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Cloning and expression of *Dazl* in *Rana pipiens*

A Thesis

Presented to the Bayer School of Natural and Environmental Sciences
Department of Biological Sciences
Duquesne University

In partial fulfillment of the requirements
for the degree of Master of Science

by

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Degree: Master of Science

Date: July 11, 2003

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Abstract

The *DAZ* gene on the human Y chromosome has been implicated in male fertility. Humans and other animals possess an autosomal version of this gene, referred to as *DAZ-like* or *Dazl*, which functions in gametogenesis and fertility. In order to expand our knowledge of *Dazl*, I cloned *Dazl* from the northern leopard frog *Rana pipiens*. To obtain the clone, I screened a *R. pipiens* ovary cDNA library with a *RpDazl*-specific PCR-generated radioactive probe. *RpDazl*, as determined by sequencing, is 3.5kb in length, with an 843 nucleotide open reading frame (ORF) and a 3' untranslated region (UTR) of 2635 nucleotides. At the amino acid level, the ORF of *RpDazl* is 51.8% identical to the ORF of *Xenopus laevis Dazl* or *XDazl* and 53.6% identical to the ORF of human *DAZL*. *RpDazl* has a characteristic RNA recognition motif (RRM) that is 73% identical to the RRM in *X. laevis*, and contains two smaller motifs within the RRM, RNP-1 and RNP-2. These two smaller motifs are almost 100% conserved across all species studied. *RpDazl* also contains a DAZ repeat, another characteristic domain of *Dazl* genes. The DAZ repeat of *RpDazl* is 69% identical to the DAZ repeat of *XDazl*. Northern blot analysis revealed a transcript length of 3.8kb and 3.3kb in two independent trials. RT-PCR was performed using template cDNA, made from *R. pipiens* ovarian RNA, and 23 sets of primers that spanned the cloned sequence. These data authenticated continuity over greater than 3.25kb of the cDNA. Combining results of the Northern blot and RT-PCR experiments, I conclude that the cloned *RpDazl* is full length. Preliminary data were also obtained regarding the maternal localization of the *RpDazl* RNA using RT-PCR as well as a computer based sequence analysis program called RepFind. RT-PCR analysis revealed that *RpDazl* is localized to the vegetal one-third of the oocyte. RepFind revealed clusters of CAC repeats, which are known localization elements. The significance of these clusters, however, is lower than that of the clusters present in the 3' UTR of *XDazl*. Further analysis will be required to show more precise *RpDazl* localization, specifically germ plasm association.

Acknowledgements

First and foremost, I want to thank my mom. Without her love and encouragement, and the occasional disciplinary acts, I would never have known who I am or have believed in myself to make it this far. Mom, thanks for getting me through midterms and finals for all these years. Thanks for giving me all that you could, even when it meant sacrifice for you. Thanks for the words of wisdom and for holding my hand through the rough times, that usually resulted from me not listening to your words of wisdom.

I want to thank everyone in the Elinson lab, past and present, for patience and laughter. These thanks go especially to Yvonne. Yvonne- you are one of the most remarkable people I have ever met. Under your tutelage (I told you I would use that word), I gained a strong sense of friendship that no one else could have ever given me, not to mention that I now know the power of wit. Kim, thanks especially for your patience, for all your help, and for all those good times making fun of Tabby. Thanks also to Mary for showing me the ropes of cDNA library screening.

In addition to my family and labmates, I want to thank all of my friends, especially my class, Sarah, Heather, Joy, Amanda, Anne, Amy, Paul, Danielle, AJ, Pete, Marty, Justin, Christa, and all my students. You all make my life just that much better. Ron, thanks for putting up with me. I can only hope that you will continue to put up with me for the rest of my life.

Also, thanks to Pam and Judy for making the walk to the office always worthwhile. Phil, your jokes and sarcasm have taught me much, wise one.

Thanks to Dr. Trun and Dr. Doctor for serving as my committee members. You have both helped so much along the way.

In the faculty, however, there is one person that stands above all in the “thank you” department. Dr. Elinson, certainly you have taught me the value of hard work and helped me to see that science truly is an art. You are a shining example of a mentor. And although I am sorry about my grammar and my persistent use of parentheses, I sometimes think you deserved it when I think about you making fun of my defolliculation attempts.

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Introduction

In the early 1890's, before the term genetics was coined by William Bateson, August Weismann proposed the theory of the continuity of the germline. Weismann's theory stated that in animals, the body is made up of two types of cells, the body or somatic cells, which were referred to as somatoplasm, and the gamete-producing cells that he termed germplasm. Weismann described the impossibility of passing characteristics of the somatic cells to offspring, and considered that the somatic cells simply served as a home to protect and nourish the germ cells. The germ cells are solely responsible for passing genetic information of a particular organism to its offspring. Weismann's ideas contributed to the rediscovery of Mendel's laws that were published 30 years earlier.

For some animals, the distinction between the germ cells and the somatic cells is determined early in development. The cells that are destined to become the germ cells "know" their fate from their very origin. These cells "know" their fate based on certain determinants that are located and remain in those cells. Other animals, including mammals, depend on cellular signaling to tell specific cells that they are to become the germ cells.

Primordial germ cells (PGCs) are the precursor cells in developing embryos that are responsible for forming the gametes, either sperm or egg, of the organism. Generally, the PGCs are formed elsewhere in the embryo and migrate to the gonadal region during development (Saffman and Lasko, 1999). In some organisms, the determination of the PGC population is a result of the localization of specific mRNAs to a distinct cytoplasmic area termed the germ plasm (Johnson et al., 2003). Germ plasm contains the germ cell determinants in the form of a collection of both coding and non-coding mRNAs including mitochondrial large (Kobayashi et al., 1998) and mitochondrial small (Kashikawa et al., 2001) ribosomal RNAs. Other components of germ plasm that have been identified at the ultrastructural level include mitochondria, ribosomes, and electron-dense structures termed germinal granules (Houston and King, 2000).

Germ plasm and its components are responsible for germ cell fate in organisms that contain them, so that the developmental pathway is determined exclusively by cell lineage. Germ plasm is usually localized within oocytes; that is, it is maternal by nature (Saffman and Lasko, 1999). Germ plasm may also be localized at various stages of development following fertilization. Organisms that utilize germ plasm for germ cell specification and development

include the frog *Xenopus laevis* (Ikenishi, 1998), the zebrafish *Danio rerio* (Knaut et al., 2000), the nematode *Caenorhabditis elegans* (Ikenishi, 1998; Schnabel, 1991), the chicken *Gallus gallus* (Tsunekawa et al., 2000), the fruit fly *Drosophila melanogaster* (Ikenishi, 1998), and other insects (Bradley et al., 2001). This localization-dependent development differs from regulative development, in which signals of varying types and cellular interactions lead to differentiation. Germ cells arise regulatively in sea urchins, urodele amphibians or salamanders, and mammals (Johnson et al., 2001).

I. Formation of germ plasm in *Xenopus laevis*

Before the diplotene stage of meiotic prophase, there are assemblies of mitochondria that surround the nucleus, or germinal vesicle, of the oocyte. One of these clusters matures to be significantly larger than the others and is termed the mitochondrial cloud (Zhou and King, 1996b). In *X. laevis*, the RNAs that are responsible for the destiny of the germ cells are first sequestered in the oocyte in the mitochondrial cloud (King et al., 1999). The cloud always forms at the prospective vegetal side of the germinal vesicle, is the site of extreme mitochondriogenesis, and serves as the home of germ plasm components (Figure 1). These components include the germinal granules and associated matrix, as well as specific RNAs and proteins, ribosomes, and cytoskeleton (Houston et al., 1998). There is a further order of sequestration within the mitochondrial cloud, as some RNAs are isolated in the messenger transport organizer (METRO) region at the vegetal-most portion of the cloud (Figure 1A) (Kloc and Etkin, 1995; Zhou and King, 1996b).

The diplotene phase of meiosis lasts between four and eight months, and this long phase has been subdivided into six stages as the oocyte grows in the ovary (Dumont, 1972). The sub-oocytes are classified according to their external appearance and their cytoplasmic organization. Stage I oocytes have a translucent cytoplasm, making the germinal vesicle and the mitochondrial cloud easily distinguishable. Stage II oocytes take on a characteristic opaque white color, and the vitelline envelope begins to form. The mitochondrial cloud fragments at the beginning of stage II, and approximately one-third of its components migrate toward the prospective vegetal cortex of the oocyte, bringing at least eight specific mRNAs as well as large and small

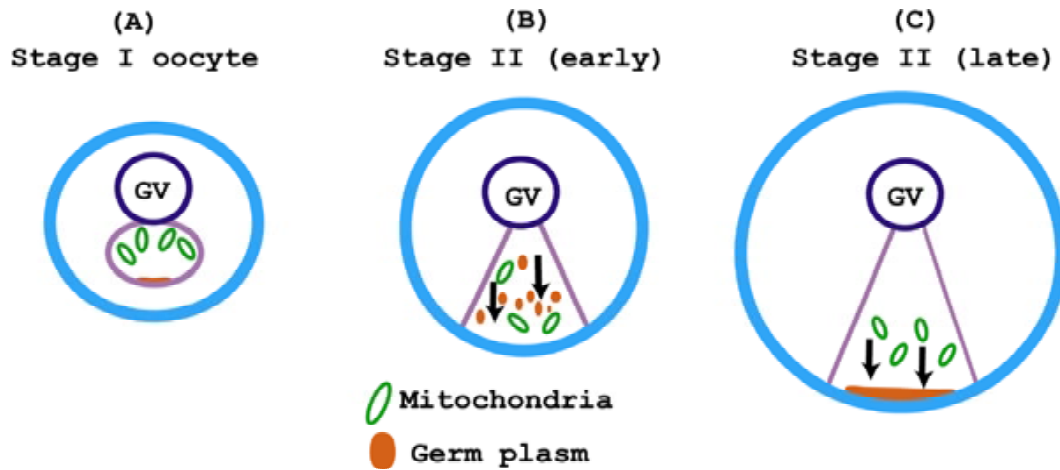


Figure 1: **METRO or Early Pathway of mRNA localization in *Xenopus laevis* oocytes.** (A) Stage I oocyte with the mitochondrial cloud (purple) located at the vegetal side of the germinal vesicle (GV). Note the prospective germ plasm (red) is localized at the vegetal-most portion of the GV—the METRO region. (B) Early stage II oocyte has a fragmented mitochondrial cloud, with the contents traversing toward the vegetal cortex (C) Late stage II oocyte with all the germ plasm and its components localized at the vegetal cortex. Drawing adapted from King et al., 1999.

mitochondrial rRNAs to that site (Figure 1B and 1C). The RNAs that follow this pattern of localization are always located in the METRO region of the mitochondrial cloud before its breakdown, and for this reason they are said to be mitochondrial cloud dependent. The pathway is called the METRO or Early Pathway. When the mitochondrial cloud fragments in early stage II oocytes, a wedge is formed in the vegetal hemisphere, which is progressively filled by a subdomain of the endoplasmic reticulum. This subdomain aids in the localization of the METRO Pathway RNAs, although the exact mechanism of the endoplasmic reticulum's involvement in the process remains unclear (King et al., 1999). An additional five or more RNAs localize vegetally in stages IV, V, or VI in the mitochondrial cloud-independent or Late Pathway. These RNAs require intact microtubules, which are not required by the METRO Pathway RNAs (King et al., 1999; Kloc et al., 2002).

II. Localized RNAs in *Xenopus laevis*

A. METRO Pathway RNAs

Xenopus laevis is the only anuran species for which RNAs localized to the germ plasm are known. Several of these RNAs will be discussed briefly as follows. *Xenopus* cytoskeletal-associated transcript, *Xcat2*, is localized to the vegetal cortex of *X. laevis* oocytes via the METRO Pathway, and encodes a protein with a zinc-finger domain that has RNA binding activity (Mosquera et al., 1993). *Xcat2* has homology to *nanos* in *D. melanogaster* (Zhou and King, 1996a). The 3' UTR of *Xcat2* is both necessary and sufficient for localization of *Xcat2* RNA to the germinal granules of the germ plasm (Kloc et al., 2000; Zhou and King, 1996a). The proposed function of the *Xcat2* protein is to coordinate regional differences in gene expression during development by acting as a translational repressor.

The protein product of DEADSouth, another METRO Pathway localized RNA, is a member of a small subfamily of DEAD-box RNA helicases (MacArthur et al., 2000). DEADSouth is related to eukaryotic initiation factor-4A (eIF4A), which unwinds RNA secondary structure in the 5' UTR of eukaryotic mRNAs and is fundamental in many organisms for protein synthesis (MacArthur et al., 2000; Rogers et al., 1999). DEADSouth is homologous to *vasa* in *D. melanogaster*, and *vasa* homologues in chicken, zebrafish, and *C. elegans* (Knaut et al., 2000; Raz, 2000; Tsunekawa et al., 2000).

Xenopus laevis short interspersed transcripts, or *Xlsirts*, are a family of noncoding RNAs (Kloc et al., 1993). They are homologous to mammalian *Xist* that is involved in X chromosome inactivation. The *Xlsirt* RNAs have 3-13 tandemly repeated sequences, 79-81 nucleotides in length, which act as localization elements. *Xlsirts* play a structural role in the oocyte, both aiding in the localization and the anchoring of RNAs, such as *Vg1* RNA, in the vegetal cortex (Kloc et al., 2002). The *Xlsirts* form a family of transcripts that are derived from both strands of the genes that encode them. The set of transcripts from the sense strand localize to the vegetal cortex utilizing the METRO Pathway, and the set of transcripts from the antisense strand are located throughout the cytoplasm (Erdmann et al., 2000). *Xlsirt* sequences can be attached to coding sequences as well as non-coding sequences (Zearfoss et al., 2003).

Xenopus laevis primordial germ cell-associated transcript, or *Xpat*, is a unique RNA whose encoded 35 kD protein has no currently known homology (Hudson and Woodland, 1998). With its known localization pattern, *Xpat* serves as a molecular marker specific to germ plasm and primordial germ cells. The 3' UTR is both necessary and sufficient for localization.

Xwnt-11 in *Xenopus* resembles other known *wnt* genes. *wnt* genes encode cysteine-rich glycoproteins that have characteristics of secreted growth factors (Ku and Melton, 1993). These growth factors are known to be involved in regional cellular communication. *Xwnt-11* may be involved in embryonic dorsal-ventral axis formation.

Xenopus laevis fatty acyl CoA synthetase, or XFACS, plays a role in the uptake of long chain fatty acids (King et al., 1999). Another RNA, called Fingers, encodes a protein that contains zinc fingers. The zinc finger domains follow a unique amino acid region that shares some homology with a protein that has transcriptional repressing activity (Kloc et al., 2002; Numoto et al., 1999).

The last of the known RNAs that utilize the METRO Pathway of localization is *Xenopus laevis Dazl* (see below).

B. Late Pathway RNAs

Several other important RNAs utilize the Late Pathway for localization. *VegT* RNA localizes to the vegetal pole using the Late Pathway, and encodes a T-box transcription factor that functions as a mesoderm-endoderm determinant (King et al., 1999; Kofron et al., 1999; Xanthos et al., 2001; Zhang et al., 1998). Depletion of maternal *VegT* results in complete loss of

both endoderm and mesoderm. *VegT* RNA also plays a role in the localization and/or the anchoring of other RNAs to the vegetal cortex (Heasman et al., 2001). With the maternal *VegT* RNA depleted, normally localized RNAs such as *Xwnt-11* and *Vg1* are no longer localized.

Vg1, which uses the same pathway as *VegT*, is a member of the TGF- β family of growth factors and is implicated in the induction of mesoderm (Forristall et al., 1995; Thomsen and Melton, 1993). *Vg1* also plays roles in dorsal/ventral polarity (Kessler and Melton, 1995) and in left/right axis formation (Hyatt and Yost, 1998).

fatvg RNA, expressed in the fat bodies of the developing embryos, is very different in that it uses parts of both the Early and the Late Pathways (Chan et al., 2001). In addition to vegetal localization, there are at least ten RNAs that localize to the animal pole of the oocyte (King et al., 1999). Several different mechanisms for their localization have been proposed based on the fact that the RNAs have different localization patterns.

III. *DAZ* and *DAZL*

One of the most interesting RNAs that employs the METRO Pathway for vegetal localization is *XDazl*. *DAZ* means “Deleted in AZoospermia”, and *DAZL* simply means *DAZ*-like. In humans, the *DAZ* gene has been mapped to the long arm of the Y-chromosome, distal Yq11, which is termed the Azoospermia Factor (AZF) region. The AZF region is subdivided into intervals referred to as AZFa, AZFb, and AZFc (Vogt et al., 1996), and candidate genes implicated in oligo- and azoospermia map to each interval (Seboun et al., 1997; Slee et al., 1999). In the AZFa region, the protein product of one gene is similar to a ubiquitin C-terminal hydrolase (Slee et al., 1999). Within the AZFb interval, a gene family is present termed the RBM or RNA binding motif family (Ma et al., 1993). However, it is difficult to pinpoint the role of this gene family due to the presence of up to 40 copies of genes and pseudogenes on the Y chromosome in humans and mice (Elliott et al., 1996; Seboun et al., 1997). The *DAZ* gene maps to the AZFc region (Slee et al., 1999; Yen et al., 1996). Interestingly, humans and greater primates are thus far the only species to possess the *DAZ* gene. Deletions in the *DAZ* gene cluster in humans has been linked to a wide range of spermatic defects, ranging from oligospermia to a complete lack of germ cells, called Sertoli cell only syndrome (Yen et al., 1996).

Humans also possess an autosomal *DAZ* homolog, as do many other organisms including *C. elegans* and mouse. This gene is implicated in the variation of phenotypes that occur as

spermatic defects in men with malfunctional *DAZ* genes, as the functions of *DAZ* and *DAZL* may be overlapping. The autosomal version, which is referred to as *DAZL*, is thought to play a role in human gametogenesis, but this role is still greatly unexplored. Human *DAZL* maps to chromosome 3, and its predicted protein product is 95% identical to that of *DAZ*. The main difference between the two genes is the presence of a repeated 24 amino acid sequence called a *DAZ* repeat, that is found only once in *DAZL* and 8-18 times in *DAZ* (Xu et al., 2001). Both protein products, as well as *Dazl* proteins in many other organisms contain a common RNA recognition motif or RNA binding domain (RRM or RBD). In addition, the *DAZ* repeat is conserved, although its function is yet to be determined (Yen et al., 1996). The greatest level of homology is in the RRM of *Dazl* proteins, resulting in a highly conserved RNA binding ability. *In vitro*, *X. laevis* *Dazl* binds to poly (G) and poly (U) RNA efficiently (Houston et al., 1998). There is also weak binding to poly (A) RNA and no binding to poly (C) RNA or ssDNA.

DAZL homologs have been discovered in a variety of metazoans, and loss of function analyses reveal an assortment of phenotypes. Mouse *Dazl* maps to chromosome 17 and is required for both spermatogenesis and oogenesis. Neither process occurs when the *Dazl* gene is non-functional (Dai et al., 2001). The human *DAZ* gene allows for partial recovery of the *Dazl* null mouse, demonstrating functional homology in the *DAZ* gene family (Slee et al., 1999). A partial rescue of the *Dazl* null mouse was also achieved by the human *DAZL* gene (Vogel et al., 2002). Mouse *Dazl* binds stretches of oligo (U) sequence that are interspersed with G or C residues (Venables et al., 2001).

In *C. elegans*, the *Dazl* gene is required for female meiosis, but it is not necessary for male meiosis (Karashima et al., 2000). In *D. melanogaster*, the *DAZ* homolog called *boule* is required for meiotic entry in spermatogenesis, and loss of *boule* leads to complete infertility as a result of cell cycle arrest just before meiosis. Partial rescue of *Drosophila* *boule* mutants was achieved by *XDazl* from *Xenopus laevis* (Houston et al., 1998). Zebrafish *Dazl* is expressed in the gonads of both males and females (Maegawa et al., 1999). *ZDazl* is localized to the vegetal cortex of oocytes as well as in the PGCs of embryos. A *ZDazl* null phenotype is not known (Xu et al., 2001). *ZDazl* protein binds *in vitro* specifically to the sequence 'GUUC' via its RRM; however, as with other *Dazl* proteins, the target RNA *in vivo* is unknown (Maegawa et al., 2002). The *DAZ* repeat of the protein binds polysomes, suggesting a function for this motif. This is the first report of a proposed function for the 24 amino acid repeat.

In *Xenopus laevis*, *XDazl* is expressed throughout the frog's lifetime. After the mitochondrial cloud fragments, *XDazl* is localized in the vegetal cortex of the oocyte in the matrix of the germ plasm (Kloc et al., 2002). After fertilization, the germ plasm is present in specific vegetal cells as distinct islands. The germ plasm is divided unequally until gastrulation, when it becomes perinuclear, and is then divided equally between daughter cells. The cells that contain the germ plasm and its components throughout early cleavage eventually differentiate to become the primordial germ cells (PGCs). The PGCs begin to migrate at the late tailbud stage, first laterally from the ventral endoderm and then dorsally to the posterior dorsal endoderm, where they accumulate and are incorporated into the lateral plate mesoderm. PGC migration continues in later stages until they reach the developing genital ridges (Houston and King, 2000; Wylie and Heasman, 1976). *XDazl* remains in the PGCs, and remains associated with the gonads in premeiotic spermatogonia and spermatocytes of the frog's testes, or in the oocytes of the developing ovary.

