Expression and Localization of *Hsp83* RNA in the Early *Drosophila* Embryo

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ABSTRACT

The *Hsp83* gene encodes the sole *Drosophila* homolog of the mammalian *Hsp90* family of regulatory molecular chaperones. *Hsp90* has been implicated in regulating the activity of several signal transduction molecules, including receptor tyrosine kinases, steroid hormone receptors, *src* family tyrosine kinases, raf kinase, Wee1 cell cycle kinase, eIF-2α, protein kinase C and casein kinases. We show that *Hsp83* is dynamically expressed during *Drosophila* oogenesis and embryogenesis. Maternally transcribed mRNA is uniformly distributed in the early embryo, but becomes localized to the posterior pole by a unique mechanism of generalized degradation and localized protection. *Hsp83* mRNA is a component of the posterior polar plasm, and the polar plasm is necessary and sufficient for the protection of the maternal RNA from degradation. *In vivo* analysis reveals that the protection is mediated by sequence elements within the 3'UTR of the *Hsp83* mRNA. Maternal *Hsp83* mRNA is taken up into the germline progenitor cells (the pole cells) as they form, and RNA is detected in the pole cells throughout embryogenesis. *Hsp83* is first expressed zygotically throughout stage 3 embryos but this RNA disappears after only 20 minutes and is undetectable in stage 4 embryos. *Hsp83* is then expressed in the anterior third of stage 5 embryos under the control of the anterior morphogen, BICOID. *Cis*-regulatory sequences necessary for transcriptional activation by BICOID map 1124 bp within the single intron of the *Hsp83* gene. *In vitro* gel shift analysis shows that BICOID binds to a site within this sequence, suggesting that *Hsp83* is a direct target of BICOID. In addition, we show that there is a later, independent phase of transcription within the anterior of the early embryo. These analyses form the basis for detailed molecular analysis of *Hsp83* functions during early embryogenesis.

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CHAPTER 1

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MECHANISMS AND FUNCTIONS OF RNA LOCALIZATION TO THE POSTERIOR POLE OF THE *DROSOPHILA* OOCYTE AND EARLY EMBRYO

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INTRODUCTION

Over a century ago, experimental embryologists postulated the existence of localized, cell fate-specifying determinants in particular regions of oocytes or embryos.[1] With the growth of *Drosophila* as a model organism of choice, first for the study of genetics and then for the analysis of developmental mechanisms, it soon became clear that the cytoplasm of its oocyte and early embryo was non-homogeneous. In particular, the posterior polar plasm was recognized as a region of differentiated ooplasm that was likely to be involved in specifying nuclei that arrived in that region to adopt germinal rather than somatic identity.[2] More recently, cytoplasmic transplantation studies demonstrated that the posterior polar plasm of the early embryo contains spatially restricted activities that are absolutely required for germ cell determination[3, 4] and abdominal pattern formation[5, 6] ("posterior" determination). During the past decade, genetic screens aimed at identifying maternally active genes involved in programming germ cell and abdominal development,[5, 7, 8] and the ability to clone and analyze genes based either on their chromosomal position[9] or on the basis of selected molecular properties,[10-14] has led to the realization that the posterior polar plasm contains several localized RNAs that encode key determinants of these developmental events as well as other localized RNAs whose functions have yet to be discovered.[13]

This chapter focuses on RNAs that are localized to the posterior pole of the *Drosophila* oocyte and early embryo. After presenting an overview of the development and structure of the ovary and early embryo, we then consider the dynamics of posterior RNA localization and the cis- and trans-acting factors involved. Finally, we discuss the developmental functions of the products encoded by these RNAs as well as the function of their localization *per se*.

DROSOPHILA OOGENESIS AND EARLY EMBRYOGENESIS

Oogenesis

The organization of the *Drosophila* ovary makes it particularly useful in the analysis of RNA localization during oogenesis. Each ovary is composed of 15-20 ovarioles, and oogenesis is ordered spatially and temporally within each ovariole (Fig. 2.1). Each ovariole contains differently aged egg chambers that are composed of germ line-derived cells surrounded by somatically-derived follicle cells. The oldest chambers are found in the posterior-most region of the ovariole relative to the body axis of the female fly. As such, all stages of oocyte development can be observed within a single ovary. Oogenesis has been subdivided into 14 morphologically distinct stages (Fig 2.1).[15] The developmental genetics of oogenesis have recently been reviewed in depth by Spradling and by Lasko.[16, 17]

The germarium lies within the anterior-most part of the ovariole and is subdivided into regions 1, 2A, 2B, and 3. Oogonial stem cells reside at the anterior of region 1 where they divide asymmetrically, giving rise to a daughter stem cell and a cystoblast. The cystoblast then undergoes four stereotypical rounds of mitosis with incomplete cytokinesis, resulting in 16 interconnected cells that comprise the germ-line component of the cyst. The cytoplasmic bridges between these cells are stabilized by structures known as ring canals. In germarial region 2, one of the two cells that has four ring canals is determined to become the oocyte, and the other 15 cells become polyploid nurse cells that will produce most of the RNAs, proteins and organelles necessary to program and support early embryogenesis. The oocyte always resides at the posterior of the cyst with the 15 nurse cells clustered at the anterior. In region 2A, mesodermally derived follicle cells begin to surround the 16 cell germline cyst, and this process is completed by the time the cyst passes through region 2B. Movement of the oocyte to the posterior of the cyst serves as the key event determining antero-posterior polarity both of the oocyte and

of the surrounding follicle cell layer.[18, 19] Cysts within germarium region 3 are also referred to as stage 1 egg chambers in the vitellarium.

Within the vitellarium of the ovariole, stages 1-7 are previtellogenic stages, characterized by additional polyploidization of the nurse cells, nurse cell synthetic activity and overall increases in egg chamber size. Until stage 8, when yolk uptake from the hemolymph begins in the oocyte, the size of the oocyte and the individual nurse cells is indistinguishable. Thereafter, the oocyte is clearly the largest cell of the egg chamber. Generalized dumping of the nurse cell contents into the oocyte commences at stage 10B/11 and finishes during stage 12, but stage specific transport of many RNAs into the oocyte occurs much earlier (see below). The nurse cells break down during the rest of oogenesis, while the oocyte continues to increase in size through stage 12 because of yolk uptake. The follicle cells begin to synthesize the vitelline membrane that surrounds the oocyte during stage 10, and the chorion (egg shell) is laid down by the follicle cells in subsequent stages. After secretion of the egg shell, the follicle cells die. The entire progression from stage 1 through stage 14, which is a mature, unfertilized egg, takes roughly 80 hr at the standard *Drosophila* culture temperature of 25 degrees Celsius.

Cytoskeletal Organization Of The Nurse Cell-Oocyte Complex

A number of cytoskeletal proteins have been isolated from *Drosophila* and a description of their subcellular localization and function during oogenesis is emerging. Here we briefly consider the cytoskeletal organization of the nurse cell-oocyte complex and its functions in RNA localization (a more detailed presentation of the cytoskeleton's role in RNA localization is given in Chapter 4). Two aspects are of particular relevance to RNA localization in the oocyte. First, since most known localized RNAs in the oocyte are synthesized in the nurse cells and transported into the oocyte, defects in nurse cell-oocyte complex structure are likely to produce secondary defects in RNA localization.

Second, dysfunction of specific components of the cytoskeletal machinery involved in RNA transport and localization will also produce defects in RNA localization.

During the incomplete cystocyte divisions in region 1 of the germarium, a structure referred to as the fusome runs through the ring canals and functions to ensure that cytokinesis is incomplete and to determine the pattern of nurse cell-oocyte interconnections. At least three distinct isoforms of ADDUCIN-LIKE protein (140, 102, 95 kD; encoded by the *Adducin-like/hu-li tai shao* locus) contribute to the fusome in the germarium, as does α-spectrin.[20, 21] Mutation of the *hts* gene results in disruption of the fusome with consequent complete cytokinesis and abnormal ovarian cyst formation.[22]

Filamentous-actin, a 60 kD isoform of ADDUCIN-LIKE/HU-LI TAI SHAO, KELCH protein (which has a domain homologous to the actin binding protein SCRUIN), and phosphotyrosine-containing protein(s) are localized to the ring canals of the nurse cell-oocyte complex.[23] The 60 kD form of ADDUCIN-LIKE is involved in the early assembly or stabilization of F-actin in the ring canals, while KELCH is involved in later, growth stages of the canals. Transport of RNA and protein from the nurse cells into the oocyte occurs through the ring canals: thus mutations in these components disrupt RNA localization within the oocyte by affecting the transport of RNAs from the nurse cells into the oocyte.

Functionally, inhibition of actin assembly by cytochalasin treatment during oogenesis results in failure of nurse cell dumping into the oocyte at stage 10B/11.[24] Genetic screens have identified "dumpless" mutants whose function is required for cytoplasmic transfer from the nurse cells to the oocyte at these stages. A subset of these loci have been cloned and shown to encode actin-associated proteins: these include *chickadee* (*Drosophila* PROFILIN), *singed* and *quail* (which, respectively, encode homologs of the actin filament crosslinking proteins FASCIN and VILLIN).[25-27] One function of the actin-based cytoskeleton in the nurse cells is to restrict the movement of nuclei, thus preventing them from plugging the ring canals during cytoplasmic

transport.[25] In addition, the actin-based cytoskeleton appears to play a role in force generation during the rapid cytoplasmic transport from the nurse cells into the oocyte, presumably by a combination of contraction of the nurse cell cortex, actin-dependent oocyte expansion and follicle cell flattening.[28]

Disruption of the fusome, ring canal integrity or the actin-based cytoskeleton all result in defects in oogenesis and indirectly affect RNA localization to and within the oocyte. However, these are secondary effects. In contrast, the microtubule-based cytoskeleton appears to be directly involved in specific transport and localization of RNAs. Disruption of microtubules with colchicine treatment during oogenesis prevents the differentiation of the oocyte within the 16 cell cyst, eliminates ooplasmic streaming in stage 10-12 oocytes and inhibits the anterior localization of the RNA encoding the anterior morphogen, BICOID (see also Chapter 3).[29, 30] During oogenesis, the formation of stage-specific polarized microtubule arrays correlates with the timing of RNA and protein localization in the oocyte (Figure 2.2).[31, 32] In region 1 of the germarium, microtubules are evenly distributed throughout all 16 cells of the cyst. In region 2A, a microtubule organizing center (MTOC) becomes localized within one of the cells, presumably the pro-oocyte. A microtubule array emanates from the MTOC and extends through the ring canals into the nurse cells. This array persists through stage 6. Since the MTOC nucleates the minus ends of microtubules, this organization reflects a polarized microtubule system. In stage 1 oocytes, the MTOC is localized in the oocyte anterior, but in stages 2-6 it relocalizes to the posterior. Therefore, the microtubule minus ends are now located at the posterior of the oocyte (Fig. 2.2A). The function of this microtubule array in RNA transport and localization is not fully defined. One possibility is that transport of RNA from the nurse cells into the oocyte at these early stages is accomplished by means of minus-end directed motors such as dynein or minus-end directed kinesins (Figure 2.2A). Certainly, dynein (specifically, the heavy chain) is present in the oocyte commencing at germarial stage 2B, and later it is localized to the

posterior pole (stage 9).[33] While posterior localization of dynein does not correlate with the apparent polarity of the microtubules at stage 9, it may have localized to the posterior during earlier stages but not have been detectable above its general distribution throughout the oocyte.

