). General solutions were prepared by technical staff.

2.2 General Microbiological techniques

2.2.1 Bacterial strains used

All plasmids were propagated in the *E. coli* strain XL1-Blue (Stratagene), genotype = *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)]*.

2.2.2 Growth of Bacterial strains

Bacterial strains were maintained on Luria Bertani (LB) agar plates or in LB medium containing appropriate antibiotics (Kanamycin at 50mg/ml or Ampicillin at 100mg/ml).

2.2.3 Bacterial transformations

Chemically competent XL1-Blue cells were thawed on ice before the addition of approximately 100ng of plasmid to 50µl cells. Cells were then incubated on ice for 30min before being heat shocked at 42°C for 45sec, and returned to ice for a further 2min. 200µl of LB medium was added before incubating the cells for 60min at 37°C, after which 100µl of the cells were plated on LB plates containing the appropriate antibiotics to select for the desired plasmid. Plates were then grown overnight at 37°C.

2.2.4 Yeast strains used

Yeast two-hybrid analysis was performed with *S. cerevisiae* strains L40ura$^-$ *(MATa,ura3-52, leu2-3,112, his3, trpΔ1, ade2, Δgal4, lys::(lexAop)-HIS3, ura3::(lexAop)-LacZ)* (Zhang *et. al.*, 1999) and Mav99 *(MATa ura3-52, leu2-3, 112, trpl-901, his3A200, ade2-101, gal4A, gal80A canlR*, *cyh2R GAL1::HIS3@LYS2 GALJ::lacZ, SPAL10::URA3)* (Vidal *et. al.*, 1996) as described previously (Zhang *et. al.*, 1999).

2.2.5 Growth of yeast strains

All yeast strains were initially grown in YPD medium or on YPD agar plates at 30°C. Yeast transformed with plasmids containing selectable markers (tryptophan (trp) and/or leucine (leu)) were grown on selective minimal media (SD + 1 x dropout mix, + 1 x histidine, + 1 x tryptophan, + 1 x uracil) without trp or leu as appropriate at 30°C.
Yeast amino acids stocks: 10 x Dropout = 200mg arginine, 300mg isolucine, 300mg lysine, 200mg methionine, 500mg phenylalanine, 2000mg threonine, 300mg tyrosine, 1500mg valine in 1l water. 100 x histidine stock = 100mg histidine in 50ml water. 100 x tryptophan stock = 100mg tryptophan in 50ml water. 100 x uracil stock = 100mg uracil in 50ml water.

2.2.6 Yeast transformations

Single colonies of the appropriate yeast strain were used to inoculate 5ml of YPD broth and incubated overnight with shaking at 30°C. 1ml of the overnight culture was used to inoculate 50ml of YPD broth that was grown shaking at 30°C until OD_{600} was between 0.6-1.0. 1ml of yeast culture was spun down at 7000rpm (10,000rcf) for 1min for each transformation. The supernatant was removed and the yeast pellet was washed first with 1ml of water and next with 1ml 0.1M LiOAc/1 x TE (10mM Tris, pH 7.5, 1mM EDTA). After washing the pellet was resuspended in 50µl 0.1M LiOAc/1 x TE and 6µl of

salmon sperm carrier DNA (Clontech) with 1μg of each plasmid to be transformed. 300μl of 40% PEG/0.1M LiOAc/1 x TE was then added to each sample, which were then vortexed and incubated at 30°C for 30min. Yeast were then heat shocked in a 42°C water bath for 15min. The yeast was pelleted by spinning at 7000rpm (10,000rcf) for 2min and resuspended in 400μl water. 150μl was then plated onto appropriately selective media.

2.3 General Recombinant DNA techniques

2.3.1 Plasmid propagation and extraction

Plasmid DNA was prepared using Qiagen prep systems as described in the manufacturer's instructions. Mini, Midi and Maxi preps were all employed as appropriate for the volume of plasmid desired. Purity was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and the DNA concentration was measured by UV absorption at 260nm.

2.3.2 Purification of Nucleic Acid

Phenol/Chloroform extraction

DNA was purified by the addition of equal volumes of 25:24:1 phenol:chloroform:isoamyl alcohol (50% buffered phenol, 48% chloroform, 0.5% 3-methyl-1-butanol pH>7.8) followed by vortexing and centrifugation (13,000rpm (16,060rcf), 10min). The top aqueous phase was collected and the DNA was ethanol precipitated.

Ethanol precipitation

DNA was precipitated by the addition of 2.5 volumes 100% molecular biology grade ethanol and 0.1 volumes 3M NaOAc.

2.3.3 Restriction enzyme digests

Restriction digests were conducted using New England Biolabs (USA) or Roche (Germany) restriction enzymes according to manufacturer's specifications using appropriate buffers as supplied. Restriction enzymes never exceeded 10% of the volume to avoid star activity produced by excess glycerol.

2.3.4 Dephosphorylation of digested plasmids

Linearised plasmid DNA for ligations was dephosphorylated using Calf intestinal alkaline phosphatase (Roche) according to manufacturer's instructions. Generally 20μg of cut vector plasmid was dephosphorylated in 100μl total volume with 1 x alkaline phosphatase buffer (Roche) and 20 Units (2μl) phosphatase.

2.3.5 Gel purification of DNA fragments

Plasmid DNA for purification was run on 1-2% agarose gels containing 0.5μl/ml ethidium bromide. The bands were visualised with UV light and cut out of the gel using a clean razor blade. DNA was purified from the gel fragments using the Qiagen Gel Purification Kit according to the manufacturer's instructions.

2.3.6 Ligation of DNA fragments

Ligations of gel purified, dephosphorylated, linearised plasmid and gel purified DNA inserts were performed overnight at 16°C using T4 DNA ligase (Stratagene) according to the manufacturer's instructions. The molar ratio of plasmid to insert was 1:3 unless a different ratio was deemed necessary.

2.3.7 PCR reaction

The standard PCR reaction used (50 µl) contained; 10 ng/µl of template DNA, 5 mM $MgCl_2$ (Roche), 1x PCR reaction buffer (Bioline), 1mM dNTPs, 2.5 units cloned Bio-X-Act Long High-Fidelity polymerase (Bioline), 50pM oligonucleotide primers. Reactions were incubated in a PTC-225 PCR machine (MJ Research) for 30 cycles with a standard annealing temperature of 60°C. PCR products were separated by gel electrophoresis and the bands of the desired product were cut out and gel purified. Standard program: 1) 5mins @ 94°C, 2) 30secs @ 94°, 3) 1min @ 60°C, 4) 1.5min @ 68°C, 5) repeat steps 2-4 30 times, 6) 10min @ 68°C.

2.3.8 Site directed mutagenesis PCR

The method used to generate the mutants was a PCR based site directed mutagenesis approach called overlap extension PCR. This allows the insertion of point mutations into DNA sequences without the need to generate single stranded DNA as in other techniques (Ling and Robinson, 1997). Tthis technique involves designing two mutagenic primers containing the mutation, partially or completely complementary to each other. Each primer is used in a separate reaction with an outer flanking primer designed to one end of the region of interest. The two halves of the amplicon are generated in this manner in two separate reactions and mixed in the next step, where they anneal in the 25-30 bp region of complementarity and prime off each other, to produce the full length product. Finally the mutagenised amplicon is amplified as normal, subcloned and sequenced to ensure that it contains the desired mutations and only the desired mutations. The mutant amplicons can then be inserted into the vector of choice.

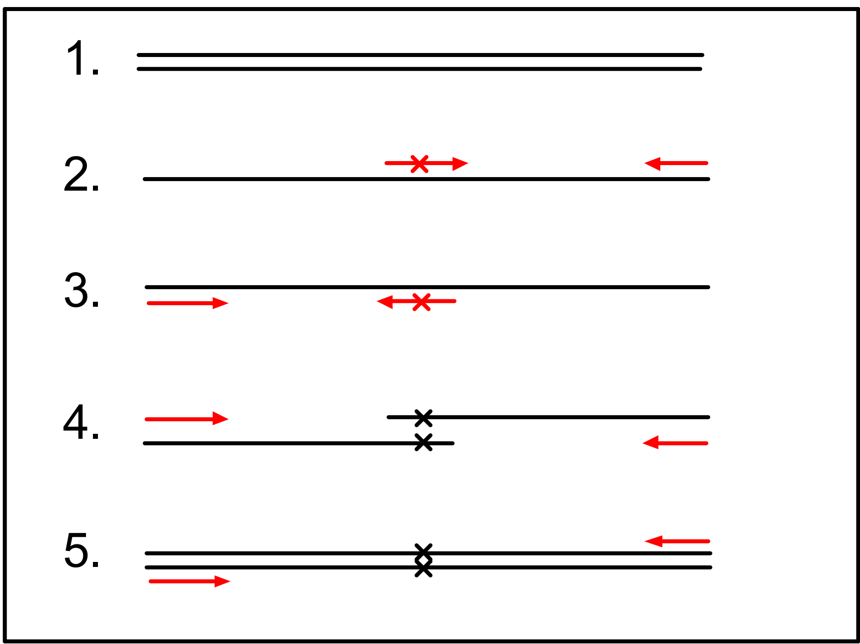


Figure 2.1: Site directed mutagenesis method.
1: original double stranded wild type sequence. 2+3: Two separate PCR reactions are used to generate overlapping fragments containing the mutation (as indicated by X). Primers are represented by red arrows; primers with mutated residues contain an X. 4: The products of the original two reactions are annealed together and the single stranded areas are filled in by a PCR reaction priming off the free ends. 5: The then mutagenised product is then amplified by in a standard manner.

The conditions for the PCR reactions are as follows: First reactions (20µl): 10 ng/µl of template DNA, 100pM oligonucleotide primers, 5mM $MgCl_2$ (Roche), 1x PCR reaction buffer (Bioline), 1 unit Bio-X-Act Long High-Fidelity polymerase (Bioline), 0.2mM dNTPs (Clontech). Reactions were incubated in a PTC-225 PCR machine (MJ Research) for 12 cycles with a standard annealing temperature of 56°C. PCR products were separated by gel electrophoresis and the bands of the desired product were cut out, gel purified and eluted in 20µl of dH_2O. Two reactions (PCR I and PCR II) were conducted for each mutant (see chap 4). Program: 1) 5mins @ 94°C, 2) 30secs @ 94°, 3) 1min @ 56°C, 4) 1min @ 68°C, 5) repeat steps 2-4 12 times, 6) 10min @ 68°C.

Second reactions (50µl): 1.5µl PCR I, 1.5µl PCR II, 5 mM $MgCl_2$ (Roche), 1X PCR reaction buffer (Bioline), 2.5 unit Bio-X-Act Long High-Fidelity polymerase (Bioline), 0.2mM dNTPs (Clontech). Reactions were incubated in a PTC-225 PCR machine (MJ

Research) for an initial 7 cycles with a standard annealing temperature of 56°C. After these initial cycles the reaction was stopped and 100pM oligonucleotide primers were added and the reactions were returned to the PCR machine for a further 28 cycles. Program: 1) 5mins @ 94°C, 2) 30secs @ 94°, 3) 1min @ 56°C, 4) repeat steps 2-4 7 times, 5) 10min @ 68°C, 6) stop and add primers 7) repeat 2-4 7 times, 8) 1min @ 68°C.

2.3.9 Agarose gel electrophoresis

0.8-1.5% agarose gels were made by dissolving appropriate amounts of agarose in 1 x TAE (90mM Tris-HCl pH 8.3, 90mM acetic acid, 2mM EDTA) by heating in a microwave for 1-2min. Gels were poured with ethidium bromide added to a concentration of 0.5µl/ml. 1/6 volume of DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was added to DNA samples before loading. Gels were run at 50-150 volts in 1 x TAE buffer for 30-90min, depending on size of the fragment to be visualized and agarose concentration of the gel. 2-log ladder (New England Biolabs) was used to estimate DNA band sizes.

2.3.10 DNA sequencing

All plasmid constructs were verified using the in-house sequencing service offered at the Human Genetics Unit. Briefly, DNA sequencing was performed using Big Dye Terminator Sequencing Kit (Applied Biosystems (UK)) using an in house protocol modified from the manufacture's instructions with a reduced enzyme concentration.

2.3.11 Quantification of Nucleic acids

DNA or RNA was quantified by measuring its absorbance spectra at 260nm using either a Nanodrop ND-1000 (Nanodrop Technologies Inc.) or an Ultraspec 3000pro (Amersham Biosciences) spectrophotometer. Quality of nucleic acids was assessed via comparing the ratios of absorbance at 260nm vs. 280nm.

2.4 General RNA techniques

2.4.1 *In vitro* transcription

Plasmids to be transcribed were linearised by digestion with restriction enzyme as follows; MS2-mDazl and MS2-U1A were cut with HindIII, pLG-MS2, pCSFV-luc-MS2 and pPV-luc-MS2 were Linearised with BglII. Linearised plasmids were purified by two phenol/chloroform extractions followed by ethanol precipitation prior to transcription.

RNA templates were prepared by T7-RNA polymerase catalysed *in vitro* transcription of linearised plasmids.

The transcription reactions were set up as follows; 0.1μg/μl of template DNA, 1X Transcription Buffer (Stratagene), 30mM DTT, 1mM ATP, 1mM UTP, 1mM CTP (Pharmacia), 7mM m7GpppG Cap (New England Biolabs) or ApppG Cap (New England Biolabs), 0.2 units RNasin (Roche) and 4 units T7 RNA polymerase (Stratagene).

The transcription reaction was incubated for 5min at 37°C in the presence of m7GpppG or ApppG to produce capped RNA. After an initial round of capping 1mM GTP (Pharmacia) was added to allow template elongation. This results in an efficiency of capping greater than 95% (R. Stipecke, personal communication). The reactions were incubated at 37°C for 90min. The reaction mixtures were digested with RNase-free RQ DNase I (Promega) for 25 in at 37°C to remove the template DNA. Synthesised RNAs were purified twice with acidic phenol/chloroform/isoamylalcohol (25:24:1) (Ambion) and passed over Chroma spin-100 DEPC-H_2O columns (Clontech) to remove short, incomplete partially transcribed RNA products and excess ^{7}mGpppG cap and nucleotides. The RNA was then ethanol precipitated and resuspended in 10μl of RNase-free distilled H_2O.

RNA integrity was confirmed by agarose gel electrophoresis. RNA concentration was determined by UV spectrophotometry.

2.5 General Protein techniques

2.5.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE separating gels were made with 10-13.5% polyacrylamide (40:1 acrylamide:bis-acrylamide), 375mM Tris-HCl pH 8.8 and 0.1% SDS. Stacking gels were made with 4% polyacrylamide, 115mM Tris-HCl pH 6.8 and 01% SDS. The gels were polymerised with 1% ammonium persulphate (APS) and 0.1% TEMED.

Samples were mixed 1:1 with 2x SDS-PAGE loading buffer (20% glycerol, 10% 2-mercaptoethanol, 4% SDS, 0.25% bromophenol blue and 160mM Tris-HCl pH 6.8) and heated for 5min at 95°C before immediate storage on ice. Samples were loaded onto the gel alongside a pre-stained Benchmark molecular weight marker (Invitrogen). Gels were run at 150 volts in SDS-PAGE running buffer (50mM Tris, 0.4M glycine and 0.1% SDS. pH 8.8) until the dye front was run off the gel (1-2 hours).

2.5.2 Fixing and drying of 35-S methionine radiolabelled gels

Separating gels were fixed in 10:10 fixative (10% methanol and 10% acetic acid) for 20-30min, then EN^3HANCE (NEN Life Science Products) for 30min. The gels were washed twice in water and dried for 1-2 hours on a Model 583 Gel dryer (Bio-Rad). Gels were then exposed overnight to Kodak film.

2.5.3 Transfer to membrane

After SDS-PAGE stacking gel was discarded and proteins were transferred onto Hybond-P nitrocellulose membrane (Amersham) using a semi-dry transfer apparatus (Millipore). Gels were transferred onto membranes surrounded by two sheets of 2mm blotting paper soaked in transfer buffer (0.25M Tris, 1.92M glycine, 20% Methanol, pH 8.3) either side of the gel/membrane. Proteins were transfer using a fixed current of 200mA for 1 hour.

2.5.4 Western blotting

Membranes with immobilised proteins were blocked in either TBST (Tris-buffered saline (TBS; 20M Tris, 137mM NaCl, pH 7.6, 0.1% Tween 20) supplemented with 5% (w/v) skimmed milk powder or in commercial blocking solution (Bohringer) dependent on the primary antibody used. Primary antibodies were diluted in appropriate blocking solution and incubated with membranes either at 4°C overnight or 1 hour at room temperature on a rotating platform. The membranes were washed three times with TBST for five minutes each wash at room temperature, again on a rotating platform before the addition of the secondary antibody diluted in TBST. The membrane was incubated with the secondary antibody for 1 hour at room temperature. Membranes were washed a further 4 times with TBST before treatment with enhanced chemiluminescence (ECL) solutions (Amersham). Membranes were blotted to remove excess fluid and wrapped in cling film before being exposed onto X-ray film (Kodak).

Antibody	Primary dilution	Secondary antibody
Anti-*Xenopus* Dazl	1:1000	Anti-mouse HRP 1:10000
Anti-DAZAP1-Rabbit1-D109	1:1000	Anti-rabbit HRP 1:10000
Anti-DAZAP1-Rabbit2-D109	1:1000	Anti-rabbit HRP 1:10000
Anti-DAZAP1-Affinity purified	1:1000	Anti-rabbit HRP 1:10000

Table 2.1: Antibodies used

The anti-Dazl antibody was a gift from Masakane Yamashita. The anti-DAZAP antibodies were generated in the course of the thesis (see below). Goat secondary anti-mouse HRP (Dako) and goat anti-rabbit HRP (Sigma) were bought commercially.

2.5.5 Generation of an antibody to DAZAP1

The generation and testing of the anti-DAZAP antibodies is described in more detail in chapter 5. Briefly polyclonal antibodies to *Xenopus* DAZAP1 were raised commercially by Covalab (France). Two rabbits were injected with two peptides corresponding to DAZAP1, GWTGQPPQTWQGYS and SGQQDFPFSQFGNAC over a three month period. Regular bleeds were collected from both rabbits as detailed in chapter 5.

2.5.6 *In vitro* Transcription and translation (TNT)

Transcription/translation (TNT) reactions were conducted utilising a T7 Quick TNT system (Promega) according to manufacturer's instructions. Briefly, 2µg of miniprep DNA was incubated in TNT lysate in the presence of [^{35}S]-methionine for 60min at 30°C. Aliquots of the translation products were separated by SDS-PAGE (see section 2.5.2).

2.5.7 Immunoprecipitation

Where indicated, stage VI *Xenopus laevis* oocytes were incubated for 6 hours with [^{35}S]-methionine (GE Healthcare) to label newly synthesized proteins. Labelled or unlabelled oocytes were lysed in immunoprecipitation lysis buffer (10µl/oocyte) (20mM HEPES [pH 7.6], 10mM KCl, 1.5mM $MgCl_2$, 100mM NaCl, 0.5% Triton X-100, 0.5mM dithiothreitol, Complete Protease Inhibitor Cocktail Tablets (Roche)). The oocyte lysate was cleared by centrifugation. The volume was increased to 1ml with immunoprecipitation lysis buffer and mixed for 1 hour or overnight at 4°C with an anti-PABP antibody or anti-ePABP antibody; 30µl of protein G-Sepharose beads (Amersham

Pharmacia Biotech) was added to the lysate and mixed for 60 to 90min at 4°C. The beads were washed three times with immunoprecipitation lysis buffer. Where indicated, 200U of RNase I (Ambion) was added to the last wash and incubated at 37°C for 15min. Bound material was eluted in 30µl of SDS gel loading dye prior to SDS-PAGE and Western analysis.

2.6 *Xenopus* oocyte techniques

2.6.1 Obtaining *Xenopus* Oocytes

Female *Xenopus laevis* were euthanised by immersing in 400ml water containing a lethal dose of 4g 4-aminobenzoic acid for 15min. Once animals failed to "right" a further lethal dose of 0.4ml Phenobarbital was delivered to the heart. Death was confirmed by exsanguination by removing the head and heart. The ovaries were removed and washed in 1 x MMR buffer (5 mM HEPES, 2 mM KCl, 100 mM NaCl, 0.2 M $MgCl_2$, 0.1 M $CaCl_2$ and 0.1% Pen/Strep).

2.6.2 Microinjection of *Xenopus* oocytes

Stage VI *Xenopus laevis* oocytes were microinjected as described previously (Gillian-Daniel *et. al.*, 1998). Briefly, lobes of a dissected *Xenopus laevis* ovary were torn open using tweezers under a Leica MZ6 dissecting microscope. Individual oocytes were separated from the ovary membrane by mechanical scraping with a metal loop. Stage VI oocytes were manually sorted from others based on their size and appearance. All oocyte manipulations were conducted in MMR buffer.

Purified RNA was injected into the animal cap side of the midline of each oocyte using a Harvard apparatus (USA) microinjector and a Narishige (Japan) micromanipulator. RNAs were injected in sets of 25. Oocytes were incubated overnight at 16°C in 1 x MMR, before processing.

2.6.3 *In vivo* protein radio-labelling of oocytes

When radioactive labelling of newly synthesised proteins was required oocytes were labelled by incubation in 1ml of MMR containing 10µl/ml of [^{35}S]-methionine (*In vitro* labelling grade ^{35}S-methionine, specific activity 50 mCi/µl, GE Healthcare) overnight.

Injected and labelled oocytes were collected and pooled in groups of five. Any obviously necrotic oocytes were discarded. Oocytes were washed in fresh MMR buffer to remove any non-internalised [^{35}S]-methionine. Oocytes were resuspended in 10µl per oocyte of TE with added protease inhibitors and homogenised mechanically with pestles (Anachem). Lysates were centrifuged for 10min at 13,000 rpm (16,060rcf) in a bench top centrifuge to remove yolk proteins. The supernatant was collected and centrifuged for a further 5min at 13,000 rpm (16,060rcf). 10µl of the supernatant was added to 10µl of SDS-PAGE 2X loading buffer and heated at 95°C for 5min before being transferred immediately to ice. The samples were then analysed on a 10% SDS-PAGE acrylamide gel.

2.6.5 Luciferase and β-galactosidase reporter assays

Any obviously necrotic oocytes were discarded prior to assaying. The sets of 25 oocytes were divided into groups of five oocytes before assaying to reduce the effect of variation between individual oocytes. Lysis buffer (Tropix) was added to the oocytes in the ratio of 40µl per oocyte and the oocytes were mechanically homogenised with pestles. Luciferase activity was assayed in 5µl of oocyte lysate. The lysate was mixed with 100µl of luciferase assay reagent (LAR) (Promega), and the luciferase activity was measured with a Monolight 3010 Luminometer (Pharmingen). β-galactosidase (β-gal) activity was assayed by adding 2.5µl of oocyte lysate to 100µl of a 1/100 dilution of Galacton-Plus (Tropix) in Galacto Reaction Buffer Dilutent (Tropix). The samples were then incubated for 60min in the dark at room temperature before β-gal activity was

measured by Luminometer using Accelerator II reaction substrate (Tropix). The relative amounts of β-galactosidase activity were used to account for any variations in the amount of RNA injected and relative luciferase light activity was calculated. This was achieved by normalising each luciferase value to the level of β-galactosidase in each sample group of oocytes, therefore oocytes with low expression of both reporters were normalised relative to the oocytes showing the highest expression.

2.6.6 Progesterone induced maturation

Oocyte maturation was induced by incubating oocytes overnight in 1 x MMR containing 10μg/ml progesterone. Maturation was identified by the appearance of a white spot on the vegetal hemisphere, indicating the occurrence of germinal vesicle breakdown.

2.7 Specific techniques

2.7.1 Electromobility shift assays

2.7.1.1 Generation of radiolabelled RNA

Radiolabelled RNA was generated as described in section 2.3.1 for unlabelled RNAs but the transcription reaction was as follows; 0.1μg/μl of template DNA, 1x Transcription Buffer (Stratagene), 30mM DTT, 1mM ATP, 1mM GTP, 1mM CTP, 0.1mM UTP (Pharmacia), 7 mM m7GpppG Cap (New England Biolabs), 0.2 units RNasin (Roche), 8 units T7 RNA polymerase (Stratagene). 50μCi of ^{32}P UTP (Amersham) was also included in each reaction.

2.7.1.2 RNA-protein complex assembly

Protein preparations were pre-treated on ice with protein de-oligomerisation buffer (1mM acetic acid and 1mM DTT). Binding of protein to RNA was performed on ice in

binding buffer (0.2M Tris-HCl pH 8.5, 160mM KCl, 20mM magnesium acetate and 160mg/ml bovine serum albumin (BSA)) for 1 hr. Unlabelled competitor RNAs were added prior to ^{32}P labelled probes. Heparin (final concentration 5mg/ml) was added 1 hr after the addition of ^{32}P labelled probes. RNA-protein complex formation was analysed by non-denaturing gel electrophoresis.

2.7.1.3 Non-denaturing gel electrophoresis

Non denaturing separating gels were made with 4% polyacrylamide (30:0.5 acrylamide:bis-acrylamide) and 1 x TBE (90 mM Tris-HCl pH 8.3, 90mM boric acid, 2mM EDTA). The gels were polymerised with 0.1% ammonium persulphate (APS) and 0.04% TEMED. Loading buffer was made from glycerol with a small amount of Bromophenol-Blue sodium salt added. 1 x TBE was used as running buffer.

50ml gels were cast for each assay. Gels were run at 250V for 4-5 hrs at 4°C. Gels were fixed in 40% methanol, 10% glacial acetic acid, 50% dH_2O before being exposed to X-ray film (Kodak).

2.7.2 RNA Stability assays by QPCR

2.7.2.1 Extraction of RNA from oocytes

Groups of between 10-20 oocytes were mechanically homogenised in 10µl Tri-reagent (Sigma) per oocyte. The lysates were snap frozen in liquid nitrogen, thawed and an additional 300µl of tri-reagent was added. The lysates were centrifuged for 10min at 13,000 rpm (16,060rcf) at 4°C. The supernatant was collected and incubated at room temperature for 5 min. 100µl of chloroform was added to each sample and vortexed for 15sec before centrifugation for 15min at 13,000 rpm (16,060rcf) at 4°C. The colourless top phase was collected and RNA precipitated by the addition of 250µl of iso-propanol at room temperature for 5min preceding a third centrifugation for 10min at 13,000 rpm

(16,060rcf) at 4°C. Finally the pellets were washed twice in 75% ethanol and resuspended in 20µl of dH_2O.

2.7.2.2 Reverse transcription

cDNA was synthesized from total RNA with an OMA reverse transcription kit (Qiagen) according to the manufacturer's instructions.

2.7.2.3 QPCR

Quantitative RT-PCR analysis was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems) by SYBR green incorporation with primers 5'-GGCGCGGTCGGTAAAGTT-3' and 5'-AGCGTTTTCCCGGTATCCA-3' for luciferase and 5'-TCACGAGCATCATCCTCTGC-3' and 5'-CAGCGGATGGTTCGGATAATGC-3' for β-galactosidase. The reactions were performed in 1 X SYBR Master Mix (Applied Biosystems).

Data analysis was performed with the SDS2.1 program (Applied Biosystems), and standard curves generated from luciferase and β-galactosidase DNA standards were used to determine concentrations in the test samples. All samples were analyzed in triplicate.

2.7.3 Yeast-2-Hybrid assays

Two-hybrid protein-protein interactions were detected by β-galactosidase assays as following: Colonies from yeast transformed with “bait” and “pray” plasmids were re-streaked onto appropriate selective media (SD –trp –leu) and grown overnight at 30°C. Yeast colonies were streaked onto selective media (SD –trp –leu) plates topped with 2mm whatman filter papers and grown overnight at 30°C. Filter papers were removed from the plates and immersed in liquid nitrogen for 20 sec before being thawed at room temperature. The freeze thawed filters were placed in a petri dish atop two more

whatman filters soaked in Z-buffer (60mM Na_2HPO_4, 10mM KCl, 1mM $MgSO_4$, 50mM β-mercatoethanol, pH 7) supplemented with 150μg/ml X-gal. The dish was sealed with parafilm before being incubated at 30°C until a blue colour was observed (usually 1-4 hours).

2.7.4 Translational analysis by sucrose gradient

2.7.4.1 Pouring gradients

10-50% sucrose gradients were prepared as follows: 10% and 50% sucrose (w/v) containing 250mM KCl, 2mM $MgCl_2$, 20mM HEPES pH7.4, 0.5% NP-40, 2.5mM DTT, 0.5μg/ml Heparin were prepared. Gradients were poured by mixing equal amounts of 10% and 50% sucrose solutions using a dual chamber manual gradient pourer. Gradients were weighed for balance and left to stand overnight at 4°C.

2.7.4.2 Preparation of oocyte extracts

Oocytes were collected and injected (if desired) as before. Where appropriate oocytes were pre-treated with cycloheximide by incubating them in MMR supplemented with 20μg/ml cycloheximide at 16°C for 10min before homogenisation. Oocytes were homogenised in homogenisation buffer (250mM KCl, 2mM $MgCl_2$, 20mM HEPES pH7.4, 0.5% NP-40, 2.5mM DTT) supplemented with either 150μg/ml cycloheximide or 20mM EDTA as appropriate. 100μl of buffer was used per 10 oocytes. After homogenisation extracts were incubated on ice for 10min the centrifuged for 10min, 13000 rpm (16,060rcf) at 4°C. The supernatant from 20 oocytes was loaded onto each gradient.

2.7.4.3 Centrifugation of samples and collection of fractions

Equal volumes of oocytes extracts were loaded onto the gradients by gentle layering on top of the sucrose. Gradients were centrifuged at 30,000rpm (r-average 110,583rcf) at 4°C for 150min in a Sorval TH-641 rotor. Once spun, the gradients were divided into fractions using a Pharmacia Superfrac fraction collector and the absorbance of cytosolic protein and RNA at A254 was recorded by an inline UV monitor (Pharmacia).

2.7.4.4 TCA precipitations

The protein in the gradient fractions was precipitated by the addition of equal amounts of 20% trichloroacetic acid (TCA), before centrifugation for 15min, at 13,000rpm at 4°C. Pellets were washed twice with cold acetone and dried by placing in a hot block at 95°C for 5mins. Pellets were resuspended in SDS-PAGE loading buffer and analysed by Western blot.

2.8 Plasmids

2.8.1 Tethered fusion protein constructs

pMSPN-U1A and p**MSPN.xPABP** have been previously described (Gray *et. al.*, 2000).

pMSPN.mDazl has been previously described (Collier *et. al.*, 2005)

pMSPN.DAZAP1 was supplied by Professor Philip Cohen (MRC protein phosphorylation Unit, Dundee). Human DAZAP1 was cloned into the BamHI and SpeI of pMSPN.

pMSPN.DAZAP1.T-A was supplied by Professor Philip Cohen (MRC protein phosphorylation Unit, Dundee). Human DAZAP1 containing mutations T269A T315A was cloned into the BamHI and SpeI of pMSPN.

pMSPN.DAZAP1.T-D was supplied by Professor Philip Cohen (MRC protein phosphorylation Unit, Dundee). Human DAZAP1 containing mutations T269D T315A was cloned into the BamHI and SpeI of pMSPN.

pMSPN.Xl.DAZAP1 was created by generating a fragment containing the ORF of *Xenopus* DAZAP1 by PCR using oligodeoxyribonucleotides "5'XlPrrpNhe
" (=5' GTCAGTGCTAGCATGAACAACCAAGGGGGGAC 3') and "3'XlPrrpSpe
" (=5' GTCAGTACTAGTTCAAATCCACTCGGACAATTTCAC 3') using pET.Xl.DAZAP1 as a template. The PCR product was cut with NheI and SpeI and ligated into pMSPN vector cut with NheI and SpeI.

2.8.2 Tethered function assay reporter constructs

pLG-MS2 has been previously described (Gray *et. al.*, 2000).

pJK-350 has been previously described (Evans *et. al.*, 1994).

pLG-luc-A45, pLG-luc-MS2$_1$ and **pLG-luc-MS2$_9$** have all been previously described (Collier *et. al.*, 2005).

pPV-Luc-MS2 and **pCSFV-Luc-MS2** have been previously described (Gorgoni *et. al.*, 2005).

pPV-Luc-ΔMS2 and **pEMCV-Luc-ΔMS2** have been previously described (Bergamini *et. al.*, 2000).

pCSFV-CAT has been previously described (Ostareck *et. al.*, 2001).

pEMCV-Luc-MS2 was constructed by Mr J. Glover. A fragment containing the EMCV IRES and a fraction of the luciferase ORF cut from pEMCV-Luc-ΔMS2 with SmaI and ClaI was ligated into pLG-MS2 vector cut with HindIII (then blunt ended) and ClaI.

pCSFV-Luc-ΔMS2 was constructed by Mr W. Richardson. A fragment containing the CSFV IRES was created by PCR using oligodeoxyribonucleotides (5' GAGTCAAAGCTTCGATCCGTCGACAAGGTTAGCTC) and (3' GAGTCAAAGCTTCCCGGTTCCTCCACTCCCACTGG) using pCSFV-CAT as a template was ligated into pLG-MS2 cut with HindIIII.

2.8.3 Yeast-two hybrid constructs

pACT-hDazl, pACT-hDAZ and **pACT-mDazl** have all been previously described (Ruggiu and Cooke, 2000).

pGAD.hBOULE and **pGAD-Xdazl** have been previously described (Collier *et. al.*, 2005).

BTM.LexA and **pACT.IRP** have been previously described (SenGupta *et. al.*, 1996).

pACT.xPaip has been previously described (Gray *et. al.*, 2000).

pACT.hPaip has been previously described (Gorgoni *et. al.*, 2005).

BTM.Xl-ePABPCt has been previously described (Wilkie *et. al.*, 2005).

pGEM.tPABP and **pGEM-hPABP** were supplied by Dr B. Collier and contained the relevant ORFs cloned into pGEM.T-easy (Promega) following manufacturer's instructions.

BTM.hPABP5 was made by Mr. Ross Anderson by cutting a fragment containing the ORF of human PABP5 from pET.PABP5 and ligating it into cut BTM vector.

Vp16.PABP16 was a gift from Dr. Scott Ballantyne. This plasmid was isolated in a yeast-two hybrid screen and is a fusion of the VP-16 activation domain and contains part of RRM4 and the C-terminal region of *Xenopus* PABP1.

pGBK.BaitIII was a gift from Dr D. Cazalla and contains the N-terminal third of the SC35 splicing factor (unpublished).

pGEM.tCt A fragment containing the human testis PABP C-terminal region (amino acids 396-631) was created by PCR using oligodeoxyribonucleotides "jws5'h3ct2 " (=5'GTCAGTGAATTCCGAGCACCTCCTTCAGGTTAC 3') and "jws3'h3ct2 " (=5' GTCAGTGGATCCTTAAACAGTTGGAACACCGGTTAC 3') using pGEM-tPABP as a template. The PCR product was subcloned into pGEM.T-easy (Promega) following manufacturer's instructions.

BTM.tCt A fragment containing the human testis PABP C-terminal region (amino acids 396-631) was cut from pGEM.tCt with EcoRI and BamHI ligated into BTM vector cut with EcoRI and BamHI.

pGEM.hCt A fragment containing the human PABP1 C-terminal region (amino acids 394-633) was created by PCR using oligodeoxyribonucleotides "jws5'hct" (=5' GTCAGTGAATTCATCAACCCCTACCAGCCAGCAC 3') and "jws3'hct" (=5' GTCAGTGAATCCTTAAACAGTTGGAACAACGGTGGC 3') using pGEM-hPABP as a template. The PCR product was subcloned into pGEM.T-easy (Promega) following manufacturer's instructions.

pGAD.hCt A fragment containing the human PABP1 C-terminal region (amino acids 394-633) was cut from pGEM.hCt with EcoRI and BamHI and ligated into pGADT7 (Clontech) vector cut with EcoRI and BamHI.

