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The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells

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DAZL proteins are germ-cell-specific RNA-binding proteins essential for gametogenesis. The precise molecular role of these proteins in germ-cell development remains enigmatic; however, they appear to function in the cytoplasm. In order to directly address the function of vertebrate DAZL proteins, we have used *Xenopus laevis* oocytes as a model system. Here we demonstrate that members of this family, including Xdazl, mouse Dazl, human DAZL, human DAZ and human BOULE, have the ability to stimulate translation and function at the level of translation initiation. We show that DAZL proteins interact with poly(A)-binding proteins (PABPs), which are critical for the initiation of translation. Mapping and tethered function experiments suggest that these interactions are physiologically important. This leads to an attractive hypothesis whereby DAZL proteins activate translationally silent mRNAs during germ cell development through the direct recruitment of PABPs.

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Introduction

The misregulation of gene expression during gametogenesis contributes to infertility, a problem affecting 12–15% of couples worldwide (de Kretser, 1997). Deletions on the distal long arm of the Y chromosome in infertile men suggested the presence of a genetic factor required for fertility in this region. This factor was defined as the 'AZoospermia Factor' (AZF) (Tiepolo and Zuffardi, 1976), and subsequent molecular mapping showed that three regions of the Y chromosome, AZFa, AZFb and AZFc, are important for male fertility (Vogt *et al*, 1996). Several candidate genes mapping to these regions were found to encode RNA-binding proteins. RNA-Binding Motif Y (RBMY) was the first to be identified (Ma *et al*, 1993) and is believed to play a role in splicing (Elliott, 2000). A second RNA-binding protein was identified by examining microdeletions in the AZFc region implicated in spermatogenic failure (Reijo *et al*, 1995). This gene was named Deleted in AZoospermia (*DAZ*), and, like RBMY, is present in multiple copies (Reijo *et al*, 1995; Yen *et al*, 1997).

DAZ has two autosomal homologs *DAZL* (*DAZ*-like) and *BOULE*. While *DAZ* is only found in Old World monkeys and great apes, the *DAZL* and *BOULE* genes are widely distributed from worms to humans (Haag, 2001). It has been proposed that *BOULE* is the founder member of the family and that *DAZL* arose from *BOULE* in an ancestor of vertebrates (Haag, 2001). The *DAZ* gene subsequently originated from a duplication transposition of the *DAZL* gene (Reijo *et al*, 1995; Yen *et al*, 1997). *DAZL* family members encode proteins that contain an RNA recognition motif (RRM) and a varying number of copies of a DAZ motif. DAZL and BOULE contain a single copy of the DAZ motif, while DAZ proteins can contain 8–24 copies of this repeat (Reijo *et al*, 1995; Yen *et al*, 1997), believed to mediate protein–protein interactions (Tsui *et al*, 2000a). DAZL and DAZ are highly homologous, with BOULE being more distantly related.

Studies in model organisms support a variety of roles for this family of proteins in male and female germ cells. In *Drosophila*, disruption of *boule* results in meiotic arrest during spermatogenesis (Eberhart *et al*, 1996), while, in *Caenorhabditis elegans*, loss of BOULE function leads to meiotic arrest in oogenesis (Karashima *et al*, 2000). The disruption of the *Dazl* gene in mice results in failure to produce male and female germ cells (Ruggiu *et al*, 1997), while, in *X. laevis*, *Xdazl* appears essential for primordial germ-cell production and migration (Houston and King, 2000). Phenotypic rescue experiments with different family members suggest that they may contribute to gametogenesis by related molecular mechanisms. For instance, *Xenopus Xdazl* or human *BOULE* (hBOULE) can both partially rescue the *Drosophila boule* phenotype (Houston *et al*, 1998; Xu *et al*, 2003). Similarly, human *DAZ* (hDAZ) and *DAZL* partially rescue a *Dazl* knockout mouse (Slee *et al*, 1999; Vogel *et al*, 2002). However, these proteins are not entirely functionally redundant. For example, *Dazl* knockout mice are sterile, despite the presence of Boule (Ruggiu *et al*, 1997).

Despite the potentially important role of these proteins in human fertility, their function remains undefined. Clues to their mode of action may lie in their intracellular localization. *Drosophila* Boule is localized to the nuclei of premeiotic cells and moves to the cytoplasm at the onset of meiosis, preceding the onset of the *boule* phenotype (Cheng *et al*, 1998). hDAZ and mouse Dazl (mDazl) are present in the nuclei and cytoplasm of fetal gonocytes, while in spermatogonial cells they are found in the nuclei and later relocate to the cytoplasm during meiosis (Reijo *et al*, 2000). In contrast, Xdazl appears to be in the perinuclear cytoplasm of stage IV–VI oocytes and all spermatogenic cells, except mature spermatozoa (Mita and Yamashita, 2000).

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The ability of DAZL proteins to bind RNA and their cytoplasmic location suggests a potential role in gene regulation within this cellular compartment. However, progress into determining the function of these proteins in gametogenesis has been hampered by lack of identification of cognate mRNA substrates. A number of potential mRNA substrates have been proposed, including *twine* (cdc25C) mRNA (Maines and Wasserman, 1999), which is required for the G2/M transition of meiosis. *Twine* was initially identified as a target of *Drosophila* Boule (Maines and Wasserman, 1999), and has subsequently been suggested as a target for zebrafish DAZL (zDAZL) (Maegawa *et al*, 2002) and mDazl (Venables *et al*, 2001). Other potential mRNA targets for mDazl include Tpx-1, a testicular cell adhesion protein, GRSF-1, an RNA-binding protein involved in translation activation, TRF2, a protein involved in transcriptional regulation (Jiao *et al*, 2002), and SDAD1, a homolog of an actin depolymerization protein (Fox *et al*, 2005). In spite of these potential candidates, no complete definition of the binding sites for DAZL proteins has been achieved. mDazl has been suggested to bind two loose consensus sequences (Venables *et al*, 2001; Jiao *et al*, 2002), while for zDAZL a 'GUUC' motif is necessary but may not be sufficient (Maegawa *et al*, 2002). A complete understanding of the binding sites will first require the *in vivo* validation of specific mRNAs as targets of the DAZL family of proteins.

Importantly, mDazl and zDAZL have recently been shown to be associated with polysomes, suggesting a role in mRNA stability or translation (Tsui *et al*, 2000b; Maegawa *et al*, 2002). Further support for this hypothesis is provided by genetic experiments in *Drosophila*. In these studies, Boule was shown to be required for the translation of *twine* mRNA (Maines and Wasserman, 1999) in a manner that was dependent on the untranslated regions (UTRs) of *twine*. However, Boule has not been shown to interact with *twine* mRNA; thus, its effects on *twine* translation may be indirect.

Evidence to date suggests a potential role for members of the DAZL family in mRNA translation. Many mRNAs undergo translational regulation, normally mediated by sequences within the UTRs. These sequences are often thought to serve as binding sites for *trans*-acting factors. Translational regulation has been shown to be critical to oogenesis, spermatogenesis and early development in a wide variety of species (Gray and Wickens, 1998). While this regulation is critical, relatively few *trans*-acting factors have been identified. Mechanistic studies of a few characterized factors have shown that they typically regulate translation initiation (Wilkie *et al*, 2003). This process involves four basic mRNA-dependent steps. Firstly, recruitment of the cap-binding complex to the 5′m^{7}GpppX cap. Secondly, binding of the small ribosomal subunit and associated factors to the mRNA. Thirdly, scanning of the small ribosomal subunit to the initiator codon, and, lastly, joining of the large ribosomal subunit. Intriguingly, the 3′ end of the mRNA also plays an important role in initiation. Interactions between poly(A)-binding proteins (PABPs) bound to the poly(A) tail and factors at the 5′ end bring the ends into close proximity, effectively circularizing the mRNA. This promotes both 40S and 60S joining, leading to the formation of 80S ribosomes (Gorgoni and Gray, 2004). Potentially any point in the initiation pathway can be subject to regulation by *trans*-acting factors.