Stages 7-10A of oogenesis exhibit another reorganization of the microtubule system. The MTOC is no longer observed and the density of microtubules at the posterior decreases while increasing at the anterior, first within the anterior margin. Limited treatment with microtubule inhibitors suggests that the microtubule polarity has reversed and that the minus ends are now at the anterior (Fig. 2.2B).[31] Any microtubule-based transport of RNA to the posterior pole of the oocyte during these stages must involve plus-end directed motors. This is corroborated by the observation that a *Drosophila* kinesin heavy chain tagged with β-galactosidase localizes to the posterior of stage 9 oocytes.[34]

The signal to reorganize the microtubule array beginning at stage 7 likely derives from reciprocal cell-cell communication between the oocyte and the follicle cells that surround it. For example, it has been suggested that *spindle-C* mutations lead to the formation of a mirror-symmetric microtubule cytoskeleton in the oocyte, with the minus ends of the microtubules located at each end of the oocyte and plus ends in its center.[19] As a consequence *bicoid* RNA is localized at both poles (rather than just the anterior; see Chapter 3) and *oskar* RNA is localized in the center of the oocyte (rather than at its posterior pole, see below).[19] The *spindle-C* gene functions in the oocyte rather than the follicle cells; thus it has been speculated that it is involved in the initial communication of polarized information from the oocyte to the follicle cells.[19] As a consequence, NOTCH (a transmembrane, EGF-repeat containing protein) is expressed on the follicle cells and communicates polarity from these cells back to the oocyte; temperature sensitive *Notch* alleles produce similar defects to *spindle-C* mutations with *bicoid* RNA localized at both poles of the oocyte.[18] Finally, it has been demonstrated that mutations in PROTEIN

KINASE A also result in bipolar oocytes with mislocalized bipolar *bicoid* RNA, centrally positioned *oskar* RNA and microtubules concentrated at both oocyte poles.[35] The germ line dependence of these defects suggests that PROTEIN KINASE A is involved in intra-oocyte transduction of the NOTCH-mediated signal from the follicle cells to the oocyte.

During stages 10B-12, microtubules rearrange into a subcortical parallel array, concomitant with the onset of ooplasmic streaming.[28, 31] No known RNAs are transported and localized to the posterior pole during these stages. This might be a consequence of the absence of an appropriate microtubule-based structure and/or the difficulty of localizing RNAs during the streaming process. Consistent with such a possibility, *cappuccino* and *spire* mutations result in premature ooplasmic streaming at stages 8-10A, and they disrupt posterior RNA localization (see below).[36] A possible exception is *nanos* RNA, which begins to accumulate at the posterior of stage 12 oocytes,[37] although it seems likely that *nanos* localization begins as oocyte streaming ends.

Finally, during stages 13 and 14, ooplasmic streaming ceases and several additional RNAs become localized to the posterior pole (see below). During these stages, the subcortical microtubules are replaced by short, randomly oriented filaments that are present throughout the cytoplasm, although their density appears slightly higher near the cortex.[31] Actin is also reorganized at these stages: earlier it is present in the form of dense filaments in the cortex, but stage 14 oocytes also contain an extensive network of actin filaments deep in the cytoplasm.[31] During the early cleavage stages of embryogenesis actin and tubulin remain concentrated in the cortex, with the microtubules forming a filamentous network while actin is present in a punctate pattern.[38] Longitudinal fibers that run the length of the embryo through the yolk have also been reported.[39] These apparently have contractile activity and may contain actin, but they appear to be quite complex in structure and their composition has not been reported.

Early Embryogenesis

Drosophila embryogenesis has been extensively reviewed.[40] For the purposes of this chapter, only the first 3 hours of *Drosophila* embryogenesis will be described (Fig. 2.3). The first 13 nuclear divisions occur rapidly within a syncytium without cell division. The earliest phase of embryogenesis is referred to as the cleavage stage, the one hour period during which the first eight nuclear divisions occur in the central yolk mass. After the seventh nuclear division, the nuclei begin to migrate to the periphery of the embryo. Eighty minutes after fertilization, the first nuclei reach the periphery at the posterior pole. These nuclei bud off to form the first cells of the embryo, the presumptive germ cells, known in *Drosophila* as pole cells. The remaining nuclei complete their migration to the periphery and undergo four additional rounds of division during this syncytial blastoderm stage, which is completed just over two hours after fertilization. After the fourteenth nuclear division, membranes invaginate simultaneously around all of the somatic nuclei forming the 6000 cells of the cellular blastoderm, three hours after fertilization. Gastrulation proceeds immediately thereafter.

Since the early *Drosophila* embryo is syncytial, uniformly distributed maternally synthesized RNAs can, in principle, become localized during these stages. There is at least one such maternal RNA, the product of the *Hsp83* gene, that is localized to the posterior pole between nuclear cycles six and eight (Figs. 2.4 and 2.5).[11-13]

Organization And Function Of The Posterior Polar Plasm In The Oocyte And Embryo

The posterior polar plasm is morphologically distinct from the rest of the embryonic cytoplasm: it is a yolk free zone, located just below the cortex[2] that is morphologically similar to the germ plasm observed in other organisms.[41] Within the posterior polar plasm, electron microscopic analysis has revealed the structure of the

electron-dense, non-membrane-bound organelles known as polar granules.[42] Polar granules assemble at the posterior pole during oogenesis commencing at stage 9, and early histochemical analysis showed that they are enriched for RNA and protein.[43, 44] The polar granules appear to be assembled in part from granular material - "nuage" - that is associated with the outer nuclear envelope of the germline stem cells and then the nurse cells.[45] Upon pole cell formation, the polar granules fragment and are taken up within the pole cells.[44, 45] The polar granules then reform into larger structures that are associated with the pole cell nuclei.[45] Interestingly, recent analyses have demonstrated that at least one molecular component of the polar granules - VASA - is also present in the germ cells throughout development and exhibits a very similar distribution to that described for nuage and polar granules on the basis of cytology alone,[46] thus confirming the presumption that there is some continuity to their constituents throughout the life cycle.[45]

Several lines of evidence indicate that functional posterior polar plasm is both necessary and sufficient for germ cell determination and abdominal pattern formation. First, transplantation of posterior cytoplasm into the anterior of the embryo results in ectopic formation of functional pole cells.[3, 4] Second, U.V.-irradiation of the posterior polar plasm eradicates its ability to induce pole cell formation, with an optimal wavelength at the RNA absorption maximum, thus implying that RNA is a functional component.[47-49] Third, cytoplasmic leakage from the posterior pole results in an inability to specify abdominal pattern and pole cells.[50] Fourth, transplantation of wildtype posterior cytoplasm into embryos mutant at abdominal pattern loci rescues abdomen formation.[5, 6]

Genetic and molecular analyses are now elucidating the assembly, molecular nature and function of the posterior polar plasm. In particular, several maternal RNAs have been identified that are localized to the posterior polar plasm,[13] and two of these - *oskar* and *nanos* - behave, respectively, as the polar plasm organizing activity[51, 52] and

the abdominal cell fate determinant.[53] Consistent with the more classical-style of experiments described above, embryos that carry an *oskar* or a *nanos* transgene designed to ectopically localize that RNA to the anterior pole, form ectopic polar granules, functional anterior pole cells and a mirror-image duplicated abdomen at the anterior (in the case of *oskar*)[54, 55] or a duplicated abdomen only (in the case of *nanos*).[56] The localization and functions of *oskar* and *nanos* RNAs are considered in more detail below.

POSTERIOR LOCALIZED MATERNAL RNAs IN THE OOCYTE AND EMBRYO

Identification Of Posterior Localized Gene Products

Two general approaches have led to the identification of genes encoding maternally synthesized posterior-localized RNAs and/or proteins in the oocyte and early embryo. The first has followed the path of genetic identification of maternal effect mutants with interesting defects in germ cell and/or abdominal pattern specification, followed by molecular cloning and analysis. The second has followed a reciprocal course by first identifying gene products on the basis of their posterior localization, and then uncovering their functions by means of genetic analysis. We summarize the products identified through these strategies here and in Tables 2.1 and 2.2.

Genetic screens have focused on identification of maternal effect loci whose function is required at the posterior of the embryo for pole cell formation and/or abdominal patterning. These are known as the 'posterior' group genes[57] and currently include *cappuccino*, *mago nashi*, *nanos*, *oskar*, *pipsqueak*, *pumilio*, *spire*, *staufen*, *tudor*, *valois* and *vasa*[5-8, 58-61] Embryos derived from mothers mutant for any of the posterior group genes exhibit a loss of abdominal patterning. The posterior group can be subdivided into two classes: mutations in one class, which includes *cappuccino*, *mago nashi*, *oskar*, *spire*, *staufen*, *tudor*, *valois* and *vasa*, lack polar granules and fail to form

pole cells[7, 8, 58, 59, 62]; those in the second class, which includes *nanos* and *pumilio*, display abdominal patterning defects but have morphologically wild-type posterior polar plasm and form pole cells.[6, 60] Females carrying temperature sensitive mutations in *oskar* and *staufen* produce embryos that form normal abdomens at permissive temperatures, yet still fail to make pole cells.[5, 51] Taken together, these results demonstrate that pole cell determination and abdominal pattern specification are, in part, genetically separable pathways and that pole cell determination seems to be more sensitive to disruption than abdominal patterning.

Some of the posterior group genes produce somewhat more pleiotropic effects than those described here. For example, *cappuccino* and *spire* mutations also result in defects in dorso-ventral pattern and in blastoderm cellularization,[58] *valois* and *tudor* mutations also cause defects in blastoderm cellularization,[7] *pumilio* mutations produce zygotic lethality in addition to the maternal effect on abdominal patterning,[60] *staufen* mutations also result in defective head development,[7] and *nanos* and *vasa* also function during early oogenesis.[6, 63] While these particular functions will not be considered in this chapter, it should be remembered that the role of these genes in development is more general than might appear solely from a focus on posterior RNA localization and its functions.

Of the genetically identified posterior group genes that have been characterized molecularly, several (*oskar*, *tudor*, *pumilio* and *nanos*) encode posteriorly localized RNAs, while others (*staufen* and *vasa*) encode uniformly distributed RNAs but posteriorly localized proteins (Tables 2.1 and 2.2).[51-53, 64-66] Additional posterior localized RNAs have been identified in molecular and enhancer trap screens. These include *Hsp83*, *Cyclin B*, *mitochondrial 16S large rRNA*, *orb* and *germ cell-less* RNAs.[11, 12, 14, 67-70] Genetic analyses and antisense RNA experiments are beginning to elucidate the functions of these gene products. As discussed for the posterior group genes above, several of these RNAs (e.g. *Cyclin B*, *mitochondrial 16S rRNA* and *Hsp83*)

are also expressed at other times and places during development and thus do not function exclusively at the posterior pole of the oocyte and early embryo.

In all cases, analysis of the patterns of RNA localization, the cis-acting sequences that direct their transport and localization and the trans-acting factors involved in their localization and anchoring, are yielding insights into shared and unique features of posterior RNA localization in the oocyte and early embryo. We consider these studies in detail below.