XDazl RNA is present from early oogenesis, but detectable levels of the protein are not present until blastula stage, indicating that *XDazl* RNA undergoes translational repression, similar to *Xcat2* RNA (MacArthur et al., 1999). The protein is present from blastula until early tailbud, when the concentration of *XDazl* protein decreases dramatically. This is the time in development that corresponds to the migratory phase of the PGCs. Tadpoles with few or no PGCs are the result of maternal *XDazl* RNA depletion (Houston and King, 2000). Furthermore, without proper *XDazl* expression, the few PGCs that may form do not migrate to the dorsal mesentery.

An organism that has an interesting expression of *Dazl* is axolotl, *Ambystoma mexicanum*. Axolotl is a urodele amphibian or salamander, in contrast to *X. laevis* which is anuran or frog. *Dazl* is expressed maternally and during embryogenesis, but it is not localized in the manner of *XDazl* (Johnson et al., 2001). This is interesting because both animals are amphibians. *AxDazl* is not confined to specific cells until the migrating PGCs begin to approach the gonad. Based on this observation, Johnson et al. (2001) concluded germ cell determination in axolotl is regulative and does not depend on localized RNAs as in *X. laevis*. In this way, Johnson et al concluded that axolotl, with its regulative germ cell determination, seems to be more like mouse and human.

IV. Objectives

When anuran and urodele amphibians diverged from their common ancestor, did each take a separate path in germ cell determination? In other words, is the germ plasm dependent determination of germ cells in *X. laevis* the route that all anuran amphibian species follow? Recently, Johnson et al. (2003) concluded just this. Johnson's group propose that the PGCs of urodeles are determined by cellular signaling, and that the PCGs of anurans are determined by germ plasm, without exception (Johnson et al., 2003). This conclusion is the basis for my thesis research. By studying *Dazl* in another anuran species, I hope to draw conclusions regarding germ cell determination in amphibians. The Northern Leopard frog, *Rana pipiens* has germ plasm, but no RNAs localized to the germ plasm have been identified. In this research, I cloned *RpDazl*, in order to initiate an analysis of its expression.

Methods and Materials

I. cDNA library screen

A. *Rana pipiens* ovary cDNA library

Poly A+ RNA from *Rana pipiens* ovary was prepared by Kim Nath and sent to Stratagene® (La Jolla, CA) for library preparation. When received, the Lambda ZAP® II Custom Library was aliquotted and stored according to the Stratagene protocol. One aliquot was used to titer the library and determine optimal plating density. A sample was also used as a template for polymerase chain reaction (PCR) to be sure that specific genes were present in the library (primers used found in Table 1).

All *E. coli* cells for the library screen were grown as follows. A colony was used to inoculate 50 ml of LB broth that was supplemented with 10.2% maltose and 10mM MgSO₄. The culture was incubated overnight at 37°C with aeration. After incubation, the culture was transferred to a 50 ml conical tube, and the cells were pelleted by centrifugation for 10 min at 3500 rcf. The supernatant was removed and the cells were resuspended in 25 ml of 10mM MgSO₄. The cells were diluted with 10mM MgSO₄ until the desired optical density reading at 600 nm was achieved.

PCR was performed to amplify cloned inserts throughout the library screen. The standard PCR recipe contained 14 µl sterile nanopure water, 2 µl 10x PCR buffer (-MgCl₂), 0.6 µl 50 mM MgCl₂, 0.4 µl 10mM dNTPs, 2 µl template DNA, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), and 0.2 µl Taq polymerase. Template DNA for a PCR consisted of 100-500 ng of plasmid DNA or approximately 1/20 of an RT reaction cDNA product. Customarily, a master mix was prepared, especially if the same template or primers were utilized in several PCR samples. The samples were prepared in thin-walled PCR tubes and the experiment took place in a Biometra® PCR machine. Following typical parameters, the program began with an initial denaturation of 5 min at 94°C. Cycles of 50 sec at 94°C, 50 sec at 55°C, and 50 sec at 72°C were repeated 23-35 times before a 10 min final extension at 72°C. The machine was programmed to hold the samples at 4°C until a gel was ready to run for visualization of

Gene	Primer Sequence (5'-3')	Primer Source	Expected product sizes of primer sets
RpVasa	Forward: GGCGTTGAATCTAGTGCA Reverse: CAACACAAGGTAGCGCAA	Yvonne Beckham, unpublished	~300 base pairs
RpVegT	Forward: CCTTTGGACAACACTGCAGA Reverse: GACTGCAGTAAACTCAGTCT	Yvonne Beckham, unpublished	~300 base pairs
RpTFIIIα	Forward: ATGCGGTAAAGGCTTTGTGA Reverse: AATGACTAGCTGCCAAGTGT	Gene sequence obtained from database (GenBank Acc # M85211), primers designed manually	399 base pairs
RpDazl	Set A: Forward (FA): CTGGTGTGTCGAAGGGAT Reverse(RA): GCTTGCTTGGTATGCCAA Set B: Forward (FB): CGAAGGGATATGGTTTTGTCT Reverse (RB): CCACCTTGAAACATCCT	Primers designed manually from 386 bp sequence obtained by Yvonne Beckham	FARA: 317 base pairs FARB: 337 base pairs FBRA: 308 base pairs FBRB: 328 base pairs

Table 1: **Primer sequences and sources as well as expected product sizes for several specific *Rana pipiens* RNAs.** These primers were used in a PCR experiment for using *R. pipiens* ovary cDNA library as a template, demonstrating that all RNAs were present in the library (Fig 2). The same primers were used in a PCR experiment using cDNA that was prepared from *R. pipiens* ovarian RNA, demonstrating maternal expression of the RNAs (Fig 15).

the PCR results. Some parameters of the PCR program were adjusted pending factors such as the primer annealing temperatures and expected product sizes. All primers were ordered from Integrated DNA Technologies (IDT) and were diluted in sterile nanopure water to a stock of 1 $\mu\text{g}/\mu\text{l}$. Working stocks (10 μM) were made from this suspension as needed.

To each PCR product, 2 μl of tracking dye was added. Also, a DNA ladder was prepared by adding 0.5- 2.0 μg ladder, 16-17 μl sterile water, and 2 μl tracking dye to a sterile microcentrifuge tube. The ladder was one of several that were utilized; 100 base pair ladder (Roche Molecular Biochemicals, Indianapolis, IN), Lambda DNA BstE II digest (New England BioLabs® Inc., Beverly, MA), or 1 Kb Plus ladder (Invitrogen™, Carlsbad, CA). A 1% agarose gel was prepared and supplemented, after melting, with 2 μl of ethidium bromide (stock concentration of 10 mg/ml) per 50 ml of gel. The gel was poured and allowed to solidify. Samples were loaded in the gel wells via a pipette and the gel was run in an electrophoresis apparatus containing 0.5x TBE buffer at 100 volts. After electrophoresis was complete, the gel was viewed using a transilluminator and photographed.

B. Primary library screen

a. Plating and filter lifts

The library was plated at optimal conditions as determined by the titering, resulting in approximately 45,000 plaques per 14-cm plate. This was carried out by allowing 11 μl of a 1:1000 dilution of the library in SM Buffer (Stratagene Lambda ZAP® II Library Instruction Manual) to infect 600 μl of XL1 Blue MRF' *E. coli* cells, diluted in MgSO_4 to an OD_{600} of 0.48 for 15 min at 37°C. The genotype of the XL1 Blue MRF' strain *E. coli* cells is: $\Delta(\text{mcrA})$ $\Delta(\text{mcrCB-hsdSMR-mrr})$ 173 endA1 supE44 thi-1 recA1 gyrA96 reIA1 lac [F' proAB lacI^qZAM15 Tn10 (Tet^r)]. The infected cells were combined with 8ml of NZY top agarose (Stratagene Lambda ZAP® II Library Instruction Manual) that had been melted and cooled to 55°C, and the mixture was poured immediately onto NZY agar plates. The plates were swirled to spread the infected cell mixture evenly. The twenty-nine 14-centimeter plates from 29 identical tubes of library/ cells that were poured were inverted and incubated at 37°C for 7.5 hrs. After the elapsed time, plaques, or areas that represent where the phage has lysed the *E. coli* cells, had formed on the plates. At this time, the plates were moved to 4°C for storage.

Filter lifts were then performed on 24 plates following the protocol given by Stratagene®. Two rounds of lifts were carried out for each of the 24 plates and several were selected for a third filter lift. For a filter lift, a nylon membrane (Hybond™-NX; Amersham Pharmacia Biotech, Piscataway, NJ) was carefully laid on the plate and left for 2 min (first round), 4 min (second round), or 6-8 min (third round). During this time, a hypodermic needle filled with India ink was used to mark the filters and plates in an asymmetric manner so that orientation could be maintained throughout the screen. After the proper time had elapsed, the membrane was carefully lifted from the plate and placed through a series of solutions. First, the membrane was washed for two minutes in a denaturant solution (0.5N NaOH/1.5M NaCl). Second, the membrane was washed for 5 min in a neutralization solution (0.5M Tris buffer pH 8.0/ 1.5M NaCl) in order to stop the denaturing process, and finally rinsed twice in 2x SSC. The DNA was irreversibly bound to the membrane by ultraviolet crosslinking (Spectrolinker XL-1000 UV Crosslinker; 1200 x100 µJ/cm²). Once the membranes dried completely, they were wrapped in foil and stored at room temperature for future use.

b. *RpDazl* probe preparation

A DNA *RpDazl* probe for screening the ovary cDNA library was prepared by radioactive PCR. The plasmid that was utilized was obtained from Yvonne Beckham and contained 386 bp of *R. pipiens Dazl* that she had previously isolated by degenerate PCR. The recipe for PCR probe labeling with ³²P was as follows: 2µl 10x PCR buffer (-MgCl₂)(Invitrogen™), 5µl ³²P-dCTP, 3µl 10µM dNTP mix, 0.6µl 50 mM MgCl₂, 1µl 10µM forward primer, 1µl 10µM reverse primer, 100 ng plasmid DNA, and 0.2µl Taq polymerase. The volume of the reaction was adjusted to 20µl with sterile nanopure water. The PCR was run at 30 cycles with a denaturing temperature of 94°C, an annealing temperature of 54.8°C, and an extension temperature of 72°C.

A scintillation counter was used to determine the total number of counts and the percentage of the radiolabeled nucleotides incorporated. One microliter of the PCR product was added to a scintillation tube that contained 9ml of Ultima Gold™ scintillation fluid (PerkinElmer, Inc., Boston, MA). The remainder of the PCR product was then purified using the ProbeQuant G-50 Micro Column system (Amersham Pharmacia Biotech). One µl of the resulting purified probe, whose total volume was approximately 66µl, was added to another scintillation tube containing 9ml of scintillation fluid. Along with a blank tube containing only

scintillation fluid, the counts per min (cpm) of each tube were determined. The total counts of the probe were calculated by multiplying the cpm of the purified probe by the total volume obtained in microliters. The percentage of radiolabeled nucleotide incorporation, which was used to judge the success of the PCR, was determined by dividing the purified probe cpm by the PCR product cpm before purification and multiplying by 100. The percentage of radiolabeled nucleotide incorporation varied slightly, with the highest being 11.7%. The lowest was 0.6% incorporation, but the total volume of the probe did not matter as the total counts served as the main parameter for probe use. This meant that multiple PCR products could be combined to obtain the counts necessary for hybridization.

c. Filter hybridization

The total number of counts needed for hybridization to the library filters was determined by the milliliters of hybridization buffer that is required for the procedure, which in turn is determined by the area in square centimeters of the filters to be hybridized. For the primary screen, it was established that 1.25×10^8 total counts would be needed. Five independent PCR reactions were required to obtain this number of counts.

With the DNA facing downward, the filters were placed one at a time in a Tupperware container that contained 62.4ml of hybridization buffer. Final concentrations of the hybridization buffer ingredients were 50% deionized formamide, 2x Denhardt's solution, 0.5% sodium dodecyl sulfate, 5x SSPE (Sambrook, Fritsch, and Maniatis, 1989), and 100 μ g/ml heat denatured salmon sperm DNA. The filters were rocked gently in a hybridization oven for 2 hrs at 45°C. Towards the end of the prehybridization period, 62.4ml of fresh hybridization buffer were preheated to 45°C in a water bath. Also, fresh salmon sperm DNA and the probe were combined and denatured by heating for 10 min at 98°C. As soon as the probe and salmon sperm DNA were denatured, they were chilled immediately on ice for 10 min. When the prehybridization had finished, the filters were carefully removed from the container and the buffer was discarded. The fresh, preheated buffer was added to the container, the probe/salmon sperm DNA solution was placed into the buffer via pipette, and the container was swirled gently. The filters were then placed into the solution one at a time with the DNA side facing downward. The filters were rocked gently in a 45°C hybridization oven overnight.

The following day, the filters went through a series of washes before they could be exposed to X-ray film. The filters were removed and the radioactive hybridization buffer was disposed of in the proper waste container. To the now empty container, 250ml of 1x SSPE, 0.5% SDS was added, and the filters were replaced one at a time, avoiding bubbles. The filters were rocked at room temperature for 15 min. During this time, 250ml of 1x SSPE, 0.5% SDS was placed in a new container and preheated in the hybridization oven at 45°C. After the room temperature wash, the filters were moved one at a time to the new container with the pre-warmed wash solution and were rocked gently at 45°C for 20 min. Following a second twenty-minute wash at 45°C, the filters were rinsed briefly in 2x SSC to remove as much SDS as possible.

The filters were placed, DNA side down, on Saran wrap-covered filter paper. The entire setup was covered in another piece of Saran wrap, avoiding bubbles, and the filters were placed into an autoradiography cassette. The cassette was taken to the dark room where a sheet of medical X-ray film (Fujifilm, Tokyo, Japan) was placed on the filters, and the cassette was sealed and labeled. The cassette was exposed for 71 hrs at -80°C with an intensifying screen, after which the film was developed. After this first exposure, it was noted that some of the filters were too dark, so a second exposure was performed for 20 hrs. To find potentially positive plaques, the film was examined on a light box. Plates were lined up on top of their respective filters using the India ink marks that were placed on the plates during the filter lifts. Anywhere that there was a dark spot on the film that corresponded with an isolatable plaque on the plates, the plaque was picked. To isolate a plaque, the wide end of a glass pipette was used to cut out the portion of agar that contained the plaque. The agar piece was placed into a microcentrifuge tube that contained 500 µl SM buffer. Once all the potentially positive plaques were picked and placed into the tubes, 20 µl chloroform was added to each tube and the tubes were vortexed to release the phage particles into the buffer. These tubes were stored at 4°C, where they can remain for up to six months. The 18 plaques that were picked between the two exposures were checked by PCR using 2 µl of the phage-containing buffer as template and *RpDazl* exact primers that had been designed previously. Five samples amplified by PCR and were selected for the secondary screen.

C. Secondary library screen

NZY agar plates, 9 cm diameter, were poured and allowed to solidify. Fresh XL1 Blue MRF' *E. coli* cells were grown and the optical density optimized as with the primary screen. Dilutions of 1:100 and 1:1000 in SM buffer were made of the primary screen's resultant phage that amplified by PCR. Ten microliters of the phage dilutions were allowed to infect 200 μ l of the *E. coli* cells as for the primary screen. The infected cells were combined with 4 ml of NZY top agarose that had been melted and cooled to 55°C, poured immediately onto the NZY agar plates. The plates were incubated at 37°C until plaques formed (8.5 hrs). It was decided that the 1:1000 dilution of the phage was best for the secondary screen due to the number of plaques that formed, 100-200 per plate being optimal. Filter lifts were performed as before.

Six filters were selected for the hybridization, all of the 1:1000 filters and one of the 1:100 filters, and it was calculated that 15.6 ml of hybridization buffer and 7.8×10^6 - 1.6×10^7 total cpm were needed for the secondary screen. The radioactive probe was prepared by PCR as before, and enough counts were obtained in one 50 μ l PCR reaction, in which the volumes of all ingredients were simply multiplied by 2.5. The same procedure was followed for the prehybridization and the hybridization that was followed for the primary screen, only reducing the size of the Tupperware container used and utilizing smaller volumes of solutions. The radioactive filters, after washing, were exposed on X-ray film for approximately 20 hrs.

Using the five plates that contained the 1:1000 dilutions of the phage and the corresponding film, three plaques were picked from each plate. The fifteen plaques were picked using the narrow end of a glass pipette and the resultant agar piece was placed in a microcentrifuge tube with 500 μ l SM buffer. As before, 20 μ l chloroform was added and the tubes were vortexed. The seven samples that produced the sharpest bands by PCR were selected for the tertiary screen and were named A-G.

D. Tertiary library screen

Each of the seven potentially positive phage from the secondary screen was diluted in SM buffer to two different dilutions, 1:100 and 1:200. Ten μ l of each was allowed to infect 200 μ l of XL1 Blue MRF' *E. coli* cells. Plates were poured and incubated in the same manner as for the secondary screen. Both dilutions of the plugs resulted in well-isolated plaques, so filter lifts were performed on all plates and one filter for each was selected for hybridization. The 1:100

dilution plate was used for samples A, E, and F; the 1:200 dilution was selected for samples B, C, D, and G, based on the number and arrangement of the plaques. One 50 μ l PCR reaction resulted in a probe with 3.24×10^7 counts and between 9.1×10^6 and 1.8×10^7 counts were needed with the 18.2 ml of hybridization buffer to be used. Prehybridization, hybridization, washes and overnight film exposure were performed as per the secondary screen. The number of plaques was counted on each plate, and the number of positives from the film was counted to determine the percentage of positives. One plaque from each plate was picked as they were for the secondary screen, and all seven amplified by PCR.

E. Preparation of a concentrated phage stock

To prepare a concentrated phage stock, the plugs of interest (A-G) had to be replated in such a manner that confluent lysis was achieved. In duplicate, 20 μ l of the undiluted tertiary screen phage in SM buffer was allowed to infect 200 μ l of XL1 Blue MRF' *E. coli* cells for 15 min at 37°C. Four ml of NZY top agarose was added to the infected cells, and they were plated as before. The fourteen resultant plates were incubated overnight to achieve complete lysis.

To each lysed plate, 5 ml of SM buffer was added, and the plates were swirled to distribute the buffer evenly. These were incubated overnight on an orbital shaker at 4°C. With elution being complete the following day, as much of the buffer as possible was pipetted off the plates and into a 15 ml conical tube, combining the duplicates. The plates were set at an angle and allowed to sit for 10 min to allow any residual buffer to settle on one side, which was then carefully pipetted into the corresponding conical tube. One ml of chloroform was added to each tube, and the tubes were vortexed for 30 seconds each. Once the lysed cells settled to the bottom of the tube, they were centrifuged for 10 min at 3000 rpm. The phage supernatant (the top of three distinct layers) was carefully moved to a new tube where 25 μ l chloroform was added. After a short vortex, the concentrated phage stocks were stored at 4°C in the dark.

F. Single clone excision

The cloned inserts of unknown size were in the multiple cloning site of a pBluescript phagemid packaged in a linear Lambda Zap II phage. The single clone excision will result in pBluescript phagemid in filamentous phage that can be plated via bacterial transformation. Cells and protocols were supplied with the Lambda ZAP II cDNA library (Stratagene). Previous to the

experiment, the ExAssist helper phage was titered and amplified according to the Stratagene protocol. For the excision itself, 200 μ l of XLI Blue MRF' cells at an OD₆₀₀ of 1.0 were combined with 250 μ l of concentrated phage stock and 1 μ l of the amplified ExAssist helper phage in a Falcon 2059 polypropylene tube. All tubes were incubated for 15 min at 37°C, after which 3 ml of LB broth was added. The tubes were incubated in a 37°C bacterial shaker for 6 hrs, and in a 65°C water bath for 20 min, followed by centrifugation at 5000 rpm for 15 min. The supernatant was carefully moved to a new polypropylene tube. This stock was placed at 4°C for storage. SOLR *E. coli* cells were grown overnight in LB broth, supplemented with 0.2% maltose and 10mM MgSO₄. The genotype of the SOLR strain *E. coli* cells is: e14'(McrA') Δ (mcrCB-hsdSMR-mrr) 171 sbcC recB recJ uvrC umuC::Tn5 (Kan^r) lac gyrA96 relA1 thi-1 endA1 λ^R [F' proAB lacI^qZ Δ M15] Su⁻ (nonsuppressing). The cells were pelleted on the following day, resuspended in 10mM MgSO₄, and diluted to an OD₆₀₀ of 1.0. LB plates supplemented with filter-sterilized ampicillin were poured and allowed to solidify. In seven microcentrifuge tubes, 100 μ l of the phage supernatant from the excision experiment (samples A-G) were combined with 200 μ l of SOLR cells and in another set of tubes, 10 μ l of the phage supernatant (A-G) were combined with the same volume of cells. All of the tubes were incubated at 37°C for 15 min, and 200 μ l of this mixture was plated on the LB-Amp plates. The mixture was spread evenly over the plate with a cell spreader and the plates were inverted for overnight incubation at 37°C. Resultant colonies were picked and used to inoculate 5 ml of LB-Amp broth in 2059 polypropylene tubes, which were incubated overnight in a 37°C bacterial shaker.