To address the biological function of vertebrate DAZL family proteins, we have systematically examined the potential roles of these proteins in mRNA translation using *X. laevis* oocytes as a model system. We demonstrate that members of the DAZL family of proteins, including Xdazl, mDazl, human DAZL (hDAZL), hDAZ and hBOULE, have the ability to stimulate translation of reporter mRNAs to which they are tethered. Using a yeast-two-hybrid approach and coimmunoprecipitation of endogenous proteins, DAZL family proteins were shown to interact with PABPs. Importantly, mDazl stimulates 80S formation, consistent with a role of PABPs, and deletion of the PABP-interacting domain completely abrogates mDazl function. Furthermore, mDazl stimulates the translation of nonadenylated and adenylated mRNAs differentially, implicating a role of factors bound to the poly(A) tail. Our results suggest a mechanism in which DAZL proteins stimulate translation of target mRNAs by recruiting PABPs.

Results

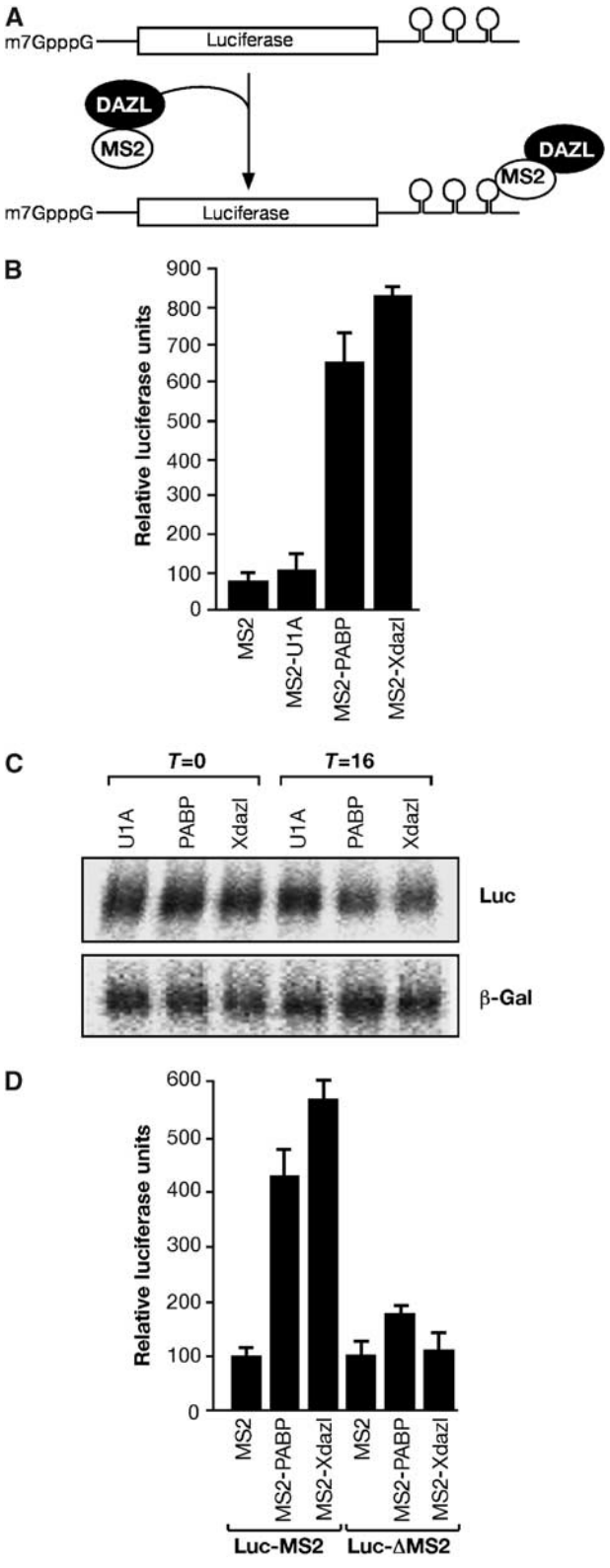
Xdazl protein can stimulate translation in X. laevis oocytes

The potential of DAZL proteins to regulate translation was tested directly by using the tethered function assay (Gray *et al*, 2000). This approach allows the ability of putative translational regulators to be assessed without prior knowledge of their *in vivo* targets, and is depicted in Figure 1A. Briefly, this assay has two components: a luciferase reporter containing RNA recognition sites for the bacteriophage MS2 coat protein within its 3′ UTR, and a fusion protein of MS2 coat protein with the protein of interest. The interaction of the MS2 coat protein with its target RNA-binding sites brings the protein of interest to the reporter mRNA.

Reporter mRNAs were injected into the cytoplasm of stage VI oocytes previously injected with mRNAs encoding MS2, MS2-U1A, MS2-PABP or MS2-Xdazl. Throughout this work, luciferase activity was normalized against β-galactosidase (β-Gal) activity by coinjection of a β-Gal reporter mRNA, which lacks MS2 sites. Tethered PABP1 (MS2-PABP) was used as a positive control. PABP1 is a known activator of translation in stage VI *Xenopus* oocytes and its effects on translation can be clearly seen (Gray *et al*, 2000; Figure 1B) when compared to the tether protein alone (MS2) or to a negative control U1A (MS2-U1A). Like PABP and DAZL proteins, U1A is an RRM-containing protein, but functions in mRNA splicing rather than translation. This shows that stimulation of luciferase expression is not a general property of tethered RRM-containing proteins (Gray *et al*, 2000).

Interestingly, when Xdazl protein (MS2-Xdazl) was tethered, it profoundly stimulated luciferase expression (Figure 1B). Typically, stimulation was eight-fold, seldom being below 5 or above 11. ^{35}S-methionine labeling of the oocytes showed that all the fusion proteins were expressed (Supplementary Figure 1). In order to show that Xdazl was not having an effect at the level of mRNA stability, Luc-MS2 and β-Gal mRNAs were injected in the presence of MS2-U1A, MS2-PABP or MS2-Xdazl, and their levels were monitored over time by Northern blot. Luc-MS2 was not stabilized by the presence of MS2-Xdazl over the course of the experiment (Figure 1C). These results strongly suggest that the increase in luciferase activity is due to differences in the translation efficiency and not due to the stability of Luc-MS2 mRNA.

To determine whether translational stimulation by tethered DAZL proteins occurred only in *cis*, we assayed the effects of MS2-Xdazl on Luc-ΔMS2 mRNA. This mRNA differs from Luc-MS2 mRNA only in that it lacks MS2-binding sites in its 3′ UTR. Translation of Luc-ΔMS2 was not stimulated by MS2-Xdazl (Figure 1D). Taken together, these results show that Xdazl can stimulate the translation of mRNAs to which it is bound.