Dynamics Of Posterior RNA Localization

The temporal aspects of RNA localization to the posterior pole of the oocyte and/or early embryo exhibit a great deal of variation (Table 2.1 and Fig. 2.4). For example, a particular RNA might exhibit localization to the posterior of the oocyte only during the middle part of oogenesis (*tudor*), from mid-oogenesis through the early cleavage stages of embryogenesis (*oskar*), from late oogenesis through early embryogenesis (*16S rRNA*), from late oogenesis through pole cell formation with uptake into the pole cells (*germ cell-less*), only in the embryo (*Hsp83*) or a biphasic pattern with localization during the middle part of oogenesis and again during early embryogenesis (*orb*).

tudor

tudor RNA is first detected within the presumptive oocyte in region 2 of the germarium.[64] In stage 1 and 2 oocytes, it surrounds the oocyte nucleus. It is localized to the posterior half of stage 4-7 oocytes. The level of *tudor* RNA decreases during stages 4-8, and it is not detected in later stage oocytes or in the embryo. Thus, *tudor* RNA is localized to the posterior of the oocyte for about half of oogenesis, starting roughly a quarter of the way through oogenesis and ending about three-quarters of the way through. In contrast to *tudor* RNA, TUDOR protein is concentrated at the posterior of stage 9

oocytes and this distribution is maintained through the cleavage stages of embryogenesis when TUDOR protein is taken up into the budding pole cells.[54]

orb

orb RNA is first detected in the presumptive oocyte in region 2 of the germarium.[67, 71] The nurse cells also show low levels of the *orb* RNA. *orb* RNA becomes concentrated at the posterior of stage 2-7 oocytes. In stage 8-10 oocytes, however, *orb* RNA is localized exclusively in the anterior of the oocyte, and *orb* RNA is distributed evenly in stage 11-14 oocytes. Cleavage stage embryos are enriched at the posterior in *orb* RNA, but it is also detectable at low levels throughout the embryo. *orb* RNA is incorporated into the pole cells where it persists until pole cell migration begins. The remaining RNA is degraded within the syncytial blastoderm. The distribution of *orb* RNA roughly correlates with that of its encoded protein, which possesses an RNP/RRM-type RNA-binding motif.[72, 73] ORB protein begins to preferentially accumulate in the oocyte of germarial cysts in region 2 and is concentrated in a posterior-to-anterior gradient in the cortex of stage 1 through 7 egg chambers. In stages 8 and 9, ORB protein is found throughout the cortex of the oocyte with the exception of the anterior cortex; it becomes concentrated in the anterior cortex of the oocyte at stage 10. ORB protein distribution in later stage oocytes or early embryos has not yet been reported. The *orb* gene and its functions in RNA localization are discussed in detail in Chapter 6.

oskar

oskar RNA is first detected in all 16 cells of the cyst in region 2 of the germarium, but is enriched within the presumptive oocyte.[51, 52] This enrichment in the oocyte persists during stages 1-7 of oogenesis and, during early stage 8, *oskar* RNA localizes to its anterior margin (Fig. 2.4). *oskar* RNA is then localized to the posterior during late stage 8 and, by stage 9, it can only be detected at the posterior pole of the oocyte. This

posterior localization persists throughout the remainder of oogenesis and the early cleavage stages of embryogenesis.[51, 52] Delocalization of *oskar* RNA then occurs, and the RNA is still detectable at least five hours after fertilization.[51] OSKAR protein is concentrated at the posterior pole of cleavage stage embryos in a pattern very similar to that of *oskar* RNA.[55] No analyses of OSKAR protein distribution during oogenesis have been reported.

nanos

nanos RNA may be transcribed as early as region 1 of the germarium and is readily detectable in stage 5 egg chambers where it is enriched in the oocyte.[37] During stages 7 and 8, *nanos* RNA is localized to the anterior margin of the oocyte but this distribution is lost by stage 9. Stage 10 nurse cells express *nanos* RNA at high levels, and it is then transported into the oocyte (Fig. 2.4) where its posterior localization is first detected at stage 12 and persists through stage 14. *nanos* RNA is concentrated at the posterior pole of early embryos although there is evidence that it is distributed generally throughout the embryo at lower levels;[53] the posterior-localized RNA is taken up into the pole cells when they form. NANOS protein is first detected in regions 1 and 2A of the germarium with the 4 and 8 cell cysts staining strongly (region 1) and the 16-cell cysts staining weakly (region 2A). NANOS protein is undetectable in stage 1-2 egg chambers, accumulates at low levels in egg chambers during stages 3-6, and high levels are expressed in the nurse cells at stage 10.[37] At no point of oogenesis is NANOS protein observed in the growing oocyte.[37] Translation of the posterior-localized *nanos* RNA begins shortly after fertilization, with levels increasing during the cleavage stages. A gradient of NANOS protein is generated with a peak at the posterior pole and extending through the posterior half of the embryo.[37, 56, 65, 74] This gradient plays a key role in programing abdominal pattern formation (see below).

pumilio

pumilio RNA is present in all 16 cells of the cyst in the germarium and through stage 4 of oogenesis.[65, 66] No RNA is detected in stage 5-8 oocytes. *pumilio* transcription resumes in stage 9 nurse cells, and the RNA is transported into the oocyte at stages 10B/11-12. Barker et al. have reported that, while *pumilio* RNA exhibits a low but general distribution, it is enriched at the posterior pole of early cleavage stage embryos.[65] However, Macdonald has reported that *pumilio* RNA is uniformly distributed at these stages with variable enrichment at the posterior.[66] No analyses of PUMILIO protein distribution during oogenesis have been reported. PUMILIO protein is uniformly distributed throughout the cortex of cleavage and syncytial stage embryos.[66] On the basis of genetic experiments it was originally suggested that *pumilio* is actually involved in transporting the 'posterior' signal from the posterior pole to the abdominal region;[60] however, it is now clear that this cannot be so since *pumilio* mutations have no effect on the distribution of *nanos* RNA or NANOS protein.[65] Recent results suggest that the uniformly distributed PUMILIO protein functions specifically in the posterior by interacting with the spatially-restricted NANOS protein (see below).[75]

mitochondrial 16S rRNA

High levels of *mitochondrial 16S rRNA* are first detectable in stage 1 egg chambers, and this accumulation continues at high levels in the nurse cells and at lower levels in the oocyte of stages 2 through 10A.[68] Nurse cell accumulation continues through stages 10B and 11, with accumulation in the oocyte commencing at stage 10B. By stage 12, *16S rRNA* is present at high uniform levels throughout the oocyte, and it is presumed that concentration at the posterior pole occurs during stages 13 and 14.[68] *16S rRNA* remains concentrated at the posterior of early cleavage stage embryos.[13, 68] While the uniformly distributed *16S rRNA* is likely to be that found in the mitochondria throughout the oocyte and embryo, the posterior-concentrated transcripts may be

cytoplasmic.[11, 68, 76, 77] The cytoplasmic *16S rRNA* is not incorporated into the pole cells when they form.[13, 68]

Cyclin B

The *Cyclin B* gene encodes two differentially spliced transcripts, 2.3 kb and 2.7 kb in length.[78] The 2.3 kb transcript is enriched in the oocyte beginning in the posterior germarium and through stage 7 of oogenesis. No *Cyclin B* RNA is detected within the egg chamber at stage 8. The 2.7 kb RNA is then transcribed in stage 9-10 nurse cells, is transported into the oocyte during stages 10B/11-12, and becomes enriched in the posterior during stages 13-14. In addition to its concentration at the posterior pole of cleavage stage embryos, *Cyclin B* RNA is localized around the somatic nuclei.[78] The posterior-localized RNA is taken up into the pole cells when they form. No analyses of CYCLIN B protein during oogenesis have been reported. However, it has been shown that the *Cyclin B* RNA at the posterior pole and in the pole cells of the embryo is translationally repressed, and that translation does not begin until just before the pole cells that have been incorporated into the embryonic gonad begin to divide (about 17 hr after fertilization, and over 30 hr after synthesis of the *Cyclin B* transcripts).[79]

germ cell-less

germ cell-less RNA is first transcribed in stage 8 nurse cells, is transported into the oocyte during stages 10B/11 and 12, and presumably becomes localized to the posterior during stages 13 and 14.[14] *germ cell-less* RNA remains restricted to the posterior pole of the cleavage stage embryo and is taken up into the pole cells when they bud. *germ cell-less* apparently encodes a nuclear pore-associated protein that is concentrated on the nuclei of pole buds and pole cells.[80] GERM CELL-LESS protein distributions during oogenesis have not yet been reported.

Hsp83

In contrast to all of the other posteriorly localized RNAs considered to this point, maternally synthesized *Hsp83* RNA is not localized to the posterior pole until well after fertilization (late cleavage stage embryos), and it is subsequently incorporated into the pole cells (Figs. 2.4 and 2.5).[12] *Hsp83* RNA is first detectable in regions 2 and 3 of the germarium and in stage 1 through 5 egg chambers where it accumulates in all 16 germline cells.[12] It is absent from stages 6 through 8, is then transcribed at high levels in stage 9-10 nurse cells and is transported into the oocyte along with the other nurse cell contents during stages 10B/11-12.[12] *Hsp83* RNA is uniformly distributed in late stage oocytes and early cleavage stage embryos. During embryonic nuclear cycles 7 and 8, *Hsp83* RNA rapidly becomes concentrated to the posterior pole and it is incorporated into the pole cells when they form. Quantitative whole mount RNA in situ hybridization analysis and Northern blots have demonstrated that the amount of *Hsp83* RNA remains constant at the posterior pole, but that *Hsp83* transcripts are degraded elsewhere in the embryo (Fig. 2.5).[12] This is the first example of an RNA that is localized by a mechanism of generalized degradation and localized protection (see below). No analyses of HSP83 protein distribution have been reported.

COMPONENTS OF THE POSTERIOR RNA LOCALIZATION MECHANISM

While the dynamics of the spatial and temporal localization of the posterior localized RNAs described above are distinct for each RNA, there are certain common features to their localization mechanisms. In six instances, the cis-acting sequences that tag these RNAs for posterior localization have been identified. In five of the six cases (*Cyclin B*, *germ cell-less*, *nanos*, *orb*, *oskar*) the 3'UTR of the RNA is necessary and sufficient for posterior localization, while in the sixth case (*Hsp83*), the 3'UTR programs

posterior protection and 5'-sequences program degradation. In addition to requiring cis-acting localization tags, trans-acting factors must interact with these tags to mediate RNA localization and/or anchoring. Directly or indirectly, such translocation and anchoring factors must interact with the cytoskeleton. Examination of RNA localization in mutant backgrounds has revealed that, in all instances, integrity of the posterior polar plasm is crucial for posterior RNA localization and anchoring in the late stage *Drosophila* oocyte and early embryo. In some instances, the localized RNAs themselves encode key components of the polar granules (e.g. *oskar*) while in other instances polar granule-associated RNAs (e.g. *nanos*, *pumilio*, *Hsp83*, *16S RNA*) encode products that are not required for polar granule integrity and/or function. We describe below what is known about each of these components of the posterior RNA localization machinery, beginning with cis-acting tags, then trans-acting factors and, finally, the assembly of polar granules and polar plasm as a unique cytoplasmic organelle with several associated localized RNAs and proteins.

Cis-Acting Tags In The Posterior Localized RNAs

Two transport and localization processes are a prerequisite for posterior localization of maternally synthesized RNAs in the *Drosophila* oocyte and early embryo. First, since most known localized RNAs are synthesized in the nurse cells, they must be transported into the oocyte. For RNAs that are not localized until late in oogenesis (e.g. *germ cell-less* and *Cyclin B*) nurse cell-oocyte transport might be accomplished as part of the bulk dumping of the nurse cell contents into the oocyte beginning at stage 10B/11 of oogenesis and thus might not require any special tags. However, for RNAs that are transported into the oocyte in advance of the dumping process (e.g. *oskar*, *orb*, *tudor*) there must be some mechanism by which they are recognized by the machinery that specifically moves certain RNAs into the oocyte. Second, once in the oocyte, the RNAs must be localized to the posterior pole and then anchored there. Again, since the RNAs

are synthesized in cells distinct from that in which they are localized, they must carry information that targets them for posterior localization.

In order to investigate the cis-acting sequences required for transport or localization, a transgene is tagged with a reporter sequence (usually part of the *E.coli lacZ* gene). If the reporter sequence fused to the full length transcript is localized in the normal fashion in transgenic flies, two classes of experiments can be conducted. First, sequences within the localized RNA can be deleted or mutated in order to define which are necessary for localization (i.e. the mutated fusion RNA cannot localize). Second, parts of the RNA that are sufficient to confer localization on the reporter can be defined. Using these strategies, it is also possible to map parts of the RNA that are necessary and sufficient for distinct aspects of the overall localization pattern or process and to define whether these elements are unique or redundant within that RNA.