BTM.hCt A fragment containing the human PABP1 C-terminal region (amino acids 394-633) was cut from pGEM.hCt with EcoRI and BamHI and ligated into BTM vector cut with EcoRI and BamHI.

pGAD.hDAZL A fragment containing the ORF of human DAZL was created by PCR using oligodeoxyribonucleotides "JWS5HDE" (=5' GTCAGTGAATTCATGTCTACTGCAAATCCTGAAAC 3') and "JWS3HDB" (=5' GTCAGTGGATCCTCAAACAGATTTAAGCATTGCCC 3') using pACT.hDAZL as a template. The PCR product was cut with EcoRI and Bam HI and ligated into pGADT7 (Clontech) vector cut with EcoRI and BamHI.

pGAD.hDAZ A fragment containing the ORF of human DAZ was created by PCR using oligodeoxyribonucleotides "JWS5HDZE" (=5' GTCAGTGAATTCATGTCTGCTGCAAATCCTGAG 3') and "JWS3HDZB" (=5' GTCAGTGGATCCTCAGTCTCTTCTCTGGATTAAAC 3') using pACT.hDAZL as a template. The PCR product was cut with EcoRI and Bam HI and ligated into pGADT7 (Clontech) vector cut with EcoRI and BamHI.

pGAD.hBOULE.106-173 A fragment containing amino acids 106-173 of human BOULE was created by PCR using oligodeoxyribonucleotides "hB106FEco" (=5' GTCAGTGAATTCGAAAAACTTAATTATAAGG 3') and "hB173RBam" (=5' GTCAGTGGATCCTGAAGGCCAAGGCGGTGG 3') using pGAD.hBOULE as the template. The PCR product was cut with EcoRI and Bam HI and ligated into T7 (Clontech) vector cut with EcoRI and BamHI.

pGBK.mDazl.99-166 A fragment containing amino acids 99-166 of mouse Dazl was cut from pAS.mD99-166 with NdeI and BamHI. The resulting fragment was ligated into pGBKT7 (Clontech) that had been cut with NdeI and BamHI.

pGBK.mDazl.129-166 A fragment containing amino acids 129-166 of mouse Dazl was created by PCR using oligodeoxyribonucleotides "5mD129" (=5' GTCAGTGAATTCTTGATTTTTAATCCTCTTCCTCC 3') and "JWS399B" (=5' GTCAGTGGATCCCTGAACATACTGAGTGATAGG 3') using pGAD.mDAZL as the template. The PCR product was cut with EcoRI and Bam HI and ligated into pGBKT7 (Clontech) that had been cut with EcoRI and BamHI.

pGBK.mDazl.139-166 A fragment containing amino acids 139-166 of mouse Dazl was created by PCR using oligodeoxyribonucleotides "5mD139" (=5' GTCAGTGAATTCTTCCAGAGTGTTTGGAGTAGTAG 3') and "JWS399B" (=5' GTCAGTGGATCCCTGAACATACTGAGTGATAGG 3') using pGAD.mDAZL as the template. The PCR product was cut with EcoRI and Bam HI and ligated into pGBKT7 (Clontech) that had been cut with EcoRI and BamHI.

pGBK.mDazl.149-166 A fragment containing amino acids 149-166 of mouse Dazl was created by PCR using oligodeoxyribonucleotides "5mD149" (=5' GTCAGTGAATTCGAGACTTACATGCAGCCTCC 3') and "JWS399B" (=5' GTCAGTGGATCCCTGAACATACTGAGTGATAGG 3') using pGAD.mDAZL as the template. The PCR product was cut with EcoRI and Bam HI and ligated into pGBKT7 (Clontech) that had been cut with EcoRI and BamHI.

pGEM.mDazl.M3.E/B was made using the mutagenic PCR method detailed in section 2.3.8. The initial PCR reaction was conducted using oligodeoxyribonucleotides "JWS5M6" (=5' GCTGAGACTGTCATGCAGCCTCC 3') and "JWS3M6" (=5' GGAGGCTGCATGACAGTCTCAGC 3') using pGAD.mDAZL as the template. The second PCR reaction used oligodeoxyribonucleotides "JWS5MDE" (=5' GTCAGTGAATTCATGTCTGCCACAACTTCTGAG 3') and "JWS3MDB" (=5' GTCAGTGGATCCTTAGTAGAGATGATCAGATTTAAGC 3'). The resultant fragment was subcloned into pGEM.T.easy as per manufacturer's instructions.

pGEM.mDazl.M2,3.E/B was made using the mutagenic PCR method detailed in section 2.3.8. The initial PCR reaction was conducted using oligodeoxyribonucleotides "JWS5M5" (=5' GAGTAGTCCAGCTGCTGAGAC 3') and "JWS3M5" (=5' GTCTCAGCAGCTGGACTACTC 3') using pGAD.mDAZL as the template. The second PCR reaction used oligodeoxyribonucleotides "JWS5MDE" (=5' GTCAGTGAATTCATGTCTGCCACAACTTCTGAG 3') and "JWS3MDB" (=5'

GTCAGTGGATCCTTAGTAGAGATGATCAGATTTAAGC 3'). The resultant fragment was subcloned into pGEM.T.easy as per manufacturer's instructions.

pGEM.mDazl.M1,2,3.E/B was made using the mutagenic PCR method detailed in section 2.3.8. The initial PCR reaction was conducted using oligodeoxyribonucleotides "JWS5M4" (=5' GTTCCAGAGTGTTGTGAGTAGTCC 3') and "JWS3M4" (=5' GGACTACTCACAACACTCTGGAAC 3') using pGAD.mDAZL as the template. The second PCR reaction used oligodeoxyribonucleotides "JWS5MDE" (=5' GTCAGTGAATTCATGTCTGCCACAACTTCTGAG 3') and "JWS3MDB" (=5' GTCAGTGGATCCTTAGTAGAGATGATCAGATTTAAGC 3'). The resultant fragment was subcloned into pGEM.T.easy as per manufacturer's instructions.

pGAD.mDazl.M3 pGEM.mDazl.M3.E/B was cut with EcoRI and BamHI. The resulting fragment was ligated into pGADT7 (Clontech) that had been cut with EcoRI and BamHI.

pGAD.mDazl.M2,3 pGEM.mDazl.M2,3.E/B was cut with EcoRI and BamHI. The resulting fragment was ligated into pGADT7 (Clontech) that had been cut with EcoRI and BamHI.

pGAD.mDazl.M1,2,3 pGEM.mDazl.M1,2,3.E/B was cut with EcoRI and BamHI. The resulting fragment was ligated into pGADT7 (Clontech) that had been cut with EcoRI and BamHI.

MSPN.mDazl.M3 A fragment containing the mDazl.M3 ORF was created by PCR using oligodeoxyribonucleotides "M1674" (=5' CATGCACAATTGATGTCTGC CACAACTTCTGAGGCTCC 3') and (=5' CATGCAACTAGTTTAGCAGAGATGATCAGATTT 3') using pGEM.mDazl.M3.E/B as the template. The PCR product was cut with MfeI and SpeI and ligated into pMSPN vector cut with MfeI and SpeI.

MSPN.mDazl.M2,3 A fragment containing the mDazl.M2,3 ORF was created by PCR using oligodeoxyribonucleotides "M1674" (=5' CATGCACAATTGATGTCTGC CACAACTTCTGAGGCTCC 3') and (=5' CATGCAACTAGTTTAGCAGAGATGATCAGATTT 3') using pGEM.mDazl.M2,3.E/B as the template. The PCR product was cut with MfeI and SpeI and ligated into pMSPN vector cut with MfeI and SpeI.

MSPN.mDazl.M1,2,3 A fragment containing the mDazl.M1,2,3 ORF was created by PCR using oligodeoxyribonucleotides "M1674" (=5' CATGCACAATTGATGTCTGC CACAACTTCTGAGGCTCC 3') and (=5' CATGCAACTAGTTTAGCAGAGATGATCAGATTT 3') using pGEM.mDazl.M1,2,3.E/B as the template. The PCR product was cut with MfeI and SpeI and ligated into pMSPN vector cut with MfeI and SpeI.

2.8.4 Other plasmids

pMSCU-CAT has been previously described (Ostareck *et. al.*, 2001).

pET.PABP5 was constructed by Mr Ross Anderson. A fragment containing the ORF of human PABP5 was created by PCR using oligodeoxyribonucleotides (5' GGATCCTTATGGGGAGCGGGGAGCC) and (3' GCGTGGACTCAGCACCTGCGCCTGGCCT) using IMAGE clone BC0631B as a template. The PCR product was digested with BamHI and SalI and ligated into pET.28c_+ that had been cut with BamHI and SalI.

pET.Xl.DAZAP1 was the kind gift of Paul W Huber (University of Notre Dame, IN, USA) and contains the coding sequence of *Xenopus* DAZAP1 inserted into the NdeI and Sal I sites of pET-23b.

Chapter 3: Characterisation of Dazl's role in translation initiation

3.1 Introduction

The Dazl family proteins are a family of RNA-binding proteins essential for gametogenesis. Prior work by a number of laboratories has suggested a conserved role in translational regulation in a variety of species. Dazl was shown to associate with polysomes in mice and zebrafish (Maegawa *et. al.*, 2002; Tsui *et. al.*, 2000b), suggesting a role in mRNAs actively undergoing translation. The *Drosophila* homologue of Dazl, Boule, was shown to be required for the translation of *twine* mRNA (Maines and Wasserman, 1999). Subsequently, a role in translational activation was directly established in this laboratory when a variety of Dazl family members were shown to be capable of translational regulation in *Xenopus* oocytes (Collier *et. al.*, 2005).

A number of putative vertebrate targets for this translational activity have been recently identified in a variety of species (Fox *et. al.*, 2005; Jiao *et. al.*, 2002), with *in vivo* evidence being available for two mouse targets, the mouse vasa homologue (*Mvh*), and *SYCP3* (Reynolds *et. al.*, 2005; Reynolds and Cooke, 2005). Both proteins are essential for mouse gametogenesis and share aspects of the Dazl phenotype in males.

This chapter explores the model proposed for Dazl-mediated stimulation of translation. In this model Dazl is recruited to the 3'UTRs of translationally inactive mRNAs and in turn recruits PABP that then interacts with 5'UTR bound factors, circularising the mRNA and enhancing translation. This is in keeping with the sucrose gradient analysis in the presence of cycloheximide that shows Dazl stimulates translation at the stage of initiation (Collier *et. al.*, 2005). As PABP has various effects on initiation and interacts with multiple factors (see section 1.5.2.1), it is not clear which factor and consequently which step in translation initiation a DAZL/PABP complex would stimulate.

The aim of this chapter is to enable a better understanding of when and how Dazl stimulates translation and the model proposed by Collier *et. al.* To this end, a definition of the stage in translation initiation that Dazl acts upon will be sought. This will be

useful in aiding the understanding the nature of the translational stimulation provided by Dazl. The data presented in this chapter will also help in understanding the effect of PABP recruitment by Dazl as PABP has been proposed to affect initiation at multiple points. It will be interesting to note whether Dazl stimulates all the stages that have been previously described for PABP or whether it has a more focused role.

3.2 The tethered function assay

The nature of the translational stimulation mediated by Dazl will be further investigated using tethered function assays with mRNAs that utilise a subset of canonical translation factors to initiate translation. A tethered function approach was utilised as target mRNAs had not been definitively identified at the time of the work.

The tethered function assay has become an established assay in understanding the function of RNA-binding proteins. It enables functional analysis of RNA binding proteins even in the absence of knowledge of their endogenous target sites for binding and allows functional domains to be mapped (Coller *et. al.*, 1998).

The key component of this assay is the use of a tether protein of unrelated function to bring the protein of interest to a reporter mRNA. The tether is used in lieu of the protein's own RNA binding activity. This has a number of advantages; by using an unrelated binding site it is possible to control exactly where the protein is localised to on the mRNA that means the assay is very specific with no known incidence of cross reaction *in vivo* (Keryer-Bibens *et. al.*, 2007). It also avoids any interference from the endogenous protein if it is present in the system. Because the tether brings the protein of interest to the reporter RNA it is not necessary to know the RNA binding sequence of the protein, which is of great benefit as frequently the RNA targets for RNA binding proteins that are identified genetically are unknown. This was the case for Dazl when it was first investigated for its translational activity.

There are a number of different tethering systems available. These include the RNA-binding domain of the λ phage antiterminator protein N with its specific λ-N binding site (boxB) (De Gregorio *et. al.*, 2001) and the human iron regulatory protein (IRP) that recognises a stem-loop known as the iron-responsive element (Gray and Hentze, 1994a). Perhaps the most widely utilised system is the bacteriophage MS2 coat protein that recognises defined stem-loops known as MS2 binding sites.

The particular assay that was chosen for use in this study was the MS2 tethered function system, as this has been utilised for the study of a number of translational regulators in stage VI *Xenopus* oocytes including Dazl. This assay has two components; a fusion of the protein of interest with the bacteriophage MS2 coat protein and a luciferase reporter mRNA that contains three MS2 RNA recognition sites in its 3'UTR (see figure 3.1).

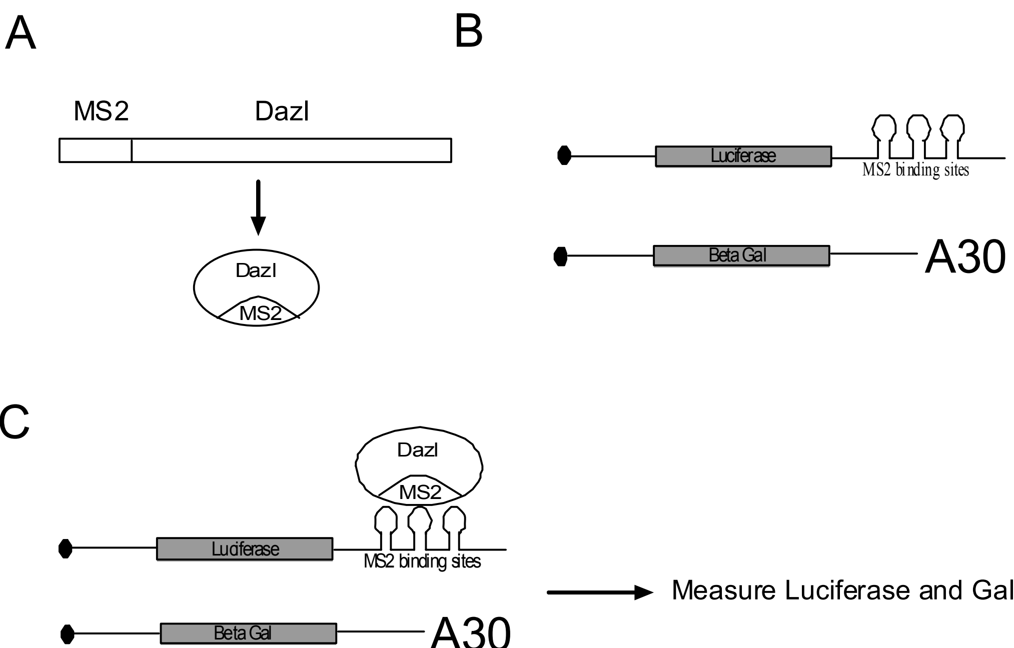


Figure 3.1: MS2 tethered function assay.
A: RNA encoding MS2-fusion protein is injected into the cytoplasm of stage VI oocytes and incubated for 6 hours to allow fusion protein expression.
B: Oocytes are then injected in the cytoplasm with a luciferase and an internal control β-galactosidase reporter mRNAs.
C: The expressed fusion protein binds to the MS2-binding sites in the 3'UTR of the luciferase reporter thus tethering the protein to the reporter. The oocytes are then incubated overnight to allow effects on reporter translation. Lysates are then made and Luciferase and β-galactosidase activities are measured.

In all the tethered function assays in this thesis a negative control protein, U1A, is used to measure the baseline translation of the reporter mRNA bound by a tether fusion protein. U1A is a RRM containing protein that functions in mRNA metabolism but is known not to affect translation in the cytoplasm (Jovine *et. al.*, 1996). When a MS2 fusion of U1A is tethered, it produces the same level of luciferase as when the MS2 protein alone is tethered (Gray *et. al.*, 2000). This shows that stimulation of translation is not a general property of tethering RRM containing proteins (Gray *et. al.*, 2000).

The activity of the luciferase reporter is used as measure of translation. The effect of tethering proteins in the absence of changes in the mRNA levels (see section 3.11 below) is observed by assessing the changes in luciferase production. The luciferase values are always normalised to β-galactosidase levels from a co-injected β-galactosidase reporter. This reporter is polyadenylated and lacks MS2 binding sites so is unbound by the fusion proteins and thus gives a value that can be used to account for any differences in translational activity in the oocytes.

3.3 *Xenopus* oocytes as a model for vertebrate germ cells

The tethered function assays discussed above can be used in a variety of systems including cell culture, *in vitro* and in *Xenopus* oocytes. The system utilised in this thesis is stage VI *Xenopus* oocytes (unless specifically stated) that comes with a variety of advantages.

A single *Xenopus* ovary contains hundreds of oocytes at different stages of development. *Xenopus* oocytes are sub-divided into six clearly defined stages, as an oocyte progresses from stage one to stage six; it grows progressively larger until it reaches stage VI (Dumont, 1972). At this point it is regarded as a fully developed oocyte but has yet to complete meiosis (Ferrell, 1999). The next stage is the maturation of the oocyte into an unfertilized egg via the application of the hormone progesterone to the oocyte; this causes the oocyte to complete meiosis. This can be easily achieved *in vitro* and mature

oocytes are easily identified by the appearance of a white spot on the animal pole (Ferrell, 1999).

The stage VI oocytes offer a number of advantages for translation assays. These cells are large, approximately 1.3mm; which means that they are relatively easy to manipulate and microinject. It is possible to conduct biochemical assays, such as luciferase or β-galactosidase assays, in oocyte extracts derived from large numbers of oocytes that give simple experimental measurements.

RNAs injected into oocytes are stable both with the m^7GpppG cap and also with the non-functional ApppG cap analogue (Gillian-Daniel *et. al.*, 1998). Another advantage is that unadenylated mRNAs are also uniquely stable in these cells (Audic *et. al.*, 1997). This allows for a large number of experimental manipulations of reporter mRNAs. Translation in *Xenopus* oocytes is however profoundly responsive to poly(A) tail length, for instance adenylation of c-mos and cyclin B1 mRNA increases their translation 10 and 75 fold respectively (Sheets *et. al.*, 1994). This is an important asset for investigating Dazl function, in that the current model posits that Dazl utilises PABP (Collier *et. al.*, 2005), which is responsible for the translational effects of the poly(A) tail (Gray *et. al.*, 2000).

Late stage oocytes, mature eggs and early embryos are essentially transcriptionally silent (Mendez and Richter, 2001). Thus all changes in the pattern of protein synthesis come from changes in translation or turnover of RNA. This is used to advantage in tethered function assays as reporter mRNAs are injected into the oocyte cytoplasm thus any effects on transcription or export by multifunctional proteins will not affect the results of the translation assays.

A final advantage of oocytes in this study is that they are vertebrate germ cells. As the Dazl family proteins are primarily germ cell specific this system is a particularly relevant model system. They are also very accessible vertebrate germ cells as you can extract thousands of oocytes from one *Xenopus* ovary. This gives plenty of material to

work with, especially when compared to mice, where a super ovulated ovary would return approximately thirty oocytes. There is evidence that Dazl family members have a functional conservation across species since *Xenopus* Dazl or human BOULE can both partially rescue the *Drosophila* boule knockout (Houston *et. al.*, 1998; Xu *et. al.*, 2003). Similarly, human DAZ and DAZL partially rescue a Dazl knockout mouse (Slee *et. al.*, 1999; Vogel *et. al.*, 2002). These results indicate that looking at mammalian Dazl proteins in model germ cells can give valid functional information.

3.4 Dazl stimulates translation as shown by the tethered function assay

Published work (Collier *et. al.*, 2005) has shown that DAZL family members can stimulate translation when tethered in stage VI *Xenopus* oocytes. To confirm this finding mRNAs encoding the mouse Dazl fusion and the negative control MS2-U1A were transcribed and injected into the cytoplasm of stage VI oocytes and left for six hours to allow the fusion proteins to be expressed. Subsequently Luc-MS2 and β-gal reporters (see figure 3.1) were injected directly into the cytoplasm and oocytes were incubated overnight, this allows the fusion proteins time to bind to the luciferase reporter and modulate luciferase production. 25 oocytes were injected for each separate experimental point. After the overnight incubation any obviously necrotic oocytes were discarded and the remainder were grouped into batches of five. These oocytes were then mechanically lysed and the lysates assayed for luciferase and β-galactosidase activity using a luminometer.

A typical set of results is represented in figure 3.2, with graphs A and B showing the raw β-galactosidase and luciferase values respectively. In this case enough oocytes survived for four sets of five oocytes for both MS2-U1A and MS2-mDazl. Luciferase values were then normalised relative to β-gal activity in order to take account of any differences in translational activity between different oocytes. As can be seen from figure 3.2A β-galactosidase is largely consistent but some oocyte sets have higher or lower activity

than others necessitating a correction. A typical correction was between 10% and 30%, and any set that showed a variation greater than this was discarded. Figure 3.2C shows the result of the corrected luciferase values. These values are then averaged to give an average luciferase activity for each fusion protein normalised to β-galactosidase activity as shown in figure 3.2D. Finally, luciferase value given by the U1A control were set to one and expressed as the relative stimulation given by the protein of interest. Multiple repetitions of an experiment are conducted and the stimulation values are averaged and the standard error of that value is calculated giving the final average stimulation for the protein of interest as shown in figure 3.2E. This is the data format given in all subsequent tethered function assay figures unless explicitly stated.

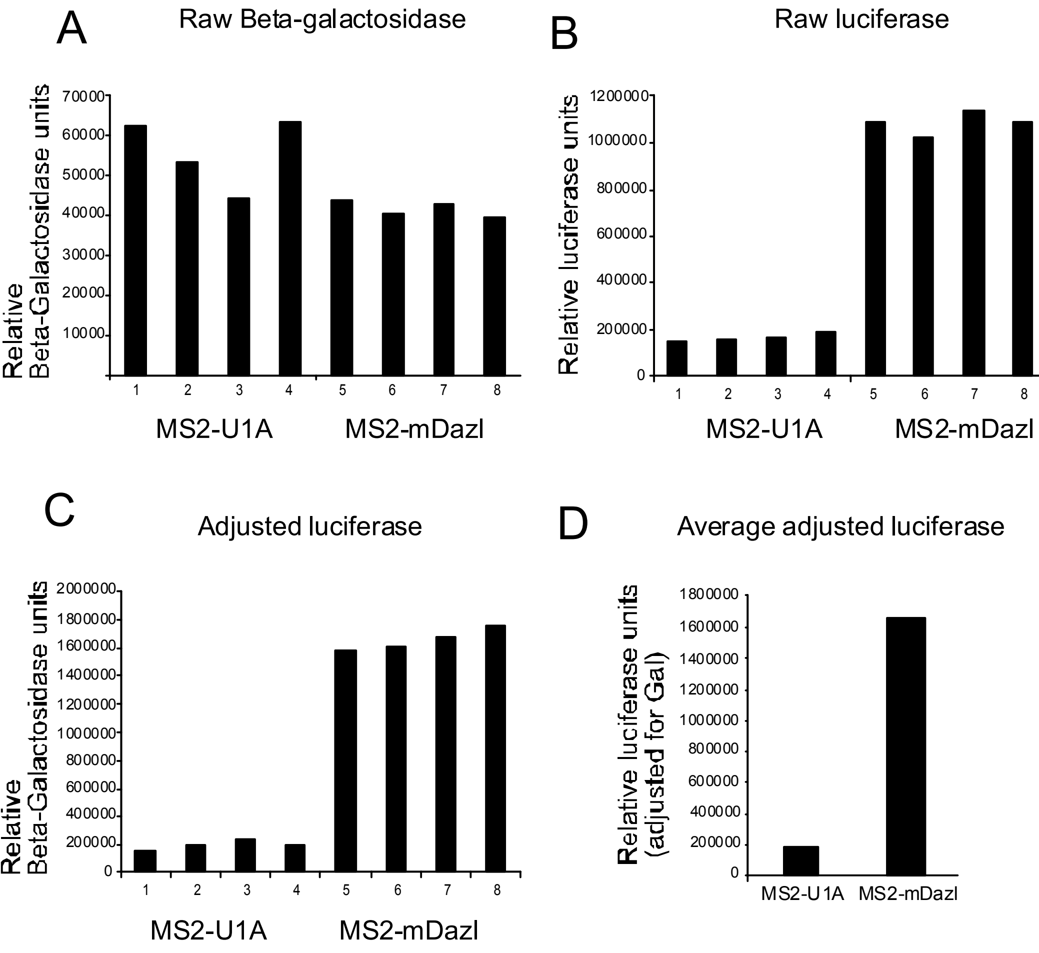


Figure 3.2: (Part 1) mDazl stimulates translation.
Mouse Dazl can stimulate translation in a tethered function assay. m^7GpppG -capped Luc-MS2 reporter (M7G-Luc-MS2) was co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-mDazl. A: Surviving oocytes were sorted into sets of five (1-8). Each set was assayed for β-galactosidase activity. The relative β-galactosidase activity for each set of oocytes was plotted as shown. B: Oocytes sets were also assayed for luciferase activity. The relative luciferase activity for each set was shown. C: Raw luciferase activity was normalised to β-galactosidase activity to give an adjusted luciferase value for each set. D: The average value of luciferase activity normalised to β-galactosidase activity was calculated for MS2-U1A and MS2 mDazl and plotted as shown.

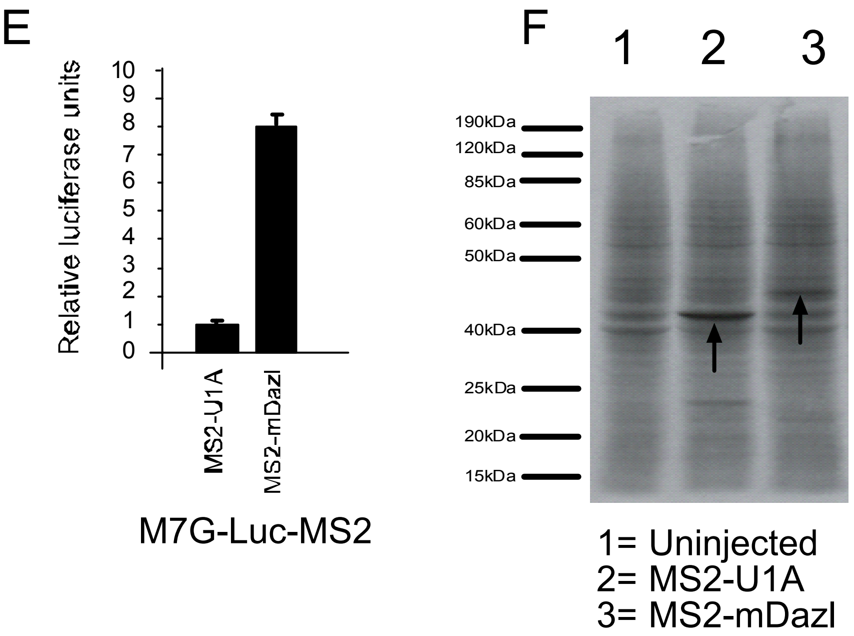


Figure 3.2: (Part 2) mDazl stimulates translation.
E: The average adjusted luciferase values from part D were used to show the ability of mDazl to stimulate translation. Average relative luciferase units were plotted with MS2-U1A values set to one. The average of six separate experiments is shown. Standard error between experiments is shown as error bars. F: The MS2-fusion proteins are expressed in oocytes. Oocytes were injected with fusion-protein mRNAs and incubated in buffer containing ^{35}S-methionine for 6 hours and protein lysates made. An oocyte's worth of lysate was separated by SDS-PAGE and analysed by autoradiography. Arrows indicate the fusion protein bands.

To ensure that the fusion proteins were expressed as expected, stage VI oocytes were injected with RNAs encoding MS2-mDazl and MS2-U1A and new protein production was monitored by metabolically labelling the oocytes with ^{35}S-methionine. Protein extracts from oocytes were made and analysed using SDS-PAGE. Bands corresponding to MS2-U1A protein and MS2-mDazl protein can be observed in the relevant lanes (figure 3.3F). The MS2-U1A band has a higher intensity than the MS2-mDazl band but this is consistent with the number of methionines in the two proteins with U1A containing seventeen and Dazl containing only six.

Consistent with published results, Dazl stimulates luciferase expression (figure 3.2E). It has been shown previously that this stimulation of luciferase by mDazl is a specific *cis* effect that requires the presence of the MS2 binding sites in the 3'UTR of the Luc-MS2 reporter (Collier *et. al.*, 2005) and is not mediated by changes in mRNA stability.

Confirmation of these observations is presented in figure 3.9. Typically the level of translational stimulation observed for mouse Dazl was eight-fold but varied between different injections with stimulation values of between 4 and 11 observed over the course of these experiments. This variation is thought to be due to natural variations in the translational activity of oocytes. It has been noticed that the quality of the oocytes regarding survival and the levels of stimulation seen varies greatly. These effect have been observed when other proteins such as SLBP and PABP were studied using the same system. Particular times of year are also known to produce poor quality oocytes.

It is thought that the *Xenopus* have an internal circadian clock and may be sensitive to subtle changes in day/night cycle or temperature despite being kept in a temperature and light controlled environment. Oocyte quality varies with the seasons of the year and it is thought that these changes could represent the frog's life style in the wild. For example, there is often a noticeable drop in quality in autumn that could be related to the breeding season for wild *Xenopus*. External influences can also affect oocyte quality, for example in recent years there has been an outbreak of a fungus (Batrachochytrium dendrobatidis) infecting amphibian populations around the world. This fungus is known to affect germ cell quality, so any contamination in animal stocks could conceivably affect experimental results. Another extremely important external influence is the quality of the water the *Xenopus* live in. In particular is ensuring the removal of any chloramine (that is added to water as a disinfectant) as it is toxic to amphibians.

As the Xenopus populations that are supplied for laboratory use are not inbred strains there may be an effect of genetic background on translation experiments. In fact it is known that suppliers regularly collect wild-type frogs for their colonies. This means that both oocyte quality and possibly translational activity could vary greatly between different animals and could account for much of the variation seen in my experiments, especially as other factors such as water quality and housing conditions are actively kept as consistent as possible.

3.5 The variable reporter tethered function assay

Although it has been previously established that Dazl can stimulate translation, little is known about the mechanism or nature of this stimulation. Previous work in our lab utilising sucrose gradients has demonstrated that Dazl stimulates translation initiation and an interaction with a pleiotropic initiation factor, PABP, has been shown (Collier *et. al.*, 2005). To understand the mechanism of Dazl mediated translation stimulation further it is necessary to distinguish whether early or late steps in initiation are targeted and what initiation factors are required for regulation.

Translation initiation can be divided into four basic mRNA dependant steps (see Chapter 1.3.1 for more detail on translation initiation). These are; first cap binding, second 43S complex joining, third scanning and fourth 60S subunit joining.

To investigate which of these initiation steps is affected a variable reporter tethered function assay was used. This approach has been previously utilised in oocytes to investigate a different translational activator, the stem-loop binding protein (SLBP) (Gorgoni *et. al.*, 2005), and was instrumental in identifying that this protein functions in an early stage of the cap-dependant initiation pathway, targeting the cap-binding complex.

The technique is a modification on the standard tethered function assay in oocytes, employing the same fusion proteins but a series of modified luciferase reporters, shown in figure 3.3.

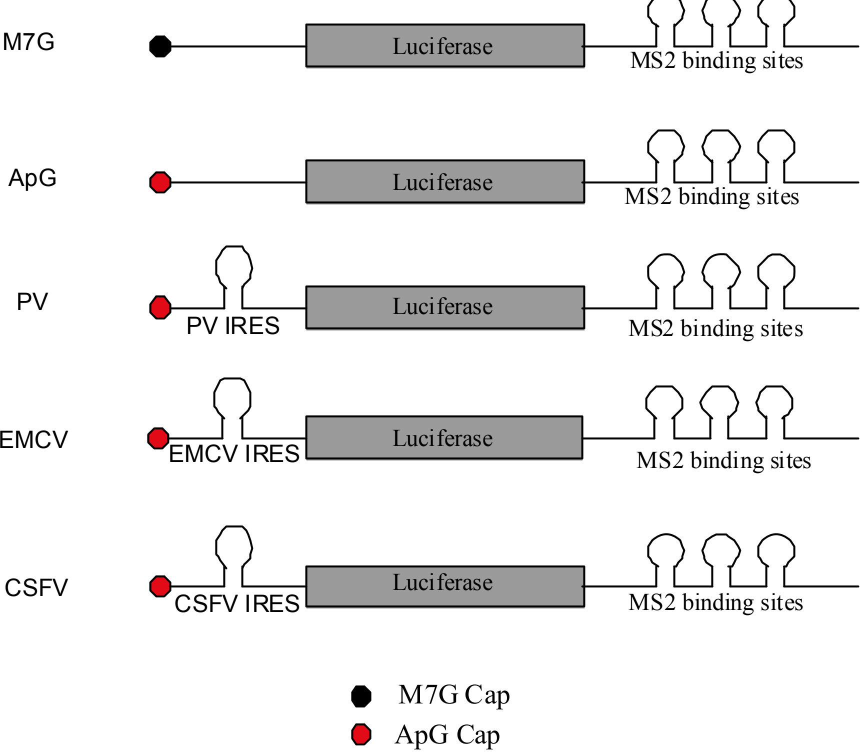


Figure 3.3: The varied tethered function assay reporters.
The first reporter, here referred to as m7G, is the standard reporter used in figure 3.2. This reporter is capped with the m^7GpppG cap that is found on endogenous mRNAs. The second reporter, ApG, is capped with an artificial ApppG cap, which is non-functional regarding its capacity to bind eIF4E. Three other ApG capped reporters contain an IRES is their 5'UTRs. The 'PV' reporter contains the Poliovirus IRES, the 'EMCV' reporter the encephalomyocarditis IRES and the 'CSFV' reporter contains the Classical Swine Fever Virus. The ApG cap minimises any cap-dependent translation from these IRES-containing mRNAs.

Reporters are capped with either an m^7GpppG cap, which is the endogenous functional cap, or an artificial ApppG cap. Both these caps act to stabilise the RNA and prevent 5' exonucleases from digesting the injected reporters but they differ significantly in function (Gillian-Daniel *et. al.*, 1998). In inhibition assays, injected m^7GpppG cap analogue could reduce the activity of a reporter by 98.5% whereas the effect of ApppG was much lower at 6.5% indicating the difference in the ability to recruit translational machinery between the two (Gillian-Daniel *et. al.*, 1998).

Some of the variable reporters contain an IRES in the 5'UTR. These are areas of specific RNA sequence that form secondary RNA structures consisting of RNA stems and loops. These three-dimensional structures provide protein and ribosomal-RNA

binding sites and allow for differing mechanisms of translation initiation between the different IRESs. The initiation mechanism of each IRES will be discussed in detail below.

3.6 Dazl Stimulation is not cap dependent

As discussed earlier, the mRNA dependent steps of translation can be subdivided into different steps the first of which is commonly accepted to be cap-binding. This process can be said to be initiated when the cap binding complex, eIF4F, binds the m^7GpppG cap through the action of the cap binding protein, eIF4E. When synthesising *in vitro* transcribed RNAs it is possible to cap them with a non-functional cap analogue, ApppG. Where the m^7GpppG cap structure contains of a 5′ 7-methyl guanosine nucleotide the ApppG cap contains a leading adenosine nucleotide. This difference in nucleotide is not sufficient to alter the stabilizing role of the cap structure in oocytes but it severely inhibits eIF4E/cap interaction. Thus by using a luciferase reporter capped with ApppG rather than m^7GpppG it will be possible to assess if the cap binding is stimulated by Dazl.

The mechanism of cap-independent initiation of non-IRES containing mRNAs is proposed to be as follows. A complex containing elements of the cap-binding complex still forms in the area of the cap, but it is unsure what interactions mediate this process. The 43S complex is then recruited as normal via interactions with eIF3. Scanning, AUG recognition and 60S joining then proceeds as in 'standard' translation initiation. This proposed mechanism is based on a number of experimental observations. First, an mRNA that undergoes decapping does not change preference for the selection of the 5'-proximal AUG codon (Kozak, 1989b), thus implying that such mRNAs still initiate translation by scanning from the 5'end. Second, the inefficient translation of uncapped reporter mRNAs can be rescued by addition of sufficient eIF4F (Fletcher *et. al.*, 1990). Third, protease cleavage of eIF4G does not affect the translation of these mRNAs, demonstrating independence from eIF4E (Ohlmann *et. al.*, 1995).

A luciferase mRNA with an m^7pppG cap was shown to be translated 48-fold more efficiently than the same mRNA with an ApppG-capped RNA in *Xenopus* oocytes (Gillian-Daniel *et. al.*, 1998). In my assays the difference in baseline translation (figure 3.4B and 3.4C, MS2-UIA values for ApG and M7G) between the m^7GpppG and ApppG capped luc-MS2 reporter is an approximate 100-200-fold loss of translation (note the different scales between figure 3.4B and 3.4C).