Mammalian DAZL family members can stimulate translation

Phenotypes observed in different species with loss of DAZL proteins suggest that the DAZL proteins may have distinct but related functions (Reijo *et al*, 1995; Eberhart *et al*, 1996; Ruggiu *et al*, 1997). To address this hypothesis, two mammalian DAZL proteins, mDazl and human DAZL, were tethered and assayed for their ability to stimulate translation. These proteins are 57 and 55% homologous to the *Xenopus* protein, respectively, and cluster closely compared to other vertebrates (Figure 2A). As can be seen in Figure 2B, mDazl (MS2-mDazl) and to a lesser degree hDAZL (MS2-hDAZL) both stimulate translation, suggesting that translational control may be an important function of the DAZL proteins.

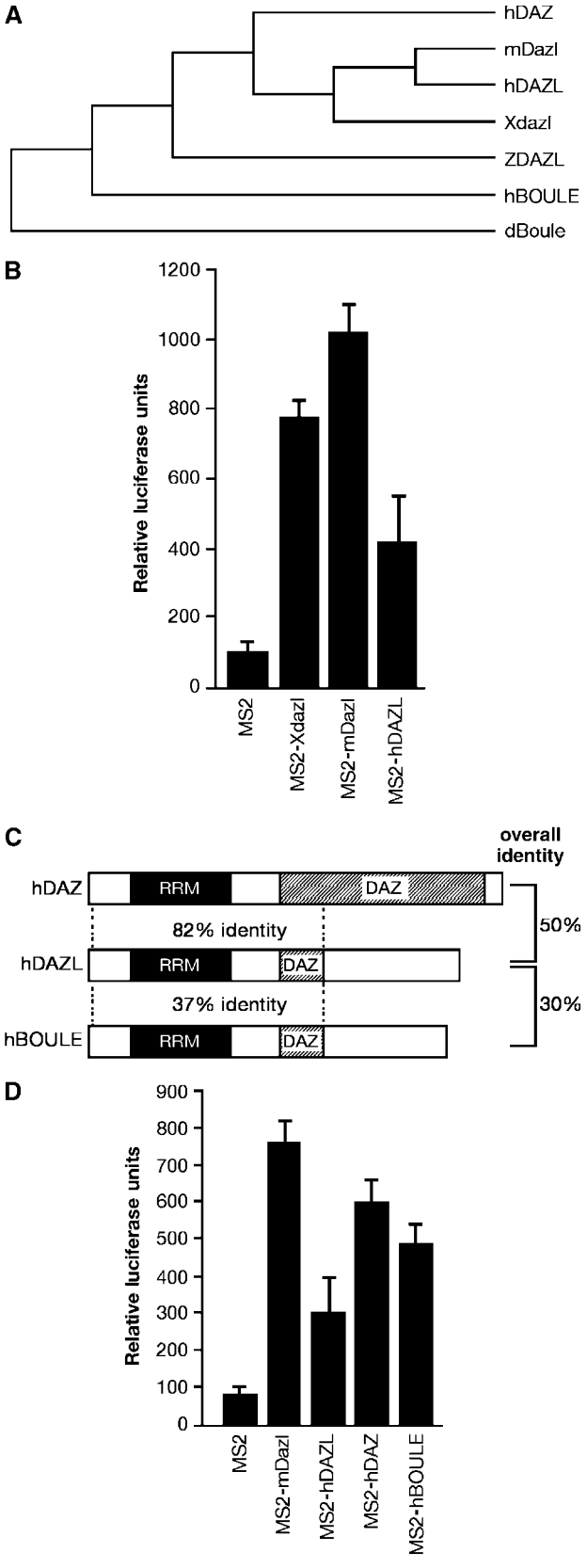
To address whether other members of this family have the ability to stimulate translation, MS2 fusion proteins of hDAZ and hBOULE were created. hDAZL is only 50% conserved with hDAZ (Figure 2C) compared to the 88% conservation observed between mDAZL and hDAZL. BOULE is the most divergent, only being 30% identical to hDAZL. Most of the conservation between these three proteins lies in the N-terminal two-thirds being 82 and 37% identical between hDAZL and DAZ or BOULE, respectively (Figure 2C). Indeed, the only significant conservation between BOULE and DAZL lies in the RRM. Despite the relatively low sequence conservation, Figure 2D clearly shows that the ability to stimulate translation has been conserved across the family. This suggests that the DAZL family may represent a family of proteins that fulfill their role in gametogenesis at least in part by controlling translation.

DAZL RNA targets may contain multiple DAZL-binding motifs

It has been proposed that mDazl protein may bind to multiple U-rich motifs (Venables *et al*, 2001), suggesting that the physiological targets of these proteins may contain multiple binding sites. Many mRNAs that undergo 3′-end-mediated regulation have multiple copies of control elements within their 3′ UTRs (Macdonald, 2001). The presence of multiple DAZL-binding sites, and thus multiple molecules of DAZL, could potentially result in further enhancement of translation of target mRNAs. Such a phenomenon has been suggested for mRNAs that undergo cytoplasmic polyadenylation. The increase in poly(A) tail length may allow recruitment of additional molecules of PABP, which leads to enhanced translation (Gray and Wickens, 1998). To directly test this hypothesis, luciferase mRNAs were engineered to contain 1, 3 or 9 MS2-binding sites, respectively (Figure 3A). Figure 3B

Figure 1 Tethered Xdazl can stimulate translation. (**A**) The tethered function assay has two components: a luciferase reporter mRNA with binding sites for the MS2 coat protein within its 3′ UTR (Luc-MS2), which is unadenylated unless stated, and a fusion protein of MS2-coat protein and Xdazl. Binding of the coat protein tethers Xdazl to the mRNA. The effects of fusion proteins on translation are measured by luciferase assay, normalized to a β-Gal. (**B**) Oocytes expressing MS2, MS2-U1A, MS2-PABP or MS2-Xdazl were coinjected with Luc-MS2 and β-Gal mRNAs. (**C**) The stability of luciferase (upper panel) and β-Gal mRNAs (lower panel) is shown by Northern blotting. RNA was extracted at 0 h ($T=0$) and 16 h ($T=16$) after incubation in oocytes expressing fusion proteins. (**D**) Oocytes expressing MS2-PABP or MS2-Xdazl were coinjected with β-Gal mRNA and Luc-MS2 or Luc-ΔMS2 mRNAs.

clearly shows that, while one MS2-binding site is sufficient to stimulate translation, the presence of three or nine binding sites further enhances translation. This is in contrast to tethered proteins such as histone stem-loop-binding protein, whose natural target mRNA only contains one binding site (Gorgoni *et al*, 2005). This suggests that cellular targets containing multiple DAZL-binding sites may be most responsive to the presence of DAZL protein.



The mechanism of DAZL stimulation

The observation that DAZL proteins can stimulate translation raises questions regarding the molecular mechanism. To date, detailed knowledge of molecular mechanisms used by translational regulators is limited (Wilkie *et al*, 2003).

To understand how DAZL proteins enhance translation, the step of translation at which DAZL acts was examined. The ability of DAZL to stimulate initiation was determined by performing tethered function assays using ^{32}P-labeled luciferase reporter mRNA in the presence of cycloheximide, which allows 80S assembly, but inhibits subsequent elongation. Linear sucrose gradients were used to resolve initiation complexes formed in oocytes expressing MS2-U1A or MS2-mDazl, and the amount of radiolabeled mRNA in each fraction was measured by scintillation counting. Our data show that the presence of mDazl protein enhances the recruitment of 80S ribosomes compared to the negative control U1A (Figure 4A): 55% of luciferase mRNA was found in the 80S ribosomal fraction in the presence of mDAZL compared to only 9% in the case of U1A (Figure 4A). Importantly, this shows that DAZL proteins stimulate translation by enhancing the rate of initiation.