It remains a formal possibility that certain cytoplasmically localized RNAs will be localized co-translationally and thus the sequence that tags them for transport and/or localization will map to the open reading frame. Such a mechanism has been defined in detail for RNAs that are targeted to the endoplasmic reticulum via a co-translational mechanism involving the amino-terminal signal sequence.[81, 82] However, to date all cases of cytoplasmic RNA localization involve tags that map to the untranslated parts of the RNAs, particularly their 3'UTRs. With regard to the posterior localized RNAs considered here, the 3'UTR has been shown to be sufficient for transport of *orb* and *oskar* RNAs from the nurse cells into the oocyte, for posterior localization of *nanos*, *germ cell-less*, *orb*, *Cyclin B* and *oskar* RNAs, and for protection of *Hsp83* RNA from degradation at the posterior of the embryo.[55, 56, 71, 78, 80, 83, 84] To date, no extensive conservation of primary sequence or of secondary structure has been observed among these 3'UTRs. Detailed analyses of *Cyclin B*, *orb* and *oskar* 3'UTRs have revealed that multiple, sometimes functionally redundant, elements exist within each 3'UTR , and that individual steps in their localization can be correlated with specific elements. The

location of the cis-acting tags in these RNAs and their features are summarized in Fig. 2.6.

Hsp83

The 3'UTR of *Hsp83* RNA is 406 nt in length and is sufficient to confer protection at the posterior pole; deletion experiments indicate that there are redundant protection elements within the 3'UTR (Fig. 2.6).[84] The cis-acting sequences that are required for generalized degradation have not yet been mapped.

orb

orb RNA undergoes dynamic localization (see above), and dissection of its 3'UTR sequences reveals discrete, albeit redundant, sequences that are required for distinct localization steps (Fig. 2.6). Within the 1207 nt 3'UTR of *orb* RNA, a 280 nt sequence is sufficient to confer on a reporter the complex pattern of accumulation seen for the endogenous *orb* RNA within the oocyte, including transport into the oocyte, localization to the posterior of stage 2-7 oocytes, and anterior localization in stage 8-10 oocytes.[71] Subdivision of this 280 nt sequence has identified two non-overlapping elements: one is sufficient only for localization to the oocyte and the other is sufficient both for localization to the oocyte and to the posterior of stage 2-7 oocytes. A minimal element required for later anterior localization has not been identified within the 280 nt fragment. Thus, there is some redundancy, at least of sequences that can tag *orb* RNA for transport into the oocyte. *orb* is considered in detail in Chapter 6.

oskar

728 nt of the 1043 nt 3'UTR of *oskar* RNA are necessary to direct its localization.[83] Deletions within this 728 nt region indicate that there are separable elements that mediate *oskar* RNA localization to the oocyte in stages 1-6, proper

translocation from the anterior margin at stage 8 and its localization to the posterior pole in stage 9 oocytes (Fig. 2.6). Further, a 200 nt domain within this region possesses all three elements, but sufficiency of this subset for normal localization has not yet been demonstrated. Presumably, each of these discrete cis-acting tags interacts with different trans-acting factors (see also below). In fact, comparisons of the distribution of endogenous *oskar* RNA in *cappuccino* and *spire* mutants versus *staufen* mutants support this idea. In *cappuccino* and *spire* mutants, *oskar* RNA is transported into the oocyte but it is never localized therein. In *staufen* mutants *oskar* RNA accumulates normally at the anterior oocyte margin at stage 8, but it never relocalizes to the posterior and, eventually, it is degraded.[51, 52] The role of STAUFEN in *oskar* mRNA localization is summarized below and is considered in detail in Chapter 8.

Recently a U.V.-crosslinking assay has been used to identify proteins in ovary extracts that can bind to the *oskar* 3'UTR.[85] This has resulted in the purification of an 80 kD protein (OBP80 - *oskar* binding protein 80 kD) that binds specifically to three regions within the 3'UTR (referred to as OBP80 response elements or OREs; Figure 2.6).[85] Rather than functioning in *oskar* mRNA localization, OBP80 is involved in translational repression of unlocalized *oskar* RNA.[85] The OREs are conserved in the *Drosophila melanogaster* and the *D. virilis* (*virosk*) *oskar* 3'UTRs, and *D. virilis* ovary extracts contain an 80 kD protein that binds to these sites.[85] Complete rescue of *oskar* mutant phenotypes can be accomplished using an *oskar* transgene with wildtype OREs. However, an *oskar* transgene with mutant OREs rescues posterior body patterning but causes anterior pattern deletions and, sometimes, mirror-image duplications of posterior structures at the anterior.[85] ORE-defective transgenes exhibit precocious synthesis of OSKAR protein prior to posterior localization of *oskar* mRNA, causing the observed gain of function phenotypes in the anterior of the embryo.[85] Thus OBP80 normally acts through the OREs in the *oskar* 3'UTR to prevent translation of *oskar* mRNA prior to its posterior localization. The *oskar* OREs have also been shown to confer translational

repression on a heterologous mRNA, the *exuperantia* mRNA (which lacks any OREs), and it has been suggested that OBP80 may act in the post-transcriptional regulation of other ovarian RNAs with OREs in their 3'UTRs (these include *germ cell-less*, *swallow*, *vasa* and *orb*).[85]

Cyclin B

The full length *Cyclin B* 3'UTR is 776 nt in length.[78] A naturally occurring alternative splice deletes 393 nt within the 3'UTR , and only the full length 2.7 kb transcript that contains these 393 nt is ever localized at the posterior of the oocyte.[78] Utilizing an assay in which in vitro transcribed RNA was injected into early embryos, it has been shown that within this 393 nt sequence, two closely apposed regions of 94 nt and 87 nt are required for posterior localization (Fig. 2.6).[79]

Additional evidence indicates that the 3'UTR of *Cyclin B* restricts its function not only by directing posterior localization, but also by regulating translation. When epitope tagged, in vitro transcribed *Cyclin B* RNA is injected into cleavage stage embryos, it is incorporated into the pole cells, but it is not translated until late in embryogenesis when the pole cells begin to divide again in the assembled gonad.[79] Between the two posterior localization tags in the *Cyclin B* 3'UTR lies a 39 nt sequence that is necessary for this translational repression of *Cyclin B* in the pole cells (Fig. 2.6). Deletion of the 39 nt sequence allows proper localization to the pole cells, but the RNA is immediately translated. The 39 nt sequence contains a conserved NANOS response element (NRE) which is also found within the 3'UTRs of *hunchback* and *bicoid* RNAs.[86] The NREs in *hunchback* and *bicoid* RNAs are necessary for their translational repression in response to NANOS activity. Translational repression of *Cyclin B* RNA, however, does not require NANOS activity.[79]

nanos

The *nanos* RNA possesses a 866 nt 3'UTR which has been shown to be sufficient to direct posterior localization in the early embryo.[56, 74] Definition of RNA localization elements within this region has proven difficult: no individual small internal deletion eliminates localization and, reciprocally, no individual small fragment can confer localization (E. Gavis and R. Lehmann, pers. comm.). If, however, several contiguous internal fragments are combined to yield an isolated 400 nt internal domain, posterior localization does occur. These data indicate that sequences necessary to confer wild-type localization are distributed throughout this region (E. Gavis and R. Lehmann, pers. comm.).

nanos RNA has been ectopically localized to the anterior of oocytes and early embryos by means of a transgene in which the 3'UTR of *nanos* has been replaced with the 3'UTR of the anterior morphogen, *bicoid*, which directs RNAs to the anterior (the transgene is referred to as *nanos-bicoid 3'UTR*) (see Chapter 3).[56, 74] In such transgenic flies, *nanos* RNA is localized and translated within the anterior of the embryo, and an ectopic, mirror-image anterior abdomen forms (see below). Unexpectedly, this 'bicaudal' phenotype is still observed in *nanos-bicoid 3'UTR* transgenic flies that lack *nanos* activity at the posterior. This suggests that the activity of anteriorly localized *nanos* is greater than that of its normal posteriorly localized product and can function at a distance from its source (i.e. in the posterior half of the embryo).[56] Reciprocally, merely increasing the dosage of *nanos* RNA at the posterior pole does not increase its activity in the anterior. Therefore, it seemed likely that the *nanos* 3'UTR possesses sequences that modulate its activity at the posterior pole and such modulation is lost when the *nanos* 3'UTR is replaced with the *bicoid* 3'UTR. This was confirmed using a transgene that bears the *nanos* coding sequence and a chimeric 3'UTR composed of both the *bicoid* and the *nanos* 3'UTRs.[74] The RNA transcribed from this chimeric transgene is localized to the anterior, but it is not translated. In contrast, substitution of the *nanos* 3'UTR with the *α-1 tubulin*

3'UTR results in uniformly distributed chimeric RNA that is translated throughout the embryo and causes duplication of posterior structures in the anterior.[74] Thus, translational repression of endogenous *nanos* RNA is mediated through sequences in its 3'UTR; recently, it has been shown that these sequences can be separated from those that confer posterior localization (E. Gavis and R. Lehmann, pers. comm.). Thus, as in the case of *oskar* and *Cyclin B*, the 3'UTR of *nanos* RNA has distinct elements that mediate both its localization and its translation, hence ensuring proper localized activity of the posterior morphogen (see below).

Trans-Acting Factors That Function In RNA Localization

The role of the cytoskeleton and associated proteins

The organization of the cytoskeleton and associated molecules in the egg chamber and the early embryo has been presented above. Transport of RNAs into the oocyte before stage 10B/11 likely requires the integrity of the microtubules (Fig. 2.2). RNAs that are transported into the oocyte during the process of nurse cell dumping that initiates at stage 11 require a functional microfilament network since actin is required for the dumping process.

Actual posterior localization of RNA in the oocyte is dependent on the microtubule network since colchicine treatment, which destabilizes microtubules, disrupts posterior localization of several posterior-localized RNAs and proteins. After drug treatment, *oskar* and *Cyclin B* RNAs and STAUFEN protein are not localized to the posterior.[32, 34] A recent report reflects a generalized requirement for the microtubule system in RNA localization and may explain the defects seen in antero-posterior patterning in *cappuccino* and *spire* mutants.[36] The in vivo distribution of microtubules in stage 8-10A *cappuccino* and *spire* mutants resembles the distribution seen in wild-type stage 10B-12 oocytes: that is, the microtubules are prematurely arranged in subcortical

parallel arrays. This cytoskeletal arrangement is correlated with ooplasmic streaming in wild-type stage 10B-12 oocytes and, in fact, premature ooplasmic streaming is observed in stage 8-12 *cappuccino* and *spire* oocytes. This suggests that *oskar* RNA and STAUFEN protein fails to localize in these mutants because the microtubule system is not properly arranged for posterior RNA translocation and/or RNA is not properly tethered and its localization is lost during ooplasmic streaming.

It is feasible that both dynein and kinesin-related motors are involved in the transport and localization of RNAs in the oocyte, the former prior to stage 9 and the latter during and after stage 9 (Fig. 2.2). While the posterior localization of dynein and a kinesin fusion protein are suggestive of such a role (see above),[33, 34] no phenotypic effects of dynein or kinesin mutants on RNA localization have been reported. Further, no proteins have yet been demonstrated to link the cytoskeleton and its associated motors with factors that are involved in posterior RNA localization and might interact directly with the RNAs. A detailed consideration of the cytoskeleton and its role in RNA localization during *Drosophila* oogenesis is given in Chapter 4.