Tethered function assays were performed using the ApppG capped luciferase reporter with the M7G reporter acting as a positive control for Dazl-mediated stimulation. Dazl stimulated the translation of the m^7GpppG capped mRNA 8-fold (figure 3.4D) and importantly also stimulated luciferase production of the ApG reporter by 11-fold (figure 3.4D), despite the less efficient translation of this reporter (figure 3.4C).

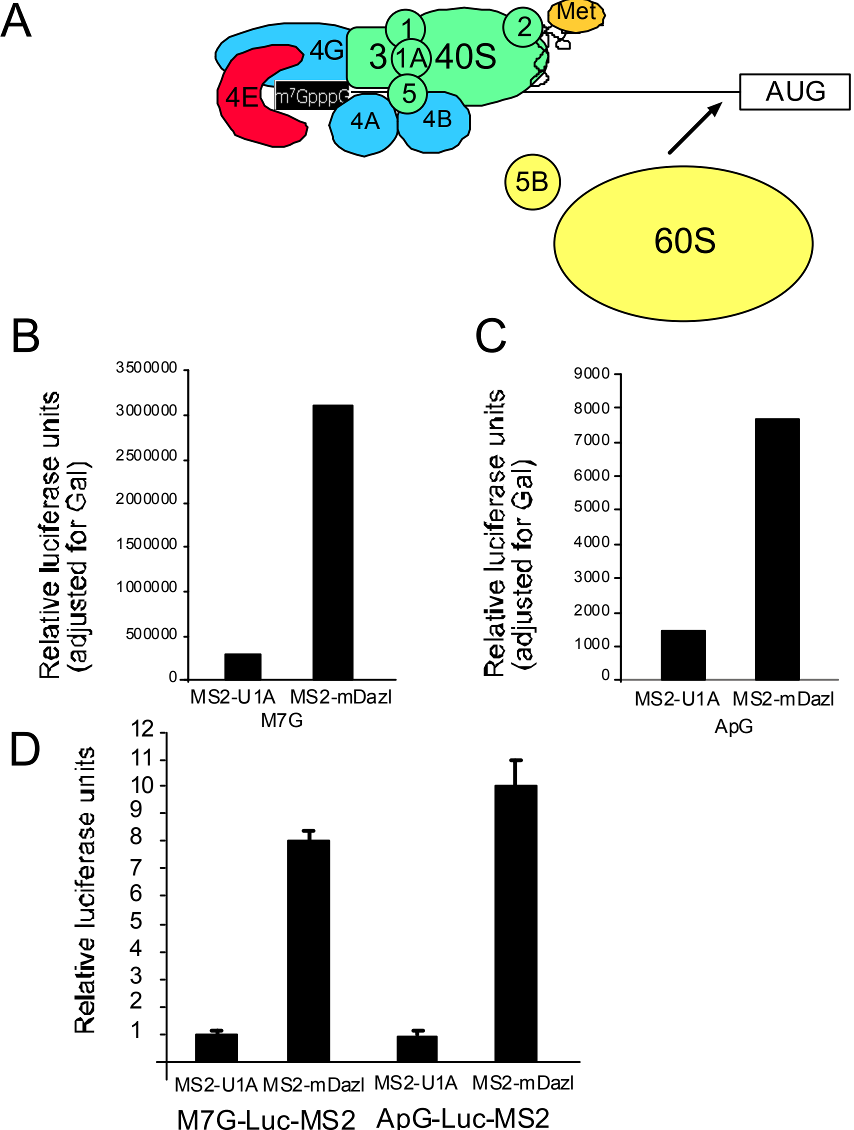


Figure 3.4: mDazl stimulates translation of the ApG-Luc-MS2 reporter.
A: The ApG-Luc-MS2 reporter initiates translation by assembling a complex at the 5'end of the RNA that does not use eIF4E and is cap independent. 4G=eIF4G. 4A=eIF4A. 4B=eIF4B. 1=eIF1. 1A= eIF1A. 2=eIF2. 3=eIF3. 5=eIF5. 5B=eIF5B. Met=initiator tRNA methionine. 40S= 40S ribosomal subunit. 60S= 60S ribosomal subunit. AUG= indicates initiation codon and start of ORF. 4E= the eIF4E protein is probably still present in the complex; but is unable to bind the ApppG cap.
B + C: MS2-mDazl can stimulate both a m^7GpppG -capped Luc-MS2 reporter (M7G-Luc-MS2) or a ApppG capped Luc-MS2 reporter (ApG-Luc-MS2). Reporters were co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-mDazl. Relative luciferase normalised to β-galactosidase activity was plotted for both reporters. Due to the extreme differences in luciferase levels the two reporters have been plotted on separate graphs with different scales. One representative experiment is shown.
D: Translational stimulation was plotted with MS2-U1A values set to one. The averages of five experiments are shown.

The ability of Dazl to stimulate the ApppG capped reporter indicates that Dazl does not stimulate translation by enhancing the interaction of the cap-binding complex to the cap because this reporter does not initiate translation by cap-binding.

3.7 Dazl can stimulate translation via the Poliovirus IRES

The results from the ApppG reporter mRNA suggests that Dazl mediated translation stimulation may be cap independent. However, as discussed above the mechanism of initiation of non-capped mRNAs is unclear. Hence to test the requirement for cap-binding more rigorously, IRES containing luciferase reporters were used. The different IRES containing reporters (figure 3.3) utilise different mechanisms of translation and also have different initiation factor requirements. By testing which of these reporters Dazl can enhance we may be able to learn more about the mechanism of Dazl's translational activity. Initially the poliovirus (PV) IRES reporter was used, the PV IRES is known to initiate translation away from the cap structure, utilising a large complex structural region within the 5'UTR.

The PV IRES does not require the eIF4E cap binding initiation factor but is thought to utilise all other initiation factors. This requirement was inferred from experiments where eIF4G was cleaved by a rhinovirus protease, thus separating the N-terminal eIF4E binding portion of eIF4G from the C-terminal domain that binds eIF3 and eIF4A. The cleavage impairs eIF4F's function on capped mRNAs but the C-terminal fragment is sufficient for the efficient translation of the PV IRES (Lamphear, 1995). The lack of requirement for eIF4E was reinforced by the insensitivity of the IRES to the over expression of the eIF4E-binding protein that sequesters eIF4E (Pause, 1994). Subsequently a strong requirement for the eIF4A/eIF4G complex was suggested by the strong inhibition of this IRES by a dominant negative form of eIF4A (Pause, 1994).

Thus, a mechanism for the translation of the PV IRES was formulated in which the IRES initiates translation away from the cap by recruiting a complex consisting of the C-

terminal of eIF4G, eIF4A and eIF4B (see figure 3.5A). This complex then recruits the 43S complex, in part through interactions between eIF4G and eIF3. The 43S complex is then thought to scan through the remainder of the 5'UTR and recognise the AUG and recruit the 60S ribosomal subunit as normal.

When compared to the luciferase levels given by the M7G reporter the PV reporter gives much lower levels with values 33-fold lower than the M7G. However, importantly this mRNA is translated more efficiently than ApppG capped mRNAs, showing that the IRES is active (see figure 3.5B). When comparing the ApppG capped reporter alone to an ApppG capped reporter with a PV IRES the average relative luciferase changes from 6,000 relative luciferase units to 11,000 in one representative experiment.

As can be seen in figure 3.5C Dazl can stimulate translation of this reporter when tethered. Typical stimulation was 11-fold compared to the 8 fold stimulation seen on the m^7GpppG capped reporter. As a specificity control, a reporter with the PV IRES but no MS2 sites (PV-Luc-ΔMS2) was employed. The stimulatory effect of Dazl required MS2 binding, as no effect on translation was observed in the absence of MS2 binding sites in the PV-Luc-ΔMS2 reporter.

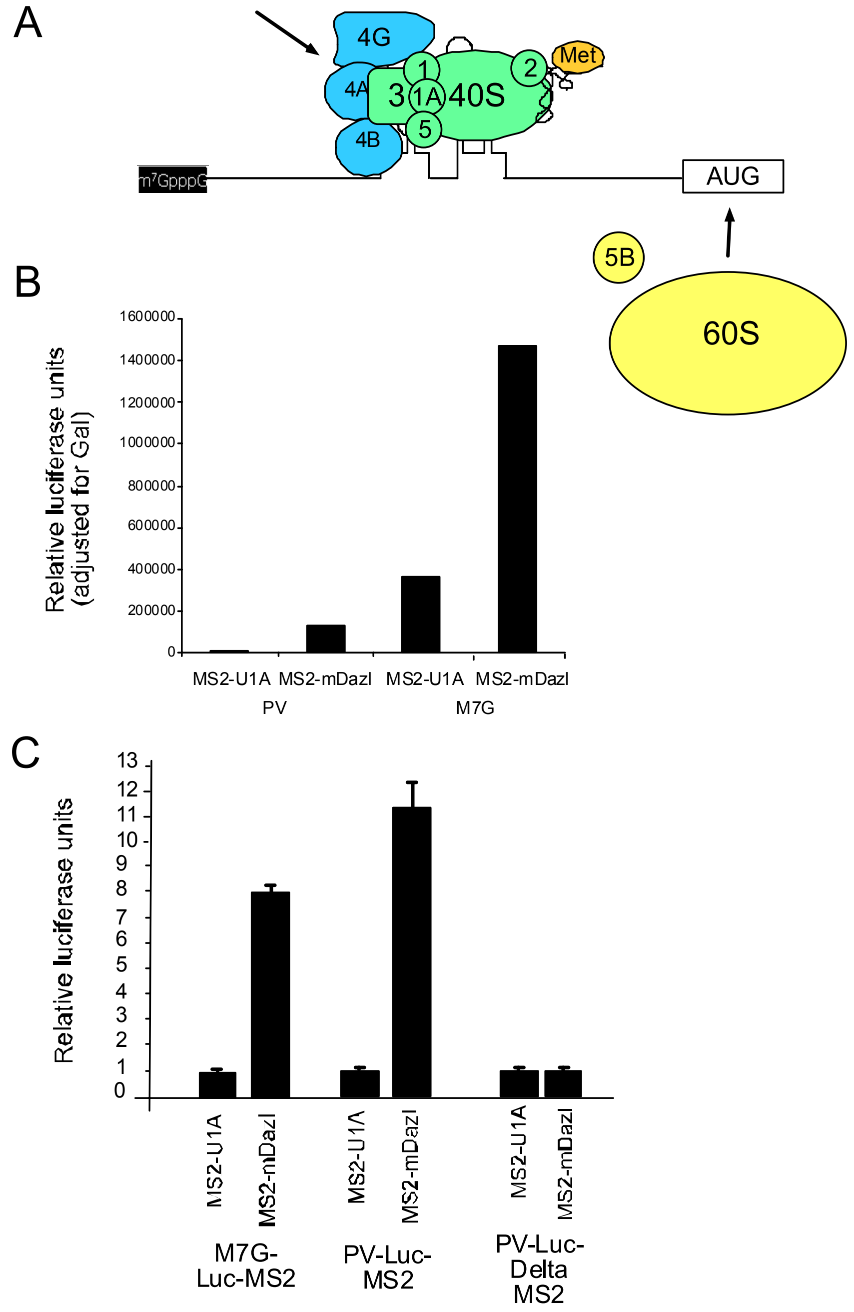


Figure 3.5: mDazl stimulates translation of the PV-Luc-MS2 reporter.
A: The PV-Luc-MS2 reporter initiates translation away from the cap and utilises all initiation factors except eIF4E. 4G=eIF4G. 4A=eIF4A. 4B=eIF4B. 1=eIF1. 1A= eIF1A. 2=eIF2. 3=eIF3. 5=eIF5. 5B=eIF5B. Met=initiator tRNA methionine. 40S= 40S ribosomal subunit. 60S= 60S ribosomal subunit. AUG= indicates initiation codon and start of ORF.
B: MS2-mDazl can stimulate a m^{7}GpppG -capped Luc-MS2 reporter (M7G-Luc-MS2) and ApppG capped PV-Luc-MS2 reporter (PV-Luc-MS2). The reporters were co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-mDazl. Luciferase activity was normalised to β-galactosidase activity and plotted.
C: Translational stimulation was plotted with MS2-U1A values set to one. The average of four experiments is shown.

Dazl's ability to stimulate the translation of this reporter further reinforces the hypothesis that Dazl does not stimulate cap-binding by eIF4E as in this reporter initiation is completely separate from the cap structure. This effectively eliminates cap binding as the target of Dazl action and suggests that it must act on 43S joining, scanning, or 60S recruitment and not at the first mRNA dependent step in initiation.

3.8 Dazl can stimulate translation via the encephalomyocarditis IRES

As discussed the PV IRES utilises the majority of canonical translation factors with the exception of eIF4E and the N-terminal fragment in eIF4G. This virus belongs to the type 1 subtype of picornaviruses (Pilipenko *et. al.*, 1989) and its mode of translation has been described as 'land and scan'. This is because the small ribosomal subunit scans to a downstream AUG from the point at which it is initially recruited by the IRES. It was therefore of interest to examine an IRES that utilises the same canonical initiation factors but whose mechanism of initiation deviates somewhat from that of the PV.

The encephalomyocarditis virus (EMCV) IRES again initiates translation away from the 5'-cap structure at a 400 nucleotide long structure that contains multiple stem loops. The initiation factors eIF4G, eIF4A, eIF4B form a complex on the stem loop structure that then recruits the 43S complex, probably through interactions between eIF4G and eIF3 (Pestova *et. al.*, 1996a). Again EMCV proteases cleave eIF4G in infected cells. The initiation AUG codon is immediately after the IRES structure and the 43S complex is localised directly to it upon recruitment. This action is sometimes referred to as a 'land and dock' initiation and does not require scanning. After this point 60S joining continues as normal.

When the luciferase levels produced by the EMCV reporter are compared to the M7G reporter with the U1A protein the EMCV reporter produces seven fold less luciferase (figure 3.6B). This indicates that the EMCV reporter is more active than both the ApG

and PV reporters but is still less active than a reporter with an m^7GpppG cap and no IRES. This is in keeping with the results of others who have shown that the EMCV IRES is more efficiently translated on oocytes than an ApppG capped luc-MS2 or PV-luc-MS2 RNA (W Richardson, unpublished).

As can be seen in figure 3.6C Dazl can stimulate this reporter when tethered though the observed stimulation is greatly reduced compared to M7G and PV (figure 3.5C) and is typically only 2.5-fold. A reporter with the EMCV IRES but no MS2 sites (EMCV-Luc-ΔMS2) was not stimulated showing that this reduced level of stimulation was specific and required MS2 binding.

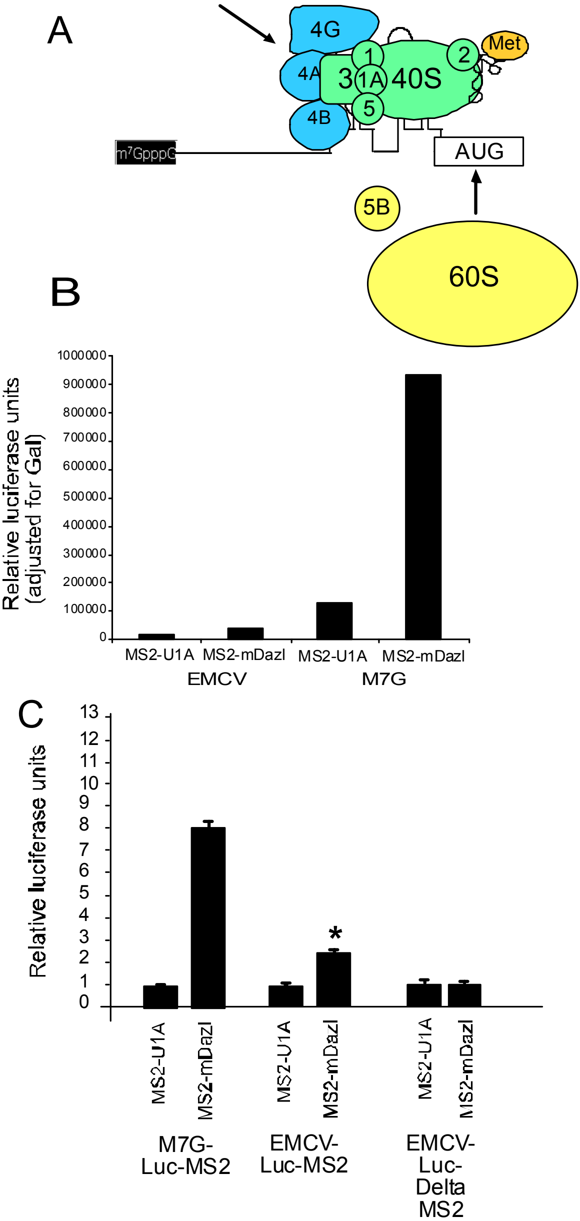


Figure 3.6: mDazl stimulates translation of the EMCV-Luc-MS2 reporter.
A: The EMCV-Luc-MS2 reporter initiates translation at the initiation codon and utilises all initiation factors except eIF4E. 4G=eIF4G. 4A=eIF4A. 4B=eIF4B. 1=eIF1. 1A= eIF1A. 2=eIF2. 3=eIF3. 5=eIF5. 5B=eIF5B. Met=initiator tRNA methionine. 40S= 40S ribosomal subunit. 60S= 60S ribosomal subunit. AUG= indicates initiation codon and start of ORF.
B: MS2-mDazl can stimulate m^{7}GpppG -capped Luc-MS2 reporter (M7G-Luc-MS2) or ApppG capped EMCV-Luc-MS2 reporter (PV-Luc-MS2). Reporters were co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-mDazl. Luciferase activity was normalised to β-galactosidase activity and plotted. * = A significant stimulation by MS2-mDazl is seen ($P < 0.0005$) compared to MS2-U1A, despite the lower stimulation compared to that achieved on the M7G-Luc-MS2 reporter.
C: Translational stimulation was plotted with MS2-U1A values set to one. The average of four experiments is shown.

This result again reinforces the hypothesis that Dazl does not stimulate translation initiation at the stage of cap binding. This reporter still initiates translation by recruiting the small ribosomal subunit in a similar way to 'classic' translation initiation, but in contrast to PV there is no scanning of the ribosomal subunit through the 5'UTR involved.

This intermediate result raises some interesting questions. Is there less stimulation because Dazl stimulates scanning and the mechanism of 43S recruitment by the EMCV IRES means that there is no scanning to stimulate. Or does the fact that Dazl can still stimulate this reporter indicate that scanning is unimportant to Dazl's stimulatory action and that Dazl stimulates 43S joining, which still occurs as 'normal' on the EMCV IRES. One sensible experiment that was not conducted was to inject the EMCV and PV reporters on the same day in order to compare the stimulation directly. I do not think that any differences would be seen as each time they were injected they were compared to the same M7G control. However it would be sensible to confirm that the differences seen in stimulation are real.

The implications of this result and that of the other reporters tested will be discussed in more detail at the end of the chapter.

3.9 Dazl can not stimulate translation via the classical swine fever virus IRES

The ability of Dazl to stimulate the PV reporter and to a lesser extent the EMCV reporter suggests that Dazl utilises factors other than eIF4E and eIF4G that are not involved in cap binding. To investigate this further a classical swine fever virus IRES was engineered into the tethered reporter mRNA (CSFV-luc-MS2).

Whilst this IRES initiates translation at a region of complex structure remote from the 5'-cap, in contrast to the PV and EMCV IRESs, it does not utilise any of the eIF4F initiation factors (Pestova *et. al.*, 1998). Instead it recruits the ternary complex consisting of the 40S ribosomal subunit, eIF2 and a Met-tRNA to the mRNA via an interaction with eIF3 and the IRES. Similar to EMCV it recruits the small ribosomal subunit directly to the initiation AUG codon, in the absence of a prerequisite for scanning. eIF1 and 1A are thought to be dispensable for the CSFV IRES action (Pestova *et. al.*, 2001). Once the small ribosomal subunit has joined the IRES AUG recognition and 60S joining is thought to proceed as normal.

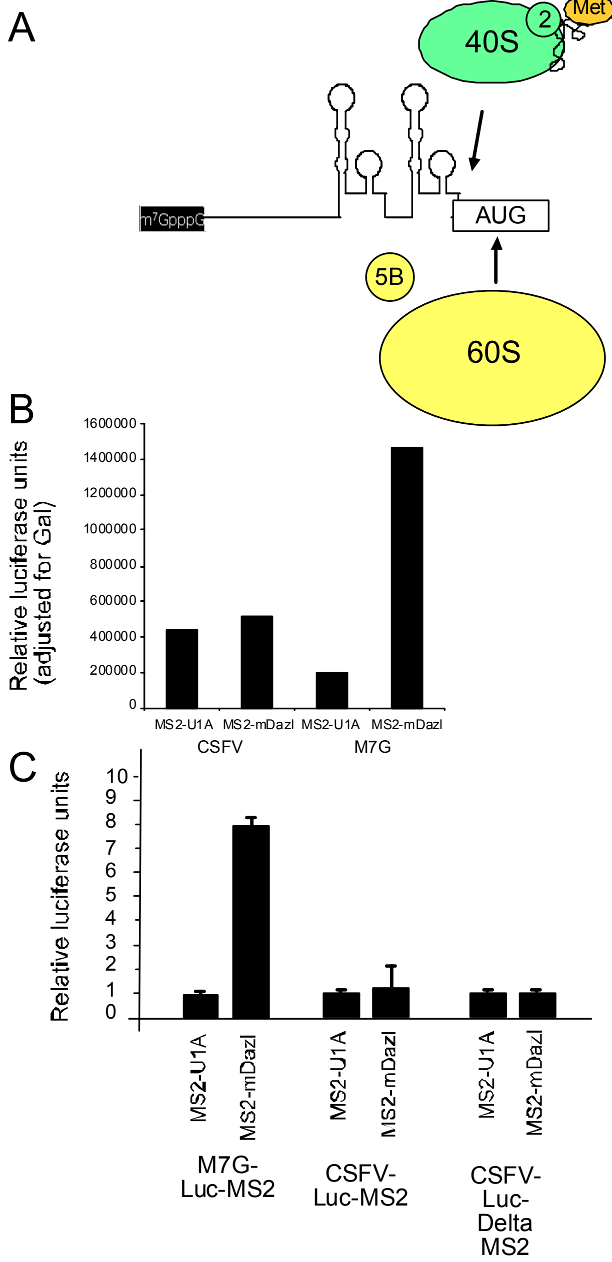


Figure 3.7: mDazl cannot stimulate translation of the CSFV-Luc-MS2 reporter
A: The CSFV-Luc-MS2 reporter initiates translation at the initiation codon and only requires eIF3, the 40S ribosomal subunit, eIF2 and a Met-tRNA eIF4E. 2=eIF2. 5B=eIF5B. Met=initiator tRNA methionine. 40S= 40S ribosomal subunit. 60S= 60S ribosomal subunit. AUG= indicates initiation codon and start of ORF.
B: MS2-mDazl can stimulate m^7GpppG -capped Luc-MS2 reporter (M7G-Luc-MS2) but cannot stimulate ApppG capped CSFV-Luc-MS2 reporter (CSFV-Luc-MS2). Reporters were co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-mDazl. Luciferase activity was normalised to β-galactosidase activity and plotted.
C: Translational stimulation was plotted with MS2-U1A values set to one. The average of four experiments is shown.

The translation achieved from the CSFV IRES was efficient when compared to the other IRES containing reporters and in fact produced more luciferase with MS2-U1A than the M7G reporter despite being ApppG capped (figure 3.7B). The translation of this mRNA is not significantly increased when m^{7}GpppG capped showing that this efficient translation is indeed due to IRES activity (tested by W. Richardson, unpublished).

As can be seen, despite the efficient translation initiated from the CSFV IRES, neither CSFV-Luc-MS2 nor CSFV-luc-ΔMS2 was regulated by tethered Dazl (figure 3.7C).

The result suggests that Dazl does not stimulate large ribosomal subunit (60S) joining as the CSFV IRES is thought to recruit this in the same manner as all the previous reporters, but suggests instead 43S joining or scanning. This result also implies that stimulation by Dazl is be mediated by an initiation factor that is required by the PV and EMCV but not the CSFV IRES.

3.10 Testing the binding affinity of MS2 for the reporters

The previous experiments show that Dazl is able to stimulate the M7G-Luc-MS2, ApG-Luc-MS2 and the PV-Luc-MS2 reporters fully and is able to partially stimulate the EMCV-Luc-MS2 reporter. In contrast Dazl is unable to stimulate the CSFV-Luc-MS2 reporter. It is attractive to conclude that this is because of the differing mechanisms of translation initiation of these reporters, only some of which can support Dazl function. However there are other purely technical explanations that must be taken into account before it is possible to reach this conclusion. One alternative explanation is that the insertion of the IRESs into the luciferase reporter constructs disrupted the binding of the fusion proteins to the MS2 sites and this was the reason for the apparent differences in stimulation. Whilst the binding sites are located far from the IRES, mRNA secondary structure is complex and is not determined purely by local folding (Shapiro *et. al.*, 2007), thus long range effects on structure have been reported (Shapiro *et. al.*, 2007).

To eliminate the possibility that unintended alterations in RNA structure may be responsible for these effects by disrupting the ability of the MS2-fusion to bind the reporter RNAs a series of electro-mobility shift assays (EMSAs) were conducted. Eliminating this possibility will further validate the conclusions drawn from the variable tethered function assay.

In these assays short MS2 containing RNA were *in vitro* transcribed in the presence of ^{32}P-UTP from the pMSCU-CAT plasmid (Stripecke and Hentze, 1992), which contains two MS2 binding sites. This RNA was incubated with purified recombinant MS2 protein (the kind gift of Dr B. Gorgoni), and the complexes resolved by native gel electrophoresis (figure 3.8A). Labelled RNA (lane 1) can be seen to shift to a higher molecular weight complex with the addition of MS2 protein (lane 2). This band shift can be reduced by adding non-radio-labelled (cold) competitor MSCU-CAT RNA to the complex (lane 3). Increasing amounts of cold full-length Luc-MS2 RNA can also eliminate the band shift (lanes 6, 7, 8) indicating that the MS2 protein is binding to the Luc-MS2 RNA leaving it unavailable to form complexes with the labelled MSCU-CAT RNA. When increasing amounts of Luc-ΔMS2 RNA, which is the same luciferase reporter lacking the MS2 binding sites, is added, there is no competition (lanes 9-11) demonstrating specificity.

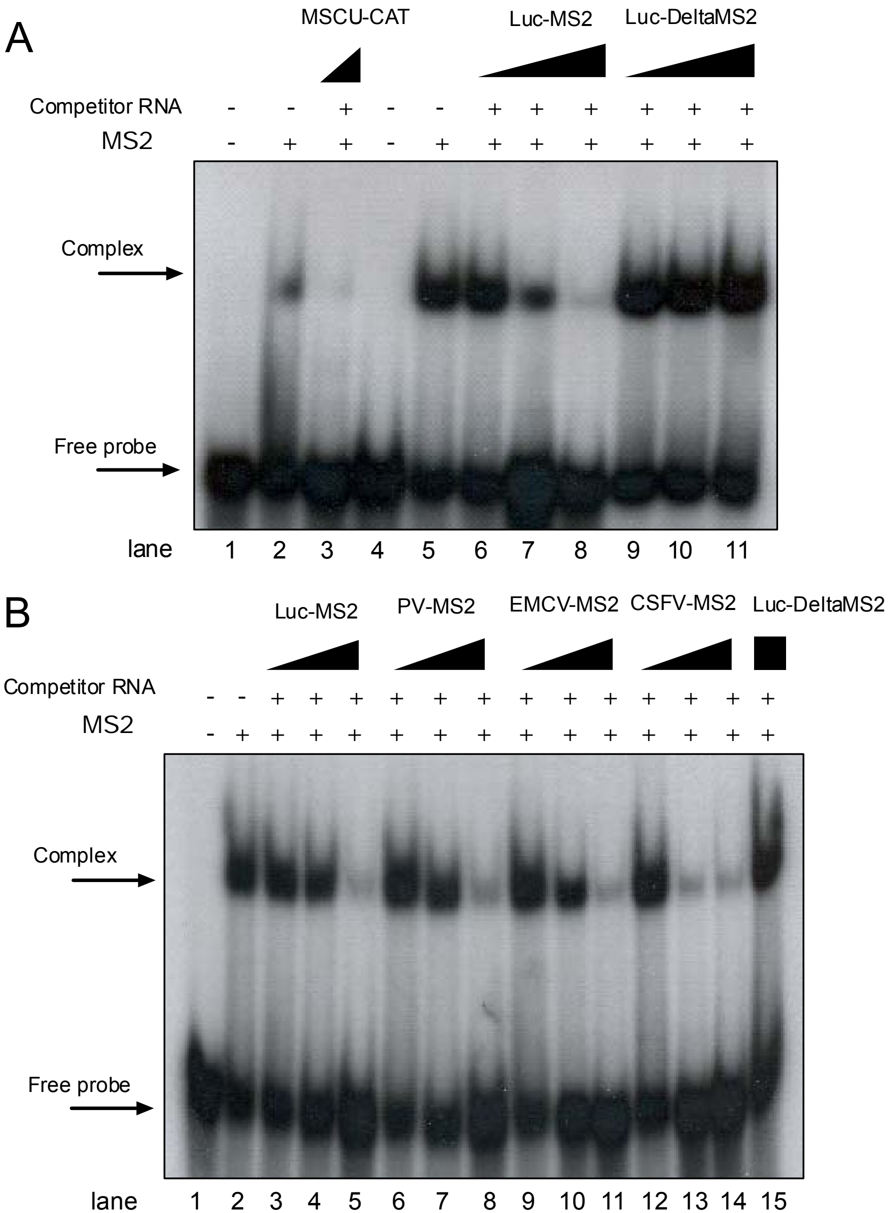


Figure 3.8: The various full-length luciferase reporters all bind MS2 with similar affinities. Short radiolabelled probes alone (lane 1, A+B) were visualized by non-denaturing gel electrophoresis. Addition of MS2 protein forms a complex with the RNA and causes a mobility shift (lane 2, A+B) as indicated by the arrow labelled 'complex'. The ability of cold full-length mRNAs to compete this shift was tested by the addition of:-

A: Increasing amounts of either unlabelled MSCU-CAT RNA (lane 3) or full-length luciferase reporter mRNAs with MS2 (Luc-MS2; lanes 6-8) or without MS2 sites (Luc-ΔMS2; lanes 9-11). Competitors were added at the following amounts; 250ng, 1000ng, 4000ng.

B: Increasing amounts of unlabelled full-length Luc-MS2 (lanes 3-5), PV-Luc-MS2 (lanes 6-8), EMCV-Luc-MS2 (lanes 9-11) and CSFV-Luc-MS2 (lanes 12-14) were added as competitors. Competitors were added at the following amounts; 250ng, 1000ng, 4000ng. Loss of the complex shows that the full-length mRNA contains correctly folded MS2 sites which can be bound by MS2.

The relative abilities of the different full-length mRNAs, Luc-MS2, PV-Luc-MS2, EMCV-Luc-MS2 and CSFV-Luc-MS2 (figure 3.8B) to compete the MS2/MS2 complex were then investigated. The band shift (lane 2) was successfully competed with increasing amounts of full-length Luc-MS2 (lanes 3-5), PV-Luc-MS2 (lanes 6-8), EMCV-Luc-MS2 (lanes 9-11) and CSFV-Luc-MS2 (lanes 12-14). Luc-MS2, PV-Luc-MS2 and EMCV-Luc-MS2 all competed effectively at the highest concentration. The CSFV-luc-MS2 competition appeared to be more effective however, with less RNA being required to compete away the shift. However this increased competition by CSFV-Luc-MS2 was not reproducible between repeats of this experiment, consistent with the absence of a linear increase in competition with the different amounts of cold CSFV mRNA (figure 3.8b, lanes 12-14). In contrast to the MS2 containing reporters, Luc-ΔMS2 (lane 15) did not compete the band shift even at the highest concentration.

These experiments indicate that the differences seen in levels of luciferase expression of the different mRNAs by DAZL are due to mechanistic differences in their translation and not due to differences in RNA binding affinity.

3.11 Dazl does not increase luciferase by stabilising the reporter RNAs

In the initial observations linking Dazl to translational activation (Collier, 2005) it was observed that Dazl did not stabilise the reporter RNAs, as shown by northern blot, and that Dazl therefore directly stimulated the translation of the luciferase RNA rather than allow higher luciferase levels by virtue of stabilisation of the reporter. Previously in this chapter, an increase in luciferase production in a tethered function assay has been interpreted to suggest that a stimulation of the translational activity of the reporter mRNA. Another interpretation of these results could be that the tethered Dazl is stabilising the reporter mRNA in the oocyte, increasing its lifespan and therefore allowing it to produce more luciferase before degradation, giving the impression of a

direct effect on translation. To rule out this possibility, in the case of the variable tethered function assay, a series of stability assays were undertaken.

As such the effect of Dazl on RNA stability on the Luc-MS2 reporter had already been investigated, however possible effects on mRNA stability for PV-Luc-MS2, EMCV-Luc-MS2 and CSFV-Luc-MS2 reporters has not been investigated and could alter the interpretation of the results from the variable reporter tethered function assays.

Several techniques are widely used for assessing the stability of injected mRNAs in oocytes; these involve recovering injected mRNAs and then measuring the amounts of different mRNAs using techniques such as analysis of labelled mRNA by gel electrophoresis, northern blotting and quantitative reverse transcription polymerase chain reaction (QRT-PCR). Due to the requirement to reduce radioactive material usage where possible and the sensitivity of QRT-PCR, it was decided to use this technique to measure stability of the above reporters in the tethered function assay.

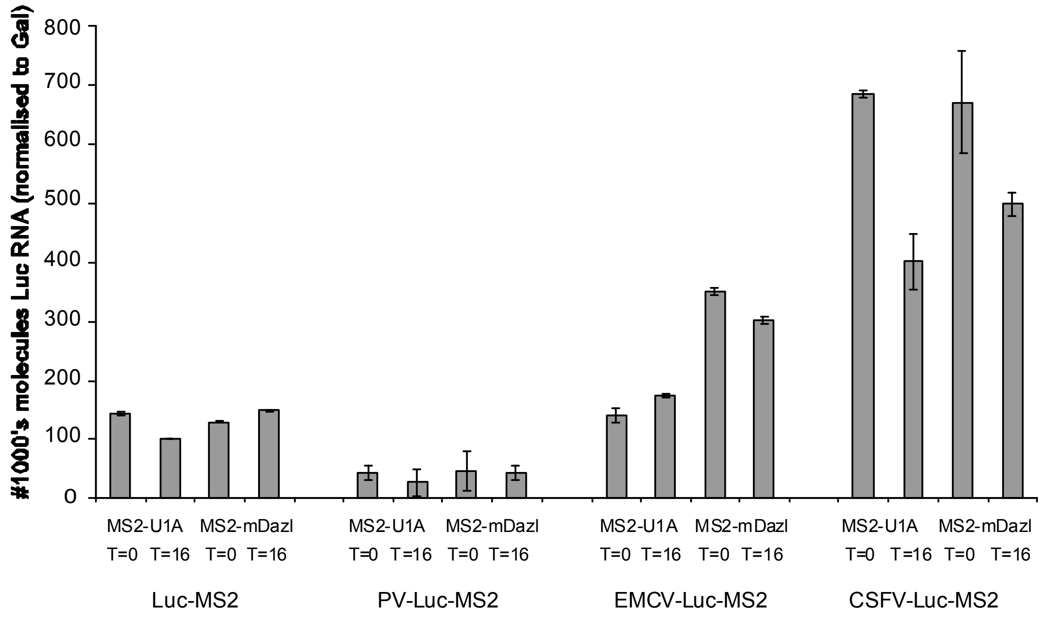
QRT-PCR has recently been used within the lab for the measurement of mRNA stability in oocytes in the presence of several different proteins (Larralde *et. al.*, 2006; Wilkie *et. al.*, 2005). This assay functions by using specific PCR primers to measure the amount of a certain transcript in a cDNA sample. The cDNA is made from RNA extracted from micro-injected oocytes both directly after injection (T=0) and at the end of the assay (T=16 hours). The assay works by using fluorescent technologies to measure the amount of PCR product. As the amount of PCR product is dependent on the amount of cDNA in the original reaction the total fluorescence is dependant on the number of molecules of the specific cDNA template that is being amplified. By including standards containing known numbers of molecules of the particular template that it being amplified it is possible to generate a standard curve for each template. This standard curve can then be used to calculate the number of molecules of a particular cDNA and thus original RNA in each sample.