Xenopus dazl interacts with a canonical translation initiation factor

One attractive model to explain the translational activation mediated by the DAZL family of proteins is that they may interact with a basal component of the translational machinery. Since our data show that mDazl enhances the recruitment of 80S ribosomes, a yeast-based approach to detect possible interactions with initiation factors was undertaken. Directed yeast-two-hybrid analysis with Xdazl and a panel of initiation factors, including domains and subunits of eIF1, eIF1A, eIF2, eIF3, eIF4A, eIF4B, eIF4E, eIF4G, eIF4H, eIF5, eIF5A, eIF5B and PABP1, was performed (data not shown). A single specific interaction was detected between the C-terminus of PABP1 (PABP-Ct) and Xdazl (Figure 4B). Several proteins with a role in translation interact with the C-terminus and RRMs 1 and 2 of PABP1 (Gorgoni and Gray, 2004). However, Xdazl does not interact with other regions of PABP1 (data not shown). Importantly, this interaction is not mediated by an RNA intermediate since the PABP-Ct cannot bind RNA (Kuhn and Pieler, 1996). Moreover, an RNA-binding mutant of Dazl that contains amino-acid substitutions important for RNA binding retains the ability to bind PABP-Ct (data not shown), confirming the RNA independence.

Figure 2 Mammalian DAZL, DAZ and BOULE proteins can also stimulate translation. (**A**) A phylogenetic comparison of some vertebrate members of the DAZL family of proteins. The tree was created using Mega 2 and is rooted using *Drosophila* Boule. (**B**) Oocytes expressing MS2, MS2-Xdazl, MS2-mDazl or MS2-hDAZL or (**D**) MS2, MS2-mDazl, MS2-hDAZL, MS2-hDAZ or MS2-hBOULE were coinjected with Luc-MS2 and β-Gal mRNAs. (**C**) The overall identity between hDAZL and DAZ or BOULE. The majority of the conservation lies in the first 190 amino acids, including the RRM and DAZ motif.

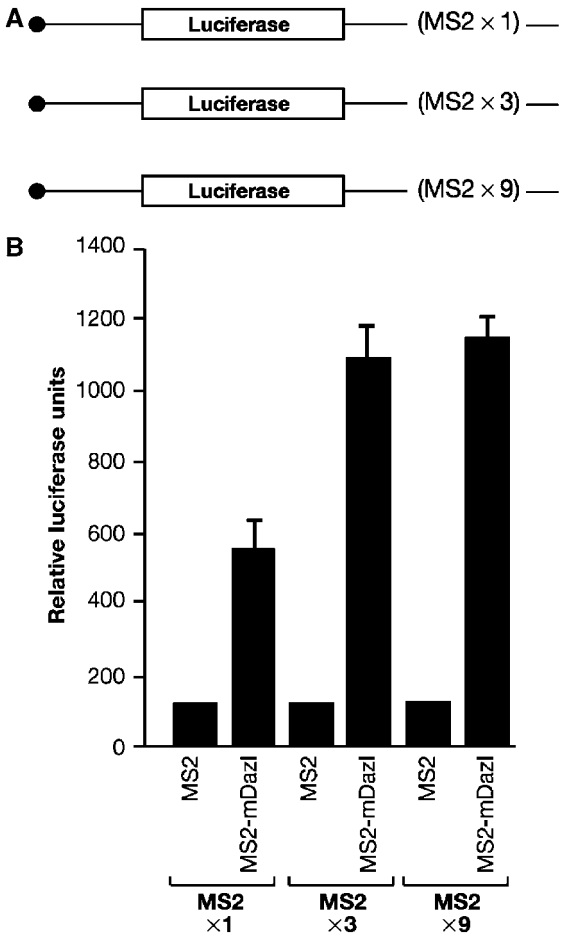


Figure 3 Recruitment of multiple Dazl molecules further enhances translation in *cis*. (**A**) Cartoon of luciferase reporter mRNAs, which vary only in the number of MS2 sites within the 3′ UTR. (**B**) Oocytes expressing MS2, or MS2-mDazl were coinjected with β-Gal mRNA and Luc-$MS2_1$, Luc-$MS2_3$ or Luc-$MS2_9$. Normalized luciferase activity is plotted with MS2 set to an arbitrary level to allow direct comparison.

Endogenous Xdazl and PABP interact

To test the physiological relevance of the Xdazl–PABP1 interaction, coimmunoprecipitation experiments were performed in *X. laevis* oocytes. Oocytes were coinjected with mRNAs encoding MS2-Xdazl and *X. laevis* PABP1, as the endogenous proteins in *X. laevis* oocytes are present at levels difficult to detect by Western blotting after immunoprecipitation (IP). A PABP1 antibody was used to pull down interacting proteins and Western blotting with an Xdazl antibody clearly shows that MS2-Xdazl was efficiently co-precipitated by PABP1 (Figure 4C, upper panel). Importantly, this is dependent on the PABP1–Xdazl interaction as MS2-Xdazl is not bound by beads alone (lane 4). Since both PABP1 and Xdazl are RNA-binding proteins, it was important to determine that they are not separately bound to the same RNA molecule. Treatment of the extracts with RNase shows that the PABP1–Xdazl interaction is not mediated by RNA (lane 3). ^{35}S labeling of oocytes prior to IP revealed that PABP1 did not co-precipitate a large number of proteins under these conditions (middle panel). In order to determine whether endogenous PABP1 and DAZL proteins interact, whole *X. laevis* testes, which express higher levels of Xdazl and PABP1, were used as a source to immunoprecipitate PABP1. Figure 4C (lower panel) clearly shows that IP with PABP1 efficiently coimmunoprecipitates Xdazl, in an RNA-independent manner, proving that these proteins interact at physiological levels.

The interaction with PABP1 is conserved

The observation that Xdazl and PABP1 can interact in *X. laevis* oocytes and testes raises the possibility that Xdazl may stimulate translation initiation by recruiting PABP1 to target mRNAs in a manner analogous to a poly(A) tail. If this interaction is physiologically important, it should be conserved for all the DAZL family proteins that can stimulate translation. To test this, DNA-binding fusions of the DAZL proteins we have shown to stimulate translation were constructed and tested in the yeast-two-hybrid system. Figure 4D shows that Xdazl, mDazl, hDAZL, hDAZ and hBOULE all interact with PABP-Ct. This further supports the biological relevance of this interaction.

A second PABP protein can interact with Xdazl

Vertebrates contain multiple PABP proteins, most of which are expressed in a tissue-specific manner. Embryonic PABP (ePABP) was recently shown to be expressed in *Xenopus* oocytes, early embryos and testis, and has a similar expression pattern in mammals (Seli *et al*, 2005; Wilkie *et al*, 2005). ePABP is present at levels higher than PABP1 in stage VI oocytes (Voeltz *et al*, 2001; Cosson *et al*, 2002; Wilkie *et al*, 2005), and, importantly, has recently been shown to be capable of stimulating translation (Wilkie *et al*, 2005) despite its divergence to PABP1. This raises the possibility that DAZL proteins may stimulate translation through ePABP as well as PABP1. Yeast-two-hybrid analysis with the C-terminal region of ePABP (Figure 4E) showed that Xdazl interacts with ePABP despite the relatively low homology between PABP1 and ePABP in this region. Due to the relative abundance of ePABP in stage VI oocytes, it was possible to test the interaction of endogenous ePABP and Xdazl in stage VI oocytes. Figure 4F shows that, like PABP1, endogenous ePABP interacts with Xdazl in an RNA-independent manner.

