Localization of Maternal RNAs in the Early Embryo of *Drosophila*

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Abstract

Maternally synthesized localized determinants play an important role in cell fate specification in early *Drosophila* embryos. Since only a third of the genes in *Drosophila* have been identified genetically, we carried out a systematic screen for polar localized maternal RNAs in the early embryo as a means of identifying novel molecules that might serve important developmental functions. Anterior– and posterior–specific directional cDNA libraries were constructed using RNA purified from anterior– or posterior–poles cut off early embryos. These libraries were used in a differential screen for cDNAs representing polar-localized maternal RNAs. Five such clones were identified, encoding *cyclin B* RNA, *Hsp83* RNA, *28S rRNA*, *mitochondrial CO1* RNA and *mitochondrial 16S lrRNA* (*16S* RNA). Maternal *Hsp83* transcripts are localized to the posterior pole of the early embryo by a novel mechanism involving generalized RNA degradation and local protection at the posterior. This protection is dependent on the integrity of the polar plasm, suggesting that *Hsp83* RNA is a component of the polar plasm. Results from antisense oligodeoxynucleotide injection experiments suggest that *Hsp83* is required for the formation/maintenance of germ cells. The *16S* RNA is highly concentrated at the posterior pole of newly fertilized *Drosophila* eggs, a process dependent on the integrity of the polar plasm. The localization pattern of *16S* RNA does not correlate well with either the distribution or the activity of mitochondria in early embryos. Taken together with previously published data, these results suggest that the*16S* RNA is exported from the mitochondria into the posterior polar plasm and that it functions in pole cell formation. In addition to the localized RNAs identified in the differential screen, a novel anteriorly localized RNA was identified. This mRNA encodes a *Drosophila* homolog of mammalian adducin, a membrane-

cytoskeletal protein that functions in the assembly of the spectrin-actin network. A comparison of the spatial distribution of *bicoid* and *Adducin-like* transcripts in maternal effect RNA-localization mutants indicates that different genetic pathways exist for localization of mRNAs to the anterior pole of the oocyte and early embryo.

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CHAPTER 1

This chapter will be submitted to *Bioessays* as an invited minireview.

Localized RNAs and their Functions

Running title: Localized RNAs

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Introduction

The eukaryotic cell is partitioned by membranes into spatially and functionally discrete subcellular organelles. In addition, the cytoplasm itself is partitioned into discrete subregions that carry out specific functions. Such compartmentation can be achieved by localizing proteins and RNAs to different subcellular regions. This minireview will focus on localized RNAs with a particular emphasis on RNA localization mechanisms and on the possible biological functions of the localization of these RNAs. In recent years, an increasing number of localized RNAs have been identified in a variety of cell types among many animal species. Emphasis here will be on localized RNAs in the most intensively studied systems — the *Drosophila* and *Xenopus* eggs.

Localized RNAs

Known localized RNAs are listed in Table 1. Most of these RNAs fall into one of the three classes. The first class consists of maternal RNAs that are localized in oocytes and/or early embryos. Maternally synthesized cytoplasmic determinants localized to discrete regions of the oocyte are known to play a key role in cell fate specification during embryogenesis in a variety of animal species.[(1)] It is therefore not surprising that many maternal RNAs have been found to be localized in *Drosophila*, *Xenopus* and ascidian eggs, and that a number of these have been shown to play an important role in programing aspects of early embryogeness. Since the emphasis in *Drosophila* has been on genetic identification of products that play important roles in early embryonic cell fate specifiaction, many of the localized maternal RNAs in the *Drosophila* egg and/or early embryo have been identified as the products of a subset of these genes. Recently, however, molecular screens for polar-localized maternal RNAs in *Drosophila* have resulted in the identification of additional anterior- and

posterior-localized transcripts.[2] In addition, a number of genes identified in other ways have been shown to encode localized maternal RNAs in the early *Drosophila* embryo. In *Xenopus*, the Vg1 mRNA, which encodes a TGF-ß homologue, was identified in a molecular screen for localized RNAs and has been implicated in induction of mesodermal cell fates.

A second class of localized RNAs is found in highly polarized somatic cells, such as neuronal, epithelial and muscle cells, suggesting that they might be involved in establishing and/or maintaining the polarity of these cells, or in fulfilling spatially restricted functions carried out by these cells.

The third class includes transcripts of a number of *Drosophila* segmentation genes that are localized to the apical periplasmic region of the early *Drosophila* embryo. The roles of the proteins encoded by these RNAs, in controlling aspects of cell fate in the embryo are known in great detail. However, while hypotheses regarding the biological significance of apical localization of their RNAs have been presented, its functional significance is poorly understood.

The localized RNAs listed in Table 1 encode products with a wide variety of potential biochemical functions. These include transcription factors, growth factors, cytoskeletal components, a cell cycle regulator, a cytoplasmically-active regulatory chaperone, and an RNA binding protein. Although most of the localized RNAs are protein-encoding messenger RNAs, there is now one example in which a mitochondrially-encoded ribosomal RNA appears to be transported out of the mitochondria and localized to the polar granules in the posterior polar plasm of the *Drosophila* egg and early embryo.[3, 4]

Mechanisms of RNA localization

Any RNA localization mechanism is likely to incorporate two aspects. First, there is likely to be a signal intrinsic to the the RNA that determines its

cytoplasmic destination, whether it be the encoded amino acid sequence itself or a non-translated "tag". 2. There must exist a trans-acting cellular machinery that interacts with this signal to direct and maintain the RNA's localization. Our current knowledge of each of these aspects of the RNA localization mechanism is presented below.

Cis-acting localization signals

In all cases where the sequences that are responsible for tagging an RNA for localization have been mapped, the localization signals have been located to 3' untranslated regions (3' UTRs). Best known is a 630 nucleotide (nt) segment of the *bicoid* UTR which was shown to be necessary and sufficient for anterior localization of the *bicoid* RNA in *Drosophila* eggs.[5] This localization signal can be folded into a stereotypical secondary structure that appears to be conserved among several *Drosophila* species that are evolutionarily divergent as far as 60 million years.[6] A 163-nt 3' UTR region has been found to be sufficient for apical localization of *even-skipped* transcripts in the early embryo.[7] Similarly, a 340-nt 3' UTR sequence has been found to be sufficient for vegetal pole localization for the Vg1 RNA.[8]

The signals for posterior localization of the *nanos* RNA, for posterior (as well as perinuclear localization) of the *cyclin B* RNA, for apical periplasmic localization of the *fushi-tarazu* and *hairy* RNAs, and for transient anterior localization of the *K10* RNA have also been mapped to their respective 3' UTRs, although less precisely.[7, 9-11]

Trans-acting localization machinery

The cytoskeletal network is likely to play a role in RNA localization. Association of mRNAs with the cytoskeleton has been documented[12-14] and

both microtubules and microfilaments have been implicated in RNA localization. Transport and subsequent anchoring of actin mRNA to lamellipodia of chicken embryonic fibroblasts is dependent on microfilaments, but not on microtubules or intermediate filaments.[15] Translocation of the initially uniform Vg1 RNA to the vegetal pole of the *Xenopus* oocyte is microtubule-dependent while the subsequent maintenance of its localization is microfilament-dependent.[16] Cytokeratin has also been implicated in anchoring the Vg1 RNA at the vegetal pole, but its role is unclear.[17, 18] Apical localization of transcripts of the pair-rule gene *fushi-tarazu* requires the integrity of microfilaments.[19] Microtubules are involved in localizing the *bicoid* RNA to the anterior pole of the oocyte.[20] The recent finding that an RNA encoding a membrane-cytoskeletal protein is localized to the anterior pole of the *Drosophila* oocyte and early embryo, suggests that this protein (adducin) might be function in establishing an asymmetric cytoskeletal network in the *Drosophila* egg.[21]

Other *trans*-acting factors that are likely to act as intermediates between localized RNAs and cytoskeletal proteins remain quite elusive. *Drosophila* offers an excellent system in which to identify such factors by genetic analysis. Indeed, a number of genes have been identified whose protein products are involved in localizing RNAs in the *Drosophila* oocyte and/or early embryo (Table 2). Three genes — *exuperantia*, *swallow* and *staufen* – have been shown to be required for successive and distinct phases of *bicoid* RNA localization during oogenesis.[22, 23] Because of the limited phenotypic effects of *exuperantia* mutations, it has been suggested that the *exuperantia* protein might interact specifically, and possibly directly, with *bicoid* RNA early in its localization.[24, 25] *swallow* mutant embryos have additional defects to those expected solely from delocalization of the *bicoid* RNA, suggesting that it might encode a component of the localization apparatus with somewhat more general functions.[22, 26] The

third protein, encoded by *staufen*, is required both for anterior *bicoid* RNA and for posterior RNA localization, indicating that it too may function as a more general factor in different RNA localization pathways.[27] Interestingly, of the three genes, only mutations in *swallow* affect localization of another anterior RNA, *Adducin-like*, implying that there are different pathways for both the localization and maintenance phases of anterior mRNA localization.[21] Products of a number of the "posterior" group genes appear to be components of the *Drosophila* posterior polar plasm and/or to be required for proper assembly and localization of the polar plasm. Table1 shows that a subset of these genes encode posterior-localized RNAs; Table 2, that a number of the "posterior" group genes affect localization of posterior RNAs and are, thus, candidates for *trans*-acting factors involved in posterior RNA localization.

Localization dynamics

Several models have been suggested for how the *cis*-acting signals and *trans*-acting factors interact to achieve RNA localization.[28] mRNAs could be localized by targeting of the nascent polypeptide they encode, in which case the cis-acting sequences required for RNA localization would overlap (completely or partially) with the open reading frame, and translation per se would be a prerequisite for RNA localization. Alternatively, non-coding sequences within the RNA (e.g. the 3' UTR) may be recognized by the intracellular localization machinery. In this case, a number of possible methods could be used to localize the RNA. For example, an initially generally-distributed RNA could become localized by a mechanism involving generalized degradation combined with localized protection from degradation in a particular subcellular region. On the other hand, translocation of the RNA from the site of synthesis to the particular subcellular region, could occur by targeted transport or by passive diffusion

followed by anchoring of the RNA at that site.

The fact that many localized mRNAs coincide with the locations of their proteins product made the co-translational model very attractive. However, there have been no demonstrated examples of this mechanism. Indeed, all localization signals identified to date map to 3' UTRs (see above for *bicoid, even-skipped, nanos, fushi tarazu, hairy* and *cyclin B* in *Drosophila* and *Vg1* in *Xenopus*), indicating that protein sequences are not required for localization of these RNAs. Beyond these particualr examples, it has been demonstrated that actin mRNA localization in chicken embryonic fibroblasts is independent of protein synthesis.[29]

It appears that an RNA targeting mechanism is involved in many instances of RNA localization. Since *bicoid* RNA is transcribed in nurse cells and transported into the oocyte through the ring canals at the anterior pole of the oocyte, where it is localized, it was originally suggested that *bicoid* RNA might be trapped at the anterior pole as it enters the oocyte.[30] Later studies suggested a more complicated mechanism since *bicoid* RNA was found to be localized apically in nurse cells before being transported into the oocyte.[23] It is therefore possible that some more active targeting mechanism is involved in *bicoid* RNA localization. Targeted transport is also likely to be involved in posterior localization of RNAs such as *oskar*, *nanos* and *cyclin B* in the *Drosophila* oocyte, but this process is less well understood.[10, 31-33] Actin RNA in chicken embryonic fibroblast cells has also been shown to be transported to its destination by means of a mechanism involving microfilaments.[15]

Localization of Vg1 RNA offers an example of translocation of an initially uniform RNA by targeting/diffusion following by anchoring.[16] In contrast, maternal *Hsp83* RNA in early *Drosophila* embryos is apparently localized by a degradation/protection mechanism. High levels of *Hsp83* RNA are uniformly

distributed in the mature oocyte. Shortly after fertilization, the *Hsp83* RNA is degraded rapidly throughout the embryo except at the posterior pole, where it is protected by components of the posterior polar plasm.[34]

Functions of localized RNAs

The biochemical properties of the proteins encoded by localized RNAs are listed in Table 1. Here we consider the developmental and/or cellular significance of the localization of these RNAs per se.

In *Drosophila*, both genetic and molecular analyses have revealed the biological significance of the localization of a number of RNAs in the oocyte and early embryo.[35] The *bicoid* protein has been shown to be the localized determinant capable of specifying the anterior cell fates in the *Drosophila* embryo.[36] After fertilization, the anteriorly localized *bicoid* RNA is translated and the *bicoid* protein diffuses posteriorly to form a concentration gradient in the anterior half of the embryo.[37, 38] The *bicoid* RNA encodes a homeodomain-containing protein.[30] It has been suggested that, if the *cis*-regulatory regions of downstream genes that are regulated by *bicoid* contain *bicoid*-binding sites with different binding-affinities, the continuous information inherent in the *bicoid* protein gradient could be transduced into discrete regional cell fates.[38-41] Injection of in vitro transcribed *bicoid* RNA posteriorly induces anterior fates in the posterior cells [40], emphasizing the need to restrict high levels of *bicoid* protein to the anterior pole by anterior localization of the bicoid RNA.

The posterior morphogen, *nanos*, controls abdominal pattern by negatively regulating *hunchback* .[42-44] The *nanos* RNA is localized posteriorly and generates a posterior-to-anterior gradient of the *nanos* protein in the *Drosophila* embryo, with a peak at the posterior pole.[33, 45] *Nanos* protein acts through regulatory sequences in the 3' UTR of the maternal *hunchback* transcript to block

hunchback protein synthesis (by degrading *hunchback* RNA and/or preventing its translation) in the posterior half of the embryo.[46] This prevents posterior cells from taking anterior identities in response to *hunchback* and enables abdominal fates to be adopted. Mislocalization of the *nanos* RNA to the anterior pole of the embryo results in development of abdominal patterns in place of anterior structures;[9] again emphasizing the role of posterior localization of the *nanos* RNA.

The posterior polar plasm is a posteriorly-localized, yolk free cytoplasmic cap present in the *Drosophila* embryo, components of which are required for both pole cell determination (the pole cells are the *Drosophila* primordial germ cells) and abdominal patterning.[47-50] Many posteriorly localized RNAs in the *Drosophila* egg appear to be components of the polar plasm that are required for its assembly as well as for maintaining its integrity. For example, the *oskar* gene encodes a posteriorly localized RNA that is involved in organizing the assembly of polar plasm.[31, 32] Ectopic positioning of *oskar* RNA by mislocalization anteriorly under control of the *bicoid* 3' UTR, or by overexpression of the *oskar* RNA that results in its presence at high levels throughout the embryo, causes in ectopic *nanos* localization and pole cell formation.[51, 52] Other posterior-localized RNAs that function in pole cell formation are the *tudor*, *germ cell-less*, *16S* and *Hsp83* RNAs.[3, 34, 53, 54] *tudor* is a "posterior" group gene whose function is required for polar plasm and polar granule integrity; recent experiments indicate that the *tudor* protein is present inside the mitochondria of early cleavage stage embryos.[55] The mitochondrial 16S large rRNA appears to be transported out of mitochondria into the polar plasm where it is concentrated in the polar granules and plays a role in pole cell formation.[3, 4, 56] The posteriorly localized *germ cell-less* mRNA is translated into a protein that is concentrated around the pole cell nuclei. Antisense experiments indicate that it is required

specifically for pole cell determination.[54] The *Hsp83* RNA is localized posteriorly by a degradation/protection mechanism (see above) and antisense experiments suggest that *Hsp83* RNA/protein might be required for pole cell formation and/or maintenance of pole cell identity (D. D., S. Parkhurst and H. D. L., unpublished data).

The functions of localized RNAs in other organisms are less understood. Vg1 RNA encodes a TGF-ß-like glycoprotein and has been suggested to be involved in induction of mesodermal cell fates.[57-59] Localized RNAs in polarized mammalian somatic cells might be involved in establishing and/or maintaining cell polarity, or in fulfilling polarized functions of these cells.

Future Prospects

The next few years will likely see a further rapid growth of the list of localized RNAs. These will by identified through the analysis of the distribution of transcripts encoded by newly-cloned genes, through systematic searches for localized RNAs,[2, 60, 61] or through biochemical characterization of the components of the localization machinery. The increasing number of localized RNAs will be particularly useful as an entre into the elucidation of the molecular details of RNA localization mechanisms. Novel molecules involved in RNA localization will be identified by genetic or biochemical means and molecular interactions between RNAs, *trans*-acting factors and cytoskeletal proteins will begin to be dissected biochemically. An important question will be the extent to which the components of the RNA localization machinery among different organisms is conserved. For example, a conserved motif involved in localization of several RNAs in *Drosophila* and *Xenopus* has recently been postulated, suggesting that similar factors might be involved in localizing RNAs in different animals.[62]

A second important area of study will be the relationship between subcellular localization and the biological functions of the localized RNAs and/or their products. *Drosophila* will likely continue to lead the way in these analyses because of the facility with which genetic, molecular and histological experiments can be conducted. Already demonstrated in *Drosophila*, is the power of experiments in which the RNA in question is mislocalized to produce gain-of-function phenotypes.[9, 33, 40, 51, 52] This approach may prove useful for systems in which genetic approaches are not readily available.

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References

1 Davidson, E. H. (1986). Gene activity in early development. *3rd ed.* Orlando: Academic Press.

2 Ding, D. and Lipshitz, H. D., in prep.

3 Ding, D., Whittaker, K., and Lipshitz, H. D., in prep.

4 Kobayashi, S., Amikura, R., and Okada, M., pers. commun.

5 Macdonald, P. M. and Struhl, G. (1988). *Cis*-acting sequences responsible for anterior localization of bicoid mRNA in *Drosophila* embryos. *Nature* **336**, 595-598.

6 Macdonald, P. M. (1990). *bicoid* mRNA localization signal: phylogenetic conservation of function and RNA secondary structure. *Devel.* , 161-171.

7 Davis, I. and Ish-Horowicz, D. (1991). Apical localization of pair-rule transcripts requires 3' sequences and limits protein diffusion in the *Drosophila* blastoderm embryo. *Cell* **67**, 927-940.

8 Mowry, K. L. and Melton, D. A. (1992). Vegetal messenger RNA localization directed by a 340-nt RNA sequence element in *Xenopus* oocytes. *Science* **255**, 991-994.

9 Gavis, E. R. and Lehmann, R. (1992). Localization of *nanos* RNA controls embryonic polarity. *Cell* **71**, 301-313.

10 Dalby, B. and Glover, D. M. (1992). 3' non-translated sequences in *Drosophila cyclin B* transcripts direct posterior pole accumulation late in oogenesis and peri-nuclear association in syncytial embryos. *Develop.* **115**(989-997), .

11 Cheung, H.-K., Serano, T. L., and Cohen, R. S. (1992). Evidence for a highly selective RNA transport system and its role in establishing the dorsoventral

axis of the *Drosophila* egg. *Devl.* **114**, 653-661.

12 Jeffery, W. R. (1984). Spatial distribution of messenger RNA in the cytoskeletal framework of ascidian eggs. *Dev. Biol.* **103**, 482-292.

13 Ornelles, D. A., Fey, E. G., and Penman, S. (1986). Cytochalasin releases messenger-RNA from the cytoskeletal framework and inhibits protein-synthesis. *Mol. Cell. Biol.* **6**, 1650-1662.

14 Singer, R. H., Langevin, G. L., and Lawrence, J. B. (1989). Ultrastructural visualization of cytoskeletal messenger-RNAs and their associated proteins using double-label *in situ* hybridization. *J. Cell Biol.* **108**, 2343-2353.

15 Sundell, C. L. and Singer, R. H. (1991). Requirement of microfilaments in sorting of actin messenger RNA. *Science* **253**, 1275-1277.

16 Yisraeli, J. K., Sokol, S., and Melton, D. A. (1990). A two-step model for the localization of maternal mRNA in *Xenopus* oocytes: Involvement of microtubules and microfilaments in the translocation and anchoring of Vg1 mRNA. *Devol.* **108**, 289-298.

17 Pondel, M. D. and King, M. L. (1988). Localized maternal messenger-RNA related to transforming growth-factor messenger-RNA is concentrated in a cytokeratin-enriched fraction from *Xenopus* oocyte. *Proc. Natl. Acad. Sci. USA* **85**, 7612-7616.

18 Klymkowsky, M. W., Maynell, L. A., and Nislow, C. (1991). Cytokeratin phosphorylation, cytokeratin filament severing and the solubilization of the maternal messenger-RNA Vg1. *J. Cell Biol.* **114**, 787-797.

19 Edgar, B. A., Odell, G. M., and Schubiger, G. (1987). Cytoarchitecture and the patterning of fushi tarazu expression in the *Drosophila* blastoderm. *Genes & Dev* **1**, 1126-1247.

20 Pokrywka, N. J. and Stephenson, E. C. (1991). Microtubules mediate the localization of *bicoid* RNA during *Drosophila* oogenesis. *Devel.* **113**, 55-66.

21 Ding, D., Parkhurst, S. M., and Lipshitz, H. D. (1993). Different genetic requirements for anterior RNA localization revealed by the distribution of *Adducin-like* transcripts during *Drosophila* oogenesis. *Proc. Natl. Acad. Sci. USA* , in press.

22 Frohnhöfer, H. G. and Nüsslein-Volhard, C. (1987). Maternal genes required for the anterior localization of *bicoid* activity in the embryos of *Drosophila*. *Genes and Dev* **1**, 880-890.

23 St Johnston, D., Driever, W., Berleth T., R., S, and Nüsslein-Volhard, C. (1989). Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. *Develop.* ***107 (Supplement)***, 13-19.

24 Macdonald, P. M., Luk, S. K. S., and Kilpatrick, M. (1991). Protein encoded by the *exuperantia* gene is concentrated at sites of *bicoid* messenger-RNA accumulation in *Drosophila* nurse cells but not in oocytes or embryos. *Genes. Dev.* **5**, 2455-2466.

25 Marcey, D., Watkins, W. S., and Hazelrigg, T. (1991). The temporal and spatial-distribution pattern of maternal *exuperantia* protein — evidence for a role in establishment but not maintenance of *bicoid* messenger-RNA localization. *EMBO J* **10**, 4259-4266.

26 Stephenson, E. C. and Mahowald, A. P. (1987). Isolation of *Drosophila* clones encoding maternally restricted RNAs. *Dev. Biol* **124**, 1-8.

27 St Johnston, D., Beuchle, D., and Nüsslein-Volhard, C. (1991). *staufen*, a gene required to localize maternal RNAs in the *Drosophila* egg. *Cell* ***66***, 51-63.

28 Gottlieb, E. (1990). Messenger RNA transport and localization. *Curr. Op. Cell Biol.* **2**, 1080-1086.

29 Sundell, C. L. and Singer, R. H. (1990). Actin messenger-RNA localizes in the absence of protein-synthesis. *J. Cell Biol.* **111**, 2397-2403.

30 Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G.,

Noll, M., and Nüsslein-Volhard, C. (1988). The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J.* **7**, 1749-1756.

31 Ephrussi, A., Dickinson, L. K., and Lehmann, R. (1991). *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell* ***66***, 37-50.

32 Kim-Ha, J., Smith, J. L., and Macdonald, P. M. (1991). *oskar* mRNA is localized to the posterior pole of the *Drosophila* oocyte. *Cell* ***66***, 23-35.

33 Wang, C. and Lehmann, R. (1991). Nanos is the localized posterior determinant in *Drosophila*. *Cell* ***66***, 637-647.

34 Ding, D., Parkhurst, S. M., and Lipshitz, H. D. , submitted.

35 Lipshitz, H. D. (1991). Axis specification in the *Drosophila* embryo. *Curr. Op. Cell Biol.* ***3***, 966-975.

36 Frohnhöfer, H. G. and Nüsslein-Volhard, C. (1986). Organization of anterior pattern in the *Drosophila* embryo by the maternal gene *bicoid*. *Nature* **324**, 120-125.

37 Driever, W. and Nüsslein-Volhard, C. (1988). A gradient of *bicoid* protein in *Drosophila* embryos. *Cell* **54**, 83-93.

38 Driever, W. and Nüsslein-Volhard, C. (1988). The bicoid protein determines position in the *Drosophila* embryo in a concentration-dependent manner. *Cell* **54**, 95-104.

39 Driever, W., Thoma, G., and Nusslein-Volhard, C. (1989). Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the *bicoid* morphogen. *Nature* **340**, 363-367.

40 Driever, W., Siegel, V., and Nüsslein-Volhard, C. (1990). Autonomous determination of anterior structures in the early *Drosophila* embryo by the *bicoid* morphogen. *Develop.* ***109***, 811-820.

41 Struhl, G., Struhl, K., and Macdonald, P. M. (1989). The gradient morphogen *bicoid* is a concentration-dependent transcriptional activator. *Cell* **57**, 1259-1273.

42 Hülskamp, M., Schröder, C., Pfeifle, C., Jäckle, H., and Tautz, D. (1989). Posterior segmentation of the *Drosophila* embryo in the absence of a maternal posterior organizer gene. *Nature* **338**, 629-632.

43 Irish, V., Lehmann, R., and Akam, M. (1989). The *Drosophila* posterior-group gene *nanos* functions by repressing *hunchback* activity. *Nature* **338**, 646-648.

44 Struhl, G. (1989). Differing strategies for organizing anterior and posterior body pattern in *Drosophila* embryos. *Nature* **338**, 741-744.

45 Barker, D. D., Wang, C., Moore, J., Dickinson, L. K., and Lehmann, R. (1992). Pumilio is essential for function but not for distribution of the *Drosophila* abdominal determinant Nanos. *Genes Dev.* **6**, 2312-2326.

46 Wharton, R. P. and Struhl, G. (1991). RNA regulatory elements mediate control of *Drosophila* body pattern by the posterior morphogen *nanos*. *Cell* **67**, 955-967.

47 Boswell, R. E. and Mahowald, A. P. (1985). *tudor*, a gene required for assembly of the germ plasm in *Drosophila melanogaster*. *Cell* ***43***, 97-104.

48 Schüpbach, T. and Wieschaus, E. (1986). Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. *Roux's Arch. Dev. Biol.* **195**, 302-317.

49 Lehmann, R. and Nüsslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation and embryonic polarity require the localised activity of *oskar*, a maternal gene in *Drosophila*. *Cell* **47**, 141-152.

50 Manseau, L. and Schüpbach, T. (1989). *cappuccino* and *spire*: two unique maternal-effect loci required for both anteroposterior and dorsoventral patterns of

the *Drosophila* embryo. *Genes Dev.* ***3***, 1437-1452.

51 Ephrussi, A. and Lehmann, R. (1992). Induction of germ cell formation by *oskar*. *Nature* **358**, 387-392.

52 Smith, J. L., Wilson, J. E., and Macdonald, P. M. (1992). Overexpression of *oskar* directs ectopic activation of nanos and presumptive pole cell formation in *Drosophila* embryos. *Cell* **70**, 849-859.

53 Golumbeski, G. S., Bardsley, A., Tax, F., and Boswell, R. E. (1991). *tudor*, a posterior-group gene of *Drosophila melanogaster*, encodes a novel protein and an mRNA localized during mid-oogenesis. *Genes Dev.* ***5***, 2060-2070.

54 Jongens, T. A., Hay, B., Jan, L. Y., and Jan, Y. N. (1992). The *germ cell-less* gene product: A posteriorly localized component neccessary for germ cell development in *Drosophila*. *Cell* **70**, 569-584.

55 Bardsley, A., McDonald, K., and Boswell, R., pers. comm.

56 Kobayashi, S. and Okada, M. (1989). Restoration of pole-cell forming ability to u.v.-irradiated *Drosophila* embryos by injection of mitochondrial lrRNA. *Develop.* **107**, 733-742.

57 Weeks, D. L. and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in *Xenopus* Eggs codes for a growth factor related to TGF-β. *Cell* **51**, 861-867.

58 Dale, L., Matthews, G., Tabe, L., and Colman, A. (1989). Developmental expression of the protein product of Vg1, a localized maternal mRNA in the frog *Xenopus laevis*. *EMBO J.* **8**, 1057-1056.

59 Tannahill, D. and Melton, D. A. (1989). localized synthesis of the Vg1 protein during early *Xenopus* development. *Develop.* **106**, 775-785.

60 Rebagliati, M. R., Weeks, D. L., Harvey, R. P., and Melton, D. A. (1985). Identification and cloning of localized maternal RNAs from *Xenopus* eggs. *Cell* **42**, 769-777.

61 King, M. L. and Barklis, E. (1985). Regional distribution of maternal RNA in the amphibian oocyte. *Dev. Biol.* **112**, 203-212.

62 Gottlieb, E. (1992). The 3' untranslated region of localized maternal messages contains a conserved motif involved in messenger-RNA localization. *Proc. Natl. Acad. Sci. USA* **89**, 7164-7168.

63 Suter, B., Romberg, L. M., and Steward, R. (1989). *Bicaudal-D*, a *Drosophila* gene involved in developmental asymmetry: localized transcript accumulation in ovaries and sequence similarity to myosin heavy chain tail domains. *Genes Dev.* **3**, 1957-1968.

64 Thomas-Cavallin, M. and Ait-Ahmed, O. (1988). The random primer labeling technique applied to *in situ* hybridization on tissue sections. *J. Histochem. Cytochem.* **36**, 1335-1340.

65 Ait-Ahmed, O., Bellon, B., Capri, M., and Joblet, C. (1992). The *yemanuclein-alpha* — a new *Drosophila* DNA-binding protein specific for the oocyte nucleus. *Mech. Devel.* **37**, 69-80.

66 Macdonald, P. M. (1992). The *Drosophila pumilio* gene: an unusually long transcription unit and an unusual protein. *Develop.* ***114***,, 221-232.

67 Lantz, V., Ambrosio, L., and Schedl, P. (1992). The *Drosophila orb* gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. *Develop.* **115**, 75-88.

68 Whitfield, W. G. F., Gonzalez, C., Sanchez-Herrero, E., and Glover, D. M. (1989). Transcripts of one of the two *Drosophila cyclin* genes become localized in pole cells during embryogenesis. *Nature* **338**, 337-340.

69 Lehner, C. F. and O'Farrell, P. H. (1990). The roles of *Drosophila cyclins A* and *B* in mitotic control. *Cell* ***61***, 535-547.

70 Raff, J. W., Whitfield, W. G. F., and Glover, D. M. (1990). Two distinct mechanisms localize *cyclin B* transcripts in syncytial *Drosophila* embryos.

Develop. ***110***, 1249-1261.

71 Edgar, B. A., Weir, M. P., Schubiger, G., and Kornberg, T. (1986). Repression and turnover pattern *fushi tarazu* RNA in the early *Drosophila* embryo. *Cell* **47**, 747-754.

72 Ingham, P. W., Howard, K. R., and Ish-Horowicz, D. (1985). Transcription pattern of the *Drosophila* segmentation gene *hairy*. *Nature* **318**, 439-445.

73 Macdonald, P. M., Ingham, P. W., and Struhl, G. (1986). Isolation, structure and expression of *even-skipped*: a second pair-rule gene of *Drosophila* containing a homeobox. *Cell* **47**, 721-734.

74 Gergen, J. P. and Butler, B. A. (1988). Isolation of the *Drosophila* segmentation gene *runt* and analysis of its expression during embryogenesis. *Genes Devl* **2**, 1179-1193.

75 Tepass, U., Speicher, S. A., and Knust, E. (1990). *crumbs* encodes a EGF-like protein expressed on apical membranes of *Drosophila* epithelial-cells and required for organization of epithelia. *Cell* **61**, 787-799.

76 Baker, N. E. (1988). Embryonic and imaginal requirements for *wingless*, a segment-polarity gene in *Drosophila*. *Dev. Biol* **125**, 96-198.

77 Banerjee, U., Renfranz, P. J., Pollock, J. A., and Benzer, S. (1987). Molecular characterization and expression of *sevenless*, a gene involved in neuronal pattern formation in the *Drosophila* eye. *Cell* **49**, 281-291.

78 Weeks, D. L. and Melton, D. A. (1987). An mRNA localized to the animal pole of *Xenopus* eggs encodes a subunit of a mitochondrial ATPase. *Proc. Natl. Acad. Sci. USA* **84**, 2798-2802.

79 Jeffery, W. R., Tomlinson, C. R., and Brodeur, R. D. (1983). Localization of actin messenger RNA during early ascidian development. *Dev. Biol.* **99**, 408-420.

80 Lawrence, J. B. and Singer, R. H. (1986). Intracellular localization of

messenger RNAs for cytoskeletal proteins. *Cell* **45**, 407-415.

81 Pomeroy, M. E., Lawerence, J. B., Singer, R. H., and Billings-Gagliardi, S. (1991). Distribution of myosin heavy chain mRNA in embryonic muscle tissue visualized by ultrastructural *in situ* hybridization. *Dev. Biol.* **143**, 58-67.

82 Cheng, H. and Bjerknes, M. (1989). Asymmetric distribution of actin mRNA and cytoskeletal pattern generation in polarized epithelial cells. *J. Mol. Biol.* **210**, 541-549.

83 Trapp, B. D., Moench, T., Pully, M., Barbosa, E., Tennekoon, G., and Griffin, J. (1987). Spatial segregation of mRNA encoding myelin-specific proteins. *Proc. NAtl. Acad. Sci. USA* **84**, 7773-7777.

84 Garner, C. C., Tucker, R. P., and Matus, A. (1988). Selective localization of messenger RNA for cytoskeletal protein MAP 2 in dendrites. *Nature* **336**, 674-676.

85 Tucker, R. P., Garner, C. C., and Matus, A. (1989). *In situ* localization of microtubule-associated protein mRNA in the developing and adult rat brain. *Neuron* **2**, 1245-1256.

86 Kleiman, R., Banker, G., and Steward, O. (1990). Differential subcellular localization of particular mRNAs in hippocampal neurons in culture. *Neuron* **5**, 821-830.

87 Bruckenstein, D. A., Lain, P. J., Higgins, D., and Fremeau, J., R. T. (1990). Distinct spatial localization of specific mRNAs in cultured sympathetic neurons. *Neuron* **5**, 809-819.

88 Hook, T. C., Newcomb, P. M., and Herman, I. M. (1991). β actin and its mRNA are localized at the plasma membrane and the regions of moving cytoplasm during the cellular response to injury. *J. Cell Biol.* **112**, 653-664.

Table 1. Localized RNAs

Species	RNA	Localization Pattern	Product function	Reference
Drosophila	*bicoid*	Anterior pole of oocyte and early embryo	Transcription factor	(30)
	Adducin-like 56D	Anterior pole of oocyte and early embryo	Cytoskeletal component	(21)
	Bicaudal-D	Anterior pole of oocyte (stage 8 to 10)	Cytoskeletal component	(63)
	fs(1)K10	Anterior pole of oocyte (stage 8 to ?)	?	(11)
	yemanuclein-a	Anterior pole of oocyte (stage 8 to 10)	DNA binding protein	(64, 65)
	oskar	Posterior pole of oocyte and early embryo	?	(31, 32)
	nanos	Posterior pole of oocyte and early embryo	?	(33)
	pumilio	Posterior pole of early embryo	?	(45, 66)
	tudor	Posterior pole of oocyte and early embryo	?	(53)
	orb	Posterior pole of oocyte and early embryo	RNA binding protein	(67)
	germ cell-less	Posterior pole of early embryo	?	(54)
	mt 16S rRNA	Posterior pole of early embryo	?	(3)
	Hsp83	Posterior pole of early embryo	Cytoplasmic chaperone	(34)
	cyclin B	Posterior pole of oocyte and early embryo, anterior pole of early embryo and perinuclear regions of early embryo	Cell cycle regulator	(2, 68-70)
	fushi-tarazu	Apical periplasmic layers of syncytial blastoderm	Transcription factor	(71)
	hairy	Apical periplasmic layers of syncytial blastoderm	Transcription factor	(72)
	even-skipped	Apical periplasmic layers of syncytial blastoderm	Transcription factor	(73)
	runt	Apical periplasmic layers of syncytial blastoderm	?	(74)
	crumbs	Apical regions of blastodermal and epithelial cells	EGF-like growth factor	(75)
	wingless	Apical regions of blastoderm and imaginal disc cells	Intercellular signaling molecule	(76)
	sevenless	Apical regions of developing photoreceptor cells	Transmembrane receptor tyrosine kinase	(77)
Xenopus	Vg1	Vegetal pole of oocyte	TGF-b-like growth factor	(60, 61)
	An2	Animal pole of oocyte	Mt ATPase subunit	(78)
Ascidian	actin	Ectoplasm and yellow crescent cytoplasm of oocyte	Cytoskeletal component	(79)
Chicken	actin	Lamellipodia of mobile fibroblasts and myoblasts	Cytoskeletal component	(80)
	myosin heavy chain	Peripheral regions of developing muscle cells	Cytoskeletal component	(81)
Mouse	actin	Apical end of epithelial cells	Cytoskeletal component	(82)
	myelin basic protein	Myelinated fibers in brain	Membrane structural protein	(83)
Rat	MAP-2	Dendrites of neurons	Cytoskeletal component	(84-87)
Bovine	actin	Plasma membrane and moving cytoplasm in injured endothelial cells	Cytoskeletal component	(88)

Table 2. Genes involved in RNA localization in *Drosophila* oocyte and early embryo

Localization	RNA	Genes required for localization									Reference
		exuperantia	*swallow*	*staufen*	*cappuccino*	*spire*	*oskar*	*vasa*	*tudor*	*valois*	
Anterior	*bicoid*	+	+	+							(23)
	Adducin-like	–	+	–	–	–					(21)
	fs(1)K10	?	?	?	+	+					(11)
Posterior	*oskar*			+	+	+	+	–	–	–	(31, 32)
	nanos			+	+	+	+	+	?	?	(31, 33)
	pumilio			+	?	?	+	+	?	?	(45)
	germ cell-less			+	+	+	+	+	+	+	(54)
	mt 16s rRNA			+	+	+	+	+	+	+	(3)
	Hsp83			+	+	+	+	+	+	+	(34)
	cyclin B			+	+	+	+	+	–	–	(70)

CHAPTER 2

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A molecular screen for polar-localized maternal RNAs in the early embryo of *Drosophila*

Running title: *Drosophila* polar-localized RNAs

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Abstract

Localized maternally-synthesized RNAs and proteins play an important role in early animal embryogenesis. In *Drosophila*, genetic screens have recovered a number of maternal effect loci that encode localized products in the early embryo. However, only a third of *Drosophila*'s genes have been genetically mutated. Consequently, we conducted a molecular screen for polar localized RNAs in the early *Drosophila* embryo in order to identify additional maternal molecules that carry out spatially-restricted functions during early embryogenesis. Total RNA was purified from anterior or posterior poles cut off early *Drosophila* embryos. These RNAs were used to construct directionally cloned anterior- and posterior-cDNA libraries which were used in a differential screen for cDNAs representing maternal RNAs localized to one or other pole of the embryo. Five such clones were identified, representing *cyclin B* RNA, *Hsp83* RNA, *28S ribosomal* RNA, *mitochondrial cytochrome c oxidase subunit one* RNA and *mitochondrial 16S large ribosomal* RNA. Mutations in the loci encoding these RNAs have not been recovered in genetic screens, confirming that our molecular approach complements genetic strategies for identifying maternal molecules that carry out spatially-restricted functions in the early embryo. We consider the possible biological significance of localization of each of these species of transcripts as well as the mechanism of their localization, and discuss the potential use of our cDNA libraries in screens for rarer localized RNAs.

Key words: differential cDNA screen, *Drosophila*, RNA localization, maternal RNA, *cyclin B*, *Hsp83*, *28S ribosomal* RNA, *mitochondrial cytochrome c oxidase subunit one*, *mitochondrial 16S large ribosomal* RNA

Introduction

A fundamental question in developmental biology is the extent to which molecules that function in early embryogenesis are asymmetrically distributed in the unfertilized egg and subsequently become partitioned into particular cells (reviewed in Davidson 1986). In *Drosophila*, cytoplasmically-localized determinants, in the form of localized maternal RNAs and proteins, play a key role in embryonic cell fate specification (reviewed in Lipshitz 1991). Most of these genes have been identified in genetic screens for maternal effect female-sterile mutations, followed by their molecular cloning and analysis of the spatial distributions of their encoded products. For example, anterior cell fates are programed by the *bicoid* gene, which is transcribed in the nurse cells of the ovarian follicles and its RNA transported into the developing oocyte where it is localized to the anterior pole (Frigerio et al. 1986; St Johnston et al. 1989). Anterior localization of *bicoid* RNA is crucial to the production in the anterior half of the embryo of a *bicoid* protein gradient which is then transduced into discrete regional cell fates (Driever and Nüsslein-Volhard 1988a; 1988b; Driever and Nüsslein-Volhard 1988b; Driever et al. 1989; Struhl et al. 1989; Driever et al. 1990). Similarly, a number of maternal genes involved in specifying abdominal and/or germ cell fates (so-called "posterior" cell fates) encode posterior-localized products (reviewed in Lipshitz 1991; Lasko 1992). These include *nanos* RNA and protein, *oskar* RNA, *pumilio* RNA, *tudor* RNA and protein, *vasa* protein and *staufen* protein (Wang and Lehmann 1991; Ephrussi et al. 1991; Kim-Ha et al. 1991; Macdonald 1992; Barker et al. 1992; Golumbeski et al. 1991; A. Bardsley, K. MacDonald and R. Boswell, pers. comm; Lasko and Ashburner 1988; 1990; Hay et al. 1988a; 1988b; 1990; St Johnston et al. 1991).

While the genetic approach has been spectacularly successful, it is

becoming clear that in *Drosophila melanogaster*, as well as *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, only one third of the transcription units can be correlated with genetically identified loci (Bossy et al. 1984; Olson 1991; Sulston et al. 1992). These results suggest that genetic screens have failed to define many of the maternal molecules that function in the early embryo, and so a strategy designed to complement the genetic ones seems timely. Specifically of interest here is the possibility that molecular searches for polar-localized maternal RNAs might reveal additional molecules with spatially restricted functions. Particularly encouraging is the fact that maternally synthesized molecules localized to either the anterior or the posterior embryonic pole, but that were not recovered in genetic screens, have recently been identified serendipitously (Ding et al. 1993a; Lehner and O'Farrell 1990; Raff et al. 1990; Lantz et al. 1992; Jongens et al. 1992). To date there have, however, been no systematic molecular screens for polar-localized maternal RNAs; consequently, we decided to begin such a search for cDNAs representing maternal RNAs that are localized to either the anterior or the posterior pole of the *Drosophila* oocyte and/or early embryo.

Here we report the construction of anterior- and posterior-specific cDNA libraries, their characterization and their initial use in a differential screen for cDNAs representing polar-localized maternal RNAs in the early *Drosophila* embryo. Five classes of cDNAs representing localized RNAs were identified, none of which had been identified in genetic screens. Of the genes encoding the five localized RNAs, one - *cyclin B* - had previously been cloned and shown to encode a posterior-localized RNA (Lehner and O'Farrell 1990; Raff et al. 1990). While the genes encoding the remaining four RNAs (*Hsp83* RNA, *28S rRNA* , *mitochondrial 16S large rRNA* and *mitochondrial cytochrome c oxidase subunit one* RNA) had been cloned previously (Whitfield et al. 1989; Lehner and O'Farrell 1990; Garesse 1988; Hackett and Lis 1983; Blackman and Meselson

1986; Tautz et al. 1988; Kobayashi and Okada 1990), none had been known to encode localized RNAs. We discuss the possible biological significance of localization of these RNAs, their mechanism of localization, as well as the potential use of our cDNA libraries in subtractive screens to identify less abundant polar-localized RNAs.

Materials and Methods

Isolation of anterior and posterior RNA from early embryos

Embryos staged between 0 and 1 hour post-fertilization were collected from a population cage containing well-fed wild type (Canton-S) flies. The embryos were washed with water until yeast-free, dechorionated in 50% bleach (2.5% sodium hypochlorite in water) for 45 seconds, followed by another wash with water to remove residual bleach. The embryos were transferred to a drop of inert halocarbon oil (HC-56; Halocarbon Products Corp., Hackensack, N.Y.) on a standard glass microscope slide that could be fitted into a small guillotine designed for transecting embryos (Fig. 1). The guillotine consists of a metal base and sides with a vertical slot the width of a single-edged razor blade. Up to two hundred embryos could be lined up across the slide, all oriented with their antero-posterior axes orthogonal to the blade. The preparation and orientation of the embryos took about 30-45 minutes. Thus, the age of the embryos at freezing ranged from 30 to 105 minutes. The guillotine with slide was then placed on dry ice until the embryos and oil were chilled to -70°C and a prechilled blade was used to cut off the anterior fifths or posterior fifths of the aligned embryos (Fig. 1). The high viscosity of the -70°C oil enabled an entire strip of embryo-poles to be picked up with a precooled dissecting probe and transferred to an Eppendorf tube kept on dry ice. In this way, anterior poles were cut from approximately

the restriction sites at the ends could be cleaved because they were unmethylated.

7,500 embryos and posterior poles were cut from another 7,500 embryos. The anterior and posterior embryo poles were stored at -70°C until RNA was extracted and purified as described by Meyerowitz and Hogness (1982).