Plasmid DNA was purified from each preparation the following day using Promega's Wizard® Plus Miniprep DNA purification system. The 11 resultant samples were set up for a restriction enzyme digest to determine the size of the cloned insert that each phage/plasmid contained. Typically, a restriction enzyme digest consists of 1 μ g of DNA, 1 μ l or 15 units of each restriction enzyme used, 2 μ l of the appropriate buffer, and the reaction is brought up to 20 μ l with sterile nanopure water in a microcentrifuge tube. The concentration of the DNA was determined by a spectrophotometer reading at OD₂₆₀. For these samples in particular, the concentration of four samples could not be determined, and 2 μ l was the chosen volume for the digest. The enzymes that were chosen to digest the pBluescript plasmid were *Apal* and *SpeI*, to which 10x L buffer (Takara Bio Inc., Japan) was determined to be appropriate. The tubes were incubated for 1 hr at 37°C, during which time a 1% agarose TBE gel, with 10 μ g ethidium

bromide per 25 ml gel for visualization, was poured and allowed to solidify. After the incubation period, 2 μ l of tracking dye was added to each sample and the gel was loaded and run at 100 volts/cm. Nine of the eleven samples were transformed into XLI competent cells. For a bacterial transformation, 1-2 μ l of DNA was combined with at least 100 μ l of competent cells and incubated on ice for at least 30 min. The mixture was heat shocked at 42°C for 45 seconds, after which the volume was brought up to 1 ml with SOC media or LB broth. The transformation solution was incubated at 37°C for 1-2 hrs. At this point the cells can be plated on LB-Amp plates. The entire volume was not plated on the same plate; 200 μ l proved a sufficient volume in order to obtain well-isolated colonies. Colonies that resulted from this transformation were used to inoculate 5 ml of LB-Amp broth, and were incubated overnight at 37°C in a bacterial shaker.

Plasmid DNA was purified from these preparations the following day again using Promega's Wizard[®] Plus Miniprep DNA purification system (Madison, WI). Quantification by spectrophotometry could again not be used, so the resultant plasmid DNA was quantified by agarose gel electrophoresis. Onto a 1% agarose TBE gel with ethidium bromide for visualization, plasmid DNA of known concentration was loaded at several dilutions. The eleven samples were loaded at both original concentration and at a 1:3 dilution. The resulting bands were compared to the bands from the DNA of known concentration and its dilutions. Estimates of the concentrations were assigned based on band similarities in intensity.

G. DNA sequencing

All DNA sequencing was performed at the DNA Sequencing Core Facility, at the University of Pittsburgh's Molecular Medicine Institute. The samples were prepared according to the Facility's protocol. Each sequencing sample was prepared in a sterile microcentrifuge tube with 300-500 ng of DNA and 1 μ l of primer, which was brought up to 13 μ l with sterile nanopure water. One DNA sample was selected for sequencing based on insert size as determined by restriction enzyme digest, and all sequencing performed was performed on the same sample throughout. Both the top strand and the bottom strand were sequenced with primers that were designed as the experiment progressed (Table 2).

After obtaining results from one primer, a new primer was designed from the resultant sequence. The reverse complement of some sequences was utilized, and a set of

“troubleshooting” primers was designed at the end of the experiment to eliminate any areas of ambiguity. After obtaining DNA sequencing results, the sequences were aligned on AutoAssembler™ 1.3.0, a global sequence alignment program available from Applied Biosystems. From this alignment program, a consensus sequence was constructed.

Primer Name	Strand	Primer Sequence and Location (5'-3')
T7	Coding	MCS ¹ GTAATACGACTCACTATAGGGC
For1	Coding	640 CCTGGGGTGAAGTGTTGCA 660
For2	Coding	1326 GATGCTTATCTTCACTCCCT 1346
For3	Coding	1989 TGTATTGACTATTGATGCTGA 2011
For4	Coding	2667 CTA CTACTGTGCAAGCATCTACCA 2689
T3	Non-coding	MCS ¹ AATTAACCCTCACTAAAGGG
Rev1	Non-coding	2898 CATCCCAGCAATCGATATGCT 2876
Rev2	Non-coding	2261 TTGCTCTTAATTCCAGGATCA 2239
Rev3	Non-coding	1567 TACATTCCTAACTAGCCATCA 1545
F1RC	Non-coding	660 TGCAACACTTCACCCCAGG 640
F2RC	Non-coding	1346 AGGGAGTGAAGATAAGCATC 1326
F3RC	Non-coding	2011 TCAGCATCAATAGTCAATACA 1989
R1RC	Coding	2876 AGCATATCGATTGCTGGGATG 2898
R2RC	Coding	2239 TGATCCTGGAATTAAGAGCAA 2261
386F	Coding	386 TATCAGAGCATGTGGAGCA 406
3089F	Coding	3089 GTGAAATCTGGAATACATTTA 3111
2648F	Coding	2648 ATTATACAGGATGTGACACTA 2670
386R	Non-coding	406 TGCTCCACATGCTCTGATA 386
1808F	Coding	1808 AATAGGCTGTTTCCTGATA 1828

¹ MCS = multiple cloning site

Table 2: **Table of primers utilized in the sequencing of *RpDazl*.** Note, the primer location specified is in reference to the cloned insert that was sequenced. A schematic representation of the location of these primers, again according to the cloned insert, can be seen in Fig. 8.

II. Animals, ovary, and embryos

A. Animals

Adult *Rana pipiens* were obtained from the J.M. Hazen Frog Farm (Alburl, Vermont). Upon receipt, the frogs were separated according to sex and were housed in plastic boxes with approximately two liters of water and 20 ml of a saline antibiotic solution (0.13mM NaCl, 20.1mM KCl, 13.6mM CaCl₂, 5.61mM Penicillin G, 1.37mM streptomycin sulphate). The frogs were stored at 4°C until needed. The water was changed every one to two weeks.

B. Ovary

Ovaries were surgically removed from female *Rana pipiens* that were anesthetized in 1g/l 3-aminobenzoic acid ethyl ester. The skin was cut on the frog's ventral side, to one side of the midline. The muscle wall underneath was cut, exposing the body cavity. The ovary was carefully pulled out of the body using forceps. Once enough ovary was outside of the body, the exposed piece was cut with scissors and immediately placed into ROM (*Rana* oocyte media: 100mM NaCl, 5.4mM Tris, 4mM sulfadiazine, 1.6mM KCl, 1mM CaCl₂, and 1.2mM MgSO₄). The females were either sacrificed after the operation or sutured and allowed to recover from the surgery.

The isolated ovary was cut into smaller pieces of 10-20 oocytes, rinsed twice in ROM, and placed into a petri dish of fresh ROM. The ovary was stored at 4°C for up to two days. RNA was extracted from the ovarian pieces as is, but any work to isolate the animal or the vegetal hemispheres of the oocytes required that the oocytes be defolliculated. For this, the ovary pieces were placed in Defolliculating Solution (116mM NaCl, 1.34mM KCl, 9.24mM Tris and 8 mM HCl, and 1mM EDTA) for at least an hour. After the ovary had soaked in this solution, the two membranes that tightly surround the oocyte were removed with Watchmaker's forceps. The defolliculated oocytes were rinsed a few times in defolliculation solution to ensure that all of the follicle cells were removed.

Cutting of the oocytes into animal one-third or vegetal one-third was carried out in a petri dish of P10EM (100mM PIPES, 10mM EGTA, 1mM MgSO₄, pH 6.9) using a sterile scalpel blade. The oocyte was placed on its side, and the portion to be isolated was sliced with one precise cut of the blade. Only one portion of the oocyte, animal third or vegetal third, was

isolated from each oocyte and approximately ten oocytes were cut at a time before they were carefully pipetted into sterile microcentrifuge tubes. Excess liquid was removed and RNA was extracted immediately (see below).

C. Embryos

All work with the embryos was performed in various concentrations of Steinberg's solution. A 20% Steinberg's solution is 11.6mM NaCl, 1.34mM KCl, 1.66mM MgSO₄•7H₂O, 0.67mM Ca(NO₃)₂ •4H₂O, 0.92mM Tris, and 0.8mM HCl. Typically a 200% stock of this was prepared and diluted as needed.

Embryos were obtained by in vitro fertilization. Two days before the fertilization, a female *Rana pipiens* was allowed to warm to room temperature. While the female was warming, a pituitary gland, removed surgically and stored at -20°C in 900 µl 200% Steinberg's solution, was thawed and supplemented with 100 µl (10 µg) of lutenizing hormone releasing hormone (LHRH: Sigma cat. no. L-4513). The pituitary gland was macerated with Watchmaker's forceps and sheared further with a low guage hypodermic needle. A higher guage hypodermic needle was used for the actual injection. A total volume of 1 ml was injected intraperitoneally. The female was then incubated at 18°C for two days, after which she was ready to give eggs.

A male *Rana pipiens* was sacrificed. The testes were surgically removed and placed in a petri dish containing approximately 5 ml 200% Steinberg's solution, and stored at 4°C for up to three days. The female's eggs will be of high quality for this amount of time, so the same female and the same male's sperm could be used over the period of three days. Each day, a half of a testis was isolated by Watchmaker's forceps and put into 5 ml 20% Steinberg's solution. The tissue was macerated with forceps to release the sperm. This was allowed to sit at room temperature for approximately 10 min before use. The motility of the sperm was checked by microscopic examination.

Once the quality of the sperm was assured, eggs were manually squeezed from the female directly into a 9 cm petri dish. The female was held with her legs straight with one hand, and her belly was gently rubbed in an anterior to posterior fashion with the other hand. The eggs should be delivered with ease from this position into a 9-cm petri dish. Once the eggs were in the dishes (no more than approximately 100 eggs per dish), 0.5 ml sperm solution was placed

atop the eggs using a 1 ml syringe without a needle. The dishes were incubated at room temperature for 10 min and then flooded with 20% Steinberg's solution.

The fertilized eggs are surrounded by a thick coat of jelly that must be removed in order to manipulate the embryos without difficulty. The outer two layers of jelly were removed using Watchmaker's forceps and the third, lying very close to the egg, was chemically removed. Sodium thioglycolate (15 g/L), pH 8.6, solution was prepared fresh and was used to remove the final layer of jelly. The eggs were swirled very gently in a beaker of this solution for 3-4 min and rinsed in 20% Steinberg's solution 5-6 times. Once the eggs were completely rinsed, they were moved back to petri dishes of 20% Steinberg's (10-20 eggs per dish) until they were needed. The embryos were stored at various temperatures including 18°C, 15°C, and 10°C to control the rate of development. The embryos were staged according to the normal table of Shumway (1940).

III. RNA isolation

RNA was isolated from live tissues using Trizol® (Invitrogen™). The same protocol was followed for all tissues except for *Rana pipiens* ovary for which special precautions had to be taken in order to obtain quality RNA (see below). Before isolation, all equipment was rinsed with RNase AWAY® and then with Diethyl pyrocarbonate (DEPC) treated water. After the equipment was dry, the tissue that RNA was to be isolated from was placed in either sterile microcentrifuge tubes or a glass homogenizer, depending on the volume of tissue. Ten volumes of Trizol reagent were added, and the tissues were thoroughly homogenized. If the glass homogenizer was used, the homogenate was transferred to microcentrifuge tubes at 1 ml per tube. The homogenate was incubated at room temperature for 5 min before 200 µl chloroform was added per 1 ml of homogenized tissue. The samples were shaken by hand for 15 sec and incubated at room temperature for 3 min. The samples were centrifuged for 10 min at 13,200 rpm. The top layer of the three distinct resultant layers was carefully transferred to a new sterile tube where 500 µl isopropanol was added per ml of sample solution. This was incubated at room temperature for 10 min and centrifuged for 10 min at 13,200 rpm. Following this centrifugation, the isopropanol was carefully removed, leaving the RNA pellet intact. One milliliter of 70% ethanol, made with DEPC treated water, was then added to the tube and the samples were

centrifuged for 4 min to wash the pellet. The ethanol was carefully removed and the RNA pellet was allowed to air dry.

Once dry, the pellet was resuspended in 50 μ l DEPC treated water, and an equal volume of 8M LiCl was added. This mixture was incubated overnight at -20°C. The following day, the samples were thawed and centrifuged for 4 min at 13,200 rpm. The supernatant was removed by pipette, and the pellet was washed with 70% ethanol and dried as before. The final product was resuspended in 50 μ l DEPC treated water. The pellet was sometimes resuspended in a smaller volume depending on the sample size.

When extracting RNA from *Rana pipiens* ovary, several adjustments were made to the protocol. First, all incubations and all centrifugations were performed at 4°C. Regarding Trizol, 40-50 volumes, as opposed to 10 volumes, were utilized in successful extractions. Treatment with LiCl was not performed. The final pellet was resuspended in 25 μ l 100% deionized formamide as opposed to DEPC treated water.

IV. cDNA preparation

cDNA was prepared by reverse transcription (RT) of RNA samples. First, the concentration of the RNA was determined by spectrophotometry, and all concentrations were adjusted to 67 ng/ μ l using DEPC treated water. 15.67ng or 15 μ l of the RNA sample were placed in a sterile microcentrifuge tube with 2 μ l of oligo-dT primer (10 μ g/ μ l). This portion of the experiment was always carried out with two trials of the same RNA sample so that a -RT control could be performed. The oligo-dT and RNA were incubated at 70°C for 10 min and cooled on ice for 5 min. During these incubation times, master mixes were made for both the +RT samples and the -RT samples. The master mix per sample consisted of 6 μ l DEPC treated water, 8 μ l 5x First Strand Buffer, 4 μ l 0.1M DTT buffer, 2 μ l 10mM dNTPs, 0.5 μ l RNase inhibitor (HPR-I), and 2 μ l MMLV-RT for the +RT master mix or DEPC treated water for the -RT master mix. Once the master mix was added to the RNA samples, the tubes were incubated at 37°C for 60 min followed by incubation at 99°C for 10 min. The tubes were quenched on ice for several minutes, and then stored at -20°C.

V. Northern Blot

A Northern blot was performed to determine *in vivo* transcript size. Several protocols were utilized in the two successful independent trials of the Northern blot that were performed. The RNA sample and formaldehyde gel preparation, electrophoresis, and RNA transfer to a membrane was performed utilizing the NorthernMax™ kit (Ambion®, Austin, TX). Radioactive probe was constructed using the Megaprime DNA labeling system (Amersham Biosciences). Prehybridization, hybridization, and filter washes were performed according to Sambrook, Fritsch, and Maniatis (1989) with several modifications. First, both the prehybridization and the hybridization were performed at 65°C in KGB buffer, room temperature washes were simplified to brief rinses, 2x SSC was utilized for all rinses and washes, and washes were performed for 30 min each at 65°C.

For the denaturing gel, one gram of agarose was melted in 90 ml of DEPC treated water. This was cooled at room temperature for approximately 10 minutes, after which 10 ml of 10x Denaturing Gel Buffer was added. The gel was poured and allowed to solidify under the fume hood, due to the presence of formaldehyde. The 10x MOPS Gel Running Buffer that was provided with the NorthernMax kit was diluted to 1x with DEPC treated water and used to fill the electrophoresis unit. RNA samples and the RNA ladder sample were prepared at the same time in the following manner. Ten micrograms of RNA or three micrograms of the RNA ladder were mixed with 3 volumes of Formaldehyde Load Dye provided by the NorthernMax kit, and ethidium bromide (10 mg/ml) was added to a final concentration of 10 µg/ml. The samples were heated at 65°C for 15 minutes. The liquid was collected by a brief centrifugation, and the samples were placed on ice until the gel was loaded. Electrophoresis was performed at 50 volts. After the gel was removed, a sterile RNase-free razor blade was used to trim the top of the gel. A photograph of the gel was taken on a transilluminator after trimming to view the RNA.

The blotting materials were set up. Two “buildings” were erected beside each other as described next. The first “building” began with an empty pipette tip box that measured approximately 6 cm in height. To the top of this, 3 cm of paper towels that had been cut in half were added, followed by three dry sheets of Whatman filter paper that were cut to the size of the gel. Two more sheets of filter paper were wet briefly in Transfer Buffer (provided by the kit) and added to the stack, followed by a pre-wet piece of the BrightStar™-Plus membrane that was

cut to the exact size of the gel and trimmed in one corner for orientation. The gel was then carefully aligned and placed on top of the membrane. Any bubbles that resulted were removed by rolling a sterile glass pipette across the top of the gel. Three more pieces of pre-wet filter paper were added to this first building on top of the gel. Three longer pieces of filter paper had also been cut to serve as a bridge from building one to building two. These were wet and placed on building one only after the second building had been erected. The second building was placed 7-8 cm away and was constructed with containers that measured approximately 6-8 cm in height. A square Tupperware container was placed on the top and Transfer Buffer was added (0.5 ml per cm² of the gel). The three filter paper bridges were pre-wet and added to building one, one side lining up with the gel and the other side placed in the Transfer Buffer on building two. A gel casting tray was placed on the top of building one, and a weight balanced atop the tray to be sure that all the building components were evenly in contact.

The downward capillary transfer was allowed to proceed for 2 hours, after which the buildings were carefully disassembled. The membrane was rinsed briefly in 1x MOPS Gel Running Buffer. The RNA was then irreversibly bound to the membrane by ultraviolet crosslinking. A photograph of the blot was taken by on the transilluminator to ensure high quality transfer, and then the blot was stored in a 50 ml conical tube at -20°C until hybridization.

A PCR performed using the previously designed primers for *RpDazl* (set B, see Table 1) and the plasmid that was utilized in the library screen as template DNA. The PCR product was electrophoresed on a 1% agarose gel and the resulting 328bp product was excised and purified using the Qiaex II Gel Extraction kit (Qiagen Inc). The concentration of the resultant DNA was determined by spectrophotometry. The protocol from Amersham for Megaprime DNA labeling was followed, with several adjustments. The protocol calls for 25 ng of template DNA to be used, but in both successful Northern blots, a 1 µl volume or 47.5 ng of template was used. The DNA template was combined with 5 µl of the monomeric primer solution that was provided in the kit and 27 µl of sterile nanopure water in a microcentrifuge tube, and incubated at 95°C for 5 minutes. A brief centrifugation was performed to collect the liquid, and the following was added to the tube: 10 µl labeling buffer, 5 µl ³²P-labeled dCTP, and 2 µl Klenow enzyme. This mixture was incubated on a 37°C heat block for 60 minutes. The probe was purified using a ProbeQuant™ G-50 Micro column (Amersham Biosciences) according to protocol. Thirty microliters of the purified probe was added to 100 µl of salmon sperm DNA (10 mg/ml) in a new

microcentrifuge tube and this was incubated at 100°C for 10 minutes, after which the mixture was placed on ice for 1 min.

When the time for prehybridization and hybridization came, the blot was placed in a glass hybridization tube and wet briefly with 2x SSC. Ten ml of KGB hybridization buffer was added to the tube. KGB buffer was prepared by adding 5 grams polyethylene glycol (molecular weight 7000-9000) to 17.5 ml 20% SDS and 3.8 ml 20x SSPE; the solution is brought to 50 ml with DEPC treated water. The blot was put into a 65°C rotating hybridization oven for 2 hours. Approximately thirty minutes into the prehybridization period, the probe was prepared as previously described. By the time the probe was prepared, the 2-hour prehybridization period had passed, and the KGB buffer was removed and replaced. The salmon sperm DNA and probe mixture was carefully placed into the fresh KGB buffer, and the blot was hybridized overnight in a rotating hybridization oven at 65°C. The following day, the probe-containing hybridization buffer was removed and the blot was rinsed several times with 2x SSC. Two washes at 65°C were performed with 2x SSC for 30 minutes each. The amount of radioactivity on the blot was checked with a Geiger counter, comparing the edges of the blot to the area where the RNA had transferred. The blot was carefully wrapped in saran wrap, avoiding bubbles, and exposed on a phosphoimager overnight.

To graph the results, a standard curve was prepared on semilogarithmic paper by measuring on the gel photograph, the distances from the top of the gel to the middle of each of the RNA ladder bands, and graphing these measurements on the X-axis with their corresponding molecular weights on the Y-axis. In order to be sure that all measurements were as accurate as possible, my measurements for the standard curve, the total size of the gel and the blot, as well as the distance from the top of the blot to the resultant band, were averaged with the blind measurements of three colleagues before graphing. The distance to the band on the blot was adjusted using the ratio determined from the total gel/blot size measurements, and the transcript size was determined by locating the adjusted averaged measurement on the standard curve.

