Genetic and molecular analysis of the Drosophila gene *nanos*

by
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ABSTRACT

The Drosophila protein NANOS acts during three developmental stages: embryogenesis, germ cell migration and oogenesis. In the early embryo NANOS is required to regulate the translation of the maternal transcript *hunchback*. Translational regulation of *hunchback* also requires the RNA binding protein PUMILIO. PUMILIO and NANOS also act together in the primordial germ cells where their functions are required for normal migration through the embryo. The exact molecular process in which they are involved and the genes they might regulate in the germ cells are not known. In addition, NANOS is required in ovaries for the differentiation of the germ line stem cell progeny, the cystoblast. The partner and target of NANOS in this process is not known.

The NANOS protein contains two zinc fingers in its C-terminal region which are necessary for its function in embryogenesis. The molecular and genetic analysis of 68 new alleles isolated in a selective genetic screen has allowed us to identify amino acids critical for NANOS function. This analysis demonstrates the functional relevance of the zinc fingers in all aspects of NANOS function. Furthermore, a region C-terminal to the zinc fingers has been shown to constitute a novel functional domain within the NANOS protein. The "tail domain" of NANOS is required for abdominal formation and germ cell migration but not for oogenesis.

A yeast two-hybrid screen has been performed to identify proteins that interact with NANOS. This screen showed that NANOS is able to interact with itself through its N terminal region. Additionally, NANOS interacts with the translation elongation factor 1γ. This interaction is mediated by the tail domain of NANOS and might be relevant to the translational repression function of NANOS. Finally it has been shown through the two-hybrid screen and immunoprecipitation experiments that NANOS directly interacts with the RNA binding protein and translational regulator BRUNO. Both NANOS and BRUNO affect the differentiation of the cystoblast during early oogenesis. In addition, NANOS and BRUNO together can regulate the translation of a reporter gene targeted for BRUNO binding. Therefore we suggest that BRUNO is the partner of NANOS during the development of the germ line cystoblast and that they both regulate the translation of a yet unknown maternal transcript.

Thesis supervisor: Ruth Lehmann

Title: Professor of Cell Biology

Whosoever would undertake some atrocious enterprise should act as if it were already accomplished, should impose upon himself a future as irrevocable as the past.

—*Jorge Luis Borges, "The Garden of Forking Paths"*

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CHAPTER 1

Introduction

Regulation of gene expression is a universal phenomenon required for the functioning of all organisms. Genes need to be turned off and on during different stages of the cell cycle, during the response to stress factors and during the development of an organism. Additionally, certain genes need to be expressed in a specific region of a cell. Regulation of transcription is the most common and best understood method of achieving differential gene expression. Nonetheless, many situations exist in which abundant amounts of transcripts for genes that need to be regulated are already present. In this case, turn-over or modification of the transcript is an efficient method of regulation. Transcripts can also be localized to different cells or cell regions in order to achieve differential expression. A third method of post-transcriptional regulation is the control of the translation process. Translational regulation is a widely used mechanism during the earliest stages of development and oogenesis and it is also seen during other processes such as cell growth. In addition, the modification of proteins can control their function at different times and places. This thesis will study the regulation of translation during the development of the fruit fly *Drosophila melanogaster.* This introduction will review what it is known about the mechanisms of translation and its regulation during development.

mRNA FEATURES REQUIRED IN TRANSLATION

A key step in translation is the binding of the mRNA to the ribosomes. The ribosome can either enter through the 5' end of the message and "scan" the RNA in order to find the initiation codon or enter in the middle of the RNA at or upstream of the initiator codon. The "scanning" method of translation is the most common and the most relevant to the system described in this thesis. Several RNA features are important for efficient "scanning" and translation of eukaryotic mRNA: the presence and accessibility of a m^7GpppN cap, a poly(A) tail, an unstructured 5' untranslated region (5'UTR), the initiation codon and the context surrounding it.

Both the 5' m^7GpppN cap and the poly(A) tail are added to the RNA in the nucleus. A methylated G is added to the 5' end of the RNA almost immediately after RNA synthesis starts. Capping involves condensation of the triphosphate group of a GTP molecule with a diphosphate left at the 5' end of the initial transcript. Polyadenylation of the nascent RNA is preceded by a site-specific cleavage reaction 30 nucleotides upstream from a conserved AAUAAA polyadenylation signal. An uninterrupted tract of polyadenylic acid is then added by the poly(A) polymerase (Wickens, 1990). It has been seen in various in vitro and in vivo systems that the presence of m^7GpppN structure at the 5' end and a poly(A) tail at the 3' end of an mRNA can enhance translation (Gallie, 1991). Nevertheless, dependence on the m^7GpppN cap or poly(A) tail differs between systems and mRNAs.

Efficient translation also requires a 5'UTR devoid of secondary structure. The length of the 5'UTR can vary between specific RNAs, but mRNAs with very short leader sequences are translated inefficiently (Kozak, 1991). Finally, translation tends to begin at the AUG closest to the 5' terminus of the mRNA. The sequences flanking the initiation codon aid its recognition by the initiation complex. In mammalian cells GCCACC**AUG**G is the strongest consensus sequence for initiation, while in an insect organism such as Drosophila the consensus is CAAAAC**AUG** (Cavener, 1987).

TRANSLATION MECHANISMS

Translation can be conveniently divided in three main stages: 1) initiation, 2) elongation and 3) termination (reviewed in Merrick, 1992; Merrick and Hershey, 1996).

Translation initiation

The appropriate initiation of eukaryotic translation requires assembly of a pre-initiation complex between the 40S ribosomal subunit and the initiation met-tRNA (met-$tRNA_i$). The pre-initiation complex then needs to bind to the RNA, locate the initiation codon and recruit the 60S subunit to the initiation complex. The execution of these processes requires the action of at least 11 initiation factors (eIFs) and the expenditure of energy in the form of ATP and GTP hydrolysis (Figure 1). Inactive 80S ribosomes remain in equilibrium with free 40S and 60S subunits. The equilibrium is shifted towards dissociation by the action of three initiation factors. eIF6 binds to the 60S subunit and inhibits its association to the 40S subunit, which is, in turn, bound by association inhibitors eIF1A and eIF3. eIF1A also inhibits the dimerization of the 40S subunit and along with eIF3 promotes RNA binding and the association with the met-$tRNA_i$. Before it can be recruited to the 40S subunit, the met-$tRNA_i$ must form a ternary complex with eIF2 and GTP. The formation of the ternary complex is one of the most important stages in translational and therefore is a common target for regulation. For instance, phosphorylation of eIF2 is a common mechanism for repressing global translation.

The 40S subunit associated with eIF3, eIF1A and the ternary complex can be found as a 43S intermediate pre-initiation complex. The pre-initiation complex is recruited to the RNA by eIF4F, which first must bind the m^7GpppN cap and unwind any secondary structure in the 5'UTR. eIF4F is a complex formed by three initiation factors: eIF4E, eIF4G and eIF4A. eIF4E is the cap-binding protein; eIF4G binds to eIF4E, eIF4A, and eIF3 acting as a platform for initiation complex formation; and eIF4A is a DEAD box RNA binding protein that possesses ATPase and bidirectional helicase activity. eIF4F binds also to eIF4B in order to enhance the RNA helicase activity of eIF4A. Once the 43S subunit is brought to the RNA, "scanning" begins until the met-$tRNA_i$ binds the start codon. eIF5 recognizes the complex and induces the hydrolysis of the GTP carried by eIF2. This event reduces the affinity of the initiation factors for the 40S ribosome resulting in their release. eIF2 leaves the complex bound to GDP and is then recycled back to the GTP form with the action of eIF2B (Figure 1). After the

dissociation of the initiation factors, the 60S subunit joins the complex to form the 80S initiation complex and start the translation of the message.

Even though this work only deals with the translation mechanisms of eukaryotes, it is worth mentioning some of the main differences between eukaryotic and prokaryotic initiation. Transcription and translation are coupled spatially and temporally in prokaryotes but uncoupled in eukaryotes. This difference might account for the distinct character of initiation in these two classes. Identification of the initiator codon by the ribosomes in prokaryotes relies on the absence of secondary structure, which is not as important in eukaryotes due to the presence of proteins with helicase activity. In addition, while the recruitment of the ribosome to the mRNA in eukaryotes depends on interactions with a m^7GpppN cap, in prokaryotes initiation involves direct interaction between ribosomal RNA and the mRNA. Finally, only eukaryotes require the action of a specific factor, eIF5, to achieve subunit joining.

Translation elongation and termination

Elongation consists of the addition of amino acid residues to the carboxy-terminal end of the nascent peptide. Most of the knowledge on the mechanisms of elongation comes from work done on prokaryotes. Nevertheless, eukaryotic homologues for most elongation factors have been found and the processes are expected to be very similar. The elongation process requires the ribosome to interact simultaneously with several tRNAs (reviewed in Merrick, 1992; Wilson and Noller, 1998). Consequently, there are three tRNA binding sites in the ribosome. The A site is occupied by the aminoacyl-tRNA (aa-tRNA), the P site is held by the peptidyl-tRNA (pep-tRNA) and the E site is occupied by the stripped tRNA. At the end of the initiation process, the met-$tRNA_i$ is in the P site of the ribosome. The aa-tRNA carrying the amino acid to be added forms a ternary complex with elongation factor 1α (eEF1α) and GTP (Figure 2). This ternary complex then binds to the A site which induces a hydrolysis reaction by EF1α. The GDP bound form of eEF1α then leaves the ribosome. In order to bind a new aa-tRNA, the eEF1α undergoes an exchange reaction where GTP replaces GDP. This nucleotide exchange reaction is facilitated by eEF1βγ.

Once the A and P sites are occupied, the 60S ribosome catalyzes a reaction between the aa-tRNA in the A site and pep-tRNA (or the met-$tRNA_i$ in the first reaction) in the P site. This reaction produces a new pep-tRNA and a stripped tRNA. The pep-tRNA and the stripped tRNA shift their aminoacyl ends from the A and P sites to the P

and E site while the codon recognition part of both tRNAs remain in the A and P sites. Actual translocation of both tRNAs and the mRNA requires the binding of eEF2-GTP. Following GTP hydrolysis, eEF2-GDP is released and the pep-tRNA lies in the P site, the stripped tRNA is in the E site and the A site is vacant. eEF3 promotes the ejection of the stripped tRNA. The ribosome is then ready to take another ternary complex and continue peptide synthesis.

Termination occurs when a stop codon is exposed to the A site. No ternary complex is available for the stop codon. Instead, release factor eRF1 binds to the A site and promotes the hydrolysis and release of the pep-tRNA in the P site. In prokaryotes, RF4 catalyzes the release of the mRNA from the ribosome, but the corresponding eukaryotic factor has not been isolated.

THE CAP, THE TAIL AND TRANSLATION INITIATION

Almost all eukaryotic RNAs have a 5' cap structure and a poly(A) tail. The cap has been associated with diverse functions such as RNA splicing, transport, stabilization, and translation, while the poly(A) tail has been implicated in RNA stability and translation. Addition of a cap to an in vitro transcribed RNA stimulates protein synthesis in vitro and in injected oocytes. The ability of the cap to enhance translation can be explained by its role in ribosome recruitment (reviewed in Sachs, et al., 1997). As mentioned earlier, eIF4F is required for efficient initiation. This initiation factor prepares the RNA for ribosome binding by eliminating secondary structure through its subunit eIF4A. In addition, a second component of eIF4F, eIF4G, is responsible for recruiting the ribosome by directly binding to eIF3 in the 43S preinitiation complex. In order to perform these functions efficiently eIF4F must bind the RNA. RNA binding is accomplished by eIF4E, the third member of eIF4F, which specifically binds to the 5' cap structure. The interaction between the cap and the cap-binding protein of eIF4F is essential for efficient translational initiation and explains the cap's role in translation. Accordingly, adding cap analogues to in vitro assays inhibits translation, most likely by sequestering eIF4F away from the RNA (Lawson, et al., 1988). Also, inhibiting the formation of the eIF4F complex eliminates cap dependent initiation. Mutations in eIF4G that inhibit its association with the cap-binding protein affect translation in vivo (Tarun and Sachs, 1997). Additionally, a family of inhibitory proteins known as 4E-binding proteins can inhibit cap-stimulated translation initiation. These proteins bind to eIF4E and inhibit the formation of eIF4F, thus affecting translational initiation (reviewed in Sonenberg, 1996).

The role of the poly(A) tail in translation has recently been elucidated by the identification of proteins that can interact with both the tail and the initiation complex (reviewed in Gallie, 1998). A series of earlier experiments showed that the presence of a poly(A) tail at the 3' end of the mRNA could stimulate translation (Doel and Carey, 1976). The addition of a poly(A) tail to RNA can enhance its translation in Xenopus oocytes (Galili, et al., 1988). Conversely, blocking polyadenylation by mutating the polyadenylation signal results in the failure to translate the mutated RNA (McGrew, et al., 1989). A protein containing four ribonucleotide recognition motifs (RRM) known as poly(A) binding protein (PABP) binds the poly(A) tail (Adam, et al., 1986; Sachs, et al., 1986). The binding of PABP to the poly(A) tail was suggested to be responsible for poly(A) dependent translational enhancement. Accordingly, addition of excess poly(A) to an in vitro translation resulted in a reduction in translation efficiency (Munroe and

Jacobson, 1990). This inhibition could be reverted by the addition of purified PABP (Grossi de Sa, et al., 1988). In addition, genetic analysis in yeast showed that a temperature sensitive mutation in the *pab1p* gene affected translation (Sachs and Davis, 1989). The mutant yeast lacking PABP (I will use PABP to refer to both yeast Pab1p and higher eukaryotes PABP to avoid confusion) is characterized by an increase of monoribosomes, suggesting a defect in initiation. In addition, reversion analysis isolated bypass suppressors of the PABP mutation that affected components of the 60S ribosomal subunit (Sachs and Davis, 1989). All of these suppressor strains showed a decrease of relative amount of 60S subunit compared to 40S subunits. This genetic analysis led to the belief that PABP's function was to promote assembling of the 80S ribosome at the start codon. A series of experiments using reporter mRNAs containing either or both a 5' cap and a poly(A) tail in plant, yeast and mammalian cells, revealed that these two structures act synergistically to enhance translation (Gallie, 1991). The synergism observed in these experiments confirms that PABP may participate in steps at which the 5' cap is involved, such as recruitment of the preinitiation complex.

The development of a yeast in vitro translation system which showed cap and poly(A) dependence helped to further the understanding of the poly(A) tail's role in translational initiation. Adding monoclonal antibodies to PABP to the extract resulted in reduced translation of a polyadenylated reporter gene (Tarun and Sachs, 1995). This result confirmed the requirement of PABP for poly(A) dependent translation. In addition, this study verified a synergistic effect between the cap and the poly(A) tail and showed that this effect could be eliminated by the addition of antibodies against either PABP or the cap-binding protein. Therefore, both PABP and eIF4E are required for the synergistic stimulation of translation. Finally, this report showed that the poly(A) binding protein stimulates 40S subunit joining to the RNA. It was therefore suggested that mutations in 60S could compensate for the loss of 40S subunit recruitment by increasing the concentration of free 40S ribosomal subunit in the cell.

The interdependence of the 5' cap structure and the poly(A) tail for optimal translation suggests that communication between these two structures might be important for efficient translation. Biochemical studies performed with yeast extracts showed that eIF4F and PABP physically interact (Tarun and Sachs, 1996). More specifically, PABP binds to the eIF4G subunit. This interaction involves a 114 amino acid region of eIF4G adjacent to its eIF4E-binding site and requires that PABP be bound to RNA. The PABP binding site in eIF4G is required for the cap and the poly(A) tail to synergistically stimulate translation (Tarun, et al., 1997). The interaction with eIF4G maps to the second

RRM of PABP. As expected, this domain is required for efficient translation of a polyadenylated message (Kessler and Sachs, 1998). These results suggest that a physical contact between the eIF4F and PABP is necessary for the poly(A) tail to enhance translation (Figure 3).

A direct interaction between PABP and eIF4G was also reported in plant and human cells (Le, et al., 1997; Imataka, et al., 1998). In plants an additional interaction between PABP and eIF4B has been reported (Le, et al., 1997). In addition, studies using human cells revealed that PABP associates with a newly identified PABP interacting protein (PAIP-1) (Craig, et al., 1998). This protein shares homology with a part of eIF4G responsible for binding eIF4A. Accordingly, PAIP-1 binds to both PABP and eIF4A making a second connection between the poly(A) tail and the cap (Figure 3).

All of this information suggests that the poly(A) tail functions to recruit PABP to the RNA, which in turn interacts with initiation factors that are connected to the cap structure. There are various reasons why an interaction between the two ends of the RNA might be important for its function. It has been suggested that the PABP/eIF4G interaction aids in the recruitment of the 40S ribosomal subunit to the RNA hence the increase in translation efficiency with the presence of a cap and a poly(A) tail. In addition, requiring a complex between proteins bound at each end of the RNA for its translation might be a means to test for RNA integrity prior to starting translation. Alternatively, these protein-protein interactions between factors associated with the cap and the poly(A) tail might enable RNA circularization during translation. Atomic force microscopy showed that a reconstituted yeast eIF4E/eIF4G/PABP complex can circularize capped and polyadenylated RNA (Wells, et al., 1998). Circularizing the RNA may facilitate re-initiation through the physical proximity of the two termini. Support for a circular model of translation comes from work on rotavirus mRNA translation. Rotavirus mRNAs are capped but lack a poly(A) tail. The viral protein NSP3A binds to specific sequences in the 3'UTR of viral mRNAs. NSP3A has been shown to interact with cellular eIF4G (Piron, et al., 1998). In addition, the amount of PABP bound to eIF4F decreases during rotavirus infection, suggesting that NSP3A is able to recruit eIF4F away from the cellular RNAs and preferentially translate its RNA.

The mechanism used by rotavirus to block cellular translation shows that the complex formation between PABP and the initiation factors is a good target for translational regulation. For instance, based on the models described above, selectively deadenylating a message would lead to its translational silencing. In addition, global

regulation of translation by the cell or a virus can be mediated by the phosphorylation of initiation factors involved in cap-binding.

TRANSLATIONAL REGULATION IN DEVELOPMENT

In the last few years it has become clear that translational regulation is a common method of controlling gene expression. Nowhere is translational regulation more abundant and important than in oocyte and embryonic development. Early embryos are usually transcriptionally inactive, but require the expression of a great number of genes. For this purpose maternal mRNAs are stockpiled in the developing oocyte. The requirement for different proteins at different times demands that the translation of these messages be regulated. As development carries on, cells need to assume specific identities and functions. Translational regulation seems to also play a role in the specification of cell fate as seen in the case of germ cell specification in *C. elegans* and neuron development in mammalian systems. In addition, translational regulation can, in concert with mRNA localization, be involved in the formation of morphogenic protein gradients within a single cell in order to achieve axis and pattern formation in the embryo.

It has become clear that untranslated regions at both ends of the mRNA play important roles in the regulation of translation. Regulation mediated by the 5'UTR usually utilizes steric hindrance to impede the formation and scanning of the initiation complex. For instance, the regulation of the *ferritin* mRNA involves the binding of a regulatory protein to a stem loop in the 5'UTR. Protein binding to the 5'UTR stabilizes the stem loop and inhibits *ferritin* translation by impeding binding of the 40S ribosomal subunit scanning complex (reviewed in Rouault, 1996).

The examples discussed in detail in this introduction require regulatory elements in the 3'UTR of the mRNA. We now know that the mediation of translational regulation by the 3'UTR is the rule more than the exception. It has been suggested that the lack of evolutionary constraint on the 3'UTR might ease the appearance of regulatory sequences in this region of the transcript. Very small changes in the 5'UTR can strongly affect initiation and translation, while the 3'UTR can afford more modifications. In addition, the recent understanding of the influence of the poly(A) tail on translation could explain the role of 3'UTR in translational control. The proximity of some of these elements to sequences required for polyadenylation could suggest that the process involved in the addition of a poly(A) tail might be a regulatory target.

Translational regulation through elements in the 3'UTR can also utilize mechanisms that do not involve changes in the poly(A) tail. These sequences can act as signals for masking proteins that sequester the mRNA away from the translational

machinery. In addition, 3'UTR sequences can in some cases affect the initiation process. How the 3'UTR affects events occurring at the 5' end of the message is not known. Nevertheless, the fact that the poly(A) tail interacts with the cap through protein interactions might help in developing and testing models that explain the role of the 3'UTR in the regulation of initiation. All of the cis-acting elements described here associate with specific proteins. Genetic approaches in *C. elegans* and Drosophila have shown that these factors are required for the regulation imparted by the 3'UTR. The function of these proteins could be very diverse. They might displace proteins necessary for polyadenylation or for the initiation of translation or directly remove the poly(A) tail.

The examples here discussed have been chosen to provide an idea of the diversity of mechanisms and factors involved in the regulation of translation during development and cell fate determination. In addition, these examples show that the mechanisms used are conserved in a diversity of species and developmental processes. In all of these cases the cis and trans acting factors are known. Nevertheless, the actual mechanisms for the regulation are not fully understood. As new techniques are developed for the identification of proteins interacting in this process and the study of the regulated mRNA, many of the unanswered questions will surely be solved.

Masking, adenylation and translational activation in oocyte development

In the developing oocytes the transition from cell cycle arrest to mitotic division occurs upon fertilization. In species such as frogs and mice, this event is preceded by the a release from cell cycle arrest, known as oocyte maturation. In oocytes a great number of the components needed for maturation and early development are present in the form of mRNAs. Some of these mRNA must be silent until oocyte maturation or fertilization. Cyclins, for instance, play important roles after the cell cycle resumes, but their premature expression has deleterious effects. Therefore, cyclin mRNAs are not translated in early oocytes and become active after oocyte maturation. Two models have been used to explain the global silencing of these messages. The "masking" hypothesis proposes that the formation of mRNA-protein complexes (mRNP) upon transcription hides the RNA from the translational machinery (reviewed in Standart and Jackson, 1994). A second model proposes that the active and specific deadenylation of newly transcribed messages keeps them repressed until maturation induces their polyadenylation and subsequent translation (reviewed in Richter, 1996). New evidence

shows that these two models are not mutually exclusive and that the masking proteins might be involved in the deadenylation events described for various mRNAs.

The masking hypothesis was first used to explain the global regulation of mRNAs in immature oocytes. mRNPs were suggested to form after transcription in order to keep the mRNA from being recruited to the ribosomes. Upon fertilization, the masking proteins are released and the mRNA is translated. mRNPS have been isolated from Xenopus and clam oocytes and their components and properties studied in detail. Purified mRNPs from frogs and clams do not allow in vitro translation of the mRNA (Richter and Smith, 1984). Nevertheless, if the proteins are removed first by phenol extraction, the mRNA is translated. Therefore, the transcript itself is competent for translation but the proteins somehow impede its expression. Two proteins that form part of Xenopus mRNPs, mRNP3 and FRGY2, have been identified (Bouvet and Wolffe, 1994b). These two proteins form part of the Y box family of nucleic acid binding proteins. FRGY2 and mRNP3 are not sufficient to reconstitute mRNP silencing, indicating that other proteins might be required for this process (Bouvet and Wolffe, 1994b). In addition, functional mRNPs only form in vivo for mRNAs transcribed in the nucleus and will not form with in vitro transcribed and injected mRNAs (Bouvet and Wolffe, 1994b). This observation, along with the fact that FRGY2 is a transcription factor, establishes a link between transcription and the assembly of mRNPs. mRNA sequences required for masking have not been identified, but the 3'UTR seems to play an important role in the masking and unmasking steps. It has also been suggested that the global unmasking of the silenced mRNP may be mediated by phosphorylation of the masking factors (Standart and Jackson, 1994; Bouvet and Wolffe, 1994b). Further studies are required to better understand the role of the mRNPs in translational regulation and the mechanisms involved in masking of mRNAs.

In Xenopus oocytes, some of the silenced mRNAs such as cyclin, *cdk-2* and *c-mos* have a shortened poly(A) tail. With the onset of maturation, the poly(A) tail of these messages are lengthened. Cytoplasmic adenylation in the case of these mRNAs is concurrent with translational activation (Dworkin and Dworkin-Rastl, 1985; Dworkin, et al., 1985; McGrew, et al., 1989; Gebauer, et al., 1994). This observation, combined with the implication of the poly(A) tail in translation, have led to the hypothesis that silencing and activation can be achieved by changes in the poly(A) tail length. Cytoplasmic adenylation depends on two cis-acting elements in the mRNA. The first signal is the canonical AAAUAA polyadenylation signal also responsible for polyadenylation in the nucleus. A second signal necessary for cytoplasmic polyadenylation lies also in the

3'UTR, upstream of the AAAUAA hexamer. This signal, known as the cytoplasmic polyadenylation element (CPE), has a general structure of UUUUUUAU, but its composition and location vary among different mRNAs. An elegant study involving interspecies injections of mRNAs from Xenopus, Drosophila and mouse into embryos of the three species was able to show that the regulatory sequences and the trans–acting factors needed for cytoplasmic polyadenylation are likely to be conserved across species (Verrotti, et al., 1996). A protein that can bind the CPE, CPEB, has been identified in Xenopus oocytes (Hake and Richter, 1994). CPEB has two RRM motifs and is abundant in oocytes. Immunodepletion of CPEB impedes the polyadenylation of injected mRNA in mature oocytes and this phenotype is rescued by the addition of purified CPEB (Hake and Richter, 1994).

Prior to a developmental signal such as oocyte maturation these mRNAs remain in a silent deadenylated state. This silencing seems to be an active process requiring both cis and trans-acting factors. Studies of the repression of cyclin B1 before maturation in Xenopus led to the discovery that the CPE is necessary to maintain the quiescent state of the mRNA (Joel Richter, personal communication). Injection of CPE sequences into Xenopus oocytes resulted in the premature translation of Cyclin B1. This result indicates that the injected CPE can displace a translational repressor from the mRNA. In addition, this resulting premature translation is not accompanied by polyadenylation of the message. This could suggest that the determining event for translational activation at oocyte maturation might not be the addition of a poly(A) tail but the displacement of a masking factor. Notwithstanding, after maturation, polyadenylation results in an induction of translation to levels greater than seen when the masking factors are competed away in immature oocytes. In addition, mutations in the CPE affect the early masking and also the post maturation induction. This result suggests that the CPE sequences play a dual role in repression and activation. A second type of CPE has been identified in Xenopus in mRNAs that get preferentially polyadenylated after fertilization. Cl2 and activin receptor mRNAs contain an embryonic type CPE (eCPE) consisting of a poly(U) tract in their 3'UTR. Similar to CPE, eCPE is involved in masking of the RNA and in the polyadenylation of these messages after fertilization (Simon, et al., 1996). eCPE is bound by Elr1A, a member of the ELAV family of RNA-binding proteins, but its role in cytoplasmic polyadenylation has not been determined (Wu, et al., 1997). It will be interesting to learn whether CPEB also binds to these sequences and what the role of Elr1A is in the unmasking and polyadenylation of these mRNAs.

Similar evidence for the dual function of the CPE comes from studies in surf clam (*Spisula solidissima*) oocytes. Three abundant RNAs coding for cyclins A, B and ribonucleotide reductase (RR) remain in a silent state in the developing oocyte until fertilization, which in this organism triggers maturation. Cis-acting sequences responsible for the masking effect have been mapped to the 3'UTR of these mRNAs and contain poly(U) stretches similar to the CPE from Xenopus mRNAs (Standart, et al., 1990). An 82 kDa protein that binds to the masking element has been identified as a homologue of the CPEB (Walker, et al., 1996; Walker, et al., 1999). Both the cis and the trans-acting factors involved in activation seem to play a role in translational repression. Addition of antibodies against CPEB to clam oocyte lysates leads to the translation of cyclin mRNA, while the same treatment of mature eggs results in deadenylation and repression of the mRNA (Minshall, et al., 1999). This study confirms the dual role of CPEB as a masking and activation factor. It was observed that CPEB in Spisula undergoes rapid phosphorylation after fertilization (Walker, et al., 1999). Consequently, phosphorylation of CPEB may be required for the activation of mRNAs and account for the different functions of this protein.

The role of CPE sequences and their associated factors in mRNA masking is also seen in mammalian oocyte development. When the mRNA for mouse tissue type plasminogen activator (tPA) is first transcribed, it possesses a long poly (A) tail which is rapidly reduced in the growing mouse oocyte. This mRNA remains in the oocyte cytoplasm in a dormant state until the resumption of meiosis, when it acquires a long poly(A) tail. The sequences required for the masking effect and the maturation-induced polyadenylation of tPA mRNA are overlapping, and therefore known as adenylation control elements (ACE) (Huarte, et al., 1992). Injecting ACE sequences into immature mouse oocytes result in the premature translation of tPA mRNA (Stutz, et al., 1998). The translational activation of this mRNA with competitor ACE sequences does not require lengthening of the poly (A) tail. Nevertheless, a tail of at least 40-50 residues is required to see this effect. A factor binding to the ACE sequences has been identified. Injection of ACE sequences reduces the amount of this 80kD factor bound to tPA mRNA suggesting that it is responsible for the masking effect (Stutz, et al., 1998). This factor has a similar molecular weight to the Xenopus CPEB. Its binding and functional behavior would suggest that the ACE binding protein might be the mouse homologue of CPEB.

From these studies it is evident that CPE/CPEB-mediated masking and activation of oocyte mRNAs are conserved in diverse species. It has recently been suggested that

the actions of CPE sequences and CPEB might be required also in somatic tissues of a developed organism. Thus, masking and control of polyadenylation might be general mechanisms for the control of translation. In mouse brain it has been observed that αCaMKII, a protein required for synapse plasticity, gets preferentially translated in dendrites following visual stimulation. Analysis of the αCaMKII mRNA shows that it is polyadenylated upon visual stimulation (Wu, et al., 1998). In addition, αCaMKII RNA has two CPE domains in its 3'UTR, and CPEB is present in high concentrations in the dendritic layer of the hippocampus and in postsynaptic densities of the adult mouse brain. The CPEs in αCaMKII can bind CPEB in vitro and can mediate polyadenylation-induced translation in Xenopus oocytes (Wu, et al., 1998). These results implicate the CPE/CPEB-mediated mechanisms of translational control and activation in somatic cells and suggest that mechanisms known to affect translation during early development might be used at other stages to regulate gene expression in specific tissues.

Oocyte development seems to also require other silencing events that are independent of the CPE and CPEB. Some of the messages that get polyadenylated and activated after maturation get deadenylated and become translationally silent after fertilization. Xenopus mRNAs *eg1-cdk2*, *eg5*, *eg-2* and *c-mos* show this characteristic polyadenylation/deadenylation behavior. In contrast to the default deadenylation that leads to turn over of RNA messages, the deadenylation of *eg2* mRNA is a sequence specific event. Deletion of a 17 nucleotides region in the 3'UTR of this mRNA turns deadenylation into polyadenylation (Bouvet, et al., 1994a). This embryo deadenylation element (EDEN) is also present in the 3'UTR of c-mos and Eg5 mRNA and is independent of the cytoplasmic polyadenylation signals (Paillard, et al., 1998). In addition, a 53 kDa protein containing three RRM RNA binding motifs (EDEN-BP) binds to the EDEN sequences and is a necessary component of the EDEN-dependent deadenylation (Paillard, et al., 1998). Three additional AUU repeats in the c-mos 3' UTR are able to enhance EDEN-dependent deadenylation (Audic, et al., 1998). This effect was observed for two distinct RNAs containing EDEN sequences, implying that combinations of cis-acting elements may regulate stage and site-specific deadenylation in Xenopus embryos.

The concerted masking, adenylation and final deadenylation of mRNAs during oocyte maturation seems to control the translation of maternal messages. This mechanism of translational control underlines the important role that poly(A) tails play during translation. In addition, it is clear that this regulatory mechanism and the cis and trans-acting factors involved in it are conserved among diverse organisms such as clams,

frogs and mice. Nevertheless, various issues are still unresolved. The relationship between CPEB and masking factors such as FRGY2 is not understood. It is possible that these two masking mechanisms act on different messages to achieve regulation. A second possibility is that mRNPs form in the nucleus so that the mRNA is protected from translation as it is exported to the cytoplasm. Once in the oocyte cytoplasm other factors such as CPEB may reduce the poly (A) length and continue to mask the mRNA from the translation machinery. Finally, it is also possible that CPEB and mRNP factors like FRGY2 are partners in the masking of mRNAs during oocyte development.

The relationship between unmasking, polyadenylation and activation is not clear. Release of CPEB from the 3'UTR seems to be sufficient for activation, but polyadenylation of the messages insures that the translation is efficient. Immunodepleting CPEB from mature oocytes eliminates polyadenylation. This might be a consequence of a direct role of CPEB in polyadenylating the mRNA. The switch from repressor to activator perhaps induced by phosphorylation needs to be studied further. Further work on the translational regulation of mRNAs in early oocytes will allow us to better understand the processes involved in masking and activation. Experimental systems in which genetic methods are well established could be useful to identify new factors involved in these processes.

Translational regulation in *C. elegans* sex determination: tra-2 and fem-3

Sex determination in the nematode *C. elegans* is controlled by a cascade of regulatory genes (Hodgkin, 1990). Two sexual fates exist: XX animals are self-fertilizing hermaphrodites, while X0 animals are cross-fertile males. The germ line of a *C. elegans* hermaphrodite produces sperm first and then oocytes, while the male exclusively produces sperm. The *tra-2* gene directs female cell fate in the hermaphrodite worm. Tra-2 protein is thought to act as a repressor of downstream male determinants. In the male, *tra-2* mRNA is present but its translation is regulated. Dominant gain of function mutations of *tra-2* (*tra-2* (gf)) transform hermaphrodites into females which only produce oocytes (Doniach, 1986). XO animals are also feminized, producing only oocytes. This transformation corresponds to higher levels of Tra-2 protein.

All six *tra-2* (gf) mutations map to a 60 nucleotide direct repeat element (DRE) located in the 3' UTR. The direct repeats consist of two 28 nucleotide elements separated by a four nucleotide spacer (Goodwin, et al., 1993). Deletion of these repeats causes

germ line transformation. Conversely, DREs in the 3'UTR of a reporter message cause its translational repression. Disruption of the DRE does not affect the mRNA levels but causes a movement of the mRNA towards larger polysomes. Using crude worm extracts, a protein complex that binds to the DRE was identified and named DRF (Goodwin, et al., 1997b). Stages at which this complex is present correspond to the lowest levels of *tra-2* expression.

Further studies using a yeast three-hybrid screen identified the *C. elegans* protein Gld-1 as a member of DRF (Jan, et al., 1999). Gld-1 is a member of the STAR family of RNA binding proteins. In *gld-1* loss of function (*gld-1(lf)*) mutants, the oocyte germ line fails to progress through meiosis, causing an over proliferation of germ-line cells. In hermaphrodite animals, few or no sperm are made. This phenotype is consistent with a failure to regulate *tra-2* expression. Correspondingly, Tra-2 protein is over-expressed in *gld-1(lf)* while levels of *tra-2* mRNA are unchanged. In vivo and in vitro studies show that Gld-1 can direct the repression of a DRE containing reporter. The mechanism by which the translation of *tra-2* is regulated in not completely understood. The presence of DREs correlate with a short poly(A) tail while their absence is characterized by a long poly(A) tail (Jan, et al., 1997). Whether the deadenylation is the mechanism by which translation is shut down or a consequence of a different regulatory mode is not known. The identification of targets and protein interactions of the trans-acting factor Gld-1 will help to understand how the DRE/Gld-1 complex controls translation.

Evidence that this mechanism of regulation might be conserved in other organisms comes from the presence of DRE sequences in the 3'UTR of the human oncogene *gli* (Jan, et al., 1997). Consequently, these elements have been renamed *tra-2* and *gli* elements (TGE). Deletion of the TGEs increases *gli*'s capability of transforming cells. In addition, the *gli* 3'UTR binds to a protein complex from mammalian cell extracts that has a similar molecular weight to DRF. This information suggests that *gli* might be regulated in human cells in a similar way to *tra-2*'s regulation in nematodes, implicating this regulatory mechanism in higher eukaryotes. In addition, the presence of the *tra-2* TGEs in the 3'UTR of a reporter can direct deadenylation in Xenopus oocytes, emphasizing the conserved nature of TGE mediated regulation (S. R. Thompson and M. Wickens, personal communication).

Fem-3 is required for normal male development in *C. elegans*. In hermaphrodites Fem-3 instructs the first germ cells to become sperm and then its expression is turned off and oocytes are formed. Loss of *fem-3* function transforms both the hermaphrodites and males into females. Gain of function mutations result in the masculinization of the

hermaphrodite germ line. Genetic analysis shows that, in gain of function mutations, *fem-3* is able to escape the regulation to which it is normally exposed after the formation of sperm. All *fem-3* gain of function mutations fall within a five nucleotides element in the 3'UTR (Ahringer and Kimble, 1991). These mutations do not affect the levels of mRNA but result in a lengthening the poly(A) tail. Deadenylation might therefore be part of the mechanism used to repress the expression of *fem-3*.