Construction of cDNA libraries

A whole embryo cDNA library was constructed from 60 µg of total RNA isolated from 0-1 hour wildtype embryos according to an unpublished prototol provided by K. Zinn (pers. comm.). The cDNA was cloned into the Eco RI site of λgt10. The initial packaging gave approximately $8x10^6$ independent clones. After library amplification, 32 clones were randomly selected and were subjected to enzymatic amplification by means of the polymerase chain reaction (PCR) using primers complementary to sequences flanking the Eco RI site of λgt10 (see below). Agarose gel electrophoresis of these inserts indicated that the library insert frequency was 85% and that the average length of inserts was 1.1 kb.

Anterior (A) and posterior (P) specific cDNA libraries were constructed according to a method developed by M. Strathmann and M. Simon (pers. comm.) with minor modifications (see Palazzolo et al. 1990) (Fig. 2). For the anterior library, 20 µg of total anterior RNA was incubated with 3 units of DNase (RQ1 DNase, Promega) in DNase buffer (40 mM Tris pH7.5, 10 mM NaCl, 6mM $MgCl_2$, 1 mM DTT) at 37°C for one hour. After heat inactivation of DNase, RNA was copied into cDNA with an oligo(dT)-Sac I primer-adapter (5'-CCTTTGAGCTCCTTTTTTTTTT-3'). First strand synthesis was carried out using 5-methyl-dCTP in place of dCTP, while second strand synthesis used unmethylated dCTP. Apa I linkers were ligated to the double-stranded cDNA and the cDNA was double-digested with Apa I and Sac I. Thus, all internal Apa I and Sac I sites were hemimethylated and could not be cleaved by Apa I or Sac I, while the restriction sites at the ends could be cleaved because they were unmethylated.

The restriction-digested cDNA was then unidirectionally cloned into λEXLX+ vector (Palazzolo et al. 1990) that had been digested with Apa I and Sac I. The posterior cDNA library was constructed using the same procedure as for the anterior library except that an oligo(dT)-Apa I primer-adapter (5'-CCTTTGGGCCCCCTTTTTTTTTTT-3') was used to prime synthesis of first-strand cDNA, and a Sac I linker (instead of an Apa I linker) was ligated to the double-stranded cDNA before cloning into the λEXLX+ vector.

Conversion of phage cDNA libraries into plasmid libraries by Cre-loxP *automatic subcloning*

Conversion of anterior and posterior λEXLX+ cDNA libraries was according to Palazzolo et al. (1990). 4×10^6 pfu from the phage libraries were converted in host strain BM25.5 (Palazzolo et al. 1990) to give about 2×10^6 colonies. Since yields of plasmid DNA containing *loxP* sites are poor in BM25.5, the converted libraries were introduced as follows into a second host strain that does not express Cre protein (Palazzolo et al. 1990). After amplification, the plasmid libraries were grown in LB supplemented with ampicillin (60 μg/ml). Plasmid DNA was purified from the bacterial culture using a CsCl banding procedure (Sambrook et al. 1989). The purified plasmid library DNA was then transformed into host strain XL1-Blue (Strategene) by electroporation according to instructions provided by the manufacturer (BioRad). After amplification, XL1-Blue cells containing the cDNA libraries were stored in LB containing 20% glycerol at -70°C.

Probe preparation

For differential screening of the libraries, plasmid DNA purified from either the converted anterior or the converted posterior cDNA library was linearized with Apa I (for the anterior library) or Eco RI (for the posterior library). After

separation from contaminating λ-phage DNA on an agarose gel, the linearized plasmid DNA was purified. ^{32}P-labeled probe was then synthesized by single-sided PCR using primer AG5 (5'-GCTGGTACCGGATCGAATTC-3') for anterior probe and AG3 (5'-TGTTAGCAGCCGGATCAAGC-3') for the posterior probe. The 30 µl PCR reaction contained 200 ng of linearized A- or P-library DNA, 250 µM primer DNA, 200 µM each of dATP, dGTP and dTTP, 6 µM dCTP, 1 µM α-P^{32}-dCTP (3000 Ci/mmol), 10 mM Tris (pH8.5 at 1M, 22°C), 1.5 mM $MgCl_2$, 50 mM KCl, and 2.5 unit of Taq Polymerase (Cetus). The PCR reaction was carried out in a thermal cycler using the following conditions: 94°C (45 seconds), 55°C (45 seconds) and 72°C (2 minutes) for 40 cycles.

bicoid probe was prepared using a 1.7 kb Eco RI-Eco RV fragment from cDNA c53.46.6 (Frigerio et al. 1986). For probe synthesis, gel-purified DNA was labeled with α-^{32}P-dCTP (3000 Ci/mmol) by means of the random hexamer-priming method (Feinberg and Vogelstein 1983).

Primary differential screen for anterior- or posterior-cDNAs

For differential screening of the 0-1 hour whole embryo λgt10 cDNA library described above, 10^5 phage from this cDNA library were plated at a density of 2,500 phage per 150 mm plate. Phage DNA was transferred in duplicate to nylon filters (Colony/Plaque Screen, Du Pont) as instructed by the manufacturer. The filters were washed for one hour in 5x SSC, 0.5% SDS and 1 mM EDTA, followed by prehybridization for one hour at 42°C in 50% formamide, 6x SSPE, 5x Denhardt's solution, 0.5% SDS, 100 µg/ml sonicated salmon sperm DNA. The pre-hybridization solution was discarded and fresh solution of the same composition was used for hybridization. Hybridization was carried out at 42°C for 34 hours using $1x10^6$ cpm/ml of anterior- or posterior-probe. Filters were washed in 1x SSC, 0.5% SDS at 68°C and were exposed to Kodak XAR-5 X-ray

film with intensifying screens for 3 days at -70°C. Plaques that were detected exclusively or primarily by either anterior or posterior probes were picked as putative A- (designated as "LP-") or P- (designated as "SHTZ-") clones.

PCR-amplification of cDNA inserts from primary lambda phage plaques

PCR of individual lambda phage plaques was according to a protocol provided by C. Martin (pers. comm.). Briefly, single plaques were picked from agar plates with a Pasteur pipette and transferred to 200 µl of elution buffer (2.5 mM $MgCl_2$, 2.5 mM Tris pH8.0, 0.01% gelatin). After adding a drop of $CHCl_3$ and eluting the phage for at least 4 hours at room temperature, 25 µl PCR reactions were set up with 2.5 µl of each phage eluate. PCR reactions were carried out in a Cetus thermal cycler at 94°C (1.5 minutes), 55°C (2 minutes), and 72°C (2 minutes), for 35 cycles. For cDNA inserts cloned into λgt10 vectors, primers gt10-1 (5'GCAAGTTCAGCCTGGTTAAG-3') and gt10-2 (5'-GGCTTATGAGTATTTCTTCCAGGG-3') were used. For cDNA inserts cloned into λEXLX+ vectors, primers AG3 and AG5 (see above) were used.

Secondary screen of putative anterior or posterior clones by means of PCR-Southern blot analysis

Insert DNA of putative anterior- or posterior- phage clones was amplified by PCR as described above, then electrophoresed in agarose gels and transferred in duplicate to nylon filters (Nytran, Schleicher & Schuell) according to Sambrook et al. (1989). These duplicate Southern blots were hybridized with the same anterior- and posterior-probes that were used for the primary differential screen (see above) using standard protocols (Sambrook et al. 1989). cDNA clones carrying inserts that were detected exclusively or primarily by anterior- or posterior-probes were scored as potential anterior- or posterior-differential cDNA

clones.

Sorting of potential polar-localized clones by cross-hybridization

Four potential anterior clones and four potential posterior clones were randomly picked and their insert DNA was amplified by PCR (see above) followed by separation on an agarose gel and purification using Geneclean kits (Bio 101). Probes representing each of these clones were then synthesized by hexamer random priming (Feinberg and Vogelstein 1983) and were hybridized to the Southern blots representing all LP- and SHTZ-PCR inserts. Separate blots were used for different probes. Clones that carried inserts which hybridized to a particular probe were grouped into a single class. Four non-cross-hybridizing LP- and four such SHTZ-clones were then picked and the sorting cycle was repeated until all the LP- and SHTZ- clones identified in our rescreen had been classified.

RNA tissue in situ hybridization to whole-mount embryos

The final, tertiary screen, for anterior- or posterior-RNA localization involved RNA tissue *in situ* hybridization to embryos using digoxigenin-labeled probe synthesized off representative clones from each of the sorted classes of cDNAs. Whole mount RNA tissue *in situ* hybridization was based on the method of Tautz and Pfeifle (1989) modified as described in Ding et al. (1993a).

Serendipitous identification of an additional posterior-localized RNA

Four classes of sorted cDNAs (LP-134, LP-169, SHTZ-68 and SHTZ-422) were identified as polar-localized according to the primary, secondary and tertiary screening methods described above (see also Results). During preparation of PCR-probe from the LP-134 class of cDNAs, an additional insert band from contaminating phage was found and was digoxigenin-labeled for *in situ*

hybridization to embryos. It was found that this probe detected a posteriorly-localized RNA with a distribution pattern distinct from that of the LP-134 RNA. cDNA clones were isolated from the 0-2 hour embryonic cDNA library and subsequent partial sequencing of these SHTZ-CON clones was conducted (see Results).

Results

Construction of cDNA libraries specific for anterior- or posterior-poles of the early embryo

The anterior and posterior ends of a *Drosophila* egg and embryo can easily be distinguished — the anterior end possesses the micropyle (the sperm entry channel) and appears more pointed than the posterior end. This asymmetric morphology and the relatively large size of the *Drosophila* egg (approximately 600 μm in the long axis and 200 μm in the short axis) made it possible for us to rapidly orient recently fertilized eggs and to isolate anterior (A) and posterior (P) poles (roughly the anterior or posterior fifth of each embryo, see Materials and Methods). From about 7,500 A- and 7,500 P-poles, we purified roughly 100 μg each of total A- and total P-RNA. To determine whether the P-RNA was contaminated with a known anterior-localized-RNA (*bicoid*), a fraction of A- and P-RNA was used in Northern blot analysis with a probe synthesized from a *bicoid* cDNA (Sambrook et al. 1989). The *bicoid* RNA could only be detected in our A-RNA sample (data not shown). The A-RNA and P-RNA were then copied into double-stranded cDNA and directionally cloned into our λEXLX+ vector (Palazzolo et al. 1990) (Fig. 2). We included a DNase digestion step prior to reverse transcription (see Materials and Methods) in order to destroy any contaminating genomic DNA that might be enriched during the cDNA cloning

process. To characterize the A- and P-cDNA libraries, the inserts of 40 randomly picked clones (20 each from of the A- and P-libraries) were analyzed by PCR using primers flanking the cloning site. The initial library sizes, estimated insert frequencies and average insert lengths are summarized in Table 1. To determine the specificity of our A- and P-cDNA libraries, we screened the libraries with a probe synthesized from *bicoid* cDNA. As shown in Table 1, *bicoid* is at least 20 times more abundant in the A-library than in the P-library.

Differential screens for anterior- and posterior-cDNAs

Differential screens for cDNAs representing RNAs concentrated either anteriorly or posteriorly in the *Drosophila* early embryo were carried out in four steps (diagramed in Fig. 3 and described in Materials and Methods). In the first step, duplicate plaque lifts carrying 10^5 clones from a 0-1 hour whole embryo λgt10 cDNA library were differentially screened (St. John and Davis 1979; see Materials and Methods). The probes were synthesized by single-sided PCR using DNA prepared from our A- and P-cDNA libraries (Materials and Methods). In this primary screen, 176 plaques that hybridized strongly to A-probe but did not hybridize, or hybridized only weakly, to P-probe were picked as putative anterior clones (designated "LP" clones). Similarly, 407 putative posterior clones (designated "SHTZ" clones) were picked. In our secondary screen, the cDNA insert in each of the LP- and SHTZ- plaques was amplified by PCR, electrophoretically separated on agarose and blotted to nylon in duplicate. These duplicate filters were then reprobed with the same A- and P- probes that were used in our primary screen. This secondary screening step eliminated 104 LP clones and 252 SHTZ clones as false positives (Fig. 3). To identify and avoid analysis of redundant clones, we then sorted the remaining 72 LP- clones and 155 SHTZ- clones into cross-hybridizing classes. This step resulted in 19 LP- classes

and 20 SHTZ- classes (Fig. 3). In the final step of the screen, we synthesized digoxigenin-labeled probes from a representative member of each of the 39 LP- and SHTZ- cDNA classes and used these probes in RNA *in situ* hybridizations to whole mount 0-4 hour embryos, to verify localization of the RNAs. In this step, only two LP clones (LP-134 and LP-169) and two SHTZ clones (SHTZ-68 and SHTZ-422) showed polar localization in early *Drosophila* embryos (Figs. 3-7). In addition, we identified as a contaminant of a LP-134 plaque a fifth class of cDNAs (SHTZ-CON) representing a posterior-localized RNA (see Materials and Methods) (Fig. 8).

Molecular characterization of the five identified localized RNAs

We screened a 0-2 hour embryonic cDNA library (D.D. and H.D.L., unpub.) with probes made from LP-169, SHTZ-422, SHTZ-68 and SHTZ-CON to isolate additional cDNA clones. Several cDNA clones were isolated for each of the four genes and partial DNA sequences were obtained. For LP-134, partial sequence was obtained by direct sequencing of the PCR insert . Similarity searches using the FASTA program revealed that LP-134 encodes *cyclin B*, LP-169 encodes *mitochondrial cytochrome c oxidase subunit one* (*CO1*), SHTZ-68 encodes *Heat shock protein 83* (*Hsp83*), SHTZ-422 encodes the *28S large ribosomal* RNA (*28S rRNA*) and SHTZ-CON encodes the *mitochondrial 16S large ribosomal* RNA (Whitfield et al. 1989; Lehner and O'Farrell 1990; Garesse 1988; Hackett and Lis 1983; Blackman and Meselson 1986; Tautz et al. 1988; Kobayashi and Okada 1990). Polytene chromosome *in situ* hybridization using LP-134 and SHTZ-68 probes confirmed hybridization to the same cytological regions as previously reported for *cyclin B* and *Hsp83*, respectively (Whitfield et al. 1989; Lehner and O'Farrell 1990; Holmgren et al. 1979).

Expression patterns of the five identified localized RNAs

The distribution of each of the five localized RNAs in early embryos was examined using RNA tissue *in situ* hybridization to whole mount embryos. The results are shown in Figs. 4 through 8 and are described below.

Cyclin B RNA (LP-134): As expected for an anterior-RNA, *cyclin B* transcripts in early cleavage stage embryos are distributed in an anterior to posterior gradient in the anterior half of the embryo, with the highest concentration at the anterior pole (Fig. 4A). In addition *cyclin B* RNA is concentrated to the posterior polar plasm (Fig. 4A). The amount of anterior RNA decreases progressively during the syncytial blastoderm stage, while the posterior RNA is more stable and is incorporated into the pole cells as they form (Fig. 4B). At the cellular blastoderm stage, the pole cells are stained intensely with *cyclin B* probe, but *cyclin B* RNA is undetectable in somatic cells (Fig. 4C). Similar patterns of *cyclin B* transcripts have been observed in previous studies (Whitfield et al. 1989; Lehner and O'Farrell 1990). It has also been reported that low levels of *cyclin B* RNA can be detected in somatic cells at cellular blastoderm stage after prolonged staining (Lehner and O'Farrell 1990).

Mitochondrial *CO1* RNA (LP-169): In early cleavage embryos, *CO1* RNA is present at a basal level throughout the embryo with higher levels present at the anterior and posterior poles (Fig. 5A). In the early embryo, the total level of *CO1* RNA anteriorly is significantly higher than that posteriorly, consistent with identification of LP-169 as an anterior cDNA. However, it should be noted that a subset of the embryos at this stage do not exhibit anterior levels of RNA detectably higher than the basal level, but still exhibit the posterior RNA cap (Fig. 5B) (see Discussion). By the time that pole cell buds appear, the intensity of *CO1*

RNA at the posterior tip of the embryo has dropped significantly and is only slightly higher than the basal levels found throughout the embryo (Fig. 5C). By this stage the anterior concentration of *CO1* RNA has also disappeared (Fig. 5C). The pole cell buds themselves are not stained at this stage (Fig. 5C), indicating that *CO1* RNA is not taken up into the pole cells. After pole cell formation, the concentration of *CO1* RNA is uniform throughout the somatic part of the embryo. The staining level for *CO1* RNA in the pole cells is no greater than the background, indicating that there is very little, or no, *CO1* RNA in these cells. During the syncytial and cellular blastoderm stages, *CO1* RNA continues to be uniformly distributed throughout the somatic cytoplasm of the embryo but remains undetectable in the pole cells (Fig. 5D). The asymmetric distribution of *CO1* RNA in the early embryo reflects the asymmetric respiratory activity of the mitochondria at these stages (Ding et al. 1993c; Akiyama and Okada 1992); however subsequent high respiratory activity of mitchondria in the pole cells is not reflected in high levels of *CO1* RNA.

Hsp83 RNA (SHTZ-68): A detailed analysis of *Hsp83* RNA localization during oogenesis and embryogenesis in both wildtype and mutants, will be presented elsewhere (Ding et al. 1993b). Here we present a summary of maternal *Hsp83* RNA localization during wildtype embryogenesis. Maternal *Hsp83* RNA is uniformly distributed in the early embryo from nuclear division cycles 1 through 5 (for stages, see Foe and Alberts 1983; Zalokar and Erk 1976) (Fig. 6A). During cleavage cycles 6 to 8, *Hsp83* RNA is more concentrated at the posterior pole, forming a decreasing gradient towards the anterior (Fig. 6B), consistent with identification of SHTZ-68 as a posterior cDNA. It is likely that this transcript gradient is achieved by generalized turnover of the maternal *Hsp83* RNA along with the protection of posteriorly-localized *Hsp83* RNA from degradation by

components of the posterior polar plasm (Ding et al. 1993b). At the early syncytial blastoderm stage the only detectable *Hsp83* RNA is in the pole cells and a small region just beneath the pole cells (Fig. 6C). The RNA in the posterior somatic cells disappears at the time of cellularization, leaving high levels of maternally synthesized RNA only in the pole cells. At the cellular blastoderm stage, zygotic transcription of the *Hsp83* gene commences in a complex spatially regulated fashion (Ding et al. 1993b).

*28S r*RNA (SHTZ-422): The nuclear-encoded *28S r*RNA is very abundant in early embryos. The localization pattern of the *28S r*RNA as detected by whole mount RNA tissue *in situ* hybridization is somewhat variable. This may be a consequence of the fact that the localized distribution of the *28S r*RNA is most evident when the *in situ* staining reaction was limited; longer color reactions produced intense staining throughout the embryo (Fig. 7A). In most cleavage-stage embryos in which we could observe localization, there was a high concentration of *28S r*RNA posteriorly (Fig. 7B-D), consistent with identification of SHTZ-422 as a posterior-cDNA. In some of these embryos, there was also a dorsal-ventral difference in the concentration of the RNA (Fig. 7B-D). Occasionally, other distributions, such as an anterior localization pattern were observed (data not shown).

Mitochondrial *16S large r*RNA (SHTZ-CON): Mitochondrial *16S large r*RNAis a very abundant, polyadenylated RNA species (Benkel et al. 1988; Ding et al. 1993c). Details of its expression and localization during wildtype and mutant oogenesis and embryogenesis will be presented elsewhere (Ding et al. 1993c). A summary is presented here. Immediately after egg deposition, *16S r*RNA is present at high levels at the posterior tip of the embryo and at a much lower,

uniform concentration throughout the rest of the cortical cytoplasm of the embryo (Fig. 8A). During the early cleavage stages, the high posterior concentration of *16S r*RNA decreases rapidly. By the time pole cell buds appear, the intensity of *16S r*RNA at the posterior tip of the embryo has dropped significantly, becoming only slightly higher than elsewhere in the embryo. The pole buds are not labeled at this stage, indicating that the *16S r*RNA is not taken up into the pole cells. After pole cell formation, the concentration of *16S r*RNA in the cortical cytoplasm at the posterior tip is the same as that throughout the cortex of the embryo (Fig. 8B). The labeling of the pole cells for *16S r*RNA is no greater than background, indicating that there is very little *16S r*RNA in these cells (Fig. 8B). During the syncytial and cellular blastoderm stages, this distribution pattern of *16S r*RNA is retained in the somatic cytoplasm and the pole cells (Fig. 8C). The distribution pattern of 16S RNA in early embryos contrasts with that of two other mitochondrially-encoded RNAs: *ND-1* RNA is found at uniform levels (Ding et al. 1993c) and *CO1* RNA is localized both anteriorly and posteriorly in the early embryo (see Fig. 5 above).

Discussion

Technical aspects of the differential cDNA screen

We carried out our screen in four steps (outlined in Fig. 3). The first step was a differential screen of a whole egg library with probes made from A- and P-cDNA libraries. We expected that a large percentage of the primary differential clones identified in this step would be false positives, as is typical in a differential screen (Rebagliati et al. 1985, for an example). In order to facilitate the elimination of the false positives, we rescreened differential clones picked in the first step using PCR Southern blot analysis. We chose this method instead of a standard plaque

filter screen because of the higher sensitivity provided by Southern blot analysis (Palazzolo et al. 1989). This rescreen eliminated 59% of the putative A- and 62% of the putative P-clones as false positives. After our secondary screen, we used a cross-hybridization process to sort the remaining clones into 39 groups in order to avoid analysis of redundant clones. The final step was to confirm RNA localization of the isolated clones by *in situ* hybridization to whole mount embryos. Only 4 of the 39 classes exhibited localization, while the remainder showed a uniform RNA distribution. The most likely explanation of this high frequency of false-positive classes of clones is statistical fluctuations between the libraries, resulting either in the absence, or a reduction in the frequency, of clones in one of the libraries. In order to test this hypothesis we repeated the secondary rescreen of PCR Southern blots carrying the primary A- and P-cDNAs, using A- and P-probes synthesized from alternative A- and P-cDNA libraries that we had constructed in a different cDNA cloning vector. These results indicated that a significant fraction of the false-positive clones no longer appeared differential, supporting the above hypothesis (data not shown).

The results of our screen demonstrate both the limitations and strengths of such a differential screen. A major limiting factor in differential screens is their relatively low sensitivity, resulting in the inability to identify RNAs present at a frequency of less than 0.1% to 0.05% in the population of molecules from which the probe is synthesized (Lasky et al. 1980; Maniatis et al. 1982). Consequently, all five localized RNAs identified in our screen are very abundant (Lehner and O'Farrell 1990; Benkel et al. 1988; data not shown). This sensitivity limitation is strikingly exemplified by our data showing that *bicoid* cDNAs are 20 times more abundant in the A-cDNA library than in our P-library, but that their frequency in the A-library is only 0.1% (Table 1). This placed *bicoid* below the sensitivity limit of a differential screen and, indeed, *bicoid* was not identified as a localized

RNA in our screen. Other known localized RNAs, such as those encoded by the *Adducin-like-56D*, *nanos*, *oskar*, *pumilio*, *tudor, orb* and *germ cell-less* genes (Ding et al. 1993a; Wang and Lehmann 1991; Ephrussi et al. 1991; Kim-Ha et al. 1991; Macdonald 1992; Barker et al. 1992; Golumbeski et al. 1991; Lantz et al. 1992; Jongens et al. 1992), also were not identified in our screen, presumably because of their low abundance. Subtractive screens are expected to partially overcome this sensitivity limitation (see below).

In contrast to this limitation, a strength of the differential screening strategy we used was the ability to identify localized RNAs with dynamic and complex distributions in the early embryo. Indeed, the localized RNAs identified in our screen exhibit distribution patterns that include a high anterior- or posterior-polar concentration, but do not exclusively comprise such a pattern. In principle, cDNAs representing such RNAs would be excluded in a subtractive screen since exhaustive subtractive hybridization should result in sequences present at a frequency as low as 10^{-5} of the poly(A)$^+$ RNA, hybridizing to completion with homologous cDNA sequences (Sargent and Dawid 1983, for an example). Thus, differential screens such as the one reported here, are particularly useful for the identification of relatively, but not exclusively, differential clones.

The differential screen recovered localized RNAs that genetic screens failed to identify

Our differential screen, although it has by no means identified all localized maternal RNAs in the early embryo, has demonstrated the usefulness of such a systematic molecular search. Four of the five localized RNAs identified here (i.e., LP-169, SHTZ-422, SHTZ-68, SHTZ-CON) were not previously known to be concentrated at one or other pole of the early *Drosophila* embryo. In addition, none of the five localized RNAs is encoded by a genetically-identified locus.

Indeed, three of the five could not feasibly be expected to be genetically identified; one (28S rRNA) is encoded by a nuclear multigene family, and the other two (*16S r*RNA and *CO1* RNA) are encoded not by the nuclear genome but rather by the mitochondrial DNA. To date, despite extensive efforts, no mutations have been identified in the fourth gene, *Hsp83* (Wohlwill and Bonner 1991; Ding et al. 1993b). Finally, it has recently been shown that, while deletion of the *cyclin B* gene alone results in no detectable phenotype, deletion of both the *cyclin A* and *cyclin B* genes results in profound mutant effects (Lehner et al. 1992; Knoblich and Lehner 1993), suggesting redundancy of function for the *cyclin B* protein in *Drosophila*.

Thus, we have shown that molecular screens have the potential to identify molecules that genetic screens have failed to recover, providing an important complementary strategy in the analysis of the process of interest (in this case, early embryogenesis). Further, by screening on the basis of a very specific molecular phenotype (in this case spatially and temporally localized maternal RNAs in the early embryo), a limited number of molecules is recovered. This avoids a common problem that arises from less focused differential screens, namely the identification of a large number of distinct molecules without any basis for deciding which should be studied in detail.

Functional significance of localization of the five identified RNAs

Since none of the RNAs identified here are the products of genetically-identified loci, a discussion of the biological significance of their localization must of necessity be highly speculative.

Cyclin B RNA: While maternal *cyclin B* RNA exhibits dynamic localization patterns in the early embryo, the functional significance of this localization is not

known. Indeed, it has been shown that *cyclin B* protein is present uniformly along the anteroposterior axis of the early *Drosophila* embryo (Maldonado-Codina and Glover 1992). The *cyclin B* RNA that is localized to the posterior polar plasm is taken up into the pole cells when they form (Fig. 4). We assume that the localization of *cyclin B* RNA to the polar plasm and then the pole cells relates to particular functions of *cyclin B* in the control of germ cell division. However, since it has not been reported when the *cyclin B* RNA in the germ cells is translated, it is unclear when such a role would be implemented.

CO1 RNA: Mitochondria are uniformly distributed in the cytoplasm of the early embryo (Akiyama and Okada,1992; Ding et al. 1993c). However their respiratory activity as assayed by rhodamine-123 staining of living embryos is distinctly non-uniform, with highest levels localized to the posterior polar plasm, lower levels in the anterior fifth of the cortical cytoplasm, and basal levels elsewhere (Akiyama and Okada 1992; Ding et al. 1993c). The localization pattern of the *CO1* RNA correlates very well with these higher levels of mitochondrial activity at the anterior and posterior poles of the early cleavage embryos (Fig. 5), indicating that the differential distribution of the *CO1* RNA might reflect the different synthetic requirements of mitochondria in different regions of the early embryo. Indeed, a subset of early embryos exhibit only the high posterior cap of mitochondrial activity (without showing elevated levels anteriorly) (Ding et al. 1993c), correlating with the fact that a fraction of the embryos visualized for *CO1* transcripts exhibit only a high posterior transcript level without an elevated anterior level (Fig. 5B). We suspect, therefore, that the *CO1* RNA is not localized in the cytoplasm of the early embryo, but rather is present inside the mitochondria; this contrasts with the mitochondrial *16S r*RNA (see below).

Hsp83 RNA: Mammalian homologs of *Hsp83* — the *Hsp90* family of heat shock proteins - are known to be important cytoplasmically-active regulatory molecular chaperones (Lindquist and Craig 1988). We have shown that maternally transcribed *Hsp83* RNA is localized to the posterior polar plasm (Fig. 6), that it is protected from degradation by components of the polar plasm (Ding et al. 1993b), that it is then taken up into the budding pole cells (Fig. 6) and that *Hsp83* RNA is present at high levels in the germ cells throughout most of development (Ding et al. 1993b). In addition, zygotic expression of *Hsp83* occurs in a complex fashion (Ding et al. 1993b). At this point we do not know whether the protein expression pattern of *Hsp83* is also dynamically controlled spatially and temporally. Should this be the case, it is likely that the dynamic distribution of *Hsp83* might be involved in conferring spatio-temporal regulation on more generally distributed *Hsp83*-partner proteins during development (Ding et al. 1993b).

28S rRNA: An obvious potential role for the non-uniform distribution of the *28S rRNA* in the early embryo, is in conferring a spatial asymmetry to the translation of more uniformly distributed RNAs. In this regard, it is interesting that the distribution of the *28S rRNA* is asymmetric with respect to the antero-posterior axis of the early embryo (Fig. 7). The developmental relevance of such an asymmetric *28S rRNA* distribution remains to be determined, particularly since these distribution patterns are somewhat variable. Interestingly, a number of uniformly distributed maternal RNAs (e.g., *vasa* and *staufen*) have been shown to encode posterior-localized proteins in the early embryo (Hay et al. 1988a, b 1990; Lasko and Ashburner 1988 1990; St Johnston et al. 1990). However, the relative contributions to such protein localization, of asymmetric translation *per se* rather than other post-transcriptional and/or post-translational events, are not known.

16S large rRNA: The localization of the *16S* rRNA to the posterior pole of the early embryo has been studied in detail (Ding et al. 1993c). Unlike the *CO1* RNA (above), the distribution of the *16S* rRNA does not correlate well with the distribution of highly active mitochondria (Fig. 8 cf. Fig. 5). Localization of *16S* rRNA is dependent on components of the posterior polar plasm (Ding et al. 1993c). The majority of the *16S* rRNA at the posterior pole of the early embryo is likely to be extra-mitochondrial and is probably a major component of the polar granules - non-membrane bound cytoplasmic organelles that play an important role in germ cell formation and specification (Kobayashi and Okada 1989; Ding et al. 1993c; S. Kobayashi, R. Akimura and M. Okada, pers. comm.).

RNA localization mechanisms

The four cytoplasmically localized RNAs reported here will also be useful for investigating the mechanisms of cytoplasmic RNA localization *per se*, since comparative analysis of the mechanisms of localization of RNAs with similar distribution patterns can provide information about the cellular RNA-localization machinery. For example, our recent analysis of the localization of the *Adducin-like-56D* RNA to the anterior pole of the oocyte has revealed that there are multiple genetic pathways for the anterior localization of RNAs (Ding et al. 1993a).

Consistent with the existence of a variety of posterior RNA localization mechanisms, the three posterior localized cytoplasmic RNAs identified here, appear to differ in their localization mechanisms. It has been shown that localization of the *cyclin B* RNA to the posterior plasm of the early embryo occurs during late oogenesis and is dependent on sequences in the 3'-untranslated region of the RNA (Dalby and Glover 1992). In contrast, localization of maternal *Hsp83* RNA does not occur during oogenesis, but is carried out during early

embryogenesis. Further, it represents a novel localization mechanism involving a combination of generalized degradation of the initially uniformly distributed *Hsp83* RNA and local protection of *Hsp83* RNA at the posterior pole by components of the posterior polar plasm (Ding et al. 1993b). A third mechanism is implicated in posterior localization of the 16S RNA to the posterior polar plasm since it is synthesized in the mitochondria but appears to be specifically exported into the posterior cytoplasm by an unprecedented mechanism (Kobayashi and Okada 1989; Ding et al. 1993c; S. Kobayashi, R. Akimura and M. Okada, pers. comm.).

While the localization of the *cyclin B*, *Hsp83* and *16S* RNAs differ mechanistically, analyses of the genetic requirements for their posterior localization suggest that certain of the *trans*-acting factors involved in their localization may be shared. All are dependent on components of the polar plasm for their localization/protection (Raff et al. 1990; Ding et al. 1993b; c). A common feature of mutants that disrupt localization of these RNAs is that they also disrupt the polar granules, suggesting that these three RNAs may be associated with these organelles (Raff, et al. 1990; Ding et al. 1993b; c).

Identification and comparative analyses of additional *trans*-acting genes required for localization of these RNAs, as well as *cis*-acting sequences that 'tag' them for localization, are expected to provide insights into the mechanisms of intra-cellular RNA localization.

Future directions: identification of cDNAs representing rare polar-localized RNAs

We are ultimately interested in identifying rare maternal RNAs that are localized to either the anterior or the posterior pole of the early embryo, therefore we plan to follow the differential screens described here with subtractive screens,

which are significantly more sensitive (Sargent and Dawid 1983; Davis et al. 1984; Palazzolo et al. 1989). Since a large amount of RNA is needed to drive the subtractive hybridization (Sargent and Dawid 1983; Davis et al. 1984), a potential difficulty in attempting such a subtractive screen for localized RNAs in the *Drosophila* embryo, is the limiting amount of anterior and posterior RNA that can feasibly be purified directly from embryos. Two strategies could be used to overcome this limitation. In the first, enzymatic amplification (PCR) could be used to produce sufficient quantities of A- or P-single-stranded DNA/RNA (Duguid and Dinauer 1990; Timblin et al. 1990). A potential limitation of this method is the possible bias that may be introduced and amplified during the PCR process. The second strategy involves the use of a set of lambda cDNA cloning vectors that we constructed to facilitate subtractive hybridization with limited amount of starting RNA (Palazzolo et al. 1990). These vectors allow directional cDNA cloning, high frequency conversion of phage cDNA libraries into plasmid libraries, and the ability to synthesize essentially unlimited quantities of single-stranded DNA or RNA copies of the library-inserts for use in the subtraction procedures (Palazzolo et al. 1990). Our cDNA libraries are constructed in one of these vectors, λEXLX+ (Palazzolo et al. 1990; see Materials and Methods).

The specificity of our cDNA libraries as revealed by *bicoid* cDNA frequencies (see Table 1) and the successful use of the libraries in the differential screen described here, indicate that these libraries will be useful in future subtractive screens. It should be noted that library amplification also introduces biases that can be overcome partially by the use of multiple libraries constructed in different cloning vectors (see above). Since our A- and P-cDNA populations were cloned into the same site of λEXLX+, but in opposite orientations (see Materials and Methods), the versatility built into λEXLX+ will make it possible to employ a number of recent improvements to facilitate subtractive hybridization

screens for cDNAs representing rare polar localized RNAs (Pruitt 1988; Sive and St John 1988; Palazzolo et al. 1989; Rubenstein et al. 1990).

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References

Akiyama, T., and Okada, M. (1992). Spatial and developmental changes in the respiratory activity of mitochondria in early *Drosophila* embryos. *Develop.* 115, 1175-1182.

Barker, D. D., Wang, C., Moore, J., Dickinson, L. K., and Lehmann, R. (1992). Pumilio is essential for function but not for distribution of the *Drosophila* abdominal determinant Nanos. *Genes Dev.* 6, 2312-2326.

Benkel, B. F., Duschesnay, P., Boer, P. H., Genest, Y., and Hickey, D. A. (1988). Mitochondrial large ribosomal RNA: an abundant sequence in *Drosophila*. *Nucleic Acids Res.* 16, 9880.

Blackman, R. K., and Meselson, M. (1986). Interspecific nucleotide sequence comparisons used to identify regulatory and structural features of the *Drosophila hsp82* gene. *J. Mol. Biol. 188*, 499-515.

Bossy, B., Hall, L. M. C., and Spierer, P. (1984). Genetic activity along 315 kb of the *Drosophila* chromosome 2537-2541. *EMBO J. 3*, 2537-2541.

Boswell, R. E., and Mahowald, A. P. (1985). *tudor*, a gene required for assembly of the germ plasm in *Drosophila melanogaster. Cell 43*, 97-104.

Dalby, B., and Glover, D. M. (1992). 3' non-translated sequences in *Drosophila* cyclin B transcripts direct posterior pole accumulation late in oogenesis and peri-nuclear association in syncytial embryos. *Develop.* 115.

Davidson, E. H. (1986). Gene activity in early development (3rd ed.). Orlando: Academic Press.

Davis, M. M., Cohen, D. I., Nielsen, E. I., Steinmetz, M. S., Paul, W. E., and

Hood, L. (1984). Cell type-specific cDNA probes and the murine I region. *Proc. Natl. Acad. Sci. USA* 81, 2194-2198.

Ding, D., Parkhurst, S. M., and Lipshitz, H. D. (1993a). Different genetic requirements for anterior RNA localization revealed by the distribution of Adducin-like transcripts during *Drosophila* oogenesis. *Proc. Natl. Acad. Sci. USA* **90**, in press.

Ding, D., Parkhurst, S.M., and Lipshitz, H.D. (1993b) Dynamic *hsp83* RNA localization during *Drosophila* oogenesis and embryogenesis. (submitted)

Ding, D., Whittaker, K., and Lipshitz, H.D. (1993c) Mitochondrially encoded 16S large rRNA is associated with the polar plasm in early *Drosophila* embryos. (in preparation)

Driever, W., and Nüsslein-Volhard, C. (1988a). A gradient of bicoid protein in *Drosophila* embryos. *Cell* 54, 83-93.

Driever, W., and Nüsslein-Volhard, C. (1988b). The bicoid protein determines position in the *Drosophila* embryo in a concentration-dependent manner. *Cell* 54, 95-104.

Driever, W., Siegel, V., and Nüsslein-Volhard, C. (1990). Autonomous determination of anterior structures in the early *Drosophila* embryo by the *bicoid* morphogen. *Develop. 109*, 811-820.

Driever, W., Thoma, G., and Nusslein-Volhard, C. (1989). Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the bicoid morphogen. *Nature* 340, 363-367.

Duguid, J. R., and Dinauer, M. C. (1990) Library subtraction of *in vitro* cDNA

libraries to identify differentially expressed genes in scrapie infection. *Nucl. Acids Res.* 18, 2789-2792.

Ephrussi, A., Dickinson, L. K., and Lehmann, R. (1991). *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell 66*, 37-50.

Feinberg, A. P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragment to high specific activity. *Anal. Biochem.* 132, 6-13.

Foe, V. A., and Alberts, B. M. (1983). Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.* 61, 31-70.

Frigerio, G., Burri, M., Bopp, D., Baumgartner, S., and Noll, M. (1986). Structure of the segmentation gene paired and the *Drosophila* PRD gene set as a part of a gene network. *Cell* 47, 735-746.

Frohnhöfer, H. G., and Nüsslein-Volhard, C. (1986). Organization of anterior pattern in the *Drosophila* embryo by the maternal gene bicoid. *Nature* 324, 120-125.

Garesse, R. (1988). *Drosophila melanogaster* mitochondrial DNA: Gene organization and evolutionary considerations. *Genetics* 118, 649-663.

Golumbeski, G. S., Bardsley, A., Tax, F., and Boswell, R. E. (1991). *tudor*, a posterior-group gene of *Drosophila melanogaster*, encodes a novel protein and an mRNA localized during mid-oogenesis. *Genes Dev. 5*, 2060-2070.

Hackett, R. W., and Lis, J. T. (1983). Localization of the *Hsp83* RNA within a

3292 nucleotide sequence from the 63B heat shock locus of *D. melanogaster*. *Nucl. Acids Res. 11*, 7011-7030.

Hay, B., Ackerman, L., Barbel, S., Jan, L. Y., and Jan, Y. N. (1988a). Identification of a component of *Drosophila* polar granules. *Develop. 103*, 625-640.

Hay, B., Jan, L. Y., and Jan, Y. N. (1988b). A protein component of *Drosophila* polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. *Cell* 55, 577-587.

Hay, B., Jan, L. Y., and Jan, Y. N. (1990). Localization of *vasa*, a component of *Drosophila* polar granules, in maternal effect mutants that alter embryonic anteroposterior polarity. *Develop. 109*, 425-433.

Holmgren, R., Livak, K., Morimoto, R., Freund, R., and Meselson, M. (1979). Studies of cloned sequences from four *Drosophila* heat shock loci. *Cell* 18, 1359-1370.

Jongens, T. A., Hay, B., Jan, L. Y., and Jan, Y. N. (1992). The *germ cell-less* gene product: A posteriorly localized component neccessary for germ cell development in *Drosophila*. *Cell* 70, 569-584.

Kim-Ha, J., Smith, J. L., and Macdonald, P. M. (1991). *oskar* mRNA is localized to the posterior pole of the *Drosophila* oocyte. *Cell 66*, 23-35.

Knoblich, J. A., and Lehner, C. F. (1993) Synergistic action of *Drosophila* cyclins A and B during G_2-M transition. *EMBO J.* 12, 65-74.

Kobayashi, S., and Okada, M. (1989). Restoration of pole-cell forming ability to

u.v.-irradiated *Drosophila* embryos by injection of mitochondrial lrRNA. *Develop.* 107, 733-742.

Kobayashi, S., and Okada, M. (1990). Complete cDNA sequence encoding mitochondrial large ribosomal RNA of *Drosophila melanoganster. Nucleic Acids Res.* 18, 4592.

Lantz, V., Ambrosio, L., and Schedl, P. (1992). The *Drosophila orb* gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. *Develop.* 115, 75-88.

Lasko, P. F. (1992). Molecular-movements in oocyte patterning and pole cell-differentiation. *Bioassays* 14, 507 -512.

Lasko, P. F., and Ashburner, M. (1988). The product of the *Drosophila* gene vasa is very similar to eukaryotic initiation factor 4A. *Nature* 335, 611-616.

Lasko, P. F., and Ashburner, M. (1990). Posterior localization of *vasa* protein correlates with, but is not sufficient for, pole cell development. *Genes Dev. 4*, 905-921.

Lasky, L. A., Lev, Z., Xin, J., Britten, R. J., and Davidson, E. H. (1980). Messenger RNA prevalence in sea urchin embryos measured with cloned cDNAs. *Proc. Natl. Acad. Sci. USA* 77, 5317-5321.

Lehmann, R., and Nüsslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation and embryonic polarity require the localised activity of *oskar*, a maternal gene in *Drosophila. Cell* 47, 141-152.

Lehmann, R., and Nüsslein-Volhard, C. (1987). Involvement of the *pumilio* gene in the transport of an abdominal signal in the *Drosophila* embryo. *Nature 329*,

167-170.

Lehmann, R., and Nüsslein-Volhard, C. (1991). The maternal gene *nanos* has a central role in posterior pattern formation of the *Drosophila* embryo. *Develop. 112*, 679-693.

Lehner, C. F., and O'Farrell, P. H. (1990). The roles of *Drosophila* cyclins A and B in mitotic control. *Cell 61*, 535-547.

Lehner, C. F., Ried, G., Stern, B., and Knoblich, J. A. (1992) cyclins and cdc2 kinases in *Drosophila* - genetic analyses in a higher eukaryote. *CIBA Found. Symp.* 170, 97-114.

Lindquist, S., and Craig, E. (1988). The heat-shock proteins. *Ann. Rev. Genet. 22*, 631-677.

Lipshitz, H. D. (1991). Axis specification in the *Drosophila* embryo. *Curr. Op. Cell Biol. 3*, 966-975.

Macdonald, P. M. (1992). The *Drosophila pumilio* gene: an unusually long transcription unit and an unusual protein. *Develop. 114*,, 221-232.

Maldonado-Codina, G., and Glover, D. M. (1992). Cyclin A and B associate with chromatin and polar regions of spindles, respectively, and do not undergo complete degradation at anaphase in syncytial *Drosophila* embryos. *J. Cell Biol.* 116, 967-976.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Manseau, L., and Schüpbach, T. (1989). *cappuccino* and *spire*: two unique maternal-effect loci required for both anteroposterior and dorsoventral patterns of

the *Drosophila* embryo. *Genes Dev. 3*, 1437-1452.

Meyerowitz, E. M., and Hogness, D. S. (1982). Molecular organization of a *Drosophila* puff site that responds to ecdysone. *Cell* 28, 165-176.

Olson, M. (1991) Genome structure and organization in *Saccharomyces cerevisiae*. *In* "The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Genome Dynamics, Protein Synthesis, and Energetics". Cold Spring Harbor Laboratory Press, N.Y.

Palazzolo, M. J., Hamilton, B. A., Ding, D., Martin, C. H., Mead, D. A., Mierendorf, R. C., Vijay Raghavan, K., Meyerowitz, E. M., and Lipshitz, H. D. (1990). Phage lambda cDNA cloning vectors for subtractive hybridization, fusion protein expression and Cre-*loxP* automatic plasmid subcloning. *Gene 88*, 25-36.

Palazzolo, M. J., Hyde, D. R., VijayRaghavan, K., Mecklenburg, K., Benzer, S., and Meyerowitz, E. (1989). Use of a new strategy to isolate and characterize 436 *Drosophila* cDNA clones corresponding to RNAs detected in adult heads but not in early embryos. *Neuron* 3, 527-539.

Perrimon, N., Engstrom, L., and Mahowald, A. P. (1984). Analysis of the effects of zygotic lethal mutations on the germ line functions in *Drosophila*. *Dev. Biol.* 105, 404-414.