Factors that might interact directly with the RNAs

Genetic identification of mutations that result in abnormal posterior RNA localization and/or molecular characterization of gene products localized during oogenesis have resulted in the identification of several proteins that may function as trans-acting factors in posterior RNA localization and in polar plasm assembly. Several localized proteins bear molecular homologies that indicate they may interact with RNAs in their transport to and/or assembly at the posterior pole (Table 2.2). These include ORB (a RNP/RRM domain-containing RNA-binding protein), STAUFEN (a double-stranded RNA-binding protein) and VASA (a DEAD family, RNA-dependent helicase).[46, 63, 67, 87, 88]

ORB

ORB protein is present at the posterior pole and anterior margin of wild-type stage 8-10 oocytes (see above).[72, 73] Functionally, localized ORB protein is required for *oskar* RNA localization. In orb^{mel}/orb^{F343} transheterozygous mutant ovaries, ORB protein fails to localize in stage 8 and 9 oocytes and *oskar* RNA fails to localize to the posterior.[73] Since ORB is an RNA-binding protein with homology to the *Xenopus* CPEB protein that is involved in translational control, it is conceivable that there is a direct interaction at the posterior pole between ORB protein and elements in the 3'UTR of *oskar* RNA that are required to stimulate *oskar* translation (Chapter 6). OSKAR protein is required to maintain *oskar* RNA at the posterior.[51, 52] ORB is considered in detail in Chapter 6.

STAUFEN

STAUFEN protein is localized to anterior margin of stage 8 oocytes and then to the posterior of stage 9 oocytes.[89] This is directly coincident with the pattern and timing of *oskar* RNA localization, suggesting that STAUFEN may also mediate, directly or indirectly, the localization of *oskar* RNA. Since *oskar* RNA accumulates normally in the anterior margin of *staufen* mutant oocytes, STAUFEN probably functions specifically in its transport to the posterior pole.[51, 52] The amount of posterior-localized STAUFEN protein is proportional to the number of copies of *oskar* RNA; in contrast, extra *staufen* RNA simply results in the production of extra unlocalized STAUFEN protein.[90] Thus STAUFEN protein is present in excess and its movement to the posterior pole of the oocyte is dependent on its association with *oskar* mRNA. The association between STAUFEN protein and *oskar* RNA ends at egg activation which occurs upon egg laying; at this stage STAUFEN diffuses away from the posterior polar plasm.[90] Supporting this conclusion is the fact that in vitro transcribed *oskar* 3'UTR RNA, when injected into early

embryos, is unable to associate with STAUFENwhile *bicoid* 3'UTR RNA is (see Chapters 3 and 8).[90] STAUFEN is considered in detail in Chapter 8.

OSKAR

oskar encodes a novel protein so sequence homologies do not immediately suggest its function, but mutant analysis and ectopic localization of *oskar* to the anterior suggest that OSKAR protein acts as a trans-acting factor in posterior polar plasm assembly (see below).[51, 52] Nonsense mutations in *oskar* result in failure to maintain *oskar* RNA and STAUFEN protein localization at the posterior.[51, 52] When introduced into *Drosophila melanogaster* flies by P element-mediated germline transformation, the *Drosophila virilis oskar* homolog (*virosk*) causes a delocalization of the endogenous OSKAR protein.[91] This suggests that *Drosophila melanogaster oskar* RNA and OSKAR protein are anchored, directly or indirectly, to the posterior cortex, and that VIROSK interferes with the anchoring. As such, this suggests that OSKAR protein may anchor both its own RNA and STAUFEN protein at the posterior. It should be noted, however, that the polar granules are complex organelles and defects in their assembly and maintenance are not always easy to interpret (discussed below).

VASA

VASA protein is a component of the perinuclear nuage in the nurse cells and the polar granules at the posterior of the oocyte and early embryo and is necessary for posterior localization of *tudor*, *oskar*, *nanos*, *pumilio*, *cyclin B*, *Hsp83* and *germ cell-less* mRNAs and *mitochondrial 16S rRNA*.[12, 14, 37, 51, 52, 64, 65, 68, 69] Localization of VASA to the perinuclear nuage is abolished in most *vasa* mutants, but is unaffected by mutations in *cappuccino*, *spire*, *oskar* and *staufen*, genes that are required for posterior localization of VASA protein within the oocyte.[46] Thus, localization of VASA to the nuage is independent of the pole plasm assembly pathway. Initial localization of VASA

to the posterior pole of the oocyte can occur in *vasa* mutants that produce proteins severely defective in RNA-binding and RNA-unwinding activity. This indicates that initial recruitment of VASA protein to the posterior polar plasm is independent of its RNA-binding ability and thus is likely to depend on protein-protein interactions, possibly directly with OSKAR.[46] However, in contrast to wildtype, these mutant VASA proteins delocalize from the posterior pole after fertilization.[46] Thus maintenance of VASA as a component of the polar granules is dependent on its binding to RNA(s), although it remains unclear exactly what RNA(s) these might be.

Hierarchy Of Posterior Polar Plasm Assembly

Two types of analysis have led to some understanding of the pathway of assembly of the polar plasm. First, the examination of RNA and protein localization in wild-type ovaries has been used to define a temporal and spatial order to the localization and function of the gene products. Second, and more important, analysis of RNA and protein distributions in mutant backgrounds has been used to position the gene products in a functional hierarchy. More powerful than analysis in loss of function mutants, is analysis in a novel situation. For example, ectopic localization of *oskar* RNA has been particularly informative: *oskar* RNA localization to the anterior pole of the oocyte and early embryo results in ectopic anterior polar granules, polar plasm, pole cells and formation of a mirror image anterior abdomen. If other posterior group mutations are introduced into this background, it can be determined from the production of a mirror-image 'bicaudal' abdomen, or the lack thereof, whether the gene product in question functions above or below OSKAR in the hierarchy. The results of such experiments have been interpreted to uncover a stepwise pattern of polar plasm assembly at the posterior (Fig. 2.7) that is summarized below. In reality, however, the situation is somewhat more complicated than can be represented by such a functional hierarchy, and the process

might best be subdivided into at least two phases: translocation to the posterior pole and assembly/anchoring into polar granules.

Overexpression of OSKAR in flies that carry the two endogenous wild-type genes and additional copies introduced by germ line transformation have several effects.[55, 92] First, a greater number of pole cells form than in wild-type embryos. Second, the NANOS protein gradient extends more anteriorly than in wild-type, resulting either in defects in anterior patterning or a bicaudal phenotype. Third, more OSKAR, STAUFEN and VASA protein than normal is localized to posterior pole, although the total pool of STAUFEN and VASA protein remains unchanged in comparison to their wild-type levels.[55, 74] These results suggest that OSKAR is the limiting factor in polar plasm assembly.

The role of OSKAR in polar plasm assembly is most definitively shown in embryos that bear an *oskar-bicoid 3'UTR* transgene.[55] In such transgenic flies, *oskar* RNA is ectopically localized at the anterior. The phenotypic result is dominant female sterility caused by the production of embryos with a bicaudal phenotype. VASA and TUDOR proteins, *nanos* RNA and NANOS protein and *Hsp83* RNA are localized to the anterior (Fig. 2.8).[12, 54, 55] In addition, polar granules assemble and functional pole cells form at the anterior.[54, 55] These phenotypes are dependent on VASA, TUDOR and NANOS.[55] Eliminating functional VASA or TUDOR prevents the formation of ectopic pole cells and abdominal segments, while elimination of functional NANOS prevents ectopic abdominal segmentation but ectopic pole cells still form. In addition to confirming the existence of the two subclasses of posterior group mutants (Fig. 2.7), this result suggests that (i) localization of germ cell determinants and (ii) localization of the posterior morphogen, NANOS, within the posterior polar plasm, are separable events. Further, elimination of *nanos* function increases the number of pole cells that form at the posterior in wild-type embryos and ectopically in embryos that carry 6 copies of the *oskar* gene.[92] The *Drosophila virilis oskar* homologue is capable of rescuing the

abdominal phenotype of *Drosophila melanogaster oskar* mutants but not their pole cell defect.[91] This may be because an insufficient amount of OSKAR protein is anchored at the posterior. Taken together, these results suggests that NANOS acts antagonistically to the germ cell determinant(s) in the posterior, perhaps by competing for the RNA localization/anchoring machinery in the posterior.

CAPPUCCINO and SPIRE are required for the posterior translocation of STAUFEN protein at stage 8 of oogenesis, thus they reside above *staufen* in the hierarchy.[89] Posterior *oskar* RNA localization in stage 8 oocytes is genetically separable into two distinct steps: CAPPUCCINO and SPIRE are required for *oskar* RNA localization to the anterior margin and posterior pole, while STAUFEN is required only for the translocation of *oskar* RNA from the anterior margin to the posterior.[51, 52] Since STAUFEN possesses a double-stranded RNA-binding motif,[93] it is possible that it interacts directly with *oskar* (and other) RNAs to mediate their transport to the posterior pole (see also Chapter 8). Initial posterior localization of STAUFEN protein and *oskar* RNA occurs normally in $oskar^{54}$, $oskar^{084}$ and $oskar^{346}$ mutants, but this posterior localization is subsequently lost.[51, 52] Since each of these alleles are nonsense mutations that truncate the OSKAR protein,[52] this result has been interpreted as indicating that OSKAR protein is required for the maintenance of STAUFEN protein and its own RNA at the posterior.

OSKAR is required for the posterior localization of VASA protein beginning in stage 10 oocytes.[94, 95] In turn, VASA is required for the formation and integrity of the polar granules, the posterior localization of TUDOR protein and of *nanos*, *pumilio*, *Hsp83*, *germ cell-less* and *Cyclin B* RNAs.[12, 14, 37, 54, 65] Distributions of *nanos* and *germ cell-less* RNAs indicate that TUDOR and VALOIS are required not for their initial localization but for maintenance of localization.[14, 37] VASA and TUDOR can best be regarded as key components of the polar granules which are required for their formation and integrity, but they are not participants in the translocation of RNAs to the posterior

pole. The role of VALOIS is unclear at this point since observed mutant phenotypes suggest that it might serve a maintenance function, but analysis in a situation where OSKAR assembles ectopic anterior polar granules and polar plasm suggest that VALOIS functions upstream of OSKAR in the polar granule asssembly pathway. It does appear, however, that the remaining posterior RNAs (*nanos*, *pumilio*, *16S rRNA*, *Hsp83*, *germ cell-less* and *Cyclin B*) do not encode components of the polar granules that are required for their assembly or maintenance, but rather are associated with the polar granules. This association serves to restrict these RNAs to the posterior pole of the embryo with potentially important functional consequences (see below).

FUNCTIONS OF POSTERIOR LOCALIZED RNAS

The functions of the posterior localized RNAs have been touched on above in regard both to the role of OSKAR in the assembly of the germ plasm and the role of NANOS as the posterior morphogen that programs abdominal development. Here we elaborate somewhat on the foregoing and also consider why these RNAs must be localized rather than more generally distributed.