A protein that specifically binds to the *fem-3* regulatory sequences was identified using a yeast three-hybrid screen (Zhang, et al., 1997). The FBF protein is specifically expressed in the germ line and is required for the sperm/oocyte switch. FBF contains eight repeats in its C terminal region which are homologous to the RNA binding domain of the Drosophila protein PUMILIO (PUM) (Barker, et al., 1992; Macdonald, 1992; Murata and Wharton, 1995; Zamore, et al., 1997). PUM and FBF belong to a large family of proteins containing this newly identified RNA binding domain (Zamore, et al., 1997). PUM is required in the Drosophila embryo in order to, along with the posterior determinant NOS, repress the expression of *hunchback* (*hb*) RNA (Wang and Lehmann, 1991; Barker, et al., 1992). This repression correlates with a deadenylation of *hb* mRNA and depends on specific sequences in the *hb* 3'UTR known as nanos response elements (NREs) (Wharton and Struhl, 1991; Wreden, et al., 1997). The PUM RNA binding domain binds specifically to the NREs (Zamore, et al., 1997). NREs and the *fem-3* regulator sequences show some homology, but it is likely that FBF and PUM might have distinct RNA specificity. Since PUM requires the action of a second protein, NOS, in order to repress the translation of *hb*, it is further possible that FBF interacts with other proteins to control *fem-3* expression. The products of the *C. elegans mog* genes are good candidates for FBF functional partners, since they are also required for the spermatogenesis to oogenesis switch (Graham, et al., 1993b).

The Drosophila protein PUM is also active in other developmental processes such as oogenesis and germ cell migration (Lin and Spradling, 1997; Forbes and Lehmann, 1998). PUMILIO's targets during these developmental stages are not known. Furthermore, NOS does not seem to act as PUMILIO's partner in all of its regulatory functions (Forbes and Lehmann, 1998). This observation opens the possibility that PUM and perhaps FBF act to target specific RNAs for regulation by binding to 3'UTR sequences and then recruit other factors that impose translational silencing.

Translational regulation in cell fate specification: 15-lipoxygenase

Mammalian red blood cells lose their nucleus during development, thus generating new proteins through new transcription is not possible. Therefore, as in oogenesis and embryonic development, translational regulation is used to change the protein content of the cell at different stages. An important step in the differentiation of mammalian reticulocytes into erythrocytes is the destruction of the mitochondria. The enzyme 15-lipoxygenase (Lox) catalyzes the degradation of lipids and is unique in its ability to attack intact phospholipids which makes it instrumental in mitochondria destruction. *lox* mRNA is the second most abundant message in bone marrow erythroid precursor cells. Nevertheless, the protein is not expressed until reticulocytes undergo the last steps of maturation in the peripheral blood. Rabbit's *lox* mRNA silencing depends in 10 tandem repeats of a pyrimidine-rich 19 nucleotide motif located in its 3' UTR and known as the differentiation control elements (DICE) (Ostareck-Lederer, et al., 1994). These repeats bind a 48 KDa factor that is necessary and sufficient for translational repression of *lox* in vitro. Interestingly, these in vitro studies did not used capped or polyadenylated messages suggesting that the repression might be independent of changes in the poly(A) tail.

Recent studies have identified the 48 KDa factor as a fragment of heterogenous nuclear ribonucleoprotein K (hnRNP K). In addition, hnRNP E1 was also purified because of its ability to bind the DICE sequences (Ostareck, et al., 1997). Transfection of either or both hnRNP K and hnRNP E1 into HeLa cells specifically represses the translation of a reporter message with DICE sequences in its 3'UTR. In addition silenced *lox* mRNA forms a complex with hnRNP proteins in vivo. Fractionation of rabbit reticulocyte lysates in the absence of silencing factors show that lox mRNA is present at similar levels in the ribosome-associated and lighter fractions. Nevertheless, addition of hnRNP K and hnRNP E1 shifts the message from the polyribosomes to the monosomal fraction. These results suggest that hnRNPs might regulate translation by affecting the assembly the 80S ribosomes (Ostareck, et al., 1997). In addition, it was shown that the purified hnRNP molecules could inhibit cap-dependent as well as internal ribosomal entry site (IRES) dependent translation. Therefore, the regulation of *lox* mRNA in erythrocyte development affects steps upstream of the cap-binding steps of initiation which are needed in IRES translation. hnRNPs seem to be able to affect the recruitment of the 60S ribosomal subunit to the initiation complex. The study of translational regulation in mammalian blood cell differentiation gives evidence that regulation via the 3'UTR can affect other processes besides polyadenylation. Poly(A) independent

translational regulation could act by inhibiting translation mechanisms occurring at the 5'UTR. Binding proteins complexed to the 3'UTR might be able to affect such processes given that the 5' and 3' UTR are already interacting through PABP and eIF4F.

Translational regulatory cascade in Drosophila oogenesis and embryogenesis: *oskar*, *nanos* and *hunchback*

Early development in Drosophila is characterized by the lack of transcription. Therefore, the initial molecular cues required for axis specification and body patterning are supplied to the egg during oogenesis in the form of maternal mRNAs and proteins. The regulated expression of these messages is the consequence of regulatory cascades that include both mRNA localization and translational regulation. The advantages of Drosophila as a genetic system have allowed the dissection of these mechanisms at the molecular level. Specification of the posterior pole of the embryo and the subsequent development of the abdomen have become model systems for the study of mRNA localization and regulation. Abdomen formation in the embryo depends in the regulation of the *hunchback* (*hb*) mRNA at the posterior pole of the early syncytial embryo (Irish, et al., 1989; Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991; Wharton and Struhl, 1991). Since *hb* mRNA is present in the entire embryo this regulation requires to be spatially controlled. The localized action of *hb* regulation is the product of a complex regulatory cascade that begins during oogenesis with the regulation of the *oskar* mRNA.

Oskar (OSK) protein in the oocyte is exclusively present at the posterior pole. Its function in this region of the egg is to localize mRNAs and also to specify the cytoplasm that gives rise to the primordial germ cells (Ephrussi, et al., 1991; Ephrussi and Lehmann, 1992). *OSK* mRNA is localized to the posterior pole during oogenesis. The maternal *osk* mRNA is transported into the oocyte during early stages of oogenesis, and becomes localized to the posterior pole of the oocyte during midoogenesis. Expression of OSK protein at the posterior pole of the oocyte is coincident with its localization (Kim-Ha, et al., 1995). Mutations that disrupt *osk* localization but not its stability also affect its translation, indicating that expression of unlocalized *osk* is repressed (Rongo, et al., 1997). Localization and translational control depend on sequences in the 3'UTR. A repeated sequence motif in the 3'UTR interacts with an RRM protein called BRUNO (BRU) (Kim-Ha, et al., 1995; Webster, et al., 1997). The RNA elements responsible for this interaction are known as BRU response elements (BRE). Mutations in the BREs

cause ectopic translation of *osk* without affecting *osk* localization. In addition, BREs can direct the regulation of a reporter gene. BRU, therefore, may be involved in the translational regulation of unlocalized *osk*. BRU is a homologue of the EDEN binding protein required for specific deadenylation of Xenopus *c-mos* mRNA (see above). Interestingly, the regulation of *osk* occurs without changes in its poly(A) tail (Salles, et al., 1994; Rongo, 1996). A model that explains the different mechanisms performed by these homologous proteins is that BRU and EDEN-BP simply target specific mRNAs for regulation by binding to 3'UTR sequences and that other factors perform the specific regulatory events, that may or may not involve deadenylation. A second RNA binding protein that interacts with both the *osk* 3'UTR and BRU has been identified (Lie and Macdonald, 1999). Genetic analysis of this factor, known as APONTIC, reveals that its function is also required for the regulation of unlocalized *osk* mRNA. Additionally, evidence exists for other factors that bind to 3'UTR sequences required for repression but not for BRU binding (Gunkel, et al., 1998). Furthermore, genetic analysis of the mutation *arrest,* which corresponds to mutations in BRU, indicate that BRU regulates multiple mRNAs involved in gametogenesis (Webster, et al., 1997).

The activation of *osk* translation once it is localized is an active process that requires cis and trans-acting factors. Several gene products, including Aubergine and Staufen, have been implicated in the translational activation of *osk* (Wilson, et al., 1996). A recent study reports that sequences in the 5'UTR are required for translation of OSK and that a 68 KDa factor binds to these segments of the mRNA. Interestingly, this region is not required for OSK translation if the BRU mediated regulation is affected by BRE mutations (Gunkel, et al., 1998). This result indicates that these sequences are required for derepression and that they might facilitate the removal of the BRU repressor.

Once OSK protein is translated at the posterior, one of its functions is to localize the posterior determinant *nanos* (*nos*). Localization of *nos* to the posterior pole of the oocyte occurs during late stages of oogenesis (Wang, et al., 1994). This localization requires multiple sequences in the *nos* 3'UTR (Gavis, et al., 1996a). NOS protein is expressed in the embryo after fertilization forming a posterior to anterior gradient. Embryos lacking OSK result in the delocalization of *nos* mRNA and the lack of NOS expression. The translational control of unlocalized *nos* mRNA is independent of changes in the poly(A) tail length and is mediated through a 90 nucleotide region in the 3'UTR known as the translation control element (TCE) (Gavis, et al., 1996b). Smaug, an embryonic protein, binds to sequences within the TCE which are required for regulation of *nos*, indicating that Smaug is a translational repressor of *nos* (Smibert, et al., 1996).

Quantitative experiments have revealed that most of *nos* mRNA is not localized to the posterior, suggesting that translational regulation is the principal mechanism for posterior restriction of NOS activity (Bergsten and Gavis, 1999). In addition, translational repression and localization of *nos* seem to be mutually exclusive suggesting that factors involved in the localization somehow remove the repressor from the mRNA. Thus, the spatial restriction of *nos* mRNA is achieved by translational repression, and the localization of *nos* is only required to activate translation.

NOS function in the embryo is to restrict the expression of the transcription factor *hb* to the anterior of the embryo. HB protein produces an anterior to posterior gradient complementary to that of NOS. HB represses the transcription of genes required for the formation of the abdomen. Thus, lack of NOS protein results in the misexpression of HB at the posterior and the lack of abdominal segments (Wang and Lehmann, 1991). Two bipartite repeats in the 3'UTR of *hb*, termed NANOS response elements (NRE), are required for *hb* regulation (Wharton and Struhl, 1991). As mentioned above, PUMILIO (PUM) protein binds to these sequences and aids in the repression of *hb* translation (Murata and Wharton, 1995; Zamore, et al., 1997). Unlike NOS, PUM is present throughout the entire embryo, therefore NOS confers the spatial specificity to *hb* regulation. It has been suggested that PUM acts as a "docking protein" for factors that specifically regulate the translation of *hb*. The 3'UTR of *hb* is preferentially deadenylated in the posterior, and this process requires PUM, NOS and the NREs (Wreden, et al., 1997). The exact relationship between the removal of the poly(A) tail and the repression of translational is not known.

In summary, the concerted gene regulation, required for the patterning of the Drosophila embryo, utilizes a combination of mRNA localization and translational regulation. As in many cases of translational repression in development, the 3'UTR of the regulated message plays a critical role. In addition, in some cases regulation coincides with changes in the length of the poly(A) tail, although other mechanisms are clearly exploited during the development of Drosophila.

CONCLUSIONS

It is clear that the cap and the poly(A) tail of an mRNA play in important role during its translation. Accordingly, the removal of the poly(A) tail is suspected to be a step in the regulation of translation. The translational silencing of *cyclins* in Xenopus oocytes, of *tra-2* and *fem-3* in *C. elegans* and of *hb* in Drosophila embryos is accompanied by deadenylation. On the other hand, the regulation of *nos* and *oskar* in Drosophila oocytes and embryos and of 15-lipoxygenase in mammalian erythrocytes does not involve a change in the size of the poly(A) tail. In this case, steps in initiation or ribosomal joining might be the targets of regulation. Whether the removal of the poly(A) tail is cause or consequence of the regulation of translation is not known. It is possible that deadenylation and direct control of initiation are used in combination to reinforce the necessary quiescent state of the mRNA.

One detail that repeats itself in regulatory mechanisms from masking to initiation is the role of 3'UTRs and associated RNA binding proteins. Various studies have shown that the two ends of the mRNA can interact during translation, and that this interaction is necessary for efficient translation. Thus disrupting this interaction by competing away the linking factors or by preventing access to cis-acting sequences could influence the rate of translation. New techniques developed to find protein-protein or protein-RNA interactions will allow the determination of the global translational factors affected by the specific translational regulators.

The function of the trans-acting factors PUM and BRU presents an interesting paradigm in mRNA regulation. These two factors bind specifically to mRNA regulative elements, but require the action of other factors to impart the regulation. These proteins might act as landing pads for factors that perform specific functions during regulation such as deadenylation. In addition, both PUM and BRU seem to regulate more than one target and they might recruit different protein partners depending on the message being regulated. This modular model of regulatory factors will be discussed further throughout this thesis.

Figure 1.1 Translation initiation

Diagram of factors and steps involved in the initiation of eukaryotic translation. Adapted from (Merrick and Hershey, 1996).

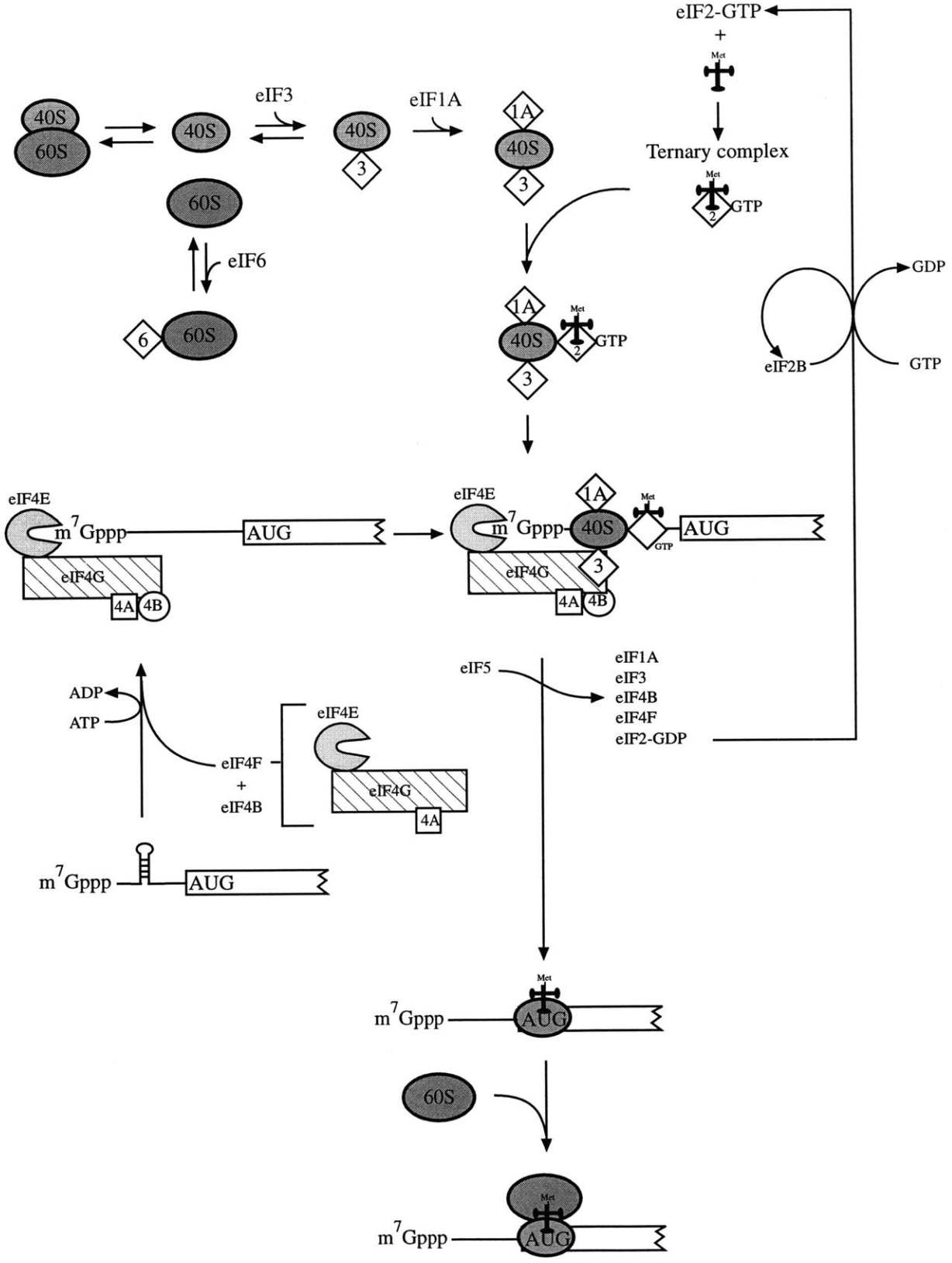

eIF2-GTP
+
Met
Ternary complex
Met
GTP
2
40S
60S
eIF3
40S
3
eIF1A
1A
40S
3
60S
eIF6
6
60S
1A
40S
Met
2
GTP
3
GDP
eIF2B
GTP
eIF4E
m7Gppp
AUG
eIF4G
4A
4B
eIF4E
1A
m7Gppp
40S
Met
AUG
GTP
3
eIF4G
4A
4B
ADP
ATP
eIF4F
+
eIF4B
eIF4E
eIF4G
4A
eIF5
eIF1A
eIF3
eIF4B
eIF4F
eIF2-GDP
m7Gppp
AUG
m7Gppp
Met
AUG
60S
m7Gppp
Met
AUG

Figure 1.2 Translation elongation

Diagram of the steps in eukaryotic translation elongation based on the hybrid state model (Wilson and Noller, 1998). Boxes labeled E, P and A refer to the ribosomal binding site for the empty, the peptidyl and the aminoacyl tRNA respectively.

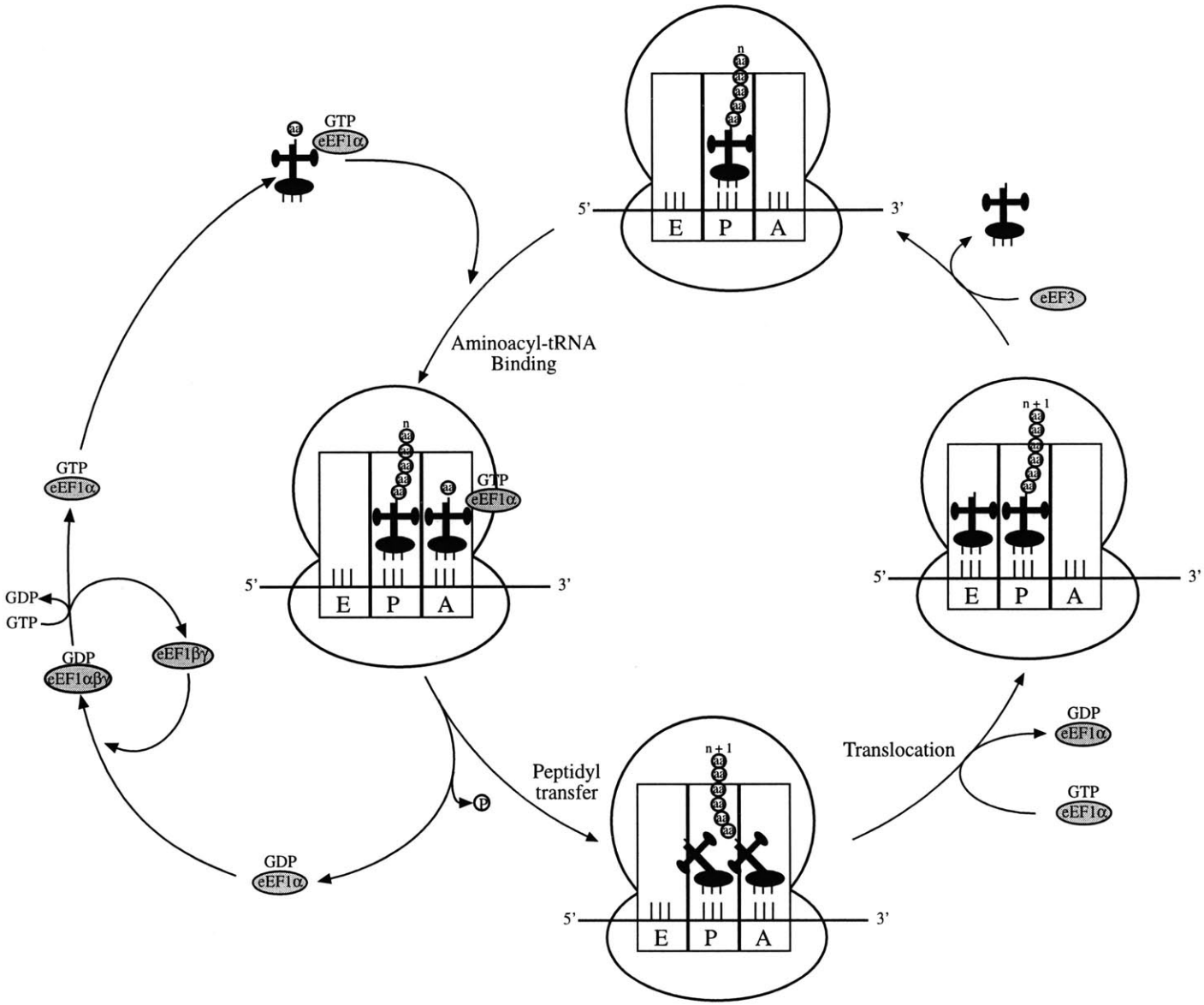
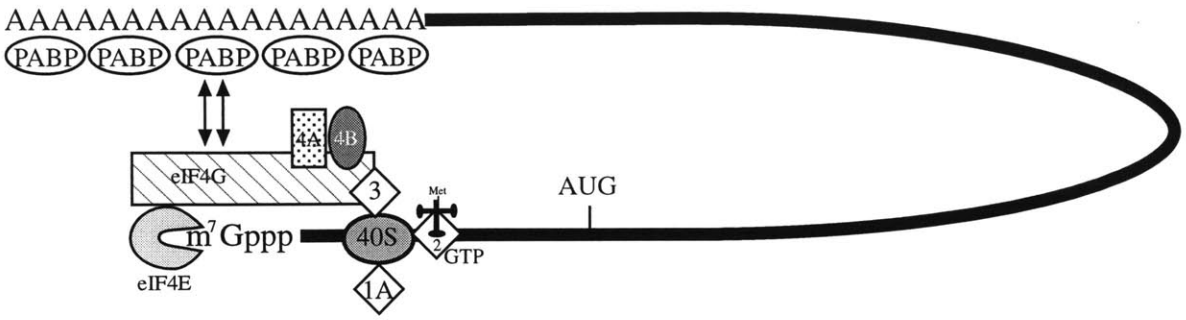

GTP
eEF1α
n
5'
3'
E
P
A
eEF3
Aminoacyl-tRNA
Binding
n + 1
GTP
eEF1α
GDP
GTP
GDP
eEF1αβγ
eEF1βγ
Peptidyl
transfer
Translocation
GDP
eEF1α
GTP
eEF1α
GDP
eEF1α

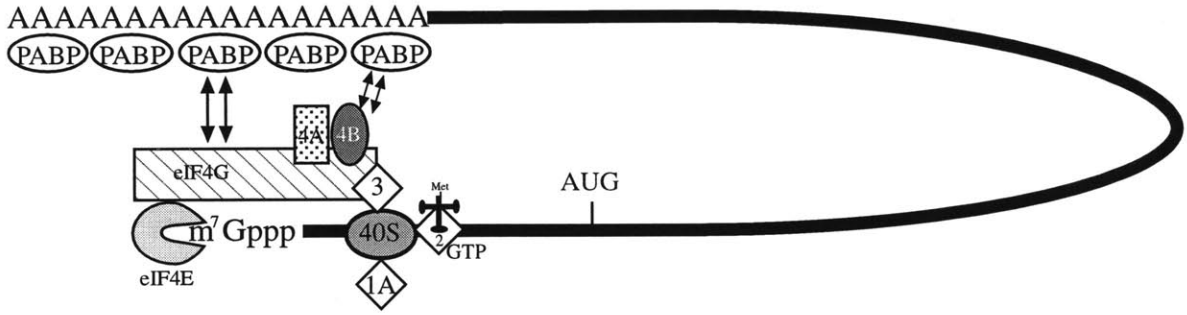
Figure 1.3 Interaction between the cap and the poly(A) tail

Models for the circularization of the mRNA in yeast, plants and mammals. Double arrows point at factors that physically interact. Adapted from (Gallie, 1998).

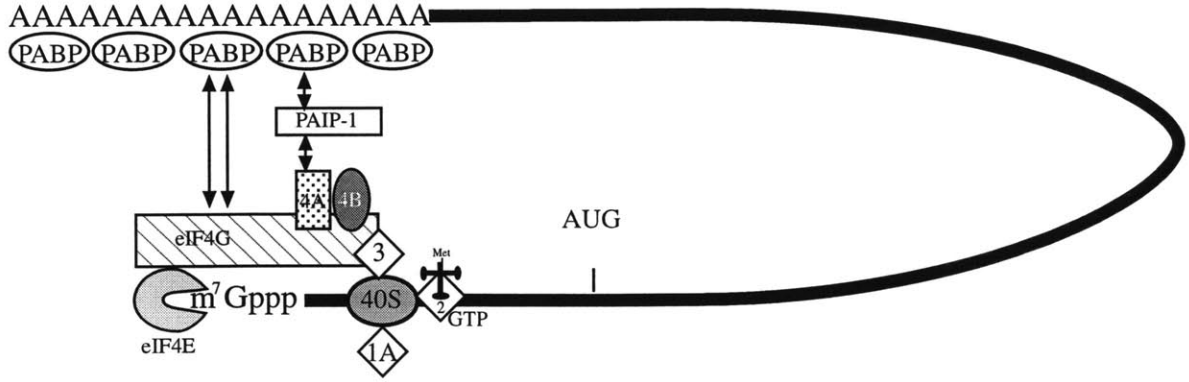
Yeast:



Plants



Mammals



SPECIFIC AIMS

The aim of this thesis is to elucidate the functional characteristics of the Drosophila protein NOS. NOS is involved in the regulation of translation of the maternal *hunchback* mRNA in the embryo. In addition, NANOS is involved in other developmental steps such as oogenesis and germ cell migration. Chapter 2 discusses a genetic and molecular analysis of the NANOS protein. A highly selective mutagenesis screen was performed to determine essential amino acids in the protein. This screen has allowed the identification of several functional domains within the NANOS protein. Chapter 3 describes a yeast two-hybrid screen performed in order to identify proteins that interact with NANOS. Two specific interactions are discussed in detail in this chapter along with tests performed to study the potential physical interaction between NANOS and PUMILIO, an RNA binding protein necessary for the NANOS dependent regulation of *hunchback.* Chapter 4 discusses experiments performed to study the physical and genetic interaction between NANOS and the RNA binding protein BRUNO. The implications of this interaction in oogenesis and in translational regulation are discussed. Finally, Chapter 5 discusses the conclusions drawn from these studies and attempts to draw a modular model for proteins involved in translational regulation.

CHAPTER 2

Isolation and characterization of new alleles of the Drosophila gene *nanos*

SUMMARY

The Drosophila protein NANOS is involved in three developmental stages: oogenesis, embryogenesis and germ cell migration. In the early embryo NANOS is required to regulate the translation of the maternal transcript *hunchback*. The mechanisms used by NANOS to regulate *hunchback* and its exact function in other stages of development are not known. The NANOS protein contains two zinc fingers in its C-terminal region that are necessary for its function in embryogenesis. Here we describe a highly selective genetic screen aimed at isolating new *nanos* alleles. The molecular and genetic analysis of 68 new alleles has allowed us to identify amino acids critical for NANOS function. This analysis shows that the zinc fingers are essential for NANOS function in all its developmental roles. Furthermore, a region C-terminal to the zinc fingers was shown to constitute a novel functional domain within the NANOS protein. This "tail region" of NANOS is required for abdomen formation and germ cell migration but not for oogenesis.

AUTHOR'S NOTE

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INTRODUCTION

Establishment of polarity along the anterior to posterior body axis of the Drosophila embryo requires the function of the maternal effect gene *nanos* (*nos*) (Wang and Lehmann, 1991). *nos* RNA is synthesized during oogenesis and becomes localized to the posterior pole of the oocyte at the end of oogenesis (Wang, et al., 1994). Upon fertilization, NOS protein is translated from the posteriorly localized RNA to form a posterior-to-anterior protein gradient. Cis-acting sequences required for localization of *nos* RNA to the posterior pole as well as sequences that prevent translation of unlocalized RNA have been mapped to the 3'UTR of *nos* RNA (Gavis and Lehmann, 1992; Dahanukar and Wharton, 1996; Smibert, et al., 1996; Gavis, et al., 1996a; Gavis, et al., 1996b). NOS acts together with *pumilio* (*pum*) to repress translation of the maternally provided transcription factor *hunchback* (*hb*) (Barker, et al., 1992; Macdonald, 1992). In the wild type, HUNCHBACK protein is distributed in a gradient reciprocal to that of NOS protein. In the absence of either NOS or PUM function, HUNCHBACK protein is translated throughout the embryo. Ectopic translation of *hb* in the posterior region leads to the transcriptional repression of genes normally required for abdomen formation. Thus, *nos* or *pum* mutant females produce embryos that lack abdomen.

Biochemical analysis has shown that a ~400 aa C-terminal region of PUM protein binds directly to a sequence motif in the 3'UTR of *hb* RNA, referred to as the NANOS Response Element (NRE) (Murata and Wharton, 1995; Zamore, et al., 1997). The NREs are necessary and sufficient for the regulation of *hb* by NOS and PUM (Wharton and Struhl, 1991). A number of experiments suggest that PUM is bound to the NRE throughout the embryo (Murata and Wharton, 1995; Zamore, et al., 1999). *hb* translation, however, is only inhibited where NOS is present. Thus, the distribution of NOS somehow determines the spatial regulation of *hb*. This conclusion is supported by experiments in which NOS was expressed at ectopic positions. In a set of experiments *nos* was mislocalized within the oocyte, by replacing the posterior localization sequences of *nos* with the anterior localization sequences from *bicoid* 3'UTR (Gavis and Lehmann, 1992). Alternatively, translational repression of *nos* was overcome by removing the *nos* 3'UTR and replacing it with the 3'UTR of *tubulin* which leads to uniform distribution of the RNA and the protein (Gavis and Lehmann, 1994). In both cases head structures were lost and replaced by more posterior structures. It was shown that ectopic expression of *nos* in addition to *hb* also affects translation of the anterior morphogen *bicoid* (*bcd*) whose function is required for the development of the head and thoracic structures. Like *hb*, *bcd* contains NRE sequences in its 3'UTR.

In addition to early pattern formation, *nos* function is also required for the development of the germ line. In the early embryo *nos* RNA and protein are taken up into the primordial germ cells as they form at the posterior pole. In the absence of NOS many aspects of normal primordial germ cell (PGCs) behavior are affected (Kobayashi, et al., 1996; Forbes and Lehmann, 1998). Germ cells fail to migrate towards the somatic gonad, which forms from mesodermal tissue. Germ cell morphology is aberrant and certain transcripts that are only expressed at later stages of normal development are expressed early in *nos* mutant germ cells. It has therefore been proposed that NOS affects the translation of a number of maternally deposited RNAs that are required for early germ cell migration (Asaoka et al., 1998). HB is not the target of *nos* in the PGCs, since the germ cell migration defect of *nos* mutant PGCs is observed even in the absence of maternal HB product (Forbes and Lehmann, 1998).

Transcription of *nos* is initiated during the first larval instar and is restricted to the germ line throughout its development. While *nos* mutant males are fertile, several aspects of female germ line development are affected in *nos* mutant females (Forbes and Lehmann, 1998). Only about 50% of *nos* homozygous mutant PGCs that reach the embryonic gonad will become germ line stem cells. In the wild type, germ line stem cells maintain themselves and produce daughter cells, termed cystoblasts. The cystoblast undergoes four round of division to give rise to sixteen interconnected cells, one of which will become the oocyte. In *nos* mutants, germ line stem cells do not maintain their stem cell character throughout the life of the female and differentiation of the cystoblast into an egg chamber is affected. As a consequence, *nos* mutant females produce only very few eggs. Targets for NOS during germ line development have not been identified. Interestingly, while *nos* shares with *pum* a role in the translational regulation of *hb* and in germ cell migration, certain aspects of the oogenesis phenotypes of *nos* and *pum* are different. This observation suggests that NOS and PUM have different partners and control separate targets during oogenesis (Forbes and Lehmann, 1998).

The specific role of NOS in translational regulation is unclear and a direct physical interaction between NOS and PUM or NOS and the *hb* NREs has not been established. NOS encodes a protein of 402 amino acids with a highly conserved C-terminal region (Curtis, 1995b). The carboxyl end includes two zinc fingers of the CCHC type, similar to those found in the nucleocapsid binding protein of HIV I (Curtis, et al., 1997). Zn finger domains similar to those of NOS have been identified in proteins from Xenopus, leech, and *C. elegans* (Mosquera, et al., 1993, Curtis, et al., 1997; Pilon and Weisblat, 1997). The function of these genes is not clear, however, due to the

respective expression pattern, a role similar to that of Drosophila NOS in pattern formation and germ line development has been proposed.

The analysis of *nos* mutants should identify domains in the protein important for NOS function. Such an analysis could also provide important information regarding the different aspects of the *nos* mutant phenotype. Only four alleles of *nos* have been described (Wang, et al., 1994; Curtis, et al., 1997). While one allele is a stop codon, three alleles map to the C-terminus. One of these alleles carries a mutation in a cysteine predicted to be involved in metal ion binding of the Zn finger and leads to a strong phenotype similar to that of complete null mutations. The other two mutations are a deletion and a point mutation in a region C-terminal to the Zn finger, termed the "tail domain". These mutants have been shown to affect *hb* translational regulation and have no effect on female germ line development. The role of mutations in the tail domain in germ cell migration has not been analyzed. In addition, injection experiments using wild-type and mutant *nos* transcripts have been used to determine regions of the NOS protein that are required for NOS function (Curtis, et al., 1997). The specific experimental design restricted the analysis to the role of NOS in *hb* regulation, while other aspects of the phenotype could not be analyzed. These experiments showed that the Zn finger region and tail domain are necessary for NOS function in *hb* regulation, while other regions of the protein seem not essential.

Here we describe a selective genetic screen that allowed us to obtain 68 new *nos* alleles. We have identified the specific lesions in each allele and have characterized the phenotype of each mutation. This analysis demonstrates the functional relevance of the Zn finger domain for all aspect of *nos* function. Our experiments further suggest that the tail domain constitutes a separate domain required for *hb* regulation and PGC migration. Implications of our analysis for the functional, structural and evolutionary aspects of *nos* are discussed.

METHODS

Fly strains

For the screen wg^{IL114} was utilized. sax^{1} *bn sp/ Cyo* $p(ry^{+t7.2}=en1)$ wg^{en11} flies were a gift from Bill Gelbart. $Nos^{RC}/Df(3R)Dl^{x43}$ was used when crossing the transgenes to a *nos* null background (Curtis, et al., 1997). Nos^{L7} carries a 7 amino acid deletion in the C terminus of NOS (Figure 2.2A).

Transgene and P-element transformation

The nos-tub3'UTR hybrid gene has been previously described (Gavis and Lehmann, 1994) and its structure is shown in figure 2.1D. It contains genomic sequences of *nos* with its 3'UTR replaced by that of *tubulin*. It also contains a haemagglutinin epitope tag at the 5' end of the coding region. A Not I fragment from pDM30*n(ha)-t* (Gavis, et al., 1996a) was inserted into the w^{+} P element vector pCaSpeR3 (Pirrota, 1998).

Injection of CaSpeR*nos-tub3'UTR* (p[*nos-tub3'UTR*]) into *yw* flies was performed as described by Spradling (Spradling, 1986). Seven independent transformed lines were established. Those carrying the transgene in the second chromosome were kept for use in the screen. Females from all transformant lines were completely sterile, producing embryos with head defects and lack of thoracic segments (data not shown). Protein expression was observed in all lines by staining embryos with anti-NOS antibodies (data not shown).

In order to test whether female sterility of the P(*nos-tub3'UTR*)transgene was 100% penetrant, 200 *yw*; P(*nos-tub3'UTR*)/*Cyo wg* females from line 198.1 were crossed to 100 OregonR males and allowed to lay eggs for 4 days. None of the eggs developed, assuring us that even in large scale this transformant line does not yield viable progeny.