The PABP interacting site is required for mDazl function

To further characterize the functional relevance of the DAZL–PABP interaction, the regions within the mDAZL protein that are responsible for this interaction were mapped by yeast-two-hybrid analysis using a series of deletion constructs (Figure 5A). mDazl is a protein of 298 amino acids that contains two conserved motifs, an RRM located between amino acids 32 and 115 and a DAZ motif between amino acids 167 and 190 (Figure 5A). Initially, a C-terminal mDazl deletion (DNT) (amino acids 1–119) and an N-terminal mDazl deletion (DCT) (amino acids 120–298) were tested. Neither interacted with PABP, suggesting that the binding site may overlap the ends of the deletions. To further define the site, either the DAZ motif (DDD) or the RRM (DDR) was deleted. Analysis revealed that deletion of the RRM, but not the DAZ motif, completely abrogated the interaction with PABP. This suggests that PABP binding requires a region between amino acids 1 and 166. To further define the minimal PABP-binding region, a series of deletion constructs encompassing this region were utilized (Figure 5A). These showed that a region between amino acids 99 and 166 was sufficient to bind PABP, while deletion from the C-terminal end to amino acid 151 abrogated binding.

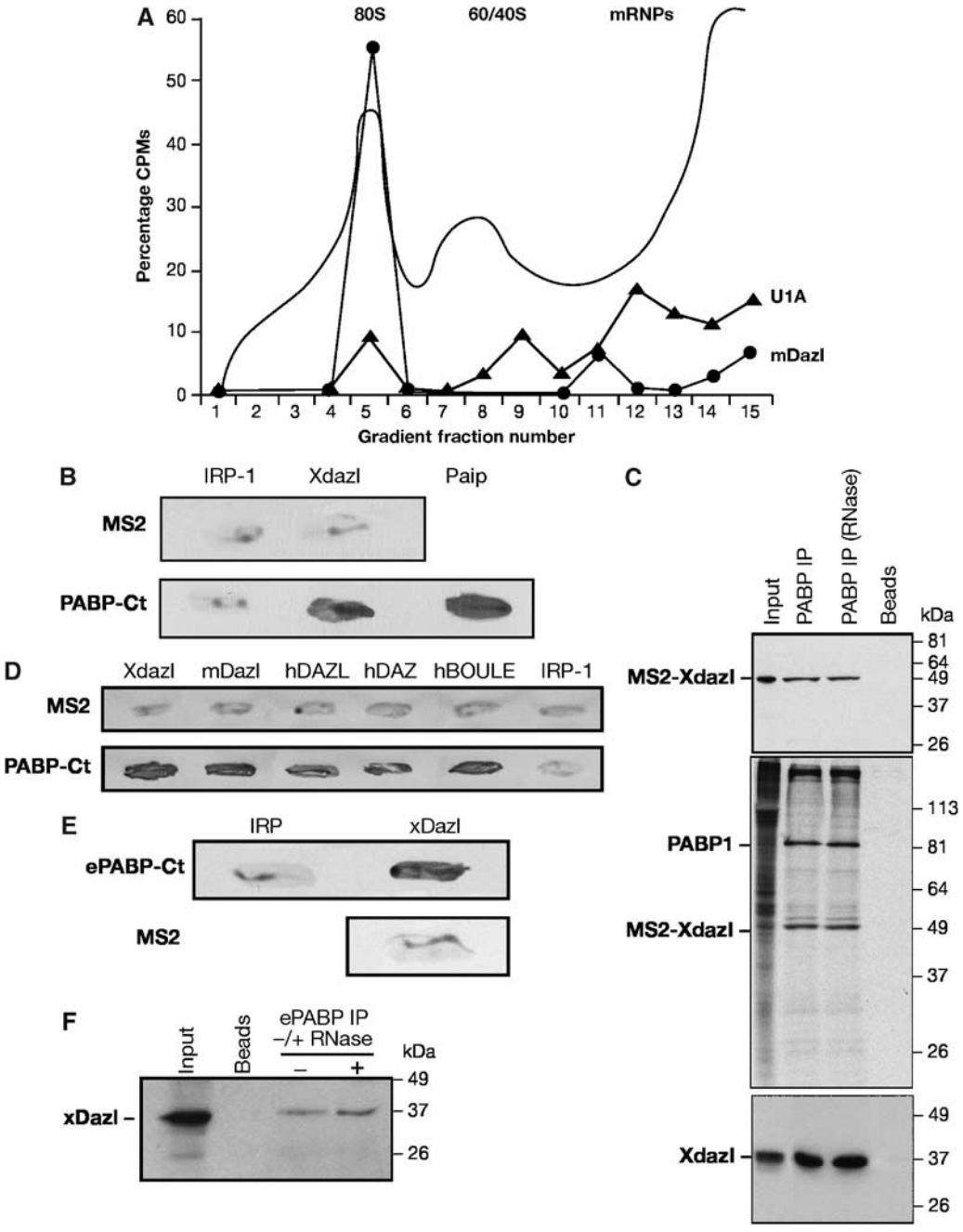


Figure 4 DAZL proteins enhance translation initiation and interact with a canonical translation initiation factor. (**A**) Sucrose gradient analysis of initiation intermediates showing the effects of mDazl (●) and U1A (▲) on the distribution of luciferase reporter mRNAs in the presence of cycloheximide. The positions of 80S ribosomes, ribosomal subunits and mRNPs are indicated. The A254 profile is represented by a curved black line. (**B**) Yeast-two-hybrid analysis with Xdazl and the C-terminus of *Xenopus* PABP1 (PABP-Ct) using qualitative β-Gal filter assays. IRP-1, an RNA-binding protein that mediates translational control, and MS2 are used as specificity controls. The interaction between *Xenopus* PABP-Ct and Paip-1 (Gray *et al*, 2000) acts as a positive control. (**C**) Oocytes injected with MS2-Xdazl and *Xenopus* PABP1 were immunoprecipitated with an anti-PABP1 antibody co-precipitating MS2-Xdazl. MS2-Xdazl was detected by Western (upper panel) blotting or by ^{35}S-methionine labeling of total oocyte proteins (middle panel). Endogenous Xdazl was detected by Western blot after IP with anti-PABP1 antibody from *X. laevis* testes (lower panel). Input: 1 μl of testis extract (**D**). Yeast two-hybrid analysis with PABP-Ct and DAZL family members using qualitative β-Gal filter assays. (**E**) Yeast two-hybrid analysis with ePABP C-terminus and Xdazl using qualitative β-Gal filter assays. (**F**) Endogenous Xdazl was detected by Western blot after IP with anti-ePABP antibody from *X. laevis* oocytes. Input: 5 μl of stage VI oocyte extract.