Pole Cell Formation

The localization of the polar granules and the polar plasm to the posterior pole of the oocyte and early embryo results in budding of pole cells from the posterior pole and restricts their formation to this location. Thus the localization of *oskar* RNA to the posterior pole, with consequent assembly of the polar granules there is crucial both in the formation of the germline at the correct location and in preventing germline cells from forming elsewhere. This is exemplified by overexpression of *oskar* RNA throughout the embryo, which results in pole cells forming antero-dorsally,[92] and by ectopic localization of *oskar* RNA to the anterior pole, which results in pole cells forming there.[55]

germ cell-less RNA is posteriorly localized in cleavage stage embryos and encodes a protein of novel sequence that is associated with nuclear pores.[14, 80] No mutations have been identified to date in the *germ cell-less* gene; therefore, the effects of *germ cell-less* antisense RNA expression, overexpression and ectopic localization to the anterior have been examined.[14, 80] Expression of *germ cell-less* antisense RNA induces sterility; fewer pole cells form and, while pole buds form initially, they regress after their second division.[14] Reciprocally, overexpression of *germ cell-less* significantly increases the number of pole cell-like cells that initially form, but these do not persist and, by the time the gonad forms, the same number of pole cells are found in the overexpressing embryos as in wild-type.[80] The levels of VASA protein and *Cyclin B* RNA at the posterior are not affected either by antisense inactivation or by overexpression of GERM CELL-LESS. Ectopic localization of *germ cell-less* to the anterior with a *germ cell-less-bicoid 3'UTR* transgene results in the formation of pole buds, but they do not pinch off to form cells.[80] The nuclei, however, resemble pole cell nuclei in their morphology and mitotic behavior. VASA protein and *Cyclin B* RNA are not localized to the anterior of such embryos, demonstrating that these, or some unidentified gene product(s), are required for the completion of pole cell formation. Nonetheless, *germ cell-less* is clearly required and sufficient to initiate pole cell formation. Given that GERM CELL-LESS protein is associated with the nuclear pores,[80] it has been suggested that GERM CELL-LESS may be necessary for the trafficking of a factor(s) into the nucleus that is required for pole cell formation. The possibility remains that GERM CELL-LESS is also required later in pole cell development.

The *mitochondrial 16S large rRNA* is concentrated at the posterior pole of early embryos in a polar plasm-dependent fashion.[11, 68, 77] This posteriorly concentrated RNA has been reported to be outside the mitochondria and to be associated with the polar granules.[76, 77] Injection of in vitro transcribed *16S rRNA* into the posterior pole of embryos that have been UV-irradiated has been reported to enable them to form pole cell-

like cells which give rise to a non-functional germ line,[76] and it has been speculated that the 16S RNA might function as part of the apparatus that specifies pole cell budding (see Chapter 9). However, there is some dispute as to the functional role of posterior-concentrated *16S rRNA* in pole cell budding, since there is no significant anterior concentrated *16S rRNA* in embryos derived from *oskar-bicoid 3'UTR* females (Fig. 2.8).[68] Since those embryos were shown to ectopically localize *nanos* RNA (Fig. 2.8) and to form functional pole cells, it was concluded that a high concentration of *16S rRNA* is not required for normal pole cell formation.[68] Those experiments could not exclude the possibility that a low level of *16S rRNA* is exported from the mitochondria at the anterior pole of eggs produced by *oskar-bicoid 3'UTR* females and that this is sufficient to implement the normal role of *16S rRNA* in pole cell budding. Okada and colleagues elaborate their model in a separate chapter in this volume (Chapter 9). The resolution of the possible role of *16S rRNA* in pole cell formation will await production of a loss of function phenotype. Unfortunately, antisense inactivation of this RNA has not succeeded in producing a phenotype;[68, 76] this leaves the question unresolved since failure to produce a phenotype could either be interpreted as further support for the conclusion that the *16S rRNA* does not fulfill the postulated function in pole cell formation or as evidence that antisense inactivation experiments often fail. Either way, the fact that the *16S rRNA* is encoded by the mitochondrial genome makes further experiments very difficult.

Pole Cell Determination

The polar granules - nucleated by posterior localization of *oskar* RNA - are required to specify the formation of the pole cells. It is also assumed that the polar granules/polar plasm contain 'determinants' that instruct these cells as to their germline identity once they have formed. To date, no single germ cell determinant has been identified; it is possible that multiple gene products may be required to specify germ cell identity. Several RNAs have been identified that are specifically localized to the

posterior and may encode products required for pole cell formation, identity and/or function. These RNAs include *Hsp83*, *Cyclin B* and *germ cell-less*.[12, 14, 78]

HSP83 is the *Drosophila* homolog of the HSP90 family of cytoplasmic regulatory molecular chaperones that have been shown regulate the activity of steroid hormone receptors, the RAF kinase, SRC-family tyrosine kinases and several other classes of proteins.[96, 97] It has been speculated that HSP83 may function in the pole cells to regulate their identity by controlling intracellular signaling pathways.[12] The recent availability of *Hsp83* mutations will soon enable these functions to be addressed directly.[98]

Because of translational repression of the posterior localized *Cyclin B* RNA (see above),[79] it is clear that *Cyclin B* has no role in the early instruction of the pole cells as to their identity. However, the relief of this repression and the production of CYCLIN B protein shortly before pole cell divisions resume in the gonad late in embryogenesis, together with the known biochemical functions of CYCLIN, suggest that CYCLIN B plays a role in programming these divisions and/or their timing.[79]

Abdominal Pattern Specification

A by-product of posterior localization of *oskar* RNA during normal development is the posterior localization of *nanos* RNA and hence the correct specification of the abdomen. The *nanos* gene was identified as encoding the so-called posterior morphogen based on the rescue of abdominal patterning defects in posterior group mutants by cytoplasmic transplantation and injection of in vitro transcribed *nanos* RNA.[5, 6, 53, 60] *nanos* RNA is localized to the posterior pole late in oogenesis, is translated during the early cleavage stages and NANOS protein is present in a gradient in the posterior half of the embryo, with its peak at the posterior pole.[53, 65, 92] In addition to promoting abdominal cell fates, NANOS functions to suppress anterior cell fates. Injection of *nanos* RNA into the anterior and ectopic expression of a *nanos-bicoid 3'UTR* transgene results

in the loss of anterior structures and their replacement with posterior structures.[53, 56] *nanos* encodes a protein with a Zn-finger RNA binding motif.[99] NANOS exerts its wild-type morphogenetic effect by post-transcriptionally repressing the translation of *hunchback* RNA, which encodes a transcription factor that promotes anterior cell fates and prevents abdominal patterning.[100] It has been shown that NANOS prevents translation of *hunchback* and *bicoid* RNA through cis-acting NANOS response elements (NREs) within their respective 3'UTRs.[86] However, recent experiments suggest that NANOS protein does not bind the NREs directly (see below).[75]

A recent report suggests that PUMILIO protein and a newly identified 55 kd protein specifically bind to NREs within the *hunchback* RNA, and this complex then recruits NANOS by protein-protein interactions.[75] UV-crosslinking and gel retardation assays with radiolabled NRE RNA and *Drosophila* embryo extracts reveal two proteins that bind in vitro: a 165 kd and a 55 kd protein. The following results identify the 165 kd protein as PUMILIO.[75] The 165 kd binding protein is absent in extracts derived from *pumilio* mutant mothers, and preincubation of wildtype extracts with PUMILIO antibodies eliminate this bound complex. In addition, PUMILIO protein produced in COS cells or reticulocyte lysates also forms the identical RNA/protein complex observed with embryo extracts. The 55 kd protein has not yet been identified. NANOS protein does not bind to the NRE RNA in vitro, nor does altering the level of NANOS protein in appropriate mutant backgrounds affect the binding of the other proteins.[75] Site directed mutations in the NRE that disrupt PUMILIO and/or the 55 kd protein binding in vitro also confer defects in abdominal patterning in vivo.[75] These results, along with the observation that PUMILIO protein is distributed uniformly in the embryo,[66] suggests that PUMILIO protein, and possibly the 55 kd protein, bind to the NREs within *hunchback* RNA throughout the embryo (and probably to the NREs in the *bicoid* 3'UTR at the anterior) but do not confer translational repression. Within the posterior, however, NANOS protein is recruited to the *hunchback* 3'UTR via protein-protein interactions with

the PUMILIO/55 kd protein complex, and this complex, in turn, inhibits the translational machinery. Thus, posterior localization of *nanos* RNA functions to restrict production of the NANOS protein gradient to the posterior half of the embryo, ensuring that abdominal development occurs in the correct spatial domain and, reciprocally, that NANOS does not repress anterior development in the anterior region of the embryo.

SUMMARY AND PROSPECTS

Localization of maternally synthesized RNAs to the posterior pole of the *Drosophila* oocyte and early embryo provides an ideal model system for the analysis of mechanisms and functions of RNA localization. Most identified posterior localized oocyte RNAs are synthesized in the nurse cells and transported into the oocyte through intercellular bridges at its anterior pole. Once within the oocyte, these RNAs are translocated to the posterior pole in a process that is likely to be mediated by microtubules and microtubule-associated motors. An alternative RNA localization mechanism has been discovered that involves a combination of generalized RNA degradation and local protection at the posterior. The cis-acting sequences and trans-acting factors involved in each step of these RNA localization processes are amenable to both genetic and molecular analysis. In addition to yielding insights into RNA localization mechanisms, studies of the posterior polar plasm of *Drosophila* are beginning to unravel the molecular basis of germ cell formation and specification, a process whose components may be conserved among metazoa (see Chapter 10). In particular, many components of the germinal granules have now been identified. In the future, systematic tests for specific protein-protein interactions will be needed to begin to define the roles of these components in the assembly and function of the germinal granules.

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Table 2.1
Dynamics of RNA localization to the posterior

Gene (homology)	Germarium	Stages 2-6	Stages 7-8	Stages 9-10A	Stages 10B-11	Stages 12-14	Cleavage Stage	Syncytial Blastoderm	References
Cyclin B (cyclin)	Region 2-3: enriched in oocyte	enriched in oocyte	St 7: enriched in oocyte St 8: no RNA	nurse cells	transport into oocyte	St 12: transport into oocyte St 13-14: localized to oocyte posterior	enriched at posterior/ dispersed throughout embryo	incorporated into pole cells/ perinuclear somatically	11, 77, 78
germ cell-less (novel)	no RNA	no RNA	St 8: nurse cells	nurse cells	transport into oocyte	localized to oocyte posterior	posterior	incorporated into pole cells	14

Hsp83 (HSP90 molecular chaperone)	Region 2-3: nurse cells and oocyte	St 2-5: nurse cells and oocyte St 6: no RNA	no RNA	nurse cells	transport into oocyte	uniform in oocyte	Nuclear Cycles 1-6: uniform Nuclear Cycles7/8: posterior to anterior gradient	incorporated into pole cells	12
mitochondrial 16S rRNA (ribosomal RNA)	no RNA	high in nurse cells, lower in oocyte	high in nurse cells, lower in oocyte	high in nurse cells, lower in oocyte	transport into oocyte	localized to oocyte posterior	enriched at posterior/ dispersed throughout embryo	not incorporated into pole cells	11, 68
nanos (Zn finger RNA binding protein)	Region 2-3: low levels in nurse cells and oocyte	enriched in oocyte	St 7: anterior margin St 8: anterior margin and nurse cells	St 9: nurse cells and oocyte St 10A: nurse cells and oocyte	transport into oocyte	localized to oocyte posterior	enriched at posterior/ dispersed througout embryo	incorporated into pole cells	37, 53, 98

orb (RNP/RRM RNA binding motif)	Region 2-3: enriched in oocyte	oocyte posterior	St 7: oocyte posterior St 8: oocyte anterior	oocyte anterior	uniform in oocyte	uniform in oocyte	enriched at posterior/ dispersed throughout embryo	incorporated into pole cells	67
oskar (novel)	Region 2-3: enriched in oocyte	enriched in oocyte	St 7: enriched in oocyte St 8: localized to anterior margin and posterior	oocyte posterior	oocyte posterior	oocyte posterior	Nuclear Cycles 1-6: posterior Nuclear Cycles 7-10: delocalized	delocalized	51, 52
pumilio (novel)	Region 2-3: nurse cells and oocyte	St 2-4: nurse cells and oocyte St 5-6: no RNA	no RNA	nurse cells	transported into oocyte	unknown	variable enrichment at posterior/ dispersed throughout embryo	variable enrichment in pole cells	65, 66

tudor (novel)	Region 2: oocyte Region 3: surrounds the oocyte nucleus	St 2: surrounds oocyte nucleus St 3: oocyte St 4-6: enriched in posterior half of oocyte	St 7: enriched in posterior half of oocyte St 8: no RNA	Stage 9: no RNA Stage 10: nurse cells	no RNA	no RNA	no RNA	no RNA	64