EMS mutagenesis and selection for *nanos* alleles

See Figure 2.2 for an outline of the screen. Males carrying the transgene p(*nos-tub3'UTR*) were balanced with a *CyO* chromosome containing a P-element homozygous lethal allele of *wingless* (*wg*). 14,400 of such males were mutagenized with a 35 mM solution of EMS (Sigma) in 1% sucrose for 24 hours using standard procedures

(Ashburner, 1989). The males were starved for 6 hours prior to mutagenesis, placing them in a bottle with a Kimwipe saturated with water. After EMS treatment, males were crossed to 14,300 *wg*/Cyo females. In the next generation, the presence of *wg* in the balancer chromosome from the males eliminates all flies except for those carrying the transgene. A total of 186,000 flies were divided in groups of approximately 200 animals, allowing them to mass mate. Only groups with a female carrying a mutation in the transgenic *nos* will gave rise to crawling larvae. 68 of about 900 groups had F2 progeny which developed to adulthood.

These surviving flies have a 50% chance of having inherited the mutant transgene. To identify those that carry the mutation, w^+ virgin females were tested for production of viable offspring by crossing them to *yw*; *wg/Cyo* males. In this manner 68 balanced heterozygous mutant lines were established. To ensure that all mutants studied were independent events, only one isolate per group was used for analysis.

Females from F2 containing two transgenes were identified by their eye color and used to test for second site suppressor. If the mutation lies within the transgene, the second transgene should be a wild-type version of the fusion gene, thus rendering the flies sterile. A second site suppressor should affect both transgenes equally, therefore allowing the females to reproduce some viable offspring.

Sequencing

Genomic DNA was isolated from each mutant line (Barker, et al., 1992). PCR was used to obtain DNA fragments of the *nos-tub3'UTR* trasgene. In order to isolate only the mutant transgene, and not the endogenous *nos* gene, we specifically amplified two fragments using primers directed against sequences in the epitope tag and the tubulin 3' UTR (Figure 2.1D). Two PCR fragments, one from the epitope tag to the *nos* intron 1 and a second from intron 1 to the 3' UTR of tubulin, were amplified from the various mutants. At least two simultaneous amplifications were performed for each fragment, in order to distinguish real mutations from those induced by PCR. We sequenced directly from the PCR fragments using either the AmpliCycle or ABI Prism systems form Perkin Elmer.

Germline clones

yw; *FRThb*^{FB}*nos*^{L7} females were crossed to *yw hsFLP*; *FRTovo*^{D} males (a gift from Claude Desplan), and their progeny heat shocked as 2nd or 3rd instar larvae to induce FLP expression (Chou and Perrimon, 1996). Larvae were heat shocked at 37°C as described by Forbes and Lehmann (Forbes and Lehmann, 1998). *yw hsFLP*; *FRThb*^{FB}*nos*^{L7}/*FRTovo*^{D} females were crossed to *nos*^{RC}/*Df(3R)Dl*^{x43}. OvoD females do not produce eggs, thus only *hb*^{FB}*nos*^{L7} homozygous germline clones will give rise to progeny.

Cuticle preparation and embryo staining

For cuticle preparations, embryos were dechorionated in 50% bleach, fixed in 1:4 glycerol and acetic acid and mounted in Hoyer's medium (Lehmann and Nusslein-Volhard, 1986).

Embryos derived from germ line clones were stained with anti-vasa to visualize the germ cells as previously described (Forbes and Lehmann, 1998).

RNA injections

To obtain *nos*^{WT} RNA, vector pN5 (Wang and Lehmann, 1991) containing the full length *nos* cDNA was linearized with Xho I and transcribed with SP6 polymerase. In order to isolate *nos*^{L7} RNA we performed the same process with a derivative of PN5 carrying the small deletion in the carboxyl terminus (Curtis, et al., 1997). The RNA was precipitated in ethanol and resuspended in water. Three concentrations were used in the injections: 250 ng/µl, 500 ng/µl and 1.3 µg/µl.

Site specific PCR mutagenesis was used to generate pN54CF1 and pN54CF2. pN54CF1 carries a mutation that changes the fourth Cys of the first finger to a Ser while pN54CF2 carries a mutation in the fourth Cys of the second finger changing it for a Ser. Both these plasmids were linearized with Xho I and transcribed with SP6 polymerase. The two RNAs were injected at a concentration of 1.5µg/µl.

A 0 to 45 minute collection of embryos laid by *nos*^{L7}/*nos*^{L7} mothers was dechorionated, lined up for injection on a coverslip, and injected from the dorsal side. Embryos were allowed to develop for 2 days at 18°C. Hatched larvae were collected and

unhatched embryos were hand devittelinized and mounted in 50% Hoyers and 50% lactic acid for cuticle preparation.

RESULTS

Isolation of new NANOS alleles

A large-scale analysis of the amino acids that are essential for NOS protein function should provide useful information regarding structurally important domains within the NOS protein. Despite a number of genetic screens designed to identify new *nos* alleles by non-complementation with the allele nos^{L7}, only 3 new alleles were identified (Lehmann and Nüsslein-Volhard, 1991; R. Lehmann, personal communication). This may indicate that NOS protein function is not easily disrupted by point mutations or the screens previously used were unsuitable to identify new alleles. We therefore designed a new scheme to identify important amino acids in NOS that allowed specific recovery of a large number of new *nos* alleles. This mutagenesis screen identifies mutations in *nos* by suppressing the dominant, female sterile phenotype that is caused by ectopic expression of *nos*. Replacing the *nos* 3'UTR with the *tubulin* 3' UTR leads to stable expression of unlocalized *nos* RNA, which is translated throughout the embryo (Gavis and Lehmann, 1994). Presence of NOS protein in the anterior of the embryos inhibits the translation of *hb* and *bicoid* (*bcd*) RNA (Gavis and Lehmann, 1992) Thus, females which carry a transgene with the NOS coding sequences fused to the 3'UTR of *tubulin* (P(*nos-tub3'UTR*)) produce embryos that lack head and thoracic structures and have duplicated abdominal segments (Figure 2.1B). These embryos are not viable thus rendering the females that carry this transgene dominant sterile. A mutation induced in the transgene that disrupts NOS function as a translational repressor of *bcd,* will revert the female sterile phenotype, thereby leading to viable offspring in the next generation (Figure 2.1C). Subsequently, the mutated transgene can be analyzed for mutations affecting NOS protein sequence.

While we are interested in identifying amino acids important for the function of NOS as a translational repressor of *hb*, the mutagenesis scheme selects for *nos* mutations that suppress NOS' ability to repress *bcd* RNA translation. A number of observations suggest that the effect of NOS on *hb* and *bcd* translational regulation are quite similar and that therefore mutations affecting the regulation of one should also affect regulation of the other. First, *bcd* regulation by NOS is dependent on sequences in the *bcd* 3'UTR that are, with regard to base composition and function, similar to the NREs in the 3' UTR of *hb* (Wharton and Struhl, 1991). Second, PUM protein, which acts in conjunction with NOS in the regulation of *hb*, is also required for translational repression of *bcd* (Lehmann

and Nüsslein-Volhard, 1991). Third, we tested whether known alleles of *nos* that were identified on the basis of their effect on *hb* regulation, also abolish *bcd* regulation. For this test we took advantage of an *oskar* (*osk*) transgene (*P(osk-bcd3'UTR*), that localizes *osk* RNA and OSK protein to the anterior pole of the egg (Ephrussi and Lehmann, 1992). In the wild type, *nos* RNA localization to the posterior is dependent on *osk* activity (Ephrussi, et al., 1991). Consequently, anterior localization of *osk* by the transgene leads to ectopic localization of the endogenous *nos* RNA and protein to the anterior. Ectopic localization of *nos* to the anterior leads to anterior deletions and posterior duplications in embryos derived from females carrying the *P(osk-bcd3'UTR)* transgene (Ephrussi and Lehmann, 1992). We placed the *P(osk-bcd3'UTR)* transgene into the background of two different, strong *nos* missense mutations, nos^{RC} *and* nos^{RD}, and analyzed the phenotype of the progeny. Embryos derived from these females showed the *nos* phenotype: head and thorax were formed normally but there was no abdomen (data not shown). This result demonstrates that a mutant NOS protein unable to repress *hb* regulation is also unable to repress *bcd* translation. Thus, a genetic screen based on the suppression of Nos-mediated regulation of *bcd* should allow identification of mutations that are also deficient in *hb* regulation.

An outline of the screen performed is shown in figure 2.2. A total of 14,400 males carrying the transgene was mutagenized with EMS and crossed to *y w* flies that also carry appropriate second chromosomal balanced markers. The use of the *wg* homozygous lethal mutation in the balancer chromosome allowed us to only obtain flies carrying the transgene in the F1 generation. Progeny of F1 females die as embryos unless the female carries a version of the transgene in which NOS function is lost. Consequently, many females could be tested simultaneously since only few would be fertile. In total 93,000 haploid genomes were tested and 68 independent mutant lines were established, this accounts for a mutagenesis rate of 1 in 1,370 haploid genomes.

Two types of repression can result in a reversion of the female sterile phenotype: intragenic and second site suppression. A simple test allowed us to distinguish between these two possibilities. In the F2 (Figure 2.2), female flies with two copies of the transgene could be easily distinguished from those carrying only one copy, since expression of the w^+ marker carried by the P(*nos-tub3'UTR*) transgene is dosage sensitive. To distinguish between intragenic and second site suppressors we analyzed the phenotype of the progeny of females that carry two copies of the transgene. If the mutation lies within one of the transgenes, the second copy of the transgene should still render the flies sterile. On the other hand, a second site suppressor should affect both

transgenes equally, therefore allowing females carrying two copies of the transgene to produce normal or phenotypically less severely affected progeny. All mutant lines were tested in this manner and none proved to carry a second site suppressor according to this test (see below). We conclude that the 68 mutation identified are likely to affect function of the *nos* transgene directly.

Molecular analysis of the alleles

Once the mutant lines were established, the transgenes were analyzed in order to identify the mutation. Each line contains both the transgene and the endogenous *nos*. Transgenic sequences were specifically isolated from the mutant lines by using primers unique to the transgene (see materials and method). We have identified mutations in 60 alleles. All but two mutations affect a single base. EMS is an alkylating agent that can add an ethyl group to many positions in all four bases (Ashburner, 1989). In Drosophila, the prevalent mutation resulting from EMS mutagenesis is a GC → AT transition. In our screen we prevalently see this type of mutation: 50 out of 58 base changes seen (Tables 1, 2 and 3). Nevertheless, we see other kinds of more unusual changes. AT → TA changes account for 6 of the 58 mutations, while the change TA→GC is seen in two mutations affecting the same amino acid (L350-R). A change in the protein coding sequence has not been detected in eight of the 68 alleles, we are presently testing the effect of these mutations on *nos* RNA and protein levels.

We have divided the mutants into three categories: nonsense mutations (Table 1), rearrangements (Table 2) and missense mutations (Table 3). In the first category we found 27 EMS induced premature stop codons (Table 1). The stop codons are distributed throughout the coding sequence and show no specific pattern.

In the rearrangement category (Table 2) we have included one deletion (nos^{272}), one insertion (nos^{581}) and a mutation that affects the intron/exon structure of the transgene ($nos^{246/526/623}$). This last mutation occurred in 3 different alleles. Similarly, the deletion and the insertion start at the same base. The deletion is in frame and eliminates 27 amino acids that affect only the first zinc finger leaving the second finger, and the end of the protein intact.

The missense mutations totaled 28 and have proven to be very informative. All the mutations affecting specific amino acids fall in the C-terminal region of the protein (Figure 3). The section of the protein affected by missense mutations spans the two Zn fingers and the last 37 amino acids that we refer to as the "tail region". The motif CCHC

is thought to be critical for zinc coordination and is characteristic for this type of Zn finger. Most cysteines and histidines in the two motifs were affected in the screen (Figure 3a) with the exception of the fourth Cys (C347 and C371) of each finger and the first Cys (C347) in the second finger. C347 is changed to a Tyr in the previously isolated allele nos^{RD} (Curtis, et al., 1997). To determine whether the fourth Cys (C347 and C371) in each Zn finger is important for *nos* function, we tested the effect of mutating the respective aminoacids directly. For this assay, C347 and C371 were mutated to Ser to generate mutated cDNAs, 4CF1 and 4CF2. RNA was prepared from wild-type and each mutant cDNA and injected into embryos from nos^{L7} females. While injection of wild-type *nos* cDNA (pN5) rescues of the abdominal phenotype of *nos* mutant embryos already at a concentration of 250ng/µl, RNAs synthesized from the mutant cDNAs were unable to rescue even at a concentration of 1.5µg/ml (Table 4). We conclude that each amino acid of the *nos* CCHC motif is important for function.

The remaining missense alleles are distributed in 4 clusters in the C terminus of the protein (Figure 2.3A). The first group (nos^{512}, $nos^{505/520}$ and nos^{672}) falls between the His and last Cys of the first finger. A second group of alleles ($nos^{585/587}$, $nos^{152/154}$ and nos^{627}) affects the region between the two fingers. Two mutations (nos^{6} and $nos^{277/560/565/579/626}$) lie in the second finger between the second Cys and the His. The second of these two mutations, Ala^{365}-Thr, was identified five times, becoming the most common mutation in the screen. The last group of mutations (nos^{295}, $nos^{3/108}$) falls into the "tail region" of NOS. Previous screens had identified two other alleles (nos^{L7} and nos^{RW}) that map to this region of the protein (Figure 2.3A).

This screen identified sixteen amino acids in the carboxyl terminal region of NOS essential for the regulation of *bcd* translation by NOS. Eight mutations change amino acids in the CCHC motif expected to have a function in the coordination of Zn, while the other 20 mutations identify amino acids in other regions of the protein, where single amino acid changes interfere with NOS function.

NOS has been shown to function during different stages of development. During embryogenesis, the primary target of NOS-dependent translational regulation is *hunchback* (*hb*), while the targets for NOS function during germ cell migration and oogenesis have not yet been identified. To determine whether specific functions of *nos* were differently affected by the new mutations, the abilities of each mutation to complement different aspects of the *nos* mutant phenotype was tested.

Effect of *nos* mutations on *hb* regulation

The new *nos* alleles were identified on the basis of their effect on *bcd* translation. We therefore devised a genetic test to study the effect of the new mutants on *hb* translational regulation. Our test rests on the following observations: First, one copy of the wild-type *P(nos-tub3'UTR)* transgene is able to rescue the abdominal phenotype of a strong *nos* mutant (Gavis and Lehmann, 1992). Due to translational repression of *bcd*, these embryos still show head structure defects and do not hatch. Second, alleles that have residual activity will show a partially rescue of the *nos* mutant phenotype, and produce embryos with a variable number of abdominal segments. We crossed all *P(nos-tub3'UTR)* transgenes into the background of a *nos* null mutant and determined the strength of the new alleles and their effect on *hb* regulation, germ cell migration and oogenesis. In the case of a mutation that affects the regulation of *bcd* but not of *hb*, the resulting embryos should develop to adulthood since the abdominal phenotype would be rescued. To perform this test we established stocks carrying the mutated transgene and the null allele nos^{RC} or a deficiency affecting *nos*. The two resulting stocks for each mutant were then crossed to each other giving rise to *nos* null flies carrying one, two or no copies of the mutated transgene. The females were then allowed to lay eggs, which were inspected for the presence of abdominal segments. The three rearrangement mutations and all of the missense mutations, except for one, when crossed to the null background, in one and two copies, give rise to embryos that are indistinguishable from *nos* mutants (Table 2.1). These embryos have normal thoracic and head structures but lack all abdominal segments. The nonsense mutations behaved identically, with the exception of the amber mutants that seemed to allow read-through, producing hatching larvae at low frequency.

Only one of the missense alleles, nos^{295}, could partially rescue the abdominal phenotype caused by the *nos* null background. Approximately 30% of the embryos laid by *nos* null females carrying one copy of the nos^{295} transgene form 2 to 8 abdominal segments. With two copies of the transgene the amount of embryos showing this level of rescue is about 50%. A previously isolated allele, nos^{RW}, shows a similarly weak phenotype (Curtis, et al., 1997). Like nos^{RW}, the mutation in nos^{295} changes an amino acid in the tail region of the protein (Curtis, et al., 1997).

The genetic analysis of these mutants demonstrates that the suppression screen was successful in identifying amino acids in the NOS protein essential for the translational repression of *hb*. In addition, we were able to obtain both strong and weak alleles.

Only mutations in the zinc finger region affect oogenesis

Females that carry *nos* null mutations produce very few eggs and show defects in early oogenesis; the number of germ line stem cells is reduced and germ line development is often arrested after cystoblast division. To examine whether the newly identified mutations also affect oogenesis, we tested the ability of the mutated transgenes to rescue the oogenesis phenotype of *nos* females carrying the null mutations nos^{RC} in trans to a deletion of the gene. For this purpose we used the same *nos* null females carrying one or two copies of the mutated transgene that were used to test the effect of the alleles on *hb* regulation (see above). All missense alleles that affect the zinc finger region are incapable of rescuing the oogenesis defect caused by a loss of NOS in the ovaries (Figure 2.3A, Table 2.3). On the other hand, the 3 alleles that affect the tail region of *nos* fully rescue the oogenesis phenotype and therefore behave very different from the alleles that map to the Zn finger region. *Nos* mutant females carrying one or two copies of these 3 transgenic alleles lay a normal number of eggs and their ovaries looked like those from wild-type females. Alleles nos^{108} and nos^{3} complement the oogenesis defect but fail to regulate *hb* appropriately. This phenotype is similar to that observed for the *nos* mutation, nos^{L7}, which deletes seven amino acids in the tail region. Two mutations, the allele nos^{RW} and the newly identified allele nos^{295}, affect abdomen formation weakly and fully complement the oogenesis phenotype. These results suggest that certain domains within the NOS protein may be required for the functions of NOS during embryogenesis and oogenesis. While the Zn finger domain seems to be necessary for all aspects of NOS function, the tail region only affects *hb* and *bcd* translational regulation.

NANOS tail domain

Five mutations have been identified that map to the tail region of the NOS protein. These mutations have in common that they specifically affect *hb* (and *bcd*) regulation, but do not interfere with normal oogenesis. One reason for this specificity could be that this region of the protein carries a function only required for the regulation of *hb*. Alternatively, these mutations may reduce NOS function. In this case, *hb* regulation would be more sensitive to reduction in NOS function than oogenesis. To distinguish between these two possibilities, we reasoned that an increase in the concentration of NOS protein with reduced activity should rescue the mutant phenotype, while such an increase of a NOS protein lacking a particular function should have no

effect. Two different means were used to increase the level of NOS protein with mutations in the tail domain. In the first test, one or two copies of the mutated transgene nos^{108} were crossed into a homozygous nos^{L7} background. The previously isolated allele nos^{L7} has a deletion in the tail region and homozygous females produce embryos that lack abdomen while oogenesis is unaffected (Curtis, et al., 1997). This genetic combination approximately doubles the amount of NOS "tail mutant" protein in the embryo (data not shown). Figure 2.4 shows the phenotype of an embryo from a mother homozygous for nos^{L7} and the transgene nos^{108}. All nos^{L7} embryos which, received either one or two copies of the nos^{108} transgene, completely lack abdomen and the filzkörper are not extended. Thus, increasing the amount of NOS protein by adding two additional copies of the *nos* mutant transgene is not sufficient to weaken the abdominal phenotype.

In the second test, we decided to increase the levels of mutant protein even further by injection of RNA that encodes protein mutant in the tail region. We introduced the seven amino acid, in frame deletion of the nos^{L7} mutation into the *nos* cDNA. RNA transcribed from this construct or a wild-type construct was injected into nos^{L7} mutant embryos. Wild-type *nos* RNA can strongly rescue the abdominal phenotype of the *nos* mutants when injected at a concentration of 250 ng/µl. In contrast, nos^{L7} RNA is incapable of any rescue regardless of the concentration used (Table 2.4). Even at a concentration as high as 1.3 µg/µl, nos^{L7} RNA was unable to rescue or weaken the abdominal defect. We conclude that mutations in the tail region of NOS render the protein completely inactive in the regulation of *hb* regardless of the amounts present. Taken together, these two experiments suggest that the carboxyl-terminal region of NOS is a functional domain required for the translational regulation of *hb* and *bcd* but not for *nos* function during oogenesis.

NANOS tail domain in germ cell migration.

It has been shown that germ cells devoid of NOS fail to migrate correctly throughout the embryo (Kobayashi, et al., 1996; Forbes and Lehmann, 1998). We therefore decided to test whether the tail region of NOS is required for the migration of germ cells. Since embryos mutant for nos^{L7} lack abdomen, germ cells are unable to associate with the somatic component of the gonad which forms from abdominal mesoderm. It is thus not possible to directly assess the role of a tail mutant such as nos^{L7} on germ cell migration. However, it was previously shown that embryos that lack maternally derived *hb* and *nos* can develop a normal abdomen (Hülskamp, et al., 1989;

Irish, et al., 1989; Struhl, 1989). In these embryos germ cell migration has been observed and it was shown that complete lack of NOS affects germ cell migration very strongly (Forbes and Lehmann, 1998). We therefore decided to test the nos^{L7} mutant in a similar experiment. Since, homozygous females for both *nos* and *hb* will not survive because *hb* is required for normal development, we obtained *hb* nos^{L7} double mutant embryos from germline clones using the *FRT/FLP/Ovo*^{D} system (Chou and Perrimon, 1996, See Materials and Methods).

Females with hb^{FB} nos^{L7} mutant germline clones were crossed to *nos* mutant males in order to have no maternal or zygotic contribution of *nos*. The resulting embryos were stained with anti *vasa* antibodies to visualized the germ cells (Figure 2.5). Germ cells form normally in these embryos and do not show any defects in migration up to stage 10 of embryogenesis. In wild-type embryos at this stage the germ cells move from the posterior midgut pocket to its basal surface. From here, the germ cells normally migrate towards the lateral mesoderm. Instead, most of the nos^{L7} mutant germ cells fail to leave the gut, forming tightly associated clusters of cells. Not all mutant germ cells stay behind in the midgut; some germ cells follow a normal migratory pattern (Figure 2.5D). The morphology of the mutant cells is normal, and the embryos go on to develop into fertile flies, confirming that some of the cells end up associated with gonadal tissues.

The phenotype observed in germ cells with NOS mutated in the tail domain resembles that of germ cells completely lacking NOS protein (nos^{BN} allele; Forbes and Lehmann, 1998). However, the nos^{L7} phenotype seems to be weaker and more restricted to the initial aspects of germ cell migration from the gut into the mesoderm. In embryos that lack NOS, more cells remain associated with each other while exiting the gut, the germ cells have aberrant morphology and do rarely contribute to the germ line. The resulting males and females are mostly sterile. This suggests that the tail domain of NOS either reduces the function of NOS for germ cell migration or this region of the protein is needed for only some aspects of germ cell migration while other aspects remain unaffected.

DISCUSSION

A highly selective screen was performed to identify essential amino acids in NOS. In total 68 new alleles were identified and characterized molecularly. All 28 missense mutations affect the carboxy-terminal region of the protein. This region harbors two CCHC type zinc fingers that were both affected in this mutagenesis. In addition, C-terminal to the Zn fingers a region of 7 amino acids, the tail domain, has been identified as a separate domain required for NOS activity during embryogenesis and germ cell migration but not for oogenesis.

The NOS protein is a novel, but evolutionary conserved protein with two unusual Zn finger motifs. Detailed mutational analysis was used to identify functionally relevant amino acids. The present screen took advantage of the dominant female sterile phenotype produced by females that carry a *nos* transgene that lacks the 3'UTR regulatory sequences required for *nos* RNA localization and translational repression. Such females are sterile because ectopic expression of NOS at the anterior causes repression of *bcd* RNA translation and consequently defects in head development (Gavis and Lehmann, 1994). Mutations induced in the transgene, which render the unlocalized protein non-functional, revert the female sterility. Thus, this screen selects for new mutations in *nos* as opposed to other screening methods that use non-complementation assays. This selection scheme allowed us to test large numbers of single F1 flies without having to establish individual lines and test those for a phenotype (Figure 2.2).

Genetic tests indicate that all 68 mutations directly affect the transgene and are not caused by second site suppressors. Given the design of the screen, it is not surprising that we did not obtain second site suppressors. A mutation in another protein necessary for *bcd* regulation by NOS would likely affect *hb* regulation as well. While such a mutation would be expected to rescue the *bcd* head defects, it may likely cause abdominal defects due to a failure in *hb* regulation. A second site mutation that would specifically affect *bcd* regulation is one that would mutate the NRE in the *bcd* 3'UTR and thereby make *bcd* non-responsive to NOS-mediated translational repression. Since, multiple base changes may be required to inactivate the NREs (Wharton and Struhl, 1991; Curtis, et al., 1997; Wharton, et al., 1998; Murata and Wharton, 1995), it is unlikely that the present screen would have uncovered such a mutation.

Genetic analysis of the mutant lines showed that all mutations that were identified due to their inability to repress *bcd* translation, also affected the ability to regulate *hb* translation. The fact that we did not find any mutations that specifically affected *bcd*

regulation strongly suggests that both regulatory functions are performed through identical mechanisms. Indeed, it has been shown that both genes are regulated by similar cis-acting sequences, the NREs and identical transacting factors, PUM and NOS (Lehmann and Nüsslein-Volhard, 1991; Wharton and Struhl, 1991; Gavis and Lehmann, 1992).

Sequence analysis of the mutated transgenes shows that all missense alleles identified map to the C-terminus of NOS. This region had already been identified as important for NOS function in repressing *hb* (Curtis, et al., 1997). The fact that no mutations affecting amino acids outside of the C-terminus were found could suggest that the C-terminus is the only functionally required region of the protein. However, injection of an RNA that encoded a truncated NOS protein that lacked 285 amino acids from the N-terminus but had an intact C-terminal region, was unable to rescue the *nos* abdominal phenotype (Curtis, et al., 1997). Thus, although necessary, the C-terminus of NOS may not be sufficient for normal function. One possibility is that a single amino acid change in the N-terminal half of NOS may not have a detectable effect on NOS function. Indeed, homologs of NOS from other insect species which are able to substitute for lack of NOS show very little conservation at the amino acid level in the N-terminal region of the protein (Curtis, et al., 1995b). At this point we can also not exclude the possibility that we have missed essential amino acids in other regions of the protein. Our screen only assayed for the effect of NOS on *bcd* regulation and may have not identified mutations specifically affecting other aspects of NOS function such as germ cell migration and oogenesis. Statistical analysis further suggests that our screen did not reach saturation. Each amino acid that can be mutated in our screen and can be identified by our selection criteria, was hit on average 1.8 times which corresponds to a saturation of about 80% according to a Poisson distribution. Further evidence for the lack of saturation is the fact that three of the cysteines that were shown to be required for Zn coordination of the NOS CCHC motif, were not identified in the mutagenesis screen, but are important for NOS function (Curtis, et al., 1997; this study).

The Zn fingers

The last 87 amino acids of NOS contain two metal binding domains of the CCHC type. It is not known what specific role the zinc fingers play in NOS. Zinc fingers of the CCHC type are not found commonly. The spacing between the Cys and His residues in NOS are unique to this protein and its homologues in insects, frogs and worms (Figure

2.3B). Other proteins, such as the HIV nucleocapsid protein (Dannull, et al., 1994), CNBP (Rajavashisth, et al., 1989) and Clipper (Bai and Tolias, 1998), have multiple copies of CCHC zinc fingers but the ligand spacing is different. All of these proteins have been implicated in binding to single stranded RNA. For instance, Clipper (Clp) is a Drosophila endoribonuclease that cleaves RNA hairpins (Bai and Tolias, 1998). This protein contains five CCCH fingers that confer the endonucleolytic function and two CCHC fingers implicated in specific RNA binding. In addition to the CCHC motif, the HIV I nucleocapsid protein and NOS share a seven amino acid spacing between the Zn fingers. Of particular interest is the fact that the fourth amino acid in this seven amino acid spacer is an Arg in both proteins. Our mutational analysis has identified this Arg351 as important for NOS function. This Arg is extremely conserved among HIV nucleocapsid proteins (De Guzman, et al., 1998) and has been shown to be required for viral genomic packaging (Ottmann, et al., 1995). In addition crystallography studies of the nucleocapsid protein bound to its RNA target showed that this Arg makes direct contact with nucleic acids (De Guzman, et al., 1998). Thus it is an intriguing possibility that this Arg plays a similar role in NOS. NOS can bind to RNA with high affinity and the ability of NOS to bind RNA resides in the C terminus (Curtis, et al., 1997). However, a specific interaction between NOS and the NREs has not been established. On the other hand, PUM protein has been shown to bind with high affinity and specificity to the NRE (Murata and Wharton, 1995; Zamore, et al., 1997; Zamore, et al., 1999). While mutations in the NRE that affect PUM binding do not affect the affinity of NOS for the RNA (Curtis, et al., 1997), a small number of nucleotides outside the conserved NRE motif have been shown to affect translational regulation of *hb* but not PUM binding (Wharton, et al., 1998). Further experiments are required to determine whether NOS contacts parts of the NREs with sequence specifictiy and whether Arg351 plays a role in such an interaction.

Evolutionary conservation

The NOS CCHC motifs show significant homology with sequences from other insects, Xenopus, leech and *C. elegans* (Figure 2.3B). While the function for the NOS homologs from frog and leech is not known, a role for the respective proteins in establishing embryonic polarity has been proposed. Both Xenopus Xcat2 and the leech homologue are expressed in the developing oocyte (Mosquera, et al., 1993; Pilon and Weisblat, 1997). In the embryo Xcat2 localizes to vegetal blastomeres, while leech NOS protein is present in cells at the animal pole which act as ectodermal precursors

(Mosquera, et al., 1993). Most amino acids mutated in our screen are conserved in these divergent species. Only two mutations, alleles *nos*$^{538/549}$ and *nos*^{512}, lead to changes in non-conserved aminoacids (V354M and S337L). Given that these two amino acids are next to His and Cys residues respectively, it is possible that these mutations affect the coordination of metal in this Zn finger. Despite the high degree of conservation between the two Zinc finger domains of Drosophila NOS and Xenopus Xcat2 the two protein regions are functionally not interchangeable. C. Wang showed that a RNA, in which the NOS Zn fingers were replaced in frame by those of Xcat2, was unable to rescue the *nos* mutant abdominal phenotype (Wang, 1995).

Three homologues of NOS have been identified in *C. elegans* (Wilson, et al., 1994; and G. Seydoux, personal communication). Nos3 is the only one that conserves the characteristic CCHC motifs with the exact spacing within and between the two fingers. Nos3 has been implicated in germ line determination (G. Seydoux, personal communication). In addition, this NOS homologue has been shown to interact in a two-hybrid screen with FBF (J. Kimble, personal communication). FBF is a *C. elegans* homologue of PUM involved in germ line sex determination (Zhang, et al., 1997). FBF binds to sequences in the 3'UTR of *fem-3* which are necessary and sufficient for its translational regulation (Ahringer and Kimble, 1991; Zhang, et al., 1997). *Fem-3* directs spermatogenesis in the hermaphrodite and its translation must be suppressed to allow the switch to oogenesis to occur (Ahringer, et al., 1992). Consequently, mutations in the *fem3* 3'UTR result in a gain-of-function phenotype in which only sperm are produced (Ahringer, et al., 1992). In addition, RNAi analysis shows that eliminating FBF has the same masculinizing phenotype as mutating the target sequences in *fem3* (Zhang, et al., 1997). These observations suggest a role for FBF and Nos3 in translational regulation similar to that performed by PUM and NOS in Drosophila. Curiously, the interaction between Nos3 and FBF occur through the N terminus of Nos3 (J. Kimble, personal communicaton; see Chapter 4). This region of the protein harbors no homology to Drosophila NOS except for its richness in the amino acids Ser and Thr. Additionally, Nos3 does not share any homology with NOS in the tail domain. Thus the tail domain of NOS might carry out functions that are unique to insect development. It is possible that NOS homologues in other organisms play a role parallel to that of NOS in germ line determination where this domain does not seem to be required.

The tail region

Mutations in a region C-terminal to the Zn fingers of NOS cause abdominal and germline migration defects without affecting the function of NOS in oogenesis, while null mutations and mutations in the Zn finger region in addition cause strong defect in early oogenesis. Our screen identified two mutations in this region, *nos*^{295} and *nos*^{3,108}, each causing a single amino acids change, (T378I) and (M379K) respectively. Previously, two other mutations affecting this region, *nos*^{L7} and *nos*^{RW} were isolated (Curtis, et al., 1997). *nos*$^{3/108}$, *nos*^{RW} and *nos*^{295} are missense mutations that map within the region deleted by *nos*^{L7}. We therefore defined the seven amino acid region deleted in *nos*^{L7} as the "tail domain". *Nos*^{RW} and *nos*^{295} are the only *nos* alleles that show a weak abdominal phenotype, which might indicate a role of this region in protein stability. On the other hand, the fact that mutations in this region seem specifically affected in their ability to regulate *hb* and *bcd* could suggest that this region constitutes a novel functional domain. Indeed, while *nos*$^{3/108}$ and *nos*^{L7} do not affect oogenesis, their ability to regulate *hb* and *bcd* translation is as strongly affected as it is in null alleles or point mutations which carry amino acid changes in the Zn finger domain. In order to distinguish between the hypothesis that mutations in the tail domain retain residual activity and the alternative hypothesis that this region of the NOS protein constitutes a separate functional domain, we altered the amount of mutant protein present in embryos. Since increasing the dosage of tail domain mutant protein does not alter the abdominal phenotype of mutant embryos, we favor the hypothesis that the tail region of NOS constitutes a separate functional domain. Interestingly, the two known targets of NOS, *bcd* and *hb* are equally affected by mutations in the tail domain. Both RNAs contain NRE sequence motifs in their 3'UTR. NOS and PUM have been shown to act together to regulate *hb* and *bcd*. The phenotypes of *pum* and *nos* mutants during oogenesis are different and it has been suggested that they act on different, yet unidentified RNA targets. The tail domain may therefore only be required for the role of NOS when it is interacting with PUM (see chapter 3 and 4).

Mutations in the tail domain affect germ cell migration differently from *nos* null mutations. Null mutations or mutations in the Zn finger region of NOS have been shown to have a dramatic effect on germ cell migration (Forbes and Lehmann, 1998). Primordial germ cells devoid of NOS have altered morphology and fail to leave the gut towards the mesoderm and tightly associate with each other in clusters (Forbes and Lehmann, 1998). Furthermore, Kobayashi and colleagues have shown that enhancer trap lines that are normally expressed in germ cells late during embryogenesis are expressed earlier in *nos* mutant germ cells (Kobayashi, et al., 1996; Asaoka, et al., 1998). This has

led to the hypothesis that some of the phenotypes displayed by *nos* mutant germ cells may be caused by the precocious expression of genes normally expressed at a later stage. Mutants in the tail domain affect germ cell migration but to a lesser extent. nos^{L7} germ cells, like germ cell lacking NOS, fail to leave the gut and form clusters. However, the clustering is not as extreme as that seen for the null mutant and germ cell morphology seems normal. Furthermore, premature gene expression has not been observed in nos^{L7} mutants (Heller and Steinmann-Zwicky, 1998). Finally, many nos^{L7} germ cells reach the embryonic gonad and the embryos develop into fertile adults.

These differences in phenotypes might indicate that nos^{L7} is a weak allele with respect to germ cell migration. Contrary to what we see in *hb* regulation, perhaps nos^{L7} retains some function in germ cell migration. Alternatively, the tail domain may just affect a subset of phenotypes observed in the null mutants. *nos* null mutant germ cells show aberrant morphology and even when some cells reach the gonad the resulting adults are sterile. Consequently, *nos* might be required in the germ cells for two independent functions: migration, which requires the tail domain, and germ cell identity, which does not require the tail domain. Problems in germ cell identity might exacerbate the migration defect, hence the greater loss of germ cells in the null mutants. Clearly the identification of *nos* germ line targets is required to further address the function of different NOS domains in germ cell migration.

In summary, our analysis of a large number of *nos* mutants has led to the following model for NOS protein function: The C terminus of NOS plays a crucial role during three developmental stages of Drosophila development: oogenesis, embryogenesis and primordial germ cell migration. NOS role during embryogenesis is to silence the translation of maternal *hb* RNA. This function requires the NOS zinc finger and tail region as well as the RNA binding protein PUM. During primordial germ cells development, PUM and NOS are required for the migratory behavior, the temporal control of gene expression, and for the differentiation of germ cells into germ line stem cells. This process requires PUM, and the Zn finger region of NOS, the function of the NOS' tail region seems to be restricted to aspects of migration. During oogenesis, PUM and NOS seem to have overlapping as well as separate functions. Only the NOS Zn finger region is necessary for NOS' function during oogenesis. NANOS homologs have been identified in a number of organisms. RNA localization studies in Xenopus and phenotypic analysis of *nos* mutants in *C. elegans* suggest a role for these NOS homologs in germ cell development. In these organisms, the region of homology is restricted to the

Zn finger motif and does not span the tail domain, suggesting that the tail domain may have been recruited later in evolution and may fulfill a more specialized role.