There are multiple different QRT-PCR fluorescent chemistries available but as one, SYBR green, had previously been used successfully in the lab (Larralde *et. al.*, 2006; Wilkie *et. al.*, 2005) I decided to use it for my experiments. SYBR green is a fluorescent dye that binds to the minor groove of double-stranded DNA. The binding increases the dye's fluorescent emission by over 100-fold. As the PCR reaction progresses more double stranded product accumulates and thus the fluorescent output increases.

Tethered function assays were conducted as before, with the Luc-MS2, PV-Luc-MS2, EMCV-Luc-MS2 and CSFV-Luc-MS2 luciferase reporters being used. However rather than collecting all the injected oocytes for luciferase and β-gal assays, half were utilised in RNA stability assays. These oocytes were snap-frozen in liquid nitrogen to preserve the mRNAs contained within.

The aim of these experiments was to measure the amount of reporter RNA in the oocytes immediately after injection at time equal zero (T=0) and then after the overnight incubation in the presence of the fusion proteins (T=16). The amount of the reporter RNA is entirely limited by the initial injection as no more can be produced. Thus this enables a measurement of the decrease in mRNA levels over the time course of the assay and whether the different fusion proteins have an effect on the kinetics of mRNA turnover. Due to the stability of mRNAs in oocytes it is unlikely that a stabilising effect of Dazl on the rate of reporter RNA decay significantly contributes to the increase in luciferase seen upon Dazl tethering, however this must be formally ruled out. Moreover, a destabilising effect of Dazl on an mRNA such as EMCV or CSFV could mask a simultaneous increase in their translation. Effects on transcription, splicing or mRNA export are not possible due to the design of the experiments where the reporter mRNAs are injected directly into the cytoplasm of the oocytes.

RNA samples were extracted from those oocytes set aside using Tri-reagent. The RNA was then reverse transcribed using specific primers to the open reading frames of the luciferase and β-galactosidase reporters. The numbers of molecules of the reporter RNAs were then calculated using a quantitative PCR (QPCR) approach. The results of these assays represent the numbers of molecules of both the luciferase and β-gal reporters in each sample (figure 3.9A) and the percentage of injected luciferase reporter at T=0 and T=16 (figure 3.9B). As the β-gal reporters are not subject to any stabilisation or decay effects inherent to fusion protein binding, they can be used to normalise RNA recovery between sets of oocytes. Therefore the number of molecules of luciferase RNA were normalised to the number of molecules of β-gal to take into account any experimental variation in numbers of RNA molecules injected.



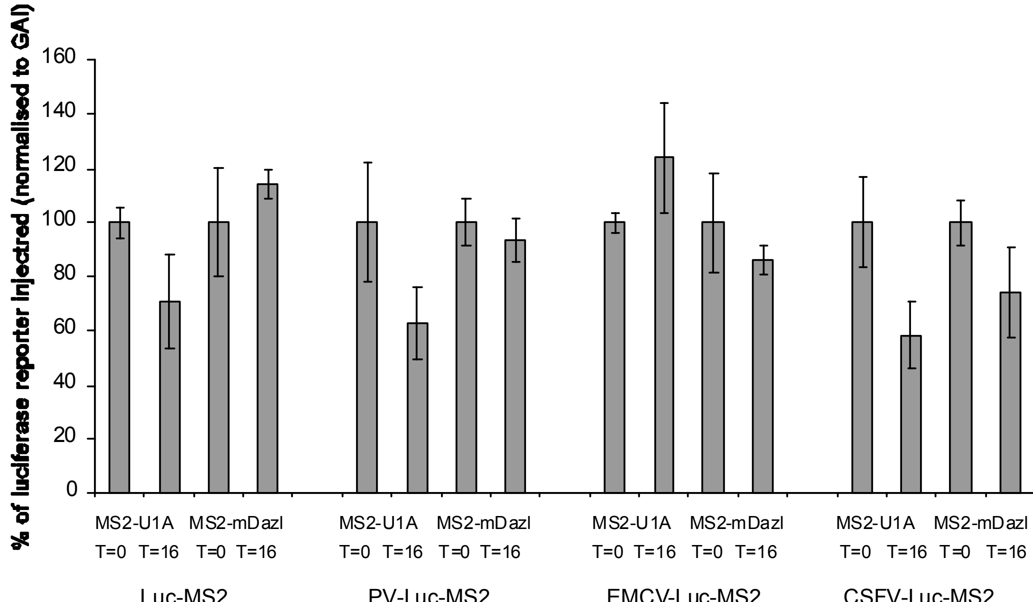


Figure 3.9: Levels of luciferase reporter in oocytes
Quantitative reverse transcription PCR was employed to measure the effect of Dazl binding on the stability of the reporters used in the variable tethered function assay over time.
A: The tethering of Dazl to the different luciferase mRNAs does not have an mRNA stabilisation effect. Oocytes expressing either MS2-UIA or MS2 mDazl were injected with one of luc-MS2, PV-luc-MS2, EMCV-luc-MS2 or CSFV-luc-MS2 and β-galactosidase. Total RNA was extracted either immediately or 16 hours post injection. Quantitative reverse transcription PCR was performed with primers directed against the reporter mRNAs. The numbers of molecules of luciferase reporter were normalised to the β-galactosidase and plotted. The data represents the averages of three repetitions with standard errors.
B: The values represented in figure 3.9A were transformed to show % of luciferase reporter injected at T=0 and T=16.

Quantitative reverse transcription-PCR showed that the levels of reporter mRNA vary at T=0 (figure 3.9A). As the experimental design of the tethered function assay is such that each oocyte should be injected with the same amount of RNA, the differences seen in numbers of molecules injected between the different sets is disturbing. As the assay involves very low concentrations of RNA being injected (7.5ng/μl) the most likely source of this variation is the quantification of the RNA preparations prior to injection. Importantly, however, the levels of individual reporter RNAs did not change significantly over the course of the experiment (figure 3.9B).

There are however changes in the abundance of mRNA in the presence of MS2-U1A compared to MS2-mDazl. This difference was not reported in the initial characterisation of Dazl mediated stimulation, and may be due to the high sensitivity of QRT-PCR versus Northern blotting, injection error or capping efficiency. Stage VI oocytes cannot decap mRNAs to promote turnover (Gillian-Daniel *et. al.*, 1998), thus changes in mRNA stability are not anticipated in this system but could reflect differences in capping efficiencies of the reporter mRNA preparations utilised in these experiments. The changes seen in the assay conducted have been quantified in table 3.1.

Luc-MS2	MS2-U1A MS2-mDazl	- 29% (+/- 17%) + 10% (+/- 5%)
PV-luc-MS2	MS2-U1A MS2-mDazl	- 37% (+/- 13%) - 6% (+/- 8%)
EMCV-luc-MS2	MS2-U1A MS2-mDazl	+ 24% (+/- 21%) - 14% (+/- 5%)
CSFV-luc-MS2	MS2-U1A MS2-mDazl	- 42% (+/- 12%) - 26% (+/- 17%)

Table 3.1: % change of luciferase reporter from T=0 to T=16

Taking these possible effects on mRNA stability/mRNA abundance into account, the effects of the fusion proteins on mRNA translation can be adjusted accordingly (see table 3.2).

	Stabilisation effect by Dazl	Original stimulation by Dazl	Stimulation adjusted for stabilisation
Luc-MS2	+ 39%	8	4.8
PV-luc-MS2	+ 31%	11	7.6
EMCV-luc-MS2	- 38%	2.5	3.5
CSFV-luc-MS2	+ 16%	1.2	1

Table 3.2: Overall effect of Dazl on luciferase levels and effect of stabilisation on translational stimulation

The data in figure 3.9A and B and tables 3.1 and 3.2 establish that changes in mRNA stability cannot account for the differences in luciferase expression in oocytes expressing MS2-Dazl compared to the control protein MS2-U1A. Thus, the adjusted stimulation values in luciferase expression in table 3.2 are solely due to translational effects. This adjustment in the absolute levels of translational stimulation does not, however, affect the interpretation of the variable tethered function assay results. MS2-mDazl stimulates the translation of capped and PV IRES-containing mRNAs and cannot stimulate the translation of CSFV IRES-containing mRNAs. The stimulation of the EMCV IRES reporter remains intermediate, but is not as reduced compared to Luc-MS2 as prior to adjustment (see chapter discussion).

3.12 Discussion

As detailed at the start of this chapter the aim of this work was to enable a better understanding of when and how Dazl stimulates translation. Previously, a model for Dazl's actions in translation was proposed (Collier *et. al.*, 2005) in which Dazl directly recruits PABP to mRNAs with short poly(A) tails in a manner analogous to polyadenylation. These PABP molecules, recruited to the 3'UTR of the mRNA, then interact with factors bound to the 5'UTR thus circularising the mRNA and enhancing translation. The key factors that PABP is thought to interact with are eIF4G, eIF4B and, via an interaction with Paip1, eIF4A. PABP is also known to interact with eukaryotic release factor three, which may promote ribosomal recycling from the 3' end of mRNAs.

It was also established using sucrose gradient analysis that Dazl stimulated translation at the stage of initiation.

One aim of this study was to investigate further the stage in initiation at which Dazl stimulated. To do this a variation on the tethered function assay was employed. Here luciferase reporters with different cap structures and in some cases containing IRESs were used. Whilst it is possible that the that the effects of Dazl on these IRES reporter mRNAs are due to residual cap-dependent translation, as bicistronic reporters were not utilised, the differences in the level of translation for the ApppG capped reporter (figure 3.4C, 3.5B, 3.6B and 3.7B) make this unlikely. Moreover, each of these IRESs are heavily secondary structured, a feature that is likely to impede scanning and thus cap-dependent translation. In addition each of the IRES containing reporters is stimulated to a different degree by Dazl, which is inconsistent with initiation through the same cap-dependent mechanism.

Thus, by looking at which reporters Dazl could and could not stimulate I gained insight into the mechanism of translational stimulation. If Dazl had been unable to stimulate the ApG or PV reporter then it would have implicated cap-binding as the step that Dazl stimulated as the key difference in the mechanism of translation initiation in these reporters is the lack of cap-binding. As Dazl stimulates the PV reporter then this suggests 40S joining, scanning, or 60S joining as the possible stages in initiation that Dazl acts on. As Dazl could not stimulate the translation of the CSFV reporter (figure 3.7), 60S joining appears unlikely as the CSFV IRES is thought to recruit the 60S subunit in the same manner to the other reporters. If Dazl did act by increasing 60S subunit joining then it would have been expected that it would have stimulated all the reporters utilised. This suggests a role in either 40S joining or scanning. As Dazl cannot fully stimulate the EMCV reporter it could be argued that perhaps Dazl affects, at least in part, scanning as it does not play a role in the translation of this reporter. Conversely, in light of the comparatively small differences in stimulation between luc-MS2 and EMCV-luc-MS2 (the adjusted values in table 3.2) it can be argued that Dazl can stimulate a reporter that does not utilise scanning as a predominant mechanism.

This is consistent with Dazl mediating the majority of its effects at the level of 43S joining. The caveat that must be considered with this conclusion is that table 3.2 combines data from all the variable tethered function assays with data from the stability experiments. As these stability experiments only represent a minor fraction of the data, it can be argued that the stability analysis should have been conducted with every tethered function assay for this conclusion to be firmly drawn. However, the stimulation in the stability experiments was representative of the experiments as a whole and I therefore consider the conclusions to be valid.

The question of why the EMCV IRES can only be stimulated to a more limited degree remains a complicated one. Perhaps the 'land and dock' mechanism of this IRES limits the rate at which 43S complexes can be loaded onto the RNA in a manner that the 'land and scan' mechanism of the PV IRES does not. With a 'land and scan' mechanism the recruited 43S complex would depart the IRES, scanning to the initiator codon where events would proceed as in 'normal' translation. In the 'land and dock' mechanism there will be additional steps occurring at the landing site, such as AUG recognition, eIF release and 60S joining before the 80S ribosome clears this site. It is envisaged that these extra events would limit the rate at which the next 43S complex could be recruited onto the mRNA and thus limit the stimulatory effect of Dazl or a poly(A) tail on this process. Alternatively, the 'intermediate' stimulation with the EMCV reporter may indicate subtle differences in the factor requirements for PV and EMCV driven translation (see below). Ultimately, sucrose gradient analysis of initiation intermediates and toe-printing will be required to fully reconcile whether 43S joining, scanning or both are affected by Dazl (see chapter 6).

The analysis in figures 3.4-3.7 also gives some indication of the initiation factors required for Dazl-mediated stimulation. Both the EMCV IRES and the CSFV IRES operate via the 'land and dock' mechanism but Dazl can only stimulate the EMCV IRES, so what are the key differences between the IRESs that could cause this effect? The EMCV IRES has been shown to require eIF2, eIF3, eIF4A and at least a portion of eIF4G protein whereas the CSFV IRES requires only eIF2 and eIF3. The role of eIF4B

with regards to these IRESs is unknown, but it was not required in the reconstitution experiments that established the factor requirements for these IRESs (Pestova *et. al.*, 1998) but its role in IRES-mediated translation in oocytes is unclear (see below). It is attractive to conclude that the reason why Dazl cannot stimulate the CSFV IRES is because it is mediated via one of the factors that the CSFV IRES does not utilise. Interestingly as stated earlier, PABP is thought to stimulate translation through interactions with eIF4G, eIF4B and, via an interaction with Paip1, eIF4A. The fact that these factors are the differentially required by EMCV and CSFV support the involvement of PABP in the model of Dazl action, which will be addressed in more detail in the next chapter.

Importantly the pattern of stimulation seen by Dazl differs to that of a translational stimulator previously investigated using the variable tethered function assay, the stem-loop binding protein (SLBP). SLBP could only stimulate the translation of the M7G reporter and failed to significantly stimulate the ApG or PV reporter (Gorgoni *et. al.*, 2005), this lead to the conclusion that SLBP stimulates an early cap-dependent stage of translation initiation; this has been subsequently verified (Cakmakci *et. al.*, 2008). This confirms that the variable tethered function assay is a useful technique and that the results obtained for Dazl are specific and not recapitulated by other regulators.

Overall the work presented in this chapter enables the model previously proposed (Collier *et. al.*, 2005) to be developed in a number of ways. First, it expands on the observation that Dazl acts at the stage of initiation to delineate the steps affected. My results suggest that Dazl stimulates initiation at the 43S joining or scanning stages (as discussed above). Second, the variable tethered function assay provides valuable insight into the initiation factor requirements of this mechanism and again expands the previous knowledge about this process. Interestingly this varies from other studied activators (Cakmakci *et. al.*, 2008; Gorgoni *et. al.*, 2005; Michlewski *et. al.*, 2008), suggesting a new model for translational regulation.

Despite giving much useful information the limitations of the tethered function assay used in this chapter must be considered. Although to date all proteins shown to regulate translation using this method have been later confirmed using other techniques (Cakmakci *et. al.*, 2008) (Michlewski *et. al.*, 2008). This widely adopted technique has recently been reviewed in detail (Keryer-Bibens *et. al.*, 2007). Because the assay uses a fusion protein there is always the possibility that the structure and therefore the function of the protein of interest is compromised in some manner (though this is common to many molecular biology assays), thus a negative outcome is hard to interpret. Another potential drawback is that sometimes the immobilisation of the tethered protein to the 3'UTR of the reporter could potentially inhibit the action of a protein that has to be able to move along RNA to function, such as a helicases. Third, expression of fusion proteins at high levels may result in disruption of the stoichometry of binding to protein partners, thus altering their function.

When considering the variable reporter tethered function assay the fact that the mechanisms of translation and the factor requirements of the various different reporters discussed above were all inferred from previously published work must be taken into account. The level of knowledge regarding the translational initiation three IRESs used here differs between IRESs. The initiation factor requirements for CSFV and EMCV were characterised by biochemical reconstitution assays *in vitro*, so a clear knowledge of factor requirement is known. However this type of experiment has not been conducted for PV, as discussed earlier. One problem with the *in vitro* reconstitution assays used to establish the factor requirements is that they do not answer the question of what is happening in the oocytes. For example it has been shown that eIF1, eIF1A and eIF4B are not required for EMCV and CSFV IRES activity. However, the effect of these proteins when on translation complexes when present (as they would be in the oocytes) is unclear. Moreover, the effect of intact eIF4G must be considered as no viral proteases are present in the oocytes.

Factors may also play a different role in initiation for different 5'UTRs. For example in the CSFV IRES eIF3 is known to contact directly with the IRES (Sizova *et. al.*, 1998)

and provide a direct link between the RNA and the small ribosomal subunit. In capped PV and EMCV driven mRNAs eIF3 is recruited to the mRNA via its interaction with eIF4G. Due to the different roles of initiation factors in the different IRESs it is uncertain how this may affect the ability of Dazl to stimulate in oocytes, for instance eIF3 subunits bound to the CSFV IRES may be unavailable for interaction with proteins such as Dazl or PABP (not suggesting DAZL directly touches eIF3) with which they could normally interact. Questions are also unanswered with the respect to the way that eIF4G interacts with IRES structures. In the PV and EMCV IRES there is a requirement for eIF4G but it is known that it is not making contact with the mRNA via eIF4E and the cap-structure. This leads to the question of exactly how is eIF4G interacting with the IRES and how does this effect the binding of other factors such as PABP. It is known that the PV and EMCV IRESs are stimulated by the presence of a poly(A) tail (Bergamini *et. al.*, 2000) but that the CSFV IRES is not (W. Richardson, unpublished).

Another factor that must be taken into consideration in the analysis of these experiments is the role of different IRES *trans*-activating factors (ITAFs) on the different IRESs. ITAFs are other non-initiation factor proteins that have been identified as important for IRES function. Both the PV and EMCV IRES require the La autoantigen and the polypyrimidine tract binding protein (PTB) and PV also requires the RNA-binding protein UNR and the poly(rC)-binding proteins PCBP1 and PCBP2. It is possible that the stimulatory potential of each IRES could be limited by the ITAF complement in the oocyte and that is the reason why reduced stimulation is seen in some cases.

This discrepancy between factors necessary for IRES translation and those actually present leads to the conclusion that their true mechanism of translation in oocytes could potentially be different from that assumed. To be entirely certain of the conclusions that have been drawn from these experiments these mechanisms must be confirmed by a second method. The best way to establish the mechanism in oocytes would to move to an oocyte extract system where factors could be manipulated by depletion and reconstitution or specific inhibitory proteins or drugs. The biggest obstacle to this approach is that it requires the production of highly pure full-length recombinant Dazl

protein, to allow the regulation to be reconstituted *in vitro*. This has proven technically difficult, despite many different techniques being employed.

Another test that could be employed to check that ITAF concentration was not limiting would be to reduce the levels of reporter RNA and see if the translational stimulation increases. The effect of reducing the concentration of reporter RNA concentration has been tested for the PV IRES reporter but not for EMCV and CSFV. The concentration of the reporter was reduced by four and this did not affect the stimulation seen. However this experiment was not sufficiently repeated so has not been shown in this thesis. This experiment would be especially important for the CSFV and EMCV reporters as in figure 3.9 it is shown that there was the highest amount of reporter present for these RNAs, thus increasing the chance of an ITAF being the limiting factor. Though it should be noted that the experiments that make up the data for the IRES stimulation experiments (figures 3.5, 3.6 and 3.6) were conducted separately to figure 3.9 so it is not known whether the higher levels of CSFV and EMCV RNA was consistent throughout the experimental series.

Chapter 4: Further characterisation of the Dazl/PABP interaction

4.1 Introduction

The previous chapter investigated the stage in translation at which Dazl stimulates translation, but also mentioned Dazl's relationship with PABP. In this chapter the nature and significance of that relationship will be explored further.

Dazl's interaction with PABP was first suggested in a previous study in this laboratory (Collier *et. al.*, 2005). It was demonstrated that a C-terminal PABP1 portion binds Dazl and that this interaction was RNA independent. This was confirmed by the co-immunoprecipitation of endogenous Xdazl with *Xenopus* laevis PABP1 and ePABP from testis and stage VI oocytes. Subsequently Xdazl and ePABP were found to be located in the same complexes in another laboratory (Padmanabhan and Richter, 2006). Other Dazl family members that stimulate translation, mouse Dazl, human DAZL, human DAZ and human BOULE were also able to interact with *Xenopus* PABP1, suggesting that this interaction may have an important role in this function.

Previous yeast 2-hybrid analysis has shown that amino acids 99-166 of mDazl are responsible for its interaction with PABP1 (Collier *et. al.*, 2005). This region was also shown to be necessary for translational stimulation (Collier *et. al.*, 2005) indicating that it and perhaps the interaction with PABP is required for Dazl's translational activity. Whilst is has been shown that reducing the 3' boundary of this region by 15 amino acids disrupts its interactions with PABP, the 5' boundary and thus minimal region for PABP-binding has yet to be established.

In this chapter the role of residues within the 99-166 region will be explored further using a combination of bioinformatics and yeast two hybrid analyses. Defining a short region or an amino acid motif that binds PABP and could be used to design a bioinformatic search for other proteins that may recruit PABP to activate the translation of mRNAs to which they are bound. A targeted mutagenesis of key residues identified by bioinformatics in Dazl will also be conducted and tested by yeast-2-hybrid assay to

assess whether the interaction with PABP is disrupted. The generation of a specific mutant of Dazl that could not bind to PABP will not only be invaluable in functional assays to further investigate the role of PABP in Dazl mediated translational stimulation but in the longer term to generate transgenic mice to assess the physiological relevance of the observed reaction between Dazl and PABP.

Mouse Dazl was chosen as the target of this analysis, as it had previously been used in the mapping of the 99-166 region and to facilitate the generation of transgenic mice to examine the physiological role of DAZL/PABP interactions in male and female fertility.

4.2 To what extent is PABP required for Dazl-mediated stimulation?

Previous work (Collier *et. al.*, 2005; Padmanabhan and Richter, 2006) has described a correlation between Dazl function and its interaction with PABP and it was proposed that Dazl's translational activity was dependent on this interaction with PABP. Indirect evidence to support this model came from the observation that the presence of a poly(A) tail on a reporter mRNA, and thus PABP recruited by an alternate means, decreased the relative stimulation by Dazl (Collier *et. al.*, 2005).

In the previous chapter the mechanism of Dazl-mediated stimulation was investigated using the variable tethered function assay. However, given the multiple effects of PABP on initiation and the lack of characterisation of its ability to stimulate the different IRESs utilised in this study, this work did not shed light on the role of PABP. If PABP is sufficient to explain the role of Dazl's translational stimulation then it would be expected that PABP would show the same pattern of stimulation as Dazl in the variable reporter tethered function assay. To test this hypothesis tethered function assays were performed with mRNAs encoding MS2-U1A and MS2-PABP and the reporter mRNAs Luc-MS2, PV-Luc-MS2, EMCV-Luc-MS2 or CSFV-Luc-MS2 described in chapter 3.

Some experiments were conducted by Dr. Barbara Gorgoni, who was examining the role of PABP in translation as stated specifically in the legend to figure 4.1A

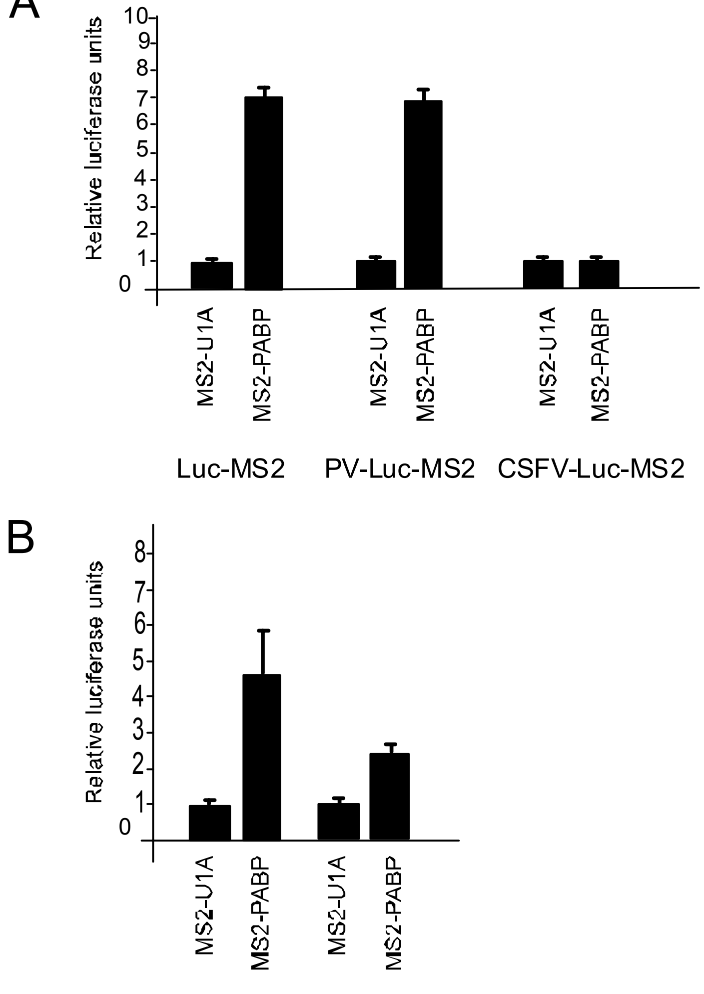


Figure 4.1: PABP pattern of stimulation in the variable reporter tethered function assay.
A: MS2-PABP can stimulate m^7GpppG capped Luc-MS2 (Luc-MS2) or ApppG capped PV-Luc-MS2 (PV-Luc-MS2), but cannot stimulate ApppG capped CSFV-Luc-MS2 (CSFV-Luc-MS2). Reporters were co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-PABP. Luciferase activity was normalised to β-galactosidase activity and the relative luciferase units were plotted with MS2-U1A values set to one. These experiments were conducted by Dr. B. Gorgoni and used with permission.
B: MS2-PABP can stimulate m^7GpppG capped Luc-MS2 (Luc-MS2) or ApppG capped EMCV-Luc-MS2 (EMCV-Luc-MS2). Reporters were co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-PABP. Luciferase activity was normalised to β-galactosidase activity and the relative luciferase units were plotted with MS2-U1A values set to one. The average of five experiments is shown.

Similar to Dazl (see chapter 3), PABP1 stimulates the Luc-MS2 reporter and the PV-Luc-MS2 reporter, approximately 7-fold. This level of stimulation of Luc-MS2 is consistent with published data that shows an average stimulation of 7-fold by PABP1 (Gray *et. al.*, 2000). Interestingly, PABP1 only stimulates the EMCV-Luc-MS2 reporter approximately 3 fold and failed to stimulate the CSFV-Luc-MS2 reporter. This is consistent with the partial stimulation of EMCV and failure to stimulate CSFV observed for Dazl (figures 3.6 and 3.7).

This result supports the hypothesis that Dazl translational stimulation is dependant on PABP, as PABP stimulates the same reporters as Dazl, showing that they share common mechanistic features. If PABP stimulated a different set of reporters to Dazl, this would have indicated that it used a different mechanism of translational stimulation to Dazl thus suggesting that it was unlikely to be Dazl's effector molecule. This is true for other regulators such as SLBP which utilise a different mechanism (Cakmakci *et. al.*, 2008; Gorgoni *et. al.*, 2005). However the result shown here is supportive of a role for PABP in Dazl's mechanism of translational stimulation. This observation will be addressed further in the chapter discussion.

4.3 The yeast two hybrid assay as an experimental system

The interaction between Dazl and PABP has been investigated previously using yeast-two hybrid assays amongst other techniques. Later in this chapter this investigation will be expanded on, further utilising this technique.

The yeast-two hybrid assay (see figure 4.2A) utilises the fact that the DNA-binding domains and activating domains of transcription factors can be separated. These domains are expressed as fusion proteins, with the proteins of interest and the interaction of the test proteins bringing the domains into proximity activating the transcription of reporter genes. These reporter genes are inactive in the absence of an activating protein domain.

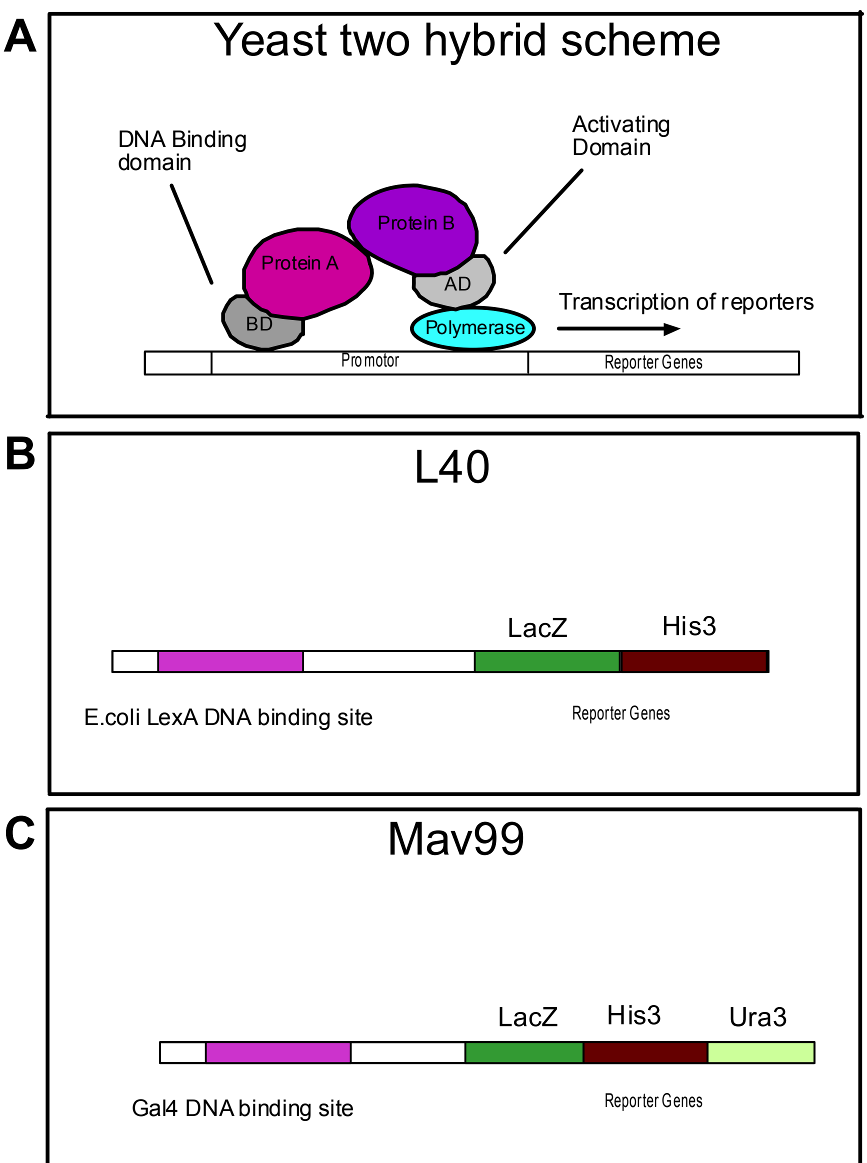


Figure 4.2: The yeast-two hybrid assay
A: General scheme of the yeast-two hybrid assay. A fusion of a DNA binding domain and the first protein of interest binds to a specific promoter upstream of reporter genes. If a second protein fused to a transcription activating domain interacts with the first this brings the activating domain into proximity of the promoter and reporter gene expression is activated.
B: The L40 yeast strain contains LexA DNA binding sites upstream of the LacZ and His3 reporters. The strain is deficient for Trp, Leu and His thus allowing for selection of transformed yeast and screening of Y2H interactions respectively.
C: The Mav99 yeast strain contains GAL4 DNA binding sites within the promoters of LacZ, His3 and Ura3 reporters. The strain is deficient for Trp, Leu and His thus allowing for selection of transformed yeast and screening of Y2H interactions respectively.

Two different yeast two-hybrid systems are utilised in this chapter. The first of these utilises fusions of the 'bait' protein with the LexA DNA binding domain. This fusion is capable of binding the LexA-binding sites that have been engineered into the promoter of several reporter genes present within the L40 (figure 4.2B) yeast strain. These reporters encode β-galactosidase and His3. The expression of β-galactosidase is detected by the presence of blue as opposed to white colonies and activation of the His3 gene allows growth on histidine deficient media. The stringency of the histidine selection can be increased by the addition of 3-amino-triazole (3-AT), which is toxic to yeast without an activated His3 gene; therefore protein interactions confer a 3-AT resistant phenotype.

The second system utilises fusions with the GAL4 DNA-binding domain. The Mav strains contain selectable markers that enable both positive and negative selection with a variable number of GAL4 binding sites within the promoter region. In these studies Mav99 (figure 4.2C) was used which contains 10 GAL4 binding sites (Vidal *et. al.*, 1996). Like L40 this strain contains the *His3* and β-galactosidase reporters, but also has the *URA3* gene. *URA3* is a selectable marker that confers lethality when the yeast is grown on plates containing 5-fluororotic acid (FOA); therefore an interaction between the proteins of interest confers a FOA-sensitive phenotype.

The different mechanisms for selecting positive and negative protein-protein interactions are summarised in table 4.1.

L40

	-ve	+ve
Lac Z	White	Blue
His	No growth on –His media No growth on 3-AT	Growth on –His media Growth on 3-AT

Mav99

	-ve	+ve
Lac Z	White	Blue
His	No growth on –His media No growth on 3-AT	Growth on –His media Growth on 3-AT
Ura 3	Growth on FOA	Death on FOA

Table 4.1: Summary of the different selection methods available to the L40 and Mav99 yeast strains
The L40 and Mav99 yeast strains have different methods to determine if two proteins tested interact. The negative (-ve) and positive (+ve) outcomes are shown for the different assays available. LacZ = β-galactosidase assay. His = Selection on His deficient media. Ura 3 = Selection on media containing 5-fluororotic acid.

It is worth noting that the activating domain plasmids can be used in both systems, the main differences being in the DNA-binding domain plasmids and in the complement of reporter genes.

The preferred system was L40 yeast with the LexA system as one of the major advantages of that system was that the LacZ reporter gene is present on a high copy-number plasmid, and therefore weak signals are more efficiently amplified than in the GAL4 system. However sometimes the Mav99/Gal4 system was used in order to be comparable to previous work. In this chapter the activation of the β-Galactosidase reporter that is present in both strains was monitored.

4.4 DAZL/PABP family interactions

The data presented in figure 4.1 provides additional evidence for the model in that DAZL utilises PABP1 and/or ePABP, at least in part, to stimulate translation in *Xenopus* oocytes. However there are multiple members of the PABP family (see introduction) in vertebrates, and an additional PABP family member, PABP4, has been recently been found in *Xenopus* oocytes (Gorgoni, manuscript in preparation). Mammals have a more

complex family of proteins with five structurally similar cytoplasmic PABPs; PABP1, ePABP, tPABP, PABP4 and PABP5. Several of these PABPs have been shown to be expressed in male or female gonads at least at the RNA level (Feral *et. al.*, 2001). This raises the possibility that several different PABP proteins may be utilised by Dazl family members within the mammalian gonad or that specific interactions occur between individual PABP and Dazl family members. As the full complement of Dazl proteins are only found in man and old world monkeys (see introduction), the ability of different family members to interact was pursued using human proteins due to their demonstrated ability to stimulate translation and interact with at least one PABP family member and the availability of expression analysis within the gonads.

In order to see the extent of potential Dazl-PABP interactions a directed yeast two-hybrid approach was undertaken. To this end human PABP1 and tPABP were PCR amplified and cloned into a DNA-binding domain vector BTM. PCR amplification of PABP4 resulted in a frameshift event and was therefore not examined. Lack of annotation of the human ePABP gene prevented the design of primers to amplify the C-terminal region of this predicted protein. BTM-PABP5 was a gift from Ross Anderson (MRC HGU). These PABP-fusions were co-transformed into L40 alongside activating domain fusions with different Dazl family members or another RNA-binding protein, IRP, which acted as a negative control. MS2 serves as a negative control for interactions mediated by Dazl family members.

	PABP1	tPABP	PABP5	MS2
hDAZL				
hDAZ				
hBOULE				
IRP				

Figure 4.3: Family interactions between Dazl and PABP.
Directed yeast two hybrids were undertaken to determine the interactions between human Dazl family members and human PABP family members. Yeast were transformed with the proteins shown and positive or negative interactions were determined using qualitative β-Gal filter assays. Yeast strain L40 was co-transformed with plasmids encoding the proteins indicated. PABP1, tPABP, PABP5 and MS2 were present in the BTM DNA binding vector. hDAZL, hDAZ, hBOULE and IRP were in present in the pGAD activating domain vector.