Pruitt, S. C. (1988). Expression vectors permitting cDNA cloning and enrichment for specific sequences by hybridization/selection. *Gene* 66, 121-134.

Raff, J. W., Whitfield, W. G. F., and Glover, D. M. (1990). Two distinct mechanisms localize cyclin B transcripts in syncytial *Drosophila* embryos. *Develop. 110*, 1249-1261.

Rebagliati, M. R., Weeks, D. L., Harvey, R. P., and Melton, D. A. (1985). Identification and cloning of localized maternal RNAs from *Xenopus* eggs. *Cell* 42, 769-777.

Rubenstein, J. L. R., Brice, A. E. J., Ciaranello, R. D., Denney, D., Porteus, H., and Usdin, T. B. (1990). Subtractive hybridization system using single-stranded phagemids with directional inserts. *Nucl. Acids Res.* 18, 4833-4842.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (2nd ed.). Cold Spring Habor: Cold Spring Harbor Laboratory Press.

Sargent, T., and Dawid, I. (1983). Differential gene expression in the gastrula of *Xenopus laevis*. *Science* 222, 135-139.

Schüpbach, T., and Wieschaus, E. (1986). Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. *Roux's Arch. Dev. Biol.* 195, 302-317.

Schüpbach, T., and Wieschaus, E. (1989). Female sterile mutations on the second chromosome of *Drosophila* melanogaster. I. Maternal effect mutations. *Genetics* 121, 101-117.

Sive, H. L., and St John, T. (1988). A simple subtractive hybridization technique employing photoactivable biotin and phenol extraction. *Nucl. Acids Res.* 16, 10937.

St Johnston, D., Beuchle, D., and Nüsslein-Volhard, C. (1991). *staufen*, a gene required to localize maternal RNAs in the *Drosophila* egg. *Cell 66*, 51-63.

St Johnston, D., Driever, W., Berleth T., R., S, and Nüsslein-Volhard, C. *(1989).*

Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. *Develop. 107 (Supplement)*, 13-19.

St. John, T. P., and Davis, R. W. (1979). Isolation of galactose-inducible DNA sequences from *Saccharomyces cerevisiae* by differential plaque filter hybridization. *Cell* 16, 443-452.

Struhl, G., Struhl, K., and Macdonald, P. M. (1989). The gradient morphogen bicoid is a concentration-dependent transcriptional activator. *Cell* 57, 1259-1273.

Sulston, J., Du, Z., Thomas, K., Wilson, R., Hillier, L., Staden, R., Halloran, N., Green, P., Thierry-Mieg, J., Qiu, L., Dear, S., Coulson, A., Craxton, M., Durbin, R., Berks, M., Metzstein, M., Hawkins, T., Ainscough, R. and Waterston, R. (1992) The *C. elegans* genome sequencing project: a beginning. *Nature* 356, 37-41.

Tautz, D., Hancock, J. M., Webb, D. A., Tautz, C., and Dover, G. A. (1988). Complete sequences of the rRNA genes of *Drosophila melanogaster. Mol. Biol. Evol.* 5, 366-376.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback. Chromosoma 98*, 81-85.

Timblin, C., Battey, J., and Kuehl, W. M. (1990) Application for PCR technology to subtractive cDNA cloning: identification of genes expressed specifically in murine plasmacytoma cells. *Nucl. Acids Res.* 18, 1587-1593.

Wang, C., and Lehmann, R. (1991). Nanos is the localized posterior determinant in *Drosophila. Cell 66*, 637-647.

Whitfield, W. G. F., Gonzalez, C., Sanchez-Herrero, E., and Glover, D. M. (1989). Transcripts of one of the two *Drosophila* cyclin genes become localized in pole cells during embryogenesis. *Nature* 338, 337-340.

Wieschaus, E. (1980). A combined genetic and mosaic approach to the study of oogenesis in *Drosophila. In Genetics and Neurobiology of Drosophila* vol. . (ed. O. Siddiqi, P. Babu, & J. C. Hall), pp. 85-94. Plenum Press.

Wohlwill, A.D., and Bonner, J.J. (1991) Genetic analysis of chromosome region 63 of *Drosophila melanogaster. Genetics* 128, 763-775.

Zalokar, M., and Erk, I. (1976). Division and migration of nuclei during early embryogenesis of *Drosophila melanogaster. J. microsc. Biol. Cell 25*, 97-106.

Figure Legends

Figure 1: The egg-cutting apparatus

For a detailed description, see Materials and Methods. The diagram is not to scale; dimensions are given.

Figure 2: Construction of the anterior library

Flow diagram indicating the steps in construction of the library. For details see Materials and Methods.

Figure 3: Differential screen for polar localized RNAs

Flow diagram indicating the steps in the differential screen, with the numbers of clones identified at each step listed. For details, see Material and Methods, as well as Results.

Figure 4: Localization of *cyclin B* (LP-134) RNA during early embryogenesis

In situ hybridization of *cyclin B* cDNA probe to wild-type embryos. (A) In an early cleavage stage embryo, maternal *cyclin B* RNA is localized in an anterior to posterior gradient through the anterior half of the embryo. In addition, *cyclin B* RNA is concentrated as a tight cap at the posterior pole. (B) By the syncytial blastoderm stage, the level of anterior *cyclin B* RNA is greatly reduced and the posterior RNA has been taken up into the pole cells. (C) At the cellular blastoderm stage, *cyclin B* RNA is present only in the pole cells. For all embryos, anterior is to the left and dorsal is toward the top of the page.

Figure 5: Localization of *CO1* (LP-169) RNA during early embryogenesis

In situ hybridization of *CO1* cDNA probe to wild-type embryos. (A) In an

early cleavage stage embryo, *CO1* RNA is present throughout the embryo, but exhibits a high concentration at the anterior pole and the posterior pole. (B) A subset of early cleavage stage embryos show high levels of *CO1* RNA at the posterior but not at the anterior. (C) When pole cells start to form, both the anterior and posterior concentrations of *CO1* RNA have dropped near to the basal levels found in the rest of the embryo. The pole cell buds (arrowhead) do not stain for *CO1* RNA. (D) At the cellular blastoderm stage, *CO1* RNA is present in the basal cytoplasm of the somatic cells; the pole cells (arrowhead) remain unstained. Embryo orientation as in Fig. 4.

Figure 6: Localization and expression of *Hsp83* (SHTZ-68) RNA during early embryogenesis

In situ hybridization of *Hsp83* cDNA probe to wild-type embryos. (A) In an early cleavage stage embryo (prior to nuclear cycle 6), maternal *Hsp83* RNA is uniformly distributed at high levels. (B) In a cycle 7 embryo, maternal *hsp83* RNA is more concentrated at the posterior pole, forming a decreasing gradient towards the anterior. (C) By the syncytial blastoderm stage, *Hsp83* RNA can only be detected in the pole cells (arrowhead) and a small region of the somatic syncytial blastoderm immediately beneath them. Embryo orientation as in Fig. 4.

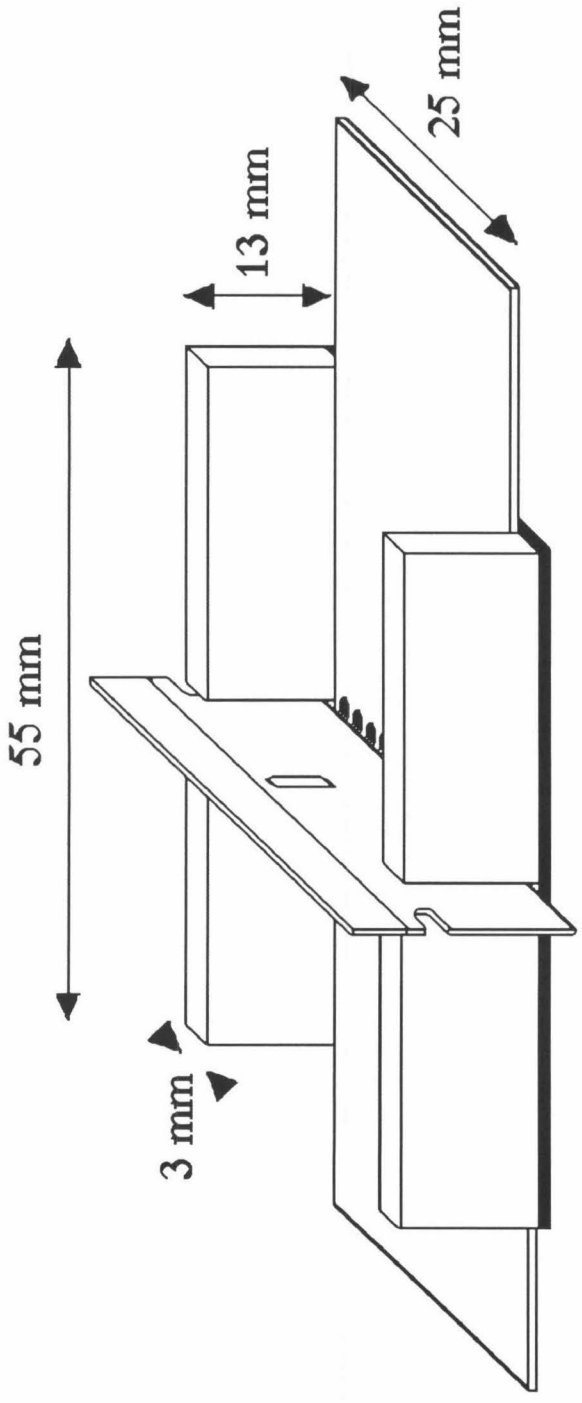
Figure 7: Localization of *28S* (SHTZ-422) *r*RNA in early cleavage stage embryos

In situ hybridization of *28S* cDNA probe to wild-type embryos. Distribution of maternal 28S rRNA in early cleavage embryos is variable (see Results). (A) an example of near uniform distribution of 28S rRNA. (B), (C) and (D) Examples of 28S rRNA localized posteriorly, but varying in their distributions along the dorso-ventral axis. Embryo orientation as in Fig. 4.

Figure 8: Localization of *16S* (SHTZ-CON) *r*RNA in early embryos

In situ hybridization of mitochondrial *16S* cDNA probe to wild-type embryos. (A) In an embryo immediately after egg deposition, *16S r*RNA is present throughout the embryo but is found at a higher concentration at the posterior pole. (B) The high concentration of *16S r*RNA at the posterior decreases rapidly during the cleavage stages such that, when pole cells form, there is only slightly more RNA at the posterior pole than in the rest of the embryo. The pole cells themselves (arrowhead) are not stained. (C) At the cellular blastoderm stage, *16S* RNA is present in the basal cytoplasm of the somatic cells, while the pole cells (arrowhead) remain unstained. Embryo orientation as in Fig. 4.

Table 1. Characterization of Anterior and Posterior cDNA Libraries		
	Anterior	Posterior
Initial Library Size (pfu)	1.8×10^7	1.4×10^7
Insert Frequency	90%	80%
Average Insert Size (bp)	900	1200
Frequency of *bicoid*	1 in 10^4	1 in 2×10^5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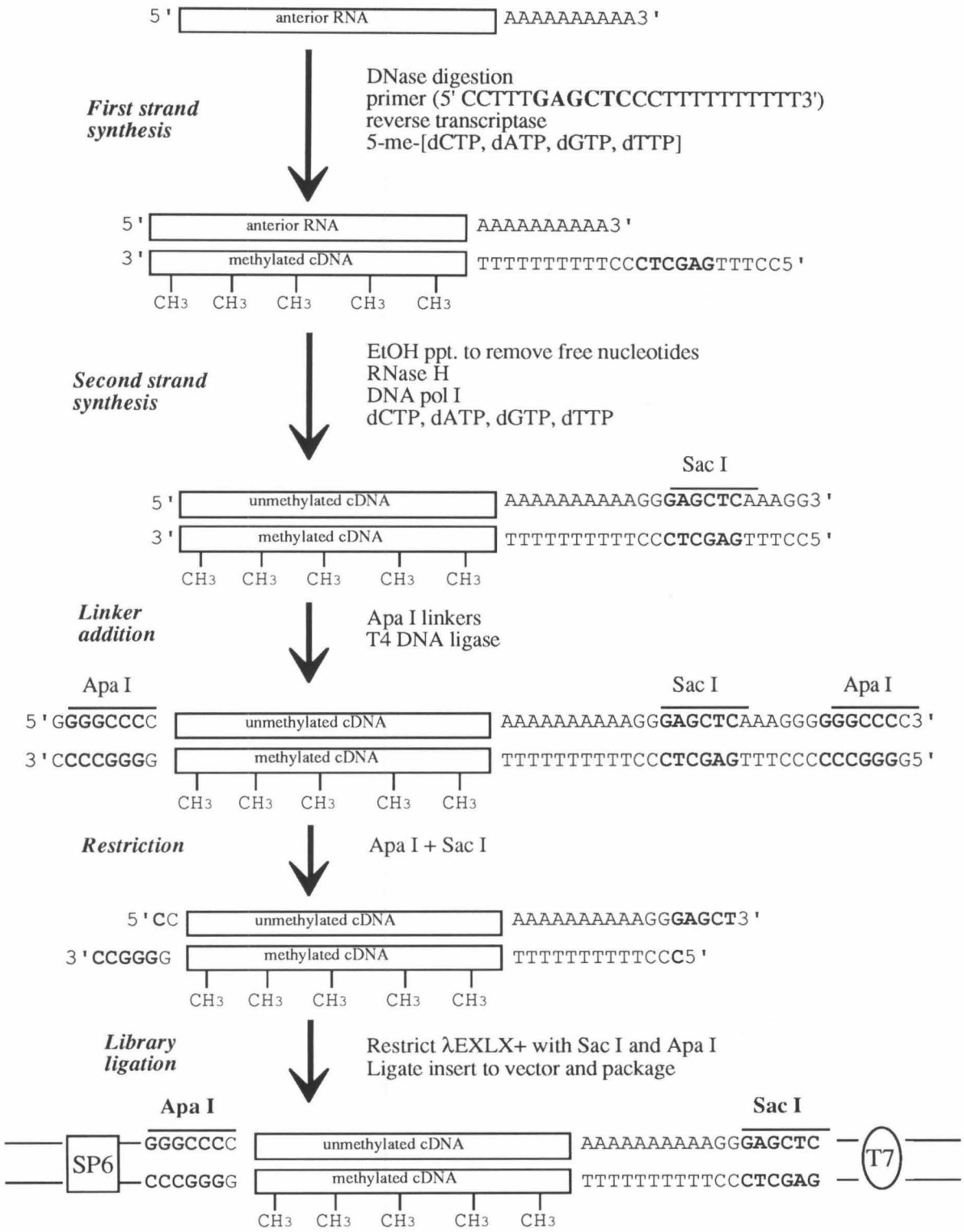
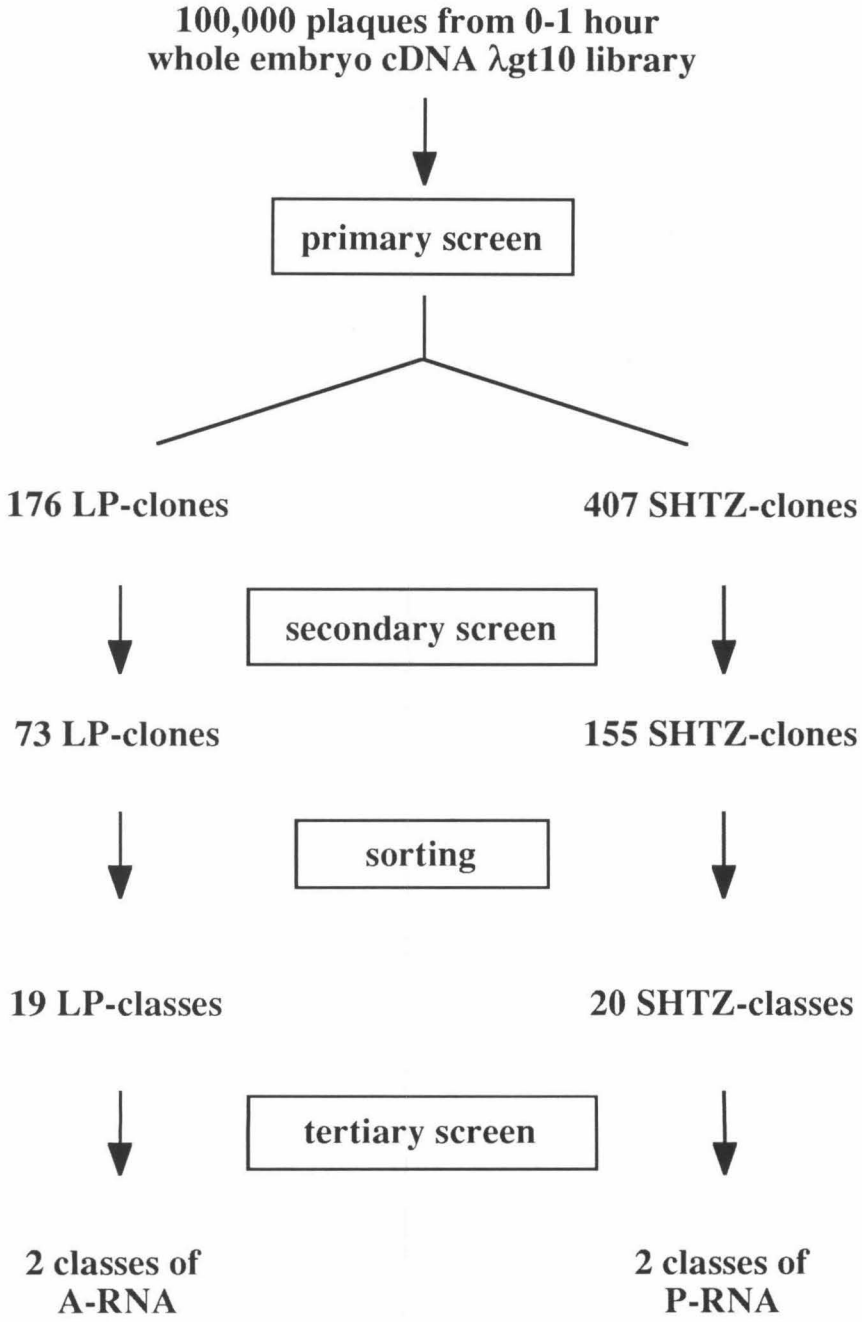
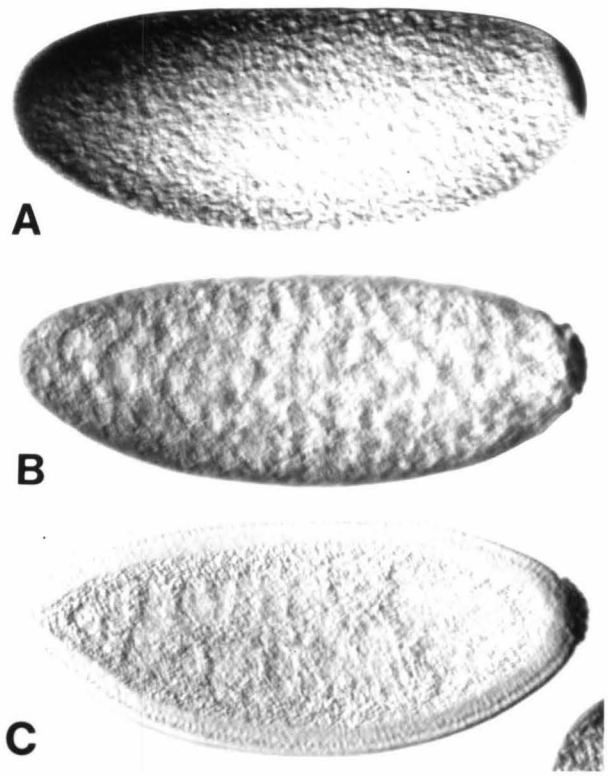
13 mm
55 mm
3 mm


5' anterior RNA AAAAAAAAAA3'
First strand synthesis
DNase digestion
primer (5' CCTTTGAGCTCCCTTTTTTTTTTT3')
reverse transcriptase
5-me-[dCTP, dATP, dGTP, dTTP]
5' anterior RNA AAAAAAAAAA3'
3' methylated cDNA TTTTTTTTTTCCCTCGAGTTTCC5'
CH3 CH3 CH3 CH3 CH3
Second strand synthesis
EtOH ppt. to remove free nucleotides
RNase H
DNA pol I
dCTP, dATP, dGTP, dTTP
Sac I
5' unmethylated cDNA AAAAAAAAAAGGGAGCTCAAAGG3'
3' methylated cDNA TTTTTTTTTTCCCTCGAGTTTCC5'
CH3 CH3 CH3 CH3 CH3
Linker addition
Apa I linkers
T4 DNA ligase
Apa I
Sac I
Apa I
5' GGGGCCCC unmethylated cDNA AAAAAAAAAAGGGAGCTCAAAGGGGGGCCCC3'
3' CCCCGGGG methylated cDNA TTTTTTTTTTCCCTCGAGTTTCCCCCCGGGG5'
CH3 CH3 CH3 CH3 CH3
Restriction
Apa I + Sac I
5' CC unmethylated cDNA AAAAAAAAAAGGGAGCT3'
3' CCGGGG methylated cDNA TTTTTTTTTTCCC5'
CH3 CH3 CH3 CH3 CH3
Library ligation
Restrict λEXLX+ with Sac I and Apa I
Ligate insert to vector and package
Apa I
Sac I
SP6
GGGCCCC unmethylated cDNA AAAAAAAAAAGGGAGCTC
CCCGGGG methylated cDNA TTTTTTTTTTCCCTCGAG
T7
CH3 CH3 CH3 CH3 CH3


100,000 plaques from 0-1 hour
whole embryo cDNA λgt10 library
primary screen
176 LP-clones
407 SHTZ-clones
secondary screen
73 LP-clones
155 SHTZ-clones
sorting
19 LP-classes
20 SHTZ-classes
tertiary screen
2 classes of
A-RNA
2 classes of
P-RNA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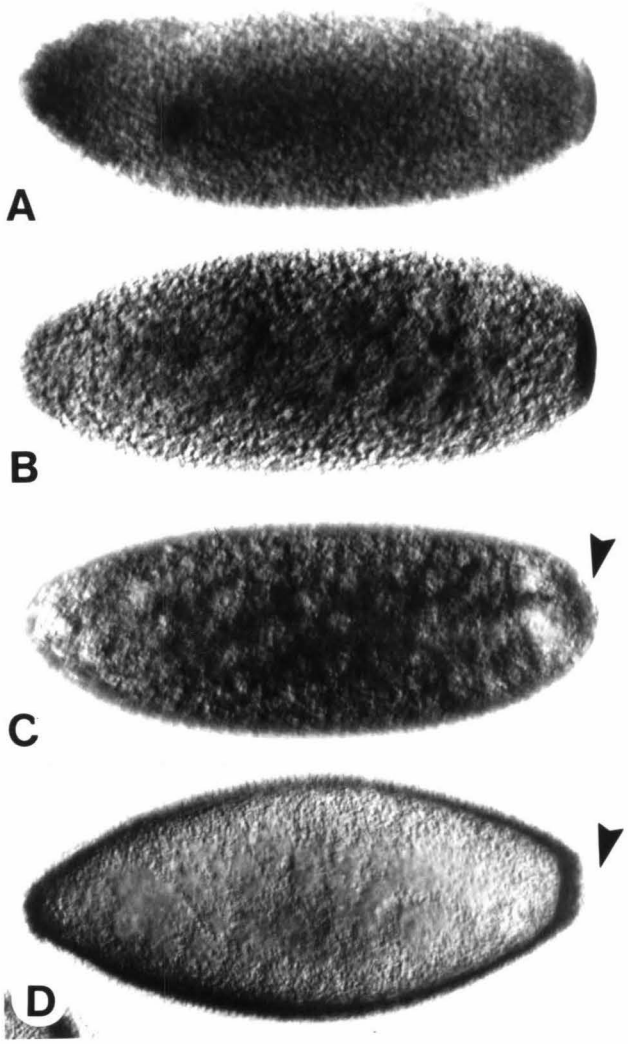
B
C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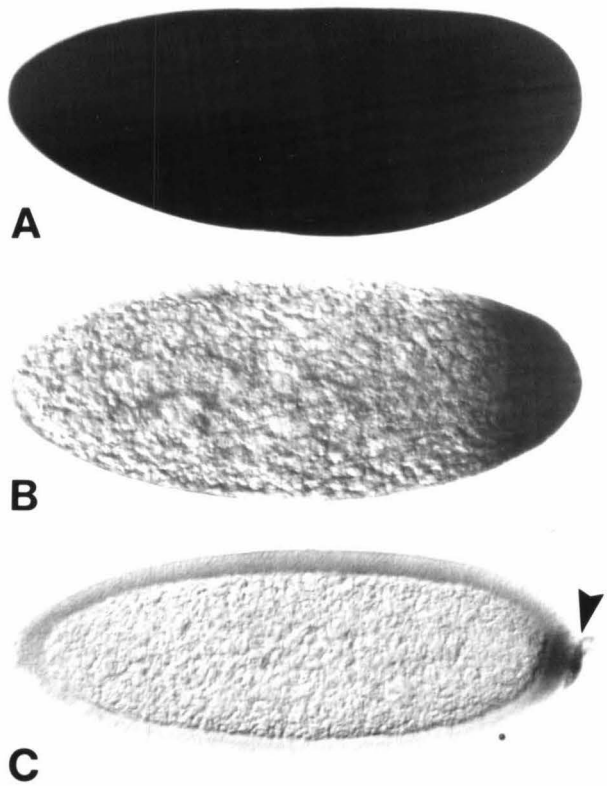
D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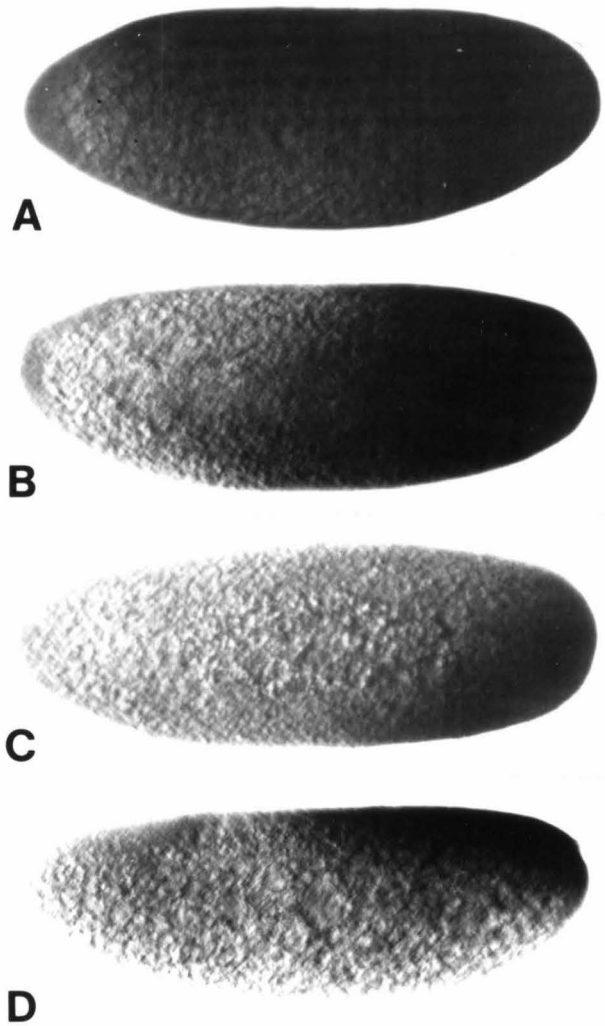
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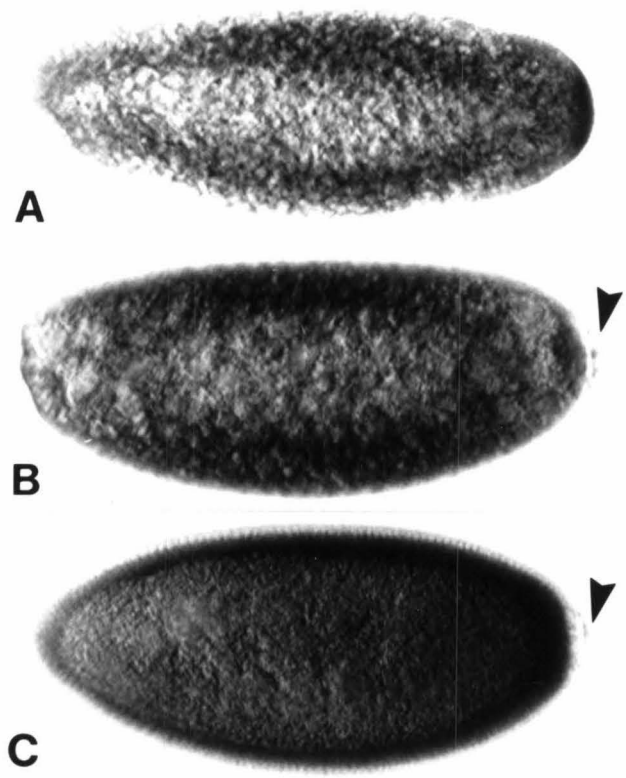
A
B
C

CHAPTER 2 - APPENDIX

This paper appeared in Gene 88, 25-36 (1990).

My contributions involved the construction and testing of the λSHLX vectors.

Phage lambda cDNA cloning vectors for subtractive hybridization, fusion protein expression and Cre-loxP automatic plasmid subcloning

(Recombinant DNA; bacteriophages λ, T7, f1 and P1; E. coli RNA polymerase; promoters; site-specific recombination)

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Abbreviations:

aa, amino acid(s); Ap, ampicillin; bp, base pair(s); Cm, chloramphenicol; ds, double-strand(ed); IPTG, isopropylthiogalactoside; kb, kilobases or 1000 bp; Km, kanamycin; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame; *ori*, origin of DNA replication; pfu, plaque forming units; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; PCR, polymerase chain reaction; ^{R}, resistant/resistance; SDS, sodium dodecyl sulfate; ss, single strand(ed); Tc, tetracyclin; wt, wildtype; [ ], denotes plasmid- or P1 phage-carrying state.

SUMMARY

We describe the construction and use of two classes of cDNA cloning vectors. The first class comprises the λEXLX(+) and λEXLX(-) vectors that can be used for the expression in *Escherichia coli* of proteins encoded by cDNA inserts. This is achieved by the fusion of cDNA open reading frames to the T7 gene*10* promoter and protein coding sequences. The second class, the λSHLX vectors, allows the generation of large amounts of ss DNA or synthetic cRNA that can be used in subtractive hybridization procedures. Both classes of vectors are designed to allow directional cDNA cloning with non-enzymatic protection of internal restriction sites. In addition, they are designed to facilitate conversion from λ to plasmid clones using a genetic method based on the bacteriophage P1 site-specific recombination system; we refer to this as automatic Cre-*loxP* plasmid subcloning. The λ arms, λLOX, used in the construction of these vectors have unique restriction sites positioned between the two *loxP* sites. Insertion of a specialized plasmid between these sites will convert it into a λ cDNA cloning vector with automatic plasmid subcloning capability.

INTRODUCTION

We have constructed a new series of phage λ cDNA cloning vectors with a number of features designed to facilitate and simplify the construction of directionally cloned cDNA libraries, their subtractive hybridization, the rapid analysis of cDNA inserts by DNA sequence analysis and the expression of fusion proteins. Both the λEXLX and the λSHLX classes of vectors are designed to allow conventional directional cDNA cloning (Palazzolo and Meyerowitz 1987; Meissner et al. 1987) or cDNA synthesis with non-enzymatic protection of internal restriction sites (M. Strathmann and M. Simon, unpub.) followed by directional cloning. In addition, they are designed to facilitate conversion from λ to plasmid clones *in vivo* using a method we refer to as automatic Cre-*loxP* plasmid subcloning. Automatic subcloning makes it possible to combine the high efficiency of cloning afforded by phage λ-derived vectors with rapid and simple conversion of each clone to a specialized plasmid (Brenner et al. 1982; Sauer and Henderson 1988; Short et al. 1988). The conversion strategy used here is based on the site-specific recombination system of the phage P1 (Sternberg et al. 1983; Sauer and Henderson 1988). Several different specialized plasmids have been inserted into λ arms in such a way that the plasmid sequences are flanked by 34 bp direct repeats known as *loxP*. The *loxP* sites are the *cis*-acting genetic elements recognized by the P1 recombinase, known as Cre. Plasmids are automatically subcloned when the *loxP*-containing λ derivatives are infected into *E. coli* strains expressing Cre.

The plasmid portions of the λEXLX expression class of vectors are derivatives of the T7 expression system developed by Studier and colleagues (Studier and Moffat 1986; Rosenberg et al. 1987; Lin et al. 1987). cDNA is directionally cloned downstream of the T7 RNA polymerase promoter between the amino-terminal portion of the T7 gene*10* and a T7 transcription terminator. A

large quantity of fusion protein is produced when cDNA plasmid clones are introduced into cells that express T7 RNA polymerase. Furthermore, these fusion proteins are often insoluble, allowing simple preparation for immunization (Lin et al. 1987). The original T7 expression vectors were based on pBR322 (Rosenberg et al. 1987). In contrast, our plasmids contain the pUC and the f1 origins of replication, thus they are expected to have a higher copy number and ss copies of the cDNA clones can easily be generated. It is thus relatively straightforward to select a cDNA clone from a λEXLX library, convert the clone to a plasmid by Cre-*loxP* automatic subcloning, sequence the insert by the dideoxy chain-termination method, alter the reading frame if necessary and induce the expression of large amounts of fusion protein.

At least two strategies have been proposed to facilitate subtractive hybridization procedures in situations where the RNA is difficult to obtain in large amounts (Palazzolo and Meyerowitz 1987; Pruitt 1988). Both approaches involve the construction of cDNA libraries from the tissues of interest, followed by the purification of DNA from these libraries to generate ss DNA or cRNA copies that can be used in the subtraction procedures. The second class of vectors that we describe here, the λSHLX vectors, have been constructed to facilitate either strategy.

It is sometimes useful to build cDNA cloning vectors for specialized reasons. To simplify construction of other vectors, we have built our vectors in a modular fashion. Specifically, we have developed a set of 38 kb λ arms in which unique restriction sites have been positioned between two *loxP* sites. After cloning a specialized plasmid between the *loxP* sites, it is straightforward to convert it into a λ cDNA cloning vector with automatic plasmid subcloning capability.

RESULTS AND DISCUSSION

(a) Initial test of Cre-*loxP* automatic plasmid subcloning

Our initial experiments asked whether the Cre-*loxP* site specific recombination system of phage P1 would be useful for automatic subcloning in λ-based cloning vectors. This was done by constructing a λgt10 (Huynh et al. 1985) derivative (λGLX) that contained plasmid sequences flanked by *loxP* sequences. To be useful for automatic subcloning, the *loxP* sites should recombine in a precise fashion to generate an autonomously replicating plasmid. Furthermore, the conversion from phage to plasmid should occur frequently enough to be useful for efficient library or clone conversion. The construction of λGLX is diagrammed in Fig. 1.

To test for conversion from phage to plasmid, *E. coli* cells that express the Cre recombinase were required. We generated a variety of P1 lysogens that express Cre; these are listed in Table I and discussed in section (e[i]) below. In our initial tests, the first of these strains to be constructed, DM103[P1] (Table I), was infected with λGLX and plated on Ap plates. pGLX1 was found to be excised precisely, recircularized *in vivo* and replicated (Fig. 1). Detailed restriction mapping of the pGLX1 plasmid as well as DNA sequence analysis of the pGLX1 region containing the *loxP* site, confirmed that pGLX1 was identical to pGLX2 except that it contained only a single *loxP* site.

(b) Universal λ arms containing *loxP* sites: construction of λLOX

Several characteristics are desirable for a set of λ arms that can accept any plasmid for Cre-*loxP* automatic subcloning. First, restriction sites that are to be placed in the multiple cloning site of the finished vector must be removed. Second, the acceptor arms must contain directly repeated *loxP* sites. Third, unique restriction sites must be positioned between the *loxP* sites so the arms can be opened for plasmid insertion. The construction of λLOX, an *acceptor meeting*

these three criteria, is outlined in Figs. 2 and 3.

(c) Construction of the λEXLX T7 gene*10* expression vectors

The plasmid portions of the λEXLX vectors consist of: (1) the T7 elements of pET3xa (the T7 promoter, the coding sequences for the amino terminal end of the product of T7 gene*10* and the T7 transcription terminator) (Rosenberg et al. 1987); (2) the essential plasmid elements of pGEM3Zf (the pUC *ori*, the f1 *ori* and the *E. coli bla* gene) (Promega, Inc.); (3) a multiple cloning site that includes *Eco*RI, *Apa*I, *Hind*III and *Sac*I restriction sites; and (4) an SP6 RNA polymerase promoter. The construction of these plasmids, pMP3(+) and pMP3(-), that differ solely in the orientation of the f1 *ori*, is diagrammed in Fig. 4. These plasmids were linearized by digestion with *Sma*I and ligated between the *loxP* sites in the λLOX acceptor arms, completing the construction of λEXLX(+) and λEXLX(-) (Figs. 4 and 6A).

(d) Construction of the λSHLX subtractive hybridization vectors

The plasmid portions of the λSHLX vectors, pDD1 and pDD2, were designed to have the following features: (1) a multiple cloning site that allows the construction of directionally cloned cDNAs by a number of different strategies; (2) T7 and SP6 bacteriophage RNA polymerase promoters that are positioned to face each other from the opposite ends of the cloning sites; (3) *Not*I and *Sfi*I restriction sites that are placed just outside of the RNA polymerase promoters; and (4) the essential plasmid elements of pGEM3Zf (pUC *ori*, the f1 *ori* and the *E. coli bla* gene). Since these vectors are designed for the subtraction of one cDNA library with another, two vectors, λSHLX1 and λSHLX2, have been built; these differ solely in the relative orientations of the multiple cloning sites. A more detailed explanation of this last point is given in section (h) below. The construction of the pDD1 and pDD2 plasmids is outlined in Fig. 5. These plasmids were linearized by digestion with *Hpa*I and separately inserted between

the *loxP* sites in the λLOX arms to give the final vectors, λSHLX1 and λSHLX2 (Figs. 5 and 6B).

(e) Tests of the functional elements of the vectors

Each of the four cDNA cloning vectors contains a variety of functional elements. These include: *loxP* sites, the pUC *ori*, the f1 *ori*, the RNA polymerase promoters, the multiple cloning sites and a drug resistance marker. Each of these was tested in each of the constructs to ensure that they are intact and functional. In addition, test cDNA libraries have been constructed in both the λEXLX and λSHLX vectors.

(i) Cre-*loxP* automatic subcloning: Cre-*loxP*-mediated *in vivo* subcloning requires a specialized host cell strain that produces the Cre protein. Conversion from phage to plasmid clones *in vivo* is accomplished by infecting such cells with phage and selecting for Ap^R colonies on agar plates. We have made several common cloning strains of *E. coli* lysogenic for a recombinant P1 phage ($P1Cm^Rr^-m^-$; Yarmolinsky et al. 1989) that carries a selectable marker (Cm^R) and lacks the P1 restriction-modification system (Table I). To test the frequency of conversion from phage to plasmid, both these P1 lysogens and their parental non-P1 lysogens were infected with each of the four vectors. The infected P1 lysogens were plated on agar plates containing Ap and the number of resistant colonies scored. The infected non-P1 lysogens were plated on non-selective medium and the resulting phage were counted. We define the apparent conversion frequency from phage to plasmid operationally as the ratio of the number of Ap^R colonies when plated on a P1 lysogen to the number of plaques when plated on its parental non-P1 lysogen. Representative results are shown in Table II; the apparent conversion frequency varied between 5 and 30%. Since infection of a λ lysogenic strain should favor the plasmid excision pathway over the lytic λ pathway, we tested the conversion frequency in *E. coli* strains that were

lysogenic for both P1 and λ (see Table II for representative results). In the doubly-lysogenic strains the conversion of phage to plasmid is more efficient; typical apparent conversion frequencies of 40 to 50% were observed for vectors alone, while for individual cDNA clones frequencies of from 80 to 140% were observed (Table II).

(ii) Growth of automatically subcloned plasmids: Yields of purified automatically subcloned plasmid DNA from our P1 lysogens (Table I) were often an order of magnitude lower than yields we obtained when the same DNA was transformed into the parental non-Cre-expressing *E. coli* strains (data not shown). In addition, plasmids grew with different efficiencies on the various host strains; for example, pEXLX(+) grew well on strain BM25.5 (Table I) but pEXLX(-) and pSHLX1 and 2 grew poorly. Our results suggest that there is a negative correlation between the presence of Cre recombinase in a cell and the growth of *loxP*-containing plasmids, particularly when the plasmid sequences are present at high copy number. Similar poor plasmid DNA yields were previously observed in cells in which the gene that encodes Cre was cloned and introduced into *E. coli* as part of a λ lysogen (Sauer and Henderson 1988; although not discussed in this paper, it appears from their Fig. 3 that plasmid DNA yields were reduced in their Cre-expressing strain). S. Elledge (pers. comm.) has constructed a related series of Cre-*loxP* cDNA cloning vectors to those described here; however, his automatically subcloned plasmids contain a pBR322 (rather than a pUC) *ori* and thus are not present at high copy number. In this case, he has found that there are good DNA yields from these plasmids when automatically subcloned in a λ-lysogenic host strain in which the *cre* gene is present in the lysogenized λ sequences. Should our reasoning be correct, then the production of Cre recombinase under control of an inducible promoter, rather than constitutively, should eliminate the depression of plasmid growth. We are currently exploring

such possibilities.

In the meantime, we have devised a strategy by which the site specific recombination is conducted in doubly lysogenic cells (λ and P1) and the resulting plasmid is rapidly transferred to a second host strain that does not express the Cre recombinase. The cDNA vector is introduced into an *E. coli* strain that is a P1 and λ lysogen and also contains an F' episome (strain BM25.8; see Table I) making the cell susceptible to infection by phage M13. Simultaneously, the cells are infected with M13KO7 helper phage (Yanisch-Perron et al. 1985). In these cells, site specific recombination occurs and the λ clone is converted into a plasmid clone, which is then replicated from the f1 *ori* to yield ss DNA that is packaged into infectious particles. Cells from a second host strain (XL1-blue; Bullock et al. 1987) that contains an F' episome with a Tc^R gene are added to the mixture which is then plated on selective medium containing both Ap and Tc. After incubation, colonies appear that contain the converted plasmid but do not express the recombinase. Yields of plasmid DNA from these colonies are equivalent to those obtained for pGEM3Zf in non-Cre-expressing strains (data not shown).

The above method for rescue of automatically subcloned plasmids occasionally results in deletion of part of the cDNA inserts, particularly when these are >2 kb in length. In this case, it is useful to carry out the automatic plasmid subcloning, followed by purification of plasmid DNA from the Cre-expressing cells. Enough DNA can be obtained to transform a non-Cre-expressing strain (XL1-blue). Plasmids can then be purified from these cells with excellent yields and with their cDNA inserts intact (data not shown).

(iii) Function of the f1 *ori*: We have tested the function of the f1 *ori* in all four vectors. Plasmids were recombined out of the phage λ vectors and transformed into *E. coli* cells containing an F' episome (XL1-blue). These cells were then infected with the M13 helper phage K07 and grown overnight

(Yanisch-Perron et al. 1985). Phage particles containing ss DNA were purified and the DNA extracted. The yield of single stranded DNA was approximately the same as that from control pGEM3Zf plasmids (data not shown).

(iv) Function of the SP6 and T7 RNA polymerase promoters: Chain termination DNA sequence analysis verified the presence of the consensus sequence for the SP6 and T7 RNA polymerase promoters in each of the vectors (Fig. 6). To test the function of these promoters, DNA prepared from each of the plasmids was linearized with an appropriate restriction enzyme and used as template in T7 and SP6 RNA polymerase reactions containing [α-^{32}P]CTP. The amount of cRNA synthesized was assayed by measuring the retention of radioactivity on DE-81 filters (Maniatis et al. 1982). These experiments indicated that all four plasmids contained SP6 and T7 promoters that functioned roughly as efficiently as they did in pGEM3Zf (data not shown).

(v) Expression of T7 gene*10* protein: To test the ability of pEXLX(+) and pEXLX(-) to produce large amounts of the T7 gene*10* product in cells that express T7 RNA polymerase, the plasmids were introduced into a pLysS host strain (Moffat and Studier 1987; Studier et al. 1990). This is a strain of *E. coli* that contains an IPTG-inducible T7 RNA polymerase gene on a λ lysogen. Since low levels of this polymerase are expressed even without IPTG-induction (Studier and Moffat 1986), we were able to compare the expression of gene*10* protein from pEXLX and pET3xa simply by lysing uninduced overnight cultures in an SDS-urea buffer followed by SDS-polyacrylamide gel electrophoresis. Our results demonstrated that the amounts of the gene*10* protein produced by pEXLX(+) and pEXLX(-) are approximately equivalent to those from pET3xa (data not shown).