Figure 2.1 Reversion of the dominant female sterile phenotype caused by ectopic NANOS.

A. Wild-type females lay eggs with *nos* RNA localized to the posterior via its 3'UTR. NOS protein forms a posterior to anterior gradient and regulates the translation of the maternal *hb* message in the posterior pole (Wang and Lehmann, 1991). The resulting larvae have 8 abdominal segments, 3 thoracic segments, head structures in the anterior and a telson at the posterior. B. Females carrying a *nos* message where the 3'UTR has been changed to that of *tubulin* (*nos-tub* 3'UTR) give rise to embryos with unlocalized NOS (Gavis and Lehmann, 1994). When NOS is ectopically expressed at the anterior, the normal expression of *bcd* and *hb* at this end, is repressed (Gavis and Lehmann, 1992). Consequently, the resulting larvae have head defects and lack thoracic segments and are embryonic lethal. All females carrying a *nos-tub* 3'UTR transgene will therefore be sterile. C. Inducing a mutation in the *nos-tub* 3'UTR construct so that the ectopic NOS, although still expressed at the anterior, can no longer function, will revert the dominant female sterile phenotype. Since endogenous *nos* is unaffected in this mutant, the resulting larvae will be identical to wild type. D. Schematic of *nos-tub* 3'UTR transgene (Gavis and Lehmann, 1994). The genomic sequences of *nos* were used. Open boxes indicate *nos* exons while the dashed lines are the introns. The black box depicts the hemaglutinin epitope included in the 5' end of *nos*. Black boxes with white dots are the *nos* UTR sequences, while the box with vertical lines represents the tubulin 3'UTR. Plain lines indicate flanking genomic sequences from the *nos* gene. The two pairs of primers used to specifically isolate the transgene DNA are shown as arrowheads. Primers A5' and A3' produce a 559 base-pair fragment while primers B5' and B3' amplifies a 1,102 base-pair fragment.

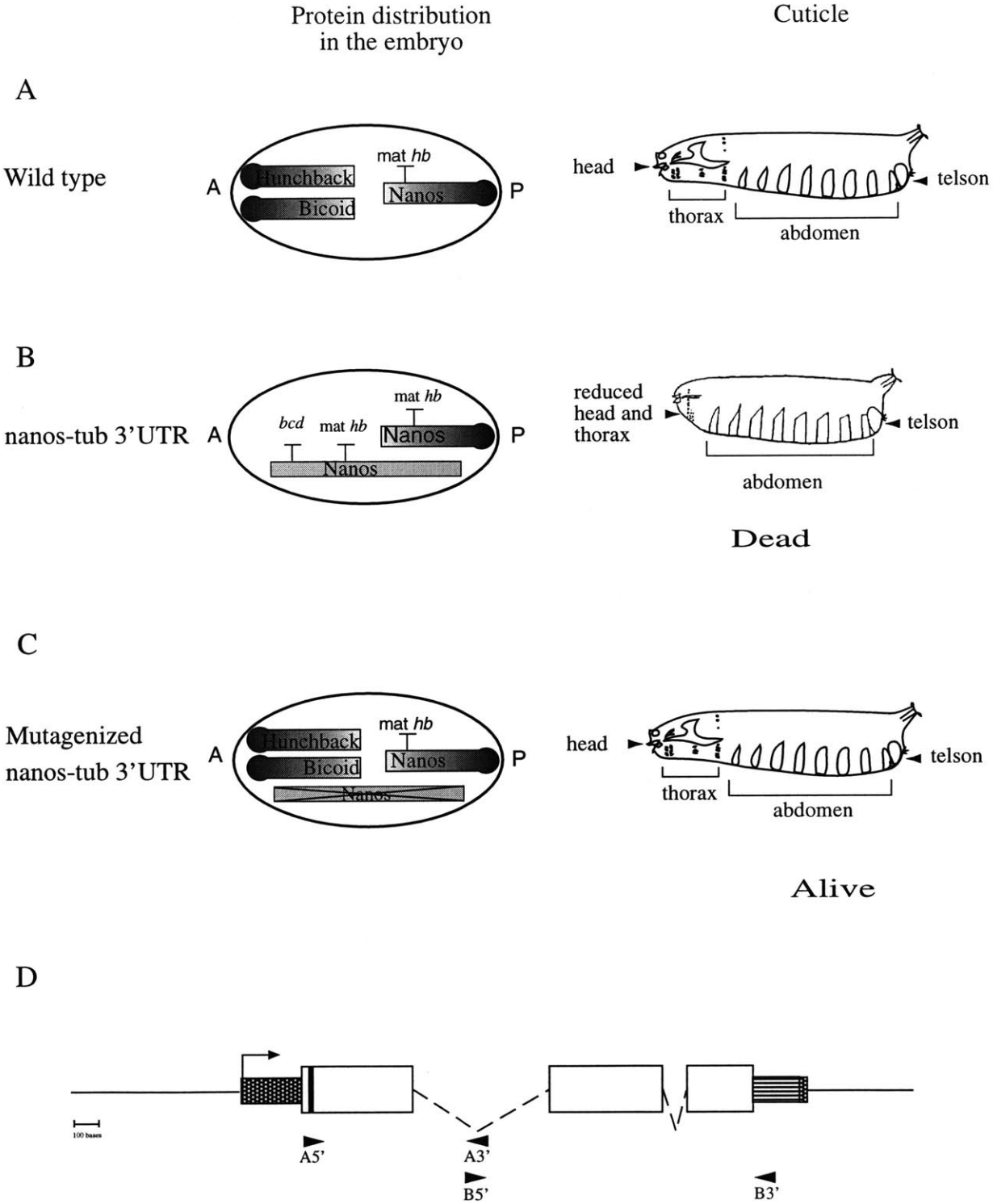

Protein distribution
in the embryo
Cuticle
A
Wild type
A
Hunchback
Bicoid
mat hb
Nanos
P
head
telson
thorax
abdomen
B
nanos-tub 3'UTR
A
bcd
mat hb
mat hb
Nanos
Nanos
P
reduced
head and
thorax
telson
abdomen
Dead
C
Mutagenized
nanos-tub 3'UTR
A
Hunchback
Bicoid
mat hb
Nanos
Nanos
P
head
telson
thorax
abdomen
Alive
D
100 bases
A5'
A3'
B5'
B3'

Figure 2.2 Isolation of stable mutant lines.

The crossing scheme used in the screen performed is shown here. Details of the strains used and the screen itself are included in the material and methods and results section. In the F1 generation all females are sterile except for those carrying a mutation that affect the transgenic NOS function. The box includes the genotype of the males and the only fertile females in this generation. A total of 14,400 males were mutagenized, 93,000 haploid genomes were tested and 68 independent mutant lines were established.

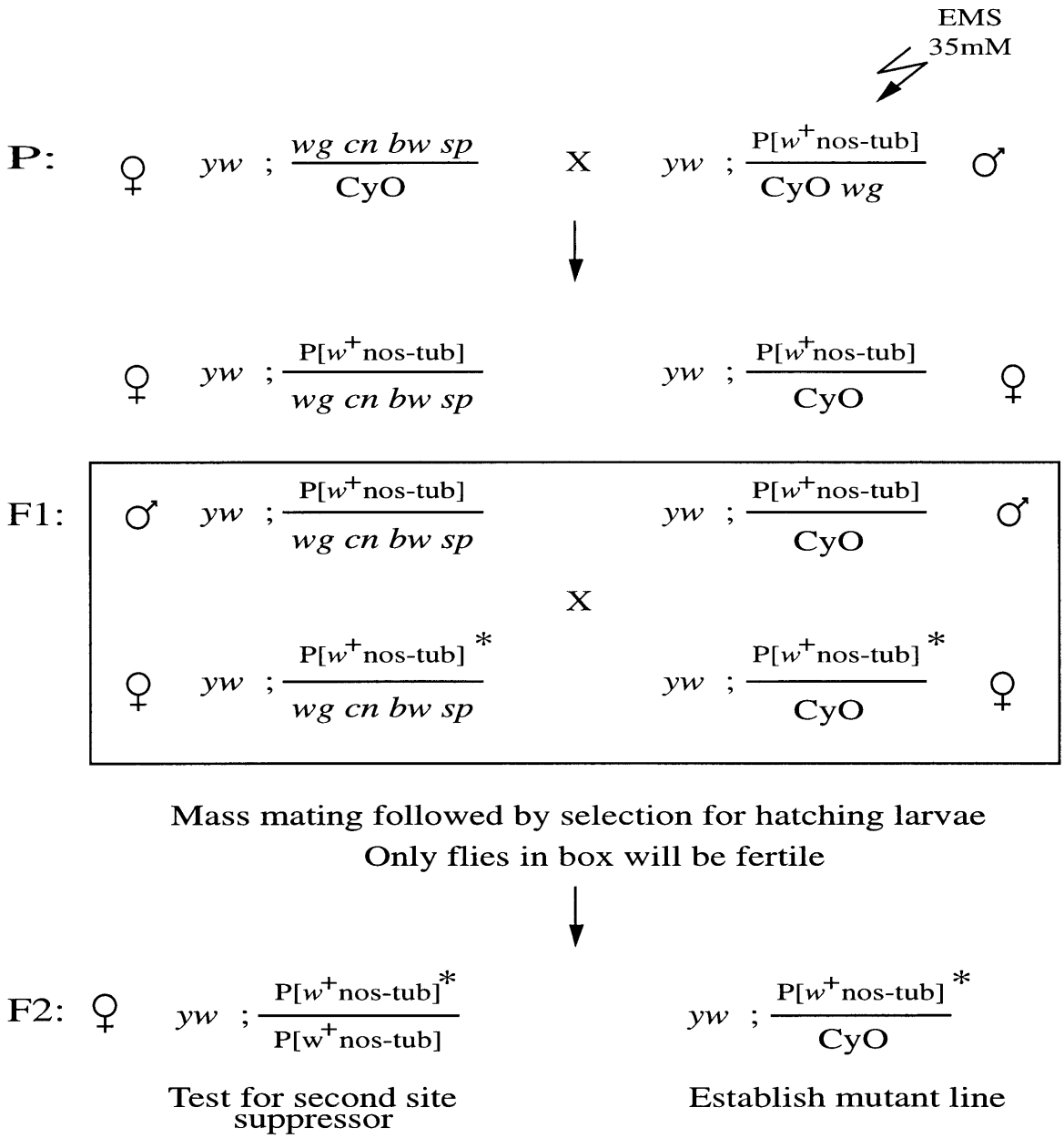

EMS
35mM
P:
♀ yw ; wg cn bw sp / CyO X yw ; P[w+nos-tub] / CyO wg ♂
♀ yw ; P[w+nos-tub] / wg cn bw sp
yw ; P[w+nos-tub] / CyO ♀
F1:
♂ yw ; P[w+nos-tub] / wg cn bw sp
yw ; P[w+nos-tub] / CyO ♂
X
♀ yw ; P[w+nos-tub]* / wg cn bw sp
yw ; P[w+nos-tub]* / CyO ♀
Mass mating followed by selection for hatching larvae
Only flies in box will be fertile
F2: ♀ yw ; P[w+nos-tub]* / P[w+nos-tub]
yw ; P[w+nos-tub]* / CyO
Test for second site suppressor
Establish mutant line

Table 2.1. Nonsense mutations

Alleles	Base change	Amino acid change
Nos 286	C(565*)→ T	Arg(102)→ Stop[1]
Nos 635	C(568)→ T	Gln(103)→ Stop[1]
Nos 641	C(598)→ T	Gln(113)→ Stop[1]
Nos 554	C(625)→ T	Gln(122)→ Stop[1]
Nos 607	C(673)→ T	Gln(138)→ Stop[1]
Nos 517	C(1302)→ T	Gln(165)→ Stop[1]
Nos 539	C(1314)→ T	Gln(169)→ Stop[1]
Nos 524	T(1337)→ A	Tyr(177)→ Stop[2]
Nos 243 Nos 253 Nos 254	C(1390)→ T	Gln(194)→ Stop[2]
Nos 197	C(1401)→ T	Gln(198)→ Stop[1]
Nos 111 Nos 204 Nos 258	C(1404)→ T	Gln(199)→ Stop[1]
Nos 264	C(1410)→ T	Gln(201)→ Stop[1]
Nos 212	C(1461)→ T	Gln(218)→ Stop[1]
Nos 558	G(1549)→ A	Trp(247)→ Stop[1]
Nos 594	T(1594)→ A	Leu(262)→ Stop[1]
Nos 139	C(1596)→ T	Gln(263)→ Stop[1]
Nos 592 Nos 595 Nos 620	C(1671)→ T	Gln(288)→ Stop[1]
Nos 568	A(1719)→ T	Lys(304)→ Stop[1]
Nos 122 Nos 183	C(1912)→ T	Gln(344)→ Stop[3]
Nos 263	A(1999)→ T	Lys(373)→ Stop[1]

* The number of the base mutated corresponds to the numbering given by C. Wang for the genomic sequence of *nos* (Wang and Lehmann, 1991) with one correction. An extra three bases, AGA, were found between bases 1410 and 1411. Consequently, His (201) is replaced by Gln and Ile.

[1] Amber

[2] Ochre

[3] Opal

Table 2.2. Rearrangements

Alleles	Base change	Mutation
Nos 246 Nos 526 Nos 623	G(1827)→ A	Last G of intron 2
Nos 272	Δ 1722-1775	In frame deletion, amino acids 305-331
Nos 581	13 bases insert	Between bases 1722-1723

Table 2.3. Missense mutations

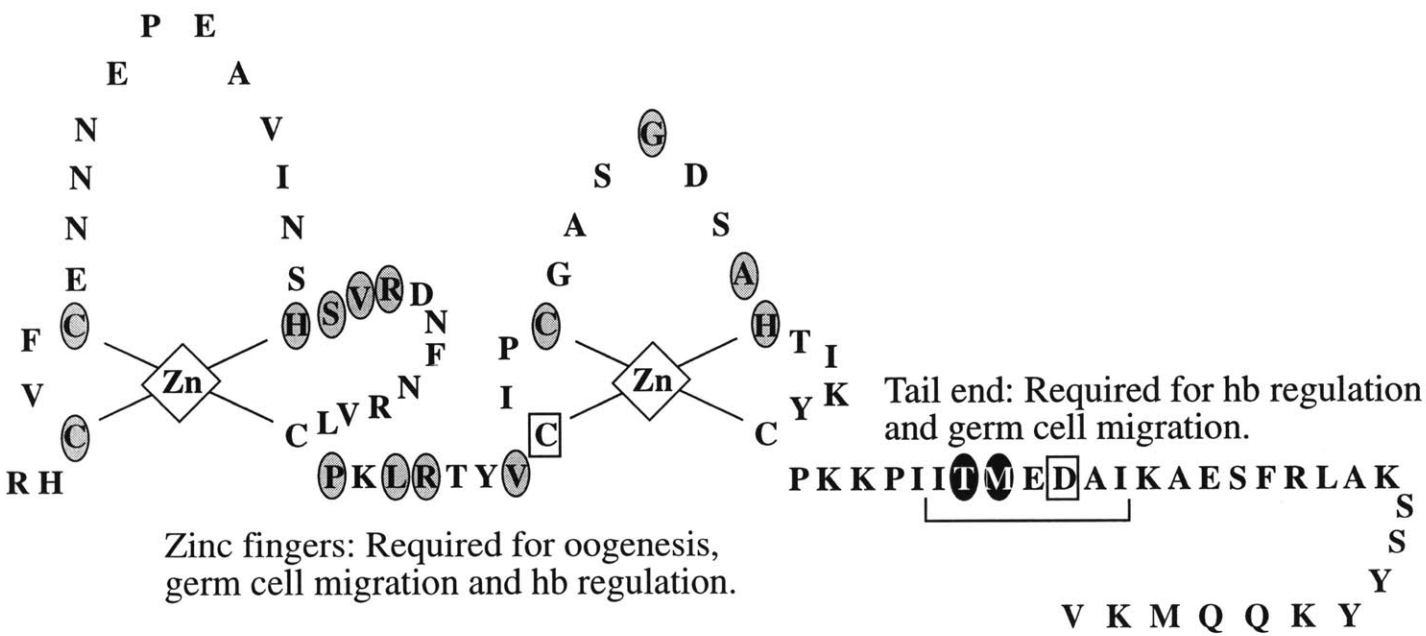
Alleles	Base Change	Amino acid change	Abdominal phenotype	Oogenesis phenotype
Nos 273 Nos 516 Nos 599	G(1841)→ A	Cys(320) → Tyr	Strong	Strong
Nos 18	A(1849)→ T	Cys(323)→ Ser	Strong	Strong
Nos 506	C(1888)→ T	His(336)→ Tyr	Strong	Strong
Nos 512	C(1892)→ T	Ser(337)→ Leu	Strong	Strong
Nos 505 Nos 520	T(1895)→ A	Val(338)→ Glu	Strong	Strong
Nos 672	G(1898)→ A	Arg(339)→ Gln	Strong	Strong
Nos 585 Nos 587	C(1924)→ T	Pro(348)→ Ser	Strong	Strong
Nos 153 Nos 154	T(1931)→ G	Leu(350)→ Arg	Strong	Strong
Nos 627	G(1934)→ A	Arg(351)→ Gln	Strong	Strong
Nos 538 Nos 549	G(1942)→ A	Val(354)→ Met	Strong	Strong
Nos 165 Nos 614	G(1955)→ A	Cys(358)→ Tyr	Strong	Strong
Nos 6	G(1967)→ A	Gly(362)→ Glu	Strong	Strong
Nos 277 Nos 560 Nos 565 Nos 579 Nos 626	G(1975)→ A	Ala(365)→ Thr	Strong	Strong
Nos 19	C(1978)→ T	His(366)→ Tyr	Strong	Strong
Nos 295	C(2015)→ T	Thr(378)→ Ile	Weak	WT
Nos 3 Nos 108	T(2018)→ A	Met(379)→ Lys	Strong	WT

Strong indicates complete failure to rescue: embryos showed no abdominal segments and females laid very few eggs and their ovaries looked devoid of mature eggs. Weak abdominal phenotype means that approximately 50% of embryos showed between 2 to 8 abdominal segments. When WT oogenesis phenotype is indicated, females null for *nos* carrying a copy of this mutated transgene were able to lay normal amounts of eggs and their ovaries looked wild type.

Figure 2.3 Missense mutations in the carboxy-terminal region of NANOS.

A. The last 87 amino acids of NOS are depicted with the proposed configuration of the two zinc fingers (Curtis, et al., 1997). Gray and black circles show amino acids mutated in the screen described here. The two amino acids with open boxes have been mutated in previous screens. The C with the box is changed to T in nos^{RD} while the D is mutated to N in nos^{RW} (Curtis, et al., 1997). Bracket in the tail of the protein points at the 7 amino acids deleted in the previously described nos^{L7} allele (Curtis, et al., 1997). The two amino acids in a black box, along with nos^{RW} and nos^{L7}, do not affect NOS function in oogenesis. B. Alignment of the C terminus of NOS from *Drosophila melanogaster* (D. mel.) and *virilis* (D. vir.), *Musca domestica* (Musca), *Chironomus samoensis* (Chiron.) (Curtis, 1995b), from leech *Helobdella robusta* (*H. ro*) (Pilon and Weisblat, 1997), from the *Xenopus laevis* protein Xcat-2 (Mosquera, et al., 1993) and the *Caenorhabditis elegans* protein NOS 3 (Wilson, et al., 1994). For NOS 3 only the two zinc fingers are shown, since 118 amino acids follow this domain. Three C. elegans homologues have been identified and all three have a role in germ line development (C. Seydoux, personal communication). NOS 3 is the only putative homologue that contains both zinc fingers with the exact spacing seen in Drosophila NOS. Outside the C terminal region there is little homology between all shown proteins (Curtis et al., 1995). Asterisks indicate the Cys and His involved in forming the two Zn fingers. Arrows point to amino acids that have been affected in this and previous screens.

A


Zn
Zn
Tail end: Required for hb regulation and germ cell migration.
Zinc fingers: Required for oogenesis, germ cell migration and hb regulation.

B

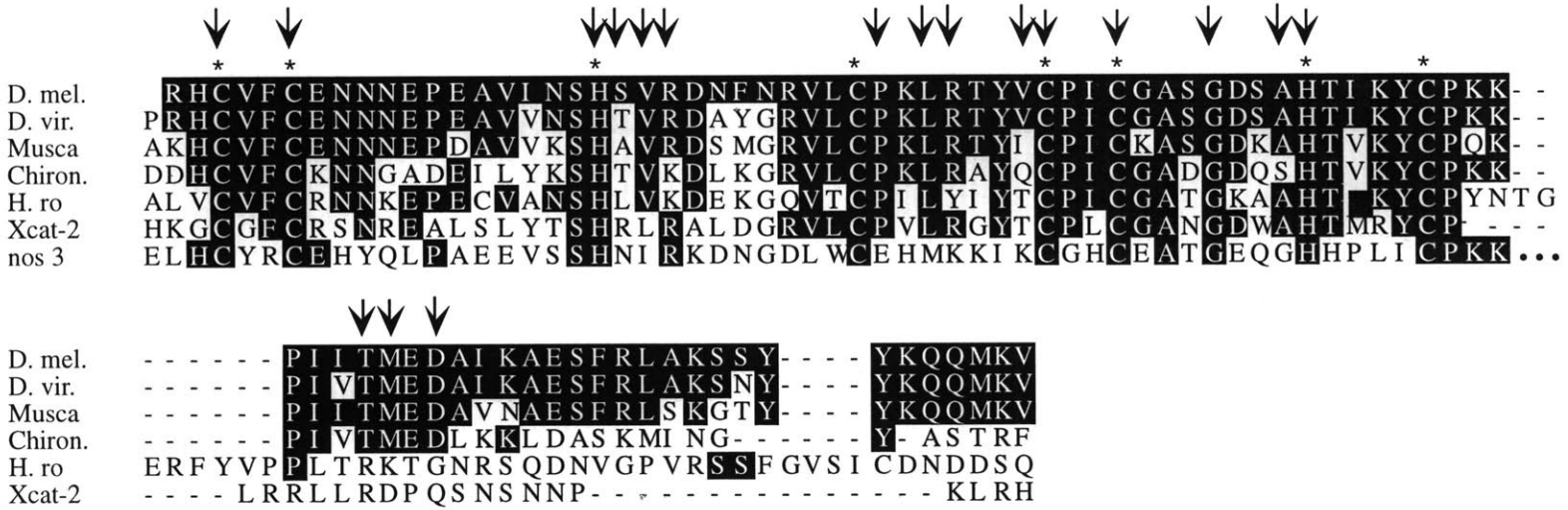

D. mel.
D. vir.
Musca
Chiron.
H. ro
Xcat-2
nos 3

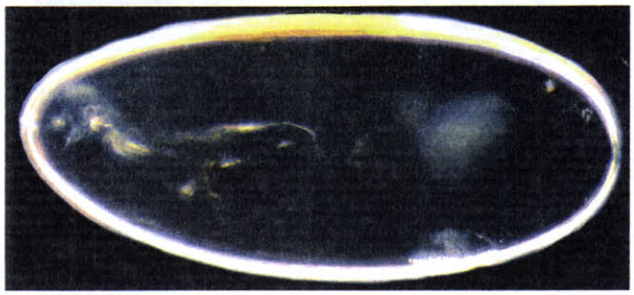
Figure 2.4 Multiple copies of NANOS carrying a mutation in its tail region do not rescue the abdominal phenotype.

Cuticle preparations were made from embryos derived from females with 2, 3 or 4 copies of *nos* carrying a mutation in the tail region. These embryos look identical to those from females null for *nos*. In all these embryos we can see no abdominal segment, a ventral scar and unextended filtzkörper, all typical characteristics of strong *nos* mutant embryos.

Wild Type

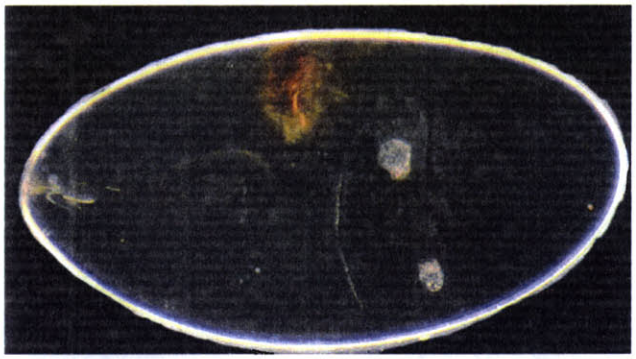
No Nanos

$$\frac{nos^{RC}}{Df(3R)Dlx}$$



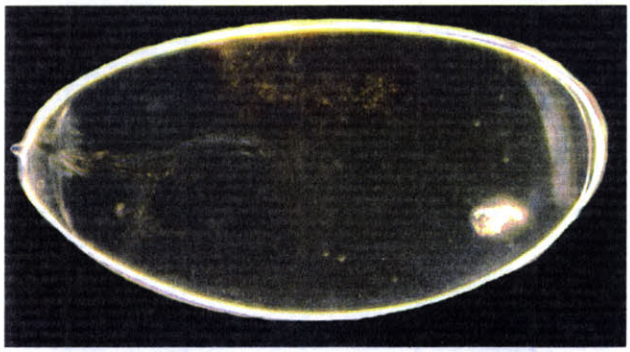
2X Nos tail mutant

$$\frac{nos^{L7}}{nos^{L7}}$$



3X Nos tail mutant

$$P(nos\text{-}tub3'UTR)^{108} \; ; \frac{nos^{L7}}{nos^{L7}}$$



4X Nos tail mutant

$$\frac{P(nos\text{-}tub3'UTR)^{108}}{P(nos\text{-}tub3'UTR)^{108}} \; ; \frac{nos^{L7}}{nos^{L7}}$$

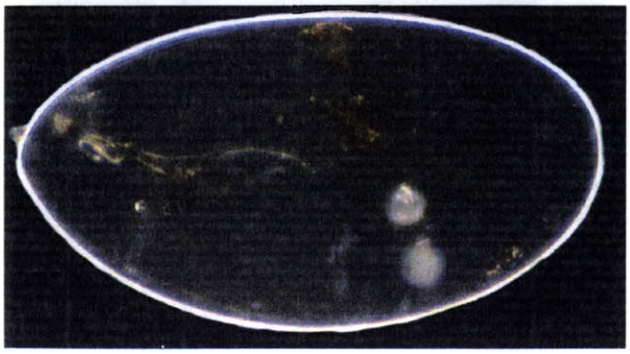


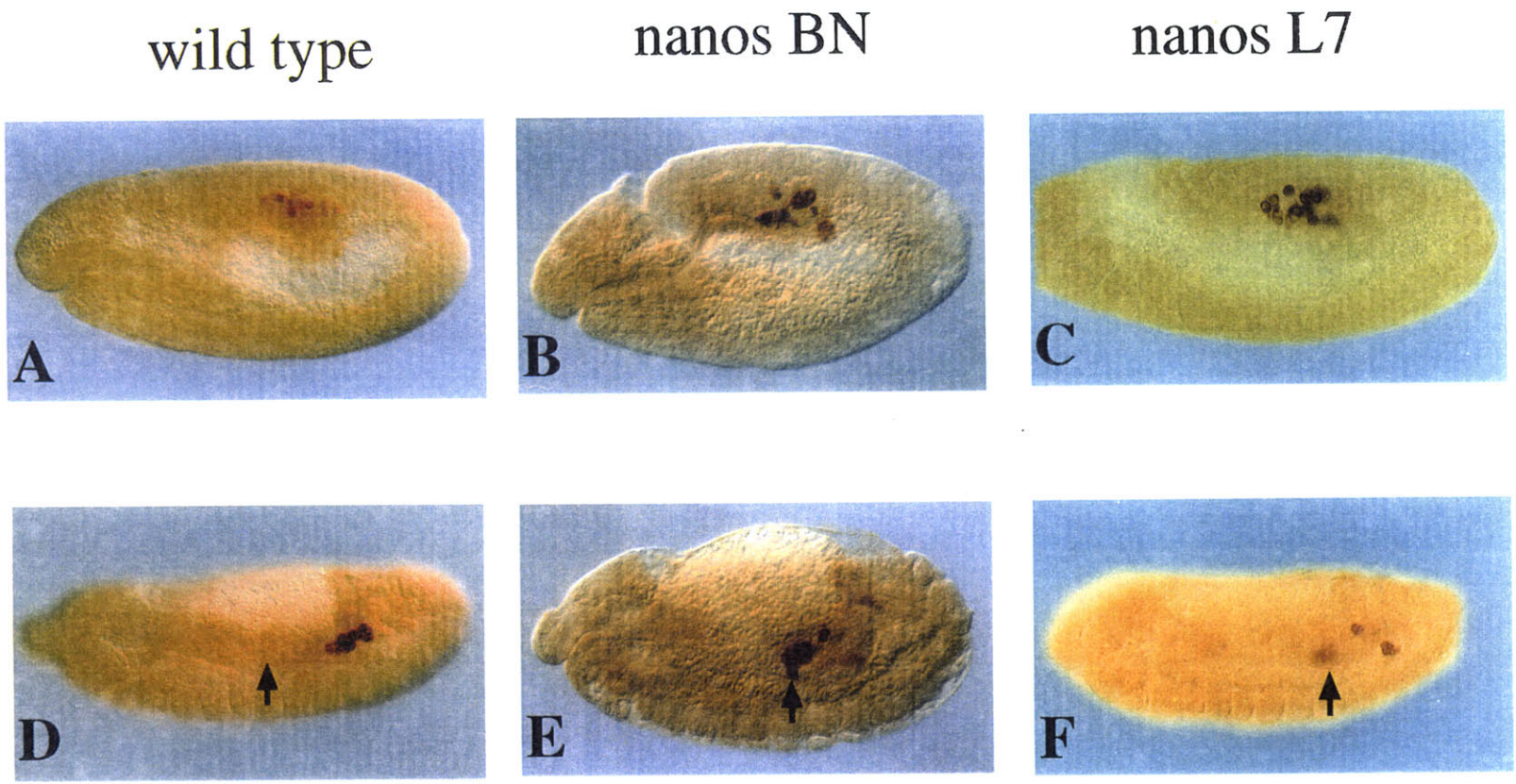
Table 2.4. RNA Injection into *nos*^{L7} embryos

RNA	# abdominal segments			% overall rescue	% strong rescue	n
	0	1-5	6-8			
nos^{WT} 250 ng/µl	10	9	49	85	72	68
nos^{WT} 500 ng/µl	7	10	58	91	77	75
nos^{WT} 1.3 µg/µl	5	6	39	90	78	50
nos^{L7} 250 ng/µl	65	0	0	0	0	65
nos^{L7} 500 ng/µl	72	0	0	0	0	72
nos^{L7} 1.3 µg/µl	53	0	0	0	0	53
4CF1 1.5 µg/µl	113	0	0	0	0	113
4CF2 1.5 µg/µl	96	0	0	0	0	96
Uninjected	20	0	0	0	0	20

Strong rescue refers to embryos with 6-8 segments. 4CF1 has fourth Cys in first finger changed to a Ser. 4CF2 has fourth Cys in second finger mutated to a Ser.

Figure 2.5 NANOS tail region required for germ cell migration.

A-F Embryos stained with anti-Vasa antibody to show germ cells in brown. Arrows point at the midgut in panels D, E and F. *Nanos*^{BN} mutants have no *nos* RNA or protein in the embryo while *nanos*^{L7} mutant embryos carry Nos protein with a deletion in the tail domain. A. In a wild-type embryo at stage 11, germ cells move from the midgut towards the lateral mesoderm. B and C. In embryos derived from *hb nos*^{BN} or *hb nos*^{L7} germline clones the cells clump together in the midgut and fail to migrate to the mesoderm. D. In a wild-type stage 13 embryo, the germ cells associate with the gonadal mesoderm at the posterior of the embryo where together they will form the gonad. E and F. At stage 13, in an embryo from the *hb nos*^{BN} or *hb nos*^{L7} maternal clones we see several clumps of cells, with a significant one still in the posterior midgut.


wild type
nanos BN
nanos L7
A
B
C
D
E
F

CHAPTER 3

Identification of NANOS protein-protein interactions

SUMMARY

The Drosophila posterior determinant NANOS functions in the embryo to repress the translation of the maternal transcript *hunchback*. Translational regulation of *hunchback* also requires the RNA binding protein PUMILIO. The mechanism by which translation of *hunchback* is controlled is not fully understood. In addition, NANOS acts in the ovaries and the primordial germ cells where its function might not require PUMILIO. Here we describe a yeast two-hybrid screen performed to identify proteins that interact with NANOS during its various functions. This screen showed that NANOS is able to interact with itself through its N terminal region. Additionally, NANOS interacts with the translation elongation factor 1γ. This interaction is mediated by a 7 amino acid stretch in the NANOS C-terminus that is essential for *hunchback* regulation. Also, we were not able to detect a direct interaction between NANOS and PUMILIO using the yeast interaction trap system.

INTRODUCTION

Translational regulation, along with RNA localization, is essential in the development of many organisms to achieve localized protein expression (Curtis, et al., 1995a). In Drosophila the correct patterning of the embryo depends on the translational regulation of the maternal RNA *hunchback* (*hb*). In order to understand the mechanisms by which an RNA is regulated it is essential to know all the factors involved in such regulation.

To ensure correct development of the abdomen, maternal *hb* expression needs to be eliminated from the posterior region of the embryo. While *hb* RNA is detected through the entire length of the embryo, HB protein is exclusively seen at the anterior pole (Tautz and Pfeifle, 1989). This observation suggests that *hb* RNA is regulated at the level of translation. Two factors, NANOS (NOS) and PUMILIO (PUM), are required for correct expression of HB and the formation of abdominal segments (Wang and Lehmann, 1991; Barker, et al., 1992). In the absence of either gene function, HB is translated throughout the embryo and abdomen-specific gene expression is repressed. NANOS seems to dictate the positional aspects of this regulation since its distribution is complementary to that of HB. On the other hand, PUMILIO protein is found throughout the entire embryo. Regulation of *hb* translation requires cis-acting elements in the *hb* 3'UTR known as NANOS Response Elements (NRE) (Wharton and Struhl, 1991). PUM has been shown to bind specifically to the NREs (Murata and Wharton, 1995; Zamore, et al., 1997).

The mechanisms by which translational regulation of *hb* occurs are not completely understood. It has been shown that in Drosophila *hb* is deadenylated in the posterior of the embryo (Wreden, et al., 1997). Consistent with the genetic information known, this shortening of the poly(A) tail is dependent on NOS, PUM and the NREs. It is not known whether the deadenylation is the cause, a consequence, or a parallel process to a direct regulation of translation. Also unknown is the level of association of NOS with the PUM-*hb* complex. We have been unable to detect a direct interaction between NOS and PUM. Potentially NOS could be contacting the RNA since it has a high affinity for nucleic acids, but no specificity for *hb* RNA has been demonstrated (Curtis, et al., 1997). Identifying factors that interact with NOS might allow us to understand how regulation occurs. One possibility is that additional proteins could either act as a link between NOS and the poly (A) tail, interact with the translation initiation machinery or confer RNA specificity to NOS.

In addition to its role in embryogenesis, NOS acts during oogenesis and is required for the migration of the germ cells (Kobayashi, et al., 1996; Forbes and Lehmann, 1998). These two processes also require PUM. Nevertheless, in oogenesis NOS and PUM seem to carry out overlapping, but at least partially independent functions (Forbes and Lehmann, 1998). Thus, it is possible that both NOS and PUM may interact with different partners and affect separate targets during oogenesis.

In order to identify proteins that interact with NOS and understand the mechanisms of NOS function, we performed a yeast-two hybrid screen. This interaction trap system allows us to screen quickly for interacting proteins expressed in different Drosophila tissues and at different stages of Drosophila development. In addition it allows us to directly test interactions with known proteins such as PUM. Through several screens we have been able to identify interactions with the elongation factor 1γ (EF-1γ), the Drosophila RNA binding protein BRUNO (BRU) and NOS itself. This chapter discusses preliminary studies performed on the interaction between NOS and EF-1γ and on the dimerization of NOS. The interaction between NOS and BRU is discussed in Chapter 4.

Plasmids and yeast strains.

All parental yeast two hybrid plasmids and strains were a gracious gift from Dr. Russ Finley (Gyuris, et al., 1993). pEG202 includes the yeast *his3* gene and sequences coding for amino acids 1-202 of the LexA protein followed by multiple cloning sites. LexA is constitutively expressed from an *adh*1 promoter. *PSH18-34* is the reporter plasmid encoding a *lacZ* gene under the control of LexA operators. This reporter plasmid carries the *ura3* gene. pJG4-5 carries the *trp1* gene and the 88 residue acidic activator under the control of the Gal1 promoter. The activation domain is followed by unique EcoRI and XhoI sites. These sites are used to introduce cDNAs in order to express activator-tagged proteins.