Interestingly, interactions were not observed between all family members. For instance whilst each of the DAZL family members interacted with tPABP (forming blue colonies), none interacted with PABP5 (white colonies). Interactions with PABP1 varied dependent on Dazl family member with DAZL and BOULE interacting whilst DAZ did not. The ability of DAZ to interact with tPABP shows that it is active in this assay, although differences in expression level may contribute to its apparent inability to interact with DAZ, as the relative levels of expression have not been determined. Interpretation of results based purely on yeast-two hybrid must be cautious and confirmation by a second *in vitro* method such as GST-pull-down would be required, and ultimately a demonstration of endogenous interactions in cells. Nevertheless a failure to interact with PABP5 is consistent with the absence of a C-terminal domain in this protein, which was the region of *Xenopus* PABP1 that was demonstrated to interact with DAZL (Collier *et. al.*, 2005).

The finding that only two family members interact with PABP1 is intriguing, and given the similarity between PABP1 and tPABP suggests a high degree of specificity. This raises the possibility that certain PABPs mediate specific roles within germ cells as dictated by which Dazl family proteins they can interact with (see chapter discussion).

4.5 The PABP binding 99-166 region is conserved across Dazl family members

A major goal of this chapter is to characterise the residues within mouse Dazl required for binding PABP, and to examine the functional relevance of this interaction. To this end, a comparison between the region of mouse Dazl known to bind PABP and other Dazl proteins was performed. This comparison was restricted to Dazl family members that have been shown to stimulate translation and to bind PABP (Collier *et. al.*, 2005).

The protein sequence of the 99-166 region of mouse Dazl and the corresponding regions of human DAZL, human DAZ, human BOULE and *Xenopus* dazl were retrieved from the Genbank gene database at the National Centre for Biotechnology Information (NCBI) website and were aligned using the ClustalW software (Higgins *et. al.*, 1996).

This comparison (figure 4.4) shows that there is a large block of homology at the N-terminal end of the amino acid 99-166 region. This corresponds to part of the RRM domain, a conserved domain present in many RNA-binding proteins. Interestingly, a number of RRM domains have been shown to mediate protein-protein interactions in addition to RNA binding (Maris *et. al.*, 2005). Thus it is possible that amino acid residues in this area could contribute to PABP binding. The structure of RRM domains bound to RNA has been solved for a number of proteins (Maris *et. al.*, 2005), and this has shown that one face binds RNA whilst the other face is involved in protein-protein interactions. To exclude conserved residues most likely to mediate RNA-binding rather than protein-protein interactions the RRM region of the Dazl proteins were aligned to UIA, another RNA binding protein for which the structure bound to RNA is solved

(Oubridge *et. al.*, 1994). This analysis excluded residues 101, 107, 108, 110, 111, 112, 113, 114 and 115 (shown in pink in figure 4.4). This analysis was performed *in silico* by Dr P. Gautier, a bioinformatician.

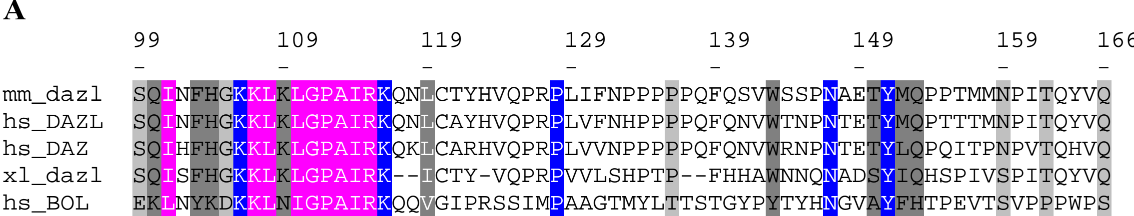


Figure 4.4: Conservation of the 99-166 region of mDazl
ClustalW alignment of the amino acid sequences of the 99-166 region of mouse Dazl and equivalent regions of human DAZL, human DAZ, *Xenopus* dazl and human BOULE. A number of conserved residues can be identified in the region as indicated by different coloured shading. Blue shading = identical residue, Dark grey shading = conserved substitutions, Light grey shading = semi-conserved substitutions, Pink shading = conserved residues recognised as part of the RRM
mm_dazl = Mouse Dazl. hs_DAZL = Human Dazl. hs_DAZ = Human DAZ. Xl_dazl = *Xenopus laevis* dazl. hs_BOL = human BOULE

Apart from the RRM, relatively few fully conserved residues are present in this region that might potentially be key PABP-binding amino acids and these remaining residues are marked in blue in figure 4.4.

However, it has not been established whether other Dazl family members utilise this '99-166' region for binding PABP and this must be established before the analysis of key residues can proceed further.

Human BOULE is the most divergent of these proteins and inclusion of it in the alignments excludes many amino acids, which are conserved or similar (e.g. amino acids 120, 124, 125, 127, 134, 136 between the other proteins). Thus it was decided to determine whether the analogous region of human BOULE (106-173), is responsible for its interaction with PABP. Given the suitability of the yeast-two hybrid assay for identifying the short PABP-binding region in mouse Dazl, the same approach was utilised for the 106-173 region of human BOULE.

4.6 The 106-173 region of BOULE interacts with PABP

To test if the region of human BOULE corresponding to 99-166 of mouse Dazl is responsible for interactions with PABP, plasmids containing fusions of the GAL4 activation domain with amino acids 106-173 of human BOULE were cloned.

Yeast-two-hybrid analysis with this plasmid was undertaken to test its ability to interact with the LexA fusion of human PABP1Ct (see figure 4.5). Activating domain fusions of human PAIP and IRP were included as positive and negative controls respectively for interactions with the PABP1 C-terminus. A fusion of the LexA DNA-binding domain and the bacteriophage MS2 protein was used as a negative control for interaction with the BOULE protein fragment.

Figure 4.5: Directed yeast two hybrid of the interaction of the human Boule 106-173 region with human PABPC1
PABP1 can interact with the 106-173 fragment of BOULE as demonstrated by directed yeast-2 hybrid assay. Yeast strain L40 was co-transformed with the proteins shown and positive or negative interactions were determined using qualitative β-Gal filter assays. Negative control interactions between PABP1 and IRP and MS2 and BOULE 106-173 were included to ensure that the fusion proteins were not self activating. A positive control interaction between PABP1 and PAIP was included to ensure that the PABP1 fusion protein was able to interact with a known partner.

This analysis revealed a clear interaction between the 106-173 region of human BOULE with PABP1. Importantly, this shows that the PABP binding region originally identified in mouse Dazl is conserved between different Dazl family members and across species. In light of this result it was decided to not test the corresponding region of human DAZL or DAZ or *Xenopus* Dazl (see chapter discussion).

4.7 Fine mapping of the 99-66 region

The region of interest as it stands is 68 amino acids long, which is still a relatively large region in terms of protein-binding sites and the conserved amino acids are dispersed throughout this region. In order to further define the minimal region that is capable of binding PABP1 a yeast-two-hybrid approach was employed, using progressively smaller fragments of mouse Dazl. As shortening the 3' end of the 99-166 region by 15 residues abrogates its ability to interact with PABP (Collier *et. al.*, 2005), it was decided to focus on N-terminal deletions.

In order to be consistent with the preceding work (Collier *et. al.*, 2005) where the 99-166 fragment was cloned into the GAL4 DNA-binding domain vector (pGBKT7) the relevant fragments of the mouse Dazl protein were cloned into pGBKT7 and tested against the C-terminal region of *Xenopus* PABP1. Initially two shorter fragments of Dazl were cloned, the first containing 37 amino acids consisting of the 129-166 region and the second containing the 27 amino acids representing the 139-166 region. Schematic representations of these areas are shown in figure 4.6.

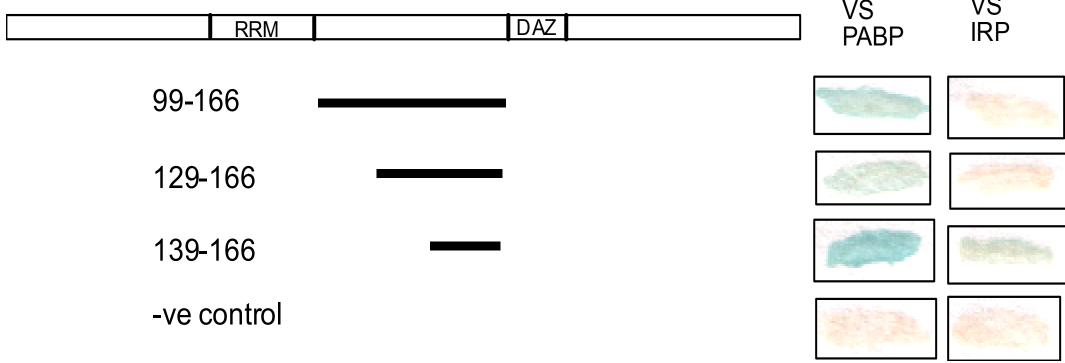


Figure 4.6: Directed yeast two hybrid of the interaction of Dazl fragments with *Xenopus* PABP1 Ct.
Directed yeast two hybrids were undertaken to determine the interactions between mDazl fragments and PABP. The mDazl 99-166, 129-166 and 139-166 fragments can all interact with PABP. Negative control interactions with a known non-interactor IRP were included to show if the mDazl fragments self activated. Yeast strain Mav99 was co-transformed with the proteins shown and positive or negative interactions were determined using qualitative β-Gal filter assays. Negative control interactions between PABP1 and IRP with MS2 were included to ensure that the fusion proteins were not self activating.

Directed yeast-two-hybrid analysis in Mav99 confirmed that the 99-166 region of mouse Dazl is sufficient to support an interaction with PABP1 (compare 99-166 versus PABP to the negative control IRP). When the region of Dazl was reduced to amino acids 129-166 the yeast were still blue indicating the interaction was maintained although they appear lighter in colour than amino acids 99-166. As the relative levels of the fusion proteins were not determined by Western blotting, it cannot be concluded whether this apparent reduction on β-Galactosidase levels is due to a reduction in binding affinity.

When the region was reduced further to amino acids 139-166, the yeast were dark blue upon assay, indicating a substantial interaction. However, the corresponding IRP control for the 139-166 fragment is light blue suggesting a degree of self-activation. However, as the signal seen with PABP1 is considerably stronger than the IRP signal, this result was considered to be significant. This suggests that the minimal region for PABP-binding can be further narrowed to amino acids 139-166.

4.8 Identification and mutagenesis of key residues in Dazl

With the knowledge that the critical region for interacting with Dazl has been narrowed down to amino acids 139-166 the alignments of the 99-166 region were re-examined, with the aid of Dr P. Gautier.

The structure of this region, which does not share significant homology with proteins outside the DAZL family, has not been solved by crystallography or protein nuclear magnetic resonance spectroscopy (protein NMR). The absence of structural information for this family thus precludes the identification of residues that are likely to be in the protein surface and available for protein-protein interactions. Structural predictions were made by Dr Gautier using protein explorer (www. protein explorer.org, Copyright © 2003 by Eric Martz), but revealed very limited information suggesting that the non-RRM region is a “long non globular region”, of low complexity. However, a few areas

of beta strand were predicted in human Dazl and BOULE (shown as red boxes on figure 4.7). Only these proteins were put into the prediction software as a representative test to see if it could generate any useful information.

Due to the absence of a clear structural prediction for the region, candidate residues for site directed mutagenesis were selected on a number of criteria: First they had to be located within the 139-166 region. Second the residues had to show a high degree of conservation or similarity across the proteins examined experimentally (Collier *et. al.*, 2005) indicating potential functional importance and thirdly residues were also prioritised based on their ability to direct protein/protein interactions. The analysis of the PABP binding critical region was also expanded to include other Dazl family members that had not been experimentally shown to stimulate translation. This analysis revealed that the residues identified as conserved in figure 4.4 are also conserved in a number of other species, thus suggesting a potentially important role.

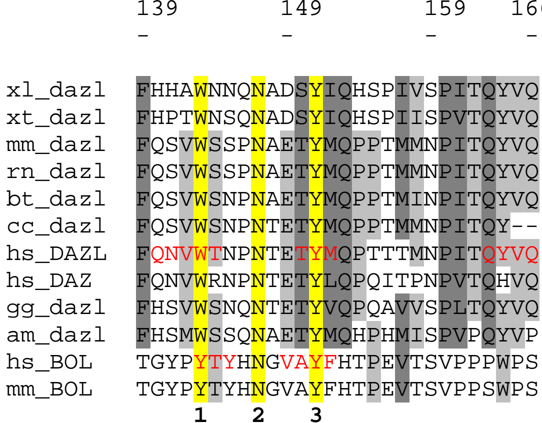


Figure 4.7: Selection of candidate residues
Expansion of the alignment of the 139-166 region to include other Dazl family members. Three residues are identified as possible candidates for mutagenesis as indicated in Yellow. Xl_dazl = *Xenopus laevis* dazl. Xt_dazl = *Xenopus tropicalus* dazl. mm_dazl = Mouse Dazl. rn_dazl = Rat Dazl. cc_dazl = Dog Dazl. hs_DAZL = Human DAZL. hs_DAZ = Human DAZ. gg_dazl = Chicken Dazl. am_dazl = *Amphioxus* Dazl. hs_BOL = Human BOULE. mm_BOL = Mouse Boule. Yellow = Suggested as mutational candidates within the 139-166 region. Dark grey= conserved substitutions. Light grey= semi-conserved substitutions. Red residues denote area of complexity identified (see main text). NB. In this alignment areas of conserved and semi-conserved substitutions are shown regardless of if they are fully conserved throughout all protein aligned.

The new alignment as shown in figure 4.7 was used to identify three residues that were considered as the best candidates to take forward into the planned mutagenesis scheme. These residues were considered to be the most likely to be crucial for Dazl-PABP protein-portion interactions based on their conservation and amino acid type. The three residues are shown in yellow in figure 4.7 and summarised in table 4.2 below.

Mutation number	Amino Acid number	Current codon	Current residue	Mutated codon	Mutated to
1	W143	TGG	Tryptophan	GTG	Valine
2	N147	AAT	Asparagine	GCT	Alanine
3	Y151	TAC	Tyrosine	GCT	Valine

Table 4.2: Mutagenesis scheme in mouse Dazl

The residues were mutated to alanines and valines on the basis that these uncharged residues do not typically support protein-protein interactions. The choice of alanine or valine was dictated by the minimal number of base changes in the codon. Where all the bases had to be changed, for example Y151, valine was chosen as the default.

The mutations were inserted cumulatively, from the C-terminal end of the protein, in case one mutation alone was not capable of abrogating the Dazl/PABP interaction. The first construct (M3) contained mutation #3 alone, the second (M2,3) mutations #2 and #3 and the third (M1,2,3) mutations #1, #2 and #3, as detailed in figure 4.8. If the triple mutant abrogated binding it was then planed to test mutations 1 and 2 together and 1 and 2 alone to identify the importance of each amino acid.

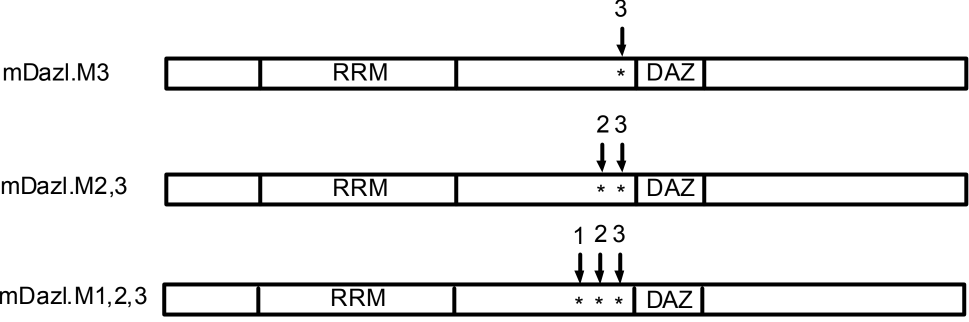


Figure 4.8: Dazl mutagenesis scheme.
Schematic of Dazl mutagenesis scheme showing location of residues to be mutated as indicated by *. RRM = RNA recognition motif. DAZ = location of the DAZ domain.

The method used to generate the mutants was a PCR based site directed mutagenesis approach called overlap extension PCR. This allows the insertion of point mutations into DNA sequences without the need to generate single stranded DNA as in other techniques (Ling and Robinson, 1997). The method is described in detail in chapter 2.

The three Dazl mutants were generated as described in chapter 2, cloned into the GAL4 activation domain plasmid pGADT7 and tested on their ability to interact with the C-terminal region of *Xenopus* ePABP in a directed yeast -2-hybrid assay. ePABP was chosen for this analysis rather than PABP1 because ePABP is the most abundant protein in stage VI *Xenopus* oocytes, and had been shown during the completion of this thesis to be capable of stimulating translation and was therefore regarded as the predominant PABP available to Dazl in these oocytes with which to interact and stimulate translation.

Figure 4.9: Effect of Dazl mutations on interactions with PABP.
Directed yeast two hybrid experiments were conducted in yeast strain Mav99 to measure if the mDazl mutants could still interact with PABP. Yeast strain Mav99 was co-transformed with the proteins shown and positive or negative interactions were determined using qualitative β-Gal filter assays. BTM.ePBAP (Xl) was tested against pGAD.Paip, pGAD.IRP, pGAD.mDazl, pGAD, pGAD.mDazl.M3, pGAD.mDazl.M2,3 and pGAD.mDazl.M1,2,3. IRP is included as a negative control and Paip as a positive control.

Paip was included as a positive control for an ePABP-interacting protein, while IRP served as the negative control. The three Dazl mutants were compared to wild type Dazl for their ability to interact with ePABP. All the Dazls, including the three mutants, gave a strong blue signal in the filter binding assay (figure 4.9) indicating a robust interaction with PABP. This result suggests that the mutation of the 'critical' residues identified by the earlier bioinformatics did not have any significant effect on the interaction between Dazl and PABP, although quantative β-galactosidase assays were not performed.

4.9 The mutations have little effect on translation

The mutations were also tested by tether function assay to determine if they affected translation stimulation by Dazl. As the mutations did not disrupt interactions with PABP, they would be predicted to stimulate if PABP is indeed important for Dazl function. If the mutations failed to stimulate, this would raise questions with respect to the model for the mechanism of translation by Dazl.

To this end full-length Dazl constructs containing the mutations were cloned into the MS2 fusion expression vector, MSPN, and compared to wild-type Dazl in standard tethered function assays.

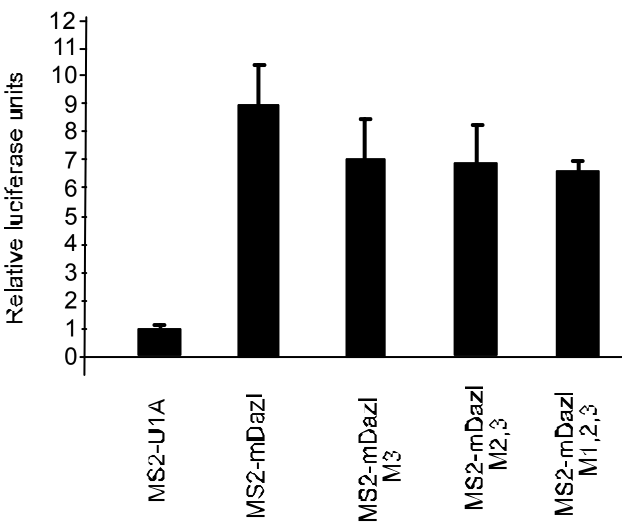


Figure 4.10: Effect of Dazl mutants on translation
The ability of the mutant Dazls to stimulate translation was measured using tethered function assays. All three mutants still stimulated translation. m^7GpppG -capped Luc-MS2 reporter (M7G-Luc-MS2) was co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A, MS2-mDazl, MS2-mDazl.M3 MS2-mDazl.M2,3 or MS2-mDazl.M1,2,3. Translational stimulation was plotted with MS2-U1A values set to one. The experiment was repeated four times and average values were plotted.

When compared to wild type mouse Dazl (figure 4.10) the single, double and triple mutants displayed slightly reduced levels of translational stimulation as shown by the mean values represented by the solid bars. However, the standard error bars overlap with the wild-type protein showing that the mutants do not give significantly lower levels of stimulation.

The fact that these mutants, which still interact with PABP, stimulate translation is consistent with the current model of Dazl action; however it does not provide additional direct support for a role of PABP.

4.10 Reassessment of the region required

As the mutations identified above did not abrogate interactions with PABP or translational stimulation it was decided to re-examine the minimal region required for PABP interaction. In section 4.3 the 99-166 region of interest was narrowed down to amino acids 139-166. Further deletion the N-terminal end of the protein fragment was tested by cloning a fragment consisting of amino-acids 149-166 being cloned into pGBKT7. This new fragment was tested for is ability to interact with PABP1 with IRP serving as a negative control (figure 4.11).

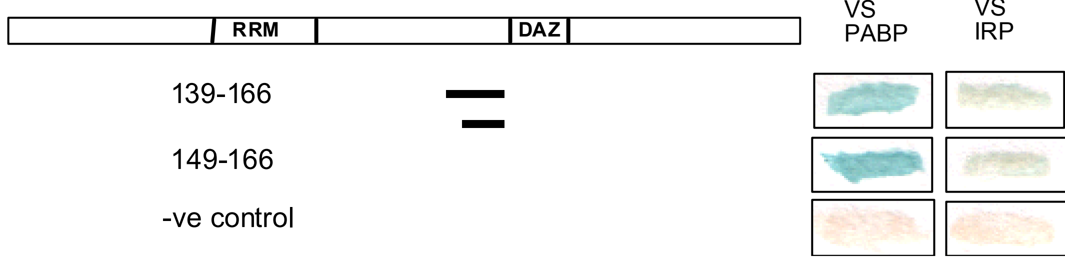


Figure 4.11: The area of interested can be narrowed to amino acids 149-166
Directed yeast two hybrids were undertaken to determine if the minimal region for interaction between mDazl and PABP could be narrowed further. The mDazl 139-166 and 149-166 fragments can both interact with PABP. Negative control interactions with a known non-interactor IRP were included to show if the mDazl fragments self activated. Yeast strain Mav99 was co-transformed with the proteins shown and positive or negative interactions were determined using qualitative β-Gal filter assays. Negative control interactions between PABP1 and IRP with MS2 were included to ensure that the fusion proteins were not self activating.

Both the 139-166 and the 149-166 fragment displayed a degree of self-activation with IRP-1. The 139-166 region gave a good strong interaction with PABP, as previously described (figure 4.6), and importantly the 149-166 fragment also showed a robust interaction with PABP over background. Taken together with the data in figure 4.6, this narrows the PABP-binding site from the original 67 amino acids (Collier *et. al.*, 2005) to just 18 amino acids. It was decided not to further narrow this region due to the propensity of these small fragments (139-166 and 149-166) to self activate in the yeast-two hybrid assay, and because it is unclear whether the natural protein folding and secondary structure of these small fragments is maintained. Moreover, this small region

of 18 amino acids is easily manageable for future analysis by point directed or saturation mutagenesis.

4.11 Design for future mutagenic approaches

With the knowledge that the minimal region for binding PABP is just a small peptide of 18 amino acids, it was decided to re-examine the alignments with a view to identifying more candidate residues.

A

```
            149       159    166
            -         -      -
mm_dazl     ETYMQPPTMMNPITQYVQ
hs_DAZL     ETYMQPTTTMNPITQYVQ
hs_DAZ      ETYLQPQITPNPVTQHVQ
xl_dazl     DSYIQHSPIVSPITQYVQ
hs_BOL      VAYFHTPEVTSVPPPWPS
              3
```

B

```
            149       159    166
            -         -      -
mm_dazl     ETYMQPPTMMNPITQYVQ
hs_DAZL     ETYMQPTTTMNPITQYVQ
hs_DAZ      ETYLQPQITPNPVTQHVQ
xl_dazl     DSYIQHSPIVSPITQYVQ
hs_BOL      VAYFHTPEVTSVPPPWPS
```

C

AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y))
DE	BLUE	Acidic
RK	MAGENTA	Basic - H
STYHCNGQ	GREEN	Hydroxyl + sulfhydryl + amine + G

Figure 4.12: Features of the minimal region
A: Yellow = Original Suggested mutational candidates. Dark grey= conserved substitutions. Light grey= semi-conserved substitutions. mm_dazl = Mouse Dazl. hs_DAZL = Human Dazl. hs_DAZ = Human DAZ. Xl_dazl = *Xenopus* laevis dazl. hs_BOL = human BOULE
B: Residues can be classified according to chemical characteristics
C: Residue characteristics as described by ClustalW program.

Figure 4.12 shows the newly identified minimal region a) including the originally identified candidate mutation #3 (marked in yellow) and b) the amino acids as sorted by

type (based on the ClustalW program). There are areas of conserved substitutions immediately flanking the Y151 residue but as seen in figure 5.6B two of the three are not conserved by amino acid type in BOULE.

This leaves three possible candidates residues that are fairly well conserved and also within the same amino acid type. These are residues Q153, N159 and Q166 in mouse Dazl. However these residues were not originally chosen from the bioinformatics survey of the 99-166 region because they were not considered sufficiently conserved to meet the selection criteria. Therefore these residues merely represent the best remaining candidates, but as these residues were not compelling enough to be selected in the original analysis, the value of mutating them is unclear.

With no clear candidates remaining it was decided that the generation of a PABP-binding deficient Dazl would be best served by a random mutagenesis scheme. Unfortunately due to time constraints this could not be undertaken during the course of the thesis (see discussion).

4.12 Discussion

This chapter focuses on the role and importance of the interaction of Dazl with PABP and how that this protein-protein relationship affected translation.

In chapter 3 the mechanism of Dazl-mediated stimulation was investigated using the variable tethered function assay. Here the role of PABP in stimulating these IRESs was investigated and importantly gave the same pattern of stimulation as Dazl (figure 4.1). This is consistent with PABP being the effector molecule for Dazl and provides further support for the proposed model. These observations are also consistent with an analysis of poly(A) tail function of different IRESs showing that an EMCV IRES was only augmented approximately three fold and PV by ten fold (Bergamini *et. al.*, 2000). As a

poly(A) tail is thought to primarily act in translation via PABP, this provides independent confirmation of my observations in figure 4.1.

The analysis of IRES stimulation in this chapter is consistent with and supports a role for PABP in translational stimulation. However both Dazl and PABP are members of multi-protein families, thus it is unclear what the full extent of interactions between the family members may be. *Xenopus* oocytes contain at least one other PABP that may also be utilised by Dazl, ePABP. In fact an interaction between species DAZL and both *Xenopus* PABP1 and ePABP was demonstrated previously (Collier *et. al.*, 2005).

However mammals are known to contain a larger complement of both Dazl and PABP proteins and only partial expression analysis at the protein level is available for either family. Within human gonads, DAZL is known to be expressed in spermatogonia, primary and secondary spermatocytes, spermatids and postmeiotic cells, DAZ in spermatogonia and spermatocytes and Boule in spermatocytes (see section 1.6.3 for details). Only the expression of PABP1 and tPABP has been examined in the gonads in human testis using in situ hybridisation these show restricted expression to the spermatocytes and spermatids for PABP1 and spermatids for tPABP (Feral *et. al.*, 2001). Mouse northern blots show expression of both PABP1 and tPABP mRNAs in spermatocytes and spermatids (Kleene *et. al.*, 1994). Northern blot and RT-PCR analysis suggests that PABPC4 and ePABP may also be present in the testis (Wilkie *et. al.*, 2005; Yang *et. al.*, 1995).

This raises the question of which PABPs are utilised by Dazl family members in the germ cells where the Dazl phenotype is evident. However, as all of these PABPs have not been observed at the protein level and it is unclear which cell-type they are expressed in within this tissue making their ability to interact with Dazl family proteins on a protein level impossible to predict until this knowledge is known.

However, PABP5 at least does not provide a good candidate for DAZL family function in these early germ cells as, at least by two-hybrid analysis, it does not interact. Whilst

this result requires confirmation by a second technique, this is consistent with the mapping of the interaction to the C-terminal region of PABP1, which is missing in PABP5.

It remains possible however that the early DAZL phenotype is not due to a lack of activation of target mRNAs through PABPs but may be due to a loss of translational repression or mislocalisation of target mRNAs as DAZL protein appear to be present in multiple complexes (see main discussion in chapter 6). This also raises the possibility that certain PABPs mediate specific roles within germ cells and should be the subject of further investigation (see chapter 6).

One of the main aims of this chapter was to further characterise the PABP-binding site within family members leading to the generation of a point mutation that is deficient in PABP binding that would have enable the categorical address of whether Dazl stimulation is entirely dependent on its interaction with PABP or whether other factors are involved. This mutant could also have been used to generate transgenic mice to determine the physiological role of the PABP-Dazl interaction in germ cells (see chapter 6). Dazl presents a better mutational candidate for such physiological analysis, as although both are members of multi-proteins families, loss of Dazl function has been shown to result in an easily identifiable loss of fertility. However, the mutation would need only to affect PABP-binding and not interaction with other protein partners or its ability to bind RNA.

Prior to this work, it was assumed that PABP-interactions were mediated by equivalent regions of the different Dazl family members. Here the prediction was tested using the most diverse equivalent of the mouse 99-166 region, human BOULE 106-173. This confirmed the importance of this region in mediating PABP interactions and allowed comparison between different family members shown to stimulate translation in our assays to identify important residues. Whilst this region was not shown to be sufficient for each family member the equivalent regions for human DAZL, human DAZ and *Xenopus* Dazl do not diverge far from the mouse Dazl 99-166, and therefore the

interaction with 106-173 of human BOULE was seen as adequate support for the alignments. These alignments identified six residues of particular interest for mutagenesis and further deletion reduced the region to 27 amino acids, resulting in three amino acids being prioritised for mutation. The selected mutations had little effect on either PABP binding or translational activity (figure 4.10 and 4.11). Whilst this is consistent with a role for PABP, it was not the desired outcome as this result neither definitively confirms or excludes or defines the extent of PABP's role in Dazl mediated stimulation of translation. However, in the absence of additional information such as structural prediction or similarity to other PABP binding proteins, these residues remain the most obvious candidates for mutagenesis.

This work presented here reduced the minimal binding site to 18 amino acids (fig 4.12), a short region that could be used for random mutagenesis. Given that there are apparently no high quality candidates for further site directed mutagenesis this approach would appear to be the most appropriate. This could be achieved by creating a library of mutations spanning this region by a low fidelity PCR in a yeast two hybrid vector. PCR conditions can be established to result in an average of only one mutation per clone in the region of interest. There is precedent for single mutations abrogating the interaction of PABP with other factors. For instance the point mutation M161A in mouse PABP1 results in a loss of interaction with eIF4G (Kahvejian *et. al.*, 2005) and single (M16) and double point mutations (M15) in the herpes simplex virus ICP27 protein appears to abrogate its interaction with PABP (O. Larralde, R. Smith, B. Clements and N. Gray, unpublished).

Mutants that disrupt interaction could be screened by yeast-two hybrid analysis using the Mav99 strain and selecting on plates containing FOA. Using *Ura3* gene as a reporter allows positive selection for mutants that do not interact, as interaction will result in lethality (Table 4.1). By varying the amount of FOA the selection can be less stringent to allow for the selection of mutations that reduce but do not fully abrogate the interaction. Clones that fail to or have significantly reduced interaction with PABP would be sequenced and defects in interaction confirmed by another means such as

GST-pull down experiments or immunoprecipitation assays of endogenous PABP in oocytes expressing wild type or mutant Dazl. This would address the ability of the mutant to disrupt binding in the context of the full length Dazl, as other regions of the protein may contribute to binding *in vivo*. For instance it has been reported that deletion of the DAZ motif from mouse Dazl reduces the interaction with PABP in oocytes (Collier *et. al.*, 2005), even though this domain is out-with the PABP binding site defined by Y2H (this will be discussed in more detail in the next chapter).

Finally, full-length mutant Dazls that fail to interact with PABP would be tested for their ability to stimulate translation in tethered function assays. If a single point mutation is not sufficient to abrogate the Dazl-PABP interaction then the mutagenesis scheme could be altered to allow multiple mutations or alternatively two or more weak mutations could be combined by site directed mutagenesis. Once a mutant had been identified it would be important to check that interactions with other protein partners was not also disrupted, as otherwise it would be impossible to attribute any affects of the mutations to PABP alone.

The results presented in this chapter imply that the binding site for PABP does not constitute an easily identifiable motif. This may not be surprising given that the PABPC binding motif PAM2, which has been identified in a number of different proteins, only has three fully conserved residues out of eighteen and has a highly variable sequence (Albrecht and Lengauer, 2004) despite being capable of binding PABP. It appears that structural features may be more important than sequence in dictating interactions with translation factors such as PABP. This trend is also demonstrated in proteins that interact with eIF4E, with Maskin, 4E-BPs and eIF4G all having a eIF4E binding region 14 amino acids long but only having two common residues as shown in figure 4.14 (Stebbins-Boaz *et. al.*, 1999).

```
        Maskin EFKLATEADFLIAA
 Human 4E-BP 2 GTRIIYDRKFLLDR
 Human 4E-BP 3 GTRIIYDRKFLLEC
 Human 4E-BP 1 GTRIIYDRKFLMEC
Human eIF-4G 1 EEKKRYDREFLLGF
Human eIF-4G 2 EGKKQYDREFLLDF
Human eIF-4G 3 DLKVKADREFLLDF
  Yeast eIF-4G HVKYTYGPTFLLQF
 Yeast 4E-BP 1 KVKPNNKIIFLPDD
    Fly eIF-4G ANEKAELEAFLEEE
```

Figure 4.14: Comparison of eIF4E binding regions.
eIF4E binding regions of *Xenopus* Maskin, Human 4E-BPs 1, 2 and 3, Human eIF4G 1, 2 and 3, Saccharomyces cerevisiae eIF4G and 4E-BP1 and *Drosophila* eIF4G. Adapted from (Stebbins-Boaz *et. al.*, 1999). Yellow indicates conserved residues.

Chapter 5: What is the function of DAZAP1 as a Dazl interactor?

5.1 Introduction

In the last chapter the importance of the interaction between Dazl and PABP was investigated. Whilst the current evidence supports a predominant role for PABP in Dazl-mediated simulation, it does not exclude a role for other factors. Several other DAZ or Dazl interacting proteins have also been described (see section 1.7.3).

One of these partner proteins is DAZ associated protein 1 (DAZAP1). This protein was identified in a yeast two hybrid screen looking for novel protein partners of the human DAZ protein and was shown via GST-pull down assays to bind to both DAZ and DAZL through the DAZ repeats (Tsui *et. al.*, 2000a). An interaction between DAZL and DAZAP1 was also demonstrated in human tissues by co-immunoprecipitation of DAZL and DAZAP1 from ovarian and testis extracts. DAZL and DAZAP were also shown to colocalise by immunohistochemical staining of human ovary sections (Pan *et. al.*, 2005). Thus several lines of evidence support an interaction between Dazl family members and DAZAP1.

DAZAP1 is evolutionary conserved across vertebrates with the gene being identified in the human, mouse, rat, *Xenopus* and Zebrafish genomes (Uniprot Knowledgebase), but appears to be absent from *Drosophila* and *C. elegans*. In contrast to DAZL family members, DAZAP1 appears to be widely expressed. In mouse both DAZAP1 RNA and protein is present in multiple tissues, although they are most abundant in the testis (Dai *et. al.*, 2001). Dazap1 was also shown to be expressed throughout oogenesis in *Xenopus laevis* (Zhao *et. al.*, 2001), consistent with a role in germ cells. However, its wide expression pattern suggests that DAZAP1 has other functions in addition to any role in Dazl-mediated regulation.

DAZAP1 contains two RNA-binding domains and a proline-rich C-terminal portion. Several studies have investigated DAZAP1's ability to bind RNA: Human DAZAP1 was shown to bind preferentially to poly(U) and poly(G) and to a lesser degree poly(A) in

(Tsui *et. al.*, 2000a). *Xenopus* DAZAP1 was shown to bind an U-rich element in the 3'UTR of *Vg1* RNA (Zhao *et. al.*, 2001) and SELEX analysis identified that mouse DAZAP1 bound RNAs containing a AAAUAG and $GU_{1-3}AG$ sequence. All these RNA binding experiments were performed *in vitro*.

The function of the DAZAP1 protein has not been investigated in detail. DAZAP1 was first identified in *Xenopus* where it was called proline rich protein (Prrp), due to the composition of the C-terminal region and based on its interaction with the RNA localization element in Vg1 mRNA. (Zhao *et. al.*, 2001) was suggested to potentially play a role in mRNA transport. DAZAP1 was found to be present in mRNP particles in mouse testis extract consistent with a potential role in the localization of Dazl-bound mRNAs; however no active role in mRNA localization has been demonstrated leaving the function of this protein unclear.