(vi) Construction of a cDNA library in λSHLX: To test the ability of the λSHLX vectors to accept cDNA molecules, a library was constructed in λSHLX2. Poly(A)$^+$RNA was isolated from a mixed-stage liquid culture of the N2 strain of

C. elegans. cDNA was prepared according to a method developed by M. Strathmann and M. Simon (pers. comm.) in which the cDNA can be directionally cloned with all of the internal sites protected (see section [g] below for details). In the construction of our library, 5 μg of poly(A)$^+$RNA was converted into ds cDNA. This material was then fractionated on a 1% agarose gel and four individual size-fractions were recovered: 0.5 - 1.0 kb (approximately 420 ng recovered), 1 - 2 kb (450 ng), 2 - 3 kb (330 ng) and greater than 3 kb (360 ng). Material from each of these fractions was then ligated into *Apa*I+*Sac*I digested λSHLX2 and the cloning mixture was packaged. Yields were between 10^6 and 10^7 pfu (e.g. the 1 - 2 kb material yielded 7.5 x 10^6 pfu).

When the 2 - 3 kb fraction library was screened at low density with probe synthesized from the *C. elegans* poly(A)$^+$RNA, almost half the clones gave positive autoradiographic signals. Over one hundred individual plaques that did not give a detectable hybridization signal were isolated and screened by the polymerase chain reaction (PCR) using oligo primers that flank the cloning sites (Saiki et al. 1988). Over 48% contained inserts greater than 1 kb in length, while 26% had smaller inserts, and 26% had no detectable insert by this assay. The selection of non-hybridizing plaques biases towards clones that are relatively rare in the mRNA population, or that have no inserts (Palazzolo and Meyerowitz 1987; Palazzolo et al. 1989). Twenty two of these λSHLX2 cDNA clones from the 2 - 3 kb fraction were converted to plasmids by the method described in section (ii) above. Restriction mapping of DNA prepared from the indivdual clones showed *Apa*I-*Sac*I inserts that matched the size of the inserts detected by the PCR test for 20 of the 22 selected clones. The remaining two converted clones showed, in one case a significantly smaller insert (0.57 kb vs. 1.75 kb by PCR) with a normal vector band, and in the second case aberrant insert and vector bands. The reason for these discrepancies has not been determined. A set of five additional clones,

selected on the basis of showing a hybridization signal with cDNA probe derived from *C. elegans* poly(A)$^+$RNA, and thus likely to contain cDNAs representing relatively abundant RNAs, were analyzed by chain-termination DNA sequence analysis. One of the clones was found to be identical to the nematode *hsp70A* gene (Snutch et al. 1988) over the 187 nt of sequence that was determined. A second clone showed a 74 out of 78 aa match to the yeast elongation factor 1-α A chain predicted protein sequence (Nagashima et al. 1986).

(vii) Construction of cDNA libraries in λEXLX: We have constructed *Drosophila* cDNA libraries in the λEXLX vectors. cDNA synthesized from poly(A)$^+$RNA purified from 0-24 h *Drosophila* embryos was cloned into λEXLX(+) and cDNA synthesized from poly(A)$^+$RNA purified from adult *Drosophila* was cloned into λEXLX(-) using the Strathmann-Simon method outlined in section (g) below. The cloning gave between 1.2 x 10^6 and 4.6 x 10^7 clones/μg poly(A)$^+$RNA. Each library was shown to contain cDNA inserts by λ plaque filter hybridization screens with ^{32}P-labelled cDNA synthesized from the same source RNA that was used in its construction. After a 2 h exposure, about 40% of the clones gave positive hybridization signals in this screen. These libraries have been screened with probes generated from selected clones isolated by Palazzolo et al. (1989) from a subtracted *Drosophila* library in which adult head cDNA was subtracted with 0-1 hr embryo RNA. These probes identified a small number of homologous clones that were plaque-purified, automatically subcloned and are currently under detailed analysis.

(f) Applications of λLOX

Almost any plasmid can be linearized and inserted between the *loxP* sites of λLOX to generate a specialized cloning vector with Cre-*loxP* automatic subcloning. Here we have constructed and tested two sets of vectors: one for expression of cDNA clones in *E. coli* and a second for use in subtractive

hybridization procedures. However, the λLOX arms can be used to construct specialized vectors containing other types of plasmids, such as shuttle vectors for yeast or different types of eukaryotic expression vectors . To use λLOX for these applications it is important first to introduce the appropriate cloning sites (such as *Hin*dIII, *Eco*RI, *Apa*I, and *Sac*I) into the plasmid of interest. This plasmid can then be linearized with a different restriction enzyme and ligated into the *Xba*I and *Sac*I sites (blunt-ended with T4 DNA polymerase) of λLOX.

(g) Methods for directional cDNA library construction

At least two strategies have been devised recently to facilitate directional cDNA cloning with the protection of internal endogenous restriction sites. Meissner et al. (1987) synthesized ds cDNA that was primed initially with oligo(dT). This was then methylated with *Bam*HI+ *Alu*I methyltransferases to protect internal *Bam*HI and *Hin*dIII restriction sites. A specialized linker was then blunt-end ligated to this cDNA; this linker contains a *Bam*HI site flanked by the last 4 nt of the *Hin*dIII recognition site. When ligated to the cDNA, the first 2 nt of the *Hin*dIII site were generated at the 3'-end by the poly(dA) sequences and a *Bam*HI site was positioned at the 5'-end of the cDNA. In the case of λEXLX and λSHLX, one would ligate a linker containing an *Eco*RI site flanked by the last 4 nt of the *Hin*dIII recognition site. The cDNA would then be digested with *Eco*RI+*Hin*dIII and cloned into the unique *Eco*RI and *Hin*dIII sites in any of the four vectors that we have constructed. We have successfully constructed 0-1 hr *Drosophila* embryo cDNA libraries in λSHLX2 using this strategy.

The second protocol was devised by M. Strathmann and M. Simon (personal communication). Briefly, an *Apa*I site is generated at the 3'-end of the cDNA by priming first strand cDNA synthesis with an oligo(dT)-*Apa*I primer-adapter. First strand cDNA synthesis is carried out using 5-methyl dCTP in place of dCTP, while second strand synthesis utilizes unmethylated dCTP. *Sac*I linkers

are ligated to the ds cDNA and the cDNA is digested with *Apa*I+*Sac*I. All internal *Apa*I and *Sac*I sites are hemimethylated and cannot be cleaved by *Apa*I or *Sac*I, while the restriction sites at the ends can be cleaved because they are unmethylated.

(h) Applications of λEXLX(+) and λEXLX(-) vectors.

It is often desirable to obtain clones in which the cDNA of interest is inserted into an expression vector so that the protein encoded by the cDNA can easily be purified for use as an immunogen. The plasmid expression vectors developed by Studier and colleagues (Rosenberg et al. 1987) offer a number of attractive features. Expression is completely silent in cells that do not contain the T7 RNA polymerase gene and is abundant in cells that do (Studier and Moffat 1986). Thus, control of gene expression is very tight, limiting problems that occur when the foreign product is lethal in *E. coli*. In addition, their vectors provide termination of transcription as well as initiation. Finally, the fusion protein is often insoluble (Lin et al. 1987), allowing a rapid and simple one-step purification. Our λEXLX(+) and λEXLX(-) vectors are λ-based versions of the pET expression vectors that additionally feature directional cDNA cloning with the protection of all the internal sites, Cre-*loxP* automatic subcloning and the ability to produce ss DNA by induction of an f1 *ori*. Recently, we have generated a monoclonal antibody to the T7 gene*10* protein product (C. Mayeda and M.J.P., unpub.) and are currently pursuing the use of this antibody in the immunopurification of expressed gene*10* fusion proteins.

The use of the λEXLX vectors for gene*10* fusion protein expression requires that the gene*10* and cDNA ORFs be in the same frame. In previous expression vectors (Young and Davis 1983) it was important to ensure that the cDNA clone was inserted into the vector in the appropriate reading frame. In the case of the λEXLX vectors, ss DNA (purified as outlined in sections *e[ii] and [iii]*

above) can be used to alter the reading frame of any individual clone by a variety of in vitro mutagenesis methods (Taylor et al. 1985; Kunkel et al. 1986) that involve priming of DNA synthesis with oligos containing small mismatches designed either to insert or to delete nucleotides.

(i) Applications of the λSHLX vectors.

Two methods have been developed for using cDNA libraries to generate the large amounts of the ss DNA required for subtractive hybridization procedures (Palazzolo and Meyerowitz 1987; Pruitt 1988). The λSHLX vectors have been designed to facilitate either strategy.

One strategy follows the hybridization selection methods of Pruitt (1988). Briefly, a cDNA library representing sample X is prepared in λSHLX1, and one representing Y is constructed in λSHLX2. Each library is then amplified and aliquots are converted to ss DNA as outlined in section (e[ii] and [iii]) above. Since the multiple cloning sites in the SHLX1 and SHLX2 vectors are in opposite orientations, the ss DNA recovered from the converted pSHLX1 library will represent the "sense" orientation and that from the pSHLX 2 library the "antisense" orientation. The ss Y-DNA can then be covalently attached to an insoluble substrate and the X-DNA can be hybridized to an excess of the Y-DNA. The soluble fraction, that should be enriched in X-specific sequences, can then be transformed into *E. coli* to give an "X minus Y" plasmid library (Pruitt 1988).

In the second strategy (modified from Palazzolo and Meyerowitz 1987 and Palazzolo et al. 1989), X- and Y-cRNA can be synthesized in vitro using as template either plasmid or phage DNA prepared from each library. Specifically, DNA from the X-library in automatically subcloned pSHLX1 can be linearized at the *Not*I site (since this enzyme has an 8 bp recognition sequence, this reduces the probability of digestion within the cloned cDNA sequences) and then used as

template for a T7 RNA polymerase reaction to make "sense" X-cRNA. Similarly, DNA from the Y-library in pSHLX2 can be linearized at the *Sfi*I site and used as template for a SP6 RNA polymerase reaction to make "sense" Y-cRNA. The X-cRNA can be reverse transcribed to make "antisense" cDNA; this is accomplished by priming cDNA synthesis with an oligo complementary to the SP6 promoter sequences present in the X-cRNA (this is preferable to the use of an oligo comprised partly of the *Not*I recognition sequence since the latter is GC-rich). This "antisense" X-cDNA can be hybridized to an excess of "sense" Y-cRNA. After subtractive hybridization the "X minus Y"-cDNA sequences can be made ds and recloned.

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REFERENCES

Brenner, S., Cesarini, G. and Karn, J.: Phasmids: hybrids between ColE1 plasmids and *E. coli* bacteriophage lambda. Gene 17 (1982) 27-44.

Bullock, W. O., Fernandez, J. M. and Short, J. M.: XL-1 blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. Biotechniques 5 (1987) 376-379.

Dieckmann, C.L. and Tzagoloff, A.: Assembly of the mitochondrial membrane system: *CBP6*, a yeast nuclear gene necessary for synthesis of cytochrome b. J. Biol. Chem. 260 (1985) 1513-1520.

Huynh, T.V., Young, R.A. and Davis, R.W.: Constructing and screening cDNA libraries in λgt10 and λgt11. In Glover, D.M. (Ed.) DNA Cloning, Vol. 1, IRL Press, Oxford (U.K.) 1985, pp. 49-78.

Kunkel, T.A., Roberts, J.D. and Zakour, R.A.: Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. 154 (1986) 367-382.

Lin, S., Dunn, J.J., Studier, F.W. and Stafford, D.W.: Expression of human factor IX and its subfragments in *Escherichia coli* and generation of antibodies to the subfragments. Biochemistry 26 (1987) 5267-5274.

Maniatis, T., Fritsch, E.F. and Sambrook, J.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1982.

Mead, D.A., Szczesna-Skorupa, E., and Kemper, B.: Single-stranded DNA 'blue' T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. Protein Engineering 1 (1986) 67-74.

Meissner, P.S., Sisk, W.P. and Berman, M.L.: Bacteriophage λ cloning system for the construction of directional cDNA libraries. Proc. Natl. Acad. Sci. USA 84 (1987) 4171-4175.

Moffatt, B.A. and Studier, F.W.: T7 lysozyme inhibits transcription by T7 RNA polymerase. Cell 49 (1987) 221-227.

Murray, N.: Lambda Vectors. In Hendrix, R. W., Roberts, J. W., Stahl, F. W., and Weisberg, R. A. (Eds.) Lambda II, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1983, pp. 677-684.

Nagashima, K., Akasai, M., Nagata, S. and Kaziro, Y.: Structure of the two genes coding for polypeptide chain elongation factor 1-alpha (EF-1-alpha) from *Saccharomyces cerevisiae*. Gene 45 (1986) 265-273.

Palazzolo, M.J. and Meyerowitz, E.M.: A family of lambda phage cDNA cloning vectors, λSWAJ, allowing the amplification of RNA sequences. Gene 52 (1987) 197-206.

Palazzolo, M.J. , Hyde, D., Raghavan, K.V., Mecklenberg, K., Benzer, S. and Meyerowitz, E.M.: Isolation and characterization of 436 Drosophila cDNA clones corresponding to RNAs expressed in the adult head but not detected in the early embryo using a new cloning strategy. Neuron 3 (1989) 527-539.

Pouwels, P.H., Enger-Valk, B.E. and Brammar, W.J.: Cloning Vectors: A Laboratory Manual. Elsevier, Amsterdam 1985, p. I-A-vi-11.

Pruitt, S.C.: Expression vectors permitting cDNA cloning and enrichment for specific sequences by hybridization selection. Gene 66 (1988) 121-134.

Raleigh, E.A. and Wilson, G.: *Escherichia coli* K-12 restricts DNA containing 5-mrthylcytosine. Proc. Natl. Acad. Sci. USA 83 (1986) 9070-9074.

Rosenberg, A.H., Lade, B.N., Chui, D., Lin, S., Dunn, J.J. and Studier, F.W.: Vectors for selective expression of cloned DNAs by T7 RNA polymerase.

Gene 56 (1987) 125-135.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A.: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239 (1988) 497-491.

Sauer, B. and Henderson, N.: The cyclization of linear DNA in *Escherichia coli* by site-specific recombination. Gene 70 (1988) 331-341.

Short, J.M., Fernandez, J.M., Sorge, J.A., and Huse, W.D.: λZAP: a bacteriophage λ expression vector with *in vivo* excision properties. Nucl. Acids Res. 16 (1988) 7583-7600.

Snutch, T.P., Heschl, M.F.P. and Baillie, D.L.: The *Caenorhabditis elegans hsp70* gene family: a molecular genetic characterization. Gene 64 (1988) 241-256.

Sternberg, N., Hoess, R. and Abremski, K.: The P1 *lox*-Cre site specific recombination system: properties of *lox* sites and biochemistry of *lox*-Cre interactions. In Cozarelli, N.R. (Ed.) Mechanisms of DNA Replication and Recombination, UCLA Symposia on Molecular and Cellular Biology, Vol. 10, Alan R. Liss, New York, 1983, pp. 671-684.

Studier, F.W. and Moffat, B.: Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189 (1986) 113-130.

Studier, F.W., Rosenberg, A.H. and Dunn, J.J.: Use of T7 RNA polymerase to direct the expression of cloned genes. Meth. Enzymol. (1990) (in press)

Taylor, J.W., Ott, J. and Eckstein, F.: The rapid generation of oligodeoxyribonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. 13 (1985) 8764-8785.

Thummel, C.S., Boulet, A.M. and Lipshitz, H.D.: Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. Gene 74 (1988) 445-456.

Yanisch-Perron, C., Vieira, J., and Messing, J.: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33 (1985) 103-119.

Yarmolinsky, M.B., Hansan, E.B., Jafri, S. and Chattoraj, D.K.: Participation of the lytic replicon in P1 plasmid maintenance. J. Bacteriol. 171 (1989) 4785-4791.

Young, R.A. and Davis, R.W.: Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. USA 80 (1983) 1194-1198.

FIGURE LEGENDS

Fig. 1. Construction and test of λGLX. The *loxP* plasmid, called pGLX2, was generated first and then inserted into the λgt10 phage arms. pGLX2 was constructed by inserting two complementary oligos that contain a *Xho*I site flanked by directly repeated *loxP* sites into the *Sma*I site of pZF18R (Mead et al. 1986). pGLX2 thus contains two directly repeated *loxP* sites that are flanked by a variety of unique restriction sites and are separated by a unique *Xho*I site. This plasmid was then digested with *Xho*I and ligated into the *Xho*I site of λgt10 to generate λGLX. The phage contains an *Xho*I site flanked by *loxP* sites. A P1 lysogenic strain (DM103[P1]) was then infected with this phage and Ap^R plasmids, pGLX1, were recovered post-conversion and found to contain a single *loxP* site. The directions of transcription are represented by arrows.

Fig. 2. Construction of λLOX, arms with *loxP* sites. λLOX is a derivative of λNM1150 (Murray 1983; Pouwels et al. 1985) in which the *Apa*I, *Hin*dIII and *Eco*RI sites have been removed and two *loxP* sites have been inserted into the *Xho* I site. Phage mλJP3 (M. Strathmann and M. Simon, unpub.) is a derivative of λNM1150 in which the *Apa*I site in the left arm has been removed and a new *Apa*I site has been introduced in the right arm (M. Strathmann and M. Simon, pers. comm.). mλJP3 and λNM1150 DNAs were mixed and digested with *SnaB*I. The digested mixture was ligated and then digested with *Apa*I to eliminate the undesirable ligation products. DNA from the resulting phage (λPH1) was digested with *Xho*I and ligated to *Xho*I-digested pGLX2 DNA (see Fig. 1 for structure of pGLX2), to introduce *loxP* sites into the λ vector. DNA from this phage (λPH2) was digested with *Sac*I+*Xba*I to remove the plasmid sequences and ligated to an 850 bp stuffer fragment of SV40 DNA taken from pC4ßgal

(Thummel et al. 1988) to maintain minimum phage genome size for efficient packaging. The *cos* ends of the resulting phage (λPH3) DNA were ligated. In order to eliminate the *Eco*RI and *Hin*dIII sites, digestion was carried out with these two enzymes (hence deleting DNA between the sites), followed by end-filling with PolIk and religation. This was followed by a second *Hin*dIII, *Eco*RI digestion to reduce background. The resultant phage (λLOX) gave large, clear plaques, did not hybridize to plasmid DNA probe and did not contain restriction sites for *Apa*I, *Eco*RI, or *Hin*dIII. After digestion of λLOX with *Xba*I+*Sac*I (and blunting the ends with T4 DNA polymerase if necessary), any linearized plasmid with appropriate ends can be ligated between the *loxP* sites. A detailed map of λLOX is given in Fig. 3. Restriction sites are marked: A, *Apa*I; H, *Hin*dIII; R, *Eco*RI; Sc, *Sac*I; Sn, *SnaB*I; X, *Xho*I; Xb, *Xba*I. ^, λLOX vectors with either orientation of the *Xba*I and *Sac*I sites are available. Figures are not to scale.

Fig. 3. Structure of λLOX. A detailed map of λLOX is shown, with the names and locations (±10 bp) of the restriction sites above. Below are shown the locations of deletions and insertions relative to wt; unshaded boxes represent wt λ DNA, black shading represents pGLX2/SV40 DNA that contains *Sac*I and *Xba*I sites not shown on the map of λLOX (see Fig. 2), and hatched shading represents the *c*I gene of phage 434. Since λLOX is derived from λNM1150, it too is Red^- (see Pouwels et al. 1985 for a detailed map and genotype of λNM1150). ΔH3/RI, deletion of DNA between the *Eco*RI and *Hin*dIII sites (see Fig. 2 legend).

Fig. 4. Construction of T7 gene*10*-cDNA fusion protein expression vectors λEXLX. The expression plasmids in λEXLX(+) and λEXLX(-) contain a plasmid backbone derived from pGEM3Zf (Promega, Inc.), an inducible promoter and protein-coding region derived from pET3xa (Rosenberg et al. 1987), and a

multiple cloning site and an SP6 RNA polymerase promoter (antisense to the protein-coding region) constructed from synthetic oligos. The two plasmids differ from each other in whether the plasmid backbone was derived from pGEM3Zf(+) in the case of λEXLX(+) or pGEM3Zf(-) in the case of λEXLX(-), and thus in the relative orientation of the f1 *ori*. The pMP1 plasmids were constructed as follows: pGEM3Zf plasmids were digested with *Pvu*II to remove the *lacZ* gene and cloning site, and were then ligated to a pair of complementary synthetic oligos containing *Hin*cII, *Hin*dIII, *Bgl*II and *Sma*I restriction sites. The resulting plasmids, pMP1(+) and pMP1(-), were digested with *Bgl*II+*Hin*cII and ligated to the small *Bgl*II-*Eco*RV fragment of pET3xa to create pMP2(+) and pMP2(-). This fragment of pET3xa contains the T7 gene*10* promoter, the coding sequence for the first 260 aa of the T7 gene*10* product, a *BamH*I restriction site, and a T7 transcription terminator (Rosenberg et al. 1987). The pMP2(+) and pMP2(-) plasmids were inserted into phage mλJ (provided by M. Strathmann and M. Simon) by homologous recombination with a fragment of the *E. coli bla* gene in the mλJ phage. mλJ is a λNM1150 derivative that contains 650 bp of the *E. coli bla* gene inserted between the *Eco*RI and *Hin*dIII sites, and also lacks the *Apa*I site present in λNM1150. Integration was detected by growth on a λ-lysogen; this requires replication of the phage DNA off the plasmid *ori*. DNAs from the resulting phages, mλMP2(+) and mλMP2(-), were digested with *BamH*I and ligated to a pair of oligos to insert *Hin*dIII, *Xba*I, and *Eco*RI restriction sites and destroy the *BamH*I site. The DNA from these phages, mλMP2P and mλMP2A, was digested with *Hin*dIII+*Eco*RI and ligated to a second pair of complementary oligos that introduced *Sac*I, *Hin*dIII, *Apa*I and SP6 RNA polymerase promoter sites, while destroying the original *Hin*dIII site. The plasmids, pMP3(+) and pMP3(-), were recovered in a λ-lysogenic strain (MP23; M. Strathmann and M. Simon, unpublished) by homologous recombination between the partial *bla* genes,

under selection for Ap^R. The pMP3(+) and pMP3(-) plasmids were digested with *Sma*I and inserted into the λLOX vector arms that had been digested with *Sac*I+*Xba*I and blunt-ended with T4 DNA polymerase, to create the final cloning vectors λEXLX(+) and λEXLX(-) (see also Fig. 6A). HcII, *Hin*cII; RV, *Eco*RV. The directions of transcription are represented by arrows.

Fig. 5. Construction of the λSHLX vectors. pCM1 (C.H.M., C. Mayeda and M.J.P., unpub.) was chosen to build the λSHLX vectors because it already contained most of the desirable plasmid elements and only required alteration of the polylinker. pCM1 contains the pUC *ori*, the f1 *ori* and the Ap^R marker derived from the *Pvu*II fragment of pGEM3Zf(+). It also contains a 1 kb fragment from pATH (Dieckmann and Tzagoloff 1985). This pATH fragment is vestigial from an earlier construction and has no relevance to the λSHLX vectors. To construct the λSHLX vectors, pCM1 DNA was digested with *Hind*III+*Sac*I. Two pairs of complementary oligos were separately ligated to generate new polylinkers with unique *Sac*I, *Eco*RI, *Xba*I, *Apa*I, and *Hind*III restriction sites between the SP6 and T7 RNA polymerase promoters. The resulting plasmid was named pDD1. A second version, called pDD2, with the orientation of the multiple cloning sites reversed relative to pDD1 was also constructed. The pDD plasmids were linearized at the *Hpa*I site and ligated to λLOX DNA that had been digested with *Sac*I+*Xba*I and blunted with T4 DNA polymerase. The resultant phage vectors were named λSHLX1 and λSHLX2 (see also Fig. 6B).

Fig. 6. Structures of the automatically subcloned plasmid portions of the (A) λEXLX and (B) λSHLX vectors. The pUC *ori*, β-lactamase gene (*bla*) and f1 *ori* are from pGEM3Zf(+) in pEXLX(+) and pSHLX, or from pGEM3Zf(-) in pEXLX(-). The T7 promoter, gene*10* protein coding fragment, and T7

transcription terminator in pEXLX plasmids are from pET3xa (Rosenberg et al. 1987). Multiple cloning sites and additional bacteriophage promoters were introduced with oligos as described in Figs. 2, 4 and 5. The directions of transcription and the orientation of the f1 *ori* are represented by arrows. The gene*10* reading frame in the pEXLX polylinker is indicated by the periods. Tϕ, T7 transcription terminator; tsp, transcription start point. Figs. are not to scale; approximate distances between restriction sites are given in bp.

TABLE I

Bacterial strains used to perform and test in vivo subcloning

Strain[a]	Based on (Ref.)[b]	Prophages[c] (Ref.)[b]	Additional properties[c]	Comments
DM103[P1]	JM103 (1)	P1Cm^{R}r$^-$m$^-$(5)	F',Tc^S, *recA*$^+$	
BH23	KW251 (2)	λ434*kan* (4)	F$^-$, Tc^R, *recA*$^+$, *mcrA*, *mcrB*(6)	
KW251[P1]	"	P1Cm^{R}r$^-$m$^-$(5)	- " -	
BM25.5	"	λ434*kan*, P1Cm^{R}r$^-$m$^-$	- " -	Robust colonies. Good DNA yields for pEXLX(+).
BH24	XL1-blue (3)	λ434*kan*	F', Tc^R, *recA*$^-$	
BH42	"	P1Cm^{R}r$^-$m$^-$	- " -	
BM25.6	"	λ434*kan*, P1Cm^{R}r$^-$m$^-$	- " -	Minute colonies. Can mobilize plasmid to new host by two-step f1 rescue.
BH28	JM101 (1)	λ434*kan*	F', Tc^S, *recA*$^+$	
BH82	"	P1Cm^{R}r$^-$m$^-$	- " -	
BM25.8	"	λ434*kan*, P1Cm^{R}r$^-$m$^-$	- " -	Can mobilize plasmid to new host by one-step f1 rescue.

[a] Lysogens for λ434*kan* were obtained by infecting 0.5 to 1.5 ml of fresh saturated culture with 10^7 to 10^8 pfu for 20 min at 37°C. Infections were spread on plates containing 50 μg Km/ml to select lysogens. To construct P1Cm^{R}r$^-$m$^-$ lysogens, 0.05 to 0.5 ml of fresh saturated culture was made 5 to 20 μM for $CaCl_2$ and then infected with 10^5 to 10^7 pfu for 20 min at 37°C. P1 lysogens were selected on plates containing 30 μg Cm/ml. Strains lysogenic for both phage were constructed serially, by infecting the λ434*kan* lysogen with P1 phage.

[b] (1) Yanisch-Perron *et al.* (1985); (2) Promega, Inc., Madison, WI (U.S.A.); (3) Bullock *et al.* (1987); (4) M. Strathmann and M. Simon (unpub.); (5) Yarmolinsky *et al.* (1989); (6) Raleigh and Wilson (1986).

[c] *kan*, gene encoding Km resistance; r$^-$m$^-$, mutant in both the restriction and modification functions of P1 (5); *mcr*, mutant in methylcytosine-specific restriction systems (6); F' markers: JM101 and 103 derivatives [F', *tra*D36, *pro*AB, *lac*I^{q}ZΔM15] (1); XL1-blue derivatives [F', *tra*D36, *pro*AB, *lac*I^{q}ZΔM15, Tn10, (Tc^R)] (3).

TABLE II

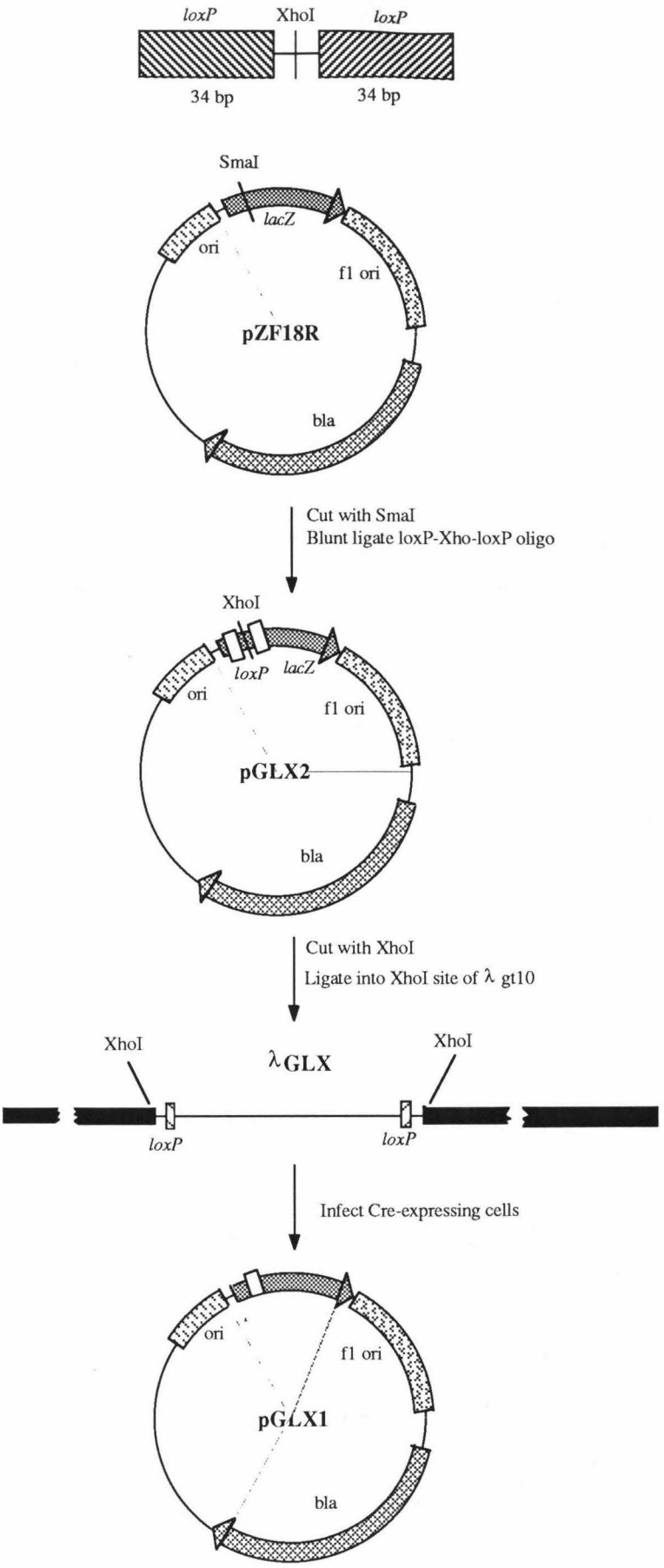
Apparent conversion frequencies of λEXLX(+) vector and λEXLX(+) cDNA clones in a P1 lysogen (KW251[P1]) and in a P1-λ double-lysogen (BM25.5).

Vector/cDNA[a]	Non-P1 lysogen[b]	Number of[b] plaques	P1 lysogen[b]	Number of[b] colonies	Conversion[c] (%)
λEXLX(+)	KW251	282	KW251[P1]	75	27
			BM25.5	117	41
λEXLX(+)	KW251	413	KW251[P1]	22	5
			BM25.5	190	46
λEXLX(+)cDNA1	KW251	315	BM25.5	372	118
λEXLX(+)cDNA2	KW251	171	BM25.5	220	129
λEXLX(+)cDNA3	KW251	171	BM25.5	144	84
λEXLX(+)cDNA4	KW251	110	BM25.5	140	127
λEXLX(+)cDNA5	KW251	364	BM25.5	332	91
λEXLX(+)cDNA6	KW251	140	BM25.5	148	106
λEXLX(+)cDNA7	KW251	392	BM25.5	396	101
λEXLX(+)cDNA8	KW251	91	BM25.5	128	141
λEXLX(+)cDNA9	KW251	290	BM25.5	0	0
λEXLX(+)cDNA10	KW251	170	BM25.5	212	125
λEXLX(+)cDNA11	KW251	104	BM25.5	88	85
λEXLX(+)cDNA12	KW251	349	BM25.5	344	99

[a] The results of two representative experiments with the λEXLX(+) vector alone are shown. The random cDNA clones were isolated from the *Drosophila* λEXLX(+) cDNA library described in section (e[vii]).

[b] In each experiment, equal volumes of appropriate phage suspension were used to infect equal volumes of saturated cultures of each *E. coli* strain. Infections were carried out for 20 min at 37°C. Infections for phage titer were plated in NZCYM top agarose. Infections for plasmid conversion were spread on plates containing Ap or carbenicillin (with or without Km and Tc; the presence of these two drugs did not influence the number of colonies).That these colonies resulted from plasmid excision was verified by growing cells from several individual colonies overnight in liquid culture, preparing rapid plasmid DNA preparations and analyzing the restriction fragments generated by digestion with diagnostic restriction enzymes (*Hinf*I for the vector alone, *Apa*I+*Sac*I for cDNA clones). In all cases tested, appropriate restriction patterns were observed (data not shown). cDNA 9 did not convert upon retesting; however, it appears to be unique in that we have seen no other such non-converting clones in several subsequent experiments. Similar apparent conversion frequencies were found for λSHLX vectors and cDNA clones as well as for other lysogenic host strains (data not shown). In all cases, fresh cultures of BM25 cells gave better apparent conversion frequencies than did either old cultures or cultures grown beyond saturation.

[c] Apparent conversion frequency is defined in section (e[i]) and is listed here as a percentage "Conversion (%)".


loxP
XhoI
loxP
34 bp
34 bp
SmaI
lacZ
ori
f1 ori
pZF18R
bla
Cut with SmaI
Blunt ligate loxP-Xho-loxP oligo
XhoI
loxP
lacZ
ori
f1 ori
pGLX2
bla
Cut with XhoI
Ligate into XhoI site of λ gt10
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λGLX
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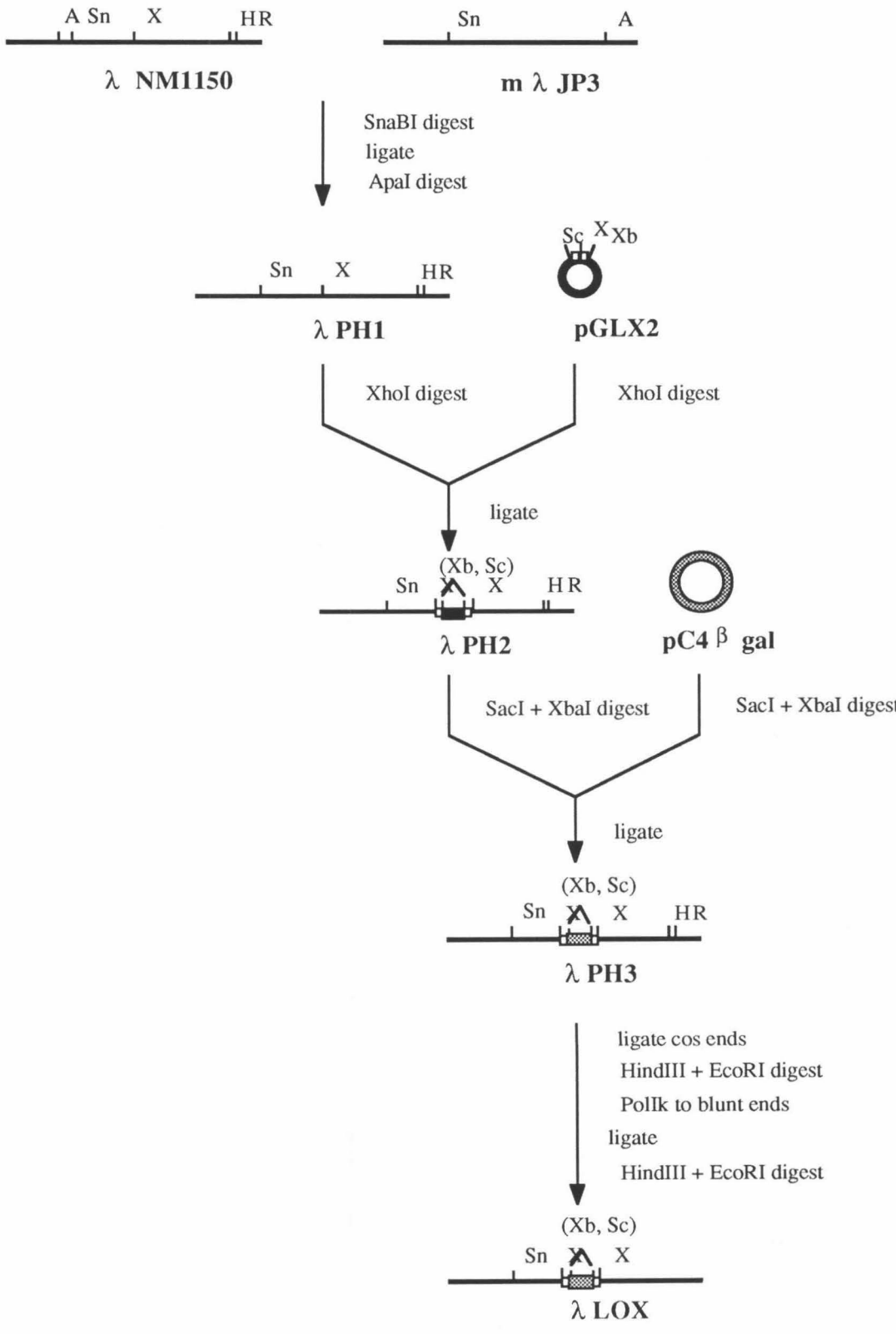
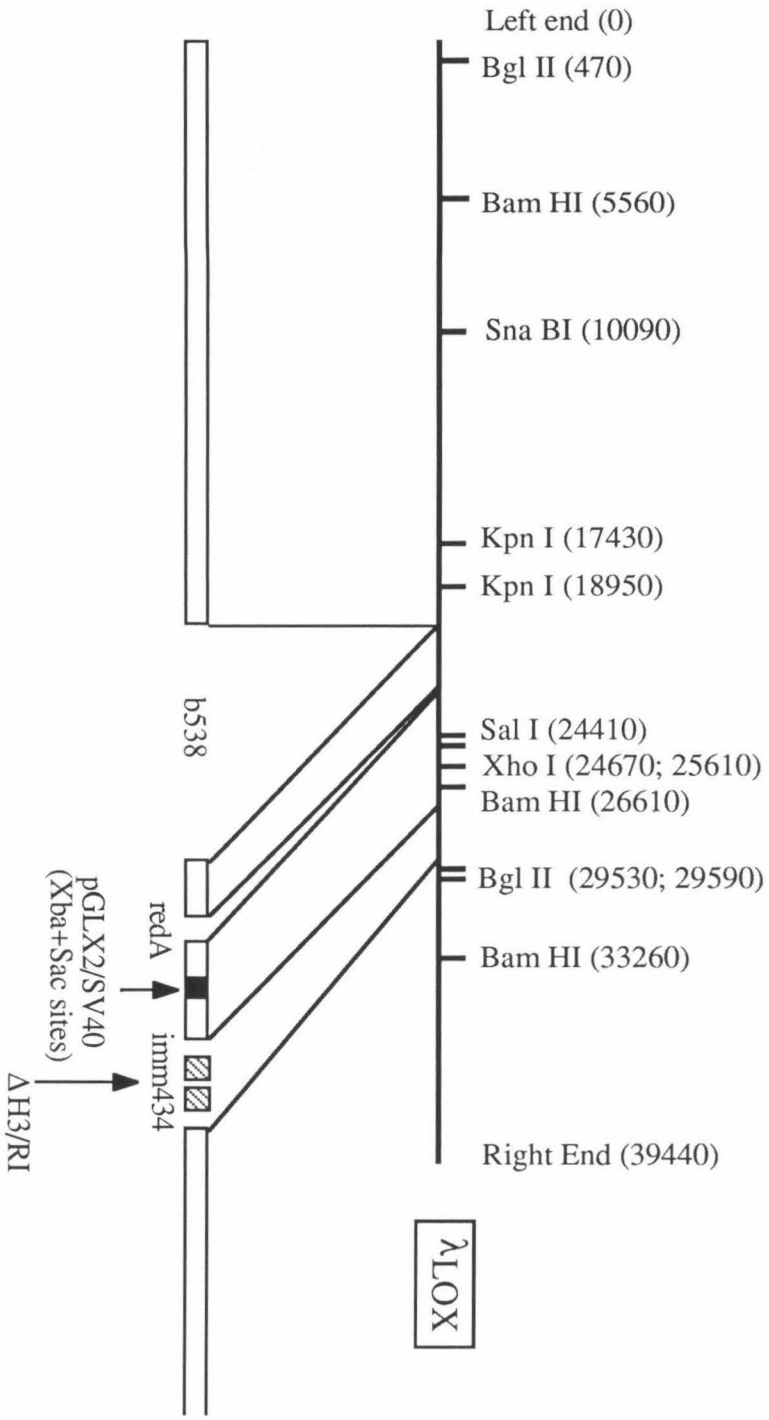
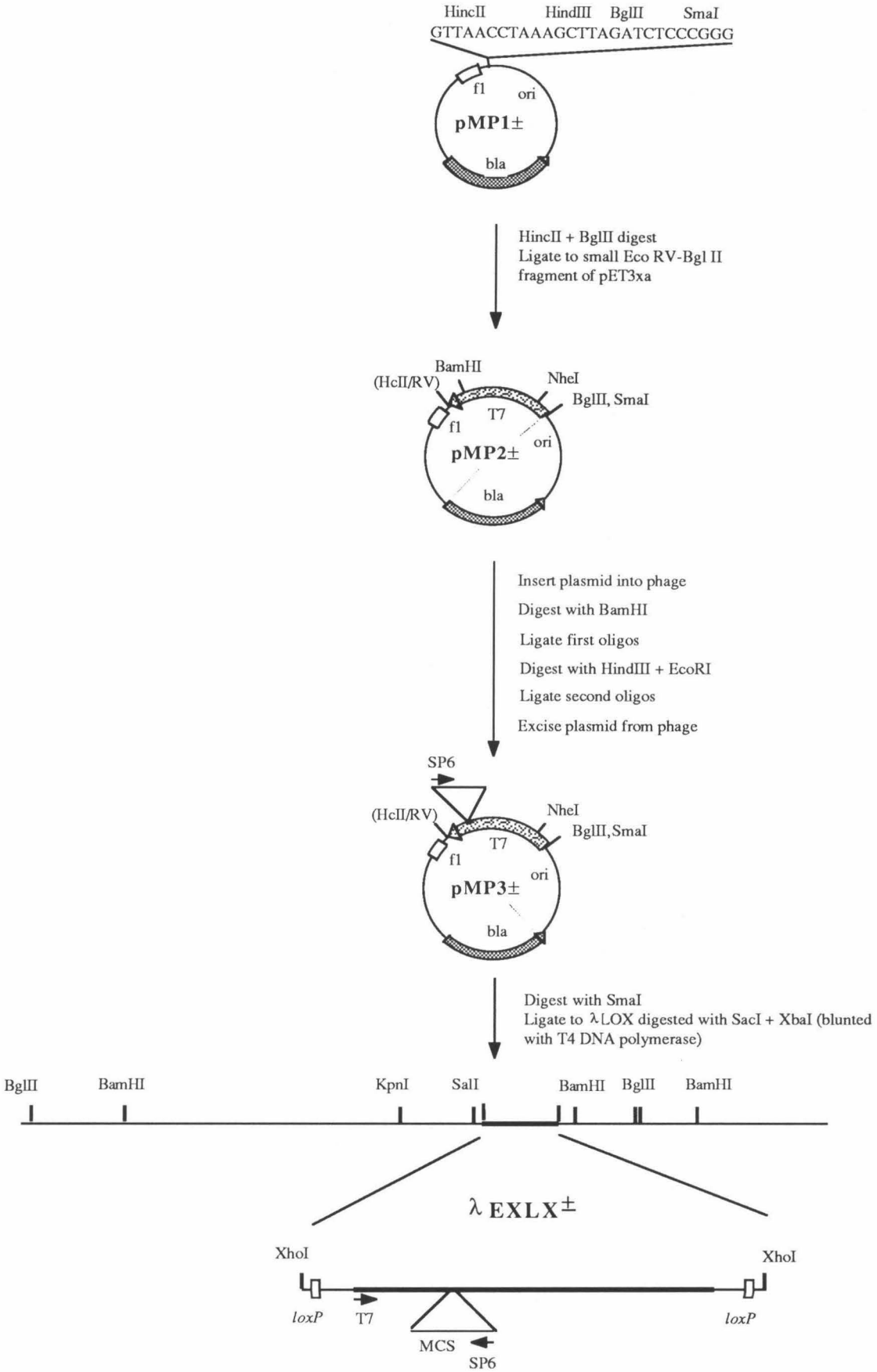
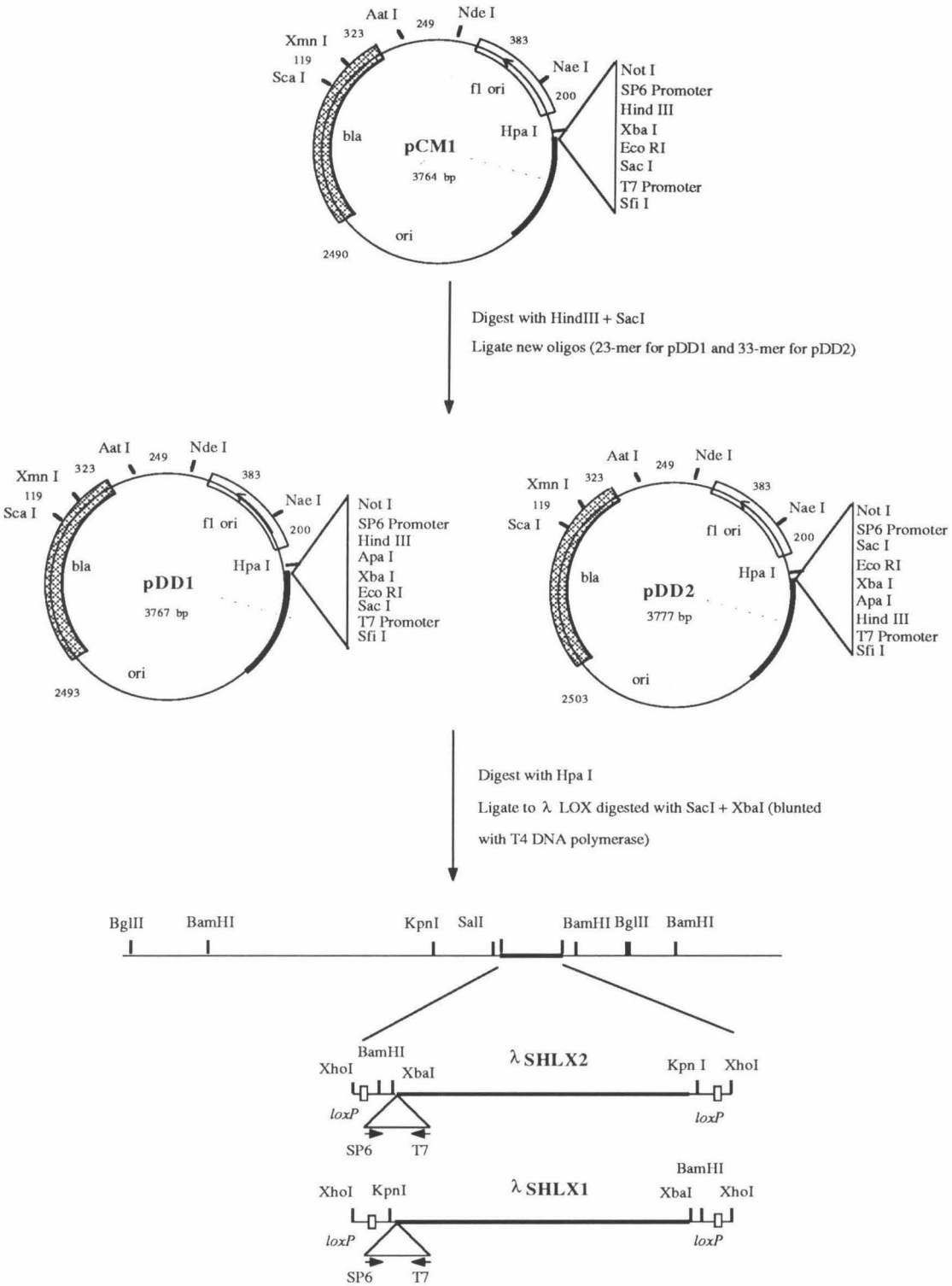
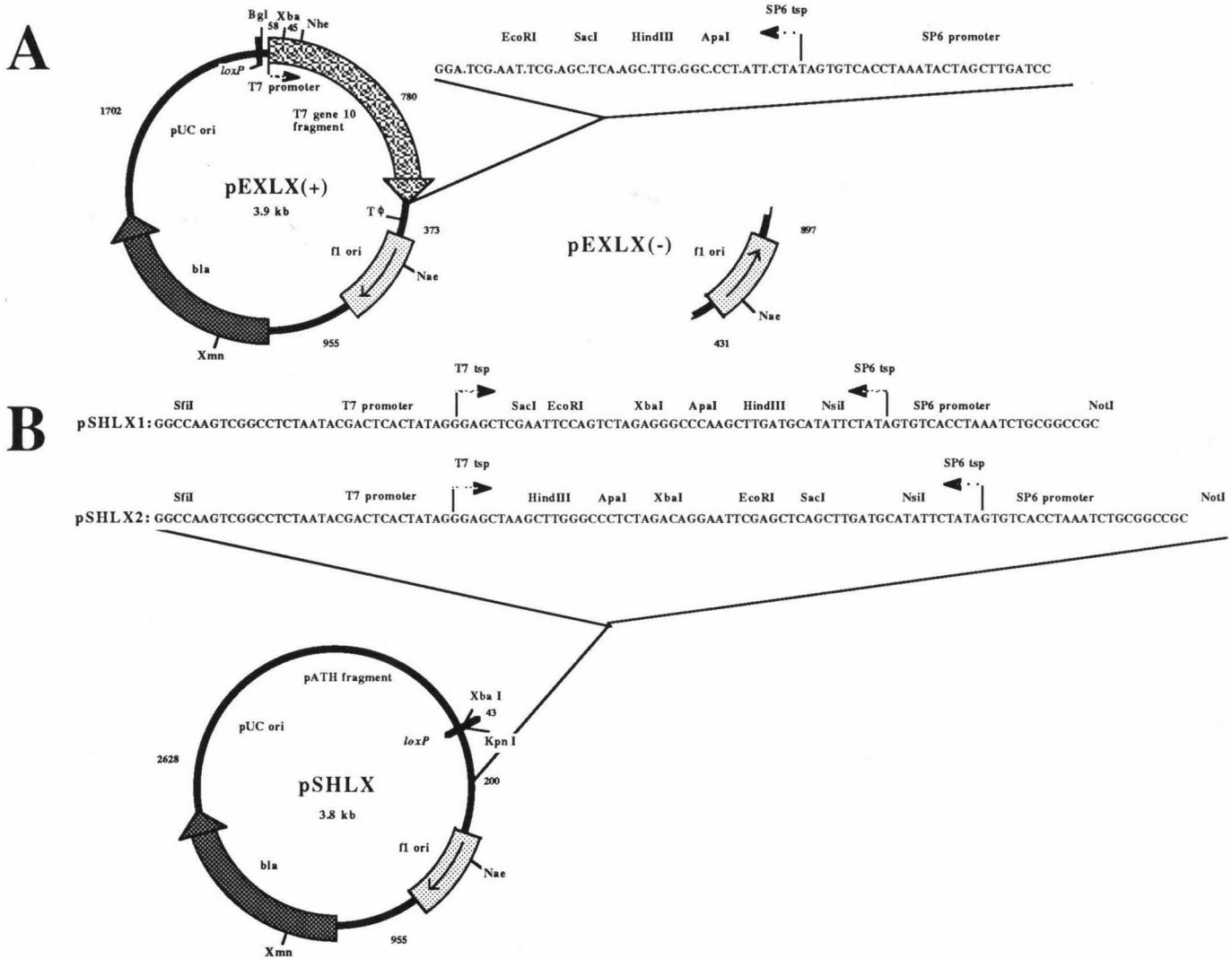
loxP
Infect Cre-expressing cells
ori
f1 ori
pGLX1
bla