Two different yeast strains where used in the course of the two hybrid screen. The actual screen was performed using yeast strain EGY48 (*MATα, his3, trp1, ura3, LexAop(x6)-LEU2*). This strain carries the Leu2 reporter gene under the control of LexA operators and requires uracil (ura), histidine (his), tryptophan (trp) and leucine (leu) in the media in order to survive. For the mating assays in addition to EGY48, yeast strain YM4271 (*MATa ura3-52, his3-200, lys2-801, ade2-101, ade5, trp1-901, leu2-3, 112, tyr1-501, gal4-Δ512, gal80-Δ538, ade5::hisG*) was utilized.

The plasmids encoding LexA fusion proteins were made by inserting the coding regions in frame at the 3' end of *lexA*. Sequences coding for full length NOS were introduced to pEG202 by inserting a BamHI to Not I fragment from pNB40-N5Bam. This last plasmid has a BamHI site replacing the starting AUG and keeps the rest of the *nos* cDNA sequence intact (D. Curtis, Unpublished data). This *nos* fragment cloned into pEG202 has the 3'UTR of *nos*.

Fusion constructs carrying fragments of the NOS protein (Wang and Lehmann, 1991) were made by inserting PCR amplified fragments into pEG202 digested with BamHI and NotI. Primers used are 25 bases long on average and contain 21 bases complementary to *nos*. The 5' primer contains a BamHI site, while the 3' primer contains a NotI site. Figure 3.3 shows a depiction of all the constructs used. "NOS N" consists of amino acids 1 to 315, "NOS C" of the last 87 amino acids and "NOS tail" of the last 29 amino acids of the protein. "NOS NI" carries amino acids 50 to 148. "NOS 114" is the last 114 amino acids.

The NOS ΔNI and NOS ΔNII deletion constructs were designed by first inserting a PCR fragment spanning the region upstream of the deleted sequence starting at the NOS start codon into pEG202 digested with BamHI and NotI. The 3' end primer used introduces an ApaI restriction site 5' of the Not I site. The resulting plasmid was then digested with ApaI and Not I and a second PCR fragment covering sequences downstream of the deletion up to the NOS stop codon was introduced. In both these constructs the deleted amino acids are replaced by two amino acids, Gly Thr, encoded by the ApaI restriction site. NOSΔNI deletes amino acids 50 to 148, while NOSΔNII eliminates amino acids 149 to 218. NOS Δ50-218 was cloned by amplifying a NotI to BamHI fragment from *pN5 Δ50-218* (Curtis, et al., 1997). NOSΔL7 which carries a deletion in the tail region of NOS was amplified from *pN5nos*^{L7} (D. Curtis, Chapter 2)

Constructs carrying *nos* mutations were generated by site directed PCR mutagenesis. Full length *nos*, *bru* and *orb* (Wang and Lehmann, 1991; Lantz, et al., 1992; Webster, et al., 1997) were isolated through PCR amplification and inserted into EcoRI and XhoI sites in pJG4-5. All PCR reactions were performed in 100 µl reactions containing 1µg DNA, 1 mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X-100, 3mM $MgCl_2$, 5µM of each primer, 0.2mM of each dNTP and 2 units of Taq polymerase. The reaction was cycled 30 times at 95°C for 30 seconds, 55°C for 45 seconds and 95°C for 2 minutes.

To sequence pEG202 constructs primers Pegga1 and Pegga2 were used. To sequence cDNA clones in pJG4-5, primers BCO1 and BCO2 were utilized.

Pegga1: 5' TCG CAA CGG CGA CTG GCT 3'
Pegga2: 3' CCC GCT TAA AGA ATA CTA 5'
BCO1: 5' CCA GCC TCT TGC TGA GTG GAG ATG 3'
BCO2: 3' AGG TTA GTT CCA ACA GCC GAA CAG 5'

The embryonic library RFYL1 and the ovary library RFLY3 were a gift from Dr. Russel Finley. OvoI was kindly given to us by Dr. Jörg Grosshans. All libraries consist of unidirectional cDNA made from poly(A) selected RNA. In the case of RFLY3 and OvoI the RNA was isolated from Drosophila ovaries while the RNA for RFLY1 came from Drosophila embryos ranging from 0 to 12 hours old. RFLY1 has 4.2 X 10^6 individual members with an insert size averaging 1 kb. RFLY3 has 3.2 X10^6 individual members and the insert size averages 800 base pairs. OvoI has 1.5 X 10^6 members and the average insert size is 1160 bp.

Two hybrid screen

All experiments involving the two hybrid system were performed as described (Finley and Brent, 1994b). In summary, yeast strain EGY48 (*ura- his- trp-*) was sequentially transformed with pSH18-34 (*ura+*) and pEG202*nos* (*his+*). The presence of these two vectors is selected on media lacking ura and his. This strain was then transformed with the various cDNA libraries (*trp+*) and selected on media without ura, his and trp. Trp+ colonies were collected and frozen. Each of the frozen cells contain the NOS bait plasmid, the LacZ reporter and a member of the cDNA library. An aliquot of the frozen cells was thawed and diluted $1:10^4$, $1:10^5$, $1:10^6$ and $1:10^7$. The dilutions were then plated on media lacking ura, his and trp to determine the density of the frozen stock. The activator tagged cDNAs are under the control of a galactose dependent promoter. In order to induce cDNA expression, aliquots that could produce 1×10^7 colonies were thawed and incubated for four hours in liquid media lacking ura, his and trp and containing galactose. The cells were then plated on media lacking ura, his, trp and leu and containing galactose to select for all the three plasmids and screen for activation of the $LexA_{op}$-*leu2* gene. Colonies growing on the lack of leucine were then streaked on –ura –his –trp plate containing glucose as a sugar source. These colonies were then replica plated onto galactose plates lacking leu or containing X-gal to re-test for the interaction and confirm it with the expression of the more stringent reporter *lacZ*. Table 1 shows the specific number of colonies screened and the positive clones obtained for each one of the three libraries used. Library plasmids were rescued in *E. coli* DH5α or KC8 cells and grouped according to insert size and restriction map. Restriction mapping was performed on PCR amplified inserts using restriction enzymes AluI and HaeII to determine groups of clones expressing the same gene. A member from each group was sequenced using primers BCO1 and BCO2.

Yeast mating

Yeast mating assays were performed as previously described (Finley and Brent, 1994a). EGY48 strain (*ura-, his-, trp-*) transformed with the pJG4-5 cDNA constructs (trp+) was selected on media lacking trp. PEG202*nos* (*his+*) and pSH18-34 *(ura+)* were introduced into yeast strain YM4271 (*ura- his-*) and selected for by growing on media lacking his and trp. EGY48 with the different cDNA clones were streaked as lines on a plate lacking trp. YM4271 strains with different LexA-NOS fusions were also streaked as lines on a plate without ura and his. Both plates were then replica plated onto one rich

media plate forming a grid with the yeast lines. This system allows us to simultaneously mate one cDNA containing strain with various strains carrying different versions of LexA-NOS. The plate is then grown overnight at 30°C to allow the formation of diploids in the areas where the two strains came into contact. This plate is then replica plated onto galactose X-gal plates lacking ura, his and trp. Since one strain can only survive the lack of ura and his and the other the lack of trp, only diploid yeast will grow and, if an interaction occurs, LacZ will be expressed.

EST clones

Clones LD05547 and GM01354 were obtained from Genome Systems. Both clones are in Bluescript SK. The two clones were sequenced in order to obtain complete sequence of the EF-1γ.

P1 filter analysis

An EcoRI to XhoI fragment from LD05547 was labeled with [32P] dCTP using the Rediprime DNA labeling system (Amersham). The probe was then purified through a column made with Bio-gel P-10 medium (Bio-Rad). The P1 filter was pre-hybridized in 10ml high phosphate buffer (HPB) (0.5M NaCl, 0.1M Na_2HPO_4 and 5mM EDTA, adjusted to pH 7.0) and 1% sarkosyl for two hours at 55°C. The filter was then incubated overnight in HPB and 1% sarkosyl with radiolabeled EF1γ probe at 55°C. The filter was then washed once in 1mM Tris-HCl pH 8.0 plus 1% sarkosyl at 55°C. This was followed by three washes in 1mM Tris-HCl at 55°C for 10 minutes. Lastly, the filter was washed in 2X SSC and 0.1% SDS for 10 minutes at room temperature. The filter was exposed to X-ray film overnight.

OvoI colony probing

Probes for *nos*, *bru*, EF-1γ, *diphosphate reductase* and clone X32 were made by DIG-labeling an EcoRI to XhoI fragment from the pJG4-5 two hybrid plasmids. The labeling reaction was done using the Dig Labeling Kit (Boehringer Mannheim).

All 69 LacZ + clones isolated from the OvoI library screen were plated on plates lacking ura, his and trp. Colonies were lifted with Hybond N nylon 0.45 micron membrane (Amersham). The filter was placed on Whatman paper filters soaked in 1M

sorbitol, 20mM EDTA and 50mM DTT for 40 minutes. The filter was then placed at –70°C for 5 minutes and thawed. Next, the filter was then placed on Whatman paper soaked in 1M sorbitol, 20mM EDTA and 200 units/ml β-gucuronidase and incubated at 37°C for 6 hours. The filter was then placed on a paper filter saturated with 0.5M NaOH for 10 minutes, on a paper filter saturated with 0.5M Tris-HCl and 6XSSC twice for 5 minutes and finally on a paper filter saturated with 2X SSC twice for 5 minutes. The test filter was air-dried and UV-crosslinked. The filter was then pre-hybridized in prehyb (5X SSPE, 50% deionized formamide, 0.1% SDS, 0.5% dry milk and 500µg/ml denatured calf thymus DNA) for 30 minutes at 42°C. The filter was incubated in 1ml prehyb plus 10µl of the probe overnight at 42°C. The filter was washed twice for 5 minutes at room temperature in 2X SSC and 0.1% and twice in same buffer at 68°C. Finally, a detection reaction was performed as described in the Dig labeling kit (Boehringer Mannheim).

Western blot

Yeast extracts were prepared by growing yeast in liquid media to an OD_{600} of 0.5, spinning 1 ml of the culture to pellet cells and then resuspending in 50µl of standard SDS loading buffer. The cells were broken by freezing on dry ice and boiling prior to loading on a 10% SDS-acrylamide gel. Proteins were transferred to a PVDF membrane. The blot was blocked in 5% milk in PBS for 2 hours. The blot was incubated overnight in 2.5 % milk in PBS and a 1:1000 dilution of rabbit antibody raised against NOS(Wang, et al., 1994). The blot was then washed in PBS and incubated in 2.5% milk in PBS with a 1:1000 dilution of AP-conjugated goat anti-rabbit sera (Jackson ImmunoResearch). Afterwards, the blot was washed with PBS and last with AP buffer (100mM NaCl, 5mM $MgCl_2$ and 100mM Tris pH 9.0). The blot was finally stained in 10ml AP buffer containing 0.375 mg/ml of NBT and 0.25 mg/ml of BCIP.

RESULTS

Two hybrid screen

In order to identify proteins that interact with NOS we performed a two-hybrid screen. We used the interaction trap system developed in yeast by Stanley Fields and Roger Brent (Fields and Song, 1989; Gyuris, et al., 1993). This system uses the binding domain of the transcription factor LexA (LexA-BD) fused to the bait protein of interest and cDNA libraries fused to a transcription activation domain (AD) (Gyuris, et al., 1993). Two reporter constructs, one coding for Leu2 and the other for LacZ, are utilized to detect interactions between the bait and a member of the library. Both of these reporters contain LexA operator sequences. If an interaction occurs between the bait protein and a member of the library, yeast containing both fusion proteins will be able to grow in media lacking leucine and will turn blue in media containing X-gal (Figure 3.1). Sequences coding for the entire NOS open reading frame were fused to LexA. This chimeric protein can not activate transcription by itself and is expressed well in yeast (Figure 3.2).

Since NOS function is required during embryogenesis and oogenesis we wanted to use libraries representative of these two stages. Consequently, we used two different cDNA libraries obtained from Drosophila ovaries and one from 0 to 12 hour old embryos (see Materials and Methods for details). Table 1 shows details of the total colonies screened and the number of positive clones isolated for each one of the libraries. In total, 229 colonies out of 5.5×10^7 were able to grow on media lacking leu. The transcription of *lacZ* is a more stringent test for an interaction. Therefore, all 229 colonies were tested for the expression of LacZ by growing them in the presence of X-gal. This test showed that 123 colonies contained library members capable of interacting with NOS.

The library plasmids from these isolates were rescued and characterized molecularly. We first grouped plasmids harboring the same cDNAs by restriction mapping using two different enzymes. Subsequently, one isolate from each one of the groups was sequenced. Table 2 shows the identity of the cDNAs isolated from libraries RFLY1 and RFLY3. Thirteen clones from the embryonic library contained the gal4 activation sequences fused to either nothing or very short sequences. In addition, sequences coding for 16sRNA were isolated many times from both libraries. 16sRNA is often isolated in two hybrid screen as a non-specific activator. Several of the cDNAs identified encode proteins involved in translation and RNA binding. These two functions

are compatible with NOS' role during development and these respective clones were analyzed further.

We first confirmed that the plasmids isolated encoded proteins that can interact with NOS. To verify the specificity of the potential interactors we performed mating assays. This test takes advantage of the fact that haploid cells of opposite mating type will fuse to form diploids when brought into contact with each other. We introduced the activation-tagged cDNA sequences into yeast of the mating type α and the bait into yeast of the mating type a. When these two strains are mated, the newly formed diploid cells will be able to activate the transcription of the two reporter genes if an interaction occurs. The strain containing the AD fused cDNA sequences was mated to three different mating type a strains: one carrying the *lexA-nos* fusion, a second one with *lexA* fused to the coding sequences of the human protein Lamin, and a third one carrying the parental pEG202 plasmid. This mating test allowed us to recreate the interaction for only three proteins (Table 2). High levels of LacZ expression were seen when diploid cells contained LexA-NOS and either BRUNO, NOS or Elongation Factor 1γ (EF-1γ) fused to the activation domain. This interaction was specific to NOS. All other proteins tested failed to show an interaction with NOS in the mating assay.

All 69 isolates from the OvoI screen were transferred to a filter and probed for the presence of *nos*, *bruno* and the elongation factor. This was done to find out quickly whether proteins identified with one library can also be identified as interactors from another library. Both BRUNO and EF-1γ were found once in the OvoI library screen (Table 2). This result confirms the validity of these interactions.

NANOS dimerizes through its N terminus

One of the three interactions verified by the mating assay was NOS itself. The *nos* cDNA isolated in the screen codes for amino acids 157-402. This fragment contains most of the areas of homology found between NOS and other proteins (Figure 3.4a) (Curtis, 1995b). In order to map the region of dimerization we performed mating assays using LexA fused to various parts of the NOS protein (Figure 3.3). All these fusion proteins were expressed at high levels in yeast and did not activate transcription in the absence of an activation domain (data not shown). In order to recapitulate the in vivo situation, we first performed mating assays using the entire NOS fused to the activation domain instead of the fragment isolated in the screen. A negative control consisting of activator tagged Orb was used in this experiment. Orb is a Drosophila protein required

during oogenesis and is not expected to interact with NOS (Lantz, et al., 1994). The NOS-NOS interaction occurs through the N terminal region of NOS, specifically through amino acids 50 to 218 (Figure 3.3). Deletion of this region (Δ50-218) disrupts the interaction (Figure 3.3). Additionally, there is an interaction between the smaller fragment NOS NI and the NOS protein. This result suggests that this 88 amino acids fragment is the dimerization domain. Nevertheless, this domain is not present in the original NOS clone isolated (3X74, Figure 3.4A). In addition, deleting this domain or the adjacent 69 amino acids does not affect the interaction. Two possibilities can explain this result. It is possible that redundant dimerization sequences exist in the protein making it difficult to eliminate the interaction through deletions. Alternatively, the interaction could occur using a different part of the N terminal region in each of the two molecules involved. For example, NOS NI could interact with the part deleted by ΔNOS NII. Unfortunately, we were unable to express a NOS NII fragment in either of the parental plasmids used in the mating assay. In order to differentiate between these two possibilities we performed the mating assays using the fragment coded by 3X74 and the different LexA fusions (Figure 3.4B). The key result from these mating assays is the lack of interaction between NOS NI and the clone isolated in the screen. The activator tagged protein coded by 3X74 does not contain the regions in NOS NI. Similarly, 3X74 does not interact with NOS ΔNII which carries a deletion in the section of the N terminus left in 3X74. Consequently, the dimerization does not occur through an interaction between non-homologous regions of the protein. These results could suggest that there are two distinct regions of dimerization (amino acids 50 to 148 and amino acids 149 to 218) in the N terminus of NOS or that redundant sequences exist in the 168 amino acid dimerization domain here identified.

Elongation Factor 1γ interacts with the tail domain

The elongation factor 1γ (EF-1γ) was isolated as an interactor with NOS in the two hybrid screen. This elongation factor was isolated from two independent ovary cDNA libraries. This factor had not been previously cloned from Drosophila. The clone obtained from library RFLY3 seems to code for the full-length protein by comparison to database sequences of the protein from Xenopus and *S. cerevisae*. Blast analysis showed that EST clones of this gene had been isolated from Drosophila. We obtained two different EST clones from the Drosophila genome project. One of these clones came from an ovary library, while the other was isolated from an embryo library. We sequenced these EST clones along with the two isolates from our hybrid screen to obtain

a consensus sequence for the *Drosophila melanogaster* EF-1γ (Figure 3.5). The clone from the OvoI two hybrid library coded for a fragment from amino acid 153 to the end of the protein (Figure 3.5). Drosophila EF-1γ consists of 431 amino acids and has the most homology to EF-1γ from *Xenopus laevis* and *Artemia salina*(Janssen and Moller, 1988). This protein contains a signature GST like domain in its N terminus (Koonin, et al., 1994). GST domains have been implicated in protein-protein interactions. However, this region is truncated in the OvoI clone (Figure 3.5). This suggests that the interaction with NOS does not depend on the GST domain of EF-1γ and that the interaction might be specific.

To determine where this gene maps in the fly genome we screened a P1 genomic filter representing the Drosophila genome. Most of the P1 clones have been mapped to particular contigs which cover about 85% of the total genome (BGDP). These contigs have in turn been mapped to specific positions in the chromosome. This experiment, therefore, allows us to obtain genomic sequence for this gene and simultaneously map the gene to a position in the genome. Knowing the position of EF-1γ allows us to search for genetic mutations in the region and to analyze deletions of this region for potential phenotypes. We used the entire cDNA as a probe to screen the filter. Six positive clones were found (Table 3). One of the clones had no mapping information, while three of them belonged to the same contig *stg*. This contig maps to region 99A5-99A8 on the right arm of the third chromosome. The other two clones mapped next to each other in adjacent contigs *ser* and *Ets97D* respectively. These two clones could represent separate copies of the EF-1γ gene. These two clones also map to the right arm of the third chromosomes fairly close to the *stg* contig. In summary, there are three copies of this gene in Drosophila, all mapping close together on the third chromosome. Other organisms, such as yeast, also contain multiple copies of this gene all lying next to each other in the chromosome (Kinzy, et al., 1994). The region where this gene maps in the Drosophila chromosomes does not harbor any obvious candidates for a mutation in the EF-1γ. Deficiency *Df(3R)3450* deletes the regions containing the gene found in the stg contig. Deficiency *Df (3R) Tl-P* takes out a large region taking out the other two potential copies of the EF-1γ. It will be interesting to see whether any of these deletions could rescue a weak *nos* phenotype by reducing the amount of EF-1γ expressed.

We next mapped the region in NOS that interacts with EF-1γ. In order to find the specific region of NOS needed for this interaction we recapitulated the yeast mating assays described earlier. EF-1γ interacts specifically with the tail region of the protein (amino acids 373 to 402, Figure 3.6). This interaction is inhibited by a seven amino acids

deletion in this region (NOSΔL7). This deletion is found in the allele nos^{L7}, which specifically affects *hb* regulation (Chapter 2). We also tested the interaction of EF-1γ and NOS protein with a specific amino acid change in the tail region known to affect NOS function (Nos^{108}, Chapter 2). The mutation found in allele nos^{108} (Met(379) to Lys change) does not seem to affect this interaction (Figure 3.7). As with full-length NOS, the tail domain fragment carrying this mutation can still interact with EF-1γ (NOS tail 108, Figure 3.7). We have also shown that two other mutations in the C-terminal region of the protein do not disrupt the interaction between full length NOS and any of the three interactors isolated in the two hybrid screens (Figure 3.7) (Chapter 2, (Curtis, et al., 1997)). In conclusion, the interaction between this translation factor and NOS occurs through a small region in the C terminus that has been implicated in the translation repression of *hb*.

NANOS and PUMILIO

NOS Function in the embryo requires the action of the RNA binding protein PUMILIO (PUM) (Barker, et al., 1992; Wreden, et al., 1997). While the genetic interaction between NOS and PUM has been well demonstrated, a physical interaction has not been observed. All the libraries used in this screen were made by priming RNA from its poly(A) tail. Given that the average insert size for all three libraries is approximately 1 Kb and that the 3'UTR of *pum* is approximately 1.2 Kb, we would not expect to find PUM protein in these screens. In order to get around this problem we tested for an interaction between NOS and PUM directly by using the yeast mating assay. The PUMILIO protein is too large to be expressed in its entirety as a two hybrid fusion. Therefore we used the minimal functional domain of PUM which consists of the RNA binding domain harbored in its C terminus (PumC, amino acids 1093 to 1533) (Zamore, et al., 1997; Wharton, et al., 1998). The RNA binding domain is able to rescue the *pum* phenotype (Wharton, et al., 1998). This region fused to the LexA binding domain can be expressed in yeast and does not activate transcription (D. Chagnovich, personal communication). We detected no interaction between the LexA-PumC and the activator tagged full-length NOS (data not shown). We also performed the inverse experiment by expressing PumC with the activation domain and NOS fused to the LexA binding domain with an identical result. In addition, these results were confirmed by simultaneously expressing the constructs in a haploid yeast strain. Nevertheless, no interaction was seen in these assays either. In summary, an interaction between NOS and the PUM minimal functional domain can not be detected by a directed yeast interaction trap assay.

NANOS and the Elongation Factor 1γ

We have identified three proteins that have the ability to interact with NOS protein. Two of these proteins, EF-1γ and BRUNO, are implicated in translation and the other is NOS itself. NOS role in the regulation of *hb* translation suggests that the interactions with EF-1γ and BRUNO might be meaningful. EF-1γ interacts specifically with the tail domain of NOS. This region is required for NOS function during pattern formation and primordial germ cell migration but not during oogenesis (Chapter 2). This domain is necessary for the regulation of *hb* translation, since its absence or a mutation in it renders NOS incapable of regulating *hb*. A deletion in this region isolated as allele nos^{L7} shows no function in *hb* regulation and is able to disrupt the interaction with the EF-1γ in yeast. Nevertheless, a mutation affecting a single amino acid in the tail region that shows an identical phenotype to nos^{L7} does not affect the interaction with the elongation factor. This result might indicate that this interaction is not specific or that the yeast interaction trap system is not sensitive enough to detect the effect of a single amino acid change. It is also possible that other amino acids in the tail region might be more important in interacting with the elongation factor.

The elongation factor 1 (EF-1) complex is formed by three subunits α, β and γ and is involved in protein synthesis by binding aminoacyl-tRNA to 80S ribosomes under GTP hydrolysis (Moldave, 1985). EF-1βγ carries out the nucleotide exchange of EF-1α-GDP to EF-1α-GTP. EF-1γ has been suggested to act as a catalyst in the nucleotide exchange activity carried out by the β subunit (Janssen and Moller, 1988). The exchange reaction has been suggested to be a rate limiting step in the elongation stage of translation. An interaction between NOS and an elongation factor suggests that NOS might be regulating translation by affecting the elongation rate. Potentially, NOS could recruit EF-1γ away from the elongation machinery, stalling translation. This idea is not supported by previous experiments performed to study the translation status of the *hb* RNA. Polysome gradient profiles of WT embryos, *nos* mutant embryos and embryos with NOS at both the anterior and posterior pole were analyzed and compared to each other (Wang, 1995). This experiment showed that as more NOS is present and less HB protein is expressed, *hb* RNA shifts to the monosomal fraction. This is indicative of translational regulation at the level of initiation. If *hb* were regulated during elongation, as less HB protein is expressed more of the RNA should be trapped in the polysomes.

What role an interaction between NOS and an elongation factor might play in the regulation of *hb* is not known. It is possible that EF-1γ carries out a function other than translational elongation. In yeast, approximately half of this subunit is in the EF-1 complex, while the rest is free in the cytoplasm. In addition, deletion of all three EF-1γ in yeast has no effect on viability and translation (T.Goss Kinzy, personal communication). Also , in *Artemia salina*, EF-1γ has been suggested to interact with membrane and cytoskeletal structures (Janssen and Moller, 1988). Further experiments, using mutations in EF-1γ should reveal whether the interaction observed in the two hybrid assay reflects a functional connection between NOS and PUM and the translation machinery.

NANOS dimerization domain

The third interactor isolated suggest that NOS might be able to dimerize. We had no previous evidence that dimerization might occur. We do not know at the moment whether this interaction is required for NOS normal function. This interaction seems to occur through the N terminus of NOS. This region of the protein has not been affected in a mutagenesis screen (Chapter 2). In addition, the deletion Δ50-218 shown here to disrupt this interaction, was shown to be able to partially rescue the abdominal phenotype of a *nos* null embryo in an RNA injection assay (Curtis, et al., 1997). The amounts injected are expected to exceed the normal amounts of RNA present in an embryo. Therefore, the RNA injection assay might be able to overcome the need for dimerization by having higher levels of the protein present. Potentially, this interaction is more critical in other functions of NOS where it is not known whether the N terminus plays any role. It will be interesting to see what the effect of this deletion in proteins expressed at normal levels would be on the different roles of NOS.

NANOS and PUMILIO

The only protein known to interact genetically with NOS is PUM. PUM is active in many of the developmental processes where NOS is required: oogenesis, embryogenesis and germ cell migration (Forbes and Lehmann, 1998). Co-expression of both factors and their similar phenotypes suggests that their functions are closely connected. Previous efforts to show a direct physical interaction between these two proteins have failed. We took advantage of the yeast two hybrid system used here to find NOS interactors to directly probe for a PUM-NOS interaction. Unfortunately, this assay was unable to detect direct binding between these two proteins. It is possible that an

interaction between NOS and PUM is not direct or that it requires other factors or the presence of specific RNA sequences to occur. New modifications of the two hybrid system that can use more than two factors or RNA linkers will prove useful to further test this interaction. In addition, we failed to see interactions between PUM and any of the proteins interacting with NOS (data not shown). As more information becomes available on the processes involved in the regulation of *hb* and other targets by NOS and PUM we might learn what role the interaction between NOS and EF-1γ might play in this process.

Figure 3.1 NANOS interaction trap.

Full-length NOS protein fused to LexA binding domain is used as a bait in this interaction trap. A. If no interaction occurs between NOS and a member of a cDNA library carrying an activation domain, transcription of the reporters does not occur. B. When an interaction occurs with an activator-tagged protein, the transcription of two reporters is activated. The yeast cell hosting this interaction will be able to grow in media lacking leucine, and will form blue colonies in the presence of X-Gal.

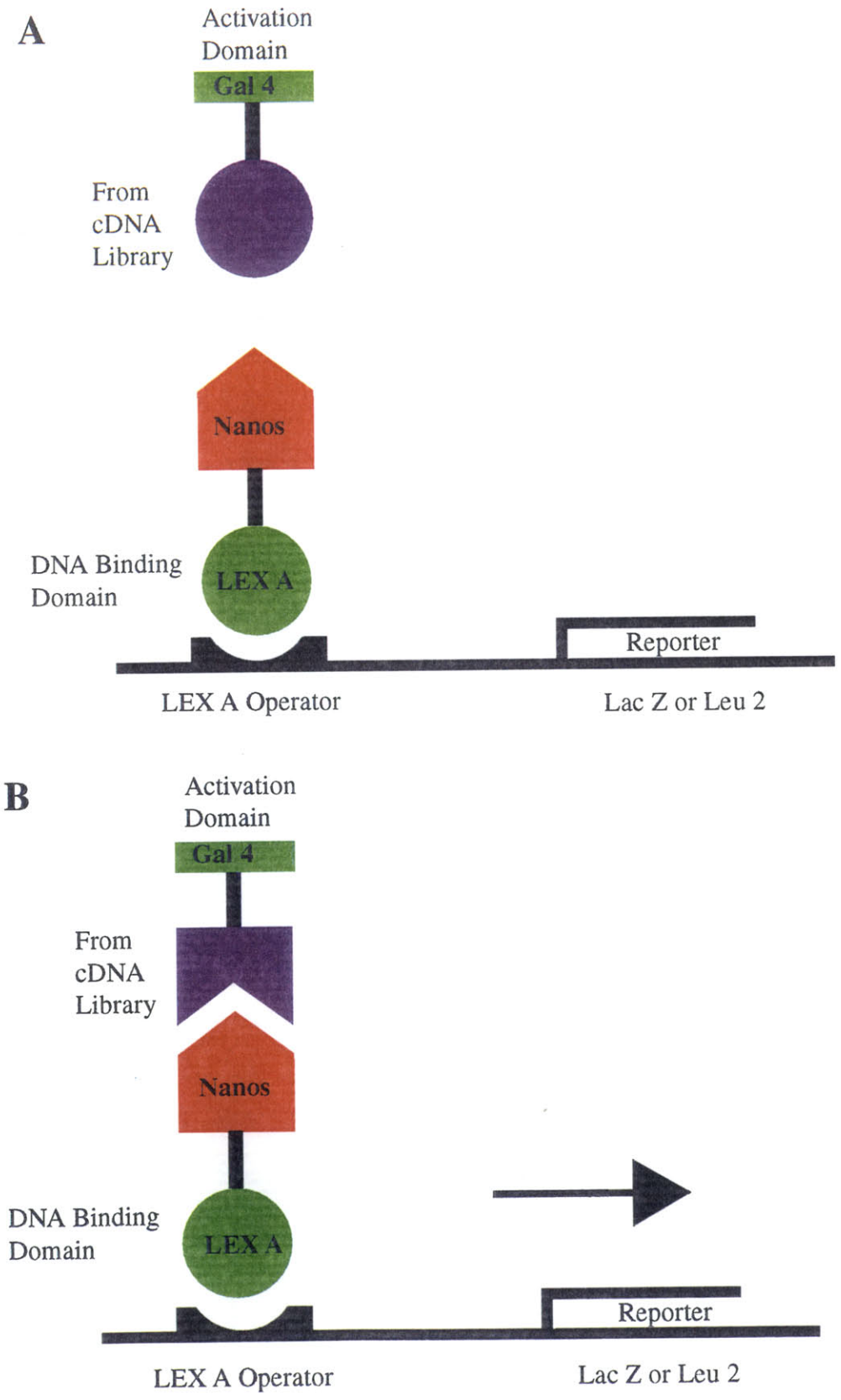
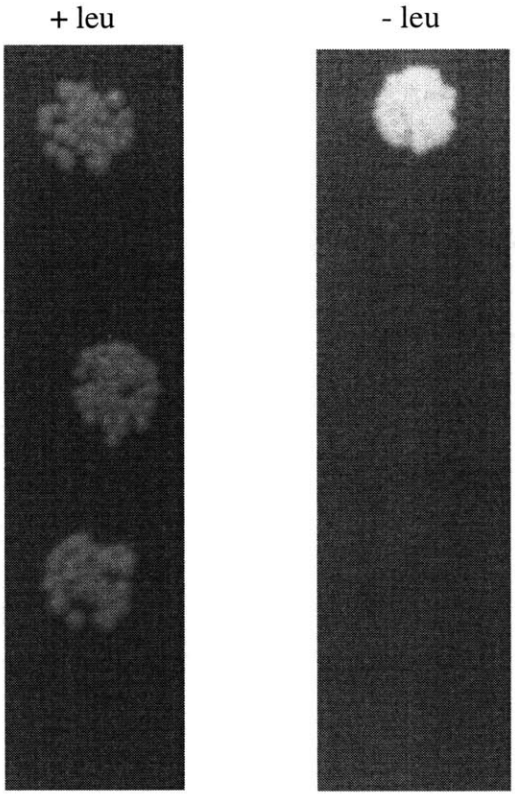

A
Activation Domain
Gal 4
From cDNA Library
Nanos
DNA Binding Domain
LEX A
Reporter
LEX A Operator
Lac Z or Leu 2
B
Activation Domain
Gal 4
From cDNA Library
Nanos
DNA Binding Domain
LEX A
Reporter
LEX A Operator
Lac Z or Leu 2

Figure 3.2. LexA-NOS does not activate transcription and is expressed in yeast.

A. Yeast cells carrying the LexA-NOS construct and no activation domain were plated on both Leu+ and Leu- plates along with yeast carrying control plasmids. pSH17-4 codes for LexA fused to the activation domain of Gal4 and is capable of directly activating the expression of *leu*. pRFHMI encodes a LexA fusion incapable of activating transcription of the reporter genes. Yeast carrying the LexA-NOS fusion do not grow in the absence of leucine, showing that this fusion protein cannot activate transcription. B. Antibodies against the tail region of NOS detect a protein of expected size, (NOS 40 kDa + LexA 30 kDa = 70 kDa) in yeast extract of two independent isolates containing the pLexA-nos fusion plasmid. MW is high molecular weight standards.

A


+ leu
- leu
pSH17-4
pRFHM1
pLexANos

B

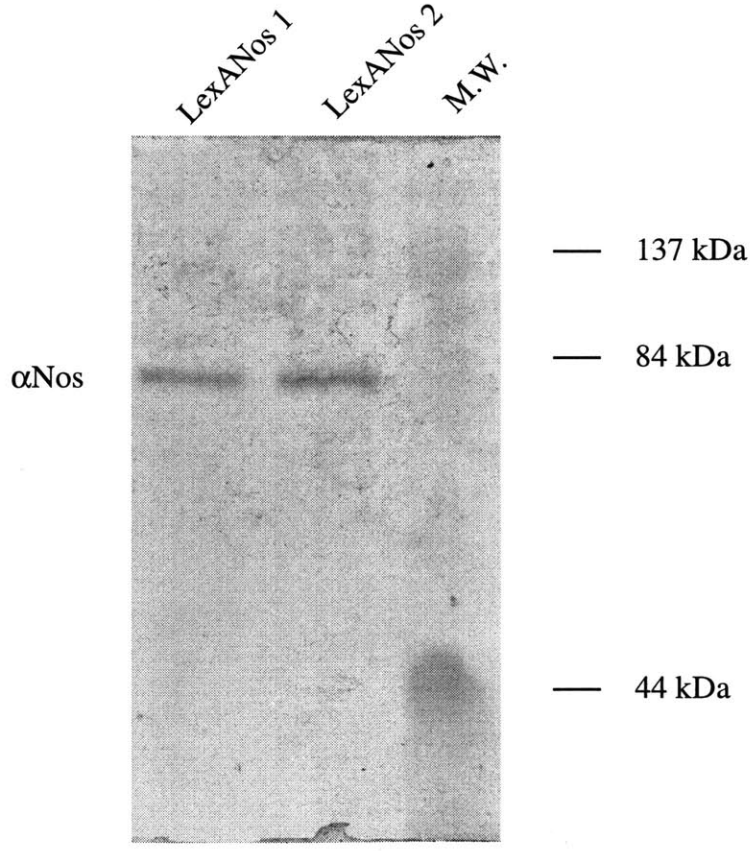

LexANos 1
LexANos 2
M.W.
αNos
137 kDa
84 kDa
44 kDa

Table 3.1. Results from three independent yeast two hybrid screens.

CDNA Library	RFLY 1 Drosophila embryo 0-12 hours	RFLY 3 Drosophila ovary	Ovo I Drosophila ovary
Complexity	4×10^6	3.2×10^6	1.6×10^6
Library + clones	2.9×10^7	1×10^7	1.3×10^6
Colonies screened	3×10^7	2×10^7	1.5×10^7
Leu + colonies	56	88	85
LacZ+ colonies	21	33	69

Library + refers to amounts of colonies containing the LexA-NOS plasmid and a member of the library. These colonies were frozen and an aliquot was thawed and plated on leu- media to find interactors. The number of cells in the aliquot determine the number of colonies screened.