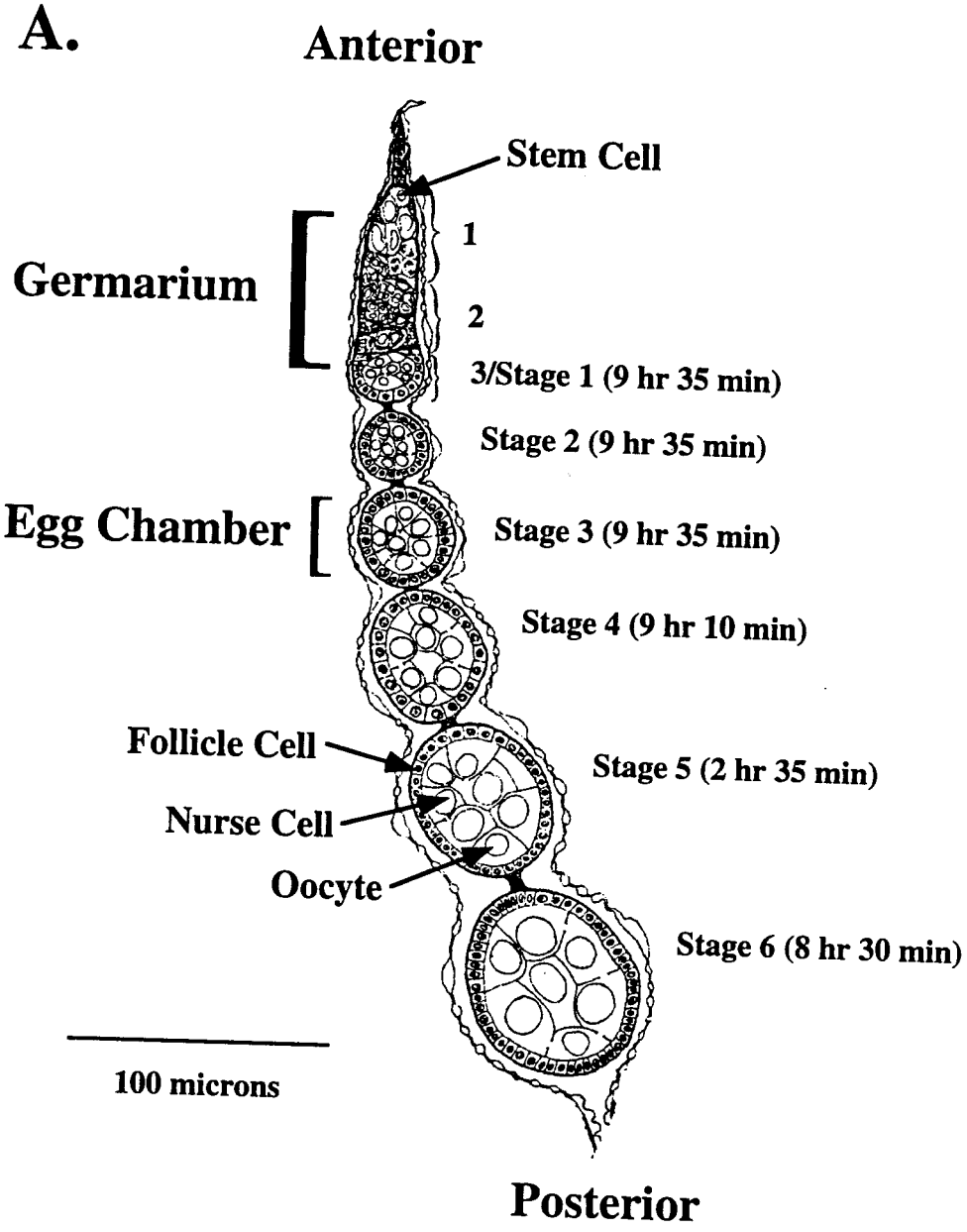
Table 2.2
Distribution of proteins required for posterior polar plasm assembly

Gene (homology)	Germarium	Stages 2-6	Stages 7-8	Stages 9-10A	Stages 10B-11	Stages 12-14	Cleavage Stage	Syncytial Blastoderm	References
ORB (RNP/RRM RNA binding motif)	Region 1: low levels detected Region 2-3: enriched in oocyte	St 2-4: enriched at oocyte posterior St 5-6: posterior and cortex	St 7: posterior and cortex St 8: anterior, posterior and cortex	St 9: anterior, posterior and cortex St 10: enriched at the anterior	not reported	not reported	not reported	not reported	72
OSKAR (novel)	not reported	not reported	not reported	not reported	not reported	not reported	enriched at posterior	not reported	55
STAUFEN (double-stranded RNA-binding protein)	not detected	St 2: not detected St 3-6: oocyte	St 7: oocyte St 8: oocyte anterior margin and posterior	oocyte posterior	nurse cells and oocyte posterior	transported into oocyte	enriched at posterior and anterior/ dispersed throughout embryo	not incorporated into pole cells/ dispersed throughout embryo	88

TUDOR (novel)	nurse cells and oocyte	St 2-3: perinuclear in nurse cells and oocyte St 4-5: perinuclear in nurse cells and anterior margin of oocyte St 6: perinuclear in nurse cells and evenly distributed in oocyte	perinuclear in nurse cells and evenly distributed in oocyte	enriched at oocyte posterior	not reported	not reported	enriched at posterior/ surrounds somatic nuclei	incorporated into pole cells/ surrounds somatic nuclei	54
VASA (DEAD family, RNA-dependent helicase)	nurse cell nuage and cytoplasm	nurse cell nuage and cytoplasm	St 7: nurse cells St 8: transport into oocyte	St 10A: localized to oocyte posterior	localized to oocyte posterior	localized to oocyte posterior	posterior	incorporated into pole cells	46, 93, 100

FIGURES

Figure 1. *Drosophila* oogenesis. The stages of *Drosophila* oogenesis are schematized. (A) A single ovariole, including the germarium at the anterior and stage 1-6 egg chambers, is shown. (B) Stage 7-14 egg chambers are represented. Approximate times spent in each stage are shown in parentheses.[15] Stem cell divisions occur within the anterior tip of the germarium, and incomplete mitotic divisions giving rise to the 16 cell germline cyst occur within region 1. The single presumptive oocyte lies at the posterior of the cyst. Within region 2, mesodermally derived follicle cells surround the cyst. Egg chambers are composed of the oocyte at the posterior, the 15 nurse cells clustered at the anterior and a layer of somatic follicle cells. During pre-vitellogenic stages 1-6, the size of the oocyte is indistinguishable from that of the nurse cells. Yolk uptake by the oocyte from the hemolymph commences at stage 7, and the size and morphology of the oocyte is clearly different from that of the nurse cells. Bulk dumping of the nurse cell contents into the oocyte occurs at stage 10B/11. Commencing in stage 10, the follicle cells lay down the vitelline membrane and subsequently direct the formation of specialized structures, such as the dorsal appendages and chorion. Figure after ref.[15]


A.
Anterior
Stem Cell
Germarium
1
2
3/Stage 1 (9 hr 35 min)
Stage 2 (9 hr 35 min)
Egg Chamber
Stage 3 (9 hr 35 min)
Stage 4 (9 hr 10 min)
Follicle Cell
Stage 5 (2 hr 35 min)
Nurse Cell
Oocyte
Stage 6 (8 hr 30 min)
100 microns
Posterior

B. Anterior Posterior

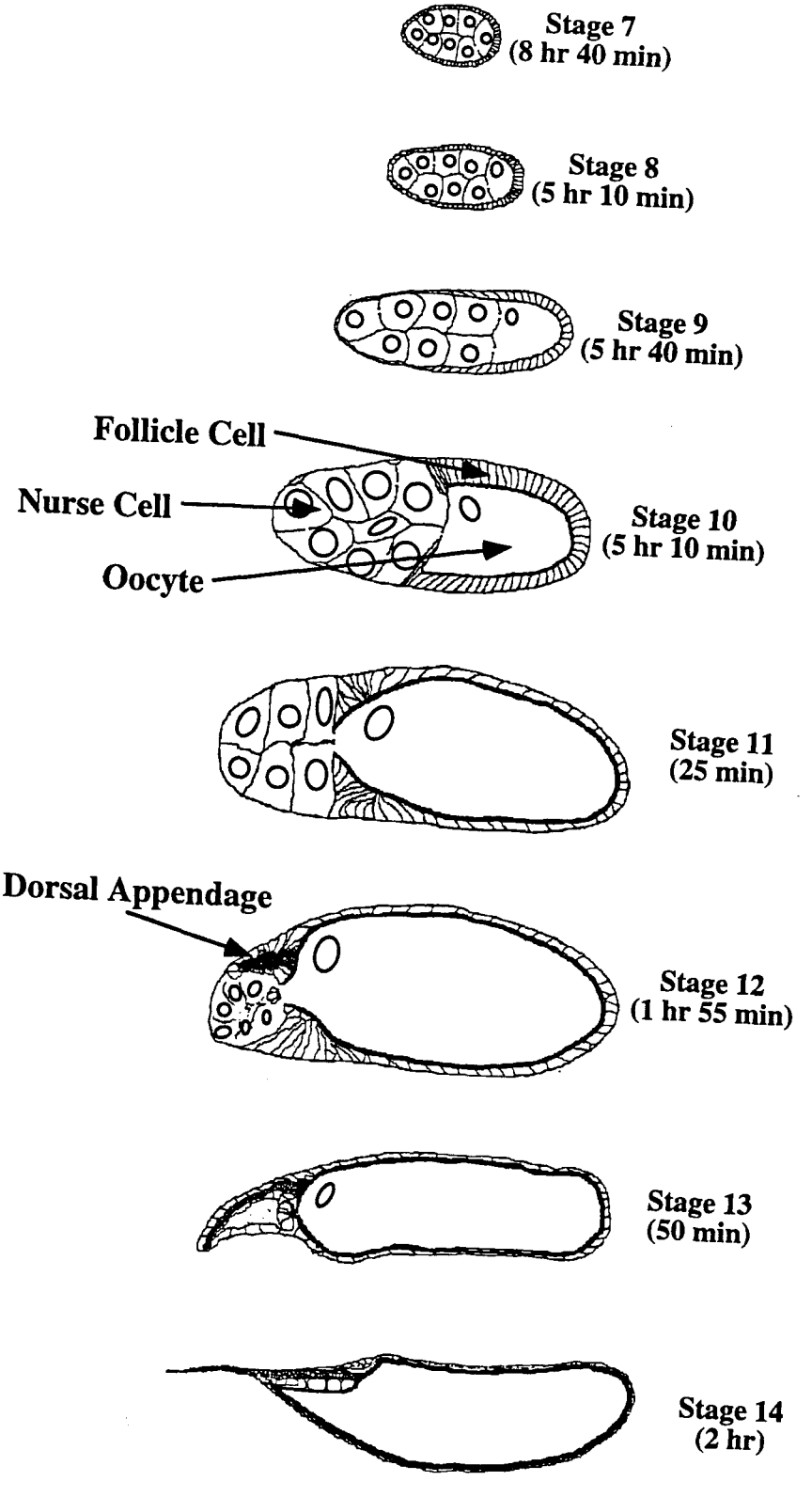
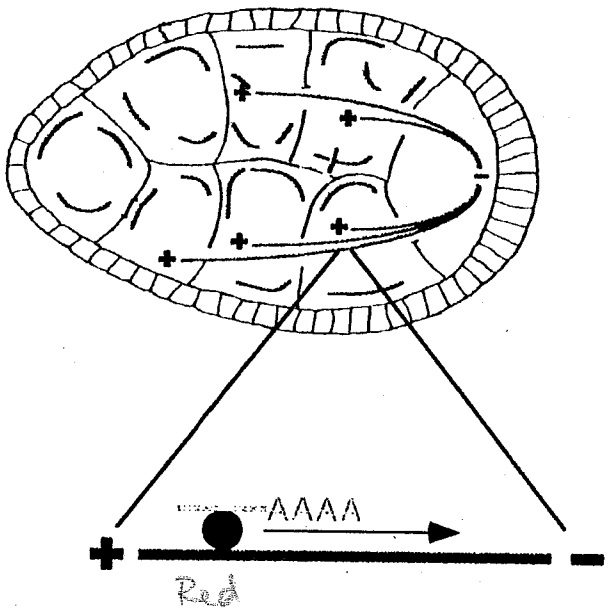
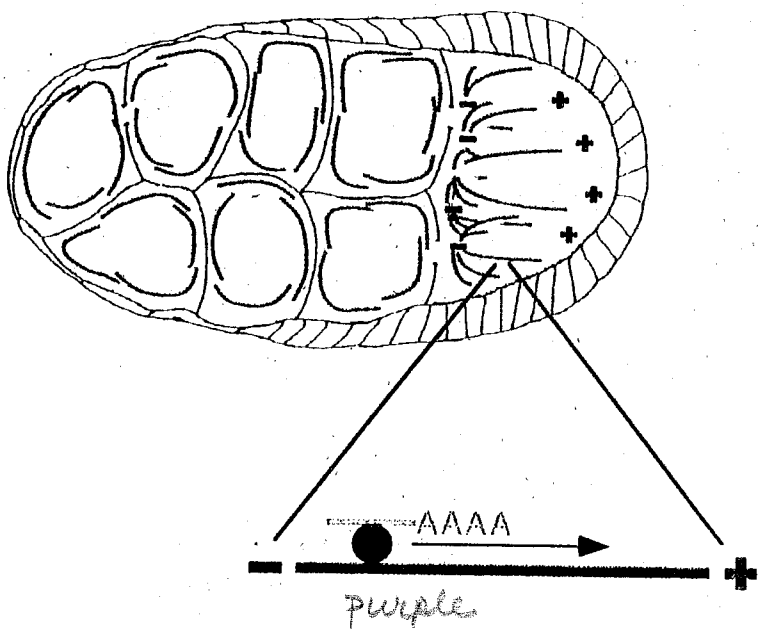