Results

I. cDNA library screen

A. *Rana pipiens* ovary cDNA library

a. Verifying presence of cDNAs by PCR

The presence of *RpVasa*, *RpVegT*, *RpTFIII α* , and *RpDazl* in the Lambda ZAP[®] II Custom cDNA library of *Rana pipiens* ovary was examined by PCR. Table 1 shows the sequences and sources of primers used. For *RpDazl*, two primer sets had been designed from the sequence obtained by Y. Beckham, A and B, and all combinations of the primer sets were tested. The library was positive for these cDNAs (Fig. 2). For *RpDazl*, primer set B produced the brightest band, and so this set was utilized in PCRs for the remainder of the experiments.

b. Titering and plating

The titer reported by Stratagene for the library was 5.9×10^9 plaque forming units (pfu)/ml; the experimental titer was determined to be 4.5×10^9 pfu/ml. The library was plated using different volumes of several dilutions to determine optimal plating density. At optimal plating density, the resultant plaques were positioned close together, but not touching each other. Eleven μ l of a 1:1000 dilution of the library in SM buffer plated on a 14 cm NZY agar plate produced the plating conditions that were used for the primary library screen.

B. Primary library screen

The library was plated at the optimal plating conditions on 29 14-cm NZY agar plates. Optimal plating conditions yielded ~45,000 plaques per 14-cm plate. Twenty-four plates were selected for filter lifts. The first round of filter lifts resulted in filters 1-24, and the second round resulted in filters 1a-24a. Plates 1, 5, 9, 13, 17, and 21 were selected for a third filter lift and were named set "b". The following filters were selected for hybridization: 2, 2b, 5, 5b, 9, 9b, 13, 13b, 17, 17b, 21, and 21b. Because duplicates were chosen for hybridization, the six filters that were selected meant that ~270,000 plaques were screened for the primary library screen. For hybridization, 125 μ l hybridization buffer per cm² of filter is required, so for these 12 filters, 62.4 ml of hybridization buffer was needed. At 5×10^5 - 1×10^6 counts/ml buffer, 3.12×10^7 - $6.24 \times$

10^7 total counts in a radioactive DNA probe were needed to proceed. The *RpDazl* DNA probe was prepared through five independent radioactive PCR reactions, using the *RpDazl* FBRB primers, and plasmid DNA template containing the 386 nt clone obtained by Y. Beckham that was in pPCR-Script Amp SK(+) plasmid. The counts per minute (cpm) were determined for each reaction using a scintillation counter. The five reactions combined yielded 4.1×10^7 total counts, and hybridization of the resultant radioactive probe to the 12 filters selected for the primary library screen was performed. After exposure to X-ray film, potentially positive plaques were selected by examining the film on a light box and picking isolatable plaques that had a corresponding positive signal on the film. The primary screen filters were exposed to X-ray film twice, once for 71 hrs, and a shorter exposure of 20 hrs. Plaques were isolated and named from the plate from which they originated. From the first exposure, 10 plaques were picked: 2a, 2b, 5a, 5b, 9a, 9b, 13a, 13c, 17a, and 17b. From the second exposure, 8 potentially positive plaques and one plaque that was to serve as a control were picked, which had an isolatable plaque but no corresponding positive signal on film: control-C9, 5c, 9c, 9d, 13e, 13f, 21a, 21b, 21c. All plaques were isolated and resuspended in SM buffer as described previously. A 2 μ l sample of each isolated plaque was used as template in a PCR reaction using *RpDazl* primers (FBRB). A schematic flow chart demonstrates the plaques isolated from the primary library screen and which amplified by PCR and were subsequently selected for the secondary library screen (Fig. 3). This flow chart shows the same information for the secondary and tertiary screens as well. Plaques 9a, 13a, 17a, 5b, and 9c amplified in this experiment (Fig. 4). These 5 plaques were selected for the secondary library screen.

C. Secondary library screen

The five plaques selected from the primary screen were plated on 9-cm NZY agar plates at two dilutions, 1:100 and 1:1000. Filter lifts were performed on all ten plates, and six of the resultant filters were selected for hybridization. The filters of the plated 1:1000 dilution of all five samples, 9a, 13a, 17a, 5b, and 9c, as well as the 1:100 dilution of 5b were selected. To hybridize these 6 filters, 15.6 ml hybridization buffer was needed as well as a DNA probe that contained 7.8×10^6 - 1.6×10^7 total cpm. One 50 μ l radioactive PCR reaction yielded counts in the required range, and hybridization was performed as described previously. The filters were exposed to film for 20 hours. Using the five plates on which the 1:1000 dilutions of each sample

were plated, three plaques were isolated from each plate. The resuspended plaques were named as follows according to their plate of origin: 9a1, 9a2, 9a3, 13a1, 13a2, 13a3, 17a1, 17a2, 17a3, 5b1, 5b2, 5b3, 5b3, 9c1, 9c2, and 9c3. A 2 μ l sample of each isolated plaque in buffer was used as template in a PCR reaction using *RpDazl* primers, the results of which are shown in Figure 5. The seven samples that produced the sharpest bands (5b3, 9a1, 9a2, 9c3, 13a1, 13a2, and 17a1) were renamed A-G and selected for the tertiary library screen.

D. Tertiary library screen

Samples A-G were plated on 9-cm NZY agar plates at two dilutions, 1:100 and 1:200. The resultant plaques, in this screen, were countable. The number of plaques on each plate and the plate dilution whose filters were selected for hybridization are shown Fig. 6a. A DNA probe that contained 9.1×10^6 - 1.8×10^7 total cpm in 18.2 ml of hybridization buffer was needed to perform the hybridization on the seven chosen filters. One 50 μ l PCR reaction yielded enough counts, and the filters were hybridized and exposed to film as before. The number of positives on the film was counted and compared to the total number of plaques on the plates to determine a percentage of positives that each sample generated (Figure 6a). One plaque was picked from each plate, named A-G, and 2 μ l of each sample, after resuspension, was used as a template in a PCR reaction. All seven samples were amplified by PCR using *RpDazl* primers (Fig. 6b).

E. Sequencing sample selection

Concentrated phage stocks were prepared for each sample, A-G, from the tertiary library screen. A single clone excision was performed on each of these samples, and the resultant pBluescript phagemids were plated using SOLR cells. Eleven colonies were isolated from the plating: 1 from plate A, 3 from plate B, 2 from plate D, 2 from plate E, 2 from plate F, and 1 from plate G. The colonies were used to inoculate LB-Amp broth, and plasmid DNA was purified from the resultant cultures. A restriction enzyme digest was performed to determine the insert size of each sample in order to select the sample(s) that would be sequenced. The insert sizes ranged from 800-850 bp to above 3kb (Fig. 7). Sample 3, which had an estimated insert size of >3kb, was selected for sequencing, and was used to transform XLI competent cells. Colonies from the transformation inoculated into LB-Amp broth, and plasmid DNA was purified

from the resultant cultures. The concentration of the *RpDazl* plasmid DNA was determined by agarose gel examination to be ~100ng/μl.

F. DNA sequencing

DNA sequencing was performed on the selected sample using primers that enabled both the top, or coding, strand and the bottom, or non-coding, strand to be sequenced completely. The primers were designed as the sequencing progressed. See Table 2 for sequencing primer information and Fig. 8 for a schematic representation of the primer locations. The contiguous sequences were aligned and assembled into a consensus sequence using AutoAssembler™ 1.3.0, a global sequence alignment program available from Applied Biosystems. The entire consensus sequence of the clone contains the entire open reading frame of *RpDazl* and a 3' UTR of 2635 nt (Fig. 9).

The sequences of other available *Dazl* ORFs were obtained through the NCBI database, and, using the alignment program Clustal W, the *RpDazl* ORF was aligned with the other ORFs to examine homology at the nucleotide level. Homology among all the organisms can be seen starting at the *RpDazl* nucleotide 69. This is the RNA recognition motif, or RRM, which is approximately 200 nucleotides in length in the 5' region of the ORF (Fig. 10). The percent nucleotide identity of the ORF between *RpDazl* and the other species is approximately 60% and the percent amino acid identity is approximately 50% (Fig. 11a). Clustal W alignments of the translated *RDazl* ORF with *Xenopus laevis Dazl* (*XDazl*) and *Ambystoma mexicanum Dazl* (*AxDazl*) show amino acid identities at both ends of the putative protein (Fig 11b,c). Known domains are highlighted. *Dazl* proteins of several species were aligned using the Clustal W program to demonstrate the high level of conservation in the RRM (Fig.12). Another putative domain in *RpDazl*, the DAZ repeat, is shown as compared to the known DAZ repeat of *XDazl*, as published by Houston et al (1998) (Fig. 12b).

II. Size of the *RpDazl*

Because the 3' UTR of the *RpDazl* clone obtained is unusually long, a Northern blot was performed to determine whether the transcript length is equivalent to the clone size. RNA was obtained in two independent isolations from *R. pipiens* ovary, and two Northern blots were

performed (Fig. 13). The transcript size from the first Northern blot was 3.8 kb, and from the second blot was 3.3 kb. It should be noted that a measurement difference of 1 mm, when analyzed on the standard curve, made a difference in transcript size of 300-500 base pairs. Although the measurements were made as accurately as possible, this is most likely the source of error.

PCR was also utilized to show that there is *in vivo* continuity over the length of the *RpDazl* clone. Primers that were utilized in the sequencing of the clone, as well as two new primers, one 3' primer and one 5' primer, were used in this experiment. All of the primer sets utilized in this experiment amplified *RpDazl* plasmid DNA (data not shown). cDNA, prepared from *R. pipiens* ovary RNA, was then used as the template for PCRs, using the same primer sets. The results show continuity over greater than 3.25 kb of the clone obtained from the cDNA library screen (Fig. 14). Other primer sets were utilized in the same manner over the length of the clone using *R. pipiens* ovary cDNA as a PCR template. The following primer sets yielded bands of the expected size, listed by the exact nucleotides amplified followed by the product size in parentheses: 386-1346 (960), 386-1567 (1181), 386-2261 (1875), 386-2898 (2512), 640-1346 (706), 640-1567 (927), 640-2261 (1621), 640-2898 (2258), 1326-1567 (241), 1326-2011 (685), 1326-2261 (935), 1326-2898 (1572), 1808-2261 (453), 1808-2898 (1090), 1989-2898 (909), 2239-2898 (659), 2296-3266 (970), 2037-3028 (991) (data not shown). Controls were run in these experiments, some –RT controls, and some controls for primer interaction. Combining the results of the library screen, Northern blots, and the PCR continuity experiment above, it was concluded that the cloned *RpDazl* insert was full length.

III. Maternal expression of *RpDazl*

RT-PCR was utilized to confirm that *RpDazl* is expressed maternally. cDNA was prepared from RNA that had been extracted from the ovary of *R. pipiens* and PCR was carried out using the primers in Table 1. Amplification of *RpTFIII α* , *RpVasa*, *RpVegT*, and *RpDazl* occurred, verifying maternal expression (Fig. 15).

IV. Localization of *RpDazl*

A. Animal versus vegetal PCR

Information regarding the localization of *RpDazl* was determined by RT-PCR on selected portions of oocytes, examining the results for animal versus vegetal expression; this can provide insight as to whether or not *RpDazl* is maternally localized in the same manner as *XDazl*. The animal one-third and vegetal one-third of *Rana pipiens* oocytes were isolated and RNA extracted. The RNA was reverse transcribed to cDNA, and PCR was performed on the samples using *RpTFIII α* expression as a loading control. *RpDazl* is expressed mainly in the vegetal one-third of the oocyte, even when the *RpTFIII α* loading control shows that there was more animal one-third cDNA utilized in the experiment (Fig. 16).

B. RepFind

It has been shown that clusters of repeat sequences, specifically CAC or CAC-containing motifs, characterize the localization elements in the 3' UTR of many localized RNAs (Betley et al., 2002). RepFind is a computer-based program that searches a sequence for repeat clusters (Betley et al., 2002). Using this program to analyze the 3' UTR of *RpDazl*, clusters of CAC repeats were detected (Fig.17). These clusters, however, were not significant according to the program. A highly significant P-value is considered to be less than or equal to 2.1×10^{-6} . RepFind determined a P-value for the 3' UTR of *RpDazl* to be 3.21×10^{-4} . Using the same parameters to examine the 3' UTR of *XDazl*, a P-value of 6.37×10^{-7} was determined. The 3' UTR of zebrafish *Dazl*, *DEADSouth* of *X. laevis* are also shown in Fig. 17. *AxDazl*, mouse *Dazl*, chicken *Dazl*, and *C. elegans Dazl* were examined, and no significant clusters of CAC repeats were found, even under the least stringent conditions possible.

It must be mentioned, however, that when the parameters are made more stringent, the clusters of CAC repeats in the 3' UTR of *XDazl* are no longer significant. At these more stringent conditions, namely when using a database that contains more sequences, a significant P-value is considered to be less than or equal to 5×10^{-7} . The P-value for the 3' UTR of *XDazl* under these conditions is 2.9×10^{-6} , and for the 3' UTR of *RpDazl* is 7.68×10^{-4} . The implications of these results will be discussed in detail later.

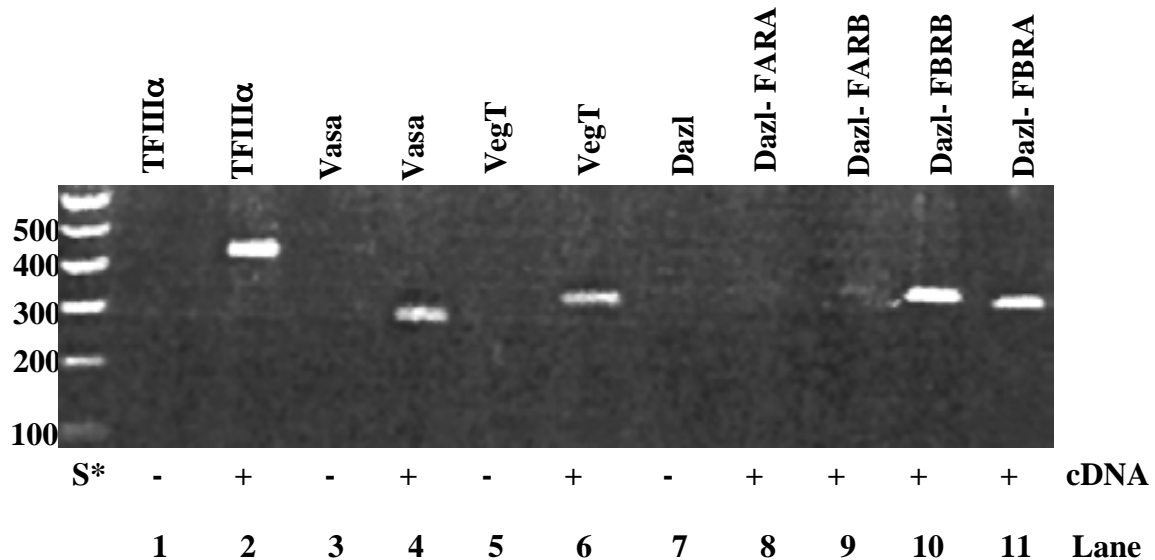

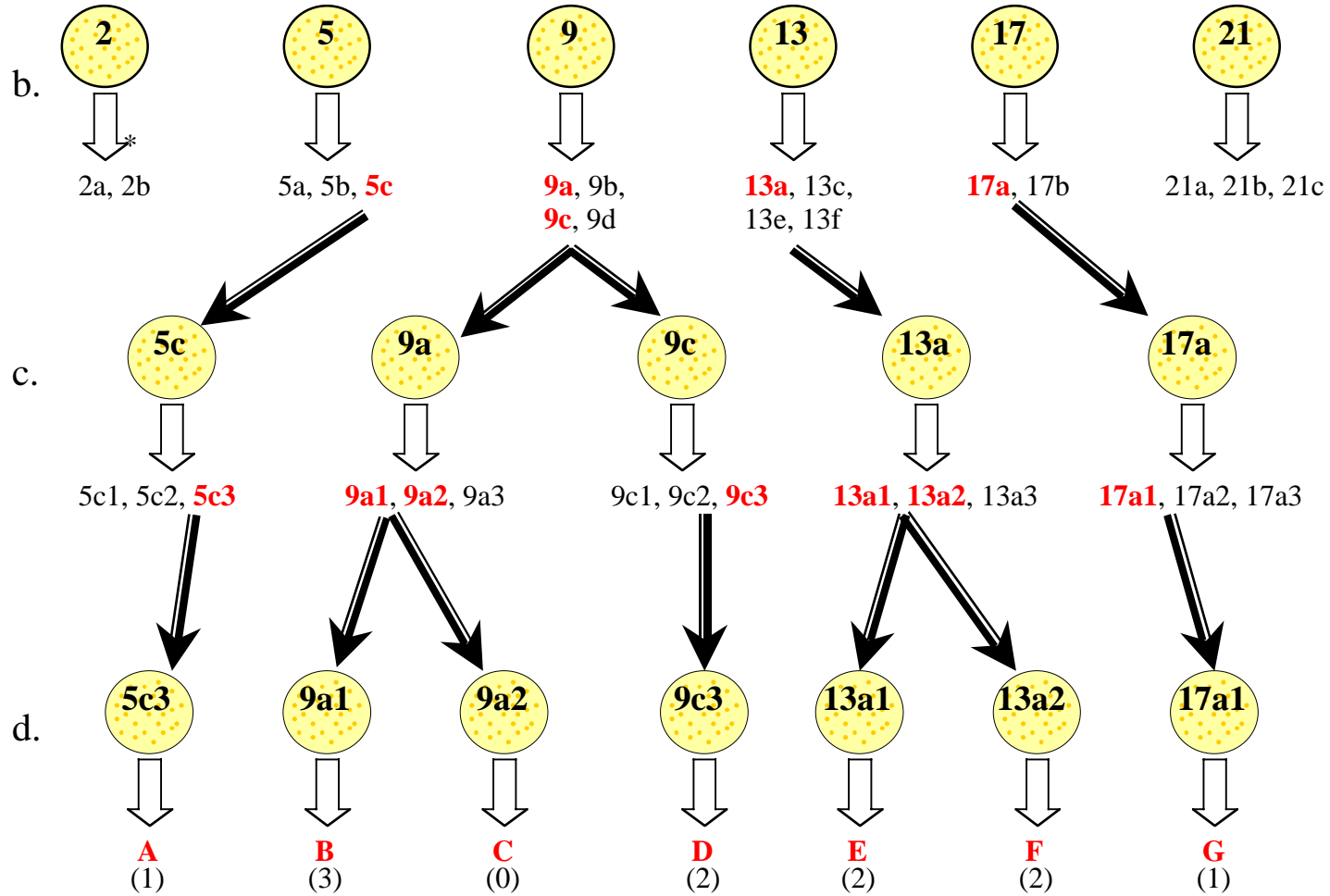
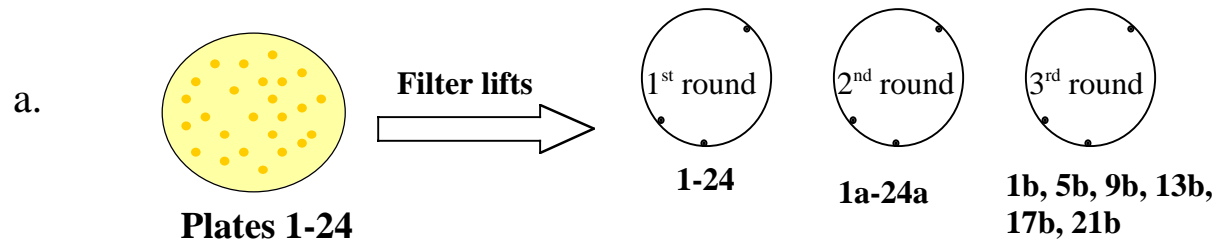


Figure 2: **PCR amplification of maternal RNAs, using the *Rana pipiens* ovary cDNA library as a template.** Primer information, including sequence and expected product sizes can be found in Table 1. Lanes 1, 3, 5, and 7 are PCR samples that contain the indicated primers, but no cDNA template. TFIII α is a regulatory element of 5s rRNA transcription (Nolte et al., 1998). *Vasa* and its homologs are members of a subfamily of DEAD-box RNA helicases that play important roles in translation. *VegT* encodes a T-box transcription factor that in *X. laevis*, is important for the specification of both mesoderm and endoderm as well as in the localization of other RNAs such as *Vg1*. *Dazl* plays a role in gametogenesis and fertility. In *X. laevis*, *Dazl* is important for primordial germ cell (PGC) specification and migration. Lane 7 is a PCR sample with two sets of *Dazl* primers (A and B), but no cDNA template. Information regarding both *Dazl* primer sets is located in Table 1. Lanes 8, 9, 10, and 11 are combinations of the two primer sets showing the expected *Dazl* product amplification with three of the four combinations; FBRB, FBRA, and a faint band from FARB. The MgCl₂ in this PCR reaction was reduced to one-third the amount normally utilized, and 35 cycles were performed with an annealing temperature of 50° C. All other temperatures and reagent concentrations were as are listed in the Methods and Materials.