If the ability to interact with PABP is required to stimulate translation, then loss of the PABP interaction domain on mDazl should abrogate the translational enhancement. To address this, several MS2-mDazl deletions were constructed, injected into oocytes and were expressed at comparable levels (data not shown). Deletion of the minimal PABP-binding site (Δ99–166) completely abrogates the function of mDazl, implicating the PABP-binding region as an important domain. Deletion of the DAZ motif (Δ167–190), which is not absolutely required for PABP binding, significantly reduces but does not prevent the ability of mDazl to stimulate translation, suggesting a more minor contribution of this

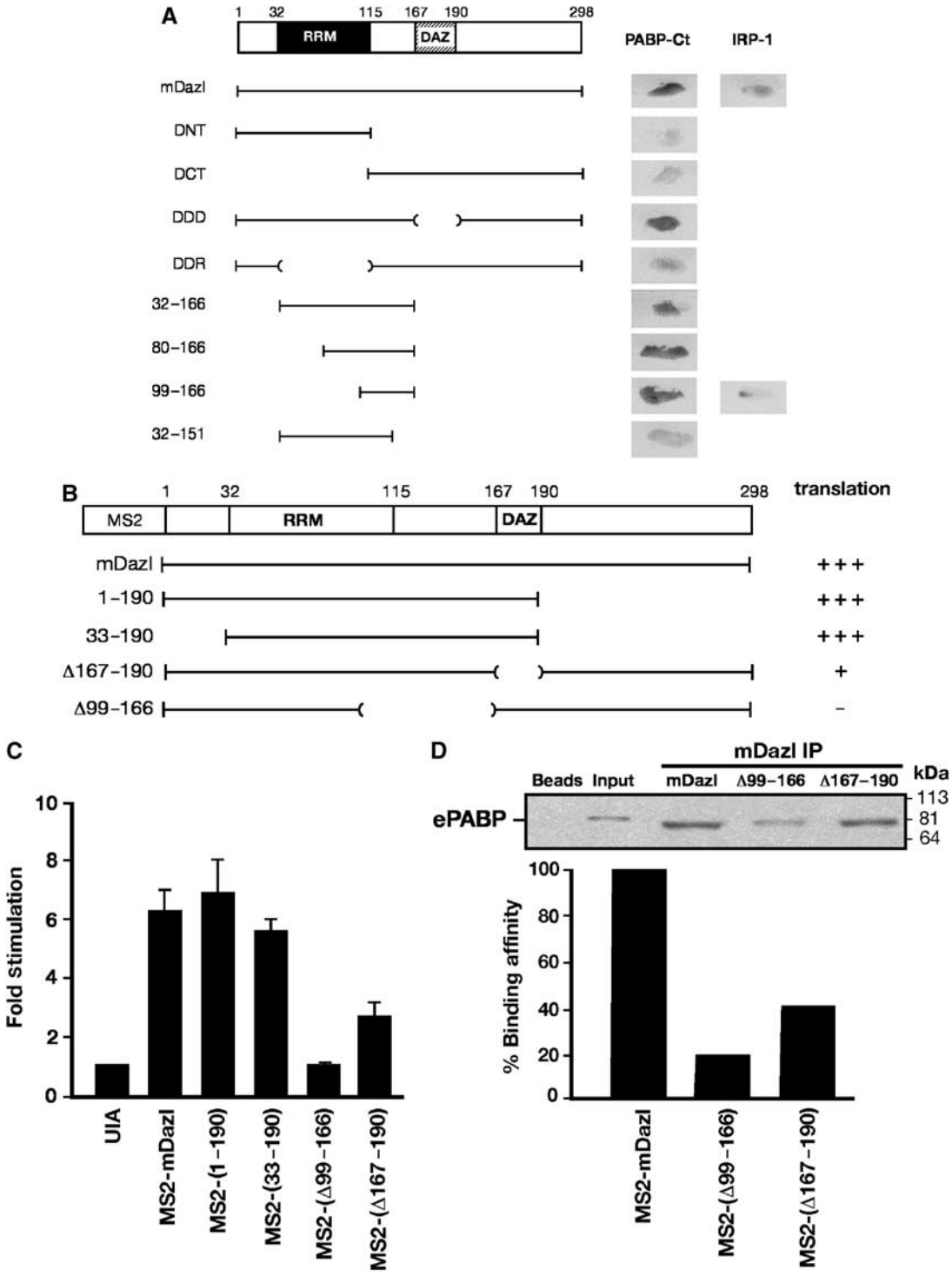


Figure 5 Mapping of the minimal PABP–mDazl interaction domain and analysis of the minimal activation domain required for mDazl stimulation. (**A**) On the left, mDazl deletion constructs are represented. Black lines indicate sequences that are present in construct. Brackets denote internal deletions. Qualitative β-Gal filter assays generated by yeast-two-hybrid analysis are shown on the right. Where shown, IRP-1 is used as a specificity control. (**B**) Representation of mDazl deletion constructs. Black lines indicate sequences that are present in constructs. Brackets denote internal deletions. The data shown in panel C are summarized on the right. (**C**) Oocytes expressing MS2, MS2-mDazl or the deletion constructs of mDazl shown in (B) were coinjected with Luc-MS2 and β-Gal mRNAs, and relative stimulation is shown. (**D**) Upper panel, coimmunoprecipitation of ePABP from oocytes expressing MS2-mDazl, or the deletion constructs of mDazl shown in (C). Lower panel, quantitation of ePABP binding to MS2-mDazl deletions is shown.

region (Figure 5B and C). IP of expressed MS2-mDazl and Western blotting for ePABP suggest that this loss of function is due to a reduction in PABP binding (Figure 5D), although this region is not within the minimal binding site, as defined by yeast-two-hybrid analysis.

In contrast, deletion of other regions of mDazl does not significantly affect mDazl function, including the C-terminal two-thirds or the N-terminal region of mDazl (Figure 5B and C). This defines the minimal region of mDazl required for translational activation as between amino acids 33 and 190.

Interestingly, homology searches reveal that this region is the most highly conserved between all DAZL, DAZ and BOULE proteins. Thus, the PABP-binding region is completely encompassed within the region required for translational activation. However, the full-length translation activation domain, as defined by tethered function assay, is larger than the minimal PABP-binding site.

Translational stimulation by DAZL proteins is poly(A) tail sensitive

Polyadenylation plays a vital role in translational control (Macdonald, 2001). During gametogenesis, many stored mRNAs possess relatively short poly(A) tails. Cytoplasmic polyadenylation occurs at discrete developmental time points during oogenesis and spermatogenesis (Wickens *et al*, 2000; Kashiwabara *et al*, 2002) and is required for the activation of specific mRNAs, presumably in part by recruiting additional PABP molecules (Figure 6A). Other mRNAs undergo polyadenylation as a secondary consequence of translational activation (Gray and Wickens, 1998).

Our data lead us to propose a model in which DAZL proteins function by directly recruiting PABP in a manner analogous to polyadenylation (Figure 6B). One prediction of this model is that DAZL proteins should stimulate the translation of mRNAs with short poly(A) tails more than those with long poly(A) tails, as long poly(A) tails are sufficient to bind many molecules of PABP. To test this directly, the effect of DAZL on the translation of nonadenylated and polyadenylated mRNAs was examined. Two Luc-MS2 reporter mRNAs were used that were identical, except that one possessed a substantial poly(A) tail (Luc-MS2-pA) (Figure 7A). Figure 7B clearly shows that the translation of both mRNAs is stimulated by the presence of MS2-mDazl and that the mRNA possessing a poly(A) tail and bound by mDazl is translated most efficiently. More importantly, the relative stimulation of the nonadenylated mRNA by MS2-mDazl was greater than its adenylated counterpart (Figure 7C: 8.5-fold compared to 2.8-fold). Thus, the presence of tethered Dazl can function analogously to a poly(A) tail in stimulating translation, providing strong support for the hypothesis that Dazl mediates its effects through PABP. Moreover, it raises the interesting possibility that DAZL proteins may activate the translation of mRNAs with relatively short poly(A) tails. This would allow their activation in the absence of cytoplasmic polyadenylation.