The observation that DAZAP1 interacts with the DAZ motif is of interest, not only because this motif is only present in this family suggesting a specific functional role, but also as this motif has been suggested to be required for the association of Dazl with polysomes (Tsui *et. al.*, 2000b). This is perhaps consistent with the deletion of the DAZ motif reducing the extent of stimulation by DAZL in tethered function assays and *in vitro* (Collier *et. al.*, 2005). As Dazl was tethered in one of these studies, this suggests that this reduction was not due to the proposed ability of this motif to bind RNA but due to disruption of the structure of Dazl or due to this motif serving as binding site for a protein that contributes to Dazl-stimulatory activity. Yeast two-hybrid analysis suggested that PABP does not bind the DAZ motif although PABP-binding although appeared somewhat reduced in oocytes expressing a DAZ deleted version of DAZL (Collier *et. al.*, 2005). Thus it is possible that the DAZ motif forms part of a structure that can influence the efficiency of PABP-binding. Alternatively, this motif may influence Dazl function through its interaction with DAZAP1. In this chapter the possible role of DAZAP1 in Dazl mediated translational activation will be explored.

5.2 Generation of Anti-DAZAP1 antibody

The interaction between DAZ/DAZL and DAZAP1 was established with human and mouse proteins but has not been shown to extend to non-mammalian species such as *Xenopus*. Thus to understand the potential translational role of DAZAP1 in *Xenopus* oocytes, it was deemed necessary to determine whether these proteins interact in these cells. An antibody was previously generated against *Xenopus* DAZAP1 (Zhao *et. al.*, 2001), by another lab however it is no longer available. Thus it was decided to raise a new anti-Dazap1 antibody. To this end, two rabbits were immunised with two peptides specific to *Xenopus* laevis DAZAP1 over a three month period at Covalab (France).

The first peptide corresponded to amino acids 218- 231 (GWTGQPPQTWQGYS) and the second peptide corresponded to amino acids 338-352 (SGQQDFPFSQFGNAC). The locations of these peptides with respect to the RRM motifs of DAZAP1 protein are represented in the diagram in figure 5.1A and their specific locations are shown in 5.1B. These peptides were chosen based on their estimated immunogenic activity. Other potential regions of good immunogenic potential were identified in N-terminal half of the protein near the RRMs but were discounted as BLAST analysis of the protein databanks suggested an increased risk of cross-reactivity with other proteins (data not shown).

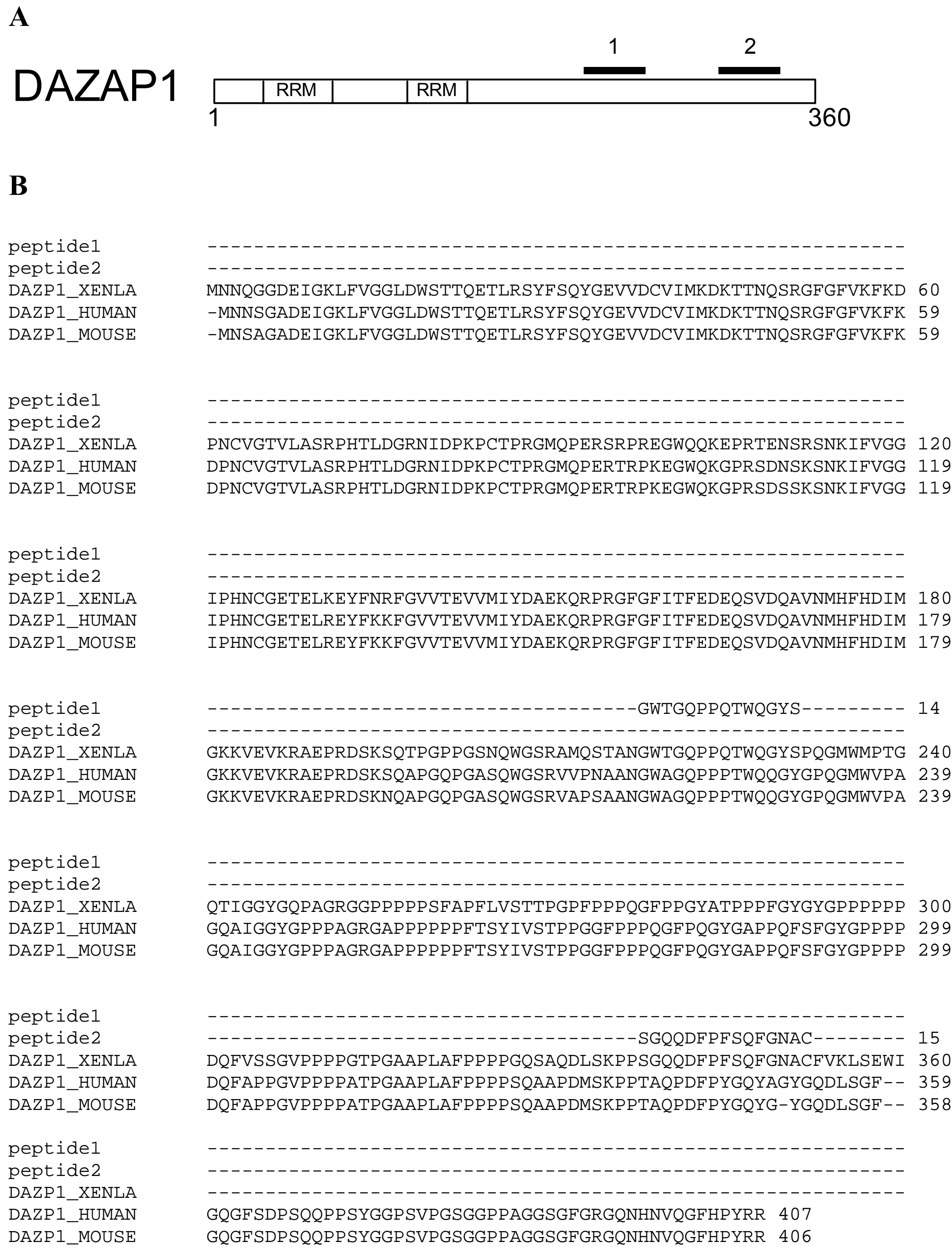


Figure 5.1: Generation of an anti-DAZAP1 antibody
A: Schematic diagram of location of peptides injected to make anti-DAZAP1 antibody. The position of the RRMs is marked and the positions of the peptides used to generate the antibody are shown.
B: ClustalW alignment of protein sequences of *Xenopus* DAZAP1, Human DAZAP1 and Mouse DAZAP1 and sequences of peptides 1 and 2.

The protocol for the generation of the antibody was designed and supplied by Covalab and is shown in table 5.2. The antigen injections consisted of *in vitro* synthesised peptide tethered to Keyhole limpet hemocyanin (KLH) as a carrier.

Day	Protocol
0	**Pre-immune bleed (4 - 5 ml)**
0	**Injection** (1 ml / rabbit) 0.5 ml antigen + 0.5 ml complete Freund's adjuvant
21	**Injection** (1 ml / rabbit) 0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant
42	**Injection** (1 ml / rabbit) 0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant
53	**Test bleed (4 - 5 ml)**
54	**Dispatch of sera (D0 & first test bleed)**
63	**Injection** (1 ml / rabbit) 0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant
74	**Test bleed (12 - 15 ml)**
75	**Dispatch of sera**
81	**FINAL BLEED**
	Immunopurification & Titration
89	**Dispatch of final bleed, affinity purified antibodies**

Table 5.1: Initial immunisation protocol for generation of anti-DAZAP1 antibody

The rabbits were bled at day 53 (D53) and at day 74 (D74) post immunisation and the antibody titre determined by ELISA at Covalab. The results of this assay are shown in table 5.2.

Peptide	Bleeding	Rabbit 1	Rabbit 2
Peptide 1	D53	1000	2000
	D74	1000	2000
Peptide 2	D53	1000	500
	D74	1000	500

Table 5.2: Immunogenic activity of D53 and D74 bleeds against peptides 1 and 2.
The immunogenic activity of D53 and D74 bleeds against peptides 1 and 2 was tested by ELISA. Immunogenicity was defined as follows: Titre < 500: no immunoreactivity. 500 ≤ Titre < 2000: low immunoreactivity. 2000 ≤ Titre < 8000: good immunoreactivity. Titre ≥ 8000: very good immunoreactivity

As can be seen from table 5.1 the antibody titre for both peptides in both rabbits was low at both D53 and day D74 with the highest titre being for peptide 1 in rabbit 2. However, a titre of 2000 is the lowest value included in the ‘good’ immunoreactivity bracket. The D53 and D74 bleeds were tested for their ability to detect DAZAP1 in stage VI oocyte extracts by Western blotting however results were inconclusive (data not shown). This coupled with the relatively low immunoreactivity of the bleeds led to the antibody schedule being lengthened to 109 days with a further peptide injection as shown in table 5.3.

Day	Protocol
0	**Pre-immune bleed (4 - 5 ml)**
0	**Injection** (1 ml / rabbit) 0,5 ml antigen + 0,5 ml complete Freund's adjuvant
21	**Injection** (1 ml / rabbit) 0,5 ml antigen + 0,5 ml incomplete Freund's adjuvant
42	**Injection** (1 ml / rabbit) 0,5 ml antigen + 0,5 ml incomplete Freund's adjuvant
53	**Test bleed (4 - 5 ml)**
54	**Dispatch of sera (D0 & first test bleed)**
63	**Injection** (1 ml / rabbit) 0,5 ml antigen + 0,5 ml incomplete Freund's adjuvant
74	**Test bleed (12 - 15 ml)**
75	**Dispatch of sera**
109	**FINAL BLEED**
	Immunopurification & Titration
117	**Dispatch of affinity purified antibodies**

Table 5.3: Revised immunisation protocol for generation of anti-DAZAP1 antibody

At day 109 (D109) the rabbits were sacrificed and the final bleeds were collected and the sera from them were extracted. A portion of these sera from rabbits 1 and 2 were pooled and affinity purified using an immunogen-affinity resin with the two original peptides immobilised on a sepharose column. This is designed to only select antibodies from the sera that can bind the peptides and therefore eliminate other antibodies from the sera, thus reducing background and increasing the effective antibody concentration.

The pooled sera's antibody titre was determined by ELISA at Covalab. The results of this assay are shown in table 5.4. As can be seen the immunogenicity is still in the range defined as low immunoreactivity.

Peptide	Titre
Peptide 1	1000
Peptide 2	1000

Table 5.4: Immunogenic activity of pool of D109 sera against peptides 1 and 2.
The immunogenic activity of the pooled sera against peptides 1 and 2 was tested by ELISA. Immunogenicity was defined as follows: Titre < 500: non immunoreactivity. 500 ≤ Titre < 2000: low immunoreactivity. 2000 ≤ Titre < 8000: good immunoreactivity. Titre ≥ 8000: very good immunoreactivity

The unpooled sera was not tested at this time as Covalab decided that as the titre did not change from D53 to D74 that it would not be worth testing the unpooled sera. In retrospect it would be desirable to have this information but it was not supplied by the company.

Both the D109 sera and the affinity purified antibodies were tested for their ability to detect DAZAP1 (figure 5.3). Their ability to detect endogenous (lane 1) and overexpressed DAZAP1 in stage VI *Xenopus* oocytes were measured (lanes 2 to 4).

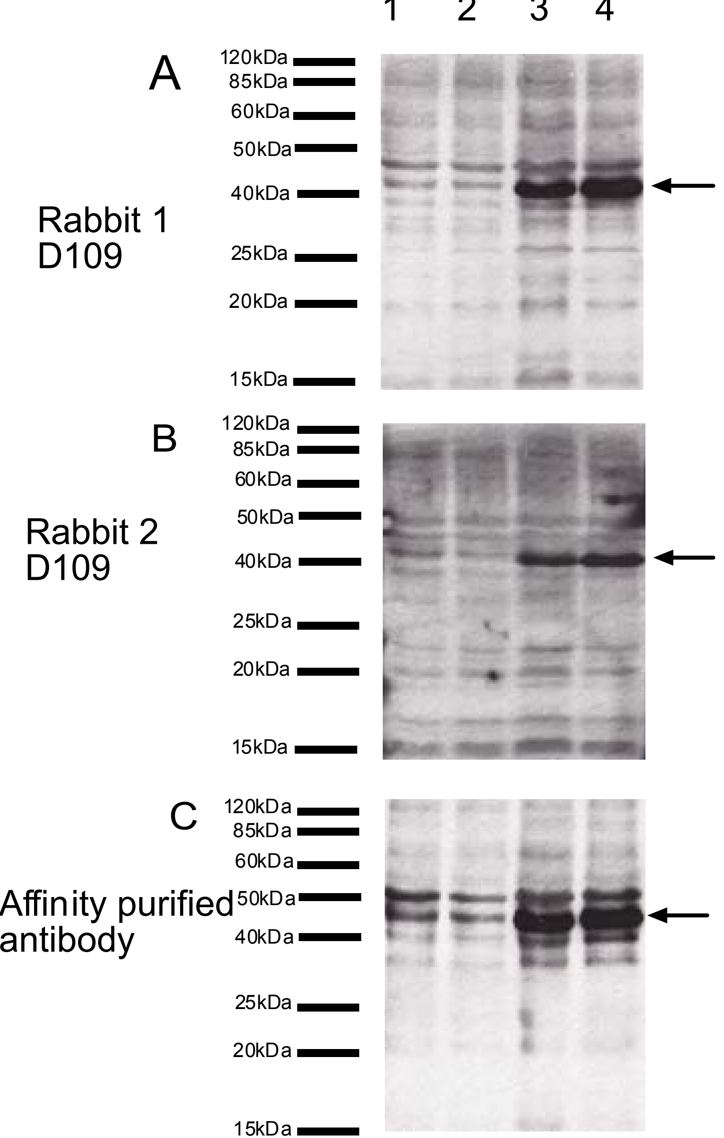


Figure 5.2: Testing of the anti-DAZAP1 antibody.
Western blots were used to test the specificity of the anti-DAZAP1 D109 sera and purified antibody. One oocyte's worth of total protein extract from either uninjected oocytes or oocytes overexpressing a DAZAP1 plasmid was separated by SDS-PAGE on a 10% gel in each lane. The oocytes used were: Lane 1= endogenous oocyte extract. Lane 2= MSPN.DAZAP1 injected oocyte extract. Lane 3= Pet.DAZAP1 injected oocyte extract. Lane 4= MSPNΔMS2. DAZAP1 injected oocyte extract. (3) and (4) should result in over expression of DAZAP1 that migrates at the same size as the endogenous protein.
A: Western blotting with D109 serum from rabbit 1 (1/1000 dilution) detects a band the predicted size of DAZAP1 as indicated by an arrow.
B: Western blotting with D109 serum from rabbit 2 (1/1000 dilution) detects a band the predicted size of DAZAP1 as indicated by an arrow.
C: Western blotting with affinity purified antibody (1/1000 dilution: 0.156µg of antibody) detects a band the predicted size of DAZAP1 as indicated by an arrow.

As can be seen from figure 5.2 both the sera antibodies and the affinity purified antibodies can detect a protein band that runs just above the 40KDa marker. The predicted size for DAZAP1 is 39229Da (Swiss-Prot database, www.expasy.org/sprot/). DAZAP1 appears to migrate slightly higher than the predicted molecular weight. This may be due to the amino acid composition of the protein or post-translational modification. The band is enhanced in lanes 3 and 4 that contain non-MS2-fused over-expressed DAZAP1. A band corresponding to this size is also observed in lane 1 for all three antibodies indicating consistent with the presence of endogenous DAZAP1 as previously reported (Zhao *et. al.*, 2001), although a fainter band of this size can also be detected in pre-immune (See figure 5.3). No clear band the size of MS2-DAZAP1 is observed. Data in figure 5.7B suggest that this is most likely due to experimental error, although steric occlusion of the antibody recognition site cannot be formally excluded, although the peptides to which the antibodies were raised are located in the C-terminal of the protein, distant from the N-terminal MS2 fusion making this very unlikely.

A doublet is seen in the blots with the rabbit 1 and the affinity purified antibody. The doublet is consistent with previously published results (Zhao *et. al.*, 2001) where a similar doublet was observed throughout oogenesis. Possible explanations include the possibility that the protein undergoes a post-translational modification, or that there are alternative splice forms of the gene. Finally, the *Xenopus laevis* is a tetraploid organism and the annotation of its genome is incomplete, so there could be multiple forms of the same gene. As rabbit 2 had very low immunogenicity for peptide two (see table 5.1) it is conceivable that the reason why it can only detect one of the doublet bands is that recognising the upper band is dependent on antibodies to peptide two and rabbit 2 did not produce these antibodies. However, the upper band in this doublet does not always appear to increase in intensity upon expression of exogenous DAZAP1, thus this may not correspond to DAZAP1 (see also figure 5.3 below).

As the D109 serum seemed to recognise a wider variety of proteins, it was decided to characterise the affinity purified antibody further. First the affinity purified antibody

was compared to pre-immune sera to investigate the specificity of the signal in the 40kDa range.

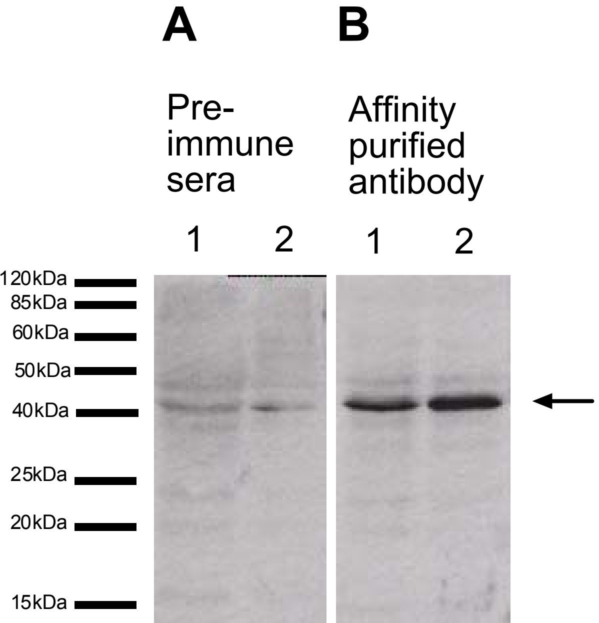


Figure 5.3: Comparison of the anti-DAZAP1 antibody to pre-immune sera
One oocyte's worth of total protein extract from oocytes expressing either Pet.DAZAP1 (Lane 1) or MSPNΔMS2 (Lane 2) was separated by SDS-PAGE on a 10% gel.
A: Western blotting with pre-immune sera (1/1000 dilution) does not detect a strong band.
B: Western blotting with affinity purified antibody (1/1000 dilution: 0.156μg of antibody) detects a band the predicted size of DAZAP1 as indicated by an arrow.

When the affinity purified antibody is compared to pre-immune sera (figure 5.3) a faint signal in the pre-immune blot corresponding to the size that DAZAP1 (approx 40KDa) is observed. This indicates that there are some antibodies in the sera that react with proteins of the same size as DAZAP1 (or even that recognize DAZAP1) prior to peptide inoculation. However, there is a marked and clear difference between the pre-immune sera and the immuno-purified antibody with respect to the lower band within the doublet that is greatly enhanced in the presence of exogenous DAZAP1- suggesting that the immuno-purified sera recognizes DAZAP1 expressed in oocytes.

Although the experiments presented above did give some indication of the ability of the D109 sera and the affinity purified antibody to recognise DAZAP1 they did have some

flaws that should be addressed in the future. First, a pre-immune control blot should have been presented for each of the sera and second the protein concentration of the sera should have been measured so that a comparison of the amount of immunoglobulins used in each blot could have been made. Without doing these controls it is difficult to truly assess the relative abilities of the sera and purified antibody to recognise DAZAP1. In addition the ability of the affinity purified antibody to recognise DAZAP1 appears to differ between figure 5.2 (panel C) and figure 5.3 with a clearer signal being seen in figure 5.3. The levels of background also differ between the two figures. However, there were also consistencies between the two test experiments with a band of the correct size for DAZAP1 always being observed as significantly stronger than the background. Because of this it was decided to continue with the affinity purified antibody and use it in some of the future experiments in this chapter despite the problems. It was felt that the signal was strong enough and specific enough to justify its use.

5.3 The anti-DAZAP1 antibody generated can be used for immunoprecipitations

The affinity purified antibody was tested for its ability to immunoprecipitate (IP) radiolabelled *Xenopus* DAZAP1 synthesised *in vitro* using the coupled transcription and translation (TNT) system (Figure 5.4, panel A). An empty vector and a vector encoding the human PABP5 were included alongside DAZAP1 as specificity controls. The radiolabelled proteins translated from each of these are shown in lanes 1-3. The DAZAP1 TNT reaction produced a doublet of bands around 45KDa in length (lane 3). Immunoprecipitations from all three TNT reactions were conducted with antibodies against DAZAP1 and PABP5 as a control. The anti-PABP5 antibody was a gift from R. Anderson. The IP with the anti-DAZAP1 antibody recognised the protein from the DAZAP1 TNT with high efficiency (lane 9) and the IP with the anti-PABP5 antibody recognised the protein produced by the PABP5 TNT (lane 5). Neither IP recognised anything significant from the empty TNT reaction (lanes 4 and 7), though there is a faint

band in lane 4 that could be protein spilt over from lane 5. There appeared to be a minor cross-reactivity between the PABP5 and DAZAP1 antibodies (lanes 6 and 8). Based on the peptides used to raise these antibodies and the divergent nature of these proteins, this was surprising. However, due to the nuclease treatment of TNT extracts, the only radiolabelled proteins present will be derived from the plasmids used to program the lysates. Thus, it is possible that this represents precipitation of the proteins in the buffers used or proteins bound to the beads in a non-specific manner suggesting that the washing protocol was not sufficient. However, as the signal is weak compared to the specific reactions (lanes 5 and 9) it did not change the conclusions drawn from this experiment.

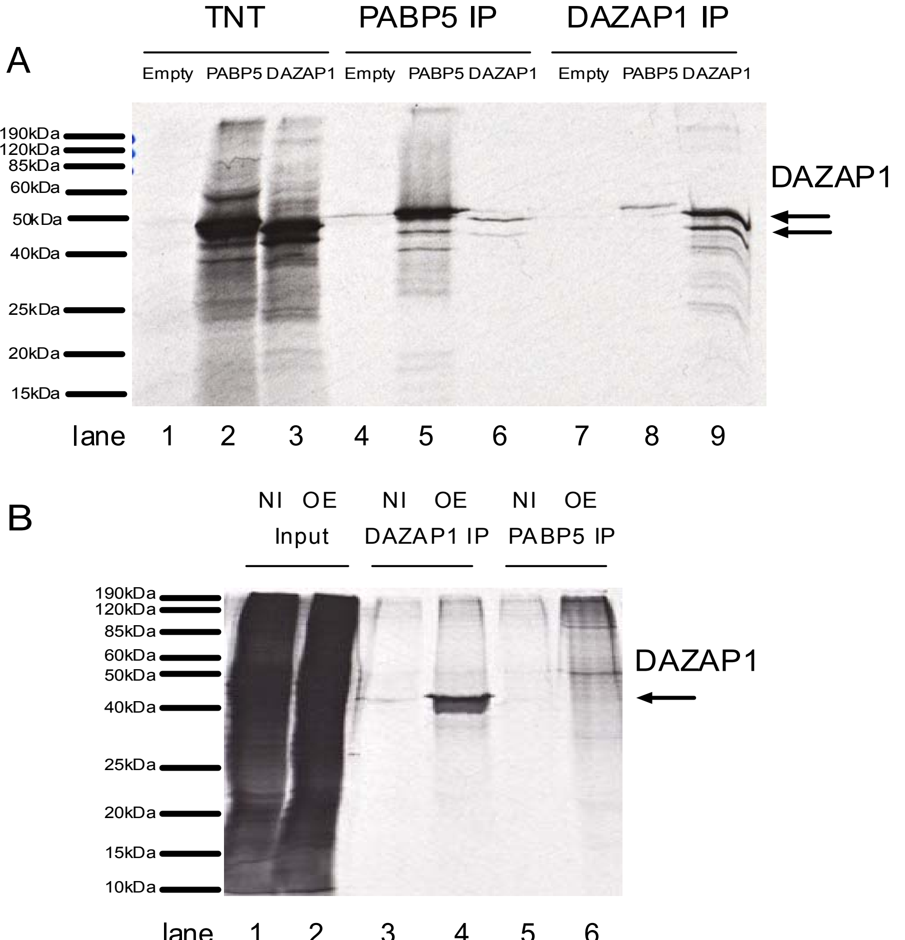


Figure 5.4: The anti-DAZAP1 antibody can immunoprecipitate radiolabelled DAZAP1
A: The ability of the anti-DAZAP1 antibody to IP protein from TNT reactions was tested. An IP with anti-PABP5 antibody was included as a positive control. Lanes 1-3: input (5µl of TNT reaction). Lanes 4-6: IP with anti-PABP5 antibody (TNT input into IP = 20µl). Lanes 7-9: IP with anti-DAZAP1 antibody (TNT input into IP = 20µl).
B: The ability of the anti-DAZAP1 antibody to IP protein from metabolically labelled oocytes was tested. Oocytes are either not injected (NI) or over expressing *Xenopus* DAZAP1 (OE). Lanes 1-2: input (1 oocyte's worth of extract). Lanes 3-4 IP with anti-DAZAP1 antibody (input into IP = 5 oocyte's worth of extract). Lanes 5-6 IP with anti-PABP5 antibody (input into IP = 5 oocyte's worth of extract).

The ability of the anti-DAZAP1 antibody to immunoprecipitate endogenous or overexpressed *Xenopus* DAZAP1 in oocytes was also determined. To this end, stage VI *Xenopus* oocytes were collected and half injected with an mRNA encoding DAZAP1 (OE) and the other half remained uninjected (NI). The oocytes were incubated for six hours with 35S-methionine to radiolabel newly synthesised proteins. After the incubation period extracts were made from the OE and NI oocytes. The DAZAP1

antibody was used to immunoprecipitate proteins from these extracts. Samples of the protein extract (Input) and the proteins recognised by the IP were resolved by SDS-PAGE (figure 5.4, panel B) and identified by autoradiography. The anti-DAZAP1 antibody efficiently recognised a protein from the overexpressed extract (lane 4) and a very faint signal was seen in the NI extract (lane 3) representing the endogenous protein. The anti-PABP5 antibody was included as a negative control (lane 6) and produced some of the same bands, but importantly not at the same size as the strong band in lane 4, suggesting that this band represented immunoprecipitated DAZAP1.

5.4 DAZAP1 and Dazl interact in *Xenopus* oocytes

The availability of this antibody permitted the testing of an interaction between Dazl and DAZAP1 in *Xenopus* oocytes similar to that described for human protein *in vitro* (Tsui *et. al.*, 2000a) and in human ovarian tissue (Pan *et. al.*, 2005).

Stage VI oocytes were collected and half left not injected (NI) and the other half was injected so that they over-expressed DAZAP1 (OE). Following incubation, protein extracts were made form both sets of oocytes. IPs were conducted using the affinity purified anti-DAZAP1 antibody and pre-immune (day 0) sera as a negative control. A sample of each protein extract and the IPs were separated by SDS-PAGE (figure 5.5) and Western blotted for the presence of Dazl.

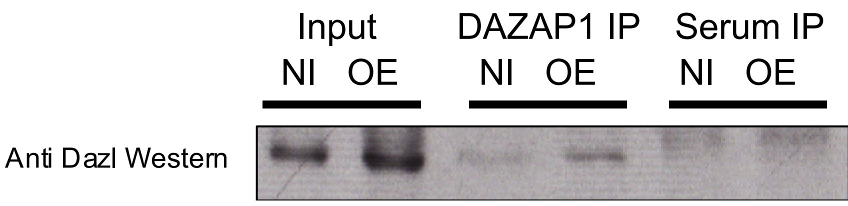


Figure 5.5: Co-immunoprecipitation of Dazl with DAZAP1 in *Xenopus* oocytes. Protein was immunoprecipitated from either uninjected (NI) oocytes or from oocytes over expressing DAZAP1 (OE) with affinity purified anti-DAZAP1 antibody (lanes 3-4) or with pre-immune sera (lanes 5-6) at 1/1000 dilution. Lanes 1-2 show input protein (1 oocyte's worth of extract). Dazl was detected by Western blot after IP with anti-Dazl antibody at 1/1000 dilution. The experiment has not been repeated.

Dazl protein could be clearly seen in the input lanes for both not-injected and DAZAP1 overexpressing samples, though the presence of more Dazl protein in the OE lane indicates that the protein concentrations of the two extracts differed slightly. Importantly, the IP with the affinity purified anti-DAZAP1 antibodies appeared to co-precipitate Dazl as indicated by the presence of a Dazl band in lanes 3 and 4. More Dazl was recovered from the cells expressing exogenous DAZAP1, suggesting that DAZAP1 concentrations may be limiting compared to Xdazl, however this may also be a direct result of the difference in protein concentration between the lysates. The IP with the pre-immune sera (lanes 5 and 6) did not isolate proteins of the same size as the Dazl, suggesting that the pull down is specific to the anti-DAZAP1 antibody.

Overall this result could be taken to suggest that *Xenopus* DAZAP1 and Dazl interact in oocytes. However this result is still in the preliminary stages with only one repetition performed and needs to be confirmed before any firm conclusions can be drawn. As discussed in section 5.2 the antibody was suboptimal and repeating this experiment with either a much better antibody or another method would be desirable, though this was not possible given the time frame of this project. Other methods that could be used to confirm the interaction include using tagged proteins so more effective antibodies to the tags can be used for co-IP or GST pull downs. Despite the preliminary nature of the result observed here it does give an indication that an interaction is possible, which enables the consideration of the potential implications of this observation.

5.6 Does DAZAP1 associate with translational machinery?

Having established that DAZAP1 and Dazl may interact in oocytes this raises the possibility that DAZAP1 may participate in DAZL-mediated translational regulation. In mouse testis DAZAP1 was shown to associate with the mRNP fraction and not localise to actively translating polyribosomes, under the conditions and developmental stage used. Moreover, a recent *in vitro* study (Morton *et. al.*, 2006) using human DAZAP1, suggested that DAZAP1 was an inducer of RNA instability and blocked the ability of Dazl family proteins to stimulate translation. These results are consistent with DAZAP1 as a negative rather than a positive regulator of mRNA translation.

To determine whether DAZAP1 is associated with mRNP complexes or ribosomes in *Xenopus* oocytes, sucrose gradient analysis was undertaken. Stage VI *Xenopus* oocytes were treated with cycloheximide, a drug that prevents peptidyl transfer and prevents ribosome run-off during extract preparation. Translation complexes were resolved on 10-50% sucrose gradients, and A254 absorbance was used to track the position of polysomes, ribosomes and mRNPs. In stage VI oocytes 80S ribosomes, some of which may be associated with mRNA rather than empty couples, are predominantly observed with few clear polysomes observed in A254 profiles. This preponderance of monosomes is due to the large number of ribosomes that have been synthesised in preparation for fertilisation. Though extraction of mRNA from the fractions heavier than the 80S shows that ribosomal RNAs are present (Personal communication, B. Gorgoni) at least in the three fractions preceding the 80S peak, though it is unknown if these are di-somes, light polysomes or merely heavier monosome complexes.

Following fractionation, proteins were precipitated from the 10 fractions using trichloroacetic acid (TCA) and resolved by SDS-PAGE. The fractions were then Western blotted with the affinity purified anti-DAZAP1 antibody.

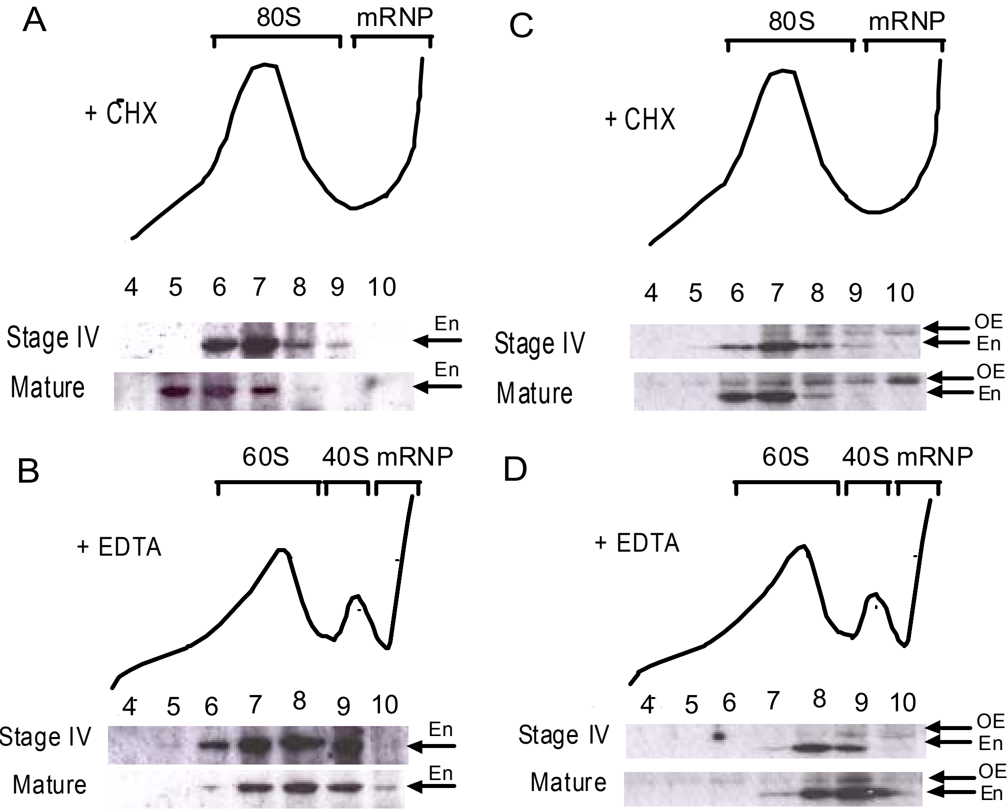


Figure 5.6: Sucrose gradient analysis of DAZAP1 in *Xenopus* oocytes.
The association of DAZAP1 with translational machinery was assessed by sucrose gradient analysis. Each figure shows the A254 absorbance trace (upper panel) and the precipitated DAZAP1 from fractions detected by Western blot using anti-DAZAP1 antibody (1/1000 dilution). Only fractions 4-10 are shown as these fractions contain detectable DAZAP1. Locations of endogenous (En) and over expressed (OE) DAZAP1 are indicated.
A: The localisation of endogenous DAZAP1 was assessed in extracts from stage VI and mature *Xenopus* oocytes treated with cycloheximide (CHX) (Panel A) or EDTA (Panel B). The positions of 80S ribosomes or 60S and 40S ribosomal subunits and mRNPs are indicated.
C: As in A, but with oocytes over expressing His-tagged DAZAP1
D: As in B, but with oocytes over expressing His-tagged DAZAP1

In stage VI oocytes extracts treated with cycloheximide DAZAP1 is mainly localised to fractions 6, 7 and 8 (figure 5.6A), being most abundant in fraction 7. These fractions correspond to an area of the gradient that consists of free 80S ribosomes and mRNAs bound by one or a limited number of ribosomes (light polysomes). DAZAP1 is not located in heavy polyribosomes and only a minority is present in the mRNP fraction associated with non-translated mRNAs. Treatment of extracts with EDTA releases mRNAs from ribosomes and the polysomes and 80S peak are lost, resolving into the 40 and 60S subunits. In the presence of EDTA the majority of DAZAP1 co-sediments with

ribosomal subunits. This may reflect an incomplete release of the RNA by EDTA but the loss of the 80S peak suggests that the EDTA treatment was effective. Alternatively this may reflect a direct interaction of DAZAP1 with ribosomal subunits or its presence in heavy mRNPs (see discussion). Taken together this suggests that DAZAP in stage VI oocytes is found in an area associated with 80S ribosomal subunits and light polysomes rather than light mRNP fraction as previously reported for mouse testis. The presence of DAZAP1 in 80S complexes could be consistent with a role in active translation on short or infrequently initiating mRNAs undergoing efficient elongation. Alternatively, it is also consistent with very heavy mRNPs associated with repression or repressed mRNAs associated with a single 80S ribosome, as has been described for a limited number of uORF containing mRNAs (Wang and Sachs, 1997).

During oocyte maturation, there is reprogramming of cellular translation, with the activation of mRNAs required for meiotic maturation and a silencing of many housekeeping genes (Mendez and Richter, 2001). Given the knockout phenotype of Dazl in the majority of model organisms and the report that repression by Pumilio of Dazl bound mRNAs in stage VI oocytes is relieved during maturation (Padmanabhan and Richter, 2006), the association of DAZAP1 with the translational machinery was also examined following maturation. In mature oocyte extracts treated with cycloheximide DAZAP1 is mainly localised in fractions, 5, 6, and also fraction 7 (figure 5.6 A and C). This represents a shift towards heavier complexes heavier than the 80S that likely corresponds to light polysomes. As maturation leads to the activation of translation for many mRNAs this result is consistent with DAZAP1 associating with actively translating ribosomes. As with the stage VI oocyte extract EDTA treatment results in most of the DAZAP1 associating with both the 40S and 60S peaks.