S*= marker lane indicating product sizes in base pairs (bp)

Figure 3: Schematic of *R. pipiens* ovary cDNA library screen. (a) The library was plated on twenty-four 14-cm plates and three sets of filter lifts were performed, resulting in two complete sets of filters and a third incomplete set. Six filters from the first round of filter lifts and their duplicates from the third round were selected for hybridization for the primary library screen. (b) Eighteen phage-containing plaques were isolated from the six plates utilized for the primary library screen, the names of which can be seen below each plate. Those indicated in red type are those that amplified by PCR using *RpDazl* primers (Fig. 4) and were subsequently plated for the secondary library screen. (c) The five plates utilized for the secondary library screen yielded 15 potentially positive phage-containing plaques. The seven that yielded the brightest bands in a PCR using *RpDazl* primers (Fig. 5) were selected, as indicated by the red type, and plated for the tertiary library screen. (d) One phage-containing plaque was isolated from each of the seven plates utilized in the tertiary library screen and all amplified in a PCR using *RpDazl* primers, as indicated by the red type (Fig. 6). A-G underwent a single clone excision experiment and were used to transform SOLR strain *E. coli* cells. The number of colonies isolated from each sample as a result of the bacterial transformation is shown in () below each sample A-G. Restriction enzyme digests were performed on the 11 resultant plasmid DNA samples to determine cloned insert size, results of which can be seen in Fig. 7.


 = Filter lift, hybridization, X-ray film exposure, and
 plaque isolation



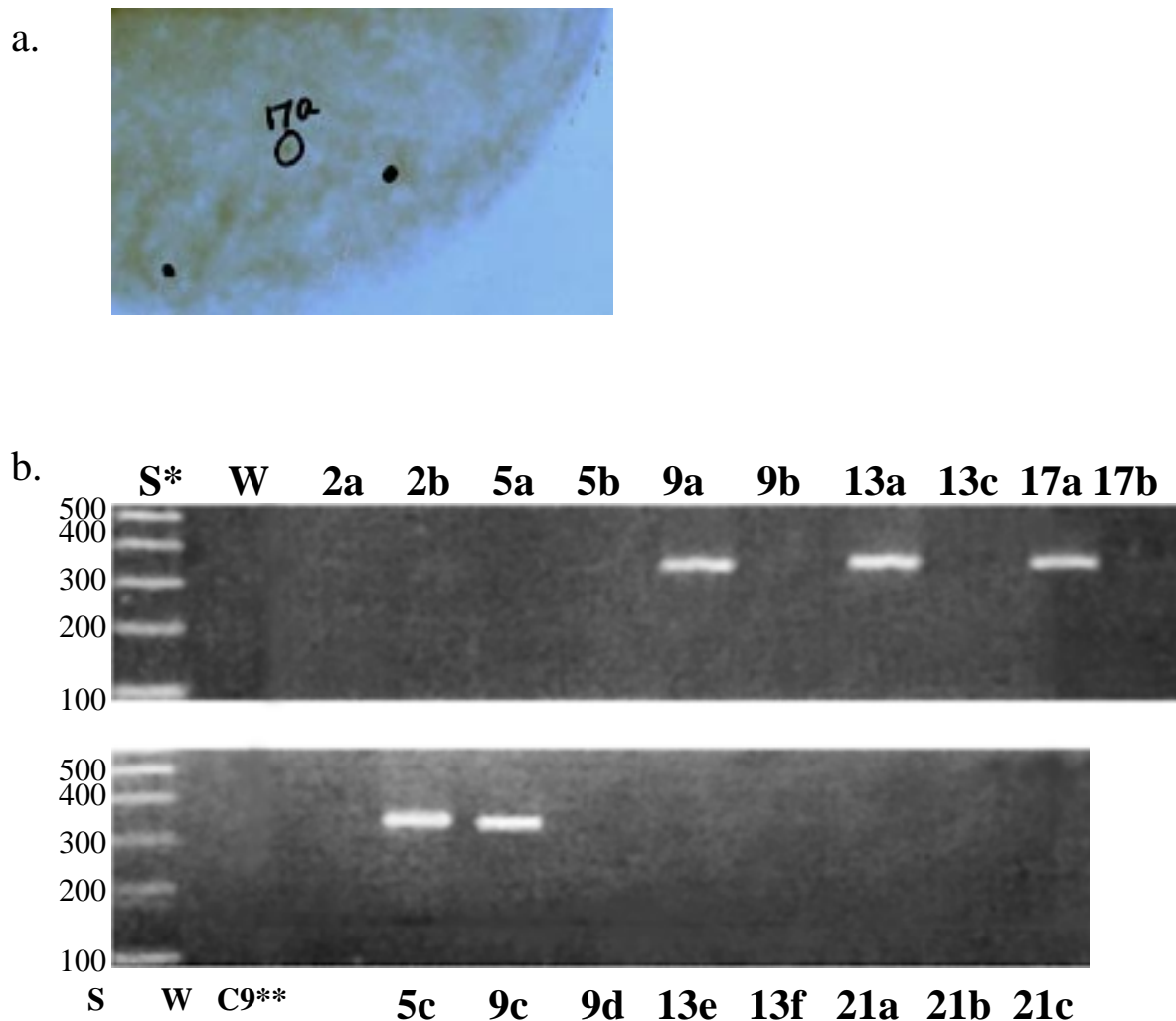


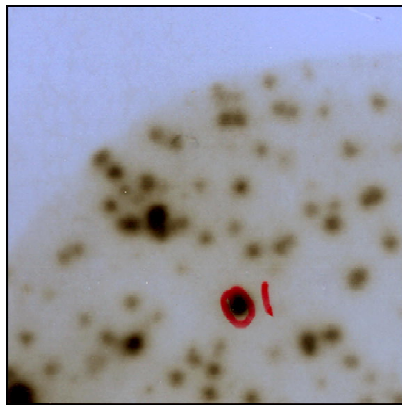
Figure 4: **PCR amplification using *RpDazl* primers of five phage-containing plaques isolated from the primary library screen.** (a) A portion of a film showing the signal from potentially positive *Dazl* clones. When this film was aligned with its corresponding plate, plaques that generated positive signals, such as the signal circled in black on the film, were isolated and resuspended in buffer. (b) A sample of the phage-containing buffer of each of the 18 potentially positive plaques was used as a template for PCR using *RpDazl* primers (set B, see Table 1). The five samples that amplified a product of expected size were selected and plated for the secondary library screen.

W (water)= control samples run with no cDNA

S*= marker lane indicating product sizes in base pairs (bp)

C9**= negative control; plaque isolated with no positive signal on the corresponding film

a.



b.

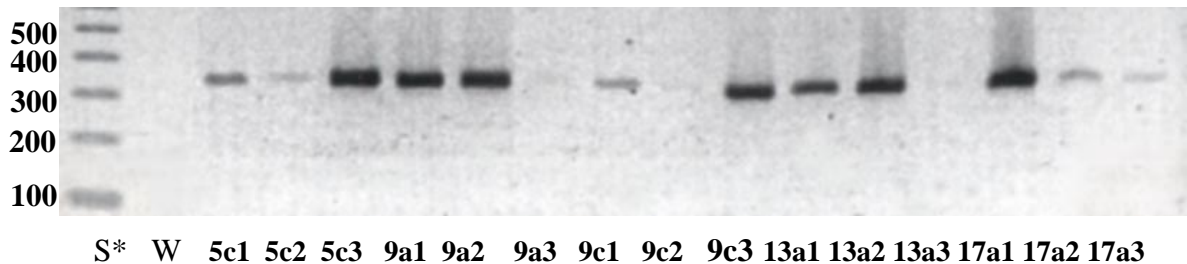


Figure 5: **PCR amplification using *RPDazl* primers of seven phage-containing plaques isolated from the secondary library screen.** (a) A portion of a film showing the signal from potentially positive *Dazl* clones. When this film was aligned with its corresponding plate, plaques that generated positive signals, such as the signal circled in red on the film, were isolated and resuspended in buffer. (b) A sample of the phage-containing buffer of each of the 15 potentially positive plaques was used as a template for PCR using *RpDazl* primers (set B, see Table 1). The seven positive samples were selected and plated for the tertiary library screen. Other samples, with a smaller amount of product of expected size, were not selected. Note that the gel is a color reversal of the original gel to increase visibility of the bands.

W (water)= control samples run with no cDNA

S*= marker lane indicating product sizes in base pairs (bp)

a.

Plate	Plaque count 1:100 dilution	Plaque count 1:200 dilution	Positive count: film of hybridized filter	Percent Positives
5c3	23	15	18	78%
9a1	81	24	20	83%
9a2	86	34	30	88%
9c3	42	26	24	92%
13a1	14	9	7	50%
13a2	29	20	28	97%
17a1	46	28	28	100%

b.

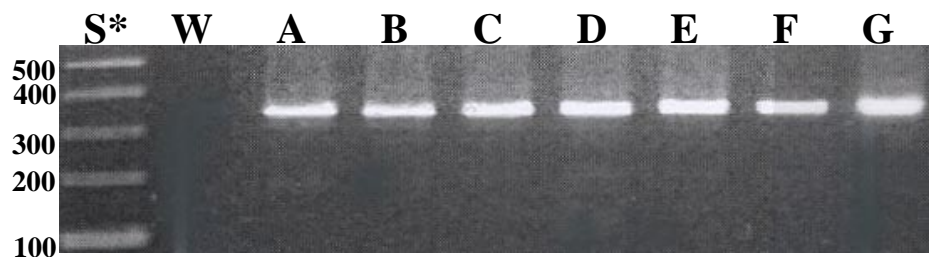


Figure 6: PCR amplification using *RpDazl* primers of seven phage-containing plaques isolated from the tertiary library screen. (a) Chart showing the number of plaques formed on each tertiary screen plate at two dilutions. The plaque count highlighted in turquoise represents the plate whose filter was selected for hybridization. The number of positive signals on the resultant films were counted (fourth column) and the percent of positives, film signals/ number of plaques, is represented in the fifth column of the chart. One plaque was isolated from each plate, resuspended in buffer, and named A-G. (b) A sample of the phage-containing buffer of each of the 7 potentially positive plaques was used as a template for PCR using *RpDazl* primers (set B, see Table 1). All seven samples amplified a product of expected size and were used for a single clone excision, followed by bacterial transformation. Eleven colonies resulted and plasmid DNA was purified from each.

W (water)= control samples run with no cDNA

S*= marker lane indicating product sizes in base pairs (bp)

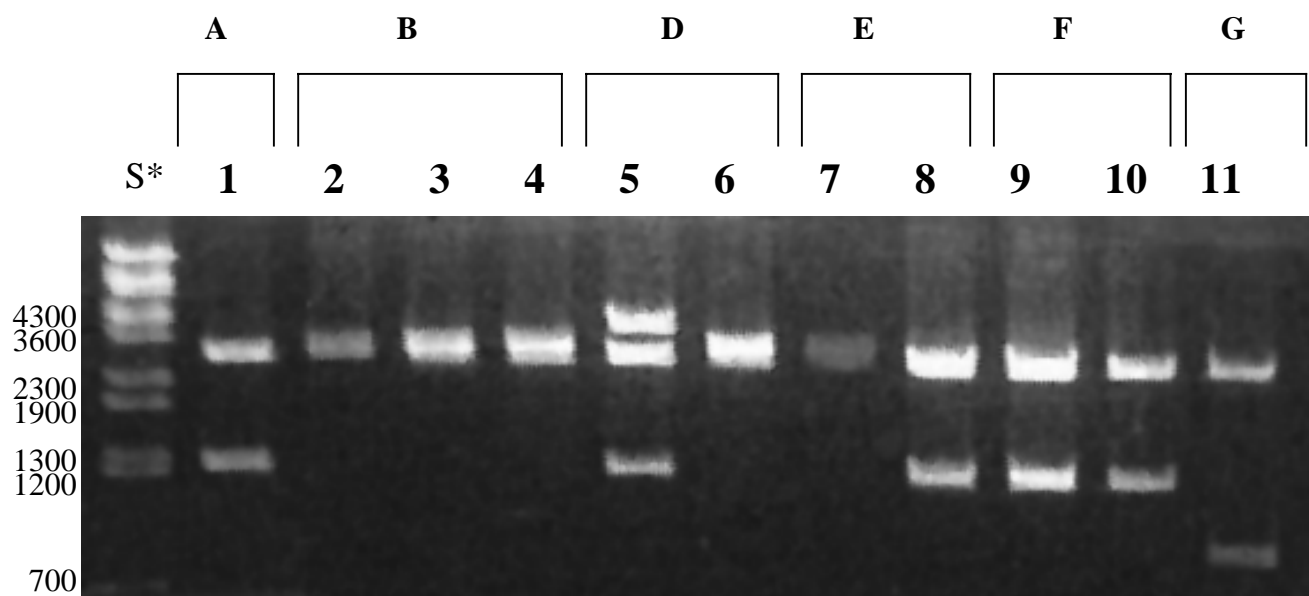
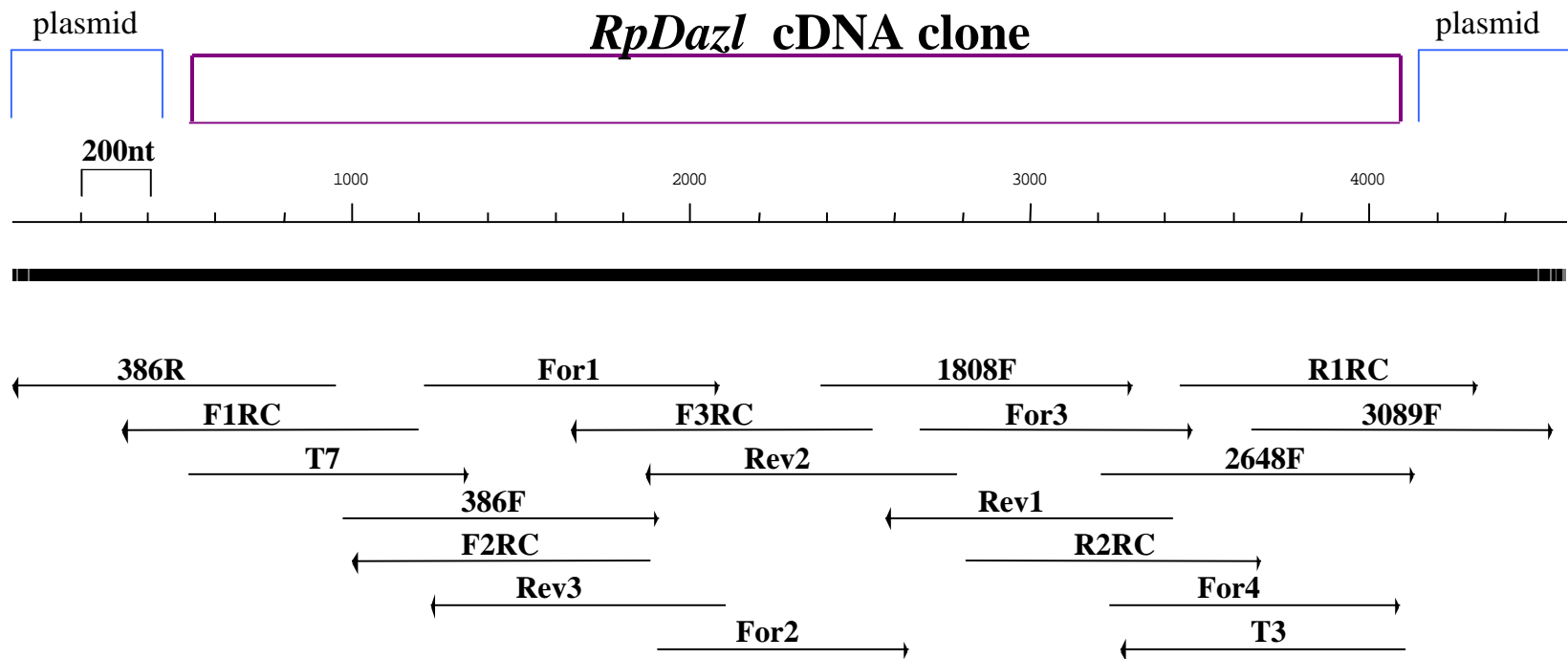


Figure 7: **Restriction enzyme digests reveal cloned insert sizes ranging from ~800 bp to >3kb.** Single clone excision and bacterial transformation yielded 11 colonies from which plasmid DNA was purified. Samples 1-11, whose origin is given above the gel (A-G)(see Fig. 3), were digested with *SpeI* and *Apal* to determined cloned insert size. Samples 1, 8, 9, and 10 appeared to contain an insert of ~12-1300 bp. Samples 2, 3, 4, and 6 appeared to be doublets, with insert sizes >3kb. Sample 5 yielded two bands, one >3kb and one of ~12-1300 bp. Sample 7 appeared to be an empty plasmid, and Sample 11 appeared to have an insert of ~800-850 bp. Based on insert size and plasmid DNA concentration, Sample 3 was selected for DNA sequencing.

S* = marker lane indicating product sizes in base pairs (bp)