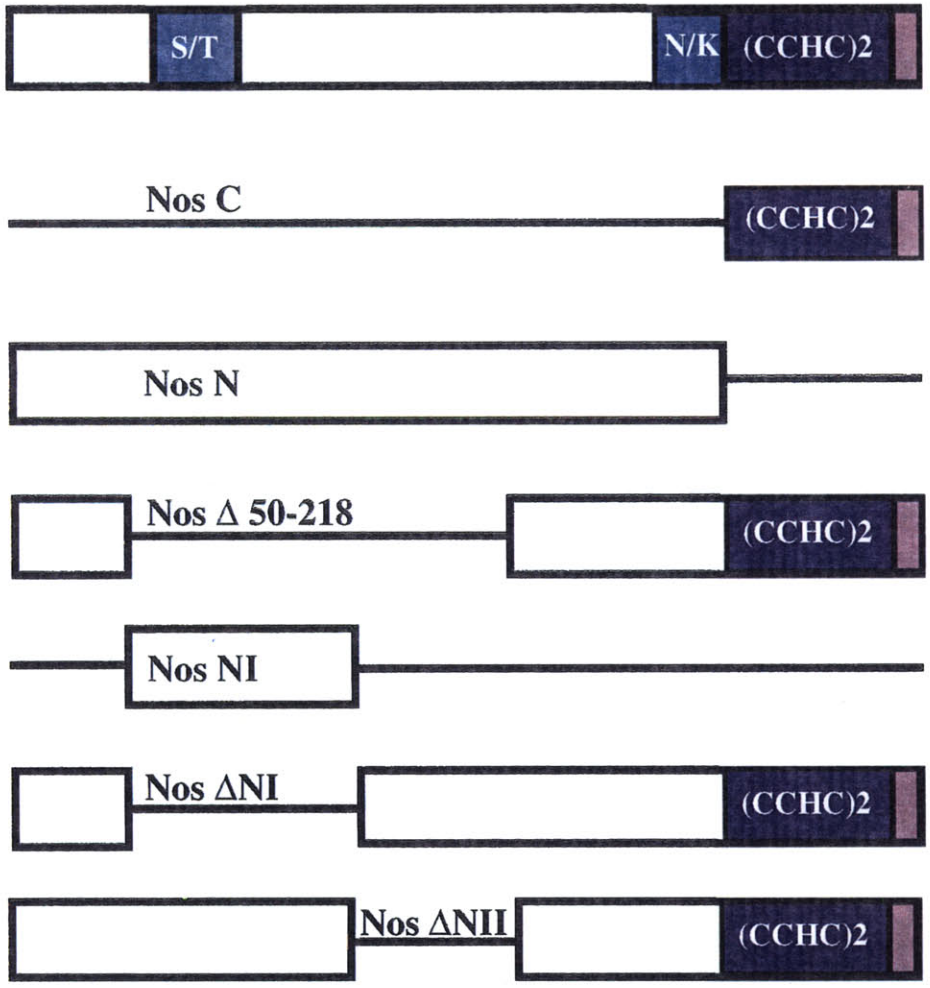
Table 3.2. Identity of clones isolated in the two-hybrid screen.

	Clone Name	Library	Isolates	Size of Insert	Mating
Elongation factor 1γ	3X57	RFLY3 OvoI	2	1.8 kb	+
Drosophila BRUNO	3X33	RFLY3 OvoI	2	1.1 kb	+
Drosophila NANOS aa 157-402	3X74	RFLY3	1	1.7 kb	+
Drosophila 16s rRNA	X14 X30 3X2	RFLY1 RFLY3	17	1.4 kb 1.1 kb 0.8 kb	-
Drosophila Ribonucleoside diphosphate reductase	3X24	RFLY3	7	1.5 kb	-
Drosophila ribosomal protein S7	3X67	RFLY3	2	1.0 kb	-
Drosophila PORIN	X8	RFLY1	2	1.4 kb	-
Protein containing double stranded RNA binding domain	X20	RFLY1	1	1.5 kb	-
Drosophila HEMOMUCIN	X31	RFLY1	1	2.5 kb	-
Drosophila EST	X32	RFLY1	1	1.5 kb	-
Drosophila ribosomal protein DL11	3X18	RFLY3	1	1 kb	-
Drosophila Minute (1) 1B protein ribosomal protein L36	3X23	RFLY3	1	0.8 kb	-
Zinc finger protein $5x(CX_2CX_{12}HX_3HX_7)$	3X52	RFLY3	1	1.7 kb	-
snRNP N/B	3X77	RFLY3	1	1.7 kb	-
?	3X14	RFLY3	1	1.0 kb	-
?	3X42	RFLY3	1	0.4 kb	-
?	3X62	RFLY3	1	1.0 kb	-
?	3X73	RFLY3	1	1.0 kb	-

Proteins that do not indicate Drosophila have not been cloned in flies. ? indicates that the sequences in the plasmid isolated showed no homology to sequences in the database. Libraries RFLY3 and OvoI were produced from ovary RNA. RFLY1 was made from 0-12 hour embryos. For mating results a + indicates that LacZ expression was seen in diploid yeast containing this clone and the LexA-Nos fusion.

Figure 3.3. NOS dimerizes through its N terminus.

Left column depicts different NOS fragments used in this mating assay. The second column shows LacZ expression of diploid yeast carrying AD-NOS fusion and the NOS fragments shown at the left fused to LexA. The last column shows diploid yeast which carries AD fused to the Drosophila protein Orb and NOS fragments fused to LexA. All diploid strains are grown on the same plate containing X-Gal. Blue yeast is indicative of an interaction. Colored boxes in the NOS protein indicate regions conserved among other insect NOS (Curtis, et al., 1995)


S/T
N/K
(CCHC)2
Nos C
(CCHC)2
Nos N
Nos Δ 50-218
(CCHC)2
Nos NI
Nos ΔNI
(CCHC)2
Nos ΔNII
(CCHC)2

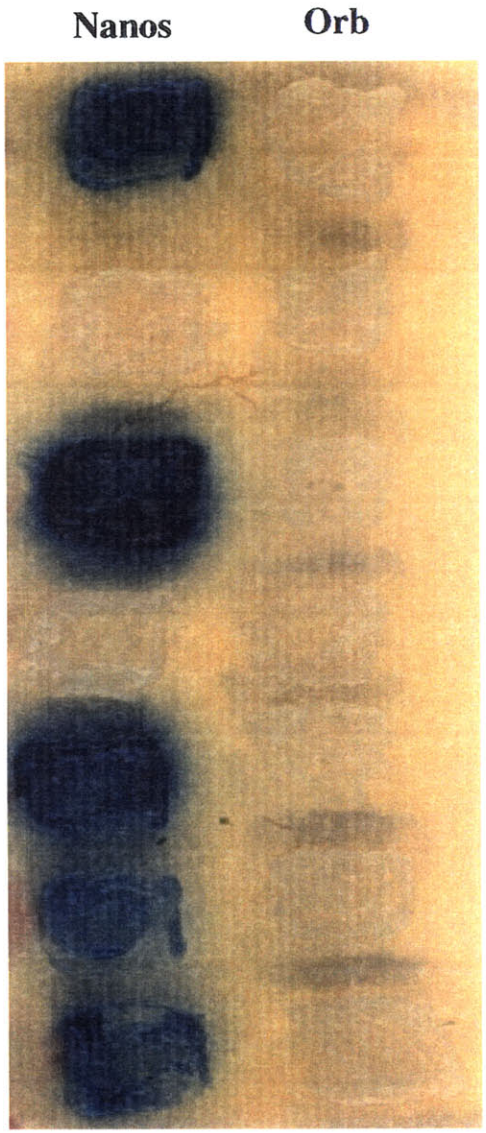
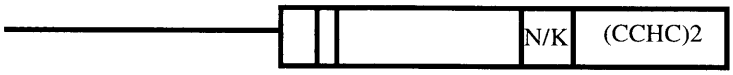

Nanos
Orb

Figure 3.4. Mapping the NANOS dimerization domain.

A. 3X74 is the NOS fragment isolated in the two hybrid screen. This fragment contains the complete conserved Zn-fingers and C-terminal tail domain. B. Results from mating assays between yeast carrying NOS fragments fused to the LexA binding domain and activator-tagged full-length NOS or the isolated clone 3X74. +++ indicates high levels of LacZ expression. When – is used no expression of the reporter was detected when plated in X-Gal media. The first construct in the left column depicts full length NOS with the previously described regions of homology in boxes (Curtis, 1995b).

A


3X74
N/K
(CCHC)2

B

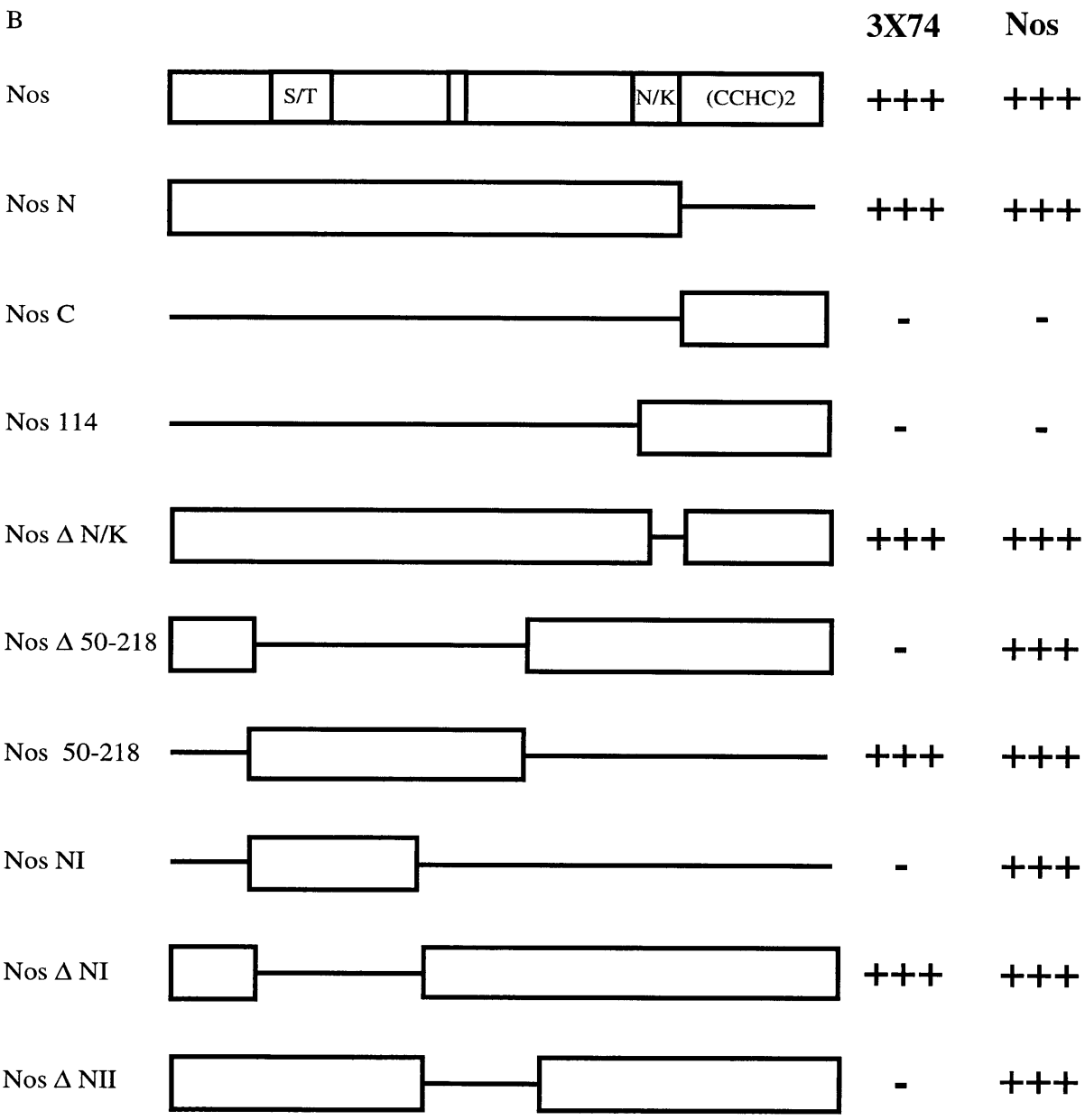

3X74
Nos
Nos
S/T
N/K
(CCHC)2
+++
+++
Nos N
+++
+++
Nos C
-
-
Nos 114
-
-
Nos Δ N/K
+++
+++
Nos Δ 50-218
-
+++
Nos 50-218
+++
+++
Nos NI
-
+++
Nos Δ NI
+++
+++
Nos Δ NII
-
+++

Figure 3.5. *D. melanogaster* Elongation Factor 1γ.

Sequence of the cDNA coding for EF-1γ. Vertical line shows where the clone isolated from the OvoI library begins. In bracket is the GST-like domain found in many EF-1γ.


GGCACGAGACGATTTAAACGGCGAATCCACCATGGTGAAAGGAACTCTGTACACTTACCCCGAGAACTTCCGGGCCTACAAGGCGCTCATCGC
93
Met Val Lys Gly Thr Leu Tyr Thr Tyr Pro Glu Asn Phe Arg Ala Tyr Lys Ala Leu Ile Ala
AGCCCAGTACTCCGGAGCCCAGGTGAAAGTGGCCGACAACTTCAAGTTCGGCGAGACCAACAAGTCGGCTGAGTTCCTCAAGAAGTTCCCCGG
186
Ala Gln Tyr Ser Gly Ala Gln Val Lys Val Ala Asp Asn Phe Lys Phe Gly Glu Thr Asn Lys Ser Ala Glu Phe Leu Lys Lys Phe Pro Gly
TGGCAAGGTGCCCGCCTTTGAGACCGCCGAAGGACAGTACTTGAGCGAGTCCAATGCCATCGCCTACCTGCTGGCCAACGAGCAGCTGCGCGG
279
Gly Lys Val Pro Ala Phe Glu Thr Ala Glu Gly Gln Tyr Leu Ser Glu Ser Asn Ala Ile Ala Tyr Leu Leu Ala Asn Glu Gln Leu Arg Gly
CGGAAAGTGCCCCTTCGTGCAGGCCCAGGTCCAGCAGTGGATCTCCTTCGCCGACAACGAGATTGTGCCTGCCTCCTGTGCCTGGGTCTTCCC
372
Gly Lys Cys Pro Phe Val Gln Ala Gln Val Gln Gln Trp Ile Ser Phe Ala Asp Asn Glu Ile Val Pro Ala Ser Cys Ala Trp Val Phe Pro
CCTGCTGGGCATTCTGCCGCAGCAGAAGAACAGCACTGCCAAGCAAGAGGCCGAGGCTGTGCTGCAGCAGCTCAACCAGAAGCTGCAGGACGC
465
Leu Leu Gly Ile Leu Pro Gln Gln Lys Asn Ser Thr Ala Lys Gln Glu Ala Glu Ala Val Leu Gln Gln Leu Asn Gln Lys Leu Gln Asp Ala
CACCTTCCTGGCCGGCGAGAGGATCACATTGGCCGACATTGTGGTCTTCAGCAGTCTGCTCCACCTGTACGAGTACGTCCTGGAGCCCAGTGT
558
Thr Phe Leu Ala Gly Glu Arg Ile Thr Leu Ala Asp Ile Val Val Phe Ser Ser Leu Leu His Leu Tyr Glu Tyr Val Leu Glu Pro Ser Val
GCGCAGTGCCTTCGGCAACGTGAACCGCTGGTTCGTCACCATCCTCAACCAGAAGCAGGTCCAGGCCGTCGTCAAGGACTACAAGCTGTGCGA
651
Arg Ser Ala Phe Gly Asn Val Asn Arg Trp Phe Val Thr Ile Leu Asn Gln Lys Gln Val Gln Ala Val Val Lys Asp Tyr Lys Leu Cys Glu
GAAGGCCCTGGTCTTCGACCCCAAGAAGTACGCCGAGTTCCAGGCCAAGACCGGAGCCGCCAAGCCCCAGCAGCAGGCTCAGCAGCAGAAGCA
744
Lys Ala Leu Val Phe Asp Pro Lys Lys Tyr Ala Glu Phe Gln Ala Lys Thr Gly Ala Ala Lys Pro Gln Gln Gln Ala Gln Gln Gln Lys Gln
GGAGAAGCCCAAGGAAAAGAAGGAGGCGCCCAAGAAGGCTGCCGAGCCCGCCGAGGAGTTGGACGCCGCCGATGAGGCCCTGGCCGCCGAGCC
837
Glu Lys Pro Lys Glu Lys Lys Glu Ala Pro Lys Lys Ala Ala Glu Pro Ala Glu Glu Leu Asp Ala Ala Asp Glu Ala Leu Ala Ala Glu Pro
CAAGTCCAAGGACCCCTTCGATGCGCTGCCCAAGGGCACCTTCAACTTCGATGACTTCAAGCGCGTGTACTCCAACGAGGACGAGGCCAAGTC
930
Lys Ser Lys Asp Pro Phe Asp Ala Leu Pro Lys Gly Thr Phe Asn Phe Asp Asp Phe Lys Arg Val Tyr Ser Asn Glu Asp Glu Ala Lys Ser
CATTCCCTACTTCTTCGATAAGTTCGATGCCGAGAACTACTCGATCTGGTTTGGCGAGTACAAATACAACGAGGAGCTGTCCAAGGTGTTCAT
1023
Ile Pro Tyr Phe Phe Asp Lys Phe Asp Ala Glu Asn Tyr Ser Ile Trp Phe Gly Glu Tyr Lys Tyr Asn Glu Glu Leu Ser Lys Val Phe Met
GTCGTGCAATCTCATCACCGGCATGTTCCAGCGTCTGGACAAGATGCGCAAGGCGGCCTTCGCCTCCGTTTGCCTGTTCGGCGAGGACGGCAA
1116
Ser Cys Asn Leu Ile Thr Gly Met Phe Gln Arg Leu Asp Lys Met Arg Lys Ala Ala Phe Ala Ser Val Cys Leu Phe Gly Glu Asp Gly Asn
CAGCACCATCTCCGGCATCTGGGTGTGGCGCGGACAGGATCTGGCCTTCACGCTCTCCCCCGACTGGCAGATCGATTACGAGGTCTACGACTG
1209
Ser Thr Ile Ser Gly Ile Trp Val Trp Arg Gly Gln Asp Leu Ala Phe Thr Leu Ser Pro Asp Trp Gln Ile Asp Tyr Glu Val Tyr Asp Trp
GAAGAAGCTCGACGCCAAGAGCGAGGAGACCAAGAAGCTGGTCACCCAGTACTTCTCCTGGTCCGGCACCGACAAGGACGGTCGCAAGTTCAA
1302
Lys Lys Leu Asp Ala Lys Ser Glu Glu Thr Lys Lys Leu Val Thr Gln Tyr Phe Ser Trp Ser Gly Thr Asp Lys Asp Gly Arg Lys Phe Asn
CCAGGGCAAGATCTTCAAGTAATCATCTCTGCCCAGCCCAGCTCCGCTCAAAGCAGCAGCCGCCCTCATTTAGACCAACAACAACAACAGCAG
1395
Gln Gly Lys Ile Phe Lys •
CAGTAACAATAAAGTTTGAGATTTAAAATGCAGGAAGAGCACAATGCCCATTTCTTAAGTTCCAACTGATAAGTACACTAAAGATATCCAATA
1488
TCTGTCGTGCTGCCTCCCACGTTTGGCGGAATCGTGTGTCTCGCCTCCTGCATTTTGTACTGGAGAATTTGTTTGTAACCGCCTAAGCATAAC
1581
ACAGCATGATATTGTCAACGGAACAGCCGCCTGCAGTCAGAAAATATTTATAAAAAATAAAAGGTTTTCTATTAATAACAGCAAAAAAAAAAA
1674
AAAAAAA
1681

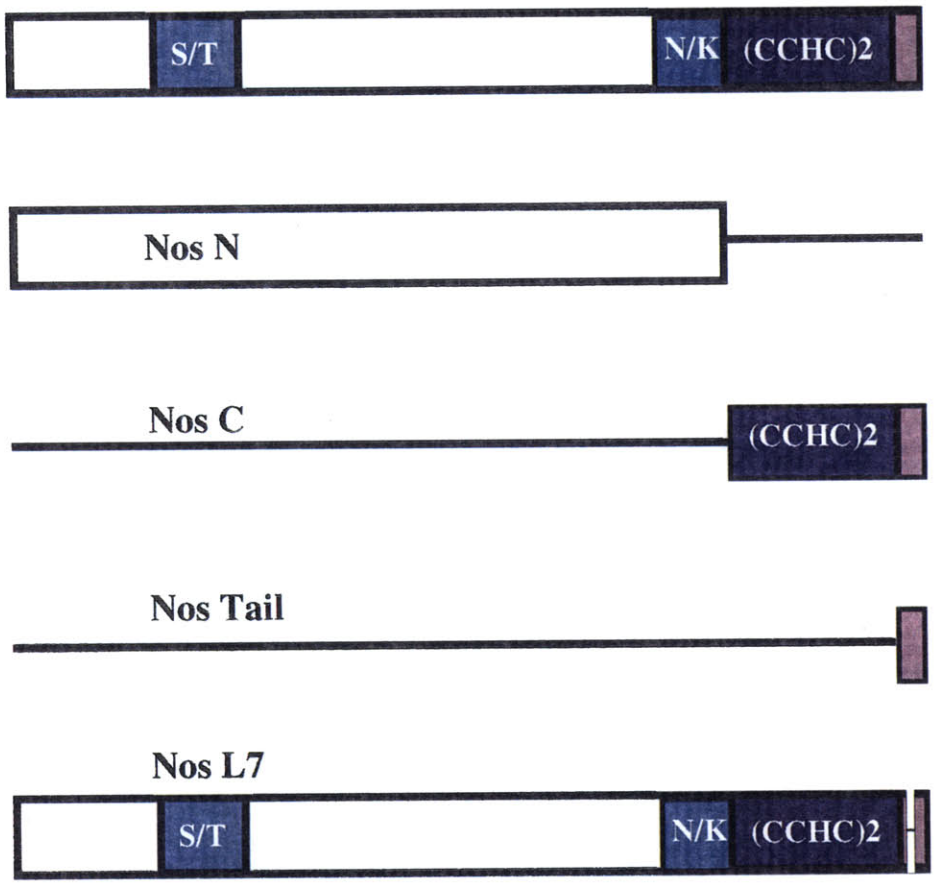
Table 3.3. P1 clones containing EF-1γ.

P1 Clone	Contig	Position	Chromosome
DS08773	ND	ND	ND
DS06138 DS05903 DS02282	stg	99A5-99A8	3(R)
DS08064	ser	99A1-99A6	3(R)
DS09080	Ets97D	97C5-D2	3(R)

ND indicates that no information is available on this particular clone.

Figure 3.6. EF-1γ interacts with the tail domain of NOS

The first column contains cartoon depictions of different NOS fragments fused to LexA. The second column shows diploid yeast formed after mating of a strain carrying AD-EF-1γ fusion to a strain carrying the NOS fragments shown at the left fused to LexA. The last column shows diploid yeast which carries AD fused to the Drosophila protein Orb and NOS fragments fused to LexA. All yeast are grown on the same plate containing X-gal. Blue yeast indicates that an interaction occurred allowing *lacZ* to be transcribed.


S/T
N/K
(CCHC)2
Nos N
Nos C
(CCHC)2
Nos Tail
Nos L7
S/T
N/K
(CCHC)2

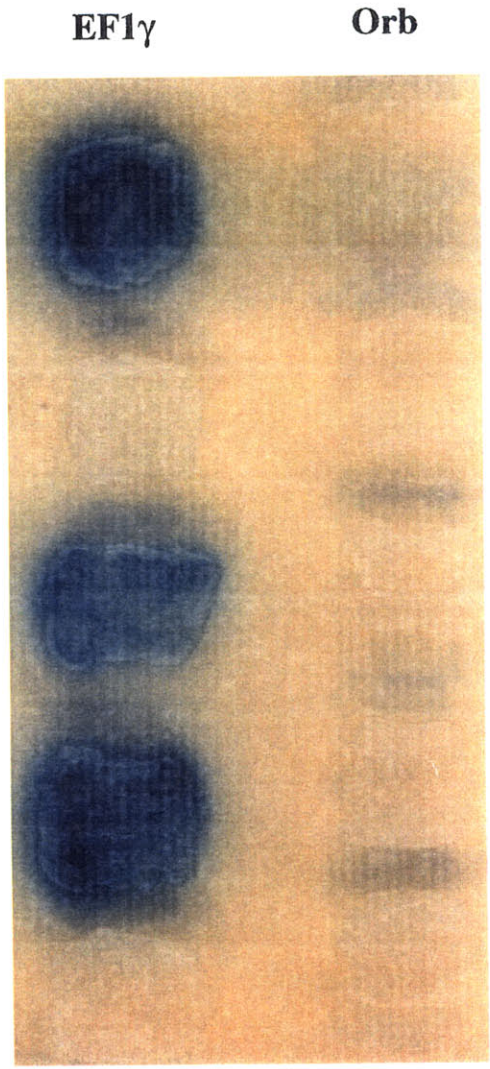
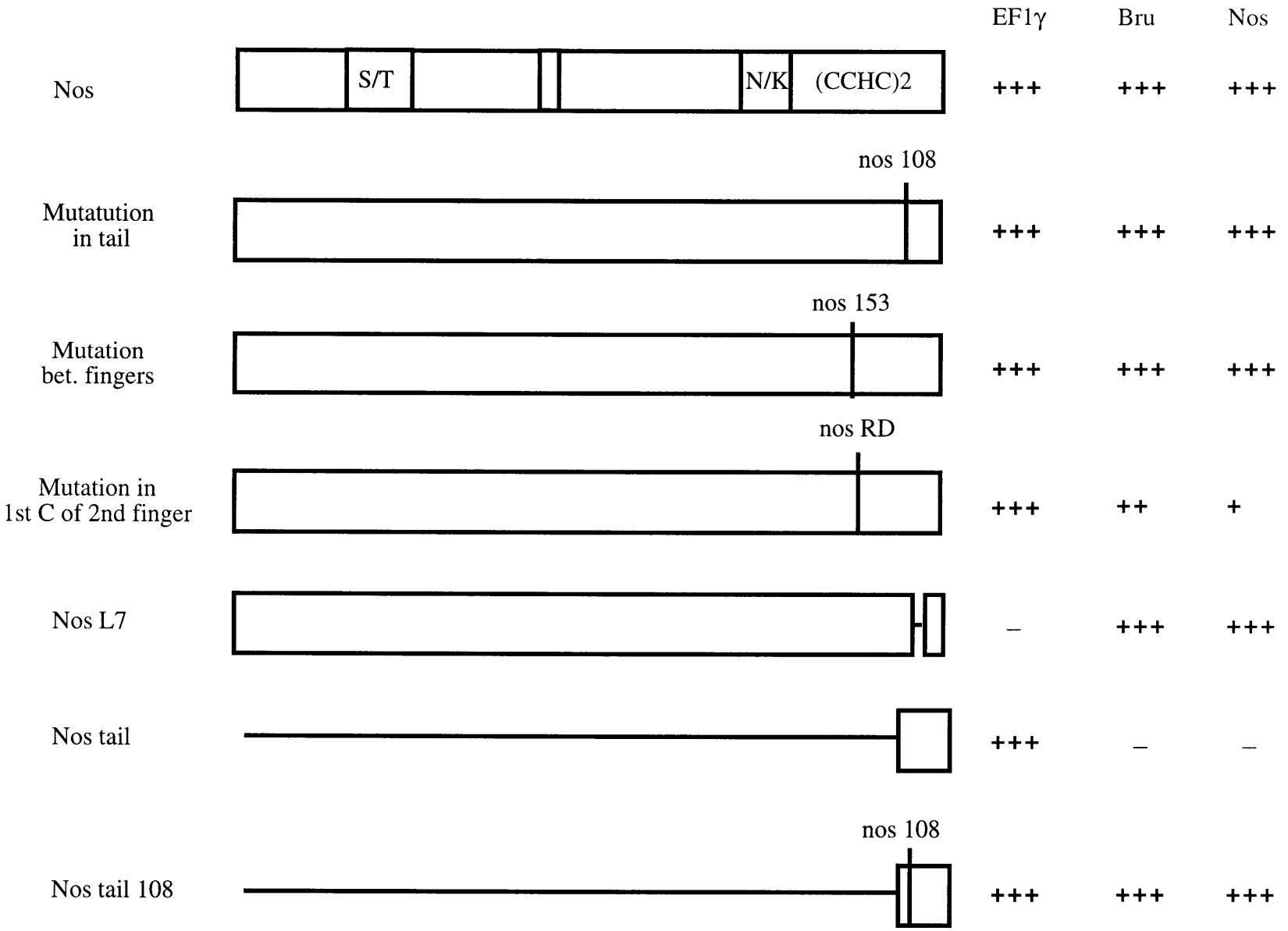

EF1γ
Orb

Figure 3.7. Effect of NOS mutations on protein-protein interactions.

LexA-NOS fusion with different mutations were used in these mating assays. These fusion proteins were expressed in diploid strains along with the three interactors identified in the screen. Full length EF-1γ, BRUNO and NOS fused to the AD were used. +++ indicates high expression of LacZ, while – indicates no detectable LacZ expression. The NOS mutations used have been shown to affect NOS function in the embryo (Chapter 2 , (Curtis, et al., 1997)).


EF1γ
Bru
Nos
Nos
S/T
N/K
(CCHC)2
+++
+++
+++
nos 108
Mutatution
in tail
+++
+++
+++
nos 153
Mutation
bet. fingers
+++
+++
+++
nos RD
Mutation in
1st C of 2nd finger
+++
++
+
Nos L7
–
+++
+++
Nos tail
+++
–
–
nos 108
Nos tail 108
+++
+++
+++

CHAPTER 4

The Drosophila posterior determinant NANOS interacts with the RNA binding protein BRUNO during oogenesis.

SUMMARY

The posterior determinant NANOS and the RNA binding protein PUMILIO act together during embryogenesis to repress the translation of the maternal mRNA *hunchback* at the posterior pole. NANOS is also required during oogenesis for the differentiation of the germ line cystoblast. At this stage, PUMILIO is not likely to be NANOS' functional partner. Here we show through a two-hybrid screen and immunoprecipitation experiments that NANOS directly interacts with the RNA binding protein BRUNO. BRUNO has been identified previously as a translational repressor of *oskar* mRNA. We find that both NANOS and BRUNO affect the differentiation of the cystoblast during early oogenesis and that *osk* is not likely to be the target for this fuction. Therefore we suggest that NANOS and BRUNO interact to regulate the translation of an unidentified transcript during the development of the germ line cystoblast. Accordingly, we show that NOS and BRU together can regulate the translation of a reporter gene targeted for BRUNO binding.

AUTHOR'S NOTE

The work described in Chapter 4 was performed in collaboration with various people in Dr. Ruth Lehmann's lab. The analysis of *nanos*, *pumilio* and *bruno* expression and phenotype in the ovaries were performed in collaboration with Dr. Alexandria Forbes. Dr. Daniel Chagnovich designed the translation assay. I adapted this injection assay and the necessary vectors for the study of NANOS and BRUNO dependent translational regulation. I also performed the two-hybrid screen and the domain mapping analysis.

This chapter will be submitted for publication as **Arrizabalaga, G., Forbes, A., Chagnovich, D. and Lehmann, R.** "The Drosophila posterior determinant Nanos interacts with the RNA binding protein BRUNO during oogenesis"

INTRODUCTION

The regulation of gene expression is a critical phenomenon necessary for the functioning of all organisms. The control of transcription is a common and well studied mode of achieving gene regulation. Nevertheless, in cells such as the developing oocyte and early embryo, transcription is silent and a large amount of transcripts are stockpiled to be used at different stages and places. Therefore, limiting the time and place of expression of these transcripts is accomplished by translational regulation and mRNA localization. The mechanisms by which the translation of specific messages are silenced are not understood. Two common features among many cases of translational regulation are the roles played by the 3' untranslated region (UTR) and RNA binding proteins (Kelley, et al., 1995; Kim-Ha, et al., 1995; Murata and Wharton, 1995; Rivera-Pomar, et al., 1996; Smibert, et al., 1996; Bashaw and Baker, 1997; Chan and Struhl, 1997; Ostareck, et al., 1997; Zamfir, 1997; Zhang, et al., 1997; Gunkel, et al., 1998; Paillard, et al., 1998; Jan, et al., 1999; Walker, et al., 1999).

A well studied phenomenon where both 3'UTR sequences and RNA binding proteins play an essential role is the masking of mRNAs previous to oocyte maturation seen in mice, frogs and clams (Hake and Richter, 1994; Stebbins-Boaz, et al., 1996; Stutz, et al., 1998; Minshall, et al., 1999; Walker, et al., 1999). Many messages in these organisms remain deadenylated and silenced until oocyte maturation when they become polyadenylated and translated (Dworkin and Dworkin-Rastl, 1985; Dworkin, et al., 1985; McGrew, et al., 1989; McGrew and Richter, 1990; Gebauer, et al., 1994). Both the silencing and the activation depend on sequences in the 3'UTR known as the cytoplasmic polyadenylation element (CPE) (Varnum and Wormington, 1990; Verrotti, et al., 1996; Minshall, et al., 1999). An RNA binding protein known as CPE binding protein (CPEB) binds to the CPE and is necessary for both masking and activation (Hake and Richter, 1994; Stebbins-Boaz, et al., 1996; Minshall, et al., 1999). How the translational repression is achieved is not known. The correlation of silencing with a short poly(A) tail and activation with a long poly(A) tail has led to the hypothesis that regulation is achieved by changes in the poly(A) tail length. The poly(A) tail is required for cap-dependent translation and through an interaction between the poly(A) binding protein and eIF4F, the poly(A) tail physically interacts with the translation initiation apparatus (Munroe and Jacobson, 1990; Gallie, 1991; Tarun and Sachs, 1995; Tarun and Sachs, 1996; Wells, et al., 1998). Consequently, it follows that elimination of the poly(A) tail can affect the rate of translational initiation. Nevertheless, a direct causative relationship between changes in poly(A) tail length and translational regulation has not been

established. Also unknown is how CPEB acts as both a masking protein and an activator. Changes in the phosphorylation state of the protein might explain the switch from repressor to activator (Walker, et al., 1996; Walker, et al., 1999). Alternatively, CPEB might interact with different cofactors to perform the two distinct functions. The study of translational regulation in systems where genetic approaches are well established will aid in the identification of other RNA binding proteins and co-factors involved in these processes.

Specification of the posterior pole of the Drosophila embryo and the subsequent development of the abdomen have become model systems for the study of both specific translational regulation and mRNA localization. Abdomen formation in the embryo depends in the regulation of the *hunchback* (*hb*) mRNA at the posterior pole of the early syncytial embryo (Hülskamp, et al., 1989). Since *hb* mRNA is present in the entire embryo this regulation requires to be spatially controlled. The localized action of *hb* regulation is the product of a complex regulatory cascade that begins during oogenesis with the regulation of the *oskar* mRNA.

OSKAR (OSK) protein in the oocyte is exclusively present at the posterior pole. Its function in this region of the egg is to localize mRNAs to this area and also to specify the cytoplasm that gives rise to the primordial germ cells (Ephrussi, et al., 1991). The maternal *osk* mRNA is transported into the oocyte during early stages of oogenesis, and becomes localized to the posterior pole of the oocyte during midoogenesis through sequences in its 3'UTR. Expression of OSK protein at the posterior pole of the oocyte is coincident with its localization (Kim-Ha, et al., 1995). Mutations that disrupt *osk* localization but not its stability also affect its translation, indicating that expression of unlocalized *osk* is repressed (Rongo, et al., 1997). Translational control depends on a repeated sequence motif in the 3'UTR, known as BRUNO response elements (BRE), and an RRM protein called BRUNO (BRU) which binds to the BREs (Kim-Ha, et al., 1995; Webster, et al., 1997). The regulation of *osk* occurs without changes in its poly(A) tail (Salles, et al., 1994; Rongo, 1996). Therefore, BRU regulates *osk* translation by an unkown mechanism.

Once OSKAR protein is translated at the posterior, one of its functions is to localize the posterior determinant *nanos* (*nos*). Localization of *nos* to the posterior pole of the oocyte occurs during late stages of oogenesis (Wang, et al., 1994). NANOS protein is expressed in the embryo after fertilization only from the localized mRNA forming a posterior to anterior gradient. Translational control of unlocalized *nos* mRNA is independent of changes in the poly(A) tail length and is mediated through a 90

nucleotide region in the 3'UTR known as the translation control element (TCE) (Gavis, et al., 1996b). Smaug, an embryonic protein, binds to sequences within the TCE which are required for regulation of *nos*, indicating that Smaug is a translational repressor of *nos* (Smibert, et al., 1996). Quantitative experiments have revealed that most of *nos* mRNA is not localized to the posterior and that translational repression and localization of *nos* seem to be mutually exclusive (Bergsten and Gavis, 1999). Therefore, the spatial restriction of *nos* mRNA is achieved by translational repression, and the localization of *nos* is only required to activate translation.