The experiment was also conducted using extracts from oocytes over expressing a His-tagged DAZAP1. This results in a slightly larger protein representing the slightly larger fusion protein being present on the Western blot in addition to the endogenous DAZAP1. The distribution of the endogenous DAZAP1 was very similar, but not identical, to the uninjected oocytes with DAZAP1 being present around the 80S peak in

stage VI and mature oocytes. An additional upper band representing his tagged DAZAP seems to localise across the gradient with a small amount in each fraction with the majority being in the mRNP fractions (note that this band is not the same as the faint upper band seen while testing the antibody, which is never seen in this experimental system).

This apparent difference between endogenous and tagged DAZAP1 could be because the target RNAs are already bound by endogenous DAZAP1 resulting in the majority of overexpressed DAZAP1 not being associated with mRNA and being located in the lighter fractions. Alternatively the N-terminal His tag could interfere with the N-terminal RRMs in DAZAP1 or other structural feature, thus inhibiting RNA binding or protein-protein interactions by the over expressed DAZAP1 resulting in its mislocalisation. There is also the possibility that DAZAP1 has a protein partner required for its normal function RNA that is only present in limited amounts in the oocyte and the additional DAZAP1 cannot participate in complexes with this protein so remains at the top of the gradients.

5.7 What is the effect of DAZAP1 in translation assays?

Previous publications have suggested that DAZAP1 is a negative rather than a positive regulator of translation. However, in *Xenopus* oocytes DAZAP1 appears to be localised with ribosomes. This result leads to the question of whether DAZAP is a regulator of translation and whether it has a positive or negative effect.

To test this directly tethered function assays were performed as the physiological mRNAs to which DAZAP1 is bound remain to be unambiguously identified (see discussion). mRNAs encoding MS2-U1A and MS2-DAZAP1 were injected followed by the β-galactosidase control and M7G Luc-MS2 reporter mRNA. The oocytes were incubated overnight and luciferase and β-galactosidase assays conducted as in earlier experiments.

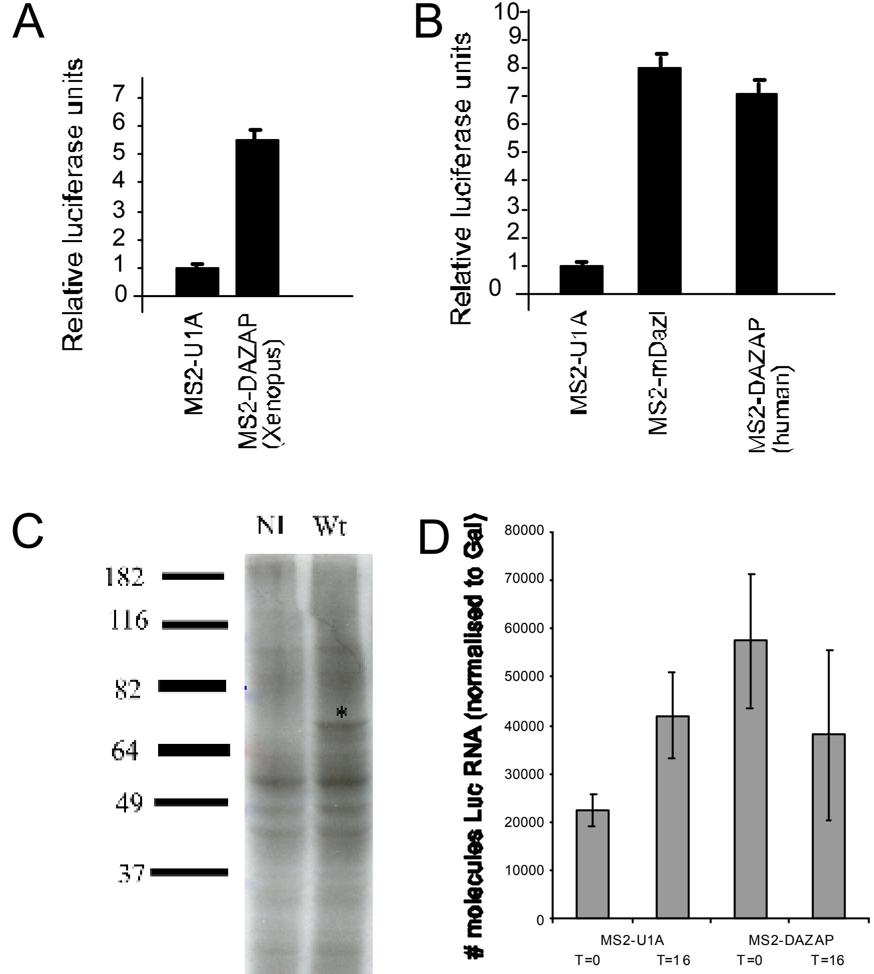


Figure 5.7: DAZAP1 stimulates translation in oocytes (part 1)
A: *Xenopus* DAZAP1 can stimulate translation. m^7GpppG capped Luc-MS2 reporter was co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-DAZAP1 (*Xenopus*). Luciferase activity was normalised to β-galactosidase activity and the relative luciferase units were plotted with MS2-U1A values set to one. The average of three experiments is shown.
B: Human DAZAP1 can also stimulate translation. m^7GpppG capped Luc-MS2 reporter was co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A, MS2-mDazl or MS2-DAZAP1 (human). Luciferase activity was normalised to β-galactosidase activity and the relative luciferase units were plotted with MS2-U1A values set to one. The average of five experiments is shown.
C: The MS2-DAZAP1 (human) fusion protein is expressed in oocytes. Oocytes were injected with fusion-protein mRNAs and incubated in buffer containing 35S-methionine for 6 hours and protein lysates made. An oocyte's worth of lysate was separated by SDS-PAGE and analysed by autoradiography. A star indicates the fusion protein band.
D: DAZAP1 does not have a stability effect on reporter mRNAs. Oocytes expressing either MS2-UIA or MS2-DAZAP1 (human) were injected with luc-MS2, and β-galactosidase. Total RNA was extracted either immediately or 16 hours post injection. Quantitative reverse transcription PCR was performed with primers directed against the reporter mRNAs. The numbers of molecules of luciferase reporter were normalised to the β-galactosidase and plotted. The data represents the averages of three repetitions with standard errors.

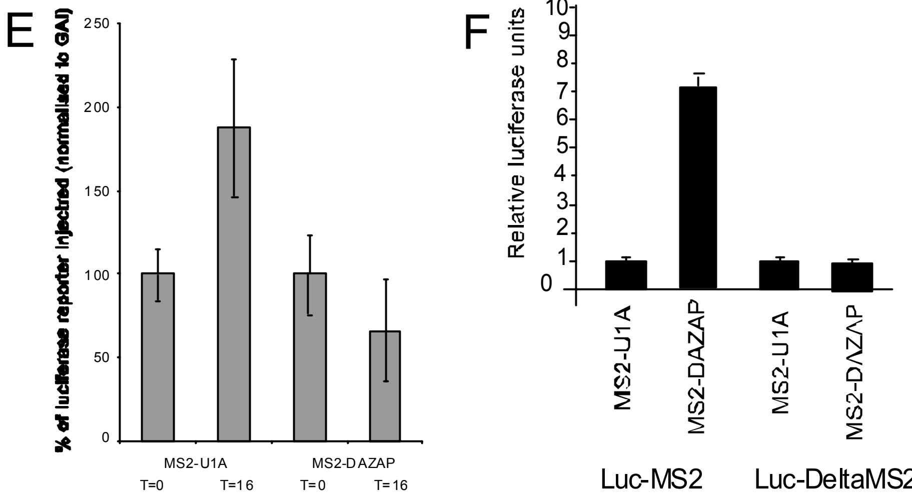


Figure 5.7: DAZAP1 stimulates translation in oocytes (part 2)
E: The values represented in figure 5.7D were transformed to show % of luciferase reporter injected at T=0 and T=16.
F: MS2-DAZAP1 cannot stimulate a reporter with no MS2 binding sites. M7G capped Luc-MS2 reporter or Luc-ΔMS2 was co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-DAZAP1 (human). Luciferase activity was normalised to β-galactosidase activity and the relative luciferase units were plotted with MS2-U1A values set to one. The average of three experiments is shown.

When tethered, *Xenopus* MS2-DAZAP1 stimulated production of luciferase 6-fold (figure 5.7A) when compared to MS2, suggesting a positive role in gene expression, consistent with the polysome analysis (figure 5.6).To see if this result was conserved across species, human DAZAP1, which was previously suggested to repress translation, was also tested by tether function. Interestingly, human DAZAP1 also stimulated translation (figure 5.7B), with a 7-fold increase, which was nearly as efficient as the positive control, mDazl, which stimulated 8-fold. To ensure that DAZAP1 fusion protein was expressed, stage VI oocytes were injected with RNAs encoding the human MS2-DAZAPl or left uninjected and new protein production was monitored by metabolically labelling the oocytes with ^{35}S-methionine. Protein extracts from oocytes were made and analysed using SDS-PAGE. A protein band corresponding to human MS2-DAZAP1 protein can be observed (figure 5.7C).

As mentioned earlier, it has been suggested that human DAZAP could be an inducer of RNA instability. Because of this and as with any reporter assay it is possible that

increased expression is due to the stabilisation of the reporter mRNA rather than a translational effect, reporter stability was measured by employing the QRT-PCR method detailed in chapter 3. The tethered function assays shown in figure 5.7B were repeated with additional oocytes. RNA was extracted from these additional oocytes and the numbers of molecules of both the luciferase and β-gal reporters in each sample (figure 5.7D) and the percentage of injected luciferase reporter at T=0 and T=16 (figure 5.6D) were measured as detailed in chapter 3. The QRT-PCR assays showed that while the tethering of MS2-U1A appeared to have a small stabilising effect on luciferase reporters levels, the tethering of DAZAP1 did not stabilise the reporters with a 33% reduction in luciferase RNA at T=16 compared to T=0. However the error of the measurement (+/-30%) is such that any drop in luciferase mRNA levels can not be said to be significant. However there is a clear lack of stabilisation, which implies that DAZAP1 does not increase luciferase levels in the tethered function assays by virtue of stabilisation and does indeed do so via a translational effect.

To test whether the increase in luciferase production seen was a specific effect of DAZAP1 binding the reporter or a more general *trans* effect a specificity control was conducted. The stimulatory effect of DAZAP1 required MS2 binding, as no effect on luciferase production was observed in the absence of MS2 binding sites in the PV-Luc-ΔMS2 reporter.

5.8 What are the interactions between DAZAP, Dazl and PABP?

So far in this chapter DAZAP1 has been shown to be localised with ribosomes and to stimulate translation in tethered function assays. These results are contrary to current theories that have suggested that DAZAP is a localisation factor or that it blocks interactions between Dazl family members and PABP preventing translational stimulation (Morton *et. al.*, 2006). The observation that human DAZAP1 shares the ability to stimulate translation shows that these differences can not be explained purely as a species specific effect. In order to resolve this problem a number of possible

models of DAZAP action were proposed that could be explored to explain the translational activity seen especially with relation to Dazl and PABP.

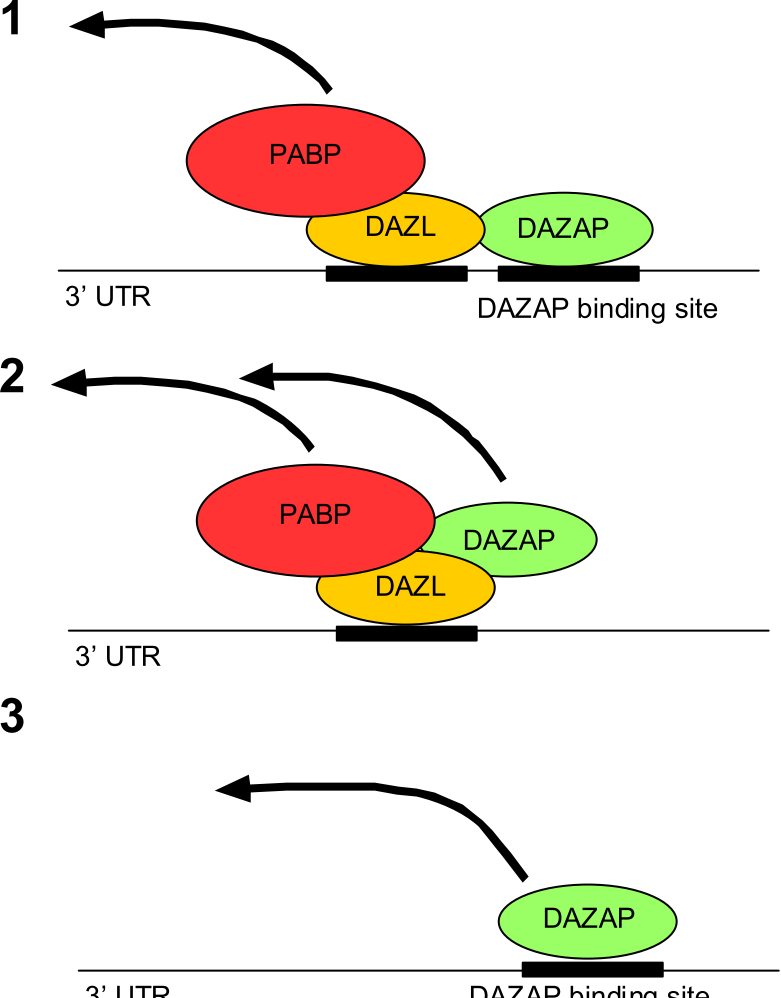


Figure 5.8: Possible models of DAZAP1 action
Dazl, DAZAP1 and PABP are as indicated. 3'UTR= 3' untranslated region
1: DAZAP1 stimulates translation via an interaction with Dazl where DAZAP1's function is to aid the recruitment of Dazl to mRNAs.
2: Dazl stimulates translation via both PABP and DAZAP1, perhaps independently rather than concurrently.
3: DAZAP1 stimulates translation independent of Dazl or PABP.

The first model is that DAZAP1 and both Dazl bind RNA as a complex and then Dazl recruits PABP that is then responsible for interacting with 5' factors and stimulating translation (figure 5.8A). The function of DAZAP1 in this complex would be to aid the

recruitment of DAZL to specific mRNAs and is consistent with the action of other 3'UTR regulatory complexes such as Nanos recruiting Pumilio (Wickens *et. al.*, 2002). This model would explain the tethered function data with DAZAP1 as tethered DAZAP1 could recruit Dazl, which in turn could recruit PABP, thus resulting in the stimulation of translation (figure 5.8B). Inconsistent with this model is the *in vitro* data using overexpressed proteins in somatic cell-lines where DAZ could not be detected in a triple complex with DAZAP1 and PABP. The second model is that DAZL utilises both DAZAP1 and PABP to directly stimulate translation (figure 5.8B). This model would explain why Dazl with the DAZAP1 binding DAZ domain removed has reduced translational activity even though it can still bind PABP (Collier *et. al.*, 2005). Again this model would require that Dazl, PABP and DAZAP form a triple complex. The third model proposes that the translational activity of DAZAP1 is unrelated to its ability to interact with Dazl and that is an independent translational regulator (figure 5.8C).

5.9 What is the number dependency of tethered DAZAP stimulation?

It was decided to further investigate the action of DAZAP1 with a variety of tethered function assays. Previously, it was shown that Dazl can stimulate the translation of mRNAs with multiple MS2-binding sites to a greater degree than an mRNA with one (Collier *et. al.*, 2005). However there was a limit to the number of binding sites that increased stimulation as increasing the number of sites from three to nine made little difference. This may relate to DAZL target mRNAs having multiple binding sites within their target mRNAs. This assay has also been conducted for the histone stem loop binding protein (SLBP). This protein binds to a single stem loop on its target mRNA and stimulates its translation. When testing the number dependency of this protein, maximal stimulation is seen with one tethered protein, reflecting its physiological role (Gorgoni *et. al.*, 2005).

To test whether DAZAP1 showed a similar dependency to Dazl, tethered function assays were performed with luciferase reporters containing one, three or nine MS2 binding sites in the 3'UTR (see figure 5.9A). The 'standard' number of MS2 sites used previously in tethered function assays in this thesis is three.

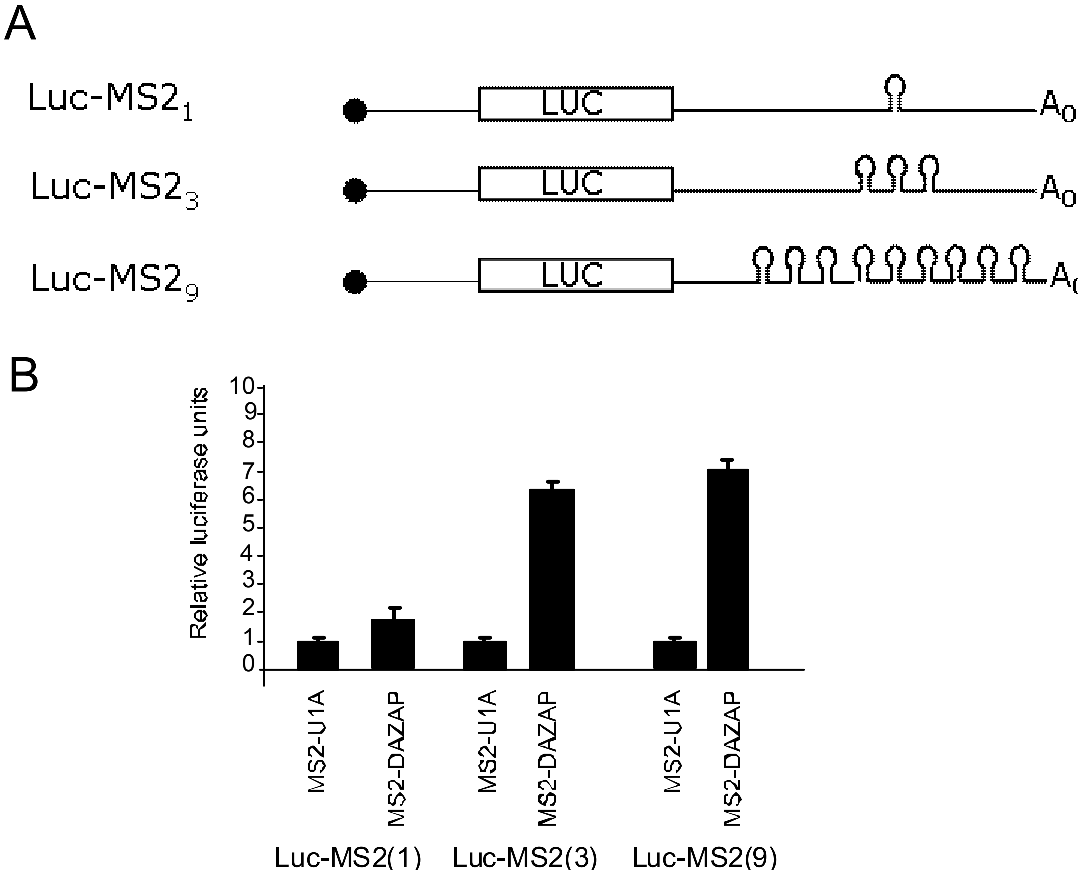


Figure 5.9: Multiple molecules of DAZAP1 stimulate translation
A: Schematic of luciferase reporter mRNAs, which vary as shown in the number of MS2 sites within the 3'UTR
B: MS2-DAZAP1 stimulates a reporter with one MS2 site at low levels and reporters with three or nine MS2 sites at high levels. Oocytes expressing MS2-U1A or MS2-DAZAP1 were co-injected with β-Gal mRNA and Luc-MS2$_1$, Luc-MS2$_3$ or Luc-MS2$_9$. Luciferase activity was normalised to β-galactosidase activity and the relative luciferase units were plotted with MS2-U1A values set to one. The average of three experiments is shown.

Figure 5.9B shows that although one-MS2 binding site is sufficient for DAZAP1 to stimulate translation, the level of stimulation is much reduced compared to three MS2-sites, with only 2-fold stimulation rather than 7-fold. Increasing the number of sites from three to nine had little effect indicating that beyond three binding sites the

translational stimulation is saturated. This pattern is similar to that of Dazl with these reporters (Collier *et. al.*, 2005).

If the pattern of stimulation of these reporters had differed from Dazl, for instance giving a pattern similar to SLBP, this would have suggested that DAZAP1's action may not be related to that of Dazl (model #3). As the pattern is similar to that of Dazl this result could suggest that DAZAP1 stimulation of translation related to Dazl function, as suggested in model #1 and 2.

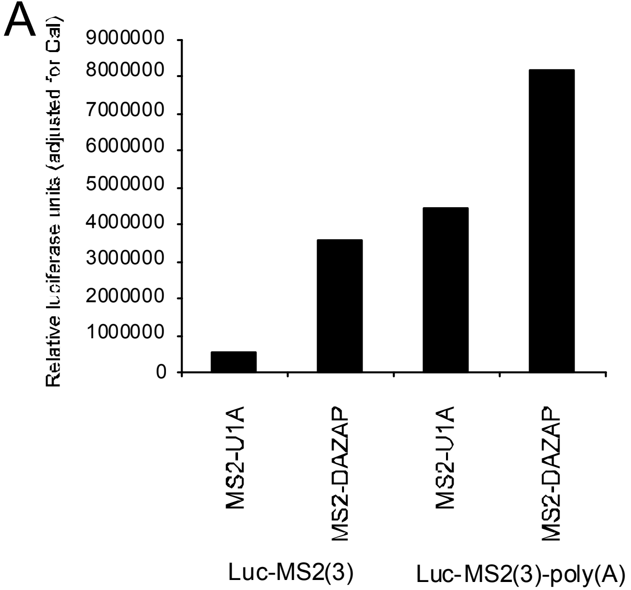
One quantative difference between DAZAP1 and Dazl is that Dazl could stimulate the reporter with a single MS2 site 4-fold where DAZAP1 could only stimulate 2-fold. One possible suggestion to account for this discrepancy is that Dazl has been shown able to dimerise (Ruggiu and Cooke, 2000) that could result in one tethered Dazl recruiting another thus having a greater effect on translation.

This experiment has also been conducted using PABP1 (N. Gray, unpublished) and the result is similar to Dazl and DAZAP and implies that multiple molecules of PABP bound to an mRNA can further enhance translation, consistent with the current models for poly(A) function. The similarity seen with all three proteins is consistent with them acting in translation via a similar pathway.

5.10 What is the role of poly(A) in DAZAP stimulation?

All the reporters used in the tethered function assays in this thesis so far have been unadenylated so the effect of tethered proteins could be seen without the complicating effects of PABP molecules bound to the poly(A) tail. When Dazl was originally investigated it was discovered that Dazl showed a reduced fold stimulation on an adenylated reporter compared to one with a poly(A) tail, although this mRNA was the most efficiently translated. As it has been proposed that Dazl's translational activity depended on recruiting PABP, this result supported that hypothesis as if there are multiple molecules of PABP bound to the poly(A) tail then the effect of bring additional molecules of PABP to the mRNA would be less marked.

To determine whether DAZAP1-mediated stimulation was also sensitive to poly(A), DAZAP1 tethered function assays were conducted as previously but using the unadenylated Luc-MS2 reporter and the Luc-MS2$_{polyA}$, with its (at least 45 base) poly(A) tail, in tandem.



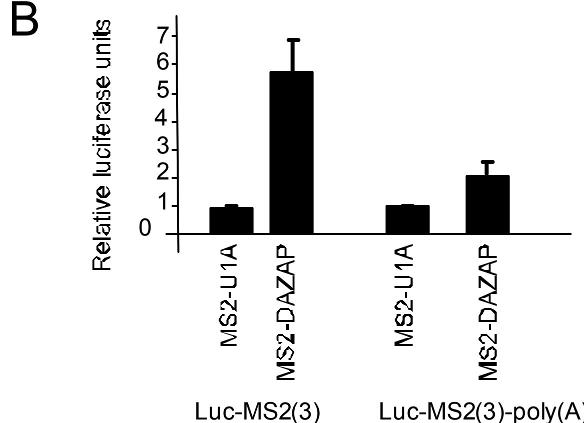


Figure 5.10: DAZAP1 shows reduced stimulation of translation in the presence of a poly(A) tail
A: DAZAP1 translational stimulation is sensitive to the presence of a poly(A) tail. Luc-MS2 reporter with no poly(A) tail (Luc-MS2) and polyadenylated Luc-MS2 reporter (Luc-MS2 $_{PolyA}$) was co-injected with β-Gal mRNA into stage VI oocytes expressing MS2-U1A or MS2-mDazl. Luciferase activity was normalised to β-galactosidase activity and plotted. One representative experiment is shown.
B: Relative luciferase units were plotted with MS2-U1A values set to one. The average of three experiments is shown.

The reporter with the poly(A) tail is translated at a much higher rate than the unadenylated reporter (figure 5.10A), but is further stimulated by DAZAP1. This is important as it shows that DAZAP1's stimulator activity is not restricted to unadenylated mRNAs but also applies to mRNAs with a physiological length of poly(A).

As can be seen in figure 5.10B the fold-stimulation of DAZAP1 is reduced by 64% in the presence of the poly(A) tail. This result suggests that at least some of the effect of tethering DAZAP1 is mediated by PABP, as adding a poly(A) tail diminishes the effect of DAZAP. The poly(A) tail added to the luciferase reporter would have had the effect of recruiting molecules of PABP to the reporter. If DAZAP1 stimulation had been insensitive to the presence of these molecules of PABP this would have indicated that it operated via a PABP insensitive mechanism. The fact that stimulation by DAZAP1 is reduced in the presence of PABP suggests that DAZAP1 may function by recruiting PABP in some manner.

This suggests model #1 as the most plausible as in this model the effect of DAZAP on translation is mediated by Dazl recruiting PABP.

5.11 What happens to DAZAP1's effect on translation during meiotic maturation?

In *Xenopus* the Stage VI to mature oocyte transition is equivalent to the G2 to meiosis 1 transition, with the end of the growth stage and the first meiotic division occurring upon maturation. Previously Dazl has been suggested to be functional during meiosis in *Drosophila* (Eberhart *et. al.*, 1996) and C. elegans (Karashima *et. al.*, 2000) and possibly mouse (Saunders *et. al.*, 2003) (see chapter 1 for more detail). In *Xenopus* Dazl has been shown to be phosphorylated during maturation and to become dephosphorylated shortly after fertilisation (Mita and Yamashita, 2000). To investigate the physiological relevance of this observation, the activity of tethered dazl was compared in stage VI and mature oocytes. This showed that Dazl stimulated translation to a significantly greater extent in mature oocytes than in stage VI (B. Gorgoni, unpublished). This experiment was also conducted for PABP1 and ePABP that also showed a significant increase in translational stimulation (Wilkie *et. al.*, 2005), although no change in the phosphorylation of these proteins is observed (Cosson *et. al.*, 2002).

To determine whether the activity of DAZAP1 was altered during maturation, tethered function assays were performed as previously, but in this case half the oocytes injected were treated with progesterone after injection. The progesterone treatment causes the oocytes to mature. Progesterone treated oocytes were collected after an overnight incubation and not at GVBD50 when half the oocytes had matured, to mirror the conditions used for DAZL and PABP1/ePABP. Maturation can be easily scored by the appearance of a white spot on the animal pole and oocytes that did not mature were discarded.

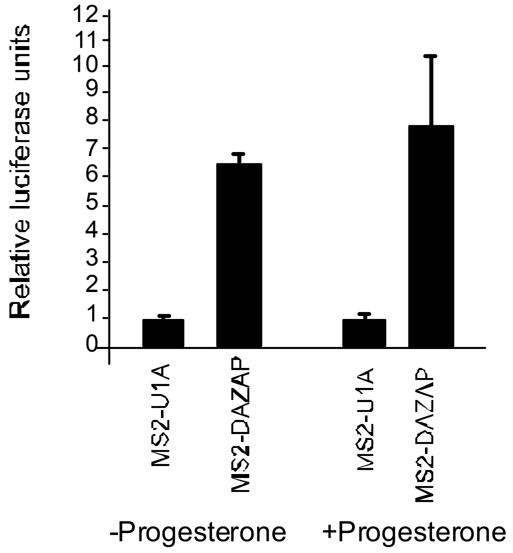


Figure 5.11: The effect of oocyte maturation on DAZAP1 translational stimulation
DAZAP1 translational stimulation is not affected by oocyte maturation.
Oocytes expressing MS2-U1A or MS2-DAZAP1 were co-injected with Luc-MS2 alongside β-Gal mRNA. Half the oocytes were treated with progesterone to induce maturation. Luciferase activity was normalised to β-galactosidase activity and the relative luciferase units were plotted with MS2-U1A values set to one. The average of three experiments is shown.

Mean DAZAP1 stimulation of translation increased slightly upon maturation (figure 5.11). However this increase was not significant as can be seen by the overlapping errors of the MS2-DAZAP1 relative luciferase units 6.5 +/- 0.5 for immature oocytes and 7.9 +/- 3.1 for mature oocytes.

Thus the action of DAZAP1 during maturation appears to differ from both DAZL and PABPs which show a 1.7-2.3 fold increase in their activity. The observation that DAZAP1 does not increase translational stimulation in mature oocytes is surprising as the previous experiment suggested that PABP may play a role in DAZAP activity, however this result appears inconsistent with this conclusion. This issue will be returned to in the chapter discussion.

5.12 DAZAP1 phosphorylation mutants do not change stimulatory activity in tethered function assays

A published study investigated the phosphorylation state of DAZAP1 and the effect of phosphorylation upon DAZAP1's interactions with DAZ (Morton *et. al.*, 2006). It was shown that a human DAZAP1 with an N-terminal truncation removing the RRMs could interact with DAZ *in vitro*. Two phosphorylation sites were identified at Thr269 and Thr315 and two DAZAP1 mutants were introduced into the truncated protein, mutating these residues to alanine or asparagine. The alanine mutant, DAZAP1-AA, mimicked the effect of hypophosphorylation and the asparagine mutant, DAZAP1-DD, mimicked the effect of hyperphosphorylation of these residues. It was demonstrated that the AA mutant could still interact with DAZ but that the DD mutant could not, suggesting that the phosphorylation of DAZAP1 causes its dissociation from DAZ. These experiments were repeated with full-length DAZAP1 mutants and the results were similar, though the DD mutant did not totally abrogate binding to DAZ, but instead was substantially reduced.

In collaboration with the authors of this study the two full-length DAZAP1 phosphomimetic mutants were investigated in tethered function assays. It was hoped that the results of this experiment could reveal the significance of the Dazl/DAZAP1 interaction in the tethered function assay.

Tethered function assays were performed using wild-type DAZAP1, DAZAP1-DD and DAZAP1-AA. Because it is known that oocyte maturation is accompanied by numerous phosphorylation events (Ferrell, 1999) and it has been suggested that DAZAP1 dissociates from DAZ upon phosphorylation the activity of these mutants were also investigated in mature oocytes.

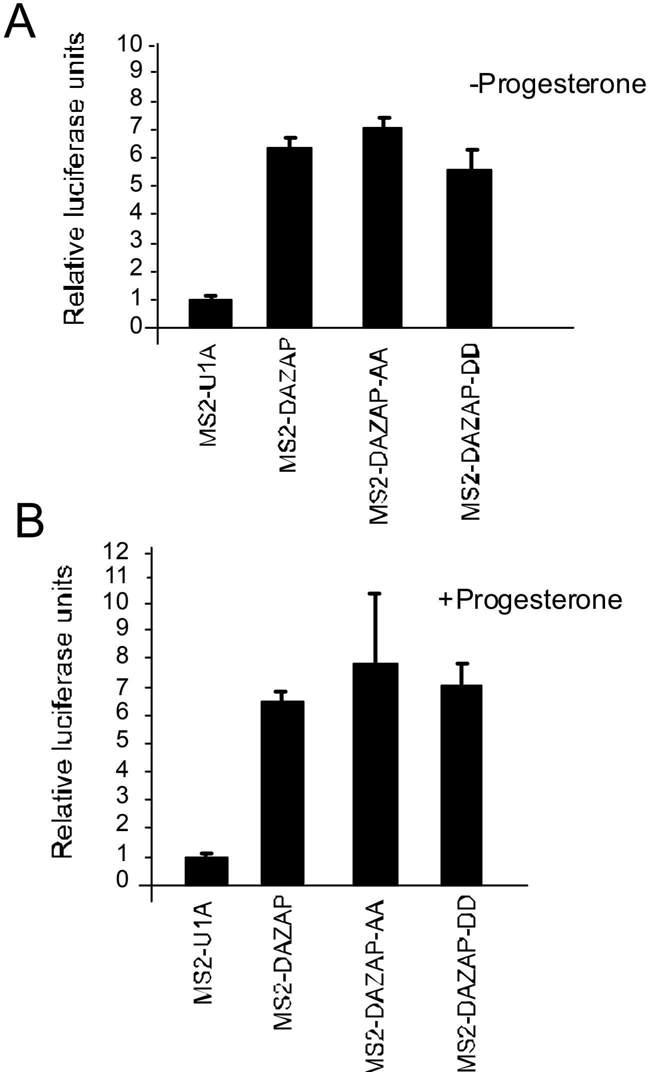


Figure 5.12: Testing the ability of phosphorylation mutants of DAZAP1 to stimulate translation.
The phosphorylation mutants of DAZAP1 do not significantly alter the pattern of translational stimulation. Oocytes expressing MS2-U1A or MS2-DAZAP1, MS2-DAZAP-AA or MS2-DAZAP-DD were co-injected with Luc-MS2 alongside β-Gal mRNA. Half the oocytes were treated with progesterone to induce maturation (B) and half were not (A). Luciferase activity was normalised to β-galactosidase activity and the relative luciferase units were plotted with MS2-U1A values set to one. The average of three experiments is shown.

When compared to the translational activity of wild-type DAZAP1 the AA and DD mutants did not differ substantially in translational activity (figure 5.12A) nor did oocyte maturation substantially alter stimulation by tethered DAZAP1 and mutants thereof (figure 5.12B).

This could be interpreted to suggest that DAZAP1 does not need to interact with Dazl to stimulate translation as it assumed that the DD mutant would not be able to interact with Dazl in the oocyte, consistent with either model 2 or 3. However, the interaction of these mutant proteins with endogenous *Xenopus* DAZL where not investigated. Alternatively as the interaction of the full-length DD mutant was not completely abolished binding *in vitro,* the residual binding may be sufficient to recruit Dazl given the expression levels of the MS2-DAZAP proteins. The implications of this experiment will be discussed in more detail in the chapter discussion.

5.13 Discussion

In this chapter the role of DAZAP1 in translation and specifically its role in translation with regards to Dazl and PABP have been investigated. Previous work had proposed different potential functions for DAZAP; the first of these was that of a RNA localisation protein (Zhao *et. al.*, 2001) and the second was that of an inducer of RNA instability or a repressor of DAZ (Morton *et. al.*, 2006). In figure 5.5 it was established that endogenous DAZAP1 and Dazl could possibly interact in *Xenopus* oocytes. This observation raised the question of whether DAZAP1 had any bearing on the model of Dazl stimulation of translation, the main focus of this thesis.

When the localisation of DAZAP1 on a sucrose gradient was explored it was found to co-sediment with ribosomes and light polysomes in stage VI oocytes and show an increased interaction with light polysomes in mature oocytes perhaps suggesting an interaction with the translation machinery. This indicates that DAZAP may have a potential role in translation, but may merely mean that it is associated with actively

translating mRNAs rather than directly promoting their translation. Such a sedimentation pattern could also be consistent with a role in stability. However, proteins that are associated with actively translating ribosomes tend to localise with polysomes fractions and then release to the mRNP fraction upon EDTA treatment so this pattern of co-localisation is not typical. One encouraging result is that in the same experimental system, over-expressed *Xenopus* PABP1, a known translational regulator localises to the same fractions as DAZAP1 (B.Gorgoni, personal communication). This recent result gives more precedence to the theory that DAZAP1 is involved with actively translating ribosomes.