Figure 8: **Schematic of primers used in *RpDazl* sequencing.** Primer names, size marker, and colored schematic above were added to an illustration provided by the global sequence alignment program, AutoAssembler™ 1.3.0. Primer information, including sequence, is located in Table 2. Arrows pointing to the right indicate coding strand sequencing, and arrows pointing to the left, non-coding strand. Nineteen primers were used in total, several of which were used to eliminate areas of ambiguity, to sequence the *RpDazl* cDNA clone of 3508 nucleotides.



```

1 ATATCGAATT CGGCACAGGG TTTGTGTTGA TGTCGGATAC AGGAAGTCCA ATGGAGGATC
61 AGCCATCTGG TTATGTGCTC CCTGAGGGGA AAGTTGTACC CAATACTGTG TTTGTTGGGG
121 GCATTGATAT CCGGATGGAT CAGCATGAAA TTAAAGACTA TTTTCAAAG TTTGGTTCAG
181 TAAAAGAGGT AAAAATAATT ACTGATCGCG CTGGTGTCTC CAAGGGATAT GGTTTGTCT
241 CCTTTTATGA TGACATCGAC GTTCAGAAGA TTTTAGATGT CAGCTTTCAT GGCAAGAGAC
301 TAAACTTGG ACCAGCTATA AGAAAGCAAA GTACATGTGC ACACATTCAA CCAAGAAGTG
361 TGGTGCTTAG CACCCAAGCA CCACAATATC AGAGCATGTG GAGCACCCCT GTCACCGATG
421 CATATGTTCA GCCTACTCCC ATGTTTAGTC CTATGGCCTC TTATATTGAG TTTGTCTCT
481 ACCCAAATC ACCAGTCATG TTGCAGCAAA TACCCTTGGC ATACCAGCAG CCAGGATGTT
541 TCCAGGTGGC TCCACAGTGG GCTGGTGGAG ATCAAAGAAG CTATGTTTTG CCTCAGGCTT
601 TAACTGGAA TTATACAGGA AATGACTTTG AGACAGCCTG TGGTGAAGTG ATTCAAGCAG
661 ACTATACAAT CCCAGAGCAA AATGCCTTAG TTTCAACAAG TCCCCAAAAG AAAATTGACC
721 GAAGCATACA AACTGCGTGA TCCTGTCTGT TTTCTGGCGA TGAAGGTTT CACAAGCCTT
781 TTATTCAAGA TGATTTTCATG AAGGACAGAA GAGTGCATCA GTTTAGAAGA AGAGCCATGT
841 TTAGGCCAC CATGGAGAAA TCTGGACTGT AAAACTGCAC CTACCACTAA TGTCCACAAT
901 GTAGCACTG TAGTTTACCT CCTAAGATTT GTTAGTAATG CTCTTCATAC AGTACCATCC
961 AGCTCAAGTT TTGGTTCGTA CATTGTGCCA GTCTATCATG AATCAGAGCT TGGACCATCA
1021 ACTGGCAATA TGCCTTTACC TCAAGTATAT CCATCTTAAA AATACGTTGG CGTAGTGTGG
1081 TATACTGTAA AACACTACCC TTGGGTTTCT GGGTTTTGTT AGATATAAAC ATTCAACTGT
1141 TGGTCTGCA TTGTTTTACA CATTTTACAC AATTTAAGC AAAGGGCACA TTTAATATTT
1201 TCAAATGTTT TTTTTTTTTC TTAAGCATGT TCTGTGTGTA AAAATGTTCT TATGTGTTCT
1261 TGTTTTTCAT AGCCAAACAC GTATGTGTTT ACTGTTTTGC CGTGCTTTT CACTCTAAT
1321 AGFTGATGC TTATCTTCAC TCCCTGTGTG CTTTGTCTCT GGGTTTTGCT TTTGGCACCC
1381 ACCTATTACC ATTCACTTGT ATGTTGTCCC TACAAATGTG TTTGCACCTA TTTATACCTC
1441 TCAGTTGTGT AATTGTCTCC ATAGTGTGTT TTATGCATCC ACTTCCACTT GTATGACCCC
1501 ATGCGTTTAA TTTTAGGCAC TCATTTCAAC TTTTTTTGGT TTGAGTGATG GCTAGTTAGG
1561 AATGTATAGT AATAAGTACT TTTTATATAT TCACCCTTTT CGTCTCTCG TTTTTCATCGG
1621 ACATAAATTT CACATTTCTG AATGTTCTAT GTATATTGTG GTTCTAAGAC ATGATTGGCT
1681 TGCCAGAATT GTTAGATATC CTGGAACAGT CTGACTTCAG CTGCAAGTTT TATTTTCCAG
1741 CTAAAAAGCC CTTTACAACA TGAGTAAACT CTTCTCACAC TGCATACAAA ACTTATACTA
1801 AATCTGTAA TAGGCTGTTT CCTGATAGCT GACAAAAGTCA AACCGAAAGC AATGCAGTGG
1861 AAAAGTGGTT CTGCTATACC ATGGAAGCTA AGAGGCAGCT TGTTTCTAT AGAAAAAGCTA
1921 TAGCTGAAAG CAAGCAAATA TGGAAAGTAA AATTCTACAG TTATAGTTCA GACAATATCT
1981 CTTGGGCCTG TATTGACTAT TGATGCTGAG CTGAGAACGC CTGCTTAGCT TGTAAACACC
2041 TTTGGCGCAA GTTAGGTGCT TCCACAGGCA GTGTTACGTT TTGCTGAGGT AACACGGCCT
2101 TTCATCACTT TGTAGTGACA TTGGTTAAG TGTATTTTGA CTATCGGTG TGTAAAGTTAC
2161 CTTCTTTTAG CAGTGTTTTC CGGTAAATTT CCATTAGTAA TAGAGCTTTT AAGACCATTT
2221 TTTTAAATA TATATGCATG ATCCTGGAAT TAAGAGCAAT GTGTGCTGTA AACGAAGTAG
2281 TGTGACTACA GAACCAAATC CTCAGTATCC CGGCTATGTT TTCCAAGGGC ATGCATTTTA
2341 TTTCTTACAT TGTAGCTGCA AATTATGTTG ATTTTAAATG GATTTTAAAG TAGTGCATTT
2401 CCGTAAACTT TTTTGAAGC AAATTGGACA AGTTACATTT TTATGCTCAG CCTGAAGACT
2461 TGTGATGAGC ATATTTTACA ATTTGCCTCA AAAATTATGGA ATTAAATTTT CCGCAGCAAG
2521 GTTAAAGTCTT GCCCAATTAA TAATATAAAT GCACAAGAAA TTGCATTCTT GCATTAGAAT
2581 TTACTGACCA TTAGATTTTC TTTGCAAGTGT GGCATAGCTT CCAAGTTAAC TGTGACGATC
2641 AATATAAAAT TATACAGGAT GTGACACTAC TGTGCAAGCA TCTACCAGTG GCTAAACATA
2701 CCTGATATCC TCCCCTGTGT TCTGGTTGCA TTTAATGTCA GCACATCTGA TAGCTTTGCA
2761 CAATCACCCA CTGCCTAATG CATGTGCATT GGTTTTACTG GTTAATGGGA ATGGTGTGGT
2821 AGAAAGTGGG AACACTTGAC GACATGTCAA AATTATGCAG CATTCTAGGA TTTGGAGCAT
2881 ATCGATTGCT TGGGATGCCA TCTAAGGATG TGCTAGTTAT TGCACTGTAG TCTGTGGAC
2941 TTTTAAACATA TTTTATACTG TCATGTTTAC TTTAAAGGGG GTATCCTACA CATGACTGGC
3001 GGCTTCATGT AGTAACATAT ATTACTACTT CCTGCAGCTT CTTGGTTTTA CTAGGTGATG
3061 AAATTGATTT TCATCTGCAG TGCATCAATT TGTGAAATCT GGAATACATT TAGTACTTGT
3121 TTTGTCTGTA ATAAAAATGT CACTCGTACC TATGTCTGCT TCTTCTTTC CTAACATTTT
3181 TTTGAGTGGG AACTGTTTAC TAGTCATTCA GCTATGTGAT CCGTGACACT TCACTATGTA
3241 AAAGTTTAAA CCCAAAATG GTGCGCTGCA ACATCATTCC GGAGTCTAAT GCTGGTGGAT
3301 TTTATTTAAA TCTGGAATCC TGTTTAAATC TATCATGACA GTGTGACTTT GTATGTACCT
3361 CTTTGGCAG TATTTAAAAA AAATCCCTTA AATGTGTCAG AATATTTCTT TGTAACTGTG
3421 TCTCTTAAATA CTGTTTTAGA TATTATTTGA CTGGAACCA ACATCTTTGT ATGACCACAT
3481 AATAAAGAAA ACCTAATTTG AAAAAAAA

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Figure 9: *RpDazl* cDNA clone sequence. Sequence from the coding and the non-coding strand were aligned using the global sequence alignment program, AutoAssembler™ 1.3.0. An open reading frame (ORF) was determined from the first methionine or ATG at nucleotide 30 to the first stop codon in the same frame (TAA at nucleotide 870). This ORF, highlighted in purple, was the only ORF of significant size determined in this or any other frame. A poly-A consensus sequence is seen at nucleotide 3481 highlighted in turquoise, 14 nt upstream of a string of adenines.

```

mouse -----ATGTCTGCCACAACCTTCTGAGGCTCCAAATTCAGCTGTCTC 41
human -----ATGTCTACTGCAAATCCTGAAACTCCAAACTCAACCATCTC 41
Gallus -----ATGTCTG---CAAATGCGGAAGCCAGTGTGGAAGTATCTC 38
Rana -----ATGTC 5
Xenopus -----ATGTCTGGAAAAGAAGAATCTT 22
Axolotl -----
Danio ATGGTTCAGGGGGTTCAGTTACCCGTGTGCTGATATGTGGTTTGTATTACAGGATATC 60

mouse CAGGGAGGCCAGCACTCAGTCTTTCATCAGCAA-CCACAAGTCAA-GGATATGTTTTGCCA 99
human CAGAGAGGCCAGCACCCAGTCTTCATCAGCTG-CAACCAGCCAA-GGCTATATTTTACCA 99
Gallus ---AGAGGATAATACCCATTCGTCA-----A-CAACCTGCCAA-GGATATGTTTTACCA 87
Rana ---GGA---TACAGGAAGTCCAATGGAGGAT-CAGCCATCT---GGTTATGTGCTCCCT 54
Xenopus ---CAAATATGCAGCCACTGCAGAGGAGGAAGCAGTTAACCAA-GGGTTTGTGTTGCCA 78
Axolotl -----
Danio CAGAAGCATCGTCAGGGTTTTCCGTCTCCCTGAAGTTGTCTAACGGTTACATTTTACCT 120

mouse GAAGGCAAAATCATGCCAAACACCGTTTTTGTGGAGGAATTGATGTTAGGATGGATGAA 159
human GAAGGCAAAATCATGCCAAACACTGTTTTTGTGGAGGAATTGATGTTAGGATGGATGAA 159
Gallus GAAGGAAAAATCATGCCAAATACAGTCTTTTGTGGTGGAAATTGATATAAGGATGAATGAA 147
Rana GAGGGGAAAAGTTGTACCCAATACTGTGTTTGTGGGGCATTGATATCCGGATGGATCAG 114
Xenopus GAGGGTGAATCATGCCAAACACAGTGTTTGTAGGAGGAATTGATATTACGATGGATGAA 138
Axolotl -----ATGCCAAATACAGTCTTCGTGGTGGAAATTGATATCAGGATGGATGAA 48
Danio GAGGGGAAAATGACGCCAACACACTGTTTCGTGCGCGGTATTGATATGAAGGTGGATGAG 180
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

mouse ACCGAAATCAGGAGTTTCTTTGCCAGATATGGCTCAGTAAAAGAAGTGAAGATAATCACT 219
human ACTGAGATTAGAAGCTTCTTTGCTAGATATGGTTCAGTGAAGAAGTGAAGATAATCACT 219
Gallus GCAGAAATTCGGAGTTACTTTGAACAATATGGTACTGTGAAGGAGGTGAAAATAATCACT 207
Rana CATGAAATTAAAGACTATTTTTCAAAGTTTGGTTCAGTAAAAGAGGTAAAAATAATCACT 174
Xenopus ATAGAAATTCGGGATTTCTTCACACGTTTTGGAAAACGTGAAGGAAGTTAAAAATCACT 198
Axolotl ATTGAAATTCGTGATTTTTTTGCAAGATTTGGTGTGTGAAAGAAGTTAAAAATAATCACT 108
Danio AACGAGATCAGGGAATTTCTTTGCCAAGTATGGCTCAGTGAAGAAGTTAAAAATCACT 240
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

mouse GATCGAACTGGTGTGTCGAAGGGCTATGGATTTGTCTCATTTTATAATGACGTGGATGTG 279
human GATCGAACTGGTGTGTCCAAAGGCTATGGATTTGTTTCATTTTATAATGACGTGGATGTG 279
Gallus GACAGAACTGGTGTTCCAAAGGGTATGGATTTGTTTCATTCCTGGACAATGTGGATGTT 267
Rana GATCGCGCTGGTGTCTCCAAGGGATATGGTTTTGTCTCCTTTTATGATGACATCGACGTT 234
Xenopus GACCGGACAGGAGTGTCCAAGGGTATGGCTTCATTTCTTTTCTGATGAGGTGGATGTC 258
Axolotl GACCGGACTGGTGTGTCTAAAGGGTATGGATTTGTTTCTTTTCATGATGATGTTGATGTG 168
Danio TATCGAGGAGGAATTTGCAAAGGATATGGTTTTCGTTTATTTTCAGTGAGGATGTTGATATC 300
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

mouse CAGAAGATAGTAGAATCACAGATAAATTTCCATGGTAAAAAGCTGAAACTGGGCCCTGCA 339
human CAGAAGATAGTAGAATCACAGATAAATTTCCATGGTAAAAAGCTGAAACTGGGCCCTGCA 339
Gallus CAAAAGATAGTAGAATCACAGATCAGTGTCCATGGAAAAAGCTGAAACTGGGACCAGCA 327
Rana CAGAAGATT-TTAGAT----GTCAGCTTTCATGGCAAGAGACTAAAACCTGGACCAGCT 288
Xenopus CAAAAGATTGTTAAGTCGCAGATAAGTTTCCATGGGAAAAAACTGAAACTGGGCCCCGCC 318
Axolotl CAGAAGATTGTTGACTCACAATAAATTTTCCAGGAAAAAACTTAAACTGGGACCAGCA 228
Danio CAGACTATCGTTGATCAGCCGATCAGTTTTAAAGGGAAAAAACTCAAACCTGGGACCTGCA 360
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

mouse ATCAGGAAACAA---AATTTATGTACTTATCATGTGCAGCCACGTCTTTGATTTTTAAT 396
human ATCAGGAAACAA---AATTTATGTGCTTATCATGTGCAGCCACGTCTTTGGTTTTAAT 396
Gallus ATTAGAAAACAAC--AAAACCTGTGTTT- TTACATGCAGCCTAGACCATTGGCTTTCAAT 384
Rana ATAAGAAAGCAA---AGTACATGTGCAC---ACATTCAACCAAGAAGTGTGGTGCTTAGC 342
Xenopus ATTCGAAAA-----ATTTGTACTT---ATGTGCAACCTAGACCTGTGGTGCTTAGC 366
Axolotl ATAAGAAAACAGCAGAACCTATGTGCTATCATGTACAACCAAGACCTATGGTGTAAAT 288
Danio ATCATGAAAGAGCGAAGTTCTCGGTCAG---TGT-CATCTCCAATGATTGGTCCATCAC 415
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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mouse	CCTCCTCCTCCACCACAGTTCAGAGTGTGGAGTAGTCCAAATGCTGAGACTTACATG	456
human	CATCCTCCTCCACCACAGTTCAGAAATGTCTGGACTAATCCAAACACTGAAACTTATATG	456
Gallus	---CCTCCTGCACCGCAATTCATAGCGTATGGACTAATCAAATAACAGAGACCTACGTG	441
Rana	---ACCCAAGCACCACAATATCAGAGCATGTGGAGCACCCCTGTCACCGATGCATATGTT	399
Xenopus	---CACCTACTCCATTCCACCA---TGCATGGAAACAATCAAATGCAGACTCTTACATT	420
Axolotl	---CCACCTGCCCCACAGTTCACAGTATGTGGAGTAGTCCAAATGCTGAAACGTACATG	345
Danio	AGTGGGTA AACCCACCCCAT --- ATATGTACTGCAGCTGCTGCTCCTCCAGGCCTGGCC	471
	* * * * *	
mouse	CAGCCTCAACCATGATGAATCCTATCACTCAGTATGTTTCAGGCATATCC---TCCTTAT	513
human	CAGCCCAACAACCACGATGAATCCTATAACTCAGTATGTTTCAGGCATATCC---TACTTAC	513
Gallus	CAGCCTCAAGCTGTGGTGAGCCCACTAACTCAGTATGTCCAGACGTATGCGTACAGTTCA	501
Rana	CAGCCTACTCCCATGTTTGTAGTCCATAGGCCTCTTATATTTCAGGCTTGCTTACCCAAAC	459
Xenopus	CAGCATCTCCCATAGTCAGCCCTATTACTCAATATGTTTCAGGCTTGCCCATATCCAAGC	480
Axolotl	CAGCACCACACATGATCAGCCAGTTCCTCAGTATGTACCGGCATATCCATACCAGAGT	405
Danio	CCACCATCACCCGTATTCAGTGGAGGAAATCAGTACATGCAGCCTTATTCTACTCCAGT	531
	* * * * * * * * * * * * * * * * *	
mouse	CCAAGTTCACCAGTTCAG-GTCA--TCACTGGATATCAGCTGCCTGTT---TATAACTAC	567
human	CCAAATTCACCAGTTCAG-GTCA--TCACTGGATATCAGTTGCCTGTA---TATAATTAT	567
Gallus	CCAGCTGTATTGATACAGCAGCAAGTTCCTGTAGGATATCAGCCAGCA---TACAACATAT	558
Rana	TC---ACCAGTCATGTTGCAGCAAATACCCTTGGCATACCAGCAGCCA---GGATGTTTC	513
Xenopus	TCTCCACCAATGGCGATTTCAGCAGATTCCTGTAGGCTGCCAACCACT---GGCTACTTT	537
Axolotl	TCTCCACCAGTAATAATTTCAGCAAATTCAGTAGGATATCAGCAGCCAACTTACAATTAT	465
Danio	CCTCCAGGAATTATGGTTCACAGGTGCCAATGAACTATGCACAGACCAGTATGCCTAT	591
	* * * * * * * * * * * * * * * * *	
mouse	CAGATGCCACCGCAGTGGCCTGCTGGAGAGCAGAGGAGTTATGTTATACCTCCGGCTTAT	627
human	CAGATGCCACCACAGTGGCCTGTTGGGGAGCAAAGGAGCTATGTTGTACCTCCGGCTTAT	627
Gallus	CAGGCTCCACCACAGTGGGTTCTGGGGAGCAAAGAACTACGTTATGCCTCCGGTTTTAT	618
Rana	CAGGTGGCTCCACAGTGGGCTGGTGAGATCAAAGAAGCTATGTTTTGCCT---CAGGCT	570
Xenopus	CAGGTATCCCCACAGTGGCCTGCCG---ATCAACGGAGCTACATGTTTCCCACACCGGCT	594
Axolotl	CAGTTGTCTCCACAGTGGGCACCAGGAGAACAGAGGGGTTACATCATTCCACAGGCTTAT	525
Danio	CAGTATCCCCTGCCACAGTGGTGTGGAGAGCAAAGGACGAGGCTTGTCAATCAGAATTTT	651
	*** * * * * * * * * * * * * * * *	
mouse	ACA-ACTGTTAATTACC-ACTGCAGTGAAGTTGATCCAG---GAGCTGATATTTTGCCCA	682
human	TCA-GCTGTTAATTACC-ACTGTAAATGAAGTTGATCCAG---GAGCTGAAGTTGTGCCAA	682
Gallus	ACT-TCAGTAAACTATC-ACTACAGTGAGG---ATCCAG---AA-----TTTATACAAA	664
Rana	TTA-ACTTGGAATTATA-CAGGAAATGACTTTGAGACAGCCTGTGGTGAAGTGTTCGAGG	628
Xenopus	TTC-ACATTCAATTACC-ACTGCTGTGATATGGATCCAAACGGAGGTGAACCTATTCCAA	652
Axolotl	GCT-GCTGTTAATTATC-ACTGTACTGAATTGGATCAACCAGGAGCTGAACCTTATCCAAG	583
Danio	GTGGATTGTGGAGTGCAGACTTTGCTAACCCCTTATGTAG-----	690
	* * * * * * * * * * * * * * * * *	
mouse	ATGAATGTTTCAGTTC---ATGATGCTGCTCCAGCTTCTGGAAATGGCCCGCAAAGAAGT	739
human	ATGAATGTTTCAGTTC---ATGAAGTACTCCACCCTCTGGAAATGGCCCAAAAAGAAAT	739
Gallus	CAGAATGTGCTGTCC---CAGAGCCACACAGATGTCTGGTAATAGTCCACAAAAAAGT	721
Rana	CAGACTATACAATCCAGAGCAAATGCCTTAGTTTC---AACAAAGTCCCCAAAAGAAA-	684
Xenopus	GAGAATATCCTATT---GATCAGACAGTGTCTGCTTCTGGAGCCAATCCACAGAAGAGAT	709
Axolotl	CAGAATTTCTGTTTATGAACTGCTCAGCCATCTTCTGGAAACAGCCCTCAAAAAAAT	643
Danio	-----	
mouse	CTGTGGACCGAAGCATAACAGACAGTGGTCTCTTGTCTGTTTAAACCCTGAGAACAGACTGA	799
human	CTGTGGACCGAAGCATACAAACGGTGGTATCTGTCTGTTTAAATCCAGAGAACAGACTGA	799
Gallus	CTGTGGACAGGAGCATACAAACAGTAGTATCCTGTCTGTTTAAACCCTGAAAACCGTCTGA	781
Rana	--ATTGACCGAAGCATACAAACAGTGTATCCTGTCTGTTTCTGGCGATGGAAGGTTCT-	741
Xenopus	ATGTGGAAATGAGTACCCAGACTATTGTATCCTGCTTGTGTTGATCCAGCAAACAATTC-	768
Axolotl	CTGTGGACCGAAGCATTCAGACAGTGTATCCTGCTTGTTCACCTCCTGAGAACAGACTGC	703
Danio	-----	

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mouse      ---GAAACTCTCTTGTACTCAAGATGACTACTTCAAGGATAAAAAGAGTACATCACTTCA 856
human      ---GAAACTCTGTTGTTACTCAAGATGACTACTTCAAGGATAAAAAGAGTGCATCACTTTA 856
Gallus     ---GGAACACCTTTGTATCACAAGAAGACTACTTCAGGGAGAGGAGGGCGCATCACTTCA 838
Rana       -----CACAAAGCCTTTATTCAAGATGATTCATGAAGGACAGAAGAGTGCATCAGTTTA 796
Xenopus    -----CATTCAATTTGTTTCACAGGAGGACTACTTAAAGGACAATAGAGTGCACTTGA 823
Axolotl    CGAGGAACTCTTTTGTATCTCAGGAGGAATACATGAAGGACAAGAGGGTGCACCAGTTTC 763
Danio      -----

mouse      GAAGAAGTC---GGGCAGTGCTTAAATCTGATCATCTCTGCTAA--- 897
human      GAAGAAGTC---GGGCAATGCTTAAATCTGTTTGA----- 888
Gallus     GAAAAGGAA---GAGCAGTGCTCAAAAGTGTTTGA----- 870
Rana       GAAGAAGAGCCATGTTTAGGCCACCATGGAGAAATCTGGACTGTAA 843
Xenopus    GGAGGAGGG---AGTCTG---TTATCA-AGAGAGTTTCCAAATGA-- 861
Axolotl    GTAGAAGTA---AAGCTGTGTTCAAACACTGTGCCAAACTGA----- 801
Danio      -----

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Figure 10: Clustal W (1.82) sequence alignment of open reading frame (ORF) of *Dazl* from selected organisms. GenBank accession numbers: mouse NM_010021; human NM_001351; *Gallus gallus* AY211387; *Xenopus laevis* AF017778; Axolotl AF308872; *Danio rerio* NM_131524. Pink asterisks below the alignment mark nucleotides that are identical among all organisms shown. Homology among the organisms can be seen starting at *RpDazl* nucleotide 69, which is within the RRM, or RNA recognition motif. Little homology is seen in the DAZ repeat among the organisms, which is found in *RpDazl* at nucleotides 444-516.