A Sn X HR
λ NM1150
Sn A
m λ JP3
SnaBI digest
ligate
ApaI digest
Sc X Xb
Sn X HR
λ PH1
pGLX2
XhoI digest
XhoI digest
ligate
(Xb, Sc)
Sn X HR
λ PH2
pC4 β gal
SacI + XbaI digest
SacI + XbaI digest
ligate
(Xb, Sc)
Sn X HR
λ PH3
ligate cos ends
HindIII + EcoRI digest
PolIk to blunt ends
ligate
HindIII + EcoRI digest
(Xb, Sc)
Sn X
λ LOX


Left end (0)
Bgl II (470)
Bam HI (5560)
Sna BI (10090)
Kpn I (17430)
Kpn I (18950)
b538
Sal I (24410)
Xho I (24670; 25610)
Bam HI (26610)
Bgl II (29530; 29590)
redA
pGLX2/SV40
(Xba+Sac sites)
Bam HI (33260)
imm434
ΔH3/RI
Right End (39440)
λLOX


HincII HindIII BglII SmaI
GTTAACCTAAAGCTTAGATCTCCCGGG
f1
ori
pMP1±
bla
HincII + BglII digest
Ligate to small Eco RV-Bgl II
fragment of pET3xa
BamHI
(HcII/RV)
NheI
BglII, SmaI
T7
f1
ori
pMP2±
bla
Insert plasmid into phage
Digest with BamHI
Ligate first oligos
Digest with HindIII + EcoRI
Ligate second oligos
Excise plasmid from phage
SP6
(HcII/RV)
NheI
BglII,SmaI
T7
f1
ori
pMP3±
bla
Digest with SmaI
Ligate to λLOX digested with SacI + XbaI (blunted
with T4 DNA polymerase)
BglII
BamHI
KpnI
SalI
BamHI
BglII
BamHI
λ EXLX±
XhoI
XhoI
loxP
T7
MCS
SP6
loxP


Aat I
Nde I
Xmn I
323
249
383
119
Sca I
Nae I
Not I
SP6 Promoter
Hind III
Xba I
Eco RI
Sac I
T7 Promoter
Sfi I
f1 ori
200
bla
Hpa I
pCM1
3764 bp
ori
2490
Digest with HindIII + SacI
Ligate new oligos (23-mer for pDD1 and 33-mer for pDD2)
pDD1
3767 bp
2493
Apa I
pDD2
3777 bp
2503
Digest with Hpa I
Ligate to λ LOX digested with SacI + XbaI (blunted
with T4 DNA polymerase)
BglII
BamHI
KpnI
SalI
XhoI
XbaI
λ SHLX2
Kpn I
loxP
SP6
T7
λ SHLX1


A
Bgl
Xba
Nhe
58
45
loxP
T7 promoter
780
1702
pUC ori
T7 gene 10
fragment
pEXLX(+)
3.9 kb
Tϕ
373
f1 ori
bla
Nae
Xmn
955
SP6 tsp
EcoRI
SacI
HindIII
ApaI
SP6 promoter
GGA.TCG.AAT.TCG.AGC.TCA.AGC.TTG.GGC.CCT.ATT.CTATAGTGTCACCTAAATACTAGCTTGATCC
pEXLX(-)
f1 ori
897
Nae
431
B
T7 tsp
SP6 tsp
SfiI
T7 promoter
SacI
EcoRI
XbaI
ApaI
HindIII
NsiI
SP6 promoter
NotI
pSHLX1: GGCCAAGTCGGCCTCTAATACGACTCACTATAGGGAGCTCGAATTCCAGTCTAGAGGGCCCAAGCTTGATGCATATTCTATAGTGTCACCTAAATCTGCGGCCGC
T7 tsp
SP6 tsp
SfiI
T7 promoter
HindIII
ApaI
XbaI
EcoRI
SacI
NsiI
SP6 promoter
NotI
pSHLX2: GGCCAAGTCGGCCTCTAATACGACTCACTATAGGGAGCTAAGCTTGGGCCCTCTAGACAGGAATTCGAGCTCAGCTTGATGCATATTCTATAGTGTCACCTAAATCTGCGGCCGC
pATH fragment
Xba I
43
pUC ori
loxP
Kpn I
2628
pSHLX
3.8 kb
200
f1 ori
bla
Nae
Xmn
955

CHAPTER 3

This chapter has been submitted for publication in *Molecular and Cellular Biology*.

Dynamic *Hsp83* RNA localization during *Drosophila* oogenesis and embryogenesis

Running title: Dynamic localization of *Drosophila Hsp83* RNA

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Key words: *Hsp83*, polar plasm, *Drosophila*, RNA localization, *bicoid*, oogenesis, embryogenesis

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ABSTRACT

Hsp83 is the _Drosophila_ homolog of the mammalian _Hsp90_ family of regulatory molecular chaperones. We show that maternally synthesized _Hsp83_ transcripts are localized to the posterior pole of the early _Drosophila_ embryo by a combination of generalized RNA degradation and local protection at the posterior. This protection of _Hsp83_ RNA occurs in wildtype embryos and embryos produced by females carrying the maternal effect mutations _nanos_ and _pumilio_, which eliminate components of the polar plasm without disrupting polar granule integrity. In contrast, _Hsp83_ RNA is not protected at the posterior pole of embryos produced by seven maternal mutants that disrupt polar granules - _cappuccino_, _oskar_, _spire_, _staufen_, _tudor_, _valois_ and _vasa_ - suggesting that _Hsp83_ RNA is a component of the posterior polar plasm that might be associated with polar granules. In addition, we show that zygotic expression of _Hsp83_ commences in the anterior third of the embryo at the syncytial blastoderm stage and is regulated by the anterior morphogen, _bicoid_. We consider the possible developmental significance of this complex control of _Hsp83_ transcript distribution.

INTRODUCTION

Cytoplasmically-localized determinants, in the form of localized maternal RNAs and proteins, play a key role in providing positional cues in the oocyte and early embryo of *Drosophila*. To date, two RNAs localized to the anterior pole of both the oocyte and the early embryo have been described: *bicoid* RNA, which encodes the anterior determinant (9, 10, 56, 58), and RNA encoding a *Drosophila* adducin homolog (8). A number of maternally active genes encode posteriorly-

localized molecules. Proteins encoded by *germ cell-less, oskar*, *vasa* and *staufen*, and RNAs encoded by *cyclin B*, *germ cell-less*, *nanos*, *orb*, *oskar*, *pumilio* and *tudor* are localized components of the posterior polar plasm, a posteriorly-located, yolk-free cytoplasmic cap that is continuous with the cortical cytoplasm of the egg and early embryo (12, 13, 17, 20, 21, 22, 25, 27, 32, 33, 34, 40, 42, 53, 57, 62). Important components of this polar plasm are the polar granules - electron-dense, non-membrane-bound organelles - that reside within 4 μm of the plasma membrane at the posterior tip of the oocyte and early embryo (24, 44, 45, 46). The polar granules are taken up into the pole cells - the primordial germ cells of *Drosophila* - as these cells bud off the posterior end of the embryo, and have been postulated to play a key role in programming them to adopt germline fates.

Many maternally transcribed genes controlling positional cues along the antero-posterior axis of the *Drosophila* embryo have been identified through the isolation of maternal effect female-sterile mutations, as well as through analysis of maternal requirements for expression of zygotic lethal loci as assayed in germline clones (5, 6, 16, 36, 38, 39, 47, 52, 54, 55, 63). A limitation of these genetic screens is that only genes which carry out non-redundant functions can readily be identified. Further, it has been estimated that only one third of the transcription units of the *Drosophila* genome have been genetically defined (4). Thus, it is likely that genetic screens have not identified all of the important components of the embryonic cell fate specification machinery. To isolate additional molecules involved in cell fate specification along the antero-posterior axis, we carried out a differential screen for cDNAs representing RNAs that are localized to either the anterior or the posterior pole of the *Drosophila* oocyte or early embryo (D.D. and H.D.L., in preparation; see Materials and Methods).

Here we report the characterization of the first posterior-localized RNA to be identified in this screen. Sequence analysis shows that it is encoded by the

Hsp83 gene, the *Drosophila* homolog of the mammalian *Hsp90* gene family (2, 19). Despite the extensive biochemical studies of this family of cytoplasmically-active regulatory molecular chaperones in mammals, yeast and *Drosophila*, little is known about their developmental functions (reviewed in 41). Mutational analyses of the *Saccharomyces cerevisiae Hsp82* genes have shown that *Hsp82* is an essential protein in yeast (3). However, no *Drosophila Hsp83* mutations have been recovered (64).

It has been known for some time that *Drosophila Hsp83* is not only heat-inducible, but that it is also expressed at high levels during normal development (30, 65, 67). Here, we show that maternal *Drosophila Hsp83* RNA is concentrated at the posterior pole of the early embryo via a localization mechanism involving a combination of generalized degradation throughout the embryo and local protection of *Hsp83* RNA at the posterior pole. Analysis of the distribution of *Hsp83* RNA in embryos produced by mutants lacking polar granules reveals that it is a component of the posterior polar plasm. *Hsp83* RNA is present at high levels in the germline cells throughout most of development, with the exception of two periods during oogenesis. In addition, *Hsp83* is transcribed zygotically in the anterior third of the embryo commencing at the syncytial blastoderm stage. This anterior zygotic expression is missing in embryos produced by *bicoid* mutant mothers, suggesting that early zygotic *Hsp83* transcription may be controlled by the *bicoid* homeodomain protein.

MATERIALS AND METHODS

Differential cDNA screen for polar-localized RNAs.

Our differential screen for cDNAs representing polar-localized RNAs will be described in detail elsewhere (D.D. and H.D.L., in preparation). Briefly, RNA

was purified from anterior or posterior poles cut off frozen embryos, and used in the construction of directionally cloned cDNA libraries in λEXLX vectors (51). The entire anterior- and posterior-libraries were converted from phage to plasmid libraries by *Cre-loxP* automatic plasmid subcloning (51), and plasmid DNA was purified from each of these libraries. Probe generated from this anterior- or posterior-DNA was used to differentially screen 10^5 plaques from a 0-1 hour whole embryo library constructed in λgt10, followed by rescreening of the clones on Southern blots, and sorting into cross-hybridization classes. From this screen, we obtained 12 members of the "SHTZ68" class of posterior clones (a frequency of 10^{-4}). These were hybridized *in situ* to whole mount early embryos (see below) to confirm that they encoded a posterior-localized RNA. Sequencing of these cDNAs followed by a sequence similarity search using FASTA as implemented in the GCG Sequence Analysis Package run at the Caltech Biology Division Sequence Analysis Facility, revealed that the "SHTZ68" cDNAs encode *Hsp83* (2, 19). The *Drosophila Hsp83* gene was cloned over thirteen years ago (23), and both its heat-inducible and its developmentally regulated expression have been studied in some detail (30, 65, 67). However, it was the recloning of *Hsp83* as the "SHTZ68" class of cDNAs that uncovered both the posterior localization of maternal *Hsp83* RNA and the anterior-restricted zygotic expression of *Hsp83*.

***In situ* hybridization to whole-mount ovaries and embryos and temporal analysis of *Hsp83* localization in the early embryo.**

Whole mount RNA tissue *in situ* hybridization was based on the method of Tautz and Pfeifle (60). Ovaries from adult females were dissected in PBS, fixed for 25 minutes in 10% paraformaldehyde or formaldehyde/50 mM EGTA/10% DMSO in PBS and washed several times in PBT (PBS plus 0.1% Tween 20). Ovaries were then rubbed gently between two frosted microscope slides in order to break apart the ovarioles and devitellinize the late egg chambers. Post-fixation,

proteinase K digestion, and refixation were as described (60). Embryos were fixed as described (60), with only minor modifications. Digoxigenin probes were labeled by random priming of DNA synthesis according to instructions from the manufacturer (Boehringer Mannheim) or by single-sided PCR-amplification according to a protocol provided by N. Patel (Carnegie Institution of Washington, Baltimore). Hybridization and detection were as described (60). Ovaries and embryos were mounted in JB4 plastic mountant for microscopy (Polysciences).

In order to analyze the time-course of *Hsp83* RNA localization, it was necessary to obtain precisely staged early embryos. Embryos were collected from well-fed wild type females at 15 to 20 minute intervals; thus, each collection contained embryos that differed only by a single nuclear cleavage cycle. The embryos were allowed to age at 25°C for different lengths of time prior to fixation in order to obtain material staged from fertilization through the completion of cellularization (0-2.5 hours after egg deposition). These embryos were then processed for *in situ* RNA hybridization with *Hsp83* probes as outlined above.

Fly strains.

Mutant embryos were obtained from females homozygous for osk^{166} (36), $capu^{HK}$, $spir^{RP}$ (47), nos^{L7}, pum^{680} (38), exu^{PJ}, vas^{PD}, $stau^{HL}$, vls^{RB}, tud^{WC8} (54), and bcd^{E1} (16). Deficiencies for *Hsp83* were provided by A. Wohlwill and J.J. Bonner (Indiana University) (64). Embryos lacking the *Hsp83* gene were derived from crosses between parent flies with the following genotypes: $Df(3L)HR218/Dp(3;3)t^{33}F19^{R}$, $Df(3L)HR298/Dp(3;3)t^{33}F19^{R}$, $Df(3L)HR370/Dp(3;3)t^{33}F19^{R}$ (64). Homozygous *hb* mutant embryos were derived from crosses between parent flies carrying $Df(3R)hb^{PTX15}$ p^{p} *e/TM3* (37).

RESULTS

Hsp83 RNA is expressed in a dynamic fashion during oogenesis.

Hsp83 RNA expression is absent in region 1 of the germarium and can first be detected in germarial regions 2 and 3 in all 16 cells of the germline cyst (Fig. 1A) (for stages, see 28). In stage 1 through 5 egg chambers in the vitellarium, *Hsp83* expression continues in all 16 cells of the nurse cell-oocyte complex (Fig. 1A) (stage 1 is equivalent to germarial stage 3). This RNA is rapidly degraded at the end of stage 5, since *Hsp83* RNA is present in stage 5 egg chambers but is undetectable in stage 6 egg chambers (Fig. 1A). It remains absent through stage 8 (Fig. 1B). High levels of *Hsp83* RNA are then expressed again beginning at stage 9, but only in the 15 nurse cells. This nurse cell expression persists through stages 10 and 11, and transport of *Hsp83* RNA into the oocyte commences at stage 10B (Fig. 1C). Thus, *Hsp83* RNA is absent from the oocyte for roughly 30 hours spanning stages 6 through 10A of oogenesis. By stage 12, when nurse cells have completely emptied their contents into the oocyte, *Hsp83* RNA is present at high levels throughout the oocyte. This high concentration of *Hsp83* RNA persists through the end of oogenesis (stage 14) (Fig. 1D).

Maternally synthesized *Hsp83* transcripts are protected from degradation at the posterior pole of the early embryo.

Maternal *Hsp83* RNA is distributed throughout the early embryo from nuclear division cycles 1 through 5 (Fig. 1E) (for stages see 14, 66). During cleavage cycles 6 to 8, *Hsp83* RNA is more concentrated at the posterior pole, forming a decreasing concentration gradient towards the anterior (Fig. 1F). *Hsp83* RNA becomes further restricted posteriorly before being taken up into the pole cells when they bud from the posterior tip of the embryo. Thus, by the syncytial blastoderm stage, the only detectable *Hsp83* RNA is in the pole cells and a small

region just beneath the pole cells (Fig. 1G). The RNA in the posterior somatic region disappears shortly after cellularization, leaving high levels of maternally synthesized RNA only in the pole cells (Fig. 1H). High levels of *Hsp83* RNA are present in the pole cells during their migration (Fig. 1I) and in the gonads of mature embryos (Fig. 1J), larvae and adults (65, 67, this study).

It is likely that localization of *Hsp83* RNA to the posterior pole of the embryo is achieved by a combination of generalized turnover throughout the embryo and protection of *Hsp83* RNA from degradation at the posterior pole. The alternative - translocation of the generally-distributed *Hsp83* RNA to the posterior pole - is unlikely since maternal *Hsp83* transcripts are present at very high levels in newly fertilized eggs and the concentration of *Hsp83* RNA at the posterior pole remains constant rather than increasing. In addition, Northern blot analysis shows that there is less *Hsp83* RNA in 2-4 hr embryos than in 0-2 hr embryos (data not shown), a result inconsistent with relocalization of a substantial fraction of the maternal RNA to the posterior pole. Embryos homozygous for deletions of *Hsp83* still show posterior localization of *Hsp83* RNA (Fig. 3A), excluding the possibility that *de novo* zygotic expression of *Hsp83* at the posterior of the embryo contributes significantly to the observed posteriorly-localized *Hsp83* RNA pool.

Zygotic transcription of *Hsp83* is restricted to the anterior third of the embryo.

At the late syncytial blastoderm stage, expression of *Hsp83* is detected in the anterior third of the embryo. This anterior expression continues through the cellular blastoderm stage (Fig. 1H), gastrulation (Fig. 1I) and most of embryogenesis. Anterior *Hsp83* expression is eliminated from embryos deleted for the *Hsp83* locus, indicating that it results from *de novo* zygotic transcription (Fig. 3A). During germband extension stages, in addition to the head and the pole

cells, *Hsp83* RNA can be detected in the neuroblasts (Fig. 1I). In mature embryos, high levels of *Hsp83* RNA can be detected in the gonads (Fig. 1J) and the central and the peripheral nervous system.

Maternally synthesized *Hsp83* RNA is a component of the posterior polar plasm.

We examined the distribution of *Hsp83* RNA in embryos derived from females homozygous for maternal mutations affecting pole cell formation and/or posterior patterning. Maternal *Hsp83* RNA is distributed normally in early cleavage stage embryos produced by seven mutants that affect the integrity of polar granules and pole cell formation (*cappuccino*, *spire*, *oskar*, *vasa*, *staufen*, *valois*, and *tudor*) (Fig. 2A, C, E, G, I, K, M). However, maternal *Hsp83* RNA is no longer protected at the posterior pole of these embryos and it is completely degraded between cycles 6 and 9, causing cellular blastoderm stage embryos to exhibit only anterior, zygotic *Hsp83* RNA (Fig. 2B, D, F, H, J, L, N). In contrast, in mutants that only affect posterior patterning but not polar granule integrity (*nanos* and *pumilio*), the posterior localization of *Hsp83* RNA is normal (Fig. 2O-R). Thus, *Hsp83* RNA is a component of the polar plasm and is protected from degradation by other component(s) of the polar plasm, possibly the polar granules themselves.

Spatially restricted zygotic expression of *Hsp83* in the anterior of the embryo is controlled by *bicoid*.

The earliest zygotic expression of *Hsp83* is detected in the anterior third of the embryo during the late syncytial blastoderm stage and this spatially restricted expression persists through the cellular blastoderm stage (Fig. 1H) and gastrulation (Fig. 1I). We have confirmed that this anterior expression is zygotic by demonstrating that it is absent in embryos which are deleted for the *Hsp83* locus (Fig. 3A). The anterior determinant, *bicoid*, a homeodomain protein present

in the early embryo in an anterior to posterior gradient (9, 10), regulates this anterior expression of *Hsp83*. In embryos that are derived from homozygous *bicoid* females, anterior *Hsp83* expression is completely abolished while posterior localization of the maternally transcribed *Hsp83* RNA is unaffected (Fig. 3B). This suggests that the *Hsp83* gene is transcriptionally activated in response to *bicoid*.

Several gene products have been shown to localize the *bicoid* RNA to the anterior pole of the oocyte and early embryo (1, 56). The most extreme delocalization of *bicoid* RNA is produced by *exuperantia* mutations; in embryos derived from *exuperantia* females, *bicoid* RNA and protein are present in a shallow gradient that extends along most of the embryonic antero-posterior axis (10, 56). In such embryos, there is a marked posterior shift in the boundary of *Hsp83* expression (Fig. 3C), consistent with the conclusion that anterior zygotic *Hsp83* expression is dependent on *bicoid*$^+$ product.

bicoid is known to directly regulate zygotic expression of the *hunchback* gene in the anterior half of the embryo (10). We examined *Hsp83* expression in homozygous *hunchback* embryos in order to determine whether the *bicoid* regulation of *Hsp83* is indirect, via zygotically expressed *hunchback*. There was no detectable effect of *hunchback* mutations on *Hsp83* anterior expression (data not shown), suggesting that *bicoid* directly regulates *Hsp83* expression. While we cannot exclude the possibility that *bicoid* acts indirectly through gene(s) other than *hunchback*, *in vitro* binding studies suggest that *bicoid* protein is capable of binding specifically to the *cis*-regulatory region of the *Hsp83* gene (S. R. Halsell, D.D. and H.D.L., unpublished).

DISCUSSION

The mechanism of *Hsp83* RNA localization.

Several RNA localization mechanisms have been suggested in *Drosophila*. First, *bicoid* (and possibly *Adducin-like*) transcripts appear to be trapped at their site of entry from the nurse cells into the anterior of the oocyte (1, 8, 15, 56). Second, posteriorly-localized RNAs (*oskar*, *nanos*, *tudor*, *pumilio*, *orb*, *germ cell-less*) are thought to be localized by being actively transported from the anterior of the oocyte and/or by cytoplasmic movement followed by trapping of these RNAs at the posterior pole (13, 17, 25, 27, 32, 42, 62). A third mechanism is implicated in maternal *caudal* RNA localization transiently to the posterior region of the embryo (43, 49). In this case, loss of *caudal* RNA commences at the anterior and proceeds posteriorly during a 30 minute period that extends through nuclear cycle 13 and part of cycle 14. By the end of nuclear cycle 14, maternal *caudal* RNA has been completely degraded throughout the embryo.

Localization of maternally synthesized *Hsp83* RNA to the posterior pole of the fertilized egg is accomplished by a combination of generalized degradation and local protection. High levels of *Hsp83* RNA are distributed throughout the mature oocyte (Fig. 1D,E). Shortly after fertilization, it is degraded rapidly throughout the embryo except at the posterior pole (Fig. 1F-H). This posterior protection of *Hsp83* RNA is likely to be accomplished by component(s) of the polar plasm, since mutations that cause both a reduction in the volume of the posterior polar plasm and disruption of the polar granules, result in a failure to protect the posterior *Hsp83* RNA from degradation (Fig. 2A-N). Degradation of *Hsp83* RNA is polarized - it commences at the anterior pole of the embryo and rapidly proceeds to the posterior pole, resulting in an antero-posterior gradient of

Hsp83 RNA (Fig. 1F). An alternative is that there are substantially higher levels of *Hsp83* RNA at the posterior than at the anterior and that degradation occurs at a uniform rate throughout the embryo commencing at nuclear cycle 6. This is unlikely since understaining of early embryos for *Hsp83* RNA reveals that it is present at essentially uniform levels along the antero-posterior axis prior to the commencement of degradation, and there is a significant decrease in the amount of *Hsp83* RNA during the first hours of embryogenesis (see Results).

While the posterior localization of *Hsp83* RNA superficially resembles that of *caudal* RNA, there are a number of differences. First, *Hsp83* RNA localization occurs well before pole cell formation while *caudal* RNA localization occurs about an hour later, after pole cell formation; second, *caudal* RNA localization is transient, while that of *Hsp83* is long-lived; third, the posterior-protection of *Hsp83* RNA is accomplished by components of the polar plasm, while the polar plasm is not involved in *caudal* RNA localization. It remains to be determined whether localization of these two RNAs is accomplished by different gene products and whether the posterior protection of *Hsp83* RNA is related to its translation, as has been proposed for *caudal* RNA (43, 49).

Functional significance of dynamic *Hsp83* expression during development.

While it has been known for some time that the *Hsp83* gene is expressed during normal development in the absence of heat shock (30, 65, 67), we have shown here that transcription of *Hsp83* is dynamically regulated during oogenesis and embryogenesis. The spatial distribution of *Hsp83* transcripts is controlled by two of the antero-posterior patterning systems that function in the early *Drosophila* embryo. The "posterior" gene family controls protection of maternal *Hsp83* RNA in the posterior polar plasm allowing its subsequent uptake into the pole cells, while the anterior determinant, *bicoid*, regulates zygotic activation of

Hsp83 transcription in the anterior third of the embryo.

Hsp83 RNA is a component of the the polar plasm that might be associated with the polar granules. Mutations that disrupt polar granule integrity result in complete degradation of *Hsp83* RNA throught the embryo (Fig. 2A - N). In contrast, in wildtype embryos or in embryos from mutants that eliminate components of the polar plasm without disrupting polar granule integrity (Fig. 2O - R), posterior protection of *Hsp83* RNA occurs normally. *Hsp83* RNA is also present in the pole cells and their germline progeny throughout embryogenesis (Fig. 1I,J), larval development (67, 65) and in the adult (with two exceptions - see below) (Fig. 1A - D) (65, 67, this study). Interestingly, the mouse *Hsp86* gene, a close relative of *Drosophila Hsp83* (50), is expressed in germ cells (7, 18, 35) as might be a human *Hsp85* gene (31), suggesting that members of the *Hsp90* gene family might serve similar functions in germ cells of diverse animal species.

Hsp83 RNA is absent from the *Drosophila* germline in stage 1 germaria and is absent from the oocyte in stage 6 through 10A egg chambers (Fig. 1). We have yet to determine the functional significance of the cessation of *Hsp83* transcription during these two periods, however they correspond to the stages during which two key cell fate decisions are made. First, it has been shown that, by early germarial stage 2A, the pro-oocyte is already different from the pro-nurse cells (28, 59), suggesting that the choice between oocyte and nurse cell fate is initiated in region 1 the germarium (28, 59). Second, communication between the oocyte and the surrounding follicle cells to specify the major aspects of its antero-posterior and dorso-ventral axes occurs during stages 6 through 8 (48). Despite directed attempts to obtain mutations in the *Hsp83* gene, none have yet been identified (64). The production of loss-of-function phenotypes either by mutational or by molecular methods (e.g., antisense inactivation, dominant negative expression) will be required to further define the developmental role of

the *Hsp83* gene.

Proteins regulated by the mammalian *Hsp90* family of molecular chaperones include steroid hormone receptors, *src*-family tyrosine kinases, eIF-2α, protein kinase C, casein kinases, actin and tubulin (reviewed in 41). Given the dynamic spatial and temporal regulation of *Hsp83* transcripts during *Drosophila* oogenesis and embryogenesis, it will be important in the future to determine whether *Hsp83* protein expression is also dynamically controlled and whether it mirrors *Hsp83* RNA. Should this be the case, then it will be necessary to identify the particular subset(s) of all possible partner proteins with which *Hsp83* interacts in the germ cells and in the anterior cells of the embryo. One such potential partner protein is the ecdysone receptor (*EcR*), which is present throughout the embryo (29). If *hsp83* were to interact with this receptor in either the anterior cells or the pole cells, the complex spatial and temporal pattern of *hsp83* expression and localization could convert the generally expressed *EcR* into a spatially and temporally regulated receptor. In addition, *D-src29A*, a *Drosophila src* homolog, is expressed throughout the somatic blastoderm (26, 61), raising the possibility that *Hsp83* regulates *src29A* activity in the anterior third of the embryo. Thus, dynamic spatial and temporal regulation of *Hsp83* might serve as a mechanism to confer spatially-regulated activation and/or repression on more generally expressed partner proteins during *Drosophila* development.

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REFERENCES

1. **Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M., and C. Nüsslein-Volhard.** 1988. The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. EMBO J. **7**:1749-1756.
2. **Blackman, R.K., and M. Meselson.** 1986. Interspecific nucleotide sequence comparisons used to identify regulatory and structural features of the *Drosophila Hsp82* gene. J. Mol. Biol. **188**:499-515.
3. **Borkovich, K.A., Farrelly, F.W., Finkelstein, D.B., Taulien, J., and S. Lindquist.** 1989. *Hsp82* is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol. Cell Biol. **9**:3919-3930.
4. **Bossy, B., Hall, L.M.C., and P. Spierer.** 1984. Genetic activity along 315 kb of the *Drosophila* chromosome. EMBO J. **3**:2537-2541.
5. **Boswell, R.E., and A.P. Mahowald.** 1985. *tudor*, a gene required for

assembly of the germ plasm in *Drosophila melanogaster*. Cell **43**:97-104.

6. **Boswell, R.E., Prout, M.E., and J.C. Steichen.** 1991. Mutations in a newly identified *Drosophila melanogaster* gene, *mago nashi*, disrupt germ cell formation and result in the formation of mirror-image symmetrical double abdomen embryos. Develop. **113**:373-384.
7. **Curci, A., Bevilacqua, A., Fiorenza, M.T., and F. Mangia.** 1991. Developmental regulation of heat-shock response in mouse oogenesis: identification of differentially responsive oocyte classes during Graafian follicle development. Dev. Biol. **144**:362-368.
8. **Ding, D., Parkhurst, S.M., and H.D. Lipshitz.** 1993. Different genetic requirements for anterior RNA localization revealed by the distribution of *Adducin-like* transcripts during *Drosophila* oogenesis. Proc. Natl. Acad. Sci. USA **90**: in press.
9. **Driever, W., and C. Nüsslein-Volhard.** 1988. A gradient of *bicoid* protein in *Drosophila* embryos. Cell **54**:83-93.
10. **Driever, W., and C. Nüsslein-Volhard.** 1988. The *bicoid* protein determines position in the *Drosophila* embryo in a concentration-dependent manner. Cell **54**:95-104.
11. **Driever, W., Thoma, G., and C. Nüsslein-Volhard.** 1989. Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the *bicoid* morphogen. Nature **340**:363-367.
12. **Ephrussi, A., and R. Lehmann.** 1992. Induction of germ cell formation by *oskar*. Nature **358**:387-392.
13. **Ephrussi, A., Dickinson, L.K., and R. Lehmann.** 1991. *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. Cell **66**:37-50.
14. **Foe, V.E., and B.M. Alberts.** 1983. Studies of nuclear and cytoplasmic

behavior in the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. J. Cell Sci. **61**:31-70.

15. **Frigerio, G., Burri, M., Bopp, D., Baumgartner, S., and M. Noll.** 1986. Structure of the segmentation gene *paired* and the *Drosophila* PRD gene set as part of a gene network. Cell **47**:735-746.

16. **Frohnhöfer, H.G., and C. Nüsslein-Volhard.** 1986. Organization of anterior pattern in the *Drosophila* embryo by the maternal gene *bicoid.* Nature **324**:120-125.

17. **Golumbeski, G.S., Bardsley, A., Tax, F., and R.E. Boswell.** 1991. *tudor*, a posterior-group gene of *Drosophila melanogaster*, encodes a novel protein and an mRNA localized during mid-oogenesis. Genes Dev. **5**:2060-2070.

18. **Gruppi, C.M., Zakeri, Z.F., and D.J. Wolgemuth.** 1991. Stage and lineage-regulated expression of two *Hsp90* transcripts during mouse germ cell differentiation and embryogenesis. Mol. Reprod. Devel. **28**:209-217.

19. **Hackett, R.W., and J.T. Lis.** 1983. Localization of the *Hsp83* RNA within a 3292 nucleotide sequence from the 63B heat shock locus of *D. melanogaster*. Nucl. Acids Res. **11**:7011-7030.

20. **Hay, B., Ackerman, L., Barbel, S., Jan, L.Y., and Y.N. Jan.** 1988. Identification of a component of *Drosophila* polar plasm. Develop. **103**:625-640.

21. **Hay, B., Jan, L.Y., and Y.N. Jan.** 1988. A protein component of *Drosophila* polar plasm is encoded by *vasa* and has extensive sequence similarity to ATP-dependent helicases. Cell **55**:577-587.

22. **Hay, B., Jan, L.Y., and Y.N. Jan.** 1990. Localization of *vasa*, a component of *Drosophila* polar plasm, in maternal effect mutants that alter embryonic anteroposterior polarity. Develop. **109**:425-433.

23. **Holmgren, R., Livak, K., Morimoto, R., Freund, R., and M. Meselson.**

1979. Studies of cloned sequences from four *Drosophila* heat shock loci. Cell **18**:1359-1370.

24. **Huettner, A.F.** 1923. The origin of the germ cells in *Drosophila melanogaster*. J. Morphol. **37**:385-423.

25. **Jongens, T.A., Hay, B., Jan, L.Y., and Y.N. Jan.** 1992. The *germ cell-less* gene product: a posteriorly localized component necessary for germ cell development in *Drosophila*. Cell **70**:569-584.

26. **Katzen, A.L., Kornberg, T., and J.M. Bishop.** 1990. Diverse expression of *dsrc29A*, a gene related to *src*, during the life cycle of *Drosophila melanogaster*. Develop. **110**:1169-1183.

27. **Kim-Ha, J., Smith, J.L., and P.M. Macdonald.** 1991. *oskar* mRNA is localized to the posterior pole of the *Drosophila* oocyte. Cell **66**:23-35.

28. **King, R.C.** 1970. Ovarian development in *Drosophila melanogaster*. Academic Press, New York.

29. **Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., and D.S. Hogness.** 1991. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell **67**:1371-1389.

30. **Kurtz, S., Rossi, J., Petko, L., and S. Lindquist.** 1986. An ancient developmental induction: heat shock proteins induced in sporulation and oogenesis. Science **231**:1154-1156.

31. **Lai, B-T., Chin, N.W., Stanek, A.E., Keh, W., and K.W. Lanks.** 1984. Quantitation and intracellular localization of 85K heat shock protein by using monoclonal and polyclonal antibodies. Mol. Cell. Biol. **4**:2802-2810.

32. **Lantz, V., Ambrosio, L., and P. Schedl.** 1992. The *Drosophila orb* gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. Develop. **115**:75-88.

33. **Lasko, P.F., and M. Ashburner.** 1988. The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. Nature **335**:611-617.

34. **Lasko, P.F., and M. Ashburner.** 1990. Posterior localization of *vasa* protein correlates with, but is not sufficient for, pole cell development. Genes Dev. **4**:905-921.

35. **Lee, S-J.** 1990. Expression of *Hsp86* in male germ cells. Mol. Cell. Biol. **10**:3239-3242.

36. **Lehmann, R., and C. Nüsslein-Volhard.** 1986. Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in *Drosophila*. Cell **47**:141-152.

37. **Lehmann, R., and C. Nüsslein-Volhard.** 1987. *hunchback,* a gene required for segmentation of an anterior and posterior region of the *Drosophila* embryo. Dev. Biol. **119**:402-417.

38. **Lehmann, R., and C. Nüsslein-Volhard**. 1987. Involvement of the *pumilio* gene in the transport of an abdominal signal in the *Drosophila* embryo. *Nature* **329**:167-170.

39. **Lehmann, R., and C. Nüsslein-Volhard.** 1991. The maternal gene *nanos* has a central role in posterior pattern formation of the *Drosophila* embryo. Develop. **112**:679-693.

40. **Lehner, C.F., and P.H. O'Farrell.** 1990. The roles of *Drosophila* cyclins A and B in mitotic control. Cell **61**:535-547.

41. **Lindquist, S., and E. Craig.** 1988. The heat-shock proteins. Ann. Rev. Genet. **22**:631-677.

42. **Macdonald, P.M.** 1992. The *Drosophila pumilio* gene: an unusually long transcription unit and an unusual protein. Develop. **114**:221-232.

43. **Macdonald, P.M., and G. Struhl.** 1986. A molecular gradient in early

Drosophila embryos and its role in specifying the body pattern. Nature **324**:537-545.

44. **Mahowald, A.P.** 1962. Fine structure of pole cells and polar plasm in *Drosophila melanogaster*. J. Exp. Zool. **152**:201-215

45. **Mahowald, A.P.** 1968. Polar plasm of *Drosophila* II. Ultrastructural changes during early embryogenesis. J. Exp. Zool. **167**:237-262

46. **Mahowald, A.P.** 1971. Polar plasm of *Drosophila* III. The continuity of polar plasm during the life cycle of *Drosophila*. J. Exp. Zool. **176**:329-344

47. **Manseau, L., and T. Schüpbach.** 1989. *Cappuccino* and *spire*: two unique maternal-effect loci required for both anteroposterior and dorsoventral patterns of the *Drosophila* embryo. Genes Dev. **3**:1437-1452.

48. **Manseau, L., and T. Schüpbach.** 1989. The egg came first, of course! Trends Genet. **5**, 400-405.

49. **Mlodzik, M., and W.J. Gehring.** 1987. Expression of the *caudal* gene in the germ line of *Drosophila*: formation of an RNA and protein gradient during early embryogenesis. Cell **48**:465-478.

50. **Moore, S.K., Kozak, C., Robinson, E.A., Ullrich, S.J., and E. Apella.** 1989. Murine 86- and 84-kDa heat shock proteins, cDNA sequences, chromosome assignments, and evolutionary origins. J. Biol. Chem. **264**:5343-5351.

51. **Palazzolo, M.J., Hamilton, B.A., Ding, D., Martin, C.H., Mead, D.A., Mierendorf, R.C, Vijay Raghavan, K., Meyerowitz, E.M., and H.D. Lipshitz.** 1990. Phage lambda cDNA cloning vectors for subtractive hybridization, fusion protein expression and Cre-*loxP* automatic plasmid subcloning. Gene **88**:25-36.

52. **Perrimon, N., Engstrom, L., and A.P. Mahowald.** 1984. The effects of zygotic lethal mutations on female germline functions in *Drosophila*. Dev.

Biol. **105**:404-414.

53. **Raff, J.W., Whitfield, W.G.F., and D.M. Glover.** 1990. Two distinct mechanisms localize cyclin B transcripts in syncytial *Drosophila* embryos. Develop. **110**:1249-1261.

54. **Schüpbach, T., and E. Wieschaus.** 1986. Maternal-effect mutations altering the antero-posterior pattern of the *Drosophila* embryo. W. Roux's Archiv. Dev. Biol. **195**:302-317.

55. **Schüpbach, T., and E. Wieschaus**. 1989. Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. Genetics **121**:101-117.

56. **St Johnston D., Driever, W., Berleth T., Richstein, S., and C. Nüsslein-Volhard.** 1989. Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. Develop. **107 (Supplement)**:13-19.