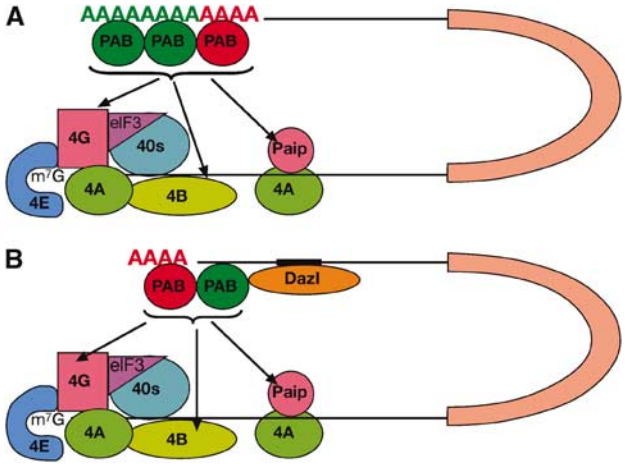


Figure 6 Model for the role of PABPs in DAZL-mediated stimulation of target mRNAs. During oogenesis and spermatogenesis, many mRNAs are stored with short poly(A) tails. (**A**) Many of these mRNAs are activated at specific times in response to cytoplasmic polyadenylation (shown as green A's). This is proposed to recruit additional PABP molecules (shown in green), which facilitates additional interactions with factors bound to the 5′ end (depicted as arrows), increasing end-to-end complex formation. (**B**) In our working model, DAZL family members directly recruit additional molecules of PABP (shown in green), 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.

Discussion

The essential role of DAZL proteins in gametogenesis has been demonstrated in a number of genetic and disruption studies (Reijo *et al*, 1995; Eberhart *et al*, 1996; Ruggiu *et al*, 1997), and at least one member of this family has been implicated in human fertility (Reijo *et al*, 1995). Thus, understanding their function at a molecular level is a priority. In this study, we have shown that members of this family of proteins may fulfill their role in gametogenesis, at least in part, by acting as translational regulators. We demonstrate that Xdazl, mDazl, hDAZL, hDAZ and hBOULE stimulate the translation of reporter mRNAs to which they are bound, and do so by enhancing translation initiation. DAZL may promote 80S ribosome assembly through its RNA-independent inter-

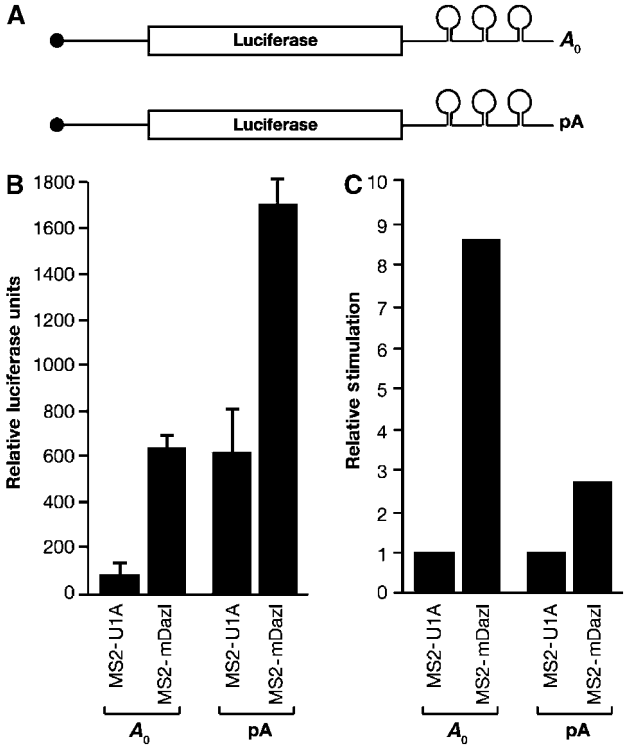


Figure 7 DAZL proteins appear to functionally substitute for poly(A) tails. (**A**) Cartoon of reporter mRNAs used. The reporters vary only with respect to their adenylation status. Oocytes expressing MS2-U1A or MS2-mDazl were coinjected with Luc-MS2 or Luc-MS2-pA alongside β-Gal mRNA. (**B**) Normalized luciferase activity is plotted. (**C**) The relative fold stimulation of MS2-mDazl compared to MS2-U1A is shown for Luc-MS2 or Luc-MS2-pA. MS2-U1A has been set to 1.

action with PABP, a known translation initiation factor: Activation by DAZL is sensitive to the presence of a poly(A) tail and the minimal mDazl–PABP-binding region was shown to be essential for translational enhancement. Our results suggest that the DAZL family proteins act as translational enhancers of specific mRNAs during gametogenesis and we provide a model for their function.

DAZL family members are translational activators

Previous studies proposed that vertebrate DAZL proteins may play a role in translation or mRNA stability due to their predominately cytoplasmic localization and association with polysomes (Tsui *et al*, 2000b; Jiao *et al*, 2002; Maegawa *et al*, 2002). Here we show that vertebrate and mammalian family members can stimulate translation *in vivo*, using *X. laevis* oocytes as a vertebrate germ-cell model (Figure 1). Our results are in agreement with a study which suggested that *Drosophila* Boule was required directly or indirectly for the expression of *twine* mRNA (Maines and Wasserman, 1999) and a recent study that revealed minor effects on translation *in vitro* (Maegawa *et al*, 2002). Given the relatively large effects observed in oocytes with tethered proteins, it raises the possibility that the smaller effects observed in the *in vitro* studies (average 1.5-fold stimulation) may have been due to low RNA-binding affinity to the target mRNAs used (Maegawa *et al*, 2002). Indeed, recent findings suggest that PUM-2, a homolog of the *Drosophila* protein Pumilio, may contribute to RNA recognition by DAZL proteins in some cases (Fox *et al*, 2005). Our results do not preclude DAZL family members having other functions.

DAZL proteins enhance translation initiation and interact with PABP

In only a few instances is the mechanism of translational regulation understood at a molecular level. However, it is clear that multiple steps can be targeted (Wilkie *et al*, 2003).

To understand the mechanism by which DAZL proteins stimulate translation, we determined the step in translation at which these proteins function. 80S ribosome assembly was found to be enhanced six-fold when mDazl was present compared to U1A (Figure 4A). This lies within the range of translation stimulation observed for DAZL proteins, providing strong evidence that initiation is the key step regulated by DAZL proteins. In order to ascertain how DAZL enhances initiation, we sought translation initiation factors that may be targeted by DAZL. This identified two PABPs as partners of DAZL (Figure 4). The physiological relevance of these interactions was substantiated by the interaction of endogenous proteins in extracts of *X. laevis* testes and stage VI oocytes.