a.

```

human      MSTANPETPNSTISREASTQSSSAATSQGYILPEGKIMPNTVVFVGGIDVRMDETEIRSF 60
Macaca     MSAANPETPNSTISREASTQSSSAAASQGYVLPPEGKIMPNTVVFVGGIDVRMDETEIRSF 60
mouse      MSATTSEAPNSAVSREASTQSSSATTSGQYVLPPEGKIMPNTVVFVGGIDVRMDETEIRSF 60
chicken    -MSANAEAQCGSIS-EDNTHSS--TTCQGYVLPPEGKIMPNTVVFVGGIDIRMNEAEIRSYF 56
Axolotl    -----MPNTVVFVGGIDIRMDEIEIRDFF 23
Xenopus    -MSGKEESSN-----YAATAEEEEAVNQGFVLPPEGEIMPNTVVFVGGIDITMDEIEIRDFF 53
Rana       -----MSDTGSPMEDQPSGYVLPPEGKVVPNVVFVGGIDIRMDOHEIKDYF 45
          :*****: *: **:
human      ARYGSVKEVKIITDRTGVSKGYGFVSFFNDVDVQKIVESQINFHGKKLLGLPAIRKQ-NL 119
Macaca     ARYGSVKEVKIITDRTGVSKGYGFVSFFNDVDVQKIVESQINFHGKKLLGLPAIRKQ-NL 119
mouse      ARYGSVKEVKIITDRTGVSKGYGFVSFFNDVDVQKIVESQINFHGKKLLGLPAIRKQ-NL 119
chicken    EQYGTVKEVKIITDRTGVSKGYGFVSFLDNVDVQKIVESQISVHGKRLLGLPAIRKQNL 116
Axolotl    ARFGAVKEVKIITDRTGVSKGYGFVSFHDVDVQKIVDSQINFHGKKLLGLPAIRKQNL 83
Xenopus    TRFGNVKEVKIITDRTGVSKGYGFISFDEVDVQKIVKSQISFHGKKLLGLPAIRKI--- 110
Rana       SKFGSVKEVKIITDRAGVSKGYGFVSFYDDIDVQKILD--VSFHGKRLLGLPAIRKQ-ST 102
          ::* *****:*****:* :*:*****: . :.***:*****
human      CAYHVQPRPLVFNHPPPPQFQNVWVTPNPTETYMQPPTTMNPIITQYVQAYPTYPNS---PV 176
Macaca     CAYHVQPRPLVFNHPPPPQFQNVWVTPNPTETYMQPPTTMNPIITQYVQAYPTYPNS---PV 176
mouse      CTYHVQPRPLIFNPPPPQFQSVWSSPNAETYMQPPTMMNPIITQYVQAYPPYPSS---PV 176
chicken    CSY-MQPRPLAFN-PPAPQFHSVWVTPNPTETYMQPQAVVSPITQYVQTYAYSSPAVLIQQ 174
Axolotl    CAYHVQPRPMVLN-PPAPQFHSWSSQNAETYMQHPHMISPVQYVPAYPYQSSPPVLIQ 142
Xenopus    CTY-VQPRPVLS-HPTP-FHHAWNNQNASYIQHSPIVSPITQYVQACPYPSSPPMAIQ 167
Rana       CAH-IQPRTVLS-TQAPQYQSMWSTPVTDAYVQPTPMFSPMASYIQACPYPNSP-VMLQ 159
          *:: :***: . : . * : : * . : : : * . * : . * : .
human      QVITGYQLPVYNYQMPQWPVGEQRSYVPPAYSAVNHYHCNEV-DPGAEVVPNECSVHEA 235
Macaca     QVITGYQLPVYNYQMPQWPVGEQRSYVPPAYSAVNHYHCNEV-DPGAEVVPNECSVHEA 235
mouse      QVITGYQLPVYNYQMPQWPAGEQRSYVIPPAYTTVNYHCSEV-DPGADILPNECSVHDA 235
chicken    QVPVGYQ-PAYNYQAPPQWPGEQRNVMPVYTSVNYHYSE--DP--EFIQTECAVPEP 229
Axolotl    QIPVGYQQPTYNYQLSPQWAPGEQRGYIIPQAYAAVNHYHCTELDQPGAELIQAEFPVHEP 202
Xenopus    QIPVGCQQPGY-FQVSPQWP-ADQRSYMFPTPAFTFNYHCCMDPNGGEPIPREYPIDQT 225
Rana       QIPLAYQQPGC-FQVAPQWAGGQRSYVLPQALT-WNYTGNDFETACGEVLQADYTIPEQ 217
          *: . * * : * .*** .:***:* * * : : : : : :

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b.

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Xdazl      ACPYPSSPPMAIQQIPVGCQQPGYFQV 27
RpDazl     ACPYPNSP-VMLQQIPLAYQQPGCFQV 26
          ***** ** : :*****: . ***** **

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Figure 12: **Amino acid alignments of Dazl.** (a) alignment of *Dazl* amino acids showing the high level of conservation in the RNA recognition motif, shaded in gray. The smaller motifs are highly conserved within the domain, RNP-1 highlighted in turquoise and RNP-2 highlighted in pink. The putative DAZ repeat (orange) is based on the DAZ repeat of *XDazl*, as defined by Houston et al (1998). (b) Although less conserved among organisms, the DAZ repeats in *XDazl* and *RpDazl* are very similar. The GenBank accession numbers for these sequences are the same as those listed in Fig. 10, with the addition of *Macaca fascicularis Dazl* X99971.

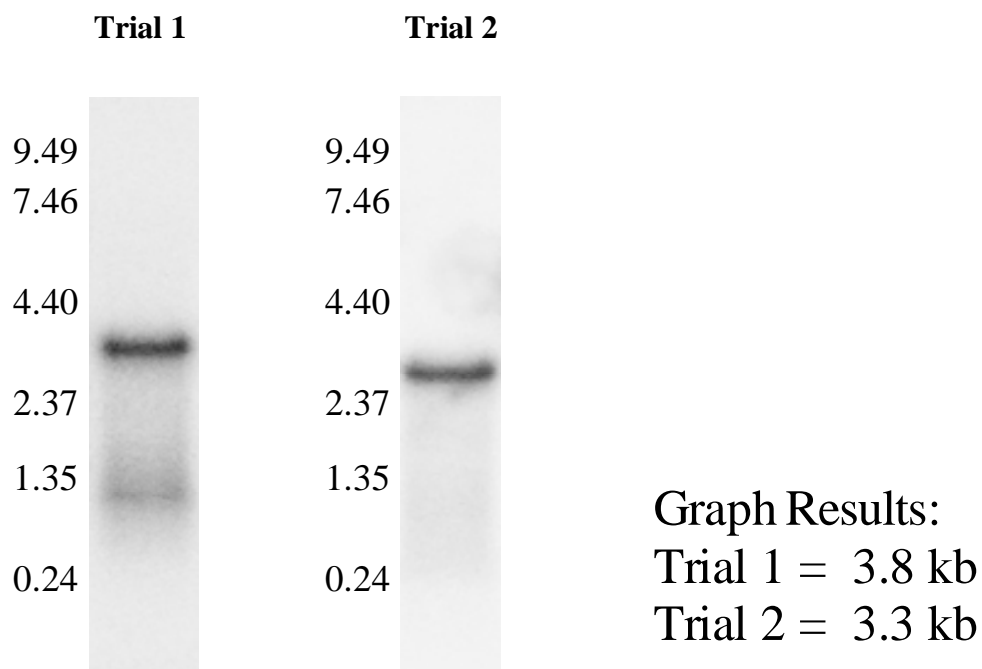
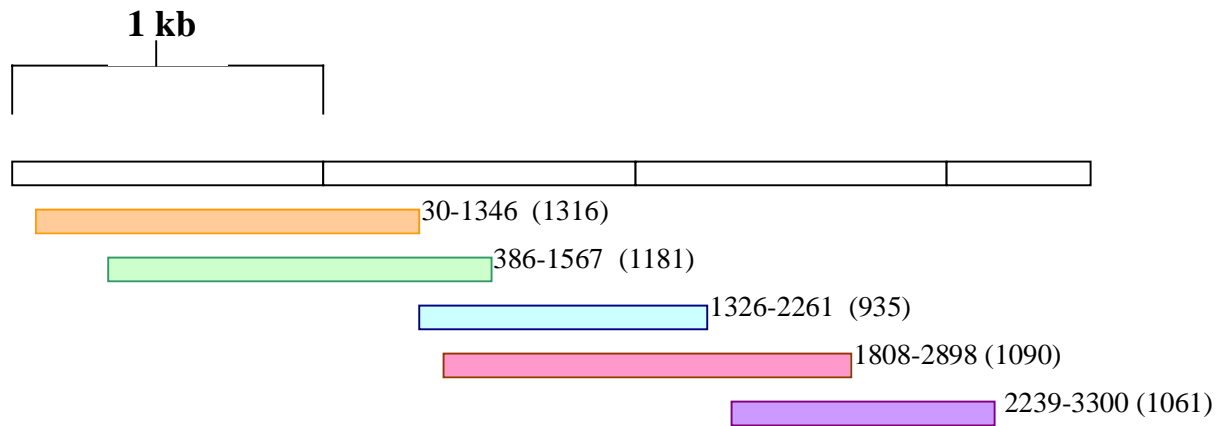


Figure 13: **Northern blot of *RpDazl* transcript.** A PCR-generated radioactive *RpDazl* DNA probe was constructed using *RpDazl* primers (set B, Table 1) and used to probe two independent blots of *R. pipiens* ovary RNA. The results of each trial were graphed as described in Methods and Materials and revealed transcript sizes of 3.8 and 3.3kb respectively. The blot lanes are shown for each trial, with approximate size markers in kilobases (kb).

a.



b.

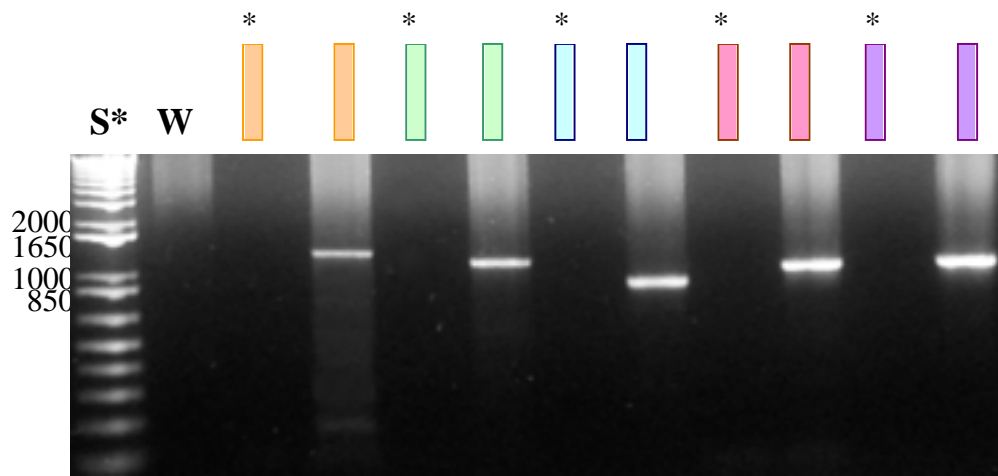


Figure 14: **PCR demonstration of *RpDazl* transcript continuity.** The cDNA utilized for this experiment was prepared from *R. pipiens* ovarian RNA. (a) Schematic of *RpDazl* clone of 3.5kb and regions to be amplified by selected. The exact nucleotides to be amplified and expected product size are given to the right of each color-coded bar. (b) 1% agarose gel showing PCR results. Colored bars above each lane represent the primer pair utilized. An * indicates controls run with primers only.

W (water)= control samples run with no cDNA

S*= marker lane indicating product sizes in base pairs (bp)

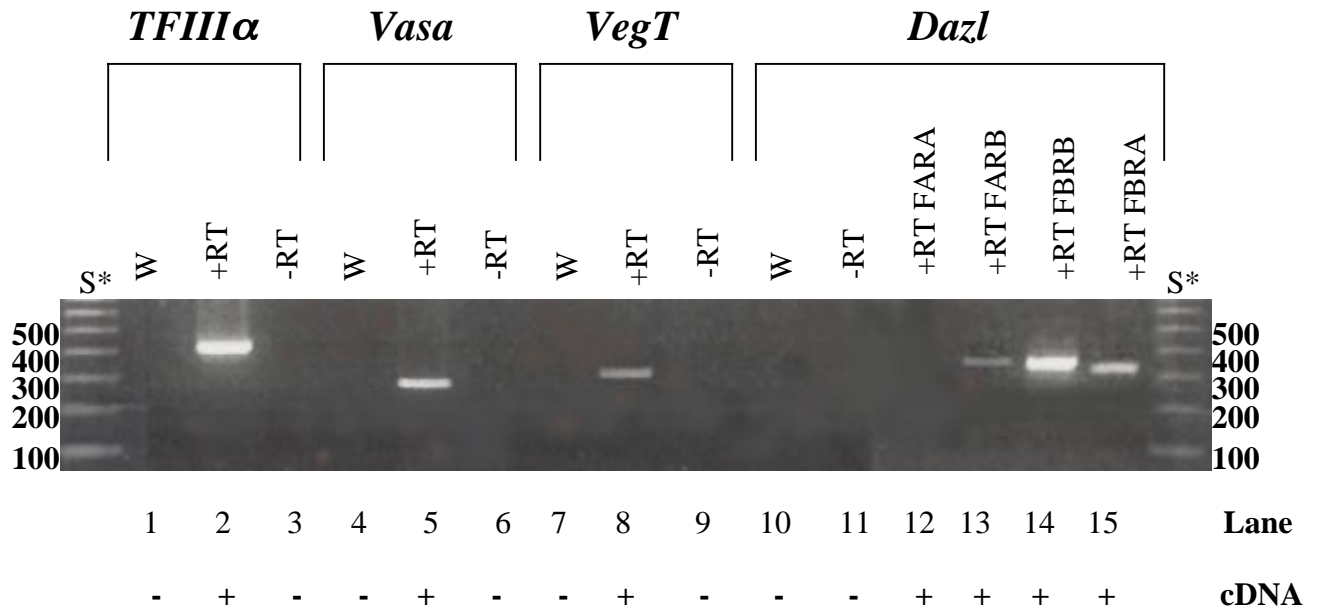


Figure15: **Maternal expression of *RpTFIIIα*, *RpVasa*, *RpVegT*, and *RpDazl*.** cDNA for this experiment was prepared from *R. pipiens* ovarian RNA, and amplified by PCR, using primers in Table 1. Product sizes are as expected. Lane number, as well as the presence or absence of cDNA in the PCR sample, are indicated below the gel. cDNAs to be amplified are the same as those described in Fig. 2.

W (water)= control samples run with the indicated primers, but no cDNA template
 S*= marker lane indicating product sizes in base pairs (bp)

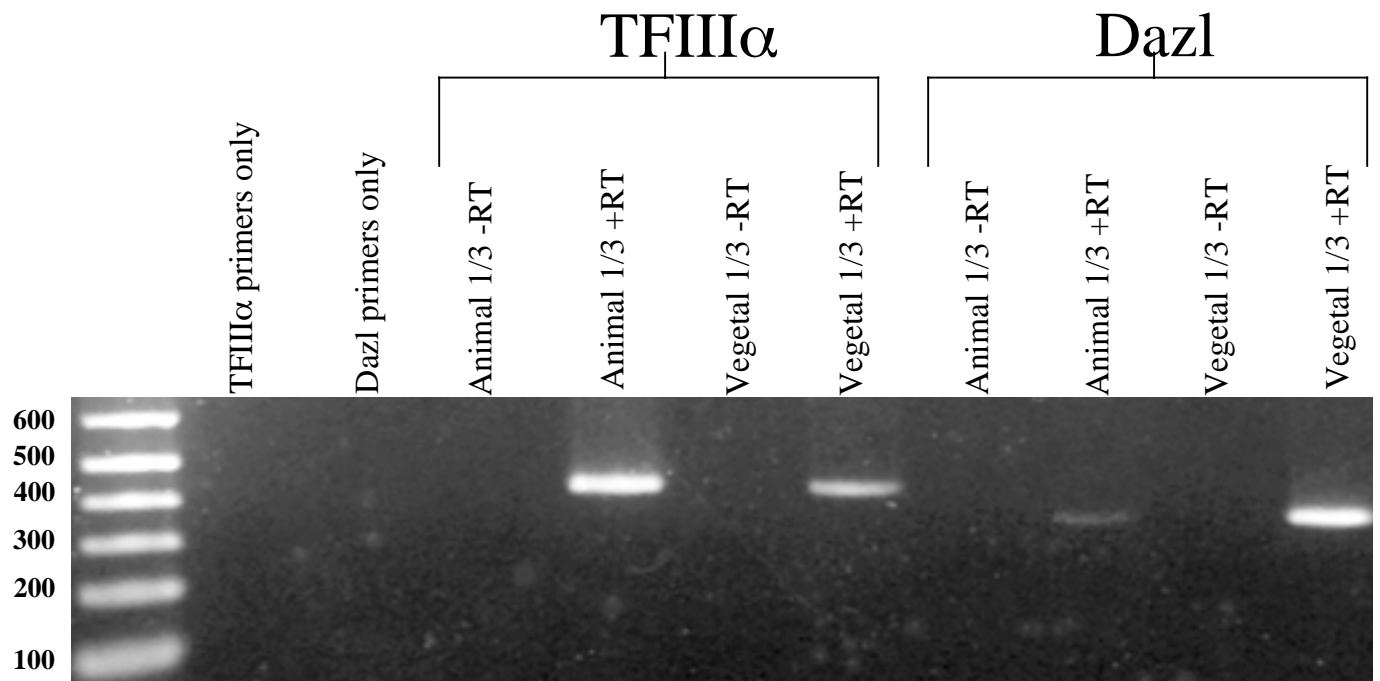


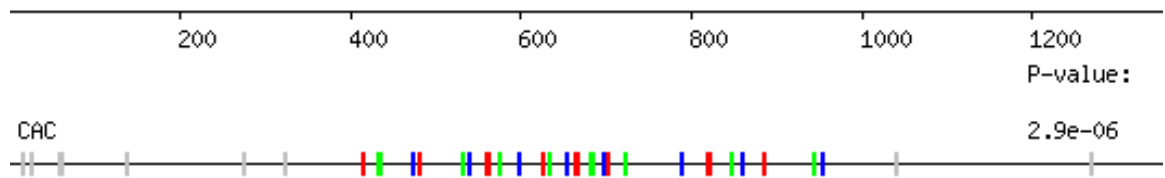
Figure 16: **Location of *RpDazl* RNA in the vegetal one-third of the oocyte.** RNA was extracted from either the animal one-third or the vegetal one-third of *R. pipiens* oocytes and cDNA was prepared by reverse transcription. Expression was examined by RT-PCR of *TFIIIα* and *Dazl*. *TFIIIα* serves as a loading control as the RNA is expressed throughout the oocyte, and *Dazl*. Control samples of RNA that were not reverse transcribed (-RT) were also run in this experiment. Information regarding the primers utilized (*TFIIIα* and *Dazl* set B) can be found in Table 1.

W* and W** (water)= control samples run with *TFIIIα* (*) or *Dazl* (**) primers, but no cDNA template

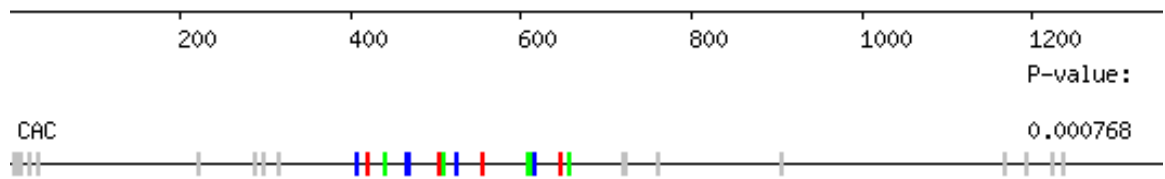
S*= marker lane indicating product sizes in base pairs (bp)

Figure 17: RepFind analysis of 3' UTRs for clusters of CAC repeats. The RepFind analyses are shown for the 3' UTR of *XDazl*, *RpDazl*, zebrafish *Dazl*, and *X. laevis DEADSouth* (panel 4). To make these analyses comparable, only the first 1352 bp of the *RpDazl* 3' UTR are shown, as this is the length of the entire *XDazl* 3' UTR. Each CAC repeat is represented by a bar, and the most significant clusters of repeats are shown as colored bars. The program shows that there are clusters of CAC repeats found in the 3' UTR of *RpDazl*, but they are less significant. Zebrafish *Dazl* RNA is localized to the vegetal cortex of oocytes and *DEADSouth* RNA localizes to the germ plasm of *X. laevis* oocytes. Both of these RNAs have clusters of CAC repeats within the 3' UTR; however, like *RpDazl*, the clusters of CAC repeats are present to a lesser extent. The 3' UTRs of *AxDazl*, as well as *Dazl* in chicken, human, mouse, and *C. elegans*. No significant clusters of CAC repeats were found.

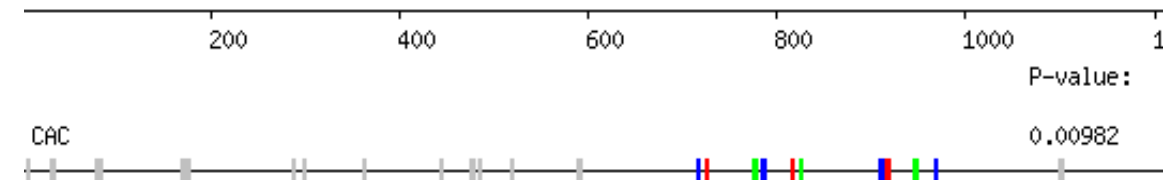
XDazl 3' UTR



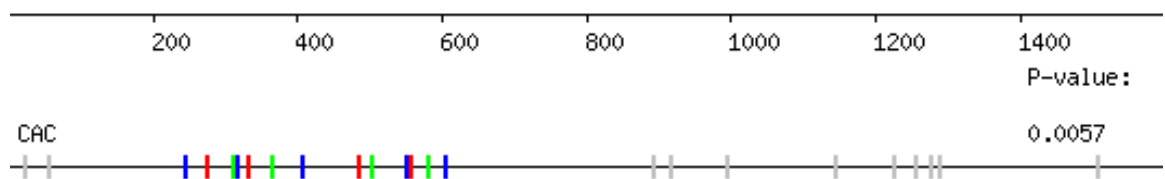
RpDazl 3' UTR



Zebrafish Dazl 3' UTR



DEADSouth 3' UTR



Discussion

A homolog of the autosomal version of the human *DAZ* gene, *DAZL*, is found in many organisms, where its protein product plays a role in gametogenesis and fertility. The exact role that *Dazl* plays varies between organisms, as demonstrated by the variation in phenotypes that result from *Dazl* null experiments. *Dazl* genes possess specific and conserved domains that suggest that they play a role in its function. The RNA binding domain suggests that the function of *Dazl* across species is to bind RNA. There are many reasons that this function is important. For one, *Dazl* may be binding other RNAs and repressing their translation until the proper time and place during development for the expression of that protein is achieved. In *X. laevis*, the expression of the *Dazl* protein, present early on, becomes undetectable during the migratory phase of the PGCs in the embryo to the developing genital ridge (Houston and King, 2000), so perhaps the PGCs cannot respond to migrational cues until *Dazl* is degraded. *Dazl* may bind to an RNA that is directly involved in PGC migration. *Dazl* may repress the translation of this RNA until there is some cellular checkpoint indicating that the PGCs are fully developed and prepared to migrate.

Another possible function of *Dazl* is translational regulation through protein-protein interaction. Proteins have been identified that directly interact with human *DAZ* and *DAZL* (Moore et al., 2003). Four of the proteins contain domains that are implicated in binding RNA. Perhaps *Dazl* interacts with other proteins to bind RNA and control translation. Mouse *Dazl* also interacts with polyribosomes (Tsui et al., 2000), further demonstrating a role in translational regulation.

In my research, *Dazl* in the anuran amphibian *Rana pipiens* was cloned by screening an *R. pipiens* ovary cDNA library. The resultant clone is 3508 base pairs and contains the entire open reading frame of *Dazl* and a substantially long 3' UTR.

I. Evidence that the clone obtained from the *R. pipiens* cDNA library is full length *RpDazl*

The first line of evidence showing that the cloned gene is the *R. pipiens Dazl* is through comparisons to known *Dazl* sequences. This was achieved by aligning sequences obtained from the GenBank database with *RpDazl*, and comparing the sequence identities. Figure 9a

demonstrates the percent identity of the *RpDazl* sequence compared to other organisms. The percent identity among organisms ranges on the nucleotide level from 41.4% to 63.8%, and on the amino acid level, from 30.4% to 53.6%. The lowest percent of identity in both cases occurred with *Danio rerio* or zebrafish, and the highest with human. With the exception of the zebrafish, the percent identity falls near 60% on the nucleotide level, and 50% on the amino acid level. These calculations are based on comparisons of the entire open reading frame of the genes and the protein products.

In *DAZ* and *DAZ-like* genes, there are specific and conserved domains that are indicative of the function of the protein product. These are the RNA recognition motif (RRM) in the amino terminus of the protein and a domain in the carboxyl terminus called a *DAZ* repeat. The highest level of conservation occurs in the RRM. RRMs are composed of 90-100 amino acids and bind pre-mRNA, mRNA, and rRNA (Burd and Dreyfuss, 1994). Two small motifs reside within the RNA binding domain, RNP-1 and RNP-2. Complex secondary structure is produced by the RRM, namely two α -helices and four β -pleated sheets in the form $\beta\alpha\beta\beta\alpha\beta$ (Burd and Dreyfuss, 1994). The small RNP motifs are located on the two central β -pleated sheets and make direct contact with RNA. In *XDazl*, the RRM is defined as 89 amino acids, which is 31% of the entire protein (Houston et al., 1998). The RRMs in *XDazl* and *RpDazl* are 73% identical, and even when comparing all seven organisms that were examined in this work, there is 47% identity in this region. Within the RRM, the two smaller motifs, RNP-1 and RNP-2, are almost 100% conserved (Fig. 10). *KGYGFVSF* and *TVFVGGI* are the consensus sequences of RNP-1 and RNP-2 respectively.

The *DAZ* repeat is not as easily defined as the RRM. Houston et al (1998) define a “consensus *DAZ* repeat” to which they compare the *DAZ* repeat of *Xdazl*, but they did not indicate the origin of this consensus sequence. The *DAZ* repeat that Houston et al (1998) defined for *XDazl* was utilized by Johnson et al (2002) as a comparison with the *DAZ* repeat of *A. mexicanum*. Xu et al (2001) defines *DAZ* repeats in several species of *Dazl*, including frog, human, and zebrafish; however, the derivations of these are not cited. Furthermore, the human *Dazl* *DAZ* repeat defined by Xu et al (2001) has 14 additional amino acids and is not an exact match to the same *DAZ* repeat as defined by Yen et al (1996). The human *Dazl* *DAZ* repeat defined by Yen et al (1996) corresponds exactly to the *DAZ* repeat obtained from the NCBI database, and although the published *DAZ* repeat of Xu et al (2001) does not match this, Xu et al (2001) is cited as a reference on the database. Throughout the literature, there are variations as

to the exact size of the DAZ repeat, as well as the exact sequence. There is obvious difficulty in the alignment of this repeat among organisms (Fig. 15). Using the DAZ repeat as defined by Houston et al (1998), a putative DAZ repeat was defined in *RpDazl*, and it is 69% identical to the DAZ repeat of *XDazl* (Houston et al., 1998)(Fig. 10b). No matter how it is defined, it is clear that the DAZ repeat is not as conserved as the RRM. It does, however, serve as an important marker motif of *Dazl* genes.

The presence of these conserved domains in my clone leads to the conclusion that the clone is in fact *RpDazl*. Upon sequence analysis, it is clear that *RpDazl* is bigger than any other known *Dazl*. A Northern blot was performed to determine the *RpDazl* transcript size (Fig. 11). Two trials yielded transcript sizes of 3.8 kb and 3.3 kb, respectively. The clone obtained from sequencing is 3.5 kb. Although an exact standard error is not assigned in either trial, a difference in measurement of 1 mm led to a change in the determined transcript size of 300-500 base pairs.

To further test the transcript length, 23 sets of primers were utilized in several PCR experiments to demonstrate continuity over the length of cDNA made from *R. pipiens* ovarian RNA. Continuity of the cDNA was authenticated over greater than 3.25 kb of the cloned sequence (Fig 12). Finally, using a web-based program for poly-A prediction, a poly-A consensus sequence was determined. The sequence, AATAAA, is located at nucleotide 3481, 12bp upstream of a string of 8 adenines. In *XDazl*, the same poly-A consensus sequence was located 12bp upstream of a string of 15 adenines (Houston et al., 1998). Based on the data obtained from the Northern blot, the PCR continuity experiments, and the prediction of a poly-A consensus sequence, I conclude that the cloned *RpDazl* is full length.

II. Unusual nature of *RpDazl*

The most distinguishing feature of the *RpDazl* clone is the size of the 3' UTR. The size of the open reading frame corresponds well with that of the open reading frame of other known *Dazl* genes, but the 3' UTR is substantially longer (Table 3).