57. **St Johnston, D., Beuchle, D., and C. Nüsslein-Volhard.** 1991. *staufen*, a gene required to localize maternal RNAs in the *Drosophila* egg. Cell **66**, 51-63.

58. **Struhl, G., Struhl, K., and P.M. Macdonald.** 1989. The gradient morphogen *bicoid* is a concentration-dependent transcriptional activator. Cell **57**:1259-1273.

59. **Suter, B., and R. Steward.** 1991. Requirement for phosphorylation and localization of the *Bicaudal-D* protein in *Drosophila* oocyte differentiation. Cell **67**:917-926.

60. **Tautz, D., and C. Pfeifle.** 1989. A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma **98**:81-85.

61. **Vincent, W.S., Gregory, R.J., and S.C. Wadsworth.** 1989. Embryonic

expresssion of a *Drosophila src* gene: alternate forms of the protein are expressed in segmental stripes and in the nervous system. Genes Dev. **3**:334-347.

62. **Wang, C., and R. Lehmann.** 1991. *Nanos* is the localized posterior determinant in *Drosophila*. Cell **66**:637-647.

63. **Wieschaus, E.** 1980. A combined genetic and mosaic approach to the study of oogenesis in *Drosophila*. Pp. 85-94. *In* O. Siddiqi, P. Babu, L.M. Hall, and J.C. Hall (eds.), Genetics and Neurobiology of *Drosophila*. Plenum Press.

64. **Wohlwill, A.D., and J.J. Bonner.** 1991. Genetic analysis of chromosome region 63 of *Drosophila melanogaster*. Genetics **128**:763-775.

65. **Xiao, H., and J.T. Lis.** 1989. Heat shock and developmental regulation of the *Drosophila melanogaster Hsp83* gene. Mol. Cell. Biol. **9**:1746-1753.

66. **Zalokar, M., and I. Erk.** 1976. Division and migration of nuclei during early embryogenesis of *Drosophila melanogaster*. J. microsc. Biol. Cell **25**:97-106.

67. **Zimmerman, J.L., Petri, W., and M. Meselson.** 1983. Accumulation of a specific subset of *D. melanogaster* heat shock mRNAs in normal development without heat shock. Cell **32**:1161-1170.

FIGURE LEGENDS

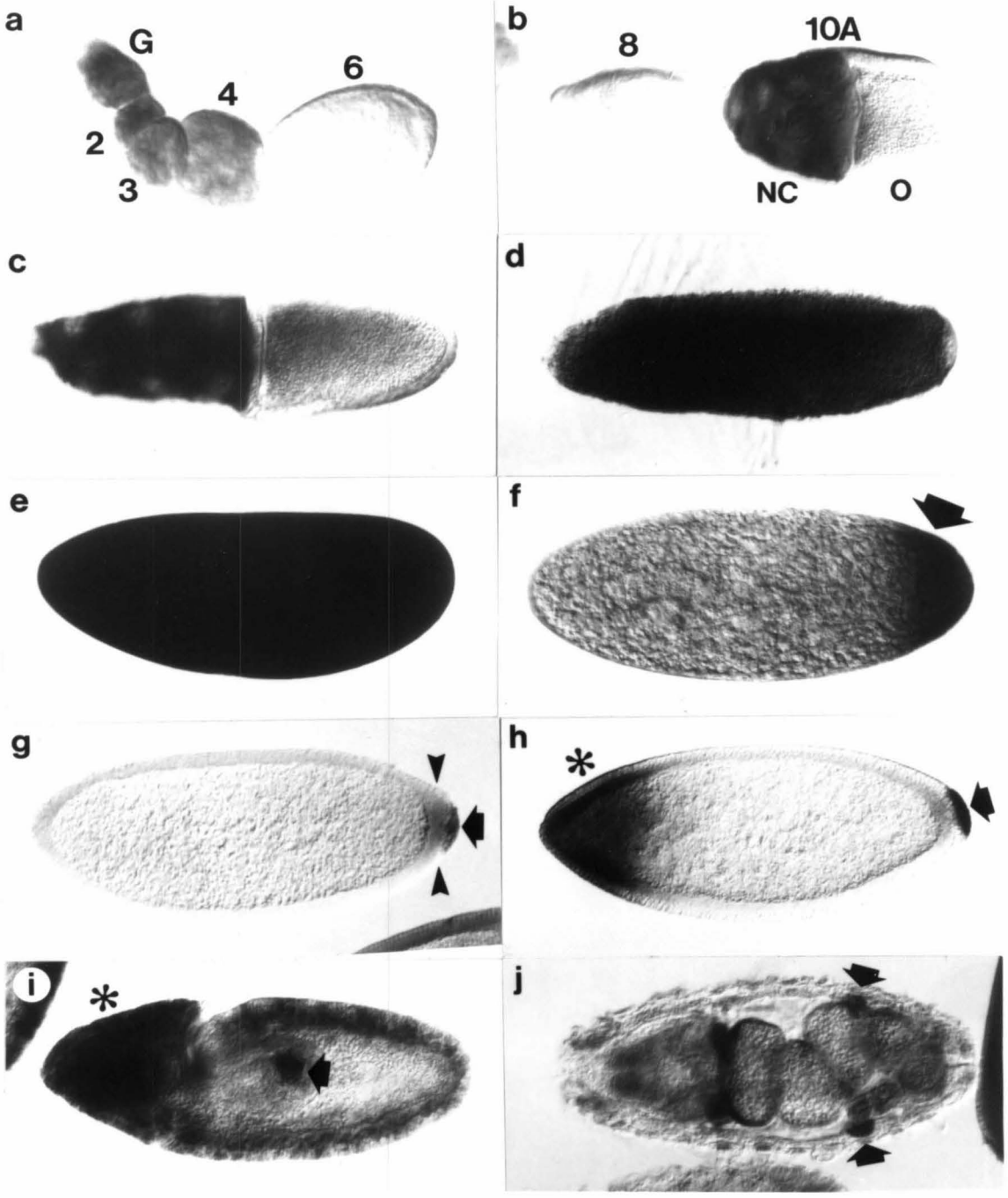
FIG. 1. Expression and localization of *Hsp83* RNA during oogenesis and embryogenesis. *In situ* hybridization of *Hsp83* cDNA probe to ovarioles and embryos from wild-type females. (A) During the early stages of oogenesis, *Hsp 83* expression is detected in all the cells in regions 2 and 3 of the germarium (G). In stage 1 through 5 egg chambers, *Hsp83* is expressed in all 16 cells of the nurse cell-oocyte complex (shown are stages 1 which is equivalent to germarial stage 3, and stages 2, 3 and 4). No *Hsp83* RNA is detected in stage 6 to 8 egg chambers (stage 6 is shown). (B) A stage 8 egg chamber ('8') lacks detectable *Hsp83* RNA, while a high level of *Hsp83* expression is found again in the nurse cells beginning in stage 9. The high level of nurse cell expression can be seen in the stage 10A egg chamber shown (NC: nurse cells; O: oocyte). (C) In a stage 10B egg chamber, nurse cells contain high levels of *Hsp83* RNA, which begin to be transported into the oocyte (O). (D) In stage 14 oocytes, *Hsp83* is distributed uniformly at high levels. (E) In an early cleavage stage embryo (before nuclear cycle 6), maternal *Hsp83* RNA is uniformly distributed and present at high levels. (F) In a nuclear cycle 7 embryo, maternal *Hsp83* RNA is more concentrated at the posterior end (arrow), forming a decreasing gradient towards the anterior. (G) By the syncytial blastoderm stage, *Hsp83* RNA is only detected in the pole cells (arrow) and a small region immediately beneath them (arrowheads). (H) At the cellular blastoderm stage, zygotic *Hsp83* transcripts are present in the anterior third of the embryo (asterisk) in addition to the maternal RNA that is present in the pole cells (arrow). (I) During germband extension, *Hsp83* RNA is present at high levels in the pole cells (arrow), the head region (asterisk) and the neuroblasts. (J) In a fully developed embryo, *Hsp83* RNA is present at high levels in the gonads (arrows). In all embryos except that shown in (J), anterior is to the left

and dorsal is up. The view in (J) is from the dorsal side, again with anterior to the left.

FIG. 2. Distribution of *Hsp 83* RNA in "posterior" class mutant embryos. *Hsp83* RNA is shown in embryos derived from females homozygous for mutations that affect the integrity of the polar granules (A - N) or that eliminate components of the polar plasm without affecting the integrity of the polar granules (O - R). These are: *cappuccino* (A) and (B); *spire* (C) and (D); *oskar* (E) and (F); *vasa* (G) and (H); *staufen* (I) and (J); *valois* (K) and (L); *tudor* (M) and (N); *nanos* (O) and (P); and *pumilio* (Q) and (R). Maternal *Hsp83* RNA is distributed normally in the early cleavage stage embryos in all mutants (A, C, E, G, I, K, M, O, Q). However, in mutants that eliminate polar granules, maternal *Hsp83* RNA is not protected from degradation at the posterior pole and can no longer be detected by the cellular blastoderm stage (B, D, F, H, J, L, N). In mutants in which the polar granules are present, *nanos* and *pumilio*, protection of maternal *Hsp83* RNA still occurs (arrows) (P, R). Note that, in all cases, the anterior zygotic expression of *Hsp83* is normal at this stage (asterisks). Also note that in addition to the anterior expression, zygotic *Hsp83* transcripts can be detected at 15-25% egg length in the embryos produced by *cappuccino* mothers (B, arrowheads). For all embryos, anterior is to the left and dorsal is up.

FIG. 3. Regulation of zygotic *Hsp83* transcription by *bicoid.* (A) A cellular blastoderm stage embryo that is homozygous for a deficiency that removes the *Hsp83* gene [*Df(3L)HR370*]. The maternal *Hsp83* RNA can be seen in the pole cells (arrow), but anterior zygotic expression is absent (cf. Fig. H). The genetic cross to produce embryos deleted for the *Hsp83* gene is given in Materials and Methods. Roughly one quarter of these embryos exhibited no anterior expression

of *Hsp83* while posterior localization of maternal *Hsp83* RNA was normal. We presume that these embryos were the ones lacking the *Hsp83* gene. (B) An embryo derived from a female homozygous for $bicoid^{E1}$, a strong *bicoid* allele. The distribution of maternal *Hsp83* RNA is identical to wild type (arrow) (cf. Fig. 1H), but the anterior zygotic transcription is completely abolished. (C) An embryo derived from a $exuperantia^{PJ}$ female. The intensity of zygotic *Hsp83* expression is reduced and extends more posteriorly than in wild type (to about 50% egg length; arrowheads) (cf. Fig. 1H). The distribution of maternal *Hsp83* RNA is normal (arrow).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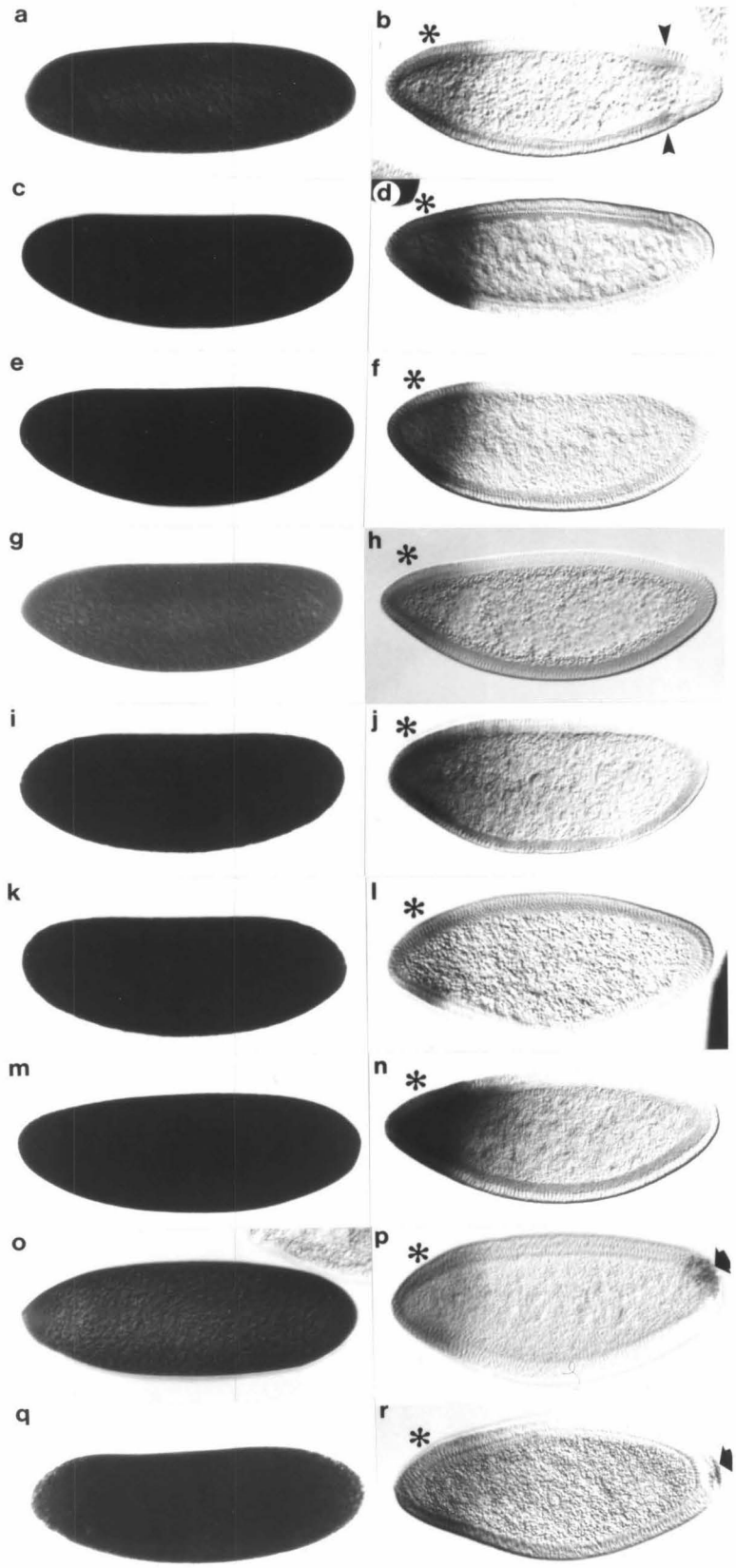
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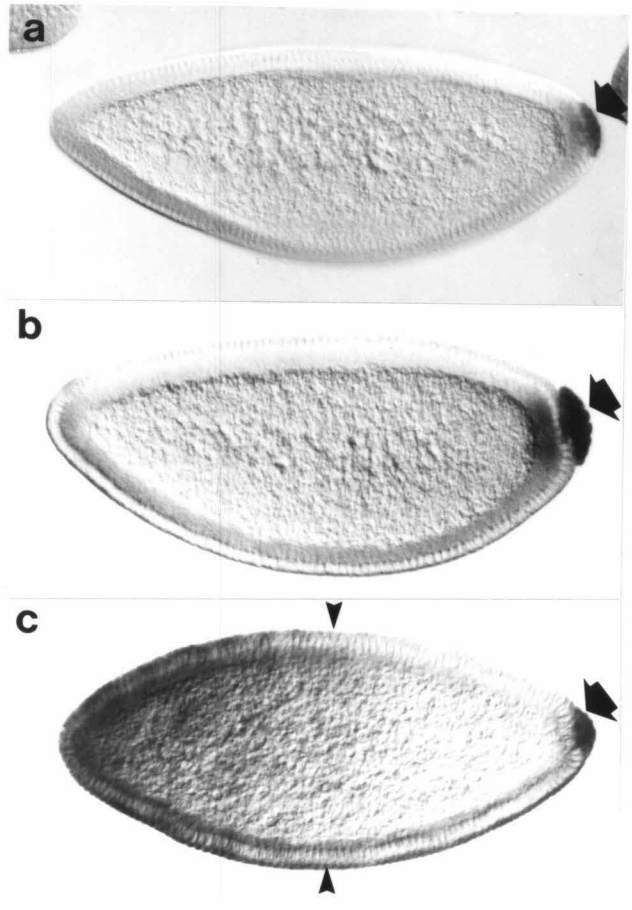
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CHAPTER 3 - APPENDIX

Anti-sense inactivation of *Hsp83*.

These experiments were conducted in collaboration with Susan M. Parkhurst.

Introduction

Since *Hsp83* RNA is a component of the posterior polar plasm (Ding, Parkhurst, and Lipshitz 1993), we suspected that *Hsp83* function might be required for pole cell formation and/or for the maintenance of pole cell identity. Since an extensive lethal mutagenesis in the 63B chromosomal region failed to yield mutations in *Hsp83* (Wohlwill and Bonner [1991] *Genetics* **128**, 763-775), we decided to carry out antisense oligodeoxynucleotide (oligo) injection experiments to address the possible functions of *Hsp83* as a component of the polar plasm.

Materials and Methods

First set of experiments:

Three antisense-*Hsp83* oligos were synthesized on a MilliGen/Biosearch Cyclone Plus DNA Synthesizer. Oligo 1 (5'-GTTACGACGCACACCGTACG-3') is complementary to a region upstream of the translation start site. Oligo 2 (5'-CATACATACAAGAGATGCCAG-3') is complementary to a region containing the translation start site. (It was later discovered that, by mistake, an oligo representing the sense strand of this region of *Hsp83* was synthesized and used in these experiments.) The third oligo (5'-GCCAGAGTCCAGCTTGCTGG-3') is complementary to the *Hsp83* sequence from codons #55 - #61. Three oligos that are not known to occur in the *Drosophila* genome (5'-CGACTCACTATAGGGAGC-3', 5'-GCTGCGGCCGCAGATT TAGGTG-3', and 5'-GGCCAAGTCGGCCTCTAATACG-3') were used as controls. For injection, a mixture of the three antisense-*Hsp83* oligos or control oligos, each at a concentration of 1 mg/ml (10^{17} molecules of each oligodeoxynucleotide/ml), were suspended in injection buffer [5mM KCl, 0.1 mM PO_4 (pH7.8)]. Embryos for injection were derived from *bw st* females. Embryos

were injected into the posterior pole (0-20% egg length) with approximately 1 nl of solution (10^{11} molecules of each oligodeoxynucleotide) at early cleavage stage (stage 2). If one assumes that *Hsp83* RNA is present at a frequency of 10^{-4} in the poly(A) RNA at the posterior pole of the early embryo (based on our frequency of recovery of *Hsp83* cDNAs from our posterior cDNA library, see Ding and Lipshitz 1993), then there are approximately 3 x 10^5 *Hsp83* RNA molecules at the posterior pole. The molar excess of antisense oligos to endogenous sense *Hsp83* RNA, can thus be estimated as between 10^5 and 10^6. After injection, embryos were allowed to develop at room temperature, and were periodically monitored under the microscope and video taped.

Second set of experiments:

This time the sequence mistake in oligo 2 was corrected. In addition, the oligos were purified through a reverse phase mini-column (Poly-Pac column, Glen Research). Injection procedures were otherwise similar to those of the first set of experiments.

Results:

First set of experiments:

Injection of the antisense-*Hsp83* oligos into the posterior of early embryos had no effect on nuclear migration or division, but efficiently diminished the ability of the embryos to form pole cells. Of 119 embryos injected with the control oligs, 10% failed to form pole cells. The other 90% underwent normal pole cell formation and somatic cellularization. In contrast, 66% of the 158 embryos injected with the anti-*Hsp83* oligos failed to form pole cells. In addition, somatic cells failed to form in the posterior region of these embryos. Injection of antisense-*Hsp83* oligos into other regions of embryos locally prevented cellularization. This experiment was repeated one time and the results were

similar.

Second set of experiments:

A total of 65 early embryos were injected with three antisense oligos. 17 embryos (26%) did not form pole cells or formed a reduced number of pole cells. The rest of the embryos formed pole cells and both the appearance and number of the pole cells seemed to be normal. No cellularization defects were observed in most of the 65 embryos. When the injected embryos were examined at the cellular blastoderm stage under the microscope, virtually all of the embryos that formed pole cells initially had lost their pole cells. We do not know whether the pole cells died or intercalated among the somatic cells, losing their characteristic morphology and adopting that of the somatic cells. Several anti-16S RNA oligos synthesized and purified the same way as for anti-*Hsp83* oligos were used for injection controls (see Ding, Whittaker, and Lipshitz 1993). In contrast to anti-*Hsp83* oligos, injection of these oligos has no affect on pole cell formation or maintenance.

Discussion

Results from the first set of experiments suggested that *Hsp83* function in the polar granules plays a role in programming pole cell formation, and that *Hsp83* is also required for cellularization throughout the embryo. This experiment was carried out twice with similar results. Somewhat different results were obtained in the second set of experiments. While again pointing to a role for *Hsp83* in pole cell determination, they suggest that *Hsp83* is not required for cellularization and possibly not for the initial formation of pole cells, but rather that *Hsp83* is required for maintenance of pole cells after they form.

We do not understand the reasons for the somewhat different outcomes of the two sets of experiments, but two differences need to be considered. First, the

oligos were prepared differently, with the second set being more highly purified through mini-columns (see Materials and Methods). It is conceivable that some contaminating substance(s) carried over from the synthesis of the first set of oligos, were responsible for their more pleiotropic affects on cellularization and pole cell formation. The second difference between the two sets of experiments is that one of the first set of three oligos used was (mistakenly) a sense oligo instead of antisense oligo. While we suspect that this is unlikely to account for the more pleiotropic results obtained in the first set of experiments, we cannot exclude the possibility that co-injection of the sense oligo somehow induced the pole cell formation and/or somatic cellularization defects.

CHAPTER 4

This chapter will be submitted for publication in *Development*.

Mitochondrially encoded 16S large rRNA is associated with the posterior polar plasm in early *Drosophila* embryos

Running title: Mitochondrial 16S RNA localization in polar plasm

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Summary

The mitochondrial 16S large ribosomal RNA is highly concentrated at the posterior pole of newly fertilized *Drosophila* eggs. This high posterior concentration decreases sharply during the first hour of embryogenesis and reaches the uniform basal level found throughout the remainder of the embryo by the time pole cells bud at one and a half hours after fertilization. At the cellular blastoderm stage the 16S RNA is uniformly distributed basal to the nuclei of all somatic cells and is present at reduced levels in, or is absent from, the pole cells. Nonyl acridine orange staining reveals that mitochondria are uniformly distributed in the cortical cytoplasm of the early embryo; thus the high concentration of 16S RNA at the posterior pole is not a consequence of a high concentration of mitochondria in the posterior polar plasm. Similarly, while rhodamine-123 staining reveals dynamic spatial and temporal changes in mitochondrial activity during early embryogenesis, these correlate only partially with the dynamics of 16S RNA expression and localization. Transcripts produced by the *ND-1* gene, which is transcribed from the same strand of the mitochondrial genome and resides just downstream of the 16S gene, do not exhibit a high posterior concentration but rather are uniformly distributed throughout wildtype early embryos. Posterior localization of 16S RNA is normal in embryos produced by mothers carrying mutations in *nanos* and *pumilio*, which affect components of the polar plasm without disrupting the polar plasm or polar granule integrity. In contrast, posterior localization of 16S RNA is abolished in embryos produced by females carrying maternal-effect mutations that disrupt the posterior polar plasm and the polar granules - *cappuccino*, *spire*, *oskar*, *staufen*, *tudor*, *valois* and *vasa*. Posterior polar mitochondria exhibit high

respiratory activity in embryos produced by these mutants but their distribution is somewhat more dispersed. Our data are consistent with the hypotheses that the 16S RNA is exported from the mitochondria into the cytoplasm, that it constitutes a major component of the posterior polar plasm, and that it functions in pole cell formation (Kobayashi and Okada 1989, *Development* 107: 733-742).

Introduction

Formation of primordial germ cells (PGCs) in many animal species ranging from worms to amphibians is dependent on localized germ plasm (Eddy 1975, for a review). In *Drosophila*, the germ plasm (also called the polar plasm) is a posteriorly-localized, yolk-free cytoplasmic cap that is continuous with the cortical cytoplasm of the egg and early embryo. An hour and a half after fertilization, pole cells (the *Drosophila* PGCs) bud from the posterior pole of the embryo, taking part of the polar plasm with them. It has been demonstrated that the polar plasm is both necessary and sufficient for pole cell formation in *Drosophila*. When components of the posterior polar plasm are disrupted either by physical means such as u.v.-irradiation or pricking, or by mutations, pole cells do not form (for reviews, see Eddy 1975; Lasko 1992). When transplanted to the anterior pole of the early embryo, polar plasm induces ectopic pole cells that can give rise to a functional germ line (Illmensee and Mahowald 1974; Illmensee and Mahowald 1976; Niki 1986). Polar plasm contains unique electron-dense, non-membrane-bound organelles (called polar granules) that have been proposed to contain the cytoplasmic determinant(s) for pole cells (Mahowald 1977).

Since *Drosophila* polar plasm is formed during oogenesis, genetic studies of the posterior polar plasm have focused on characterization of grandchildless

mutations, maternal mutations that produce progeny lacking a functional germ line. A number of such mutations have been found to affect the polar plasm. Most of these mutations disrupt both germ cell formation and the development of the abdomen [*cappuccino*, *spire* (Manseau and Schüpbach 1989), *oskar* (Lehmann and Nüsslein-Volhard 1986), *vasa* , *staufen*, *valois* (Schüpbach and Wieschaus 1986), *tudor* (Boswell and Mahowald 1985) and *mago nashi* (Boswell et al. 1991)]. Two other genes also appear to encode components of the polar plasm; these are required for abdominal patterning but not for pole cell formation [*pumilio* and *nanos* (Lehmann and Nüsslein-Volhard 1987; Lehmann and Nüsslein-Volhard 1991)]. Additional components of the polar plasm have been identified by molecular approaches. These include RNAs encoded by *cyclin B*, *orb* and *hsp83*, and protein and RNA encoded by *germ cell-less* (Lehner and O'Farrell 1990; Raff et al. 1990; Lantz et al. 1992; Ding et al. 1993b; Jongens et al. 1992). The specific functions of *cyclin B*, *orb* and *hsp83* in the polar plasm are not known. In the case of *germ cell-less*, antisense-inactivation experiments indicate that it may be specifically required for pole cell formation (Jongens et al. 1992).

It has been reported that the mitochondrial 16S large ribosomal RNA (referred to as 16S RNA hereafter) might also function as a component of the posterior polar plasm required for pole cell formation. This conclusion was based on the fact that injected in vitro transcribed 16S RNA rescues the ability of embryos to form pole cells after u.v.-irradiation of the posterior polar plasm (Kobayashi and Okada 1989).

Here, we report that, consistent with its possible role in pole cell formation, 16S RNA is highly concentrated at the posterior pole of the embryo immediately after fertilization. Further, this posterior localization of the 16S RNA is dependent on genes that are required for polar plasm integrity and pole cell formation, but

does not correlate well with either the distribution or the activity of mitochondria in early embryos. We hypothesize that the 16S RNA is exported from the mitochondria into the cytoplasm and that it serves as a major component of the polar granules in early embryos.

Materials and Methods

Identification of the 16S RNA as a posteriorly-localized RNA

A cDNA representing the 16S RNA was identified in our differential screen for cDNAs representing maternally synthesized polar-localized RNAs in the early embryo (D.D. and H.D.L., in preparation). Whole mount in situ hybridization using probes made from the cDNA revealed that it recognizes an abundant RNA concentrated at the posterior pole of early embryos. Additional cDNAs were isolated by screening a 0-1 hr embryonic cDNA library with probe made from this initial cDNA. Sequence analysis of these cDNAs indicated that they represent the mitochondrial 16S large ribosomal RNA (Garesse 1988; Kobayashi and Okada 1990).

Isolation of the ND-1 gene

Primers (both 34-mers) were designed based on the *D. melanogaster* mitochondrial DNA sequence (Garesse 1988):
ND-1-5B: 5'CATAACGAAATCGAGGTAAAGTTCCTCGAACTCA-3' and
ND-1-3A: 5'-ATACTGTTATAGTAGCTGGTTGGTCGTCTAATTC-3'.
These primers were used for enzymatic amplification (PCR) of a 485-bp internal fragment of the *ND-1* gene from total *Drosophila* DNA. The 100 µl reaction contained 1 µg of total DNA, 0.5 mM *ND-1*-5B primer, 0.5 mM *ND-1*-3A primer, 0.75 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 0.1 mg/ml gelatin,

200mM dNTP mix, and 2.5 units of Taq polymerase. The reaction was run for 1.5 minutes at 94°, 1.5 minutes at 56°, and 2 minutes at 72°, for thirty cycles using a Perkin-Elmer DNA thermal cycler. A single strong band of the predicted size was visible after separation of the reaction products on an agarose gel; the DNA in this band was purified using a GeneClean kit (Bio 101, Inc.). Sequencing of the purified DNA confirmed that it indeed comprised the expected amplified fragment of the *ND-1* gene; it was subsequently labeled with digoxigenin for use as a probe in whole mount RNA tissue in situ hybridization (see below).

Analysis of the distribution of mitochondria in early embryos

The distribution of mitochondria was assayed by staining fixed embryos with 10 mM nonyl acridine orange (NAO) (Septinus et al. 1985; Maftah et al. 1989). Embryos ranging in age from 0 to 3 hour after fertilization were washed onto Nitex, dechorionated for 2.5 minutes in freshly prepared 50% bleach (5% sodium hypochlorite diluted 1:1 in water). Following dechorionation, the embryos were gently washed with water for 2 minutes. The Nitex filter was set to dry on a Kimwipe for 4 minutes, transferred into heptane, and 4% paraformaldehyde dissolved in 1X PBS was then added. Fixation was carried out at room temperature on a shaker, followed by devitellinization in 90% methanol, 50 mM EGTA. Devitellinized embryos were collected, washed once in 100% methanol, and stored in methanol at -20°C. Prior to staining, the methanol was pipetted off and the embryos were washed twice in ethanol. Embryos were then exposed to 10 mM NAO in ethanol for 15 minutes on a shaker, in a light-proof container. The embryos were rehydrated through an ethanol series, transferred to an Eppendorf tube and, after the embryos had settled, the supernatant was removed and they were mounted in 1:1 PBS:glycerol for confocal microscopy.

Analysis of the activity of mitochondria in early embryos

Mitochondrial activity was analyzed by staining live embryos with rhodamine-123 at a concentration of 10 μg/ml in 1.1x BIM, following a permeabiliztion procedure for live embryos developed by Strecker et al. (1993). Embryos were collected and permeabilized with hexane or octane, according to Strecker et al. (1993). After a 10 minute incubation with dye, the dye was drawn off by pipette and the embryos were washed twice with fresh BIM, then covered with HC-56 halocarbon oil (Halocarbon Products Corp., Hackensack, NY). The remaining droplets of BIM were vacuumed off using a drawn out micropipette connected to a vacuum aspirator. The embryos were then mounted and examined under a Zeiss confocal microscope, using an argon 488 laser, a 10% filter, 10x objective, and a confocal aperture of P20.

RNA tissue in situ hybridization to whole-mount embryos and ovaries

Whole mount RNA tissue in situ hybridization was based on the method of Tautz and Pfeifle (1989) modified as described in Ding et al. (1993a).

Since both strands of the mitochondrial DNA are transcribed, we compared the in situ hybridization pattern obtained using randomly primed probe to that exhibited by single-sided-PCR-generated probe, for both the 16S and the *ND-1* RNAs. There were no detectable differences in the results obtained for either of the transcripts using either of these methods.

Fly strains

Mutant embryos were obtained from females homozygous for osk^{166} (Lehmann and Nüsslein-Volhard 1986), $capu^{HK}$, $spir^{RP}$ (Manseau and Schüpbach 1989), nos^{L7}, pum^{680} (Lehmann and Nüsslein-Volhard 1987). vas^{PD}, $stau^{HL}$, vls^{RB}, tud^{WC8} (Schüpbach and Wieschaus 1986).

Results

16S RNA expression and localization during oogenesis and early embryogenesis

During *Drosophila* oogenesis, four incomplete mitoses of a single cystoblast in the germarium of the ovariole lead to the formation of an interconnected 16-cell germ line cyst (King 1970). One of the 16 cells becomes the oocyte while the remaining fifteen become the nurse cells, in which most of the non-yolk components of the oocyte are synthesized and then transported into the oocyte via the connecting ring canals. 16S RNA expression is first detected in region 2 of the germarium (Figure 1A). In stage 1 to 10A follicles the 16S RNA continues to be expressed in the nurse cells and can be seen at a lower level in the oocyte (Figures 1A, B). Nurse cell expression persists through stages 10B and 11, and 16S RNA begins to be transported into the oocyte commencing at stage 10B (Figure 1C). We do not know whether this represents transport of 16S RNA that has been exported from the mitochondria into the cytoplasm (see Discussion) and/or whether it represents transport of mitochondria from the nurse cells into the oocyte as has been reported to occur at these stages (King 1970). The somatically-derived follicle cells are also heavily labeled with probe that recognizes the 16S RNA (Figure 1D). By stage 12, when nurse cells have completely emptied their contents into the oocyte, 16S RNA is present at high levels throughout the oocyte (Figure 1E). A much higher level of 16S RNA accumulates at the posterior tip of the oocyte some time between stage 12 of oogenesis and egg deposition shortly after fertilization (Figure 2A). Oocytes at stages later than stage 12 are not readily fixed and devitellinized, and we were thus unable to determine unambiguously whether 16S RNA becomes localized to the posterior pole of the oocyte before or after fertilization. However, the fact that

all embryos exhibit high levels of posteriorly localized 16S RNA immediately after fertilization (Figure 2A) suggests that this RNA is likely to be concentrated posteriorly during late oogenesis. However, we cannot rigorously exclude the possibility that the 16S RNA is rapidly synthesized and localized immediately after fertilization. Either way, the timing of posterior localization of 16S RNA is similar to that of the *germ cell-less* transcript, which becomes posteriorly localized at an undetermined time between stage 11 and egg deposition (Jongens et al. 1992).

In early embryos immediately after egg deposition, 16S RNA is present at high levels at the posterior tip of the embryo and at a much lower, uniform concentration throughout the rest of the cortical cytoplasm of the embryo (Figure 2A). During the early cleavage stages, the high posterior concentration of 16S RNA decreases rapidly. By the time that pole cell buds appear, the intensity of 16S RNA at the posterior tip of the embryo has dropped significantly, becoming only slightly higher than elsewhere in the embryo (Figure 2B). The pole buds are not labeled at this stage (Figure 2B), indicating that the 16S RNA is not taken up into the pole cells. After pole cell formation, the concentration of 16S RNA in the cortical cytoplasm at the posterior tip can be seen to be the same as that throughout the cortex of the embryo (Figure 2C). The labeling of the pole cells for 16S RNA approaches that of background, indicating that there is very little 16S RNA in these cells, certainly much less than that found in the cortical cytoplasm (Figure 2C). During the syncytial and cellular blastoderm stages, 16S RNA continues to be evenly distributed in the somatic cytoplasm throughout the embryo but not in the pole cells, where very little of this RNA can be detected (Figure 2D).

Control experiments with strand-specific probes indicate that the distribution reported here for the 16S RNA does, indeed, reflect that of this RNA rather than

RNA transcribed from the complementary strand of the mitochondrial genome (see Materials and Methods). In addition, hybridization of a genomic Southern blot with 16S probe indicated that there is no nuclear copy of the 16S RNA gene (data not shown) and, therefore, that the transcripts detected must have been synthesized in mitochondria. This last result is consistent with the data reported by Kobayashi and Okada (1989).

Mitochondria are uniformly distributed in early embryos

In order to determine whether the spatial localization pattern of 16S RNA during early embryogenesis is the consequence of an asymmetric distribution of mitochondria, fixed embryos were stained with nonyl acridine orange (NAO), a lipophilic acridine orange derivative. NAO binds to the inner mitochondrial membrane independent of the membrane potential (Septinus et al. 1985; Maftah et al. 1989). Figure 3 shows that the mitochondrial distribution in cleavage-stage embryos is uniform throughout the cortical cytoplasm. At the cellular blastoderm stage, NAO staining appears slightly more pronounced in cells at the termini (Figure 3). Pole cells are stained at a level equal to that of the cortical cytoplasm.

Thus, the high posterior concentration of the 16S RNA in early embryos is not a reflection of a high concentration of mitochondria posteriorly, but must have some other cause.

Mitochondrial activity is spatially and temporally regulated in early embryos, but does not correlate well with 16S RNA distribution

A second possible explanation for the high posterior concentration of 16S RNA is that the activity of mitochondria in the posterior polar plasm is significantly higher than in the remainder of the embryo. Consequently, we assayed mitochondrial activity in permeabilized living embryos using the voltage-

sensitive dye, rhodamine-123. In contrast to the uniform mitochondrial distribution, mitochondrial activity was found to be both spatially and temporally regulated (Figure 4). During early cleavage, there is a tight "cap" of very high mitochondrial membrane potential in the posterior polar plasm, as well as a somewhat more diffuse cap of high mitochondrial membrane potential in the anterior of the embryo (Figure 4A). By mid-cleavage (roughly 1 hour after fertilization), both the anterior and posterior mitochondrial membrane potentials subside to basal levels (Figure 4B). Once pole cells have formed, they exhibit very high respiratory activity (Figure 4C).

Thus, there is a correlation between the high respiratory activity of mitochondria at the posterior pole of early embryos, and the high level of 16S RNA found there. However, respiratory activity and 16S RNA distribution do not correlate well elsewhere, since the high level of anterior rhodamine-123 staining in early embryos does not correspond to an increased amount of 16S RNA anteriorly. Further, while pole cells exhibit high levels of rhodamine-123 staining, they actually exhibit little or no 16S RNA (see above). This uncoupling of rhodamine-123 staining and 16S RNA levels is further supported by the fact that rhodamine-123 staining is high at the posterior pole of embryos produced by females carrying grandchildless mutations while these same mutations cause a dramatic reduction in the posterior concentration of 16S RNA (see below).

Mitochondrial activity is largely unaffected by grandchildless pole cell mutations

Next we analyzed the effects on mitochondrial activity (as assayed by rhodamine-123 staining) of three grandchildless mutations that reduce the posterior polar plasm, eliminate the polar granules and prevent pole cell formation. The mutations selected were *cappuccino,* and *tudor*. *cappuccino* has been proposed to function early in polar granule assembly (see Lasko *1992* for

review). The *tudor* protein has been shown to be associated with mitochondria at certain stages of oogenesis and/or embryogenesis (A. Bardsley, K. McDonald and R. Boswell, pers. comm.) (see Discussion). In addition, we assayed the effects of the *pumilio* mutation on rhodamine-123 staining. *pumilio* RNA is concentrated in the posterior polar plasm of the early embryo (Macdonald 1992; Barker et al. 1992) and the *pumilio* protein is required for abdominal development but not for maintenance of polar granule integrity.

Rhodamine-123 staining in embryos from *pumilio* mothers was indistinguishable from that in wildtype (Figure 4 cf. Figure 3). In embryos produced by *cappuccino* and *tudor* mutant females, rhodamine-123 staining was high at the anterior and posterior poles; however, the posterior signal was less tightly localized than in wildtype embryos (Figure 4 cf. Figure 3). These results are consistent with the hypothesis that, while there is an increase in the activity of individual posteriorly located mitochondria in wildtype embryos as well as in embryos produced by these mutants, the reduction in the volume of the posterior polar plasm caused by these mutations results in the active mitochondria being less tightly localized in a posterior polar cap (see Discussion).

Thus, high posterior activity of mitochondria in early embryos is not induced by the components of the polar plasm or by the presence of polar granules per se; rather, in wildtype embryos, the highly active mitochondria may be restricted to the posterior polar plasm.

Posterior localization of the 16S RNA is disrupted by grandchildless pole cell mutations

We then examined the effects on 16S RNA distribution of these four mutations as well as an additional five maternal-effect mutations which cause defects in pole cell formation and/or posterior patterning. For all mutations that

affect the integrity of polar granules, pole cell formation and abdominal development (*cappuccino*, *spire*, *oskar*, *vasa*, *valois*, *tudor* and *staufen*), the 16S RNA is present at uniform levels throughout the embryo at early cleavage stages, but the high posterior concentration is completely abolished (Figure 5). In contrast, mutations that affect abdominal patterning but not polar granule integrity or pole cell formation (*nanos* and *pumilio*), result in embryos in which the posterior localization of 16S RNA is normal (Figure 6).

Thus, components of the polar plasm, possibly the polar granules per se, are required to establish and/or maintain the high posterior concentration of the 16S RNA in early embryos.

ND-1 *RNA is uniformly distributed in the early embryo*

To compare the distribution of an additional mitochondrially-encoded transcript to that of 16S RNA, we carried out whole mount in situ detection of the *ND-1* transcript. The *ND-1* RNA is synthesized from the same strand of the mitochondrial DNA as the 16S RNA and the *ND-1* gene resides just downstream of the 16S RNA gene, being separated from it only by the Leu (CUN) tRNA gene (Garesse 1988). In addition, Kobayashi and Okada (1989) used the *ND-1* gene as a control in their experiments (see Discussion). As shown in Figure 7, unlike the posterior-localized 16S RNA, the *ND-1* RNA is uniformly distributed throughout the early cleavage stage embryo (Figure 7A cf. Figure 2A). This indicates that the non-uniform distribution observed for the 16S RNA is not a general rule for all mitochondrial transcripts.

Discussion

We hava shown that, while both the 16S and the ND1 RNAs are synthesized inside the mitochondria, only the 16S RNA is concentrated at the posterior pole of early embryos. The dynamics of 16S RNA expression and localization do not reflect either the distribution of mitochondria or their activity. Mutations that disrupt the integrity of the posterior polar plasm, the polar granules and the ability to form pole cells have little effect on the activity of mitochondria at the posterior pole. In contrast, all of these mutations completely eliminate posterior localization of the 16S RNA. Here, we discuss the significance of these results, integrate them with the results of previous studies of the 16S RNA and the posterior polar plasm, and present a series of hypotheses about the biological significance of our findings.

Posterior localization of the 16S RNA does not correlate with mitochondrial distribution or activity in early embryos

Since the 16S RNA is encoded by the mitochondrial genome, it was possible that the higher concentration of 16S RNA at the posterior tip was a result of a higher density of mitochondria in the posterior polar plasm. Our analysis of the distribution of mitochondria in early embryos using NOA has revealed that this cannot be the case since mitochondria are uniformly distributed at this stage. Akiyama and Okada (1992) have also reported a uniform distribution of mitochondria at this stage based on analyses of sectioned material in the electron microscope.

This left open the possibility that the high posterior concentration of 16S RNA is a result, not of higher mitochondrial density, but of higher respiratory activity of mitochondria in the polar plasm. Our assay of mitochondrial activity in the

early embryo indicates that the distribution of 16S RNA in embryos does not correlate well with the activity level of mitochondria. In early cleavage embryos mitochondrial activity is indeed higher at the posterior pole, correlating with the high posterior concentration of 16S RNA. However, this high activity of mitochondria at the posterior can be shown to be uncoupled from 16S RNA localization posteriorly by an analysis of grandchildless mutations which eliminate the posterior polar plasm as a histologically distinct entity and leave the posterior cytoplasm indistinguishable from the rest of the cortical cytoplasm (Schüpbach and Wieschaus 1986). Akiyama and Okada (1992) reported that such grandchildless mutations had no effect on the high level of rhodamine-123 staining at the posterior pole. We find that these mutations eliminate the high posterior concentration of 16S RNA but that (in contrast to the results reported by Akiyama and Okada) they also subtly affect the highly active posterior mitochondria, which become more dispersed. Our data are consistent with the hypothesis that these highly active mitochondria, which would have been restricted to the posterior polar plasm in wildtype, are distributed through the posterior fifth of the cortical cytoplasm in embryos produced by these mutants. Thus, components of the posterior polar plasm may be required to keep the mitochondrial activity localized, but these components are not required to induce this higher activity per se. Were the 16S RNA similarly dispersed in these mutants, the posterior 16S RNA level should still have appeared significantly higher than that in the rest of the cortical cytoplasm. However, these mutants result in an apparently complete delocalization of the 16S RNA.

The high anterior mitochondrial activity in early cleavage embryos and in blastoderm-stage pole cells, is not reflected in increased concentrations of 16S RNA in these regions. Indeed, at the stage at which pole cells exhibit high respiratory activity, the 16S RNA is present at extremely low (essentially

undetectable) levels in pole cells.

Polar granule-mitochondrial association and the dynamics of 16S RNA posterior localization during oogenesis and early embryogenesis

An association between the polar granules and mitochondria in *Drosophila melanogaster* was reported by Mahowald (1962). He showed that precursors of the polar granules attach to the mitochondria between stages 9 and 10 of oogenesis. The polar granules increase in size during their association with the mitochondria, peaking in size during stage 14 of oogenesis. Contact between the polar granules and the mitochondria is lost shortly after fertilization. We have shown that the 16S RNA is likely to be concentrated at the posterior pole of the oocyte between stages 12 and 14 of oogenesis, and that this high concentration decays rapidly after fertilization, reaching the basal level found throughout the cortical cytoplasm by one to one-and-a-half hours after fertilization. Thus, the spatial and temporal dynamics of 16S RNA localization presented here closely mirror those of the mitochondrial-polar granule association reported by Mahowald (1962). This raises the possibility that posterior concentration of the 16S RNA reflects one facet of the association between polar granules and mitochondria (see below).