The interaction of PABPs with DAZL proteins from three species suggests an evolutionary conservation of this interaction, indicating a fundamental role. The location of the PABP-binding site within the translational activation region, and additional deletions within this region of tethered mDazl provide further support for an important role of PABP. Deletion of the PABP interaction domain (99–166) resulted in loss of translational stimulation and a significant loss in binding to endogenous ePABP, while deletion of the DAZ motif decreased, but did not completely abrogate, both the translational stimulation and binding to ePABP. This suggests that the DAZ motif is not the dominant factor in Dazl's ability to stimulate translation, and is contrary to previous studies *in vitro* (Maegawa *et al*, 2002); however, it may enhance or stabilize the Dazl–PABP interaction. Further support for the idea that PABP plays an important role in stimulation by DAZL comes from the observation that tethering PABP to 3′ UTRs is sufficient to promote translational recruitment and that DAZL, like PABP, functions during initiation (Gray *et al*, 2000; Figure 4). Additional strong support comes from experiments in which nonadenylated and adenylated reporter mRNAs were utilized. These experiments show that mDazl stimulates translation of nonadenylated mRNAs to a higher extent. This indicates that the function of DAZL is sensitive to the presence of a poly(A) tail, suggesting that poly(A) interacting factors are required for its function. We interpret this to mean that mRNAs which already have several molecules of PABP bound to their poly(A) tail are less sensitive to the addition of more PABP molecules by mDazl.

Our results suggest that PABP may play an important role in DAZL-mediated stimulation. Future work will be required to determine the relative contribution of PABP to DAZL-mediated translation and whether other factors are also important. Our results do not address the role of proteins that may function by aiding the interaction of DAZL with target mRNAs.

A model for DAZL-mediated stimulation

The majority of *trans*-acting factors that regulate translation bind to 3′ UTR elements. While the target mRNAs of different DAZL proteins require validation, in most cases sequences within the 3′ UTRs have been implicated (Maines and Wasserman, 1999; Jiao *et al*, 2002; Maegawa *et al*, 2002). Thus, we envisage that DAZL proteins normally bind the 3′ UTR of target mRNAs.

During oogenesis and spermatogenesis, specific subsets of mRNAs undergo cytoplasmic polyadenylation at predetermined times. This is a complex and precise process dependent on the presence of specific mRNA sequences and the activity of different *trans*-acting factors at these times (Richter, 1996). However, not all translationally controlled mRNAs in these cells have the sequences required or are activated at times when the factors required for adenylation are active. Thus, other mechanisms for translational activation are required.

Dazl may provide such an alternate mechanism by directly recruiting PABP to these mRNAs, without the need for polyadenylation (Figure 6). The recruitment of additional PABP molecules by DAZL increases end-to-end complex formation, enhancing 40S and 60S recruitment in a manner analogous to increases in poly(A) tail length (Wickens *et al*, 2000; Wilkie *et al*, 2003). For PABPs to fulfill this function, they must be able to interact with Dazl and components of the end-to-end complex simultaneously. The DAZL proteins interact with the C-terminal region of PABP1 and ePABP. In *Xenopus*, this domain is dispensable for interactions with important factors located at the 5′ end such as eIF4G (Gray *et al*, 2000), suggesting that DAZL–PABP–eIF4G complexes can be formed. One prediction of our model is that mRNAs with multiple DAZL-binding sites and short poly(A) tails may benefit the most from the binding of PABPs to DAZL, as seen in Figures 3 and 7. These mRNAs may later undergo polyadenylation as a secondary consequence of activation (Gray and Wickens, 1998).

Our model does not include other known interactors of DAZL proteins, as their function in translational activation remains unclear (Moore *et al*, 2003; Moore *et al*, 2004; Fox *et al*, 2005). However, recent reports suggest that PUM-2 may aid the binding of DAZL proteins to some target mRNAs (Fox *et al*, 2005), in a manner analogous to its role in Nanos binding in *Drosophila* (Wickens *et al*, 2002). However, the binding regions for PUM-2 and PABP appear to overlap (Figure 5; Moore *et al*, 2003); thus, PUM-2 may be involved in repressing mRNAs by promoting deadenylation, consistent with the roles of pumilio proteins in other species (Wickens *et al*, 2002). PUM-2 may be displaced by PABP to allow the translation of stored target mRNAs at appropriate times. Further experiments will be required to distinguish these possibilities.

Our model highlights a novel mechanism for activating the translation of mRNAs via direct recruitment of PABPs by a *trans*-acting factor. This suggests a novel role for PABP proteins in promoting the translation of mRNAs in a poly(A)-independent manner. While our results suggest that DAZL proteins share this property, it also raises the possibility that other stimulatory *trans*-acting factors may also function by recruiting PABP. For instance, the recent discovery of maskin as a protein which represses some CPE-containing mRNAs in *X. laevis* oocytes has prompted the discovery of proteins that act in a similar manner and that regulate the translation of a variety of mRNAs in different species (e.g. Wilhelm *et al*, 2003; Nakamura *et al*, 2004; Nelson *et al*, 2004). Future experiments will be required to determine if direct recruitment of PABP by RNA-binding proteins to target mRNAs is a common strategy for activating translation and whether specific *trans*-acting factors preferentially or exclusively recruit individual PABP proteins.

Regulation by DAZL family members in gametogenesis

Our results suggest that DAZL family proteins may be responsible for the enhancement of translation of specific subsets of mRNAs during germ-cell development. Since all these proteins share this property (Figure 2), they may be functionally redundant. However, evidence to date (Houston *et al*, 1998; Reijo *et al*, 2000; Xu *et al*, 2003) suggests that they most likely regulate different mRNAs, but retain the ability to target some of the same mRNAs. Given the potentially important roles of these proteins in human fertility, it is a priority not only to identify the targets that they regulate, but also to further understand their mechanism of action.

Materials and methods

Plasmids

Details of the plasmids used are available in Supplementary data.

Tethered function assays

Tethered function assays, isotopic labeling and analysis were performed as described (Gray *et al*, 2000). Error bars represent standard error in all cases and represent a minimum of three experiments.

Northern blot analysis

RNA was extracted from oocytes (Gray *et al*, 2000) and Northern blot analysis performed as described (Collier *et al*, 2002) using a random primed DNA probe generated from *Hpa*I and *Eco*RV and *Bss*HII and *Eco*RV fragments from plasmids pJK350 and pLG-MS2, respectively.

Sucrose gradient analysis of initiation complexes

X. laevis oocytes were injected with mRNA encoding fusion proteins and incubated for 6–8 h as described (Gray *et al*, 2000). Prior to injection of reporter mRNAs, 20 μg/ml cycloheximide was added to the buffer for 15 min. Post injection, oocytes were incubated in the presence of cycloheximide for 15 min to allow initiation complexes to form. Initiation complexes were resolved and analyzed as described (Gillian-Daniel *et al*, 1998).

Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed with strains L40ura$^-$ (Zhang *et al*, 1999) and Mav99 (Vidal and Legrain, 1999) as described (Zhang *et al*, 1999).

Coimmunoprecipitations

Xenopus testes and *Xenopus* oocytes (15–20) were lysed in IP lysis buffer (10 μl/oocyte) (20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 100 mM NaCl, 0.5% Tx100, 0.5 M DTT, protease inhibitors). Extracts were cleared by centrifugation and 50 μl of lysate was added to 450 μl of lysis buffer, mixed for 1 h at 4°C with an anti-PABP antibody (SJ Morley), anti-ePABP or anti-mDazl at 1:1000. In all, 30 μl of Protein-G sepharose (Amersham) was added to the lysate and mixed for 1 h at 4°C. Beads were washed three times with IP lysis buffer and bound material eluted in 20 μl of SDS gel loading dye prior to SDS–PAGE analysis.

Western blot analysis

Western blot analysis using an Xdazl antibody (1:5000) was performed as described (Mita and Yamashita, 2000). Western blots with an ePABP antibody (1:1500) were performed as described (Wilkie *et al*, 2005) and quantified by densitometry.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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