NOS function in the embryo is to restrict the expression of the transcription factor *hb* to the anterior of the embryo. HB protein produces an anterior to posterior gradient complementary to that of NOS. HB represses the transcription of genes required for the formation of the abdomen. Thus, lack of NOS protein results in the misexpression of HB at the posterior and the lack of abdominal segments (Wang and Lehmann, 1991). Two bipartite repeats in the 3'UTR of *hb*, termed nanos response elements (NRE), are required for *hb* regulation (Wharton and Struhl, 1991). PUMILIO (PUM) protein binds to these sequences and aids in the repression of *hb* translation (Murata and Wharton, 1995; Zamore, et al., 1997). Unlike NOS, PUM is present throughout the entire embryo, therefore NOS confers the spatial specificity to *hb* regulation. Unlike the regulation of *osk* and *nos*, regulation of *hb* is accompanied by a deadenylation event which requires PUM, NOS and the NREs (Wreden, et al., 1997). The exact relationship between the removal of the poly(A) tail and the repression of translation is not known.

Several of the trans-acting factors involved in this regulatory cascade play roles in other processes during development. Both NOS and PUM are required for the development of the oocyte and for the migration of the germ cells (Wang, et al., 1994; Kobayashi, et al., 1996; Forbes and Lehmann, 1998). Similarly, BRU is thought to affect other transcripts besides *osk*, during early oogenesis (Webster, et al., 1997). In addition, these factors might use different co-factors at different stages. PUM and NOS seem to have distinct and separate functions during oogenesis. APONTIC (APT), a second RNA binding protein that interacts with both the *osk* 3'UTR and BRUNO, is required for the regulation of unlocalized *osk* mRNA but does not seem to share BRUNO's function in early oogenesis (Lie and Macdonald, 1999). Here we describe a direct interaction between NOS and BRU. Both of these genes share an expression partner and phenotype in early oogenesis. In the wild-type ovary, germ line stem cells maintain themselves and produce daughter cells, termed cystoblasts. The cystoblast undergoes four rounds of division to give rise to sixteen interconnected cells, one of which becomes the oocyte. In

both *nos* and *bru* mutant females, the differentiation of the cystoblast is affected. The implications of this interaction in translational regulation and in egg development are discussed.

Yeast two-hybrid screen

All experiments involving the two-hybrid system were performed as previously described (Finley and Brent, 1994b). The interaction trap system used in this report uses the DNA binding domain of the transcription factor LexA (LexABD) fused to the entire NOS open reading frame and cDNA libraries fused to a transcription activation domain (AD). Two reporter constructs, one coding for Leu2 and the other for LacZ, are utilized to detect interactions between the bait and a member of the library. Both of these reporters contain LexA operator sequences. If an interaction occurs between the bait protein and a member of the library, yeast containing both fusion proteins will be able to grow in media lacking leucine and will turn blue in media containing β-galactosidase.

All parental yeast two-hybrid plasmids and yeast strains were a gracious gift from Dr. Russ Finley (Gyuris, et al., 1993). The bait plasmid pEG202Nos was designed by fusing the entire *nos* coding regions in frame at the 3' end of the LexA BD in the 2μ HIS3+ plasmid pEG202. Cloning of pEG202Nos was achieved by inserting a BamHI to Not I fragment from pNB40-N5Bam into pEG202. pNB40-N5Bam has a BamHI site replacing the starting AUG of *nos* and keeps the rest of the *nos* cDNA sequence intact (D. Curtis, Unpublished data). The chimeric LexA-NOS protein can not activate transcription by itself and is expressed well in yeast (Chapter 3, Figure 3.2).

Yeast strain EGY48 (*MATα, his3, trp1, ura3, LexA*$_{op(x6)}$*-LEU2*) was sequentially transformed with the 2μ TRP1+ plasmid pSH18-34, which encodes the reporter gene *lacZ* under the control of LexA operators, and pEG202nos. This strain was then transformed with three different cDNA libraries. The embryonic library RFYL1 and the ovary library RFLY3 were a gift from Dr. Russel Finley. OvoI was kindly given to us by Dr. Jörg Grosshans. All libraries consist of unidirectional cDNA made from poly(A) selected RNA and cloned into the 2μ TRP1+ plasmid pJG4-5. Expression of the library cDNA fusion is under the control of the inducible GAL1 promoter. In the case of RFLY3 and OvoI the RNA was isolated from Drosophila ovaries while the RNA for RFLY1 came from Drosophila embryos ranging from 0 to 12 hours old.

A total of 5.5 x 10^7 yeast transformants containing these three plasmids were plated in media lacking uracil, histidine, tryptophan and leucine and containing 2% galactose. The lack of leu in the media selects for yeast that can activate the expression of the *leu2* reporter. In total, 229 colonies were able to grow on media lacking leucine.

These colonies were tested for expression of the more stringent reporter gene *lacZ* by growing them in the presence of β-galactosidase. A total of 123 blue colonies were selected for further analysis. Library plasmids were rescued in *E. coli* DH5α or KC8 cells and grouped according to insert size and restriction map. Restriction mapping was performed on PCR amplified inserts using restriction enzymes AluI and HaeII to determine groups of clones expressing the same gene. All the clones were distributed in 22 groups.

To verify the specificity of the potential interactors we performed mating assays (Finley and Brent, 1994a). This test takes advantage of the fact that haploid cells of opposite mating type will fuse to form diploids when brought into contact with each other. We introduced the activation-tagged cDNA sequences into yeast strain EGY48 (*MATα, his3, trp1, ura3, LexA*$_{op(x6)}$*-LEU2*) of the mating type α and the pEG202Nos bait or control plasmids into yeast strain YM4271 (*MAT*a *ura3-52, his3-200, lys2-801, ade2-101, ade5, trp1-901, leu2-3, 112, tyr1-501, gal4-Δ512, gal80-Δ538, ade5::hisG*) of the mating type a. When these two strains are mated, the newly formed diploid cells will be able to activate the transcription of the two reporter genes if an interaction occurs. The strain containing the AD fused cDNA sequences was mated to three different versions of the EGY48 strain: one carrying the LexANos fusion, a second one with LexA fused to the coding sequences of the human protein Lamin C, and a third one carrying the parental pEG202 plasmid. Only clones from three of the groups showed a specific interaction with the NOS protein. The BRUNO group contained two clones of similar size (Figure 4.1A). One was isolated from the OvoI library while the other originated from the RLFY3 library.

Binding domain mapping

Yeast mating assays were performed as previously described (Finley and Brent, 1994a). Several constructs carrying sequences for various parts of NOS fused to LexA-BD were introduced individually into yeast strain EGY48 along with the reporter construct pSH18-34. These haploid yeast strains were mated with yeast stain YM4271 carrying either the full length BRUNO protein fused to the activation domain or activator tagged Orb. Orb is an RRM containing Drosophila protein required during oogenesis and is not expected to interact with NOS (Lantz, et al., 1994). Interactions were assayed by Lac Z expression in β-galactosidase containing media.

Fusion constructs carrying fragments of the NOS protein were made by inserting PCR amplified fragments into pEG202 digested with BamHI and NotI. Primers used are 25 bases long on average and contain 21 bases complementary to *nos*. The 5' primer contains a BamHI site, while the 3' primer contains a NotI site. Figure 4.1 shows a depiction of all the constructs used. "NOS N" consists of amino acids 1 to 315 and "NOS C" of the last 87 amino acids. "NOS NI" carries amino acids 50 to 148. The NOS ΔNI and NOS ΔNII deletion constructs were designed by first inserting a PCR fragment spanning the region from the start codon to the 5' of the deleted sequence into BamHI and NotI digested pEG202. The 3' end primer used introduces an ApaI restriction site 5' of the Not I site. The resulting plasmid was then digested with ApaI and Not I and a second PCR fragment covering sequences from the 3' end of the deleted area to the stop codon was introduced. In both these constructs the deleted amino acids are replaced by two amino acids, Gly Thr, encoded by the ApaI restriction site. NOSΔNI deletes amino acids 50 to 148, while NOSΔNII eliminates amino acids 149 to 218. NOS Δ50-218 was cloned by amplifying from a NotI to BamHI fragment from pN5 Δ50-218 (Curtis, et al., 1997). Full length BRUNO was cloned from p4004 (a gift from Paul Macdonald, (Webster, et al., 1997)) into the activation domain plasmid PJG4-5 using PCR amplification.

Fly strains

Nos^{RC} is a splice donor mutation that results in unstable RNA and appears to be a protein null (Curtis 1997). *Df(3R)Dl-FX3* is a deficiency that deletes the *nos* gene. Nos^{RC}/ *Df(3R)Dl-FX3* is the strongest combination of nos mutations and might represent the null phenotype (Forbes and Lehmann, 1998). Nos^{L7} is a seven amino acids deletion in the tail region of the NOS protein (Curtis, et al., 1997). Nos^{BN} results from a P-element insertion into the 5' region of the *nos* gene that eliminates expression of *nos* in the nurse cells, consequently *nos* RNA is lacking in the embryos (Wang, et al., 1994).

The genetic mutation *arrest* (*arrt*) corresponds to mutations in the BRUNO protein (Schupbach and Wieschaus, 1991; Webster, et al., 1997). For analysis of the BRUNO phenotype we used alleles $arrt^{WH53}$, $arrt^{PE27}$ and $arrt^{WQ47}$ (Schupbach and Wieschaus, 1991). *Df(27)Prl* carries a deletion of the *arrest* locus. Transheterozygote combination of the three alleles and each one of the alleles over the deficiency were analyzed. All combinations showed similar phenotypes with $arrt^{WH53}$ over the deficiency

showing the strongest deffect. In this report we describe the phenotype of *arrt*^{WH5}*/Df(27)Prl* in detail.

Ovary staining

Antibody staining of ovaries was done as described before (Lin, et al., 1994). BRUNO antibody α-BruB was a courteous gift from Paul Macdonald (Webster, et al., 1997). This rat anti-BRUNO antibody was used at 1:10,000 for ovary staining. For the detection of PUMILIO we used an antibody raised in rabbit against the internal section of the protein at a dilution of 1:1000 (Forbes and Lehmann, 1998). Anti-Vasa antibody (a gift from A. Williamson) was used at 1:5,000 while anti-NOS polyclonal antibody was used at 1:10,000 (Wang, et al., 1994). For the PUM and Vasa staining, a Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Labratories) was used at 1:400. For BRUNO staining, FITC-conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch Labratories) was used at 1:400. All immunostained ovaries were mounted in 1:1 PBS:Glycerol containing 2.5% DABCO (Sigma).

Immunoprecipitation and western blotting

Ovaries were dissected from Oregon R females in cold 1X PBS. 50 µl of ovaries were homogenized in 180 µl of extract buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1% aprotinin, 10 µg/ml Pefablock, 0.1 mM PMSF, 2 mM EGTA, 2.5 mM EDTA and 1% NP40). Extract was spun at maximum speed in a microcentrifuge for 5 minutes at 4^{O}C. 75 µl of the supernatant were combined with 3 µl of rabbit anti NOS antisera and incubated on ice for 2 1/2 hours. To this extract, 30 µl of a 1:1 suspension of Protein A-sepharose was added followed by 90 minutes incubation on ice with occasional mixing. The beads were rinsed with cold extract buffer and then washed 3 times for 10 minutes each in cold extract buffer. The beads were finally resuspended in 50 µl of 2X SDS buffer (125 mM Tris at pH 6.8, 4% SDS, 20% glycerol, 5 M urea and 0.01% bromophenol blue) with 10 µl of 1M DTT and boiled for 5 minutes.

20 µl of the imunoprecipitated extract was ran in a 10% acrylamide gel. Proteins were transferred semidry onto a PVDF membrane. The blot was blocked for 2 hours in Superblock blocking agent (Pierce) with 0.05% Tween 20. Anti-BRUNO antibody was used at 1:10,000 in Superblock with 0.05% Tween 20. Incubation with the BRUNO antibodies was performed overnight at 4^{O}C. HRP conjugated goat anti-rat secondary

antibody was used to detect the BRUNO protein at 1:500 in Superblock with 0.05% Tween-20. Chemiluminescent detection reagents from Amersham were used as instructed by manufacturer.

Transcription vectors

Constructs coding for the Renilla and firefly luciferase (pA25Rluc and pA25Fluc) were designed by D. Chagnovich. Both these two constructs were made using pA25, a modified version of pSP64polyA (Promega). The modification consisted in exchanging the EcoRI site for an NsiI site. This change was introduced by amplifying a fragment from pSP64polyA by PCR from the SP6 promoter to the EcoRI site, exchanging the sequences encoding for the EcoRI site for those in the NsiI restriction site. This fragment was blunted and then digested with Hind3 and ligated to pSP64polyA that had been digested with Hind3 and EcoRI after making the EcoRI end blunt. The resulting plasmid had 25 adenosine residues followed by the introduced NsiI restriction site. pA25-Rluc was made by inserting a Renilla luciferase (RLuc) fragment (pRL-null, Promega) from a NheI site 10 bases upstream of the AUG to an XbaI site 2 bases after the stop codon into the pA25 vector digested with PstI and XbaI after removing the overhangs of the PstI and NheI ends.

pA25-FLuc contains the coding sequences of firefly luciferase (FLuc) and the UTR regions of *hb*. First, a fragment of the *hb* 5'UTR (Tautz, et al., 1987) spanning from the first base of the 5'UTR to 511 bases before the start codon was PCR-amplified using primers that introduced a XhoI site at the 5' end and a BamHI site at the 3' end. This fragment was digested with XhoI and BamHI and introduced into pA25 digested with PstI and BamHI after the XhoI and PstI sites were blunted. The resulting vector was digested with BamHI and SacI. FLuc coding sequences were PCR-amplified from pGEM-luc (Promega) using primers against the SP6 promoter and the 3' end of the coding sequences including the stop codon and a KpnI site added at the 3' end. This FLuc fragment was digested with BamHI, for which a site is present just before the AUG, and KpnI. The 3'UTR fragment of *hb* was also amplified using PCR and consists of sequences from the stop codon to base 3347 of the *hb* cDNA with KpnI and SacI sites at the 5' and 3' ends respectively. This fragment was digested with KpnI and SacI, combined with the digested vector and the FLuc fragment and ligated to form pA25 FLuc.

For this report the reporter construct pA25Fluc was modified to contain BREs within the *hb* 3'UTR. The XbaI and NsiI site in the 3'UTR of *hb* were changed to SpeI and HindIII sites respectively using the Quickchange site directed mutagenesis system (Stratagene) in order to flank the NREs with unique sites. The NREs were taken out by digesting pA25Fluc with SpeI and HindIII. Three different fragments with BREs were introduced in place of the NREs (Figure 4.6A). OskAB was added as a PCR-amplified fragment spanning from nucleotides 1935 to 2142 of the osk 3'UTR (Ephrussi, et al., 1991) with SpeI and HindIII sites engineered at the ends. In order to make $OskC^2$, complementary oligo-nucleotide stretches coding for a duplication of bases 2748 to 2790 of the *osk* cDNA and including overhangs complementary to SpeI and HindIII sites at the 5' and 3' end respectively were phosphorylated, allowed to anneal, and gel purified by standard methods. $OskC^2$mut fragment was made as $oskC^2$ with oligo-nucleotide stretches that included mutations shown in Figure 4.6A.

pNBT was constructed to contain the coding sequences of BRUNO fused to the *nos* 5'UTR and the *tubulin* 3'UTR. A HindIII to BamHI fragment of the *nos* 5'UTR was digested from a modified pN5 NOS plasmid (Wang and Lehmann, 1991) in which the AUG has been mutated to a BamHI site. The digested *nos* 5'UTR fragment was introduced into pSP64polyA (Promega) digested with HindIII and BamHI. The resulting vector was digested with BamHI and SacI. A PCR fragment of the entire *bru* coding sequences and flanking BamHI and XhoI restriction sites was digested with BamHI and XhoI. A fragment amplified from the Drosophila *tubulin*α1 3'UTR (Theurkauf, et al., 1986) spans from the base after the stop codon to 22 bases after the polyadenylation signal and it is flanked by XhoI and SacI sites. This fragment was digested with XhoI and SacI and ligated simultaneously to the vector containing the *nos* 5'UTR and the *bru* fragment. For transcription reactions all constructs were linearized with NsiI and transcribed with SP6 polymerase using the mMessage mMachine transcription system (Ambion).

Injection assay

RNA injection mixtures included RLuc (100 pg/µl), either FLuc-oskAB, FLuc-$oskC^2$ or FLuc-$oskC^2$mut (100 pg/µl) and the NBT mRNA (250 µg/µl). Embryos from Oregon R females were collected for 45 minutes and dechorionated in 100% bleach for 1 minute. For each experiment, 30 embryos were injected at the anterior pole, 30 embryos at the posterior pole with the RNA mixture and 30 remained uninjected. Embryos were

incubated for 75 minutes at room temperature. Anteriorly and posteriorly injected and noninjected embryos were broken and collected separately with a pipette and placed in 30 µl of passive lysis buffer provided by the dual-reporter luciferase system (Promega). Samples were spun in a microcentrifuge at top speed for 10 minutes at $4^{O}C$ to separate extract from oil used during injection. 25 µl of each sample were collected and assayed for the activity of each one of the two luciferase reporters using the dual reporter assay system from Promega and a Berthold Lumat LB 9501 luminometer. For the injections into *nos* mutants nos^{BN} or nos^{L7} homozygous flies were used.

RESULTS

Interactor trap screen

In order to identify proteins that interact with NOS we performed a yeast two-hybrid screen using the LexA interaction trap (Gyuris, et al., 1993) (See Chapter 3). The full-length NOS protein fused to the Lex A binding domain was used as a "bait". An interaction with a member of a cDNA library fused to transcription activation domain (AD) activates reporter genes under the control of a LexA promoter. Since NOS function is required during embryogenesis and oogenesis we used two ovarian libraries and an embryonic library in separate screening experiments. Three different types of clones were confirmed to have a specific interaction with NOS (See Materials and methods and chapter 3). The factor described in this chapter was isolated once from each of the two independent ovary libraries. These two cDNAs code for the C terminal region of the Drosophila ovarian RNA-binding protein and translational control factor BRUNO. BRU is a 604 amino acids protein with three RNA Recognition Motifs (RRM). Both clones isolated contain the third RRM and most of the region between the second and third RRM (Figure 4.1A).

In order to map the BRU interaction domain within NOS we performed yeast mating assays (Brent and Finley, 1997). This assay allows us to form diploid yeast strains carrying various parts of the NOS protein fused to the LexA BD, and AD fusions of the full length BRU protein or the negative control Orb (Figure 4.1B). Full-length NOS protein is able to interact with full length BRU as well as with the BRU fragments isolated in the screen. We were unable to see an interaction between NOS and fragments smaller than Ovo 17 (Data not shown). The interaction between NOS and BRU occur through a 98 amino acid region within the N terminal section of the NOS protein. Deletion of this domain disrupts the interaction in the yeast assay.

NANOS and BRUNO share expression patterns and oogenesis phenotype

NOS protein is involved in the translational regulation of *hb* during embryogenesis, while BRU is required for the translational regulation of *osk* in the developing oocyte (Wang and Lehmann, 1991; Kim-Ha, et al., 1995). The fact that these two proteins perform similar functions at different stages of development suggests that their interaction might be relevant to their activity in vivo. BRU protein is absent in the

embryo and *osk* is regulated by BRU at stages of oogenesis when NOS is absent (Wang, et al., 1994; Kim-Ha, et al., 1995; Webster, et al., 1997). Therefore, their interaction is not likely to be occurring during either of the two known regulatory events controlled by NOS and BRU, *hb* and *osk* regulation respectively.

We performed expression analysis of both NOS and BRU in order to ask whether the proteins coexpressed at any specific stage during oogenesis. The expression patterns of both NOS and BRU during oocyte development have been previously described (Wang, et al., 1994; Webster, et al., 1997; Forbes and Lehmann, 1998). In the female germline NOS protein can be detected at low levels in the stem cells. High levels of NOS protein expression are visible in cysts in region 2 of the germarium. As oogenesis progresses, NOS protein is not detected in the growing oocyte but it is seen in the nurse cells at stage 10 of oogenesis. BRU protein can be detected in the germ cell in region 2A of the germarium. It quickly accumulates in the presumptive oocyte where at stage 6 it localizes to the posterior pole. The localization pattern of the BRU protein within the oocyte coincides with the localization of the *osk* mRNA (Ephrussi, et al., 1991; Webster, et al., 1997). During early oogenesis, NOS and BRU protein are both present at their highest levels in region 2a of the germarium (Figure 4.2). Interestingly, PUM protein is absent or low in this region of the germarium. The coincidence of their expression in early germ line cysts is consistent with an interaction between NOS and BRU.

The area in region 2 of the germarium where high levels of NANOS are present coincides with the oogenesis stage arrested in ovaries lacking NOS (Figure 4.3, (Forbes and Lehmann, 1998). Thus, NOS function seems to be required in the development of the germ line cystoblast at a very specific stage of oogenesis. We studied ovaries lacking the BRUNO protein in order to see whether it was also required at the stage when it co-expresses with NOS. Loss of BRU activity results in an arrest of germ line development in region 2 of the germarium (Figure 4.3). Germ line stem cells are maintained but give rise to a reduced number of cytoblasts, all of which fail to develop into normal cysts. Cystoblast division occurs, but cysts seem to die prior to gaining a follicle cell envelope. This is very similar to the early arrest of germ line cyst development observed in the ovaries of *nos* mutant females (Forbes and Lehmann, 1998). This expression and phenotypic analysis suggests that NANOS and BRUNO are required at the same stages and for similar processes in the development of the germline.

NANOS and BRUNO interact in ovary extracts.

In order to corroborate the idea that NOS can interact with BRU in the ovary, we performed immunoprecipitation experiments. Antisera recognizing the tail region of the NOS protein can immunoprecipitate BRU protein from ovary extract (Figure 4.4). This effect is not seen with the use of pre-immune sera or Protein A beads alone. We were unable to immunoprecipitate NOS protein with anti-BRU antibodies. The BRU antibody was made using the last 188 amino acids (Webster, et al., 1997). This section of the protein is involved in the interaction with NOS (Figure 4.1A). Thus, there might be overlap between the epitope recognized by the BRU antibody and the NOS-interacting domain, which would interfere with coimmunoprecipitation.

Translational regulation by NANOS and BRUNO

Given the fact that NOS and BRU have independent roles in translational regulation, it is possible that their interaction in the germarium is required to silence the translation of a specific mRNA. Targets for NOS or BRU during early oogenesis are not known. It is likely that the mRNA targets regulated by NOS and BRU will carry BREs in its 3'UTR in order to bring BRU to the transcript. Therefore we wanted to test whether NOS and BRU could regulate the expression of a BRE containing reporter message. To test this hypothesis we have used an injection assay developed to study NOS dependent regulation in the Drosophila embryo (Figure 4.5, Chagnovich D. and Lehmann R., in preparation). Since NOS protein is only present at the posterior pole, a message regulated by NOS would be expressed better when injected at the anterior than at the posterior. Therefore we have injected firefly luciferase (FLuc) reporter RNA with BREs in its 3'UTR at the anterior and the posterior of the embryo. This RNA was coinjected with a second reporter gene encoding the *Renilla reniformis* (sea pansy) Luciferase (RLuc) without any regulatory sequences. RLuc has unique substrate requirements and can be use to normalize the values of FLuc expression obtained in different injection experiments (Figure 4.5B). Three different versions of the BREs were used in these assays (Figure 4.6). OskAB contain the four BREs encompassed in regions A and B of the *osk* 3'UTR along with flanking sequences (Kim-Ha, et al., 1995). $OskC^2$ is a duplication of region C of the *osk* 3'UTR that harbors two BREs. A similar duplication was shown to confer translational regulation on a heterologous message (Kim-Ha, et al., 1995). The third version of the BREs consists in $oskC^2$ carrying mutations in the BREs

(oskC^{2}mut) known to disrupt BRU binding and translational regulation (Kim-Ha, et al., 1995).

The BRE containing messages are translated equally at the anterior and the posterior of the embryo (Figure 4.6B). This is indicated by an anterior to posterior ratio (A:P) of standardized Fluc expression of 1.2 for FLucoskAB and 1.1 for FLucoskC^2. This result indicates BRE containing messages are not regulated preferentially at either pole of a wild-type embryo. This is consistent with the observation that BRU protein is not present in the embryo. In order to test whether NOS could mediate regulation of BRE containing reporters when BRU is present, we coinjected the reporter RNAs with mRNA encoding for BRU. Even though no endogenous BRU protein is present in the embryo, abundant amounts of *bru* mRNA are present (Webster, et al., 1997). Therefore, some regulation is imparted on the endogenous mRNA. In order to overcome any regulation of the injected *bru* mRNA, this transcript has been modified to carry heterologous sequences in its 5' and 3'UTR not known to impart regulation (see Materials and methods). When *bru* RNA is included in the injection mixture an A:P ratio of 1.9 with FLucoskAB and 2.1 with oskC2 are detected. These results indicate that the BRE containing message is translated two-fold better at the anterior pole. This differential expression of the BRE-containing reporters is abolished in the absence of NOS and with mutations in the BREs (Figure 4.6B). In conclusion, the presence of both BRU and NOS can confer regulation to a message targeted for BRU binding.

DISCUSSION

We have identified a direct interaction between the embryonic posterior determinant NOS and the ovarian RNA binding protein BRU. These two proteins have both been implicated in translational regulation. Both NOS and BRU are required at the same stage of oogenesis for the appropriate development of the germ line cyst. We have been able to show that BRU and NOS can regulate the translation of a reporter RNA containing BRU binding sites. These results taken together suggest that NOS and BRU interact to regulate the translation of a yet unknown mRNA target in the germarium.

BRUNO, NANOS and PUMILIO may have different partners during early oogenesis

NOS protein is required at the posterior of the embryo where it regulates the translation of the maternal transcript *hb* (Wang and Lehmann, 1991). This regulation requires the action of the PUM protein which binds to specific sequences in the *hb* 3'UTR (Barker, et al., 1992; Murata and Wharton, 1995; Zamore, et al., 1997). Mutant *pum* and *nos* females also show defects in oogenesis (Wang, et al., 1994; Forbes and Lehmann, 1998). In the germarium the loss of NOS affects the maintenance of the germ line stem cells and the differentiation of the cystoblast. On the other hand, the focus of PUM function seems to be in the establishment of the stem cell lineage and not in cystoblast differentiation (Forbes and Lehmann, 1998). Accordingly, PUM is expressed at high levels in the stem cells, while NOS expression is highest in the region harboring the cystoblasts (Figure 4.2). These differences in expression pattern and phenotype led to the suggestion that NOS and PUM have different partners during their respective roles in oogenesis (Forbes and Lehmann, 1998).

Experiments performed for this report suggest that BRU is likely to be NOS partner in its function in the germarium. BRU is expressed at high levels in region 2A where NOS is expressed and PUM is absent. As in *nos* mutants, females lacking BRU show a strong arrest in stage 2A of the germarium where both are expressed in wild-type ovaries. The coincident expression and function is consistent with the direct interaction seen in yeast and ovary extract. How NOS and BRU affect the development of the germ line cyst is not known. Given that both proteins are involved in translational regulation, it is possible that their interaction is required to silence a specific transcript at region 2A in order to allow cyst development.

BRU has been identified as a translational repressor of the maternal mRNA *osk*. OSK is unlikely to be the target of a NOS-BRU interaction. *OSK* mRNA is present in the germ line all throughout oogenesis, while the protein is visible only after stage 6 of oogenesis when the RNA becomes localized to the posterior of the egg (Ephrussi and Lehmann, 1992; Rongo, et al., 1995). Therefore, *osk* is being regulated by BRU at stages when NOS protein is not present (Wang, et al., 1994; Kim-Ha, et al., 1995). In addition, we were unable to see premature translation of *osk* in *nos* mutant ovaries (Data not shown). Analysis of *bru* mutant flies suggests that BRU is likely to carry out other functions besides regulating *osk*. Loss of BRU results in early defects in oogenesis not seen in flies carrying an *osk* transgene with mutated BRUNO binding sites. Therefore, the early arrest in *bru* mutants is not likely to be the consequence of OSK misexpression. Additionally, *osk* regulation by BRU requires the action of a second RNA binding protein called APONTIC (APT) (Lie and Macdonald, 1999). Like NOS, APT binds to the BRUNO protein. Nevertheless, *bru* and *apt* mutant ovaries do not share phenotypes in the germarium (Lie and Macdonald, 1999). *Apt* mutant ovaries show a later arrest in stage 6 of oogenesis (Lie and Macdonald, 1999). All these observations are consistent with BRU having a different partner and target in early oogenesis. Further genetic studies of the oogenesis process will be helpful in identifying potential targets of a BRU-NOS dependent regulation.

The N terminus of NANOS defines a new functional domain

We have mapped the region of NOS required for the interaction with BRU to the N terminus of the protein. A highly selective screen designed to identify amino acids necessary for *hb* regulation did not reveal any essential amino acids in this area of the protein (Chapter 2). In addition, the deletion Δ50-218 shown here to disrupt the NOS-BRU interaction, was shown to be able to partially rescue the abdominal phenotype of a *nos* null embryo in an RNA injection assay (Curtis, et al., 1997). These results suggest that the N-terminal region is not required for NOS function in the regulation of *hb*. These experiments did not address the requirement of this region in NOS function in oogenesis. It is possible that the domain identified through this work as required for BRUNO binding is solely necessary during NOS' role in oogenesis. Further genetic analysis will allow us to test whether this region of the protein is indeed a functional domain specific for BRU binding.

BRUNO and NANOS affect the translation of a reporter gene

We have been able to show a BRU and NOS dependent regulation of a reporter gene through the use of a novel injection assay. Translation of BRE containing mRNA is repressed two-fold in the presence of both BRU and NOS. While the two-fold regulation is statistically significant, it is considerably less than what would be expected for a regulated mRNA. Nevertheless, several observations may account for the low repression level observed. First, since BRU is not expressed in the embryo, BRU is introduced along with the reporter genes as an mRNA. Thus, BRU needs to be translated before it can target the RNA for regulation. This delay could allow the BRE containing FLuc reporter to translate before regulation is imposed. The resulting accumulation of FLuc previous to regulation would affect the measurements making the A:P ratio closer to 1. A second caveat of this assay is the use of the *osk* BREs. It is possible that the context of the *osk* BREs is not ideal for a NOS-BRU dependent regulation. If NOS is required to contact RNA outside of the BRU binding sites, the mRNA context used will influence the levels of regulation. Regardless of these problems, we see a consistent two-fold regulation, in the presence of both NOS and BRU. The absence of either protein factor, or mutations in the BREs disrupts the translational regulation. We have not been able to assess whether this regulation is PUM dependent. Strong PUM mutants lay almost no eggs and die prematurely, making it difficult to obtain enough embryos for the experiment required. NOS and BRU seem to be acting together at a stage in oogenesis when PUM is not present, thus we would predict the regulation seen in our assay to be PUM independent.

Mechanisms of translational regulation

The mechanism that NOS and BRU might use in regulating their target is not known. Interestingly, NOS and BRU are thought to affect translation of *hb* and *osk* by different mechanisms. The regulation of *hb* coincides with a shortening of its poly(A) tail which is dependent on both PUM and NOS (Wreden, et al., 1997). On the other hand the regulation of *osk* is not accompanied with changes in its poly(A) tail (Salles, et al., 1994; Rongo, 1996; Webster, et al., 1997). Several lines of evidence suggest that these facts are not contradictory. Analysis of *hb* regulation through an injection assay similar to the one described here reveals that *hb* mRNA can be regulated by NOS and PUM independent of poly(A) tail shortening (D. Chagnovich and R. Lehmann, unpublished results). This result suggests that deadenylation of *hb* might be a consequence of the

regulation or a secondary mechanism of regulation. Therefore, NOS like BRU is capable of regulating translation independently of poly(A) tail changes. The specific mechanism of translational regulation might be accomplished through interactions with the translation initiation machinery.

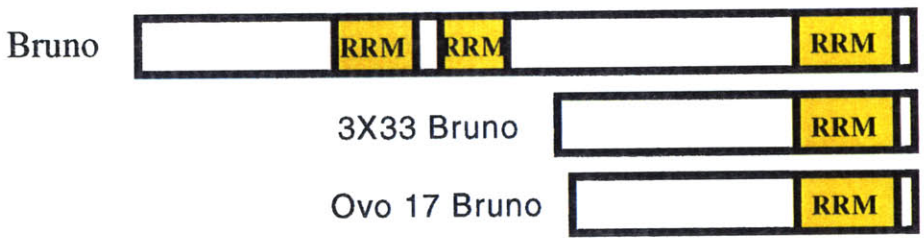
A Xenopus homologue of BRU, EDEN binding protein, is implicated in the deadenylation of specific messages such as *eg2* and *c-mos* after fertilization of the egg (Paillard, et al., 1998). This protein binds to the "embryo deadenylation elements" (EDEN) located in the 3'UTR *eg2* and *c-mos* and is necessary for deadenylation and silencing of the mRNA. It is not known whether the EDEN binding protein directly shortens the poly(A) tail or recruits deadenylation factors to the transcript. Given that BRU, which shares over 50% homology with EDEN binding protein, may not be involved in deadenylation of *osk* it is likely that these RNA binding proteins act as platforms targeting specific mRNAs for regulation by other factors.

Similar to BRU, PUM is an RNA binding protein that requires other factors such as NANOS to impart translational regulation (Murata and Wharton, 1995). Various experiments suggest that PUM is bound to the *hb* 3'UTR throughout the embryo (Murata and Wharton, 1995; Zamore, et al., 1999). HB regulation, however, is restricted to the posterior of the embryo where NOS is present. Thus, PUM targets the mRNA for repression by binding to it and NOS confers the specificity to the translational regulation. The association of NOS with different RNA binding proteins to regulate translation at different stages proposes a modular model for translational regulation (Figure 4.7): In the embryo NOS pairs with PUM to regulate the translation of *hb*. We have been unable to detect a direct interaction between these two proteins in our yeast assay (Chapter 3). During oogenesis NOS seems to interact with a different RNA binding protein, BRU. BRU is known to also associate with other factors such as APONTIC to regulate translation of *osk* at later stages of oogenesis. Hence, RNA binding proteins can exchange "co-factors" at different stages to regulate the translation of specific maternal transcripts.

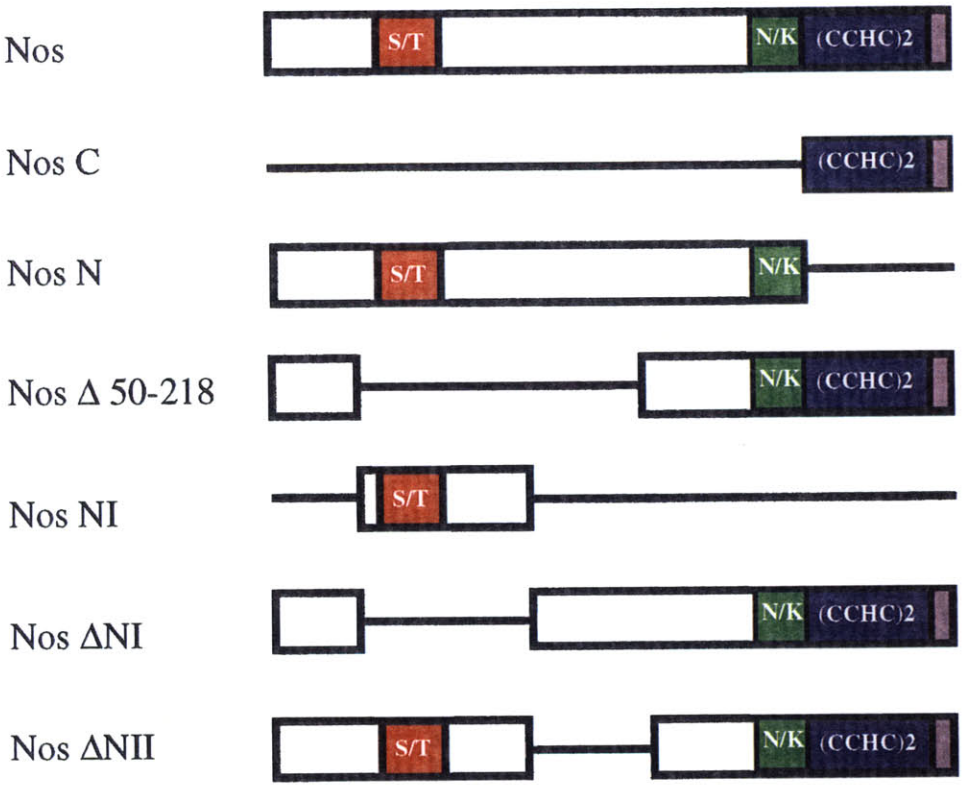
Figure 4.1 BRUNO interacts with the N terminus of NANOS

A. Comparison of full length BRU protein with the clones isolated in the two hybrid screen. Yellow blocks indicate the RRMs. 3X33 was isolated from library RFY 3 and consists of amino acids 327 to 640. Clone Ovo 17 was isolated from library Ovo I and consists of amino acids 344 to 640. B. Yeast mating assays using different parts of the NOS protein and the entire BRU protein. Left column depicts different NOS fragments fused to the LexA binding domain and used in the mating assay. The colored boxes in the full length NOS indicate the domains of homology in the protein (Curtis, 1995b). The second column shows diploid yeast carrying AD-BRU fusion and the NOS fragment shown at the left. The last column shows diploid yeast containing AD-Orb and the NOS construct in the first column. An interaction between the NOS fragment and either BRU or Orb activates the expression of Lac Z and results in blue yeast.