Is the 16S RNA exported from the mitochondria into the posterior polar plasm?

Kobayashi and Okada (1989) reported that when in vitro transcribed 16S RNA is injected into the cytoplasm at the posterior of embryos that have been u.v.-irradiated, the embryos regained the ability to form pole cells. When injected anteriorly together with u.v.-irradiated posterior cytoplasm, in vitro trancribed 16S RNA can induce pole cell-like cells at the anterior pole of these embryos. Homogenates of early embryos can be separated into two fractions – one that has

few mitochondria but contains restoration activity for pole cell formation in u.v.-irradiated embryos (the P3 fraction) and another that has many mitochondria but lacks the restoration activity (the P2 fraction) (Ueda and Okada 1982). 16S RNA was found to be significantly more abundant in fraction P3 than in P2, suggesting that 16S RNA is outside of the mitochondria in early embryos (Kobayashi and Okada 1989). Another mitochondrially-encoded transcript, *ND-1*, was found to be much more abundant in P2 than P3, as expected if *ND-1* RNA is present almost exclusively in mitochondria (Kobayashi and Okada 1989). These data suggested that the 16S RNA is an extra-mitochondrial component of the polar plasm that functions in pole cell formation (Kobayashi and Okada 1989). Interestingly, consistent with the possible presence of 16S RNA outside of the mitochondria in the cytoplasm, the *Drosophila* 16S RNA has a polyA tail which is much longer than that of its mammalian homologue and is comparable in length to those found on cytoplasmic mRNA transcripts (Benkel et al. 1988).

Our RNA tissue in situ analyses of the 16S and ND-1 RNAs support the hypothesis that 16S RNA is an extra-mitochondrial component of the posterior polar plasm. The 16S RNA is concentrated in the polar plasm, forming a tight cap at the posterior pole of early embryos, a pattern similar to that of RNAs that are likely to be components of the polar plasm (such as transcripts of *nanos*, *oskar* and *germ cell-less*) (Wang and Lehmann 1991; Ephrussi et al. 1991; Kim-Ha et al. 1991; Jongens et al. 1992). In contrast, our in situ data have shown that *ND-1* transcripts are not concentrated at the posterior pole of early embryos. Further, the dynamics of 16S RNA localization reported here mirror the time course reported by Kobayashi and Okada (1989) for the biological activity required for the restoration of pole cell formation after u.v.-irradiation. This activity is high at the posterior of early embryos but diminishes by the blastoderm stage (Kobayashi and Okada, 1989), correlating with our finding that the high posterior

concentration of 16S RNA decreases rapidly during early embryogenesis and reaches basal levels after pole cell formation.

Posterior localization of the 16S RNA is not detected in embryos derived from mothers mutant for genes that are required for polar granule integrity, pole cell formation and abdominal patterning (see below). Thus, taken together with the data of Kobayashi and Okada (1989), our results suggest that the 16S RNA might be an extra-mitochondrial component of the polar plasm that is associated with the polar granules.

Is 16S RNA a major component of polar granules?

Polar granules are electron-dense, non-membrane-bound organelles that reside within 4 μm of the plasma membrane at the posterior tip of the *Drosophila* oocyte and early embryo (Huettner 1923; Mahowald 1962; 1968; 1971a). The polar granules are taken up into pole cells as these cells bud from the posterior pole of the embryo, and have been postulated to play a key role in programming them to adopt germline fates. Mutations in the "posterior" (grandchildless-*knirps*) group genes that are required for pole cell formation all reduce the posterior polar plasm and disrupt the integrity of the polar granules (Lasko 1992, for a review). We investigated the effect of mutations in seven such genes (*cappuccino*, *spire*, *oskar*, *vasa*, *valois*, *tudor* and *staufen*) on posterior localization of 16S RNA, and found that all result in loss of the high levels of 16S RNA from the posterior pole. In contrast, *nanos* and *pumilio*, which affect abdominal development without disrupting polar granules or pole cell formation, had no effect on posterior localization of the 16S RNA. These data, thus, provide further indirect evidence supporting the possibility that the 16S RNA is not only present outside of the mitochondria in the cytoplasm but that the particular organelles with which it might be associated are the polar granules. Remarkably, proteins encoded by two

of the "posterior" group genes that are required for polar granule integrity, have been found inside mitochondria. Preliminary results suggest that some *vasa* protein is associated with mitochondria during pre-vitellogenic stages of oogenesis (W. Diehl-Jones and P. Lasko, personal communication). The *tudor* protein is found inside mitochondria (as well as in the polar granules) in early embryos (A. Bardsley, K. McDonald and R. Boswell, personal communication). These genes are, thus, candidates for proteins involved in export of 16S RNA from mitochondria and/or its assembly into the polar granules.

The 16S RNA is one of the most abundant polyadenylated RNA species in the early embryo. It represents about 10% of early embryonic polyadenylated RNA (D.D. and H.D.L, in prep.; Benkel et al. 1988). If we assume that roughly one third of this 16S RNA is localized to the posterior polar plasm, then there is about 0.1 ng of 16S RNA localized to the posterior pole of each early embryo. The mass of the posterior polar plasm is about 10 ng and, if we assume that 1% of this comprises polar granules, then the polar granules have a mass of roughly 0.1 ng. In other words, if the 16s RNA is a component of the polar granules in the early embryo, then it is likely to be a major component comprising a significant fraction of the mass of each granule. Cytochemical studies have indicated that polar granules in the newly fertilized egg contain a large amount of RNA (Mahowald 1971b) and that the RNA content of polar granules decreases rapidly during early embryogenesis such that, by the cellular blastoderm stage, the polar granules in the pole cells contain negligible amounts of RNA as assayed cytochemically (Mahowald 1971b). Consistent with our hypothesis that the 16S RNA might be a major RNA component of the polar granules in the early embryo, the dynamics of 16S RNA localization to the posterior pole (see above) correlate well with these cytochemical observations of the RNA content of polar granules (Mahowald 1971b). Recently, S. Kobayashi, R. Akimura and M. Okada

(submitted) have used electron microscopic RNA in situ hybridization to confirm that the majority of 16S RNA at the posterior pole of the early embryo is indeed in the polar granules rather than the mitochondria.

Polar granules show dynamic patterns of association with mitochondria during development (see above). In all *Drosophila* species examined except for *D. hydei*, polar granules attach to mitochondria as they form during late oogenesis and they retain this association until after fertilization when polar granules dissociate from mitochondria, fragment and disperse in the polar plasm (Mahowald 1968; 1971a). We suspect that the attachment of polar granules to mitochondria could be involved in transport of the 16S RNA out of mitochondria and its incorporation into polar granules. Whether transport of the 16S RNA also occurs in *D. hydei* where polar granules are not attached to mitochondria, remains to be seen.

Structures similar to polar granules have been found in germ plasm of other species; these include 'germinal granules' in amphibians and 'P granules' in nematodes (Mahowald and Hennen 1971; Strome and Wood 1983). Indeed, the primordial germ cells of a wide range of animals contain fibrous cytoplasmic organelles referred to as 'nuage', 'germinal granules', 'inter-mitochondrial cement' or a variety of other names. These organelles possess many of the characteristics of the polar granules, suggesting that these organelles have been evolutionarily conserved (Eddy 1975, for a review). In most species these organelles are found associated with mitochondria at some stage during germ cell development (Eddy 1975). It will be interesting to determine whether the 16S RNA is present in the germinal granules of these species when this germinal granule-mitochondrial association occurs.

Possible functions of 16S RNA in polar granules

Since 16S RNA is a major structural component of a complex ribonucleoprotein particle, the mitochondrial ribosome, we speculate that 16S RNA has been recruited into the early embryonic polar granules of *Drosophila* to play a structural role by interacting with other RNAs and/or proteins in this ribonucleoprotein complex. The cytoplasmic 28S rRNA presumably could not be recruited to the polar granules since, unlike the 16S RNA, it already has a function in the cytoplasmic compartment of the cell. Recent studies have shown that the large ribosomal RNA of *E. coli* can play a catalytic role in protein synthesis (Noller et al. 1992) raising the possibility that 16S RNA could also have a function other than a strictly structural one in polar granules.

Injection of 16S RNA into u.v.-irradiated embryos indicates that it plays a role in pole cell formation (Kobayashi and Okada 1989). A critical test of this hypothesis awaits loss-of-function phenotypic analysis of the 16S RNA. Injection into early embryos of in vitro synthesized antisense RNA complementary to 16S RNA did not give a phenotype (Kobayashi and Okada 1989). Our attempts to obtain a loss-of-function phenotype by injection into early embryos of mixtures of several antisense oligodeoxynucleotides complementary to evolutionarily highly conserved regions of the 16S RNA have also not been successful (D. D., S. M. Parkhurst, K. W. and H. D. L., unpublished observations). Different antisense-inactivation approaches are currently being tested in our laboratory. We are also using molecular and biochemical methods to identify proteins and RNAs that interact with 16S RNA. The *vasa*, *oskar* and *tudor* proteins, and other proteins and RNAs localized in the polar plasm are obvious candidates (see Lasko 1992 for review). The molecular and biochemical screens are expected to lead to the identification of additional components of the polar granules that have not been identified genetically, and therefore to further our understanding of the molecular

composition and functions of germinal granules.

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References

Akiyama, T., and Okada, M. (1992). Spatial and developmental changes in the respiratory activity of mitochondria in early *Drosophila* embryos. *Develop.* **115**, 1175-1182.

Barker, D. D., Wang, C., Moore, J., Dickinson, L. K., and Lehmann, R. (1992) Pumilio is essential for function but not for distribution of the *Drosophila* abdominal determinant Nanos. *Genes. Dev.* **6**, 2312-2326.

Benkel, B. F., Duschesnay, P., Boer, P. H., Genest, Y., and Hickey, D. A. (1988). Mitochondrial large ribosomal RNA: an abundant sequence in *Drosophila. Nucleic Acids Res.* **16**, 9880.

Boswell, R. E., and Mahowald, A. P. (1985). *tudor*, a gene required for assembly of the germ plasm in *Drosophila melanogaster. Cell* ***43***, 97-104.

Boswell, R. E., Prout, M. E., and Steichen, J. C. (1991). Mutations in a newly identified *Drosophila melanogaster* gene, *mago nashi*, disrupt germ cell formation and result in the formation of mirror-image symmetrical double abdomen embryos. *Develop.* **113**, 373-384.

Ding, D., Parkhurst, S. M., and Lipshitz, H. D. (1993a). Different genetic requirements for anterior RNA localization revealed by the distribution of *Adducin-like* transcripts during *Drosophila* oogenesis. *Proc. Natl. Acad. Sci. USA* **90**, in press.

Ding, D., Parkhurst, S. M., and Lipshitz, H. D. (1993b) Dynamic *Hsp83* RNA localization during *Drosophila* oogenesis and embryogenesis. (submitted for publication)

Eddy, E. M. (1975). Germ plasm and the differentiation of the germ line. *Int. Rev. Cytol.* **43**, 229-280.

Ephrussi, A., Dickinson, L. K., and Lehmann, R. (1991). *oskar* organizes the

germ plasm and directs localization of the posterior determinant *nanos*. *Cell* ***66***, 37-50.

Garesse, R. (1988). *Drosophila melanogaster* mitochondrial DNA: Gene organization and evolutionary considerations. *Genetics* **118**, 649-663.

Huettner, A. F. (1923). The origin of the germ cells in *Drosophila melanogaster*. *J. Morphol.* **37**, 385-423.

Illmensee, K., and Mahowald, A. P. (1974). Transplantation of posterior polar plasm in *Drosophila*. Induction of germ cells at the anterior pole of the egg. *Proc. Natl. Acad. Sci. USA* **71**, 1016-1020.

Illmensee, K., and Mahowald, A. P. (1976). The autonomous function of germ plasm in a somatic region of *Drosophila* egg. *Exp. Cell Res.* **97**, 127-140.

Jongens, T. A., Hay, B., Jan, L. Y., and Jan, Y. N. (1992). The *germ cell-less* gene product: A posteriorly localized component necessary for germ cell development in *Drosophila*. *Cell* **70**, 569-584.

Kim-Ha, J., Smith, J. L., and Macdonald, P. M. (1991). *oskar* mRNA is localized to the posterior pole of the *Drosophila* oocyte. *Cell* ***66***, 23-35.

King, R. C. (1970). Ovarian development in *Drosophila melanogaster*. New York: Academic Press.

Kobayashi, S., and Okada, M. (1989). Restoration of pole-cell forming ability to u.v.-irradiated *Drosophila* embryos by injection of mitochondrial lrRNA. *Develop.* **107**, 733-742.

Kobayashi, S., and Okada, M. (1990). Complete cDNA sequence encoding mitochondrial large ribosomal RNA of *Drosophila melanoganster*. *Nucleic Acids Res.* **18**, 4592.

Kobayashi, S., Amikura, R., and Okada, M. (1993) Presence of mitochondrial lrRNA outside mitochondria in germ plasm of *Drosophila melanogaster*. *Science* (submitted).

Lantz, V., Ambrosio, L., and Schedl, P. (1992). The *Drosophila orb* gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. *Develop.* **115**, 75-88.

Lasko, P. F. (1992). Molecular-movements in oocyte patterning and pole cell-differentiation. *Bioassays* **14**, 507 -512.

Lehmann, R., and Nüsslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation and embryonic polarity require the localised activity of *oskar*, a maternal gene in *Drosophila*. *Cell* **47**, 141-152.

Lehmann, R., and Nüsslein-Volhard, C. (1987). Involvement of the *pumilio* gene in the transport of an abdominal signal in the *Drosophila* embryo. *Nature* ***329***, 167-170.

Lehmann, R., and Nüsslein-Volhard, C. (1991). The maternal gene *nanos* has a central role in posterior pattern formation of the *Drosophila* embryo. *Develop.* ***112***, 679-693.

Lehner, C. F., and O'Farrell, P. H. (1990). The roles of *Drosophila* cyclins A and B in mitotic control. *Cell* ***61***, 535-547.

Macdonald, P. M. (1992). The *Drosophila pumilio* gene: an unusually long transcription unit and an unusual protein. *Develop.* **114**, 221-232.

Maftah, A., Petit, J. M., Ratinaud, M-H., and Julien, R. (1989) 10-N nonyl-acridine orange: a fluorescent probe which stains mitochondria independently of their energetic state. *Biochem. Biophys. Res. Comm.* **164**, 185-190.

Mahowald, A. P. (1962). Fine structure of pole cells and polar granules in *Dosophila melanogaster*. *J. Exp. Zool.* **151**, 201-215.

Mahowald, A. P. (1968). Polar granules of *Drosophila* II. Ultrastructural changes during early embryogenesis. *J. Exp. Zool.* ***167***, 237-262.

Mahowald, A. P. (1971a). Polar granules of *Drosophila*. III. The continuity of polar granules during the life cycle of *Drosophila*. *J. Exp. Zool.* **176**, 329-349.

Mahowald, A. P. (1971b). Polar granules of *Drosophila*. IV. Cytochemical studies showing loss of RNA from polar granules during early stages of embryogenesis. *J. Exp. Zool.* **176**, 345-352.

Mahowald, A. P. (1977). The germ plasm of *Drosophila*: an experimental system for the analysis of determination. *Am. Zool.* **17**, 551-563.

Mahowald, A. P., and Hennen, S. (1971). Ultrastructure of the 'germ plasm' in eggs and embryos of *Rana pipiens*. *Dev. Biol.* **24**, 37-53.

Manseau, L., and Schüpbach, T. (1989). *cappuccino* and *spire*: two unique maternal-effect loci required for both anteroposterior and dorsoventral patterns of the *Drosophila* embryo. *Genes Dev.* ***3***, 1437-1452.

Niki, Y. (1986). Germline autonomous sterility of P-M dysgenic hybrids and their application to germline transfers in *Drosophila melanogaster*. *Dev. Biol.* **113**, 255-258.

Noller, H. F., Hoffarth, V., and Zimniak, L. (1992). Unusual resistance of peptidyl transferase to protein extraction procedures. *Science* **256**, 1416-1419.

Raff, J. W., Whitfield, W. G. F., and Glover, D. M. (1990). Two distinct mechanisms localize cyclin B transcripts in syncytial *Drosophila* embryos. *Develop.* ***110***, 1249-1261.

Schüpbach, T., and Wieschaus, E. (1986). Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. *Roux's Arch. Dev. Biol.* **195**, 302-317.

Septinus, M., Berthold, Th., Naujok, A., and Zimmermann, H. W. (1985) Hydrophobic acridine dyes for fluorescence staining of mitochondria in living cells. 3. *Histochem.* **82**, 51-66.

Strecker, T.R., McGhee, S., Licking, E., Shih, S., and Ham, D. (1993). Permeabilization, staining and culture of living *Drosophila* embryos. (submitted for publication).

Strome, S., and Wood, W. B. (1983). Generation of asymmetry and segregation of germ-line granules in early *C. elegans* embryos. *Cell* **35**, 15-25.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* ***98***, 81-85.

Ueda, R., and Okada, M. (1982). Induction of pole cells in sterilized *Drosophila* embryos by injection of subcellular fraction from eggs. *Proc. Natl. Acad. Sci. USA* **79**, 6946-6950.

Wang, C., and Lehmann, R. (1991). Nanos is the localized posterior determinant in *Drosophila*. *Cell* ***66***, 637-647.

Figure Legends

Figure 1: Expression and distribution of *16S* RNA during oogenesis

In situ hybridization of *16S* cDNA probe to ovarioles from wild-type females. (A) During the early stages of oogenesis, *16S* RNA expression is detected in all the cells in region 2 of the germarium (G). From stages 1 of oogenesis on, *16S* RNA is expressed in the cytoplasm of all 16 cells of the nurse cell-oocyte complex (Shown are S1, S2, S3 and S5). (B) A stage 10A egg chamber shows high levels of*16S* RNA in nurse cells (NC), and slightly lower levels in the oocyte (O). (C) In a stage 11 egg chamber, nurse cells contain high levels of *16S* RNA, which is being transported into the oocyte (O). (D) A stage 10 egg chamber showing the somatic follicle cells which are heavily stained with *16s* cDNA probe. (E) In stage 12 oocytes, *16S* RNA is distributed uniformly at high levels throughout the oocyte.

Figure 2: Localization of *16S* RNA during early embryogenesis

In situ hybridization of *16S* cDNA probe to wild-type embryos. (A) In an embryo immediately after fertilization, *16S* RNA is present throughout the embryo, but is found at a higher concentration at the posterior pole. (B) The high posterior concentration of *16S* RNA decreases rapidly during the cleavage stages. When pole cells form, there is only slightly more RNA at the posterior end than the rest of the embryo. The pole cell buds (arrowhead) are not stained. (C) At the syncytial blastoderm, 16S RNA is uniformly distributed throughout the somatic part of the embryo. The pole cells (arrowhead) are not stained. (D) At the cellular blastoderm stage, *16S* RNA is present in the basal cytoplasm of the somatic cells while the pole cells (arrowhead) are not stained. For all embryos, anterior is to the left and dorsal is toward the top of the page.

Figure 3: Distribution of mitochondria in early embryos

Nonyl acridine orange staining of paraformaldehyde-fixed embryos. (A) In early cleavage, NAO staining shows that mitochondria are distributed throughout the cortical cytoplasm. (B) In a cellular blastoderm-stage embryo, the mitochondria are distributed throughout the cortical cytoplasm, with a slightly higher concentration at both poles of the embryo. Pole cells are stained at a level equal to that of the termini and slightly greater than that of the rest of the cortical cytoplasm.

Figure 4: Distribution of mitochondrial activity in early embryos

Rhodamine-123 staining of live embryos. (A) An early cleavage embryo shows a posteriorly localized "cap" of high mitochondrial membrane potential, and somewhat increased anterior membrane potential. (B) In mid-cleavage, mitochondrial activity is uniform throughout the cortical cytoplasm; the previously higher levels of activity at both poles have subsided to basal levels. (C) In a cellular blastoderm-stage embryo, the mitochondrial activity in pole cells is considerably higher than in any other part of the embryo.

Figure 5: Distribution of mitochondrial activity in early embryos from *pumilio*, *cappuccino* and *tudor* females

Rhodamine-123 staining of live embryos. (A) In an embryo from a *pumilio* mother, the posterior cap of mitochondrial activity is tightly concentrated, as in wild-type. (B) An embryo from a *cappuccino* mother shows a high posterior level of mitochondrial membrane potential, but the distribution is more diffuse than in wild-type. Anterior levels of mitochondrial activity are raised slightly as in wild-type. (C) In an embryo from a *tudor* female, the mitochondrial activity is high at the posterior pole but is more diffusely distributed than in wild-type. Anterior levels of mitochondrial activity appear higher than in (B) but are within the range of variation seen in wild-type embryos.

Figure 6: Distribution *16S* RNA in embryos from grandchildless-*knirps* mutant females

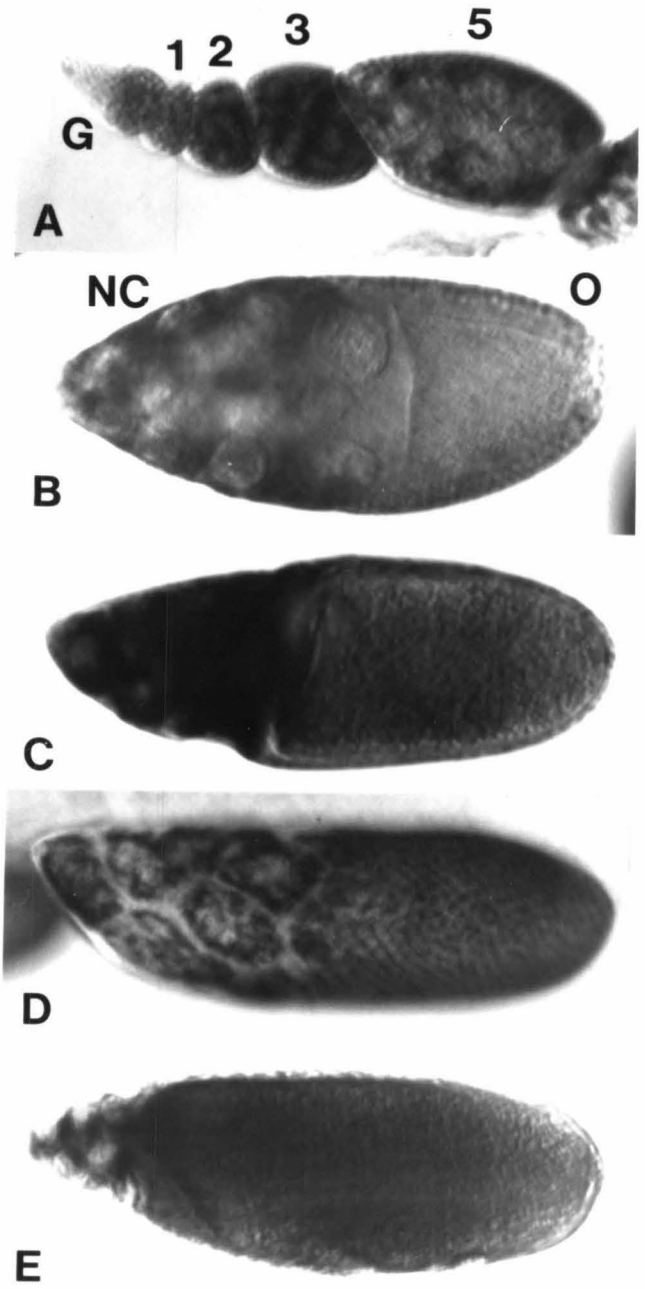
16S RNA is shown in embryos derived from females homozygous for mutations that disrupt the posterior polar plasm and the integrity of the polar granules (A-G). These are*cappuccino*: (A); *spire* (B); *staufen* (C); *oskar* (D); *vasa* (E); *tudor* (F); and *valois* (G). Maternal *16S* RNA is not present at higher levels at the posterior pole, but it is still present at uniform basal levels throughout the embryo. For all embryos, anterior is to the left and dorsal is toward the top of the page.

Figure 7: Distribution *16S* RNA in embryos from *nanos* and *pumilio* mutant females

Posterior localization of *16S* RNA is normal in early cleavage stage embryos produced by females mutant for *nanos* (A) or *pumilio* (B), which eliminate components of the posterior polar plasm without affecting the integrity of the polar granules. Shown are early cleavage stage embryos with anterior to the left and dorsal toward the top of the page.

Figure 8: Localization of *ND1* RNA during early embryogenesis

In situ hybridization of *ND1* cDNA probe to wild-type embryos. (a) In an early cleavage stage embryo, *ND1* RNA is distributed uniformly throughout the embryo. (b) At the syncytial blastoderm stage, the *ND1* RNA is distributed uniformly in the somatic part of the embryo and is present, but at a slightly lower level, in the pole cells (arrow). (c) At the cellular blastoderm stage, *ND1* RNA is present throughout the cytoplasm of the somatic part of the embryos while the pole cells (arrow) are not stained. For all embryos, anterior is to the left and dorsal is toward the top of the page.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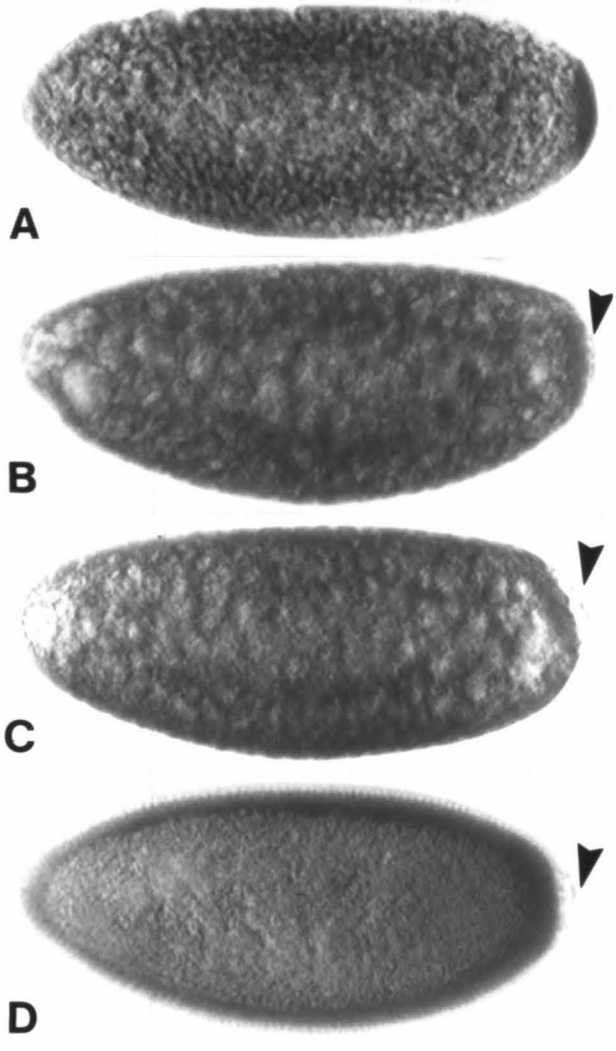
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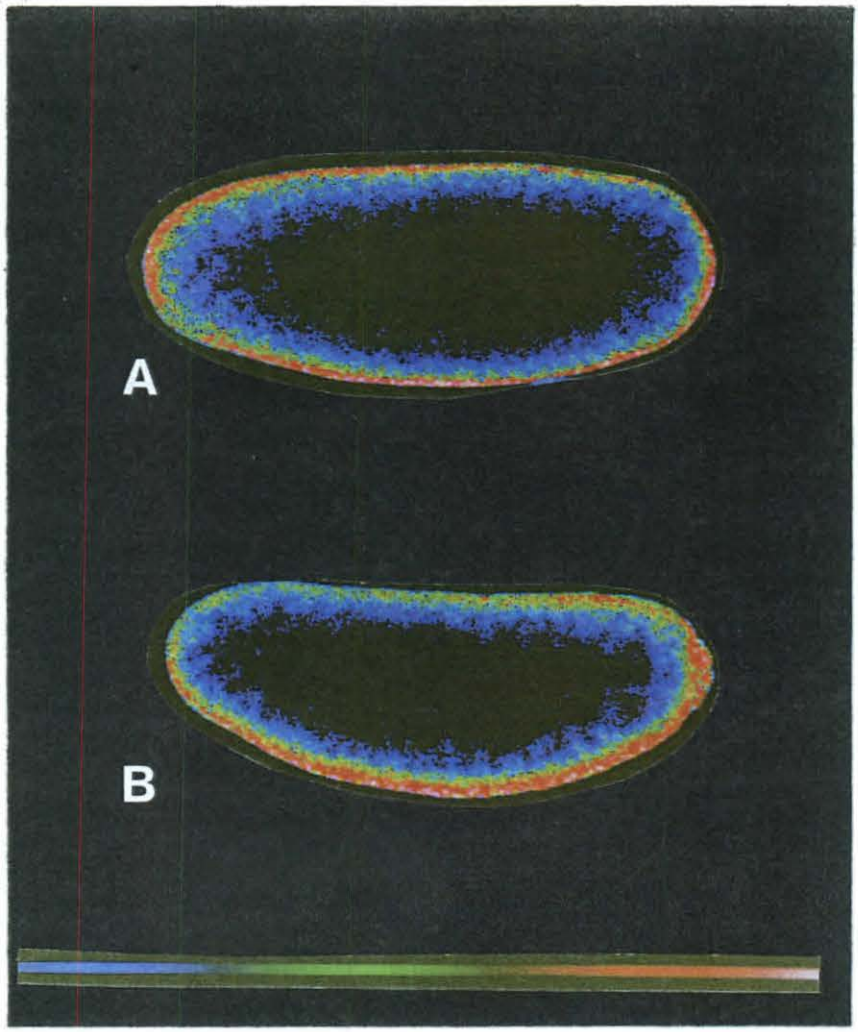
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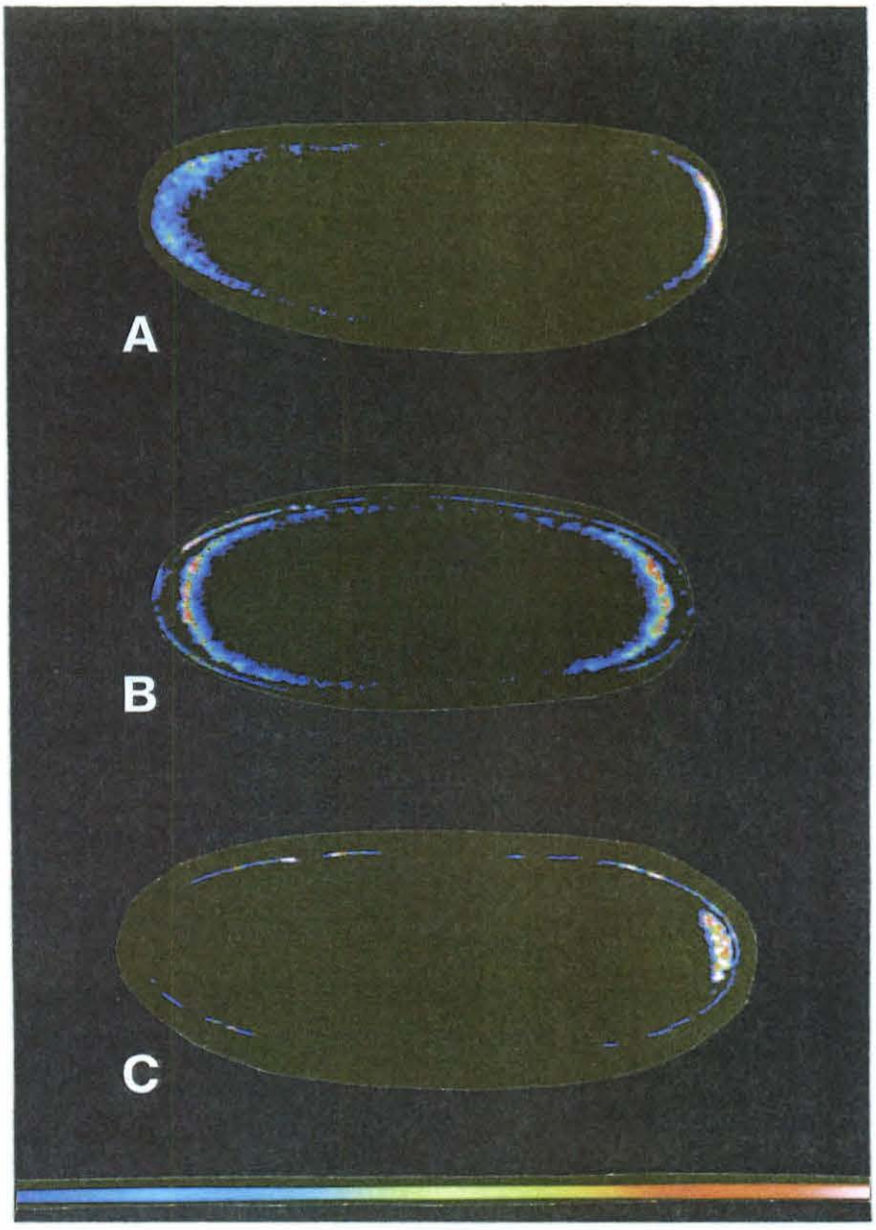
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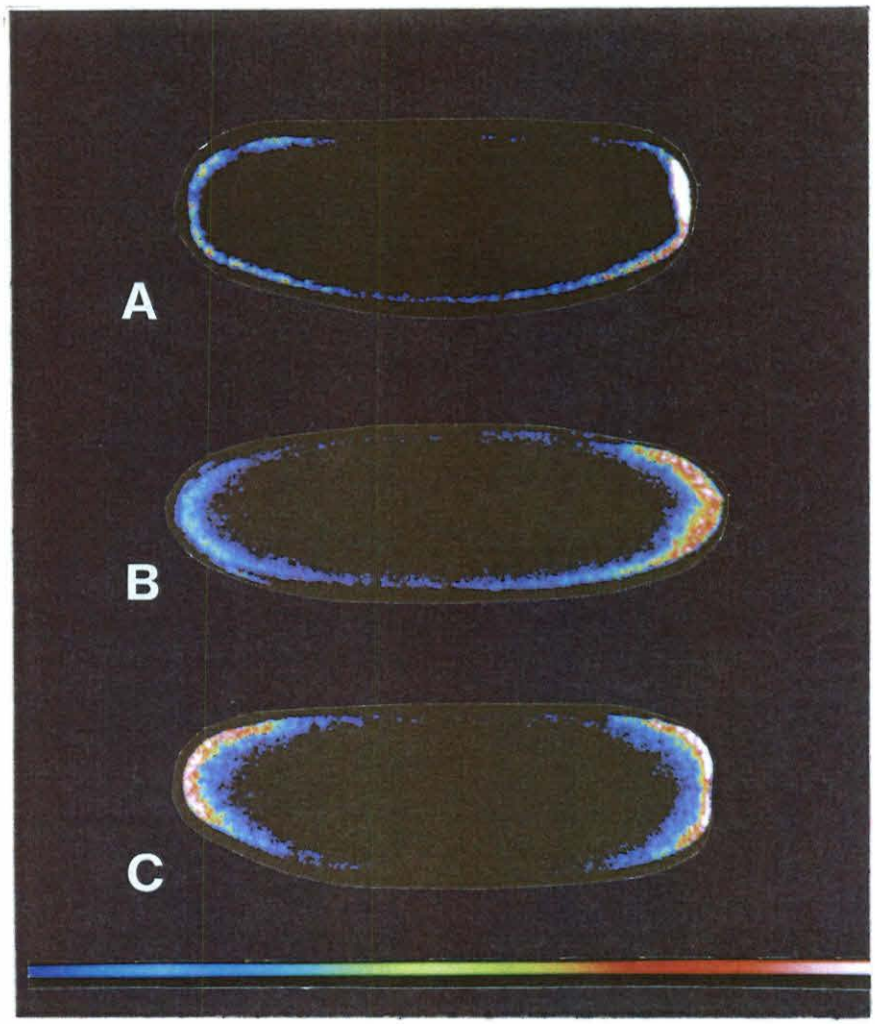
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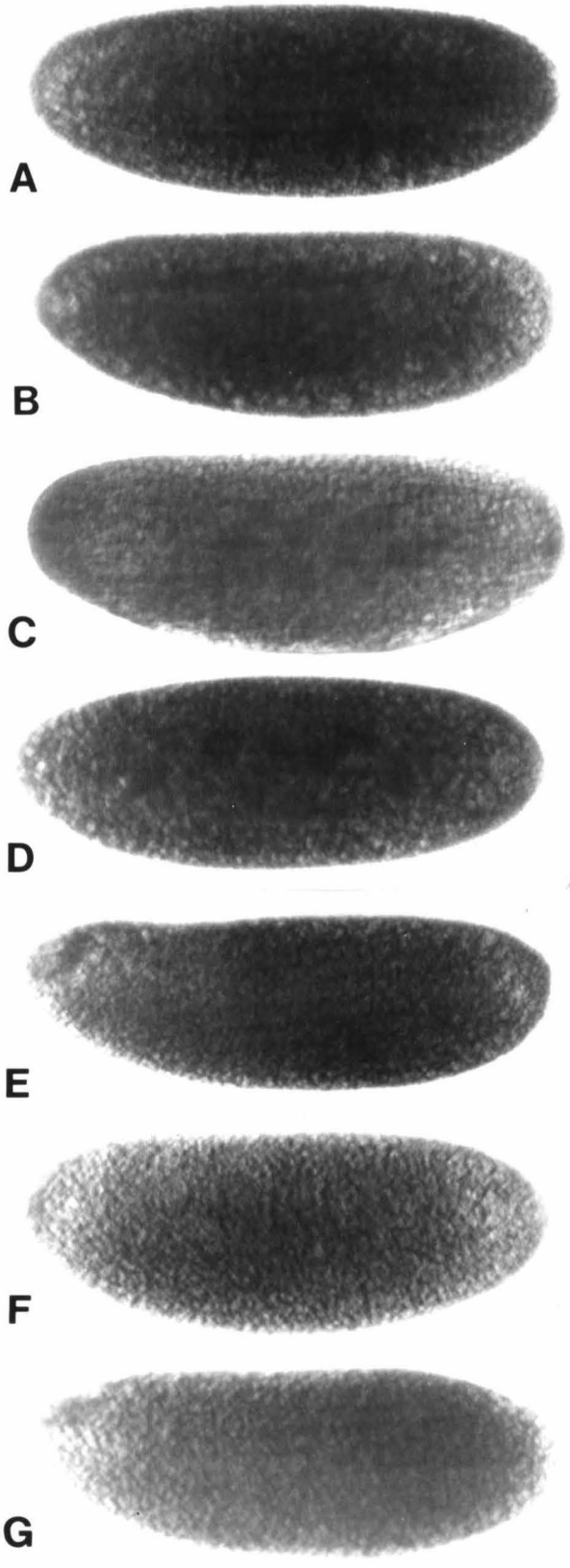
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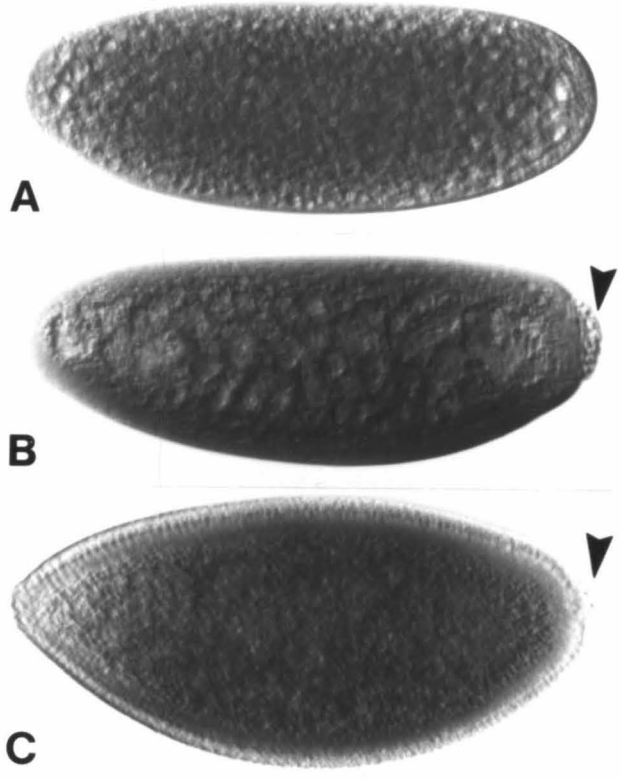
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CHAPTER 5

This chapter is in press in the *Proceedings of the National Academy of Sciences USA*, Vol. 90 (1993).

Classification: Genetics

Different genetic requirements for anterior RNA localization revealed by the distribution of Adducin-like transcripts during *Drosophila* oogenesis

(cytoskeleton/development/embryo/maternal effect genes/pattern formation)

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ABSTRACT

The proteins encoded by polar-localized mRNAs play an important role in cell fate specification along the antero-posterior axis of the *Drosophila* embryo. The only maternally synthesized mRNA known previously to be localized to the anterior cortex of both the oocyte and the early embryo is the bicoid mRNA whose localization is required to generate a homeodomain protein gradient that specifies position along the antero-posterior embryonic axis. We have identified and characterized a second mRNA that is localized to the anterior pole of the oocyte and early embryo. This mRNA encodes a *Drosophila* homolog of mammalian adducin, a membrane-cytoskeleton-associated protein that promotes the assembly of the spectrin-actin network. A comparison of the spatial distribution of bicoid and Adducin-like transcripts in the maternal effect RNA-localization mutants exuperantia, swallow and staufen indicates different genetic requirements for proper localization of these two mRNAs to the anterior pole of the oocyte and early embryo.

The asymmetric distribution of developmentally important molecules is a general phenomenon thought to contribute to proper cell and organismal polarity (reviewed in 1,2). A number of polar-localized RNAs have been identified in the *Drosophila* egg and/or early embryo. The bicoid, Bicaudal-D, *fs(1)K10* and yemanuclein-α RNAs are localized to the anterior of the oocyte (3-8). The last three of these RNAs become delocalized before the end of oogenesis; only the bicoid RNA is anterior-localized through oogenesis and into early embryogenesis.

In order to identify novel molecules that might play a role in antero-posterior axis specification in the oocyte and early embryo, we have focused on identifying maternal RNAs localized to either the anterior or the posterior pole of the *Drosophila* egg and early embryo. Here, we report the identification of a novel *Drosophila* RNA encoding a homolog of mammalian adducin (9-11), which is localized to the anterior-pole of the oocyte and early embryo. This Adducin-like RNA represents the second RNA that is localized to the anterior pole through these developmental stages. A comparison of the distribution of Adducin-like RNA with that of the other such RNA, bicoid, in mutants previously shown to result in delocalization of bicoid transcripts, has revealed different genetic requirements for anterior localization of these two RNAs.

MATERIALS AND METHODS

Identification of a Novel Anterior-Localized RNA.

While examining the embryonic expression patterns of different *Drosophila* retrotransposons by whole-mount RNA tissue *in situ* hybridization (D.D., S.M.P. and H.D.L., in preparation), we serendipitously detected an anterior-localized RNA in the early embryo using a 2.2 kb *EcoR* I - *Pst* I genomic fragment adjacent to the insertion site of a *mdg3* retrotransposon (from clone *pUC6ORC*) (12). This

EcoR I - *Pst* I fragment was used to screen a 0-4 hr embryonic cDNA library (13) and an ovarian cDNA library (D.D. and H.D.L., in preparation). Ten cDNA clones were isolated, ranging in length from 1.3 to 4.3 kb.

DNA Sequence Analysis.

Double-stranded DNA sequencing was carried out on three near full length cDNAs using a series of internal oligodeoxynucleotide primers according to D. Mathog's modification of a published protocol (14). Both strands were sequenced completely using Sequenase Version 2.0 (United States Biochemical Corp.). The GCG Sequence Analysis Package was used to assemble and analyze the DNA sequence. Similarity searches were carried out using the FASTA and the BLAST programs, and the BESTFIT and GAP programs were used to compare and align sequences found to be similar.

RNA Tissue *In Situ* Hybridization.

Whole mount RNA tissue *in situ* hybridization was based on the method of Tautz and Pfeifle (15). Ovaries from adult females were dissected in PBS, fixed for 25 minutes in 10% paraformadehyde or formadehyde/50mM EGTA/10% DMSO in PBS and washed several times in PBT (PBS plus 0.1% Tween 20). Ovaries were then rubbed gently between two frosted microscope slides to break apart the ovarioles and devitellinize the late egg chambers (a method suggested by Stephen Cohen, Baylor College of Medicine). Subsequent post-fixation, proteinase K digestion, and refixation were as described (15). Embryos were fixed according to this protocol, with minor modifications. Digoxygenin probes were labeled by random priming of DNA synthesis according to instructions from the manufacturer (Boehringer Mannheim) or by single-sided PCR-amplification according to a protocol provided by Nipam Patel (Carnegie Institution of

Washington). Hybridization and detection were as described (15), using Alkaline Phosphatase Substrate Kit II (Vector Labs). Ovaries and embryos were mounted in JB4 plastic mountant for microscopy (Polysciences).