X. laevis Dazl RNA is 2275 base pairs in length, of which 1352bp constitute the 3' UTR. The longest of the known *Dazl* genes is human *DAZL* at 3056 base pairs. Close behind, the transcript of *Dazl* from *Macaca fascicularis* or *cynDAZLA* is 3015 base pairs (GenBank Accession number X99971). It is interesting to note that Carani et al (1997) described two transcripts of *cynDAZLA*

Organism	Gene name	GenBank Accession number	Transcript Size (bp)	Exact nucleotides of ORF	Nucleotides of 3' UTR (% of total transcript)
<i>Xenopus laevis</i> (African clawed frog)	<i>Xcat-2</i>	X72340	793	16-402	391 (49%)
	<i>Xpat</i>	AJ002384	3994	105-1016	2978 (75%)
	<i>XDazl</i>	AF017778	2275	63-923	1352 (59%)
	<i>VegT</i>	U59483	2691	57-1424	1267 (47%)
	<i>Vgl</i>	M18055	2379	29-1111	1268 (53%)
<i>Mus musculus</i> (mouse)	<i>Dazl</i>	NM_010021	2937	192-1088	1849 (63%)
<i>Homo sapiens</i> (human)	<i>DAZ</i>	XM_088763	3353	295-1467	1886 (56%)
	<i>DAZL</i>	NM_001351	3056	295-1182	1874 (61%)
<i>Ambystoma mexicanum</i> (axolotl)	<i>Dazl</i>	AF308872	2037	279-1079	958 (47%)
<i>Danio rerio</i> (zebrafish)	<i>Dazl</i>	NM_131524	2166	259-948	1218 (56%)
<i>Gallus gallus</i> (chicken)	<i>Dazl</i>	AY211387	1882	174-1043	839 (45%)
<i>Macaca fascicularis</i> (cynomolgus monkey)	<i>Dazl</i>	X99971	3015	211-1098	1917 (64%)
<i>Rana pipiens</i> (Northern leopard frog)	<i>Dazl</i>	N/A	3508	30-873	2635 (75%)

Table 3: Comparison of 3' UTR sizes in various RNAs. Human *DAZ* and *DAZL*, and *Dazl* RNAs from other organisms are shown. In addition, RNAs that localize to the vegetal cortex in *X. laevis* are shown. *Xcat-2* and *Xpat* localize via the METRO Pathway, and *VegT* and *Vgl* localize utilizing the Late Pathway. The 3' UTR was defined for this work from the first nucleotide after the defined stop codon to the end of the transcript, including any polyadenylation sequence.

that were revealed by a Northern blot, one at ~3.5 kb and one at ~2.3 kb. Moreover, in addition to the published 2.1 kb zebrafish *Dazl* in GenBank (# NM_131524), Maegawa et al (1999) found smaller amounts of a 3.2 kb transcript on a Northern blot. The sequence of this larger transcript was not entered into the database, nor were its implications discussed. It seems then, that although the cloned *RpDazl* is rather large, there may be other large *Dazl* genes in existence.

The 3' UTR of RNA can play roles in localization as well as in the regulation of stability and translation (Pesole et al., 2002; Sonenberg, 1994). mRNA turnover plays a significant part in the control of gene expression, and there are several pathways leading to the degradation of eukaryotic mRNAs. One pathway involves the shortening of the poly-A tail, which leads to decapping and subsequently to 3' and 5' degradation. Another pathway involves sequence-specific cleavage (Decker and Parker, 1994). The 3' UTR can be linked to both pathways. First, there may be elements in the 3' UTR sequence that speed up deadenylation, leading to rapid degradation by exonucleases of the RNA. This would certainly be important in development, as gene expression must be tightly temporally regulated. In addition, the 3' UTR of *X. laevis Xlhbox2b* mRNA and other unstable RNAs have endonucleolytic sites (Brown and Harland, 1990); (Decker and Parker, 1994), that may speed up its degradation. The secondary structure of the 3' UTR may play a role in stability by physically blocking exonucleases and endonucleases. The long 3' UTR of *RpDazl* RNA may contribute to its stability.

An excellent example of the involvement of 3' UTR sequence in translational control lies in the *c-mos* RNA of *X. laevis*. The translational activation of *c-mos*, which plays an important role in oocyte development, is accompanied by cytoplasmic polyadenylation (Macdonald, 2001). This process involves a regulatory *cis*-acting element in the 3' UTR of the RNA. A protein recognizes this element, binds the RNA, and mediates both the activation and the repression of translation. The expression of *XDazl* indicates that it undergoes translational repression (Houston and King, 2000), so perhaps there is some *cis*-acting element in the long 3' UTR of *RpDazl*.

Localizing RNA is a process that is known to be important to animals and plants alike. The polarity created by localizing RNA is especially important in the proper development of organisms, and localized RNAs are known in many organisms. The 3' UTR of an RNA is a key player in the process of localization. RNA injections, either of coding sequence with a 3' UTR that has been truncated in a specific way or fragments of a 3' UTR attached to a reporter gene,

are typical methods utilized in the determination of localization elements (LEs). LEs are nucleotides of a 3' UTR that are essential for proper localization.

For *X. laevis Vg1* RNA, a 340 nucleotide element serves as a minimal element for localization. It is sufficient in directing RNAs to the vegetal cortex that would normally not be localized (Mowry and Melton, 1992). More specifically, there are two subelements, one at the 5' end of the LE and one at the 3' end, that contain two copies and one copy, respectively, of the repeated sequence UUUCUA. A construct with the 5' subelement and its two repeated sequences is sufficient for *Vg1* localization (Gautreau et al., 1997). UUCAC is another important sequence in the 3' UTR of *Vg1* as it is required for its binding vera protein as well as for localization (Bubunenko et al., 2002). The sequence UUCAC is also critical for *VegT* RNA localization. *X. laevis fatvg* RNA localizes to the germ plasm of oocytes in a manner that suggests its use of both the METRO Pathway and the Late Pathway. In the 3' UTR of *fatvg*, there are multiple localization elements that work most efficiently when they are all present, but can function independently (Chan et al., 1999). One of the elements, only 25 nucleotides in length, can direct RNA localization when it is present in a single copy. Interestingly, *Xcat-2* RNA has two localization elements that function to localize the RNA to two distinct places. The first 227 nucleotides localize the RNA to the mitochondrial cloud (Zhou and King, 1996a) and the next 164 nucleotides localize the RNA to the germinal granules of the germ plasm in the vegetal cortex (Kloc et al., 2000). The sequence UGCAC is a signal for RNA localization for *Xcat-2* (Betley et al., 2002).

Two small mutations in a 41 nucleotide domain of *nanos* in *D. melanogaster* results in the lack of its normal localization to the germ plasm at the posterior pole of the embryo (Bergsten et al., 2001). One of the largest known localization elements is in the 3' UTR of *bicoid* in *D. melanogaster*, at over 600 nucleotides in length (Macdonald et al., 1993; Macdonald and Struhl, 1988). Other localization elements include a 21 nucleotide sequence required by myelin basic protein RNA (Ainger et al., 1997), a 30 nucleotide region that directs the dendritic localization of α -Ca²⁺/calmodulin-dependent protein kinase II (Mori et al., 2000), and a 118 nucleotide minimal element required for localization of ASH1 RNA in *Saccharomyces cerevisiae* (Chartrand et al., 1999). Secondary and tertiary structure is suggested to be crucial in the functionality of localization elements. ASH1 RNA's localization depends on a stem-loop structure formed by the element, and the actual sequence of the element is not as important (Chartrand et al., 1999).

III. Question of localization

Cells localize RNA for many reasons. These include protecting the RNAs from other components of the cell that may cause their break down, creating polarity, and sorting and concentrating the RNAs (King et al., 1999). RNA localization and the elements in the 3' UTR that are involved are essential in many organisms for proper development. We suggest that the 3' UTR of *RpDazl* is involved in localization.

Clusters of CAC or CAC-containing motifs are in the localization elements of nearly all of the known RNAs that localize to the vegetal cortex of *X. laevis* (Betley et al., 2002). RepFind is a computer-based program that analyzes sequence for clusters of repetitive sequences. It was used to analyze the 3' UTR of *RpDazl*. The output from the program shows clusters of CAC repeats in the 3' UTR of *RpDazl* (Fig. 17); however, they are not significant according to the RepFind parameters. How physiologically important are the P-values that determine significance? Zebrafish *vasa* was analyzed with the program, and its P-value for clusters was insignificant (Betley et al., 2002). *Vasa* RNA, however is localized to the germ plasm of zebrafish oocytes (Knaut et al., 2000), and reporter transcripts fused to the 3' UTR of zebrafish *vasa* localized in *X. laevis* oocytes (Knaut et al., 2002). The P-value of *XDazl*, as shown by my work (Fig. 15), becomes insignificant when the statistical background is made more stringent. Apart from its alterable P-value, *XDazl* RNA always localizes to the germ plasm of *X. laevis* oocytes (Houston et al., 1998). Furthermore, Betley et al (2002) state that the P-values of several RNAs are highly significant such as *Xpat* and *XDazl*, but RNAs with less significant P-values, such as *DEADSouth* and *Xwnt-11*, localized when injected into young oocytes. Although the program provided valuable information regarding clusters of CACs in the 3' UTR, more definitive testing is needed to demonstrate localization of *RpDazl* RNA.

My data from RT-PCR experiments indicate that *RpDazl* RNA is vegetally localized. These data are preliminary in that the experiments do not show localization to a more distinct area than the vegetal one-third of the *R. pipiens* oocyte. *In situ* hybridization data would be needed to show germ plasm association. If this association were shown, future investigations would be directed at how the localization occurs.

Simple morphological examination of *in situ* hybridization data through the various stages of oogenesis would provide information regarding the presence of pathways that parallel

the METRO Pathway and the Late Pathway in *X. laevis*. In these endeavors, *R. pipiens VegT* RNA expression would serve as an excellent comparison to *RpDazl* expression. In *X. laevis*, the two RNAs localize by means of opposite pathways, *XDazl* by means of the METRO Pathway and *VegT* by way of the Late Pathway (King et al., 1999). If the two were expressed differently in *R. pipiens*, in such a manner that resembles the localization pattern in *X. laevis*, it would provide the necessary evidence that at least two pathways of localization exist in *R. pipiens*. *RpDazl* RNA localization to the mitochondrial cloud, specifically the vegetal-most region of the cloud, accompanied by vegetal cortex localization just after the breakdown of the cloud would indicate that *RpDazl* RNA localizes using a pathway that parallels the METRO Pathway of *X. laevis*. If *RpDazl* RNA localization resembled that of *X. laevis VegT* RNA, this would be indicative of a localization through a pathway that resembles the Late Pathway of *X. laevis*.

In *X. laevis*, localization via the METRO Pathway occurs independent of intact cytoskeletal components (Kloc and Etkin, 1995; Kloc et al., 1996; Mowry and Cote, 1999), and localization via the Late Pathway is cytoskeletal dependent (Yisraeli et al., 1990; Mowry and Cote, 1999). Knowing this, localization of *RpDazl* RNA could be assayed in oocytes treated with nocodazole or cytochalasin, which inhibit microtubules and microfilaments respectively, or both, and compared to localization in untreated oocytes.

Localization of *RpDazl* RNA in the control oocytes as well as in oocytes treated with one or both of the cytoskeletal inhibitors would point toward a cytoskeletal-independent localization pathway. This type of pathway would resemble the METRO Pathway of *X. laevis*. If localization does not occur in the treated oocytes, but it does occur in their control counterparts, the pathway by which *RpDazl* RNA is localized would be considered cytoskeletal dependent. This would parallel the localization to the Late Pathway in *X. laevis*, the pathway of *VegT* and *Vg1* localization (King et al., 1999). Testing the dependence of *RpDazl* RNA localization on the cytoskeleton would provide insight as to the pathway by which the RNA is localized.

If *RpDazl* RNA is localized to the germ plasm of the oocyte, the part of the 3' UTR required for localization could be determined. Injecting RNA of deletion mutants into oocytes and assaying localization would provide information regarding possible localization elements. Further, injections of RNAs containing 3' UTR portions attached to reporter genes would identify sequences that were sufficient for localization.

In addition to maternal expression of *RpDazl*, it will be important to shed light on zygotic expression. In *A. mexicanum*, *AxDazl* is expressed maternally and during embryogenesis, but is

not confined to specific cells until the migrating primordial germ cells begin to approach the forming gonad (Johnson et al., 2001). This is a very different story than the one that is told for *XDazl* in *X. laevis*, where localization to specific cells is maintained throughout embryogenesis (Houston et al., 1998). Because these two amphibians express *Dazl* so differently, it will be important to determine if *RpDazl* is expressed in the cells containing the germ plasm throughout development, including the primordial germ cells.

IV. Conclusion

To conclude, I cloned full length *Dazl* from the frog *Rana pipiens*. Data was obtained regarding the maternal localization of the RNA, but further studies will have to be performed to prove an association with the germ plasm of *R. pipiens*. The cloning of the entire gene is important in that *in situ* hybridizations can be performed to analyze both the maternal and zygotic expression of *RpDazl*. Functional analyses of the 3' UTR can also be performed, pending proof of localization. Loss of function analyses would also be valuable and interesting, in that *Dazl* plays different roles in gametogenesis and fertility in different organisms. It will be important to continue these studies in *R. pipiens* in order to gain insight into germ cell determination in amphibians.

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