A


Bruno
RRM
RRM
RRM
3X33 Bruno
RRM
Ovo 17 Bruno
RRM

B


Nos
S/T
N/K
(CCHC)2
Nos C
(CCHC)2
Nos N
S/T
N/K
Nos Δ 50-218
N/K
(CCHC)2
Nos NI
S/T
Nos ΔNI
N/K
(CCHC)2
Nos ΔNII
S/T
N/K
(CCHC)2

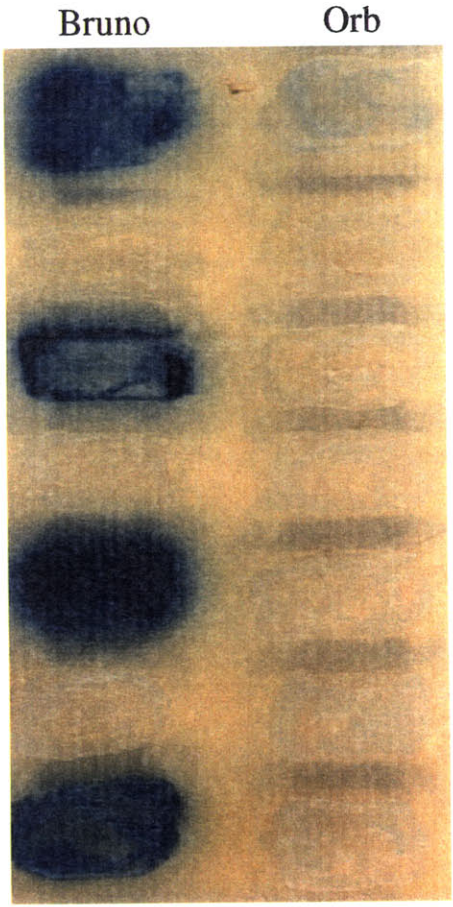
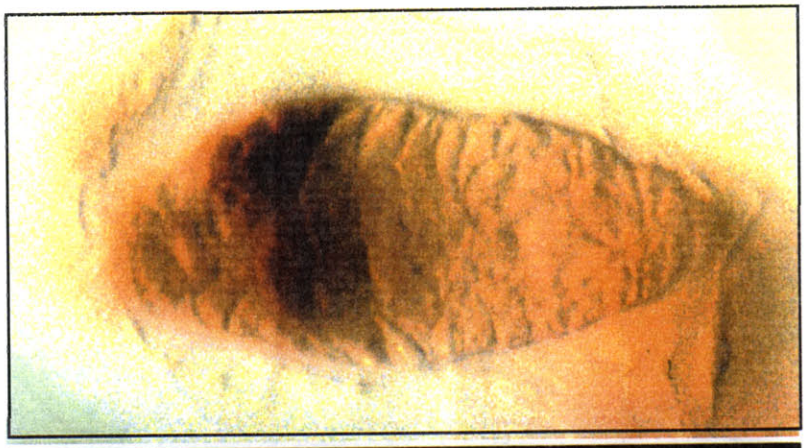

Bruno
Orb

Figure 4.2 NANOS, BRUNO and PUMILIO expression in the germarium.

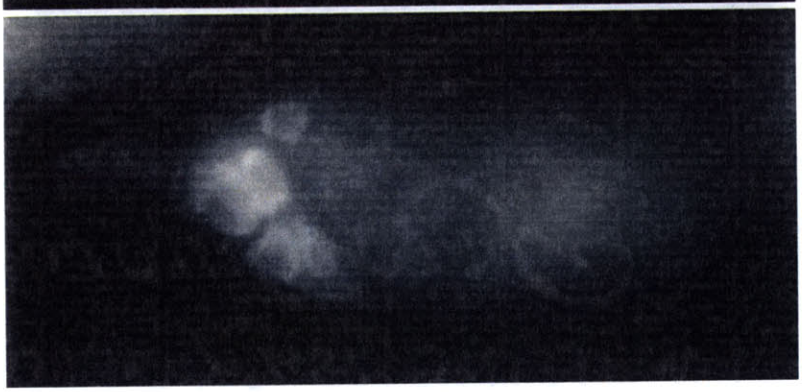
Germarium stained with antibodies raised against NOS, BRU or PUM respectively (Wang, et al., 1994; Forbes and Lehmann, 1998). Last figure is a cartoon of the germarium showing the different domains of expression.

Nanos



Bruno

Pumilio



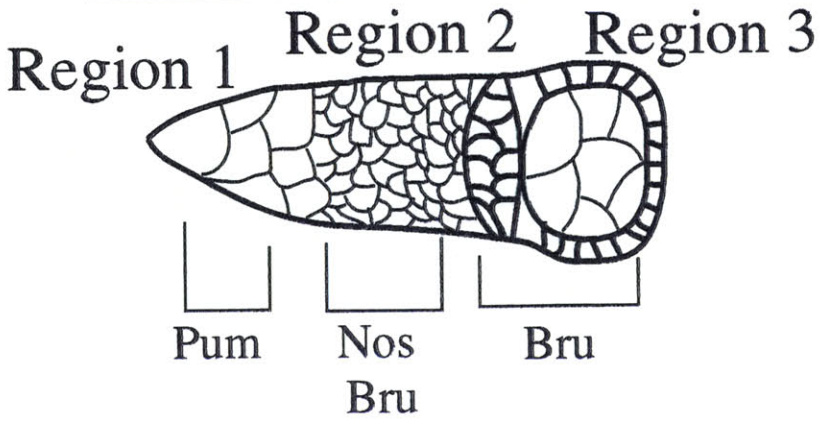


Figure 4.3 *nanos* and *arrest* mutant germarium share a phenotype.

Germarium from wild-type, *nanos* and *arrest* females are shown stained with Vasa antibodies to visualize the germline. Domains of protein expression are shown in the cartoon for reference.

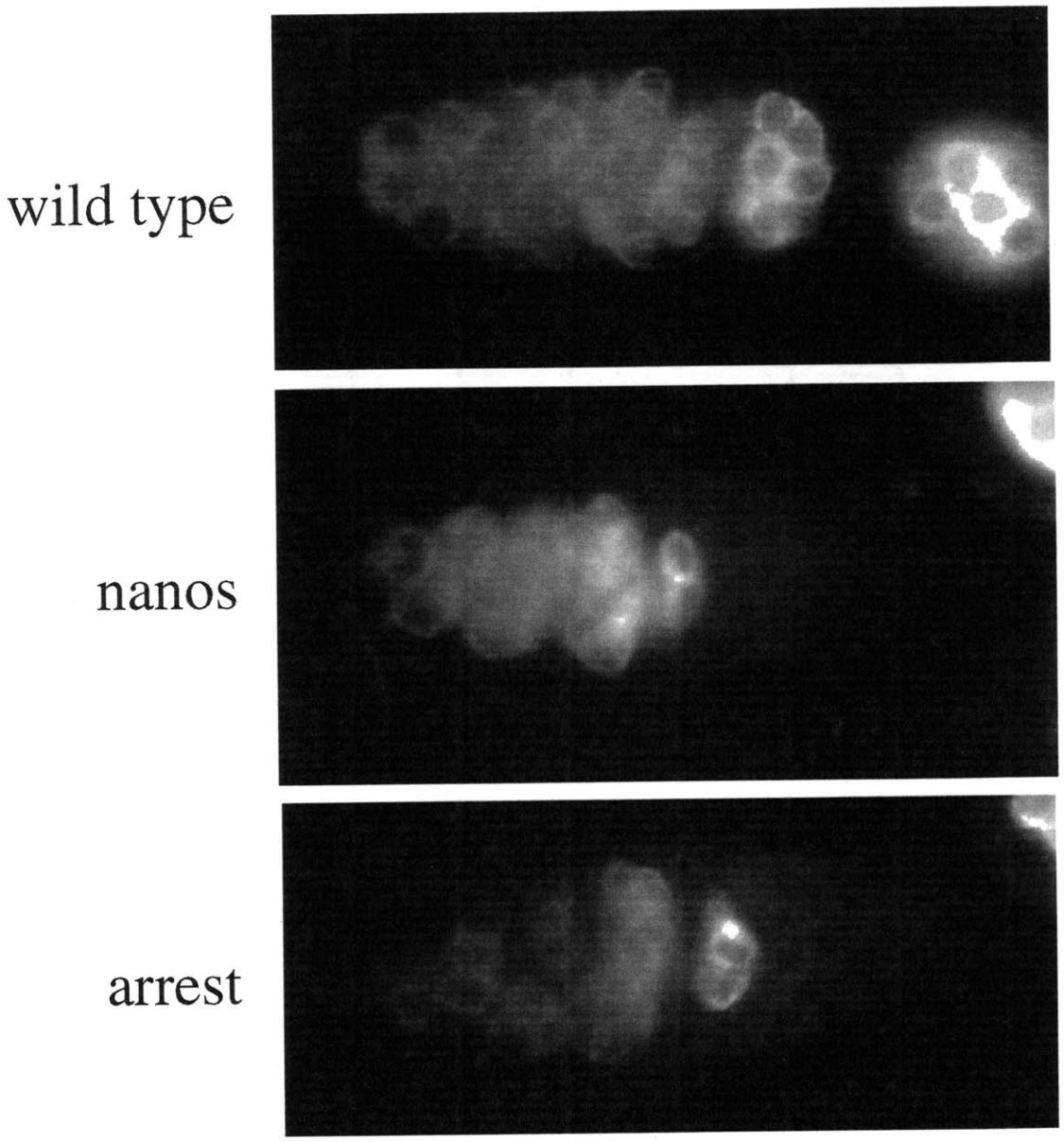

wild type
nanos
arrest
region1
region2
region3
Pum
Nos
Bru
Bru

Figure 4.4 BRUNO and NANOS coimunoprecipitate from ovary extracts.

Western blot probed with BRU antibodies. First lane shows fraction immunoprecipitated with NOS antibodies. Second lane is the sample immunoprecipitated with no antibody. Last lane shows fraction immunoprecipitated with BRU antibodies.

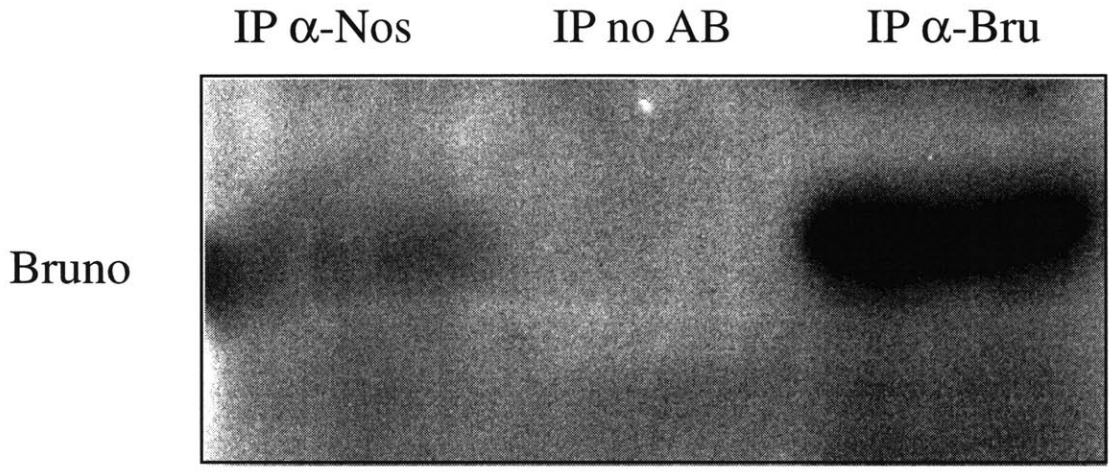
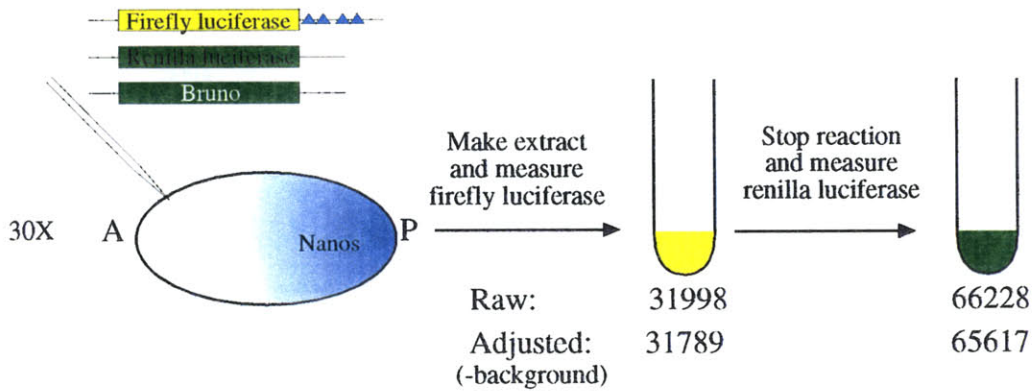

IP α-Nos
IP no AB
IP α-Bru
Bruno

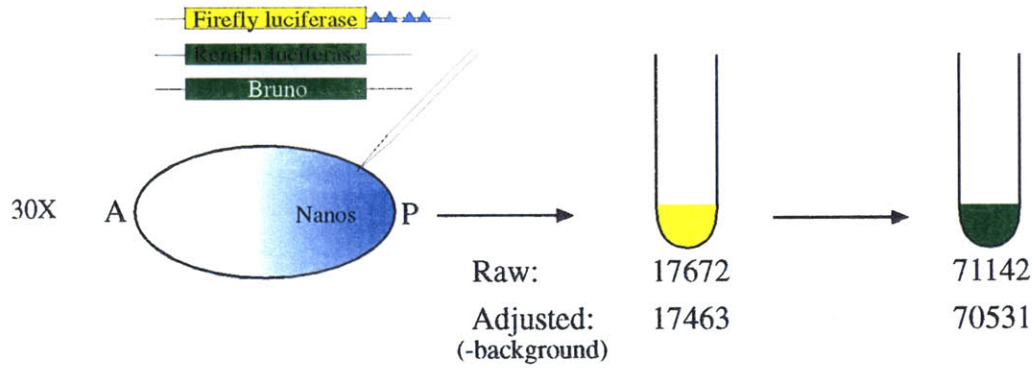
Figure 4.5 Translational activity injection assay

Figure shows a representation of the protocol followed in the injection assay. The three reporter constructs used are shown as boxes in different shades of green. The firefly luciferase reporter contains BRE sequences in its 3'UTR shown as triangles. The *Renilla* reporter is unregulated and helps to normalize the results of different injections. The *bru* construct provides BRUNO protein to the embryo. In each experiment, 30 embryos are injected into the anterior or the posterior with the three constructs. Embryos are incubated for 75 minutes, collected and broken in lysis buffer. The protein extracts are then analyzed for *Renilla* and firefly luciferase activity. Values shown are the actual values obtained for one experiment. The adjusted value is obtained by subtracting the reading of 30 uninjected embryos from the raw value. The firefly/*Renilla* ratio allows us to compare the translation activity of the BRE containing reporter when injected at the two poles. If the test reporter is translated equally at both poles the anterior/posterior ratio will be 1. A ratio of less that 1 indicates that the reporter RNA is regulated at the anterior pole, while an anterior/posterior ratio greater than 1 is indicative of regulation in the posterior pole.



Firefly / Renilla: 0.48

Anterior / Posterior: 1.92



0.25

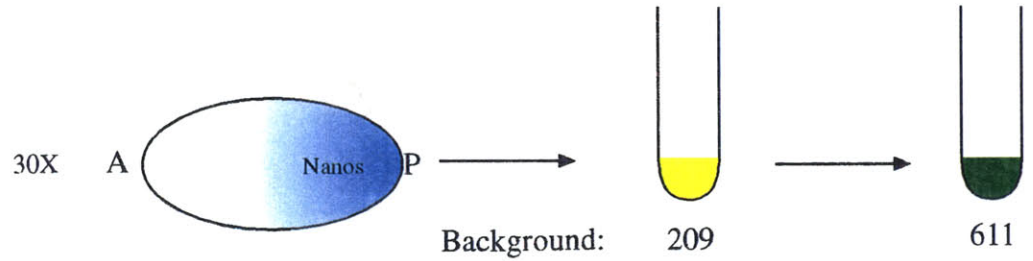
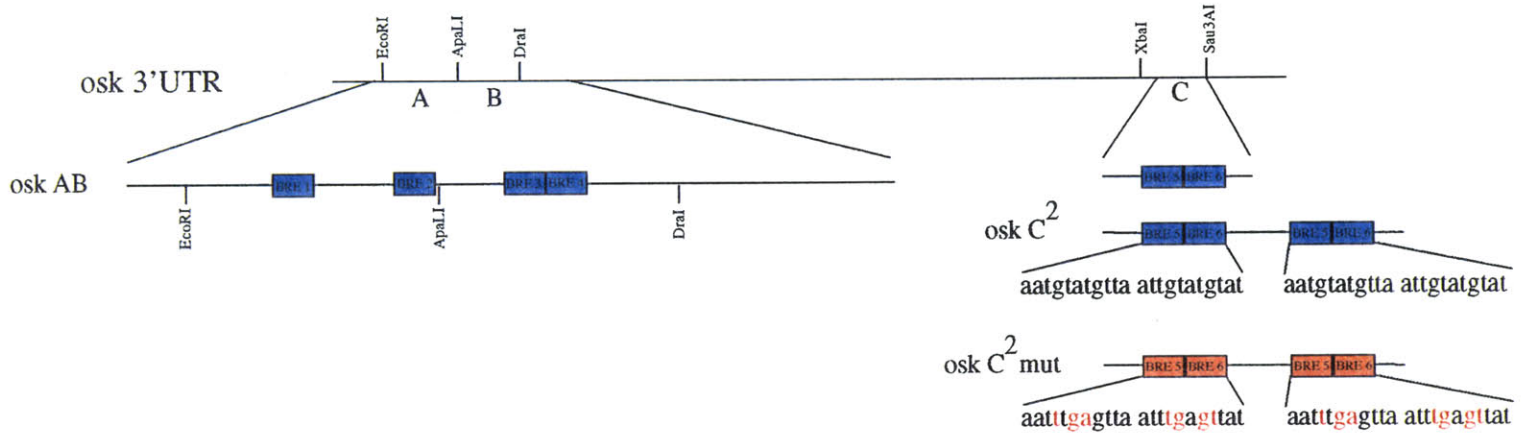


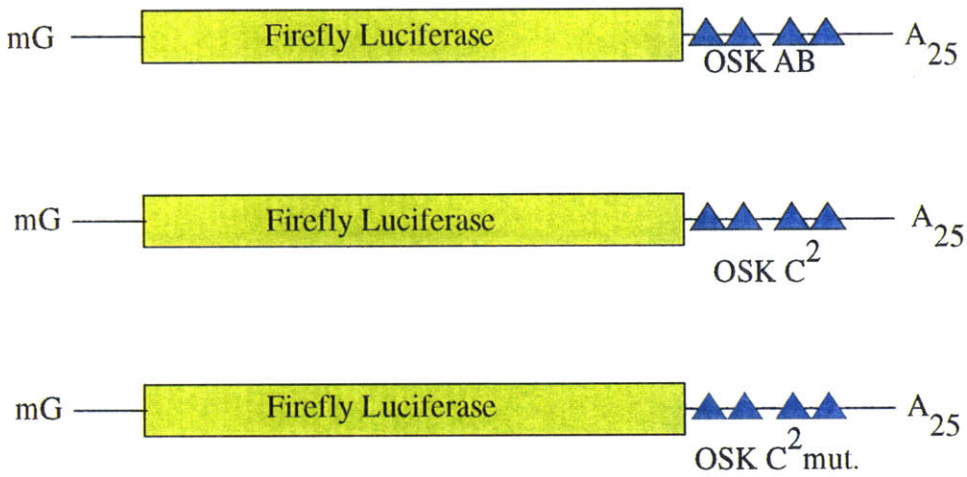
Figure 4.6 NANOS and BRUNO dependent translational regulation

A. Scheme of the *osk* 3'UTR. Regions A, B and C have been described to harbor BRU binding sites known as BREs (Kim-Ha, et al., 1995). Fragments used in the injection assay are shown in more detail. Blue boxes represent the BREs. OSK C^2 is an exact duplication of osk C that contains BRE 5 and 6. Sequences for BRE 5 and 6 are shown under their respective boxes. OSK C^2mut carries mutated BREs shown in red. The mutations in osk C^2mut are shown in red letters. B. Results for injection assays performed in wild-type or *nos* mutant embryos. In wild-type embryos the experiments were performed in the absence or presence of BRU. All A:P ratios are the average of 10 to 20 experiments.

A



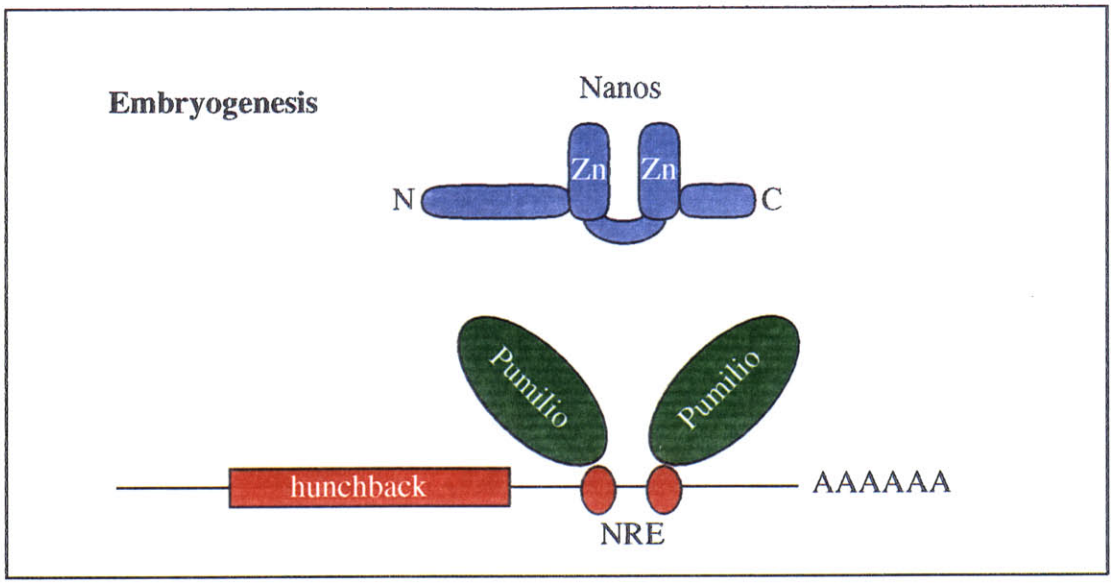
B

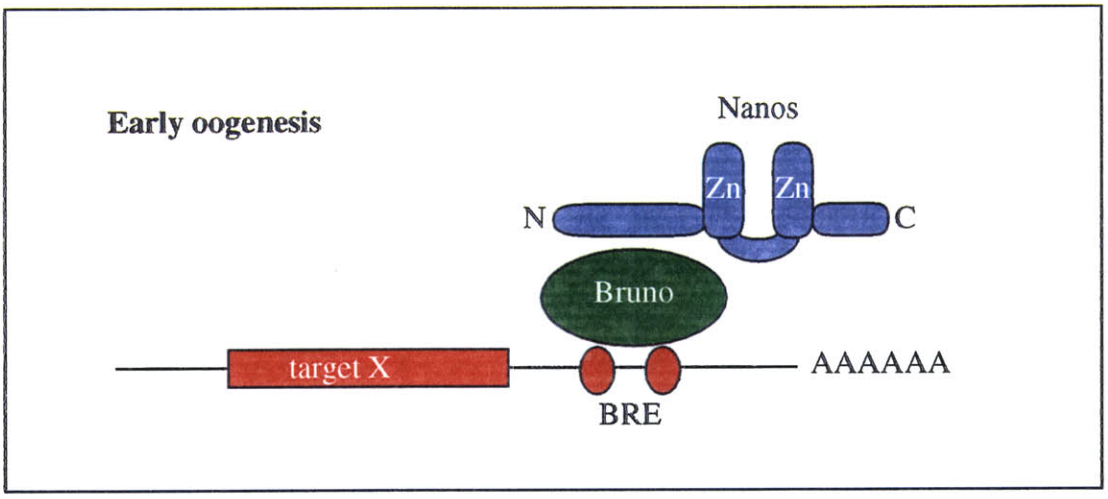


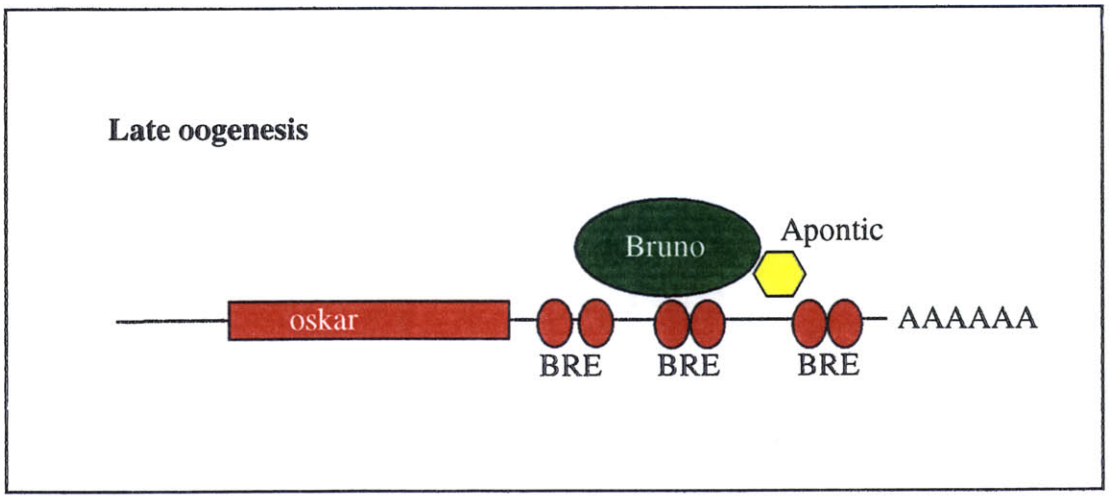
	Into wild type embryos		Into nos- embryos
	A:P -Bruno	A:P +Bruno	A:P +Bruno
OSK AB	1.2 +/- 0.3	1.9 +/- 0.3	
OSK C^2	1.1 +/- 0.2	2.1 +/- 0.5	0.93 +/- 0.15
OSK C^2 mut.		1.25 +/- 0.1	

Figure 4.7 Model for NOS, PUM and BRU roles in translational regulation

NOS role in embryogenesis entails the repression of *hb* translation. This function requires the action of the RNA binding protein PUM. In early oogenesis NANOS interacts with a different RNA binding protein called BRU. BRU is also active in late oogenesis where, along with APONTIC protein, it regulates the translation of *oskar* RNA ((Lie and Macdonald, 1999).


Embryogenesis
Nanos
Zn
Zn
N
C
Pumilio
Pumilio
hunchback
AAAAAA
NRE


Early oogenesis
Nanos
Zn
Zn
N
C
Bruno
target X
AAAAAA
BRE


Late oogenesis
Bruno
Apontic
oskar
AAAAAA
BRE
BRE
BRE

CHAPTER 5

Discussion

During oogenesis and early embryonic development, expression of new proteins is accomplished by translating stored mRNAs. Early development requires the concerted action of proteins at specific times and places in the oocyte and embryo. The timed and spatial regulation of the stored mRNA is achieved through RNA localization and translational regulation. The exact mechanisms involved in these two post-transcriptional regulatory methods are not well understood. Studies of RNA localization and translational regulation in Xenopus, *C. elegans* and Drosophila have revealed an important role for the 3'UTRs of the regulated transcripts and the RNA binding proteins that associate with them (Goodwin, et al., 1993; Hake and Richter, 1994; Bouvet and Wolffe, 1994b; Kim-Ha, et al., 1995; Murata and Wharton, 1995; Dubnau and Struhl, 1996; Rivera-Pomar, et al., 1996; Stebbins-Boaz, et al., 1996; Gavis, et al., 1996b; Bashaw and Baker, 1997; Zhang, et al., 1997; Paillard, et al., 1998; Lie and Macdonald, 1999; Walker, et al., 1999). Regulation of translation directed by sequences in the 5'UTR can be easily explained invoking a hindrance model wherein proteins can bind to the 5'UTR and block the initiation of translation (Goossen, et al., 1990; Oliveira, et al., 1993; Gray and Hentze, 1994; Bashaw and Baker, 1997). On the other hand, regulation via sequences at the 3'UTR is counterintuitive, given the separation between the regulatory sequences and the site of translation initiation.

Many studies have contributed to the idea that the poly(A) tail is important for the efficiency of translation (Munroe and Jacobson, 1990; Gallie, 1991; Gallie and Tanguay, 1994; Jacobson, 1996). Therefore, somehow the ends of the transcripts can influence each other. This hypothesis is backed by the fact that proteins associated with the poly(A) tail interact with proteins bound to the cap structure and that this association is necessary for efficient translation (Gallie and Tanguay, 1994; Le, et al., 1997; Craig, et al., 1998; Imataka, et al., 1998; Kessler and Sachs, 1998). Hence, eliminating the poly(A) tail might eliminate or reduce translation. Accordingly, short poly(A) tails are often seen in mRNAs that are not being translated (Varnum and Wormington, 1990; Huarte, et al., 1992; Bouvet, et al., 1994a; Wreden, et al., 1997; Audic, et al., 1998; Paillard, et al., 1998; Minshall, et al., 1999). It follows that proteins binding to the 3'UTR can influence translation by either deadenylating the mRNA or disrupting the interaction between the tail and the cap. Nevertheless, in cases where binding of proteins to the 3'UTR correlates with deadenylation and translational regulation, it is not known whether deadenylation is a cause or a consequence of the regulation. In addition, there are examples in which regulation through the 3'UTR does not depend on changes in the poly(A) tail (Ostareck-Lederer, et al., 1994; Salles, et al., 1994; Gavis, et al., 1996b).

Therefore, proteins bound to the 3'UTR are capable of affecting translation by mechanisms not involving deadenylation or disruption of the poly(A)-cap interaction. What those mechanisms might be is not known. The study of the proteins that bind to the RNA or proteins that act as co-factors in the regulation can help elucidate the mechanisms of translational regulation.

The Drosophila protein NANOS (NOS) is one of those co-factors involved in translational regulation and the focus of the studies described in this thesis. NOS along with the RNA binding protein PUMILIO (PUM) regulates the translation of the maternal mRNA *hunchback* (*hb*) at the posterior of the embryo (Irish, et al., 1989; Wang and Lehmann, 1991; Wharton and Struhl, 1991; Barker, et al., 1992; Murata and Wharton, 1995; Zamore, et al., 1997). Translational repression of *hb* correlates with deadenylation of the mRNA. Like repression, deadenylation is dependent on NOS, PUM and *hb* 3'UTR sequences known as NANOS Response Elements (NRE) (Wreden, et al., 1997). Nevertheless, the translation of *hb* can be regulated without changes in the poly(A) tail, suggesting that other mechanisms can be utilized to silence *hb* mRNA (D. Chagnovich, unpublished results). The specific role of NOS in either deadenylation or in other regulatory mechanisms is not known. The C-terminal region of NOS harbors two CCHC zinc fingers most similar to the zinc fingers in RNA binding proteins (Curtis, et al., 1997). NOS was shown to bind RNA, however it is unable to differentiate between wild-type NREs and specifically mutated NREs suggesting a lack of specificity (Curtis, et al., 1997). It is possible that the specificity of NOS for RNA is to sequences yet unidentified or may require the action of a second factor. Indeed, certain mutations in the NREs affect *hb* regulation but not PUM binding (Wharton, et al., 1998). We do not know whether these sequences could play a specific role in NOS binding to the RNA. In addition, most mutations in the NOS protein affect the zinc finger region, but no proteins interacting with this region have been found. This observation could suggest that this domain may be involved in RNA binding. The collection of mutations isolated in my studies could prove useful in studying this aspect of the NOS protein.

A second role for NOS could be to help PUM bind to the RNA. Nevertheless, a physical interaction between NOS and PUM has not been detected. In addition, PUM is capable of binding to the RNA in the absence of NOS with high affinity (Zamore, et al., 1997; Zamore et al., 1999). It has also been suggested that NOS could act as a deadenylase. Nevertheless, *hb* can be regulated by NOS without deadenylation (D. Chagnovich, unpublished results). A fourth possible role of NOS could be to disrupt translation by directly interacting with translation factors or with proteins binding to the

poly(A) tail. The interaction with the elongation factor 1γ (EF-1γ) could constitute the link between NOS and the translational machinery. Previous studies suggest that initiation and not elongation is the stage targeted for regulation (Wang, 1995). Nevertheless, deadenylation, which occurs during *hb* regulation, would affect initiation and any additional mechanism of regulation downstream of initiation might not be detected. The relevance of the elongation factor in the regulation of *hb* can be studied by providing the embryo with additional amounts of EF-1γ. If NOS acts by recruiting away the factor from the translation machinery, additional EF-1γ should overcome the translational regulation. This experiment could be performed in embryos expressing NOS at the anterior where it represses both *hb* and the anterior determinant *bicoid.* Due to the lack of *bicoid* at the anterior these embryos carry head defects and do not survive. If additional EF-1γ can overcome translation, injecting it at the anterior of these embryos could result in rescue of the head defects and survival of the embryo. This assay can be used for a number of translation factors that might be affected by NOS.

A similar approach, would be to randomly over-express genes in the embryo and see whether NOS-dependent regulation can be overcome. Drosophila lines have been developed in which each carries a promoter with Gal4 binding sequences randomly introduced in the genome (Rørth, 1996; Rørth, et al., 1998). Expressing the Gal4 activator in specific tissues leads to the over-expression of genes adjacent to the Gal4 promoter insertion. This approach has been successful in isolating genes that affect germ cell migration when over-expressed in those cells (M. Starz-Gaino, N. Cho, A. Forbes and R. Lehmann, personal comunication). In order to find genes that when over-expressed can overcome the regulation of *hb*, the over-expressed product has to be contributed maternally into the embryo. A newly developed Gal4 promoter region that allows UAS-tagged genes to be expressed in the germ line might make this approach feasible (Rørth, et al., 1998). This screen would allow us to identify proteins that are made limiting or recruited from the translation machinery by the action of NOS during *hb* regulation. Furthermore, this approach could help us identify other targets of NOS regulation. In the germ cells, NOS acts along with PUM to regulate the translation of unknown mRNA transcripts. Over-expressing those transcripts might overcome translational repression by having the mRNA in excess of the regulators and would cause a phenotype in the germ cells similar to that seen in the absence of either NOS and PUM. A preliminary screen using the Gal4 promoter lines and a driver expressed in the germ cells was unsuccessful in isolating any candidates for NOS targets. Nevertheless, this

screen could be modified in order to contribute the overexpressed genes maternally (Zamfir, 1997; Rørth, 1998).

During early oogenesis NOS is required for the maintenance of the stem cell progeny, the cystoblast. The results of my work strongly suggest that, during this stage of development, NOS utilizes a different RNA binding protein, BRUNO (BRU), as its partner in the translational regulation of an unknown target. In order to study the mechanisms of regulation used by NOS and BRU and to identify other factors involved in this process it is necessary to determine the target of the regulation. An approach that might answer this question is to isolate RNA sequences that bind to BRU. A yeast three-hybrid screen designed to isolate RNA binding proteins that bind to known RNA sequences has been modified to look for RNAs that can bind to a specific RNA binding protein (SenGupta, et al., 1996; SenGupta, et al., 1999). This type of screen could be used to identify RNA targets for BRU and PUM in the presence and absence of NOS.

The work described in this thesis has helped in the understanding of NOS function during different developmental stages. We have learned that the NOS protein contains several functional domains. The N terminus seems to be required for dimerization and for the interaction with BRU during oogenesis. The two zinc fingers in the C terminus are required for all of NOS functions. On the other hand the tail domain is essential for *hb* regulation and may play a role in germ cell migration but it is not required during oogenesis. In addition, this thesis describes how NOS may fulfill its different functions through interactions with different RNA binding proteins. Further studies on the NOS protein, its functional partners and its targets will allow for a clearer understanding of how the translation of specific messages is regulated during oogenesis and early development.

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