Computer-Assisted Image Analysis.

Images of embryos visualized for Adducin-like RNA by whole-mount RNA tissue *in situ* methods, were captured and digitized for computer analysis using a Dage-MTI CCD-72 Series solid-state camera (Dage-MTI, Inc., Michigan City, Indiana, U.S.A.) and an Image Grabber NUBus digitizer board (Neotech, Ltd., U.K.) installed in a Macintosh II computer. Initial processing of the image was carried out using Image Grabber software (Version 2.01). Subsequent processing and production of a pseudocolor image representing the concentration distribution of Adducin-like transcripts was carried out using Color Image public domain software (Version 1.27; written by J. Ayers and G. Fletcher, available via anonymous FTP from sumex-aim.stanford.edu).

Mutant Strains.

Adducin-like RNA distribution during oogenesis and embryogenesis was examined in a number of maternal effect mutants. The loci (and alleles) studied were exuperantia (exu^{XL1} and exu^{VL57}) (16); staufen ($stau^{D3}$ and $stau^{RY}$) (18); swallow (sww^{1}) (19,20); cappuccino ($capu^{HK3}$) (21); and spire ($spir^{RP48}$) (21).

RESULTS

Anterior Localization of a Novel *Drosophila* RNA During Oogenesis and Embryogenesis in Wildtype.

Adducin-like RNA expression is detectable during early oogenesis in region 2B

of the germarium (Fig. 1A) and continues throughout oogenesis (stages of oogenesis according to [22]). From stage 6 of oogenesis onwards, the RNA can be seen to be localized to the anterior pole of the oocyte (Fig. 1A-D). We consistently observe that, by stage 10, the RNA is concentrated antero-dorsally and extends somewhat more posteriorly along the dorsal side of the oocyte than along the ventral side (Fig. 1B,D and 2A,B). These maternally synthesized transcripts maintain their antero-dorsal position during the early cleavage stages of embryogenesis (Fig. 1E), then appear to be released and to diffuse posteriorly, thus establishing an antero-posterior RNA gradient (Fig. 1F) (stages of embryogenesis according to [23]). Maternal transcripts are lost by the syncytial blastoderm stage (Fig. 1G). Zygotic expression commences in the neuroblasts at stage 9 of embryogenesis, reaching high levels throughout the central nervous system by stage 15 (Fig. 1H).

The Anterior-Localized RNA Encodes a *Drosophila* Homolog of Mammalian Adducin.

Northern blot analyses detected a 4.4 kb poly(A)$^+$ band in RNA purified from ovaries and early embryos (data not shown). Several cDNAs between 4.2 and 4.3 kb in length were isolated and sequenced (Fig. 3A)[§] . A single long open reading frame was found that encodes a 1156 amino acid protein with a high degree of overall similarity to mammalian adducin, a membrane cytoskeleton-associated protein that is involved in regulating the association of spectrin and actin in a variety of cell-types (9-11) (Fig. 3B). Human adducin is comprised of heterodimers of α and β subunits, respectively 737 and 726 amino acids long, which exhibit 49% amino acid identity and 66% similarity to each other (10). The presumptive *Drosophila* protein identified here exhibits 38% identity and 58%

[§] The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession number L07617.

similarity to human α-adducin, and 36% identity and 56% similarity to human β-adducin, preventing us from definitively identifying it as either a *Drosophila* α- or β-homolog. The presumptive *Drosophila* Adducin-like protein is respectively 419 and 430 amino acids longer than the human α- and β-adducin subunit proteins. We refer to this new *Drosophila* gene as Adducin-like-56D (*Add*) based on its homology to mammalian adducin and its cytological map position. Reduced stringency genomic Southern blot analysis indicates that there are no additional *Drosophila* Adducin-like genes with greater than 63% similarity to the *Add* gene (data not shown). Preliminary sequence analysis of additional cDNAs suggests that variant *Drosophila* Adducin-like proteins are produced by differential splicing of the transcripts produced by the *Add* gene (data not shown). Since the probes used in our *in situ* hybridization analysis recognize common portions of the Adducin-like transcripts, we conclude that all forms of Adducin-like RNA present in the oocyte and early embryo are anterior-localized.

Effects of Maternal Mutants on Localization of Adducin-like RNA.

In order that bicoid carry out its normal developmental functions, its RNA must be localized to the anterior cortex of the egg (3,4,5,24). Localization of bicoid RNA is accomplished in at least four distinct phases and requires the products of the exuperantia, swallow and staufen genes (3,4). It has been suggested that the products of the exuperantia, swallow and staufen genes may play an accessory role in anchoring bicoid RNA to the cytoskeleton (20,25). Since Adducin-like mRNA is also anteriorly-localized in both the *Drosophila* oocyte and early embryo, we examined its distribution in embryos derived from females homozygous for the exuperantia, swallow, or staufen mutations, and compared it with that of bicoid mRNA (Fig. 4).

exuperantia activity is required prior to stage 9 of oogenesis to localize bicoid

mRNA to a perinuclear cap on the apical side of each nurse cell nucleus and in a ring at the anterior end of the oocyte (3,4,25). In contrast to bicoid, we find that Adducin-like mRNA localization proceeds normally in exuperantia mutants (Fig. 4A), showing that exuperantia plays no role in the initial stages of Adducin-like mRNA localization. In parallel experiments on embryos produced by the females from the same mutant stock, we confirmed that bicoid RNA is delocalized as expected (data not shown) (3,4).

swallow activity is required for the next phase of bicoid mRNA localization where nurse cell localization disappears and bicoid RNA becomes localized to the anterior cortex of the developing oocyte (3,4,20). In the case of bicoid mRNA, embryos from swallow mutant mothers first exhibit delocalization effects at stage 10B of oogenesis when the nurse cells are contracting and transferring their cytoplasm into the oocyte (3,4,20). In oocytes and embryos derived from swallow mutant females, Adducin-like mRNA is also delocalized (Fig. 4C-F). However, Adducin-like mRNA delocalization commences by stage 8 of oogenesis (Fig. 4C), 12 hours earlier than delocalization of bicoid mRNA. We consistently observe that delocalized Adducin-like mRNA first spreads posteriorly along the dorsal side of the oocyte (Fig. 4D), only later becoming uniformly distributed along both its dorso-ventral and its antero-posterior axes (Fig. 4E-F).

The last of the loci shown to be required for maintenance of bicoid mRNA localization is staufen, which acts to maintain bicoid mRNA in an antero-dorsal position after stage 12 of oogenesis (3,4). Neither of two strong staufen alleles had any effect on Adducin-like mRNA localization (Fig. 4B). In contrast, bicoid mRNA was delocalized as expected (data not shown) (3,4).

In addition we have found that cappuccino and spire mutations, which have been shown to result in delocalization of the *fs(1)K10* RNA (26), have no effect on anterior localization of Adducin-like RNA (data not shown).

DISCUSSION

An Anterior-Localized RNA in the *Drosophila* Oocyte and Early Embryo Encodes a Homolog of a Membrane-Cytoskeletal Protein.

The similarity between mammalian adducin and the protein encoded by the novel *Drosophila* RNA identified here, extends over the entire length of the mammalian protein, indicating that we have identified a *Drosophila* homolog of mammalian adducin. This *Drosophila* Adducin-like RNA is localized to the anterior pole of both the oocyte and the early embryo; thus, it is the first such polar-localized RNA to encode a homolog of a known cytoskeletal protein. The Bicaudal-D protein was previously shown to exhibit similarity to myosin heavy-chain and related proteins; however, this similarity is limited to the myosin tail domain (7). Further, the Bicaudal-D RNA is only transiently localized anteriorly during oogenesis (7).

Mammalian adducin is a membrane-cytoskeletal protein that promotes association of spectrin with F-actin at actin-spectrin junctions in a calcium/calmodulin regulated reaction (9-11). Adducin isoforms are present in erythrocytes, brain, liver, kidney, lung, testes, epithelial tissues and various cultured cell lines (9-11). In epithelia and cultured cells, the protein is localized to sites of cell-cell contact (11). It has been suggested that adducin functions in the assembly of the spectrin-based membrane cytoskeleton which plays a key role in orchestrating the topographic relations of integral membrane proteins within the membrane as well as in organizing integral membrane protein interactions with cytoplasmic proteins (9-11). An asymmetric distribution of the *Drosophila* Adducin-like protein might be expected to participate in the establishment and/or maintenance of an asymmetric membrane-cytoskeletal network in the nurse cell-

oocyte complex as well as in the oocyte and early embryo. Future analysis of Adducin-like protein functions will help to assess the role played by such a cytoskeletal network during oogenesis and embryogenesis.

Adducin-like and bicoid RNAs Differ in their Genetic Requirements for Anterior Localization.

Despite the similarity of the anterior localization patterns for the bicoid and Adducin-like RNAs, the genetic requirements for their proper localization differ. While exuperantia, swallow and staufen are required at different stages for the localization of bicoid RNA, only swallow functions in the localization of Adducin-like RNA to the anterior pole of the oocyte and early embryo. The initial anterior localization and the later anterior maintenance of Adducin-like RNA must, therefore, depend on some as yet to be identified components. Further studies will be needed to explore the molecular and genetic basis for the differences between bicoid and Adducin-like RNA localization mechanisms, particularly whether or not Adducin-like RNA carries a localization "tag," as is the case for bicoid RNA (27), and the identification of additional loci required for RNA localization.

Embryos from swallow mutant females exhibit abnormalities additional to those predicted solely on the basis of delocalization of bicoid RNA. These include defects in nuclear cleavage and cellularization as well as in abdominal development. Consequently the swallow protein has been proposed to play a more general role in cytoskeletal organization during oogenesis and early embryogenesis (20,25). Whether any of the developmental abnormalities in embryos from swallow mutant females are attributable to delocalization of Adducin-like RNA remains to be determined.

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REFERENCES

1. Gottlieb, E. (1990) *Curr. Opinion Cell Biol.* **2**, 1080-1086.
2. Lipshitz, H.D. (1991) *Curr. Opinion Cell Biol.* **3**, 966-975.
3. Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nüsslein-Volhard, C. (1988) *EMBO J.* **7**, 1749-1756.
4. St Johnston, D., Driever, W., Berleth, T., Richstein S., & Nüsslein-Volhard, C. (1989) *Develop.* **107(suppl.)**, 13-16.
5. Driever, W., & Nüsslein-Volhard, C. (1988) *Cell* **34**, 95-104 .
6. Haenlin, M., Roos, C., Cassab, A., & Mohier, E. (1987) *EMBO J.* **6**, 801-807
7. Suter, B., Romberg, L.M., & Steward, R. (1989) *Genes Dev.* **3**, 1957-1968.

8. Aït-Ahmed, O., Thomas-Cavallin, M., & Rosset, R. (1987) *Dev. Biol.* **122**, 153-162.

9. Gardner, K., & Bennett, V. (1987) *Nature* **328**, 359-362.

10. Joshi, R., Gilligan, D.M., Otto, E., McLaughlin, T., & Bennett, V. (1991) *J. Cell Biol.* **115**, 665-675.

11. Kaiser, H.W., O'Keefe, E., & Bennett, V. (1989) *J. Cell Biol.* **109**, 557-569.

12. Mossie, K.G., Young, M.W., & Varmus, H.E. (1985) *J. Mol. Biol.* **182**, 31-43.

13. Brown, N.H., & Kafatos, F.C. (1988) *J. Mol. Biol.* **203**, 425-437.

14. Del Sal, G., Manfioletti, G., & Schneider, C. (1989) *BioTechniques* **7**, 514- 519.

15. Tautz, D., & Pfeifle, C. (1989) *Chromosoma* **98**, 81-85.

16. Marcey, D., Watkins, W.S., & Hazelrigg, T. (1991) *EMBO J.* **10**, 4259-4266.

17. Macdonald, P.M., Luk, S.K.-S, & Kilpatrick, M. *Genes Dev.* **5**, 2455-2466 (1991).

18. St Johnston, D., Beuchle, D., & Nüsslein-Volhard, C. (1991) *Cell* **66**, 51-63.

19. Lindsley, D.L., & Zimm, G.G. (1992) *The Genome of* Drosophila melanogaster (Academic Press, San Diego).

20. Stephenson, E.C., Chao, Y.-C., & Fackenthal, J. (1988) *Genes Dev.* **2**, 1655-1665.

21. Manseau, L., & Schüpbach, T. (1989) *Genes Dev.* **3**, 1437-1452.

22. King, R.C. (1970) *Ovarian Development in* Drosophila melanogaster (Academic Press, NY).

23. Campos-Ortega, J.A., & Hartenstein, V. (1985) *The Embryonic*

Development of Drosophila melanogaster (Springer-Verlag, NY).

24. Driever, W., Siegel, V., & Nüsslein-Volhard, C. (1990) *Development* **109**, 811-820.

25. Pokrywka, M.J., & Stephenson, E.C. (1991) *Development* **113**, 55-66.

26. Cheung, H.-K., Serano, T.L., & Cohen, R.S. (1992) *Develop.* **114**, 653-661.

27. Macdonald, P.M., & Struhl, G. (1988) *Nature* **336**, 595-598.

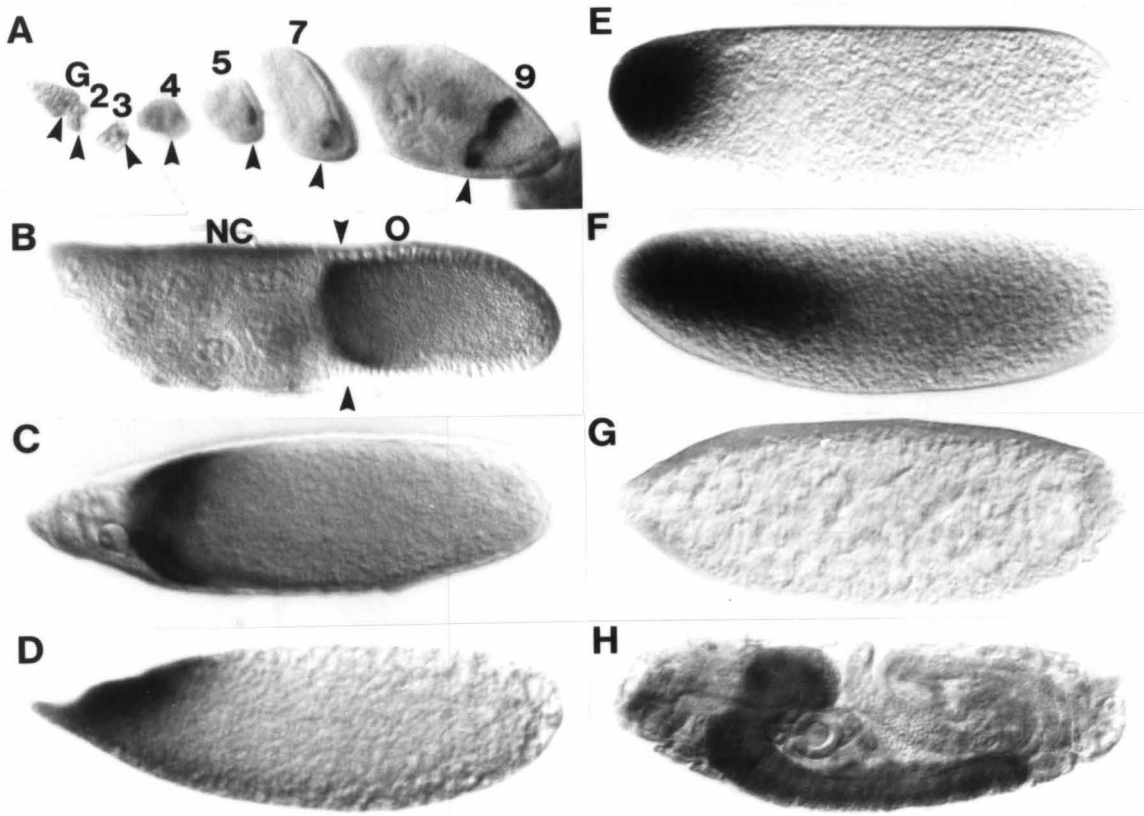
FIGURE LEGENDS

FIG. 1. Distribution of Adducin-like RNA during wildtype oogenesis and embryogenesis visualized by whole mount RNA tissue *in situ* hybridization (15). Stages are according to King (22) (oogenesis) and Campos-Ortega and Hartenstein (23) (embryogenesis). Oogenesis: (*A*) Arrowheads point to the RNA. It can first be readily detected in region 2B of the germarium ('G'). In stage 2 to 5 egg chambers ('2'-'5'), the RNA fills the most posterior cell, the oocyte. From stages 6 to 12, the RNA is localized to the anterior margin of the oocyte. This can be seen in the follicles labeled '7' and '9' in (*A*); in (*B*), a stage 10 egg chamber ('NC': nurse cells; 'O': oocyte); and (*C*), a stage 12 egg chamber. The RNA consistently extends more posteriorly along the dorsal side of the oocyte than along the ventral side and is present at its highest levels antero-dorsally from as early as stage 10 (also see Fig. 2A). (*D*) During stages 13/14, the RNA becomes even more concentrated at the antero-dorsal tip of the mature egg (also see Fig. 2B). During oogenesis, most of the RNA is concentrated at the anterior of the oocyte; however, low levels of RNA can also be seen in the nurse cells throughout oogenesis [e.g., see (*B*) which is focused on the nurse cells to show the RNA], suggesting that some or all of the RNA might be synthesized in the nurse cells. Embryogenesis: (*E*) In early cleavage stage embryos (stage 1-2), the RNA is localized antero-dorsally. (*F*) Slightly later (stage 2-3), the RNA appears to diffuse posteriorly, establishing an antero-posterior gradient. (*G*) By the syncytial blastoderm stage (stage 4), maternal RNA is no longer detectable. (*H*) Zygotically transcribed RNA reaches high levels in the CNS by stage 15/16 of embryogenesis. For all oocytes and embryos [except (*C*)], anterior is to the left and dorsal is up; (*C*) is a dorsal view.

FIG. 2. Pseudocolor images of Adducin-like RNA during oogenesis. (*A*) Image of the stage 10 egg chamber shown in Fig. 1B. Highest concentrations of RNA are represented by red and lowest by blue. The peak concentration of Adducin-like RNA is antero-dorsal and the RNA extends more posteriorly along the dorsal side of the oocyte than along the ventral side. (*B*) A pseudocolor image of the stage 13/14 egg chamber shown in Fig. 1D. Highest concentrations of RNA are represented by red and lowest by blue. The peak concentration of Adducin-like RNA is antero-dorsal. See Materials and Methods for details about the computer hardware and software used in the production of these images.

FIG. 3. Sequence of *Drosophila* Adducin-like cDNAs and comparison of *Drosophila* Adducin-like protein and human adducins. (*A*) Three near full length cDNAs (N1, N4 and R8), each greater than 4.2 kb long, were sequenced, revealing an open reading frame of 3468 bases that encodes a 1156 amino acid protein. ">" indicates the 5'-end of each cDNA. Several minor sequence variations among the cDNAs were found: one occurs in the 5'-untranslated region (DNA sequence position #57), while all the others occur in the third base of codons and thus do not affect the protein sequence (DNA sequence positions #1203, 2064, 2073, 2214 and 3096). The encoded protein sequence is shown below the DNA sequence. Following the open reading frame is a 345 bp long 3'-untranslated region. A potential polyadenylation signal (AATAAA) preceding the 3' end is underlined. (*B*) Amino acid sequence comparison of *Drosophila* Adducin-like and human α- and β- adducin (10). Identities are represented by vertical bars, similarities by colons or periods. Gaps are indicated by horizontal lines.

FIG. 4. Distribution of Adducin-like RNA during oogenesis and embryogenesis in mutants, visualized by whole mount RNA tissue *in situ* hybridization (15). Anterior localization of Adducin-like mRNA occurs normally in oocytes and embryos from exuperantia (*exu*) (*A*) and staufen (*stau*) mothers (*B*), but not in oocytes and embryos from swallow (*sww*) mothers (*C*) - (*F*). (*A*) Adducin-like RNA distribution in an embryo produced by a female homozygous for exu^{XL1}, a strong *exu* allele (16). The distribution is indistinguishable from that seen in wildtype embryos (*cf.* Fig. 1E, F). In parallel experiments bicoid RNA was assayed in embryos from these females and was found to be delocalized as expected (3,4) (data not shown). The Adducin-like mRNA distribution was also examined in ovaries of exu^{VL57} mothers and found to be normal (data not shown). (*B*) Adducin-like RNA distribution in an embryo produced by a female homozygous for $stau^{D3}$, a strong *stau* allele (18). The distribution is indistinguishable from that seen in wildtype embryos (*cf.* Fig. 1E, F). In parallel experiments bicoid RNA was assayed in embryos from these females and was found to be delocalized as expected (3,4) (data not shown). The Adducin-like mRNA distribution was also examined in ovaries of $stau^{D3}$ and $stau^{RY}$ mothers and found to be normal (data not shown). (*C*) - (*F*) Adducin-like RNA is delocalized in oocytes and embryos produced by females homozygous for sww^{1}, a strong swallow allele (19,20). (*C*) By stage 7/8 of oogenesis, Adducin-like RNA begins to delocalize from the anterior pole of the oocyte (arrowhead). (*D*) A stage 10 oocyte exhibits partial delocalization of Adducin-like RNA along the dorsal side (arrowheads). (*E*) By stage 12 of oogenesis, Adducin-like RNA is completely delocalized. This delocalized RNA persists through oogenesis and the cleavage stages of embryogenesis (*F*). In all panels, the orientation is with anterior to the left and dorsal up.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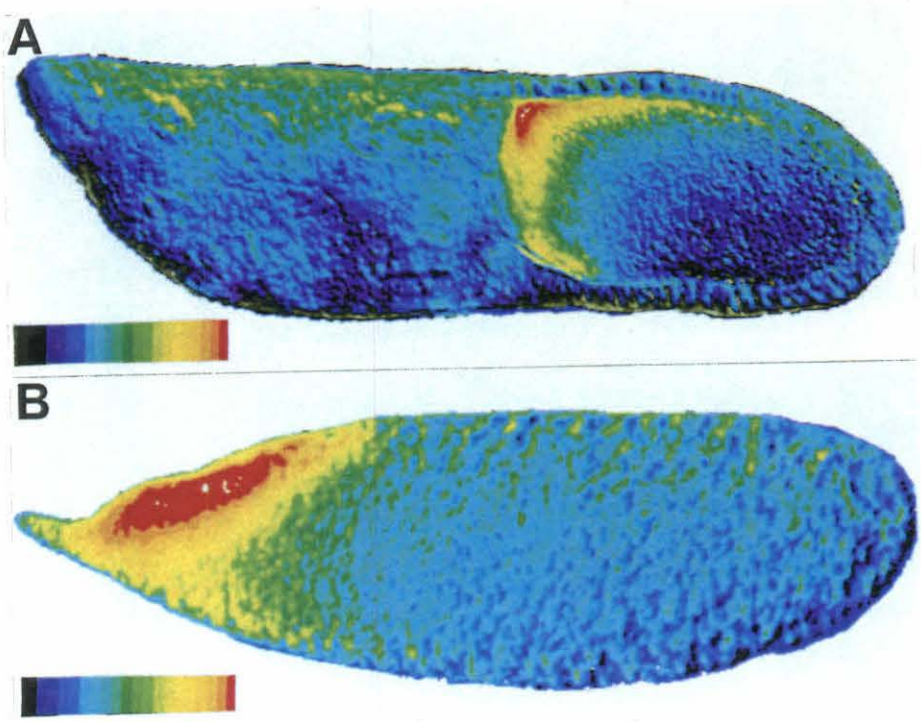
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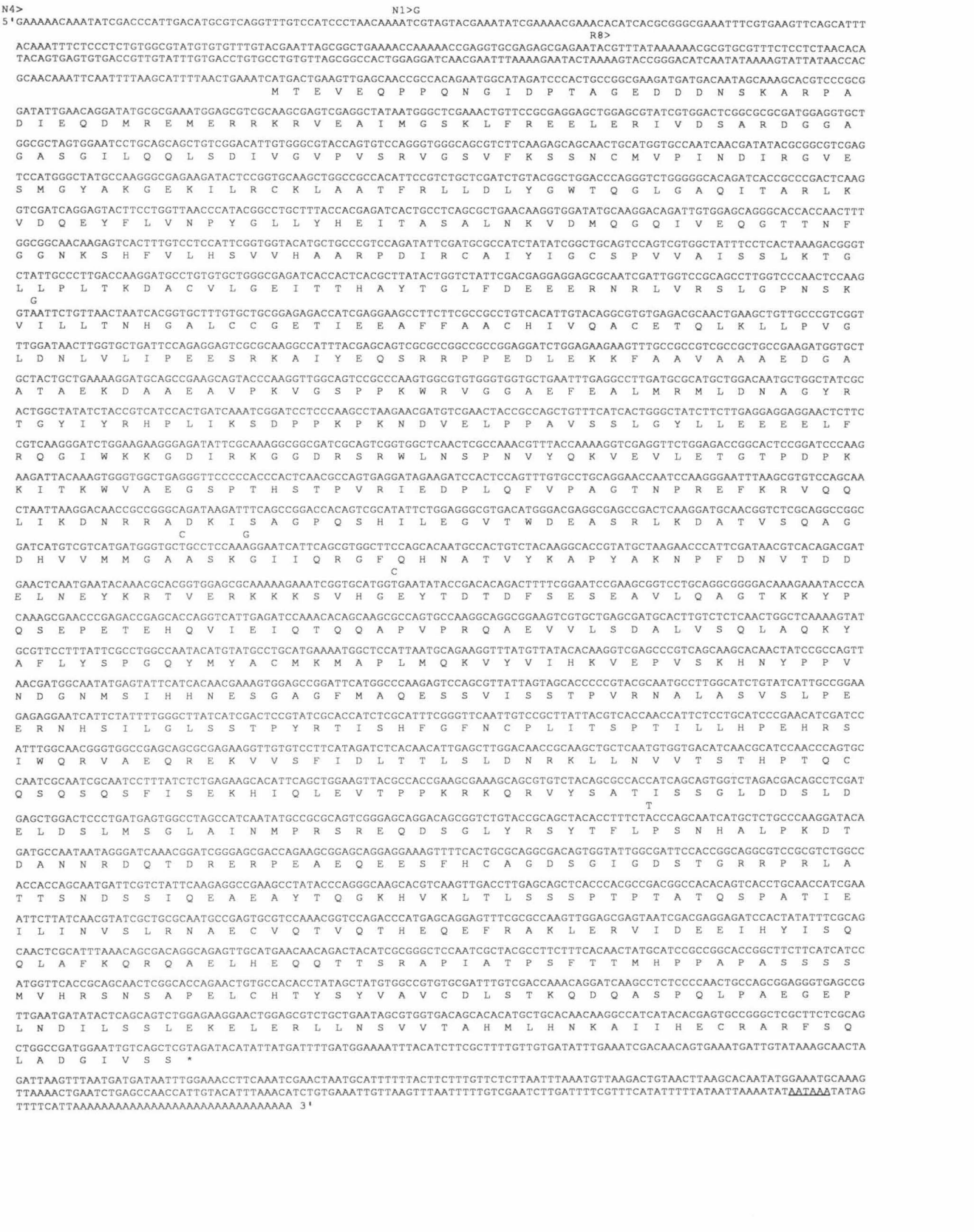
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N1>G
R8>
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241 TACAGTGAGTGTGACCGTTGTATTTGTGACCTGTGCCTGTGTTAGCGGCCACTGGAGGATCAACGAATTTAAAAGAATACTAAAAGTACCGGGACATCAATATAAAAGTATTATAACCAC
361 GCAACAAATTCAATTTTAAGCATTTTAACTGAAATCATGACTGAAGTTGAGCAACCGCCACAGAATGGCATAGATCCCACTGCCGGCGAAGATGATGACAATAGCAAAGCACGTCCCGCG
1 M T E V E Q P P Q N G I D P T A G E D D D N S K A R P A
481 GATATTGAACAGGATATGCGCGAAATGGAGCGTCGCAAGCGAGTCGAGGCTATAATGGGCTCGAAACTGTTCCGCGAGGAGCTGGAGCGTATCGTGGACTCGGCGCGCGATGGAGGTGCT
29 D I E Q D M R E M E R R K R V E A I M G S K L F R E E L E R I V D S A R D G G A
601 GGCGCTAGTGGAATCCTGCAGCAGCTGTCGGACATTGTGGGCGTACCAGTGTCCAGGGTGGGCAGCGTCTTCAAGAGCAGCAACTGCATGGTGCCAATCAACGATATACGCGGCGTCGAG
69 G A S G I L Q Q L S D I V G V P V S R V G S V F K S S N C M V P I N D I R G V E
721 TCCATGGGCTATGCCAAGGGCGAGAAGATACTCCGGTGCAAGCTGGCCGCCACATTCCGTCTGCTCGATCTGTACGGCTGGACCCAGGGTCTGGGGGCACAGATCACCGCCCGACTCAAG
109 S M G Y A K G E K I L R C K L A A T F R L L D L Y G W T Q G L G A Q I T A R L K
841 GTCGATCAGGAGTACTTCCTGGTTAACCCATACGGCCTGCTTTACCACGAGATCACTGCCTCAGCGCTGAACAAGGTGGATATGCAAGGACAGATTGTGGAGCAGGGCACCACCAACTTT
149 V D Q E Y F L V N P Y G L L Y H E I T A S A L N K V D M Q G Q I V E Q G T T N F
961 GGCGGCAACAAGAGTCACTTTGTCCTCCATTCGGTGGTACATGCTGCCCGTCCAGATATTCGATGCGCCATCTATATCGGCTGCAGTCCAGTCGTGGCTATTTCCTCACTAAAGACGGGT
189 G G N K S H F V L H S V V H A A R P D I R C A I Y I G C S P V V A I S S L K T G
1081 CTATTGCCCTTGACCAAGGATGCCTGTGTGCTGGGCGAGATCACCACTCACGCTTATACTGGTCTATTCGACGAGGAGGAGCGCAATCGATTGGTCCGCAGCCTTGGTCCCAACTCCAAG
229 L L P L T K D A C V L G E I T T H A Y T G L F D E E E R N R L V R S L G P N S K
G
1201 GTAATTCTGTTAACTAATCACGGTGCTTTGTGCTGCGGAGAGACCATCGAGGAAGCCTTCTTCGCCGCCTGTCACATTGTACAGGCGTGTGAGACGCAACTGAAGCTGTTGCCCGTCGGT
269 V I L L T N H G A L C C G E T I E E A F F A A C H I V Q A C E T Q L K L L P V G
1321 TTGGATAACTTGGTGCTGATTCCAGAGGAGTCGCGCAAGGCCATTTACGAGCAGTCGCGCCGGCCGCCGGAGGATCTGGAGAAGAAGTTTGCCGCCGTCGCCGCTGCCGAAGATGGTGCT
309 L D N L V L I P E E S R K A I Y E Q S R R P P E D L E K K F A A V A A A E D G A
1441 GCTACTGCTGAAAAGGATGCAGCCGAAGCAGTACCCAAGGTTGGCAGTCCGCCCAAGTGGCGTGTGGGTGGTGCTGAATTTGAGGCCTTGATGCGCATGCTGGACAATGCTGGCTATCGC
349 A T A E K D A A E A V P K V G S P P K W R V G G A E F E A L M R M L D N A G Y R
1561 ACTGGCTATATCTACCGTCATCCACTGATCAAATCGGATCCTCCCAAGCCTAAGAACGATGTCGAACTACCGCCAGCTGTTTCATCACTGGGCTATCTTCTTGAGGAGGAGGAACTCTTC
389 T G Y I Y R H P L I K S D P P K P K N D V E L P P A V S S L G Y L L E E E E L F
1681 CGTCAAGGGATCTGGAAGAAGGGAGATATTCGCAAAGGCGGCGATCGCAGTCGGTGGCTCAACTCGCCAAACGTTTACCAAAAGGTCGAGGTTCTGGAGACCGGCACTCCGGATCCCAAG
429 R Q G I W K K G D I R K G G D R S R W L N S P N V Y Q K V E V L E T G T P D P K
1801 AAGATTACAAAGTGGGTGGCTGAGGGTTCCCCCACCCACTCAACGCCAGTGAGGATAGAAGATCCACTCCAGTTTGTGCCTGCAGGAACCAATCCAAGGGAATTTAAGCGTGTCCAGCAA
469 K I T K W V A E G S P T H S T P V R I E D P L Q F V P A G T N P R E F K R V Q Q
1921 CTAATTAAGGACAACCGCCGGGCAGATAAGATTTCAGCCGGACCACAGTCGCATATTCTGGAGGGCGTGACATGGGACGAGGCGAGCCGACTCAAGGATGCAACGGTCTCGCAGGCCGGC
509 L I K D N R R A D K I S A G P Q S H I L E G V T W D E A S R L K D A T V S Q A G
C G
2041 GATCATGTCGTCATGATGGGTGCTGCCTCCAAAGGAATCATTCAGCGTGGCTTCCAGCACAATGCCACTGTCTACAAGGCACCGTATGCTAAGAACCCATTCGATAACGTCACAGACGAT
549 D H V V M M G A A S K G I I Q R G F Q H N A T V Y K A P Y A K N P F D N V T D D
C
2161 GAACTCAATGAATACAAACGCACGGTGGAGCGCAAAAAGAAATCGGTGCATGGTGAATATACCGACACAGACTTTTCGGAATCCGAAGCGGTCCTGCAGGCGGGGACAAAGAAATACCCA
589 E L N E Y K R T V E R K K K S V H G E Y T D T D F S E S E A V L Q A G T K K Y P
2281 CAAAGCGAACCCGAGACCGAGCACCAGGTCATTGAGATCCAAACACAGCAAGCGCCAGTGCCAAGGCAGGCGGAAGTCGTGCTGAGCGATGCACTTGTCTCTCAACTGGCTCAAAAGTAT
629 Q S E P E T E H Q V I E I Q T Q Q A P V P R Q A E V V L S D A L V S Q L A Q K Y
2401 GCGTTCCTTTATTCGCCTGGCCAATACATGTATGCCTGCATGAAAATGGCTCCATTAATGCAGAAGGTTTATGTTATACACAAGGTCGAGCCCGTCAGCAAGCACAACTATCCGCCAGTT
669 A F L Y S P G Q Y M Y A C M K M A P L M Q K V Y Y V I H K V E P V S K H N Y P P V
2521 AACGATGGCAATATGAGTATTCATCACAACGAAAGTGGAGCCGGATTCATGGCCCAAGAGTCCAGCGTTATTAGTAGCACCCCCGTACGCAATGCCTTGGCATCTGTATCATTGCCGGAA
709 N D G N M S I H H N E S G A G F M A Q E S S V I S S T P V R N A L A S V S L P E
2641 GAGAGGAATCATTCTATTTTGGGCTTATCATCGACTCCGTATCGCACCATCTCGCATTTCGGGTTCAATTGTCCGCTTATTACGTCACCAACCATTCTCCTGCATCCCGAACATCGATCC
749 E R N H S I L G L S S T P Y R T I S H F G F N C P L I T S P T I L L H P E H R S
2761 ATTTGGCAACGGGTGGCCGAGCAGCGCGAGAAGGTTGTGTCCTTCATAGATCTCACAACATTGAGCTTGGACAACCGCAAGCTGCTCAATGTGGTGACATCAACGCATCCAACCCAGTGC
789 I W Q R V A E Q R E K V V S F I D L T T L S L D N R K L L N V V T S T H P T Q C
2881 CAATCGCAATCGCAATCCTTTATCTCTGAGAAGCACATTCAGCTGGAAGTTACGCCACCGAAGCGAAAGCAGCGTGTCTACAGCGCCACCATCAGCAGTGGTCTAGACGACAGCCTCGAT
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T
3001 GAGCTGGACTCCCTGATGAGTGGCCTAGCCATCAATATGCCGCGCAGTCGGGAGCAGGACAGCGGTCTGTACCGCAGCTACACCTTTCTACCCAGCAATCATGCTCTGCCCAAGGATACA
869 E L D S L M S G L A I N M P R S R E Q D S G L Y R S Y T F L P S N H A L P K D T
3121 GATGCCAATAATAGGGATCAAACGGATCGGGAGCGACCAGAAGCGGAGCAGGAGGAAAGTTTTCACTGCGCAGGCGACAGTGGTATTGGCGATTCCACCGGCAGGCGTCCGCGTCTGGCC
909 D A N N R D Q T D R E R P E A E Q E E S F H C A G D S G I G D S T G R R P R L A
3241 ACCACCAGCAATGATTCGTCTATTCAAGAGGCCGAAGCCTATACCCAGGGCAAGCACGTCAAGTTGACCTTGAGCAGCTCACCCACGCCGACGGCCACACAGTCACCTGCAACCATCGAA
949 T T S N D S S I Q E A E A Y T Q G K H V K L T L S S S P T P T A T Q S P A T I E
3361 ATTCTTATCAACGTATCGCTGCGCAATGCCGAGTGCGTCCAAACGGTCCAGACCCATGAGCAGGAGTTTCGCGCCAAGTTGGAGCGAGTAATCGACGAGGAGGATCCACTATATTTCGCAG
989 I L I N V S L R N A E C V Q T V Q T H E Q E F R A K L E R V I D E E I H Y I S Q
3481 CAACTCGCATTTAAACAGCGACAGGCAGAGTTGCATGAACAACAGACTACATCGCGGGCTCCAATCGCTACGCCTTCTTTCACAACTATGCATCCGCCGGCAACCGGCTTCTTCATCATCC
1029 Q L A F K Q R Q A E L H E Q Q T T S R A P I A T P S F T T M H P P A P A S S S S
3601 ATGGTTCACCGCAGCAACTCGGCACCAGAACTGTGCCACACCTATAGCTATGTGGCCGTGTGCGATTTGTCGACCAAACAGGATCAAGCCTCTCCCCAACTGCCAGCGGAGGGTGAGCCG
1069 M V H R S N S A P E L C H T Y S Y V A V C D L S T K Q D Q A S P Q L P A E G E P
3721 TTGAATGATATACTCAGCAGTCTGGAGAAGGAACTGGAGCGTCTGCTGAATAGCGTGGTGACAGCACACATGCTGCACAACAAGGCCATCATACACGAGTGCCGGGCTCGCTTCTCGCAG
1109 L N D I L S S L E K E L E R L L N S V V T A H M L H N K A I I H E C R A R F S Q
3841 CTGGCCGATGGAATTGTCAGCTCGTAGATACATATTATGATTTTGATGGAAAATTTACATCTTCGCTTTTGTTGTGATATTTGAAATCGACAACAGTGAAATGATTGTATAAAGCAACTA
1149 L A D G I V S S *
3961 GATTAAGTTTAATGATGATAATTTGGAAACCTTCAAATCGAACTAATGCATTTTTTACTTCTTTGTTCTCTTAATTTAAATGTTAAGACTGTAACTTAAGCACAATATGGAAATGCAAAG
4081 TTAAAACTGAATCTGAGCCAACCATTGTACATTTAAACATCTGTGAAATTGTTAAGTTTAATTTTTGTCGAATCTTGATTTTCGTTTCATATTTTTATAATTAAAATATAATAAATATAG
4201 TTTTCATTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 3'

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H-adda 1 MNGDSRAAVVTSPPPTTAPHKERYFDRVDEN_____NPEYL____RERNMAPDLRQDFNMMEQKKRVSMILQSPAFCEELESMIQEQFKKGKNPTGLLALQQIADFMTTNVPNVYPAA
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H-addb 1 M_____SEETVPEAASP_____PPPQGQPYFDRFSEDDPEYMRLRNR__AADLRQDFNLMEQKKRVTMILQSPSFREELEGLIQEQMKKGNNSSNI__W_ALRQIADFMASTS____
H-adda 110 PQGGMAALNMSL____GMVTPVNDLRGSDSIAYDKGEKLLRCKLAAFYRLADLFGWSQLIYNHITTRVNSEQEHFLIVPFGLLYSEVTASSLVKINLQGDIVDRGSTNLGVNQAGFTLHS
D-add 89 _____GSVFKSS____NCMVPINDIRGVESMGYAKGEKILRCKLAATFRLLDLYGWTQGLGAQITARLKVDQEYFLVNPYGLLYHEITASALNKVDMQGQIVEQGTTNFGGNKSHFVLHS
H-addb 99 _____HAVFPTSSMNVSMMTPINDLHTADSLNLAKGERLMRCKISSVYRLLDLYGWAQLSDTYVTLRVSKEQDHFLISPKGVSCSEVTASSLIKVNILGEVVEKGSSCFPVDTTGFCLHS
H-adda 226 AIYAARPDVKCVVHIHTPAGAAVSAMKCGLLPISPEALSLGEVAYHDYHGILVDEE_EKVLIQKNLGPKSKVLILRNHGLVSVGESVEEAFYYIHNLVVACEIQVRTLASAGGPDNLVLL
D-add 200 VVHAARPDIRCAIYIGCSPVVAISSLKTGLLPLTKDACVLGEITTHAYTGLF_DEE_ERNRLVRSLGPNSKVILLTNHGALCCGETIEEAFFAACHIVQACETQLKLL__PVGLDNLVLI
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D-add 436 GDIRKGGDRSRWLNSPNVYQKVEVLET_____GTPDPKKITKWVAEGSPTHST_PVRIEDPLQFVPAGTNPREFKRVQQLIKDNRRADKISAGPQSHILEGVTWDEASRLKDATVSQAGD
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D-add 550 HVVMMGAASKGIIQRGFQHNATVYKAPYAKNPFDNVTDDELNEYKRTVERKKKSVHGE_YTDT_DFSESEA_VLQAGTKKYPQSEPETEHQVIEIQTQQAPVPRQAEVVLSDALVSQLAQ
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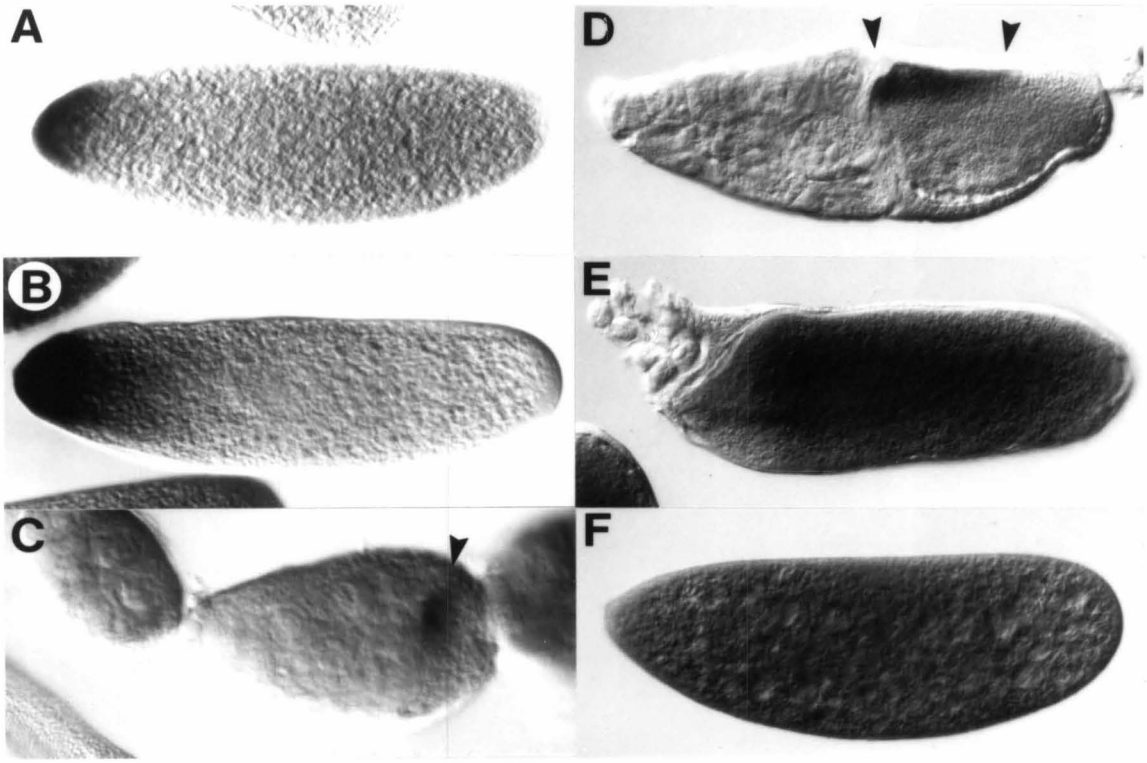
D-add 667 KYAFLYSPGQYMYACMKMAPLMQKVYVIHKVEPVSKHNYPPVNDGNMSIHHNESGAGFMAQESSVISSTPVRNA_LASVSLPEERNHSILGLSSTPYRTISHFGFNCPLITSPTILLHPE
H-addb 644 P_____EGVVVNGREEEQTAEEILSKGLSQMTTSADTDVDTSKDKTESVTSGPMSPEG_SPSKSPSKKKK_____KFRTPS_____
H-adda 733 KKSDS*
D-add 786 HRSIWQRVAEQREKVVS....
H-addb 714 ____FLKKSKKKEKVES*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