This could be a result of a lack of intact polysomes in the sucrose gradients from these experiments. It has been shown by extracting ribosomal RNA from gradient fractions that there are ribosomes in the three fractions (usually numbers 4, 5, and 6) just proceeding the 80S peak (B.Gorgoni, personal communication). However it is not clear if these represent true polysomes, or just heavier monosomes. The distribution of DAZAP1 after EDTA release could indicate that DAZAP1 interacts directly with ribosomal subunits and that hypothesis is worth investigating in the future. Another explanation for DAZAP1 localising in the same sedimentation fraction as the ribosomes could be that they are part of a 'heavy mRNP', a complex of proteins on an RNA that contains enough large proteins that it co-sediments with translational machinery, despite not actually being associated with them. Heavy mRNPs have been proposed to account for the reason that mRNAs bound by microRNAs were observed to co- sediment with a polysomes fraction (Thermann and Hentze, 2007). Given the number of proteins that interact with Dazl, i.e. DAZAP, PABP, DZIP, Dynein and Pumilio, it is possible that DAZAP can be found in a large enough protein complex to localise where it does on a gradient without being bound to RNA. This could be further investigated by releasing the ribosomes with puromycin rather than EDTA. This drug also causes the release of the ribosome from mRNA but does not disrupt other protein/protein and RNA/protein interactions in the manner of EDTA, and is therefore more specific (Xaplanteri *et. al.*, 2003). It would also be of interest to look at the gradient profile of tethered MS2-DAZAP1 on a MS2 containing RNA.

The result seen in the sucrose gradient here is directly opposed to the result for a similar experiment conducted previously (Dai *et. al.*, 2001). In this experiment post-mitochondrial mouse testis extract was analysed on a 15-45% sucrose gradient and blotted for DAZAP1. DAZAP1 was found to be localised exclusively in the mRNP fraction suggesting that it was not directly involved in translation. The disparity seen between this experiment and the results presented in this chapter could be caused by a number of factors. First, the differences seen here could be due to the species used, DAZAP1 could behave differently in mice to *Xenopus*. The effect could also be due to the tissue used, *Xenopus* oocytes represent a pure germ cell sample whereas the previous experiment was conducted in a whole testis extract. This is important as DAZAP is also present in somatic cells where Dazl is absent and may have a different role with different protein partners in these cells. There could be a developmental stage specific DAZAP1 interaction with mRNAs, especially at meiosis. The oocytes used in the experiments were either stage VI, which are all pre-meiotic or mature oocytes that are entering the first meiotic division. In an adult mouse testis not all cells would be germ cells and only a subsection would be at a particular stage of meiosis. It could be that by using a mixed lysate, Dai *et al* missed the translational activity of DAZAP1. Support for this idea comes from the finding that human DAZAP1 can also stimulate translation in these germ cells, suggesting that there is not an inherent difference between the frog and mammalian DAZAPs. These hypotheses could be investigated by looking at single cell type systems in mouse, and also by investigating if the result present in this thesis is common to all oocyte and early embryo stages, or if DAZAP1 re-localises on a sucrose gradient over time, reflecting a changing function. There could also be differing effects in different stages of oocyte or embryo development regarding the interactions of Dazl and DAZAP1.

In the gradients presented in figure 5.6 there are variations in the distribution of the endogenous DAZAP1 seen between the two gradient experiments presented. It would be expected that the endogenous protein distribution would remain constant, so what are the possible explanations? First, it could be the result of experimental variation between

the two different experiments (one with endogenous DAZAP1 alone, one with endogenous and overexpressed DAZAP1). For this reason it is only possible to compare gradients run on the same day with the same oocytes (i.e. A and B or C and D). Second, the variation could be the result of over-expressing additional DAZAP1, which may have caused the changes in the disruption of the original message. However, as these experiments were not run in parallel, this remains to be determined.

To investigate a potential active role in translation, tethered function assays were undertaken. Although the translational activity of DAZAP1 had never been directly assessed in previous work, it was found that it stimulated translation strongly when tethered, perhaps surprisingly given the previously proposed functions. One explanation is that DAZAP1 is a bona-fide stimulator of translation, and another plausible explanation is that the stimulation seen in tethered function assays could be an artefact of Dazl binding activity recruited by DAZAP1. These results lead to the development of three possible models to explain the relationship between DAZAP1 and Dazl-mediated translation (fig 5.8). The first model was that DAZAP1 acts as an RNA anchoring protein to a complex containing Dazl which through its interaction with PABP stimulates translation. The second model was that Dazl stimulates translation via both PABP and DAZAP1 and DAZAP1 has its own stimulatory activity. The third model was that DAZAP1 stimulates translation independent of Dazl or PABP. The remainder of the chapter tried to reconcile these models with the tools available at the time.

To distinguish between the models available various tethered function assays were employed. The first of these investigated the effect of tethering one or more molecules of DAZAP1 to the reporter. When DAZAP1 was investigated (figure 5.9) it was found that multiple molecules could stimulate in a similar pattern to Dazl and PABP. As this evidence is consistent with DAZAP1 acting via Dazl and PABP it supports model #1, though it must be noted that is does not rule out models #2 and 3. In the next experiment DAZAP1 showed reduced fold-stimulation of translation in the presence of a poly(A) tail (figure 5.10). This sensitivity to a poly(A) tail provides a strong argument for DAZAP ultimately using PABP as an effector for its translational activity as the

main difference in adding the tail is that the reporter would already be bound by PABP. Thus explaining the reduced stimulation as adding extra PABP molecules via DAZAP1 when there are already (poly(A) bound) PABP molecules present would have less effect then when there are no PABP molecules already present on the mRNA. This again supports model #1 as the most viable. However, other functions of the poly(A) tail independent of PABP have been described (Thoma *et. al.*, 2004) and several other proteins have been isolated from poly(A) tails in *Xenopus* oocytes (Swiderski and Richter, 1988).

So far the evidence available has most strongly supported model #1, however when the effect of oocyte maturation on DAZAP1 stimulation was investigated (figure 5.11) it was observed that translational stimulation did not increase in mature oocytes. This is different to the activity of Dazl and PABP in oocytes, which showed an increase in stimulation. This would imply that DAZAP1 does not depend on Dazl or PABP for its translational activity. However as there are so many changes in mature oocytes upon maturation it is difficult to speculate on what may be the cause of any results. There are phosphorylations of Dazl, and possibly DAZAP, which could affect their activity and interactions and also the loss of repressors, such as Pumilio, which could also have an effect. In addition, oocyte maturation causes a lot of variation in tethered function assays, as can be seen by the error bars in figure 5.11. Another aspect that could affect this result is that the oocytes were collected as mature after an overnight incubation with progesterone. There could be variation between samples and the time each set of oocytes takes to reach maturation (3-16 hours). To make this result more reliable this experiment could be repeated taking different time points, including GVBD50 and also including Dazl and PABP in parallel in order to discover the true relationship between Dazl, DAZAP1 and PABP in mature oocytes. The difficulties in assessing what is causing any changes and the experimental error in this system means that this experiment is in my opinion the least convincing of those undertaken when trying to decide on a model for DAZAP1 action.

The final investigation in this chapter utilised some DAZAP1 phosphomimetic mutants. The results of this experiment could be taken as to suggest that DAZAP1 stimulation is not dependent on binding DAZ family proteins, such as Dazl. This would indicate model #3, a function for DAZAP1 independent of its Dazl binding activity, as the most likely. However as mentioned earlier there is a lot of potential for DAZ and Dazl to interact differently with DAZAP1 so it hard to draw any reliable conclusions form these experiments. While an investigation of the interaction with DAZ is certainly interesting it is out with the scope of this thesis, which concentrates on Dazl.

Overall in this chapter there are data that support a role for DAZAP1 in Dazl mediated translational stimulation with model #1 being most consistent with the results seen. Although this may change with further investigation, what are the implications of this current model of Dazl action? This question will be addressed in chapter 6. Next the possible avenues of investigation to further clarify the activity of DAZAP1 and its relation ship with Dazl and translation will be discussed.

As previously stated further work will be required to definitively clarify the relationship of DAZAP1 with Dazl. One of the most informative techniques would be to employ further IP experiments to identify what complexes Dazl, DAZAP1 and PABP form in oocytes. These experiments were attempted during the last month of the project, but failed to yield any results in the time period available. Obviously continuing this work would be an important element of any future work on this project. A previous study (Morton *et. al.*, 2006) included a study of the complexes made by DAZ, DAZAP1 and PABP *in vitro*. This study observed that DAZ and PABP or DAZ and DAZAP1 could interact but all three could not exist as a triple complex. Applying this result to the proposed models of DAZAP1's translational activity only model three is consistent. However there are reasons why this data may not apply to this investigation. The first reason is that the previous study was conducted *in vitro* using DAZ. DAZ although related to Dazl has not been subject to much investigation. It is known to stimulate translation and bind *Xenopus* PABP1 and ePABP (Collier *et. al.*, 2005). However in this thesis it was shown that DAZ and human PABP1 may not interact (figure 4.3). DAZ

also differs from Dazl significantly in that it has multiple (9-15 depending on which of the four *DAZ* genes it is expressed from) copies of the DAZ repeat. Bearing in mind that it is via the DAZ repeat that DAZAP1 interacts with DAZ and DAZL (Tsui *et. al.*, 2000a), the requirement of this repeat for polysome association in mouse and full translational stimulation in *Xenopus* and zebrafish is not necessarily consistent with its interacting with a translational repressor. The interactions and function of DAZL and DAZ could be very different regarding DAZAP1. Another possible explanation of why triple complexes were not seen in previously published work using overexpressed proteins is that the IP conditions were too restrictive to pull out triple complexes, which may be transient and only formed on RNA or that these complexes are dependant on a germ cell environment as regulation by Dazl has failed to be recapitulated using these in somatic systems.

In order to discover the true role for DAZAP1 in Dazl's ability to stimulate translation it would be useful to map the Dazl binding site on DAZAP1. Initially large truncations or internal deletions could be used to discover which part of the DAZAP1 protein is required for the binding of Dazl. The different truncated DAZAP1 proteins could also be tested for their ability to stimulate translation in tethered function assays. This would determine whether or not DAZAP1's observed stimulatory activity is separable from its ability to bind Dazl.

If interactions with Dazl and/or PABP are proven or disproven then the other proteins that DAZAP1 binds to could be indicative of function. A screen to discover what the protein partners of DAZAP1 would be informative. So far the only protein partners identified for DAZAP1 were profillin, a protein that promotes actin polymerisation, and Mena, a microfilament-associated protein (Zhao *et. al.*, 2001). It was on the basis of these interactions that DAZAP1 was suggested to be involved in RNA transport. This ability to bind transport proteins does not preclude a role as a translational activator, indeed Dazl also binds a transport protein, dynein, reflecting the potential multifunctional roles of these proteins (see chapter 6). Establishing what the other protein partners of DAZAP1 are in oocytes could answer some of the questions

presented by the data shown in this chapter, such as why is DAZAP1 seen to associate with the ribosomal subunits, does it interact with ribosomal proteins directly? Biochemical interaction studies, for example yeast-two hybrid analysis, could be used to address this question.

The question of what RNAs DAZAP is acting upon would need to be addressed, previous work showed DAZAP1 binding to the 3'UTR of *Vg1* RNA (Zhao *et. al.*, 2001). Vg1 has not been suggested to be a target of Dazl, and does not have a sequence closely resembling the dazl binding site. This raises the question of whether DAZAP1 has separate functions depending on which RNA and proteins it is bound to. There is also the possibility that DAZAP1 may have different functions in different cell types. In *Xenopus* DAZAP1 has only been shown to be expressed in oocytes whereas in humans and mice DAZAP1 has been shown to be more widely expressed Where DAZAP1 is expressed in tissues that do not co-express Dazl family members the function could be very different.

Overall whether DAZAP1 is an activator, repressor, inducer of instability or localisation protein remains to be seen. It is also unclear if the main identified protein interaction of DAZAP1, Dazl, is significant to its function. Future investigation into these questions could provide insight into this interesting protein.

Chapter 6: Final discussion

In this thesis I have presented specific discussions in each chapter where individual results and the techniques utilised were discussed. This chapter will consider the results of the thesis as a whole and their impact on the current knowledge of Dazl as a translational regulator in gametogenesis. Potential future experimental work to expand the studies in this thesis will also be discussed.

6.1 Dazl stimulates translation initiation at 43S joining via an interaction with PABP

Before this thesis work was started a model for Dazl action was proposed in which Dazl stimulates translation by recruiting PABP to mRNAs with predominantly short poly(A) tails (Collier *et. al.*, 2005). It was also suggested that Dazl acts at the stage of translation initiation (Collier *et. al.*, 2005) consistent with the pleiotropic effects of PABP on this process.

In chapter 3 of this thesis, use of the variable reporter tethered function assay showed that Dazl most likely stimulates translation at the stage of 43S joining and in chapter 4 it was shown that PABP gives a pattern of stimulation in the variable reporter tethered function assay that is consistent with its role as an affecter molecule for Dazl. These results provide further support for a role of PABP and importantly provide the first insight into the point of the initiation pathway that Dazl stimulates.

These analyses were performed using tethered function assays, however since this work was completed several target mRNAs of mouse Dazl protein have been verified. It would be of interest therefore to extend this analysis to mRNAs containing the 3'UTR of one of these mRNAs to address the physiological relevance of my observations. The verified targets include the mouse Vasa homologue (*Mvh*) (Reynolds *et. al.*, 2005) *SYCP3* (Reynolds *et. al.*, 2007) and also *Drosophila twine* (Maines and Wasserman, 1999). The latter is in keeping with the ability to do cross-species rescue of the *Drosophila Boule* phenotype (Slee *et. al.*, 1999). In the lab, Dazl stimulates translation

from the *twine* 3'UTR 7 fold, the *Mvh* 3'UTR 5.5-fold and the Scyp3 3'UTR 4 –fold. The strong stimulation directed by the *twine* 3'UTR makes it an attractive candidate for such analysis. However, *Mvh* and *Sycp3* are both physiological targets of mouse Dazl, and point mutations in *Sycp3* have been identified that prevent Dazl-mediated stimulation, providing an excellent specificity control. However, it must be remembered that these 3'UTRs also contain other regulatory signals such as CPEs that could potentially influence the results, so mutations of these additional control elements may be required to address the influence of the Dazl protein on initiation.

The discovery that Dazl can stimulate translation at 43S joining is of particular interest as the mechanism of action of only a handful of cellular mRNA specific activators have been examined. Of these SLBP (Gorgoni *et. al.*, 2005) mediates its effects from the 3'UTR. In contrast to Dazl SLBP was shown to stimulate translation at the cap binding stage of translation initiation (Gorgoni *et. al.*, 2005).

The fact that Dazl activates at a different stage to these proteins is interesting as it suggests that it represents a new class of translational stimulatory protein and a novel mechanism of stimulating translation. Since our observations, it has also been suggested that BRCA1 may also stimulate translation by direct recruitment of PABP. Thus the model presented here may have relevance to the regulation of other mRNAs outside the germ line, and it would be of interest to determine whether other proteins also utilise PABP in a similar manner. The first step to find these other proteins is to establish what the binding site that recruits PABP consists of. The work presented in chapter 4 represents my efforts to further delineate the PABP binding motif of Dazl. At the end of the chapter some progress had been made, but the question had not been answered fully. However, the identification of a PABP binding motif and the critical amino acids for PABP binding could be crucial to identifying other translational regulators that act via PABP. It is likely that this motif would be different to the previously identify PAM2 PABP binding motif as Dazl does not contain a PAM2.

However interesting the results presented in this thesis are, the mechanism for Dazl stimulation is not yet fully defined. As mentioned in the discussion of chapter 3, although 43S joining remains the most likely stage of initiation that Dazl stimulates at, it is possible that there is also an effect on scanning but the assay used in this thesis could not distinguish further between the two stages. Ultimately, sucrose gradient analysis of initiation intermediates and toe-printing will be required to fully reconcile whether 43S joining, scanning or both are affected by Dazl. It is possible to use sucrose gradients and various different drugs to stall translation at different points and see where on a gradient a labelled RNA accumulates in the presence or absence of Dazl. While this approach could be useful it also has a number of drawbacks including the fact that it is difficult to asses cap binding effects and gives no insight into possible factor requirements, as the variable tethered function did in this study. Sucrose gradient analysis of initiation intermediates is normally performed *in vitro*, and Dazl-mediated translation appears to require a "germ-cell environment" preventing use of commercial extracts such as rabbit reticulocyte lysates. Whilst it is possible to utilise translation extracts derived from *Xenopus* oocytes these do not show robust activity, therefore the possibility of utilising *Drosophila* embryo extracts that are more robust and likely to share many of the key features required for Dazl-mediated regulation is currently being explored. Thus, it may be beneficial to utilise the *twine* 3'UTR for future variable tethered function assays to link previous work in *Xenopus* oocytes with initiation complex analysis in *Drosophila* embryo extracts.

Another pertinent question is which initiation factors does Dazl require for its role as a translational stimulator? A role for PABP has already been established (Collier *et. al.*, 2005) but the exact protein interactions that are required remain unknown, although a number of protein partners for PABP have been established it is also clear that our knowledge of PABPs partners in initiation is not complete. To further resolve the mechanism of translational stimulation beyond that presented in this thesis a series of *in vitro* experiments could be undertaken if translational stimulation can be reconstituted. This would allow the translation factor complement to be manipulated to ascertain which factors are absolutely required. For instance, to test whether the PABP is required

it could be depleted using antibodies of Paip2 (Svitkin and Sonenberg, 2004) and recombinant PABP added-back to rescue activity if lost. If PABP was shown to be required, the importance of its interaction with eIF4G for instance could be probed using viral proteases that cleave eIF4G, for example coxsackievirus B4 protease 2A (Keiper and Rhoads, 1997) or proteins that block eIF4G-PABP interactions, for example Paip2 (Karim *et. al.*, 2006) or rotavirus NSP3 (Groft and Burley, 2002). Ultimately, Dazl-mediated stimulation could be entirely reconstituted from recombinant factors in a manner analogous to how the factor requirements for particular IRESs, EMCV and CSFV were determined (Pestova *et. al.*, 1996a; Pestova *et. al.*, 1998). Again this approach would require recombinant or purified Dazl, so the development of a strategy that could generate this would be the key step in furthering this work.

An alternative approach to examine the factor requirement of Dazl could be a genetic approach where the effect of mutations that disrupt the interactions between Dazl and various initiation factors is utilised to discover which factors are important. One of the main aims of chapter 4 was to further characterise the PABP-binding site within Dazl family members leading to the generation of a point mutation that is deficient in PABP binding that would have enabled the categorical address of whether Dazl stimulation is entirely dependent on its interaction with PABP or whether other factors are involved. This mutant could also have been used to generate transgenic mice to determine the physiological role of the PABP-Dazl interaction in germ cells.

6.2 A proposed mechanism for Dazl's physiological role in translation activation

Even if the mechanism behind Dazl stimulation in terms of factor requirements and exact event in translation are discovered, there remains the question what is the physiological role of Dazl's translational activation activity. The identification of target mRNAs is an important step towards this end, but does not explain why these mRNAs are controlled by an mRNAs specific activator rather than by alternate mechanisms that

control many germ cell mRNAs. In gametogenesis many RNAs are held in the cytoplasm with short poly(A) tails (see figure 6.1A), which results in low or no translation, until they receive a signal to undergo cytoplasmic polyadenylation. Different classes of mRNAs are activated by cytoplasmic polyadenylation at different times depending on whether their protein product is required, for instance earlier or later in meiosis or following fertilisation (reviewed in (Mendez and Richter, 2001)). Regardless of the timing, newly synthesised poly(A) tails provide binding sites for molecules of PABP that then stimulate translation (figure 6.1B).

Whilst it is clear that specific subsets of mRNAs undergo this sort of polyadenylation at defined times during gametogenesis, the configuration of elements that determine this and the upstream signalling pathways remain an area of intense interest (de Moor *et. al.*, 2005; Richter, 1999). However, it is also clear that during oocyte maturation in *Xenopus* for instance, events precede the onset of the first subset of mRNAs that undergo cytoplasmic polyadenylation, some mRNAs appear to be polyadenylated in response to activation rather than being caused by activation, and other mRNAs do not require changes in poly(A) tail length for their activation. Thus these mRNAs require a different mechanism of activation. For some of these loss of a repressor protein may be sufficient but for others, especially those with a short poly(A) tail, an activator may be required to promote their efficient translation in a competitive environment where many mRNAs have undergone polyadenylation. Dazl may provide one mechanism by which these mRNAs can be activated.

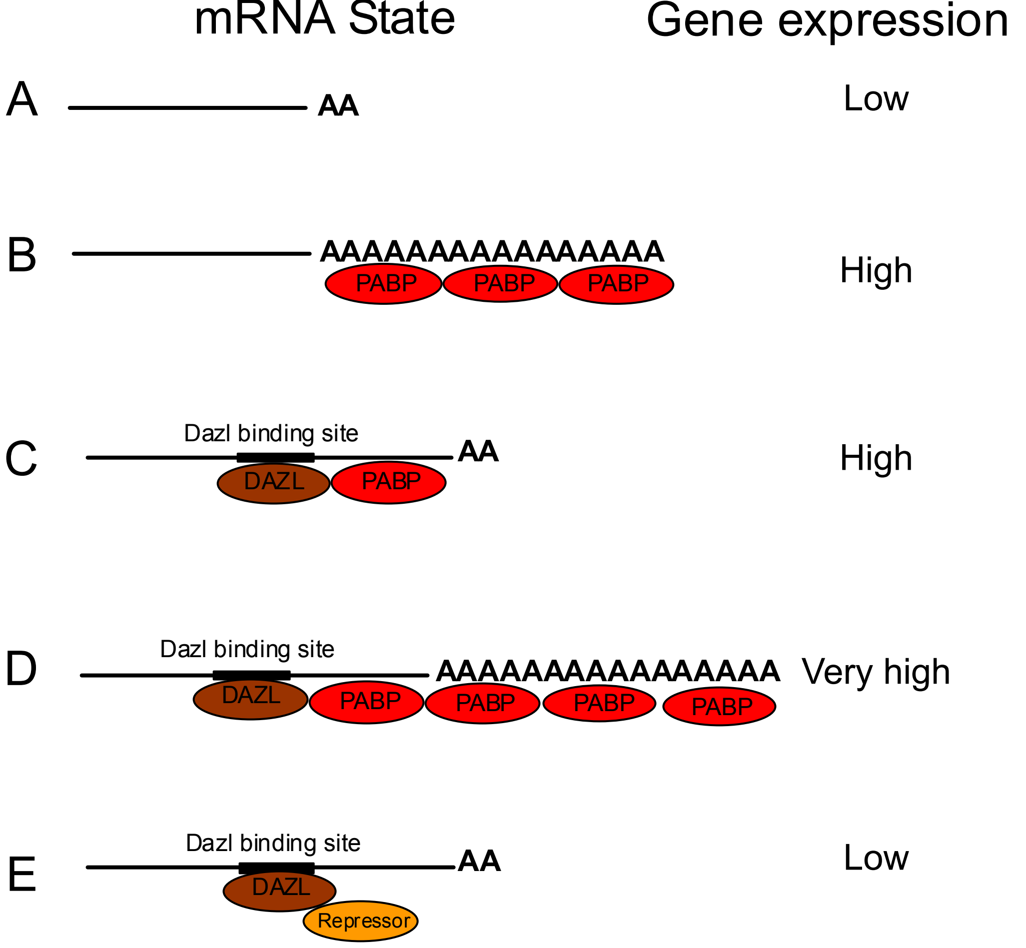


Figure 6.1: Levels of gene expression of mRNAs with and without Dazl binding sites and their response to polyadenylation.
A: mRNA with a short poly(A) tail and no Dazl binding site. Gene expression is low.
B: mRNA with a long poly(A) that is binding multiple molecules of PABP. Gene expression is high.
C: mRNA with a short poly(A) tail and a Dazl binding site, binding PABP via Dazl. Gene expression is high. Note that potential target mRNAs may contain multiple Dazl binding sites.
D: mRNA with long poly(A) that is binding multiple molecules of PABP and a Dazl binding site, binding PABP via Dazl. Gene expression is very high.
E: mRNA with a short poly(A) tail and a Dazl binding site that cannot bind PABP due to the presence of a repressor protein. Gene expression is low.

It is interesting to note therefore that the two best characterised vertebrate Dazl targets, *Mvh* (Reynolds *et. al.*, 2005) and *Sycp3* (Reynolds *et. al.*, 2007) both contain both Dazl binding sites and CPEs. In the case of *Sycp3* these CPEs have been shown to be functional and *Sycp3* has been shown to be stored with a short poly(A) tail of 20 nucleotides (Tay and Richter, 2001). Preventing the polyadenylation of Scyp3 (Tay and Richter, 2001) or blocking its activation by Dazl (Reynolds *et. al.*, 2007) both result in

heavily reduced although not completely abrogated translation of this mRNA, suggesting that both contribute to its maximal activation. Whether these events occur simultaneously or whether Dazl activation precedes cytoplasmic adenylation of this mRNA remains to be determined. However, The CPE and dazl binding site on this mRNA overlap (Reynolds *et. al.*, 2007) suggesting that both proteins are unlikely to be simultaneously bound to the mRNA.

It is interesting to speculate what is special about germ cells such that they need specific translational activator proteins such as the Dazl family members. It is known that transcription terminates during mid-spermiogenesis (Kierszenbaum and Tres, 1978) indicating that the changes to gene expression required for later spermiogenesis must be controlled in a post transcriptional manner. Perhaps the Dazl family of proteins has evolved in order to fulfill the need for control of gene expression in cells that cannot regulate gene expression via transcription due to events occurring in the nucleus.

6.3 The interactions between different Dazl and PABP members may be significant

Examining the interactions between Dazl and PABP family members (chapter 4) revealed that different Dazl family members and different PABP family members may potentially interact in a non-redundant manner (figure 4.3). This raises the question of which PABPs are utilized by Dazl family members in germ cells where Dazl is expressed. In human germ cells DAZ, DAZL and BOULE are all expressed in various different germ cells (see section 1.6.3) as are the mRNAs of PABPC1 and tPABP (Feral *et. al.*, 2001). PABP4 (Hannah Burgess unpublished) and ePABP (Wilkie *et. al.*, 2005) are also potentially expressed in germ cells whereas PABP5 is restricted to the somatic cells of the gonad at least in mouse (Ross Anderson, unpublished). Thus at least two PABP proteins with similar structure are potentially present in the cells that Dazl family proteins are expressed in. Whilst the reason underlying the expression of multiple PABPs in these cells remains unclear, one intriguing possibility raised by this work is

that whilst they share an ability to promote poly(A) mediated translation, they may differ in their ability to be recruited to specific mRNAs through proteins such as Dazl.

The exact interactions between Dazl family members in mammalian germ cells are currently impossible to determine due to the lack of evidence for which particular PABPs are expressed in which particular germ cells. Traditionally antibodies to mammalian PABPs have not discriminated between the different family members and until this problem is solved it will be impossible to determine which PABP family members have the potential to interact with which Dazl family members in germ cells. A project has been initiated (Ross Anderson and Hannah Burgess, unpublished) seeking to address this issue but at the time of writing no firm results have been established.

Thus different combinations of Dazl family members and PABPs may regulate different target mRNAs or activate at different times. Elucidating the physiological targets for different Dazl family members and investigating the differential roles of PABPs in their regulation and how the interactions are regulated will be an important aspect of truly understanding how Dazl regulates gene expression.

6.4 The role of DAZAP1 in Dazl mediated translational stimulation

In chapter 5 of this thesis it was shown that DAZAP1 can stimulate translation tethered function assays consistent with its presence on polysomes. This contrasts sharply with published models of DAZAP1 based on indirect observations of its interaction with localisation sequences, its presence in mRNPs and an inability to assemble DAZL-DAZAP1-PABP complexes *in vitro*. Whilst, I have not directly demonstrated the presence of DAZL-PABP-DAZAP1 complexes *in vivo*, a number of mechanistic approaches support the hypothesis that DAZAP1 stimulation is most likely dependent on PABP. Most strikingly is the fact that DAZAP1 showed reduced stimulation of translation in the presence of a poly(A) tail.

It is not yet clear whether this observed activity in stimulating translation is DAZAP1's primary function; many 3'UTR-binding proteins have multiple functions dependent on the partners with which they interact. As mentioned in chapter 5, deletion mapping of DAZAP1 could be utilised to determine whether binding to Dazl is required for DAZAP1s stimulatory function.

One possible outcome of this analysis is that DAZAP1 may be found to act as a co-factor for Dazl rather than having its own intrinsic function as a translational activator. As DAZAP1 has been shown to bind RNA, it may act as a 'linker' molecule, enabling Dazl to bind more efficiently to mRNA, or to a different subset of RNAs altering the specificity. Dazl binds to as yet poorly defined consensus sequences and perhaps the degenerate nature of these could be due to the separate binding of DAZAP1 to another site that helps to anchor Dazl. Such a model would be reminiscent of regulation of mRNAs in *Drosophila* by Pumilio containing complexes (Wickens *et. al.*, 2002). Moreover, some Dazl regulated mRNAs may not be dependent on an mRNA possessing a Dazl binding site but instead be regulated by a co-factor such as DAZAP1 binding before recruiting Dazl.

If DAZAP1 is found to be able to stimulate translation independent of binding Dazl, this does not rule out that it functions primarily as part of this complex in germ cells being recruited to mRNAs by Dazl, with the tether protein substituting for Dazl function in our assay. However, if DAZAP1 functions through PABP it is unlikely that it is acting as a translational stimulator in these complexes, as DAZL and PABP can interact directly. Thus is would seem most likely that this represents a function of DAZAP1 that is independent of its function from Dazl. Thus the observation that DAZAP1 could stimulate translation independent of DAZL would be potentially very interesting, suggesting that it can function as a novel translational activator in its own right. Moreover, as no interaction between DAZAP1 and any component of the translation machinery has been reported, understanding the mechanism of DAZAP-mediated

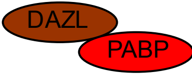
stimulation could form the basis of future work. Thus the determination of whether the action seen in this thesis is independent of Dazl is of paramount importance.

Whether DAZAP acts as a linker molecule recruiting DAZL or has translational activity in its own right, its function is unlikely to be limited to Dazl-mediated regulation. Whilst DAZAP1 is mainly expressed in the testis and ovaries in humans (Tsui *et. al.*, 2000a) (Pan *et. al.*, 2005), it has also been suggested to be expressed in other tissues at lower levels including thymus, heart, liver and muscle as shown by northern blot. In mice the DAZAP1 protein is found mainly in the testis, and also in the ovary, spleen, liver, lung and brain (Dai *et. al.*, 2001). Moreover, in *Xenopus* it has been reported as a protein bound to the Vg1 localisation element an mRNA, which has not been reported as being subject to Dazl mediated regulation. The implication of DAZAP1 being expressed in tissues that do not also express Dazl family members is that it may have multiple partners and functions and there are many possible future studies exploring this interesting protein.

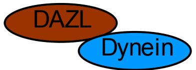
6.5 Proteins other than PABP may play a role in Dazl's translational activities

The finding that Dazl family members activate translation does not exclude them having other functions related to translation or distinct roles within the cytoplasm or nucleus. Indeed, many mRNA binding proteins interact with different partners in different cell types or developmental stages to perform different functions. Thus, it remains possible that the early phenotype seen in Dazl family member knockouts is not due to a lack of activation of target mRNAs through PABPs but a loss of translational repression or mislocalisation of target mRNAs as DAZL proteins appear to be present in multiple complexes.

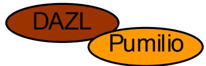
Dazl activation complex



Dazl localisation complex



Dazl repression complex



Dazl complexes with multiple partners

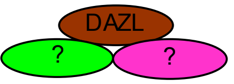


Figure 6.2: Potential Dazl functional complexes

In figure 6.2 some of the potential complexes are shown, which will be discussed in more detail below. DAZAP1 was not included as which of these categories it belongs to remains to be discovered.

6.5.1 Dazl activation complexes

The most studied type of complex so far is that of a Dazl activation complex, by which Dazl activates translation via its interaction with PABP as seen in the work that preceded this thesis (Collier *et. al.*, 2005) and further investigated in chapter 4. As mentioned above there are probably many different variations on this complex with different Dazl family members binding different PABP family members. As it is not known if all the PABP family members stimulate translation and if they do, what degree of stimulation they would give, this is another potential source of variability of Dazl function.

6.5.2 Dazl repression complexes

The functional relationship between Pumilio and Dazl is currently unclear. It is possible that Pumilio merely aids the recruitment of Dazl to mRNAs or alters direct target specificity due to the presence of Pumilio binding and Dazl binding sites within an mRNA. This would be similar to its role in *Drosophila* (Parisi and Lin, 2000). Thus, it seems likely that Pumilio may be involved in recruiting repression complex to these mRNAs, perhaps silencing them until their translation activation, as proposed for *Ringo* mRNA (see below). In this context, Pumilio may repress Dazl bound mRNAs by interacting with partners that may act independently of Dazl or directly interfere with Dazl function or Dazl itself may actively participate in translational repression when complexed with Pumilio. These questions all remain to be addressed. However, it is interesting to note that the proposed Pumilio binding site in Dazl overlaps with the PABP binding site, perhaps suggesting a mutually exclusive relationship.

In *C. elegans* an interaction between CPEB and Dazl was also reported. CPEB is a bifunctional protein involved in mRNA repression as well as cytoplasmic polyadenylation. However, the Dazl target mRNAs analysed to date do not contain the multiple CPEs indicative of repression and a complex between these proteins could not be detected in mouse. Moreover the binding sites on the only mRNA analysed (Reynolds *et. al.*, 2007) overlap suggesting that these proteins may not bind simultaneously.

6.5.3 Dazl localisation complexes

Mouse Dazl was also shown in yeast two-hybrid and GST-pull down experiments to interact in with Dynein light chain, a component of the Dynein-Dynactin motor complex that has an important role in mRNA localization and was shown to be localised in a microtubule dependent manner (Lee *et. al.*, 2006). In addition, DAZAP1, also interacts with profilin, a protein involved in localization (Zhao *et. al.*, 2001), and it is possible that it may do this whilst also complexed to Dazl.

Given the close links between mRNA localization and translation, and the presence of germplasm with many localized mRNAs in a variety of model organisms, it is tempting to speculate that Dynein may direct the localization of DAZL bound mRNAs, before Dazl dictates the levels of translation once localized.

6.5.4 Multiple interaction complexes

Rather than acting with just one protein partner as suggested in the examples above it is more likely that Dazl will form complexes of proteins with multiple members at once. An mRNA could for example initially be bound by a complex of Dazl, Pumilio, Dynein and possibly PABP. This complex could direct Dazl-bound mRNAs to specific sub-cellular regions via Dynein and repress their translation during localization or until required with Pumilio. At the appropriate time and place the Pumilio and Dynein could exit the complex and thus leave Dazl and PABP to stimulate the translation of the mRNA. The establishment of which complexes of proteins could form, on and off RNA is an interesting direction for a future project to take.

One example of a place where this sort of regulation may take place is that of the RINGO/Spy mRNA in *Xenopus* oocytes. This mRNA has been shown to be bound by Dazl, PABP and Pumilio (Padmanabhan and Richter, 2006) although it was not established if multi-protein complexes formed on the mRNA at any point. It was noted that Pumilio, was released from the mRNA upon oocyte maturation, thus potentially leaving Dazl and PABP free to activate the mRNA at that point.

6.6 Many questions about Dazl remain

Through both previous work and the data presented in this thesis progress has been made in identifying the mechanism by which Dazl stimulates translation, regarding both the role in translation initiation and also the protein partners that are involved in that process. However many fundamental questions remain about the critical role of Dazl family members in gametogenesis.

First, important questions remain pertaining to their mechanism, for instance a demonstration of the physiological role of PABP is required, as is an understanding of how this interaction promotes 43S joining and to what extent this represents a paradigm for translational activators. Secondly, many of the target mRNAs of Dazl remain to be identified, in particular the target mRNAs in the spermatogonia, spermatocytes and female germ cells where a function for Dazl is known but targets are not. Thirdly, it is also unlikely that Dazl acts in the same manner in all species, as evidenced by the differences in knockout phenotypes, so each must be investigated separately. Indeed the differences seen in the effect of a Dazl knockout caused by differences in genetic background suggest that it is unlikely that Dazl acts in the same manner even within a species and that there could be modifiers of activity beyond those explored in this thesis. Fourthly, potential roles of Dazl in other aspects of RNA biology such as translational repression, mRNA turnover and mRNA localisation remain to be explored. Fifthly, the regulation of these potentially different complexes will be important and *Xenopus* Dazl has been shown to be a phosphoprotein (Mita and Yamashita, 2000), providing a potential avenue for investigation. Lastly, the relationship between Dazl and the other Dazl family members and the extent to which their molecular functions and targets are related awaits clarification.

A detailed understanding of each of these questions will be required to fully understand how the Dazl proteins function and contribute to gametogenesis.

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