Figure 2. Polarized distribution of microtubules within egg chambers. The distribution of microtubules within egg chambers undergoes dynamic rearrangements during oogenesis (see text for details).[31, 32] (A) In stage 2-6 egg chambers, a single microtubule organizing center (MTOC) resides at the posterior pole of the oocyte, thus nucleating the minus end of microtubules that extend into all 15 nurse cells. RNA transport from the nurse cells into the oocyte at these stages may rely upon minus-end directed motors such as dynein (or minus-end directed kinesins; not shown). (B) In stage 7-10A egg chambers, a single MTOC is no longer detected, and the minus ends of the microtubules become concentrated at the anterior of the oocyte. Localization of RNAs to the posterior at these stages presumably involves plus-end directed motors such as kinesin.

A. Stages 2-6



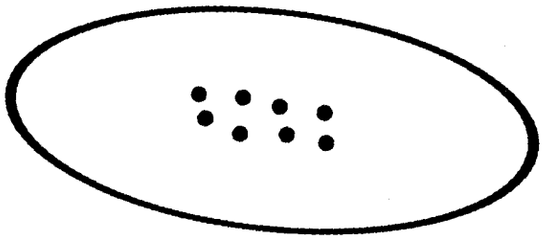
B. Stages 7-10A



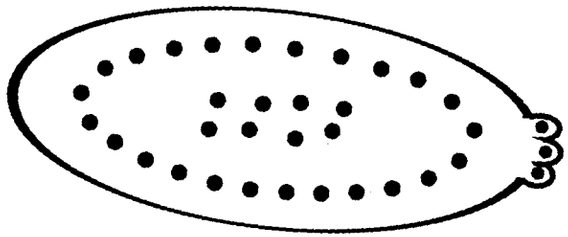
Red ● **Dynein** ┈┈AAAA **Localized RNA**

Purple ● **Kinesin** ━━━ **Microtubule**

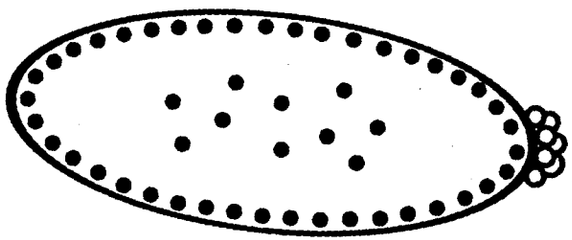
Figure 3. *Drosophila* embryogenesis. The first 3 hours of *Drosophila* embryogenesis are schematized. (A) During the cleavage stages the first 8 nuclear divisions occur within the central yolk mass of the embryo. At the end of the cleavage stage, the nuclei migrate to the periphery with nuclei first arriving at the posterior pole. (B) At the pole bud stage, these nuclei bud off in the pole cells, the primordial germ cells. (C) During the syncytial blastoderm stage, the remaining nuclei arrive at the periphery and undergo four additional rounds of mitosis. (D) In order to form the cellular blastoderm, membrane invaginates around the nuclei, thus simultaneously forming 6000 somatic cells. Anterior is shown on the left and posterior on the right. The approximate times, post-fertilization, for each stage at 25 degrees Celsius are shown.[40]



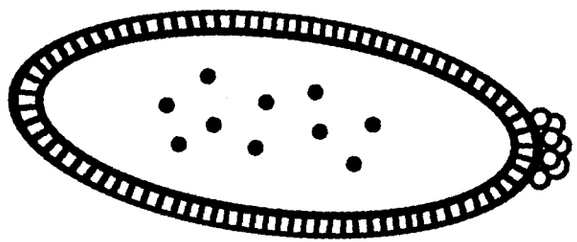
A. Cleavage Stages
(0-1 hr)



B. Pole Bud Stage
(1 hr- 1 hr 20 min)



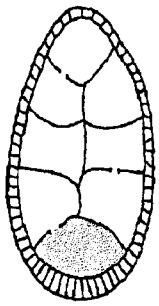
C. Syncytial Blastoderm
(1 hr 20 min-2 hr 10 min)



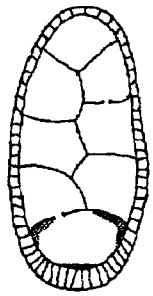
D. Cellular Blastoderm
(2 hr 20 min-2 hr 50 min)

Figure 4. Dynamics of posterior RNA localization. Representative stages during which three RNAs become posterior localized are shown. (A) *oskar* RNA is enriched in stage 7 oocytes. During stage 8, the RNA transiently localizes to the anterior margin of the oocyte and then translocates to the posterior. In stage 9 oocytes, the RNA is detected only at the posterior pole. (B) *nanos* RNA is synthesized at high levels in stage 10 egg chambers. The RNA is transported into the oocyte during bulk nurse cell dumping in stage 10B/11. During stage 12 the RNA is localized to the posterior of the oocyte. (C) *Hsp83* RNA is synthesized in the nurse cells and is uniformly distributed in stage 12-14 oocytes and early cleavage stage embryos. At the late cleavage stage (nuclear cycle 7/8), *Hsp83* RNA is distributed in a posterior to anterior gradient. The RNA is incorporated into the pole cells and is not detected elsewhere at the syncytial blastoderm stage. See the text for additional stages at which these RNAs are expressed. The egg chambers and embryos are not drawn to scale.

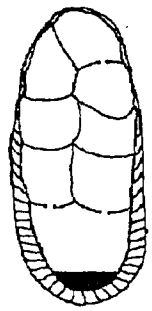
A. *oskar*



Stage 7

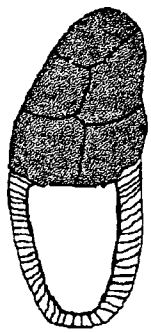


Stage 8

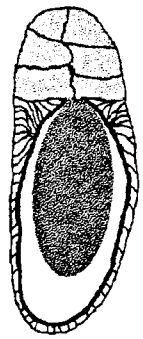


Stage 9

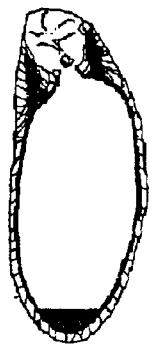
B. *nanos*



Stage 10

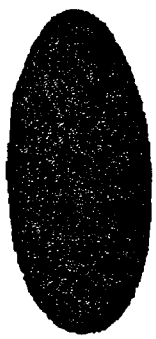


Stage 11

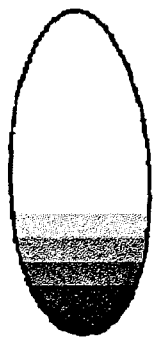


Stage 12

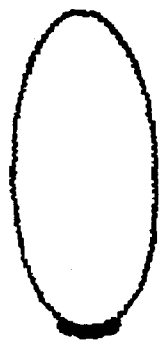
C. *Hsp83*



Early Cleavage Stage

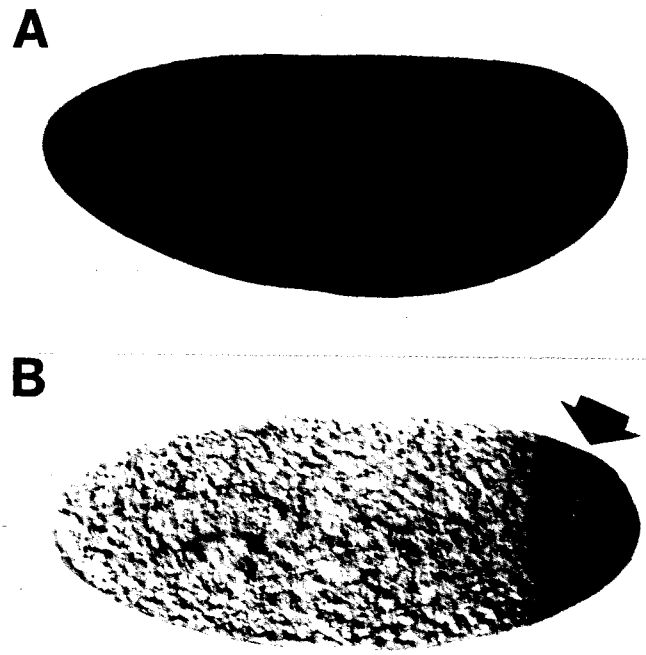


Late Cleavage Stage



Syncytial Blastoderm

Figure 5. Whole mount in situ hybridization analysis of *Hsp83* RNA distribution. *Hsp83* RNA distribution in wildtype embryos is shown in whole mount in situs. *Hsp83* RNA becomes localized to the posterior by a unique mechanism of generalized degradation and localized protection. (A) An early cleavage stage (nuclear cycle 2/3) embryo has a high uniform distribution of *Hsp83* RNA. (B) At the late cleavage stage (nuclear cycle 7/8) the RNA becomes concentrated at the posterior pole. (C) Quantification of the level of *Hsp83* RNA at nuclear cycles 2/3 and 7/8 is represented graphically. The concentration of RNA remains constant at the posterior pole of late cleavage stage embryos but drops throughout the rest of the embryo. Figure reprinted from ref. [12] by permission of the American Society for Microbiology.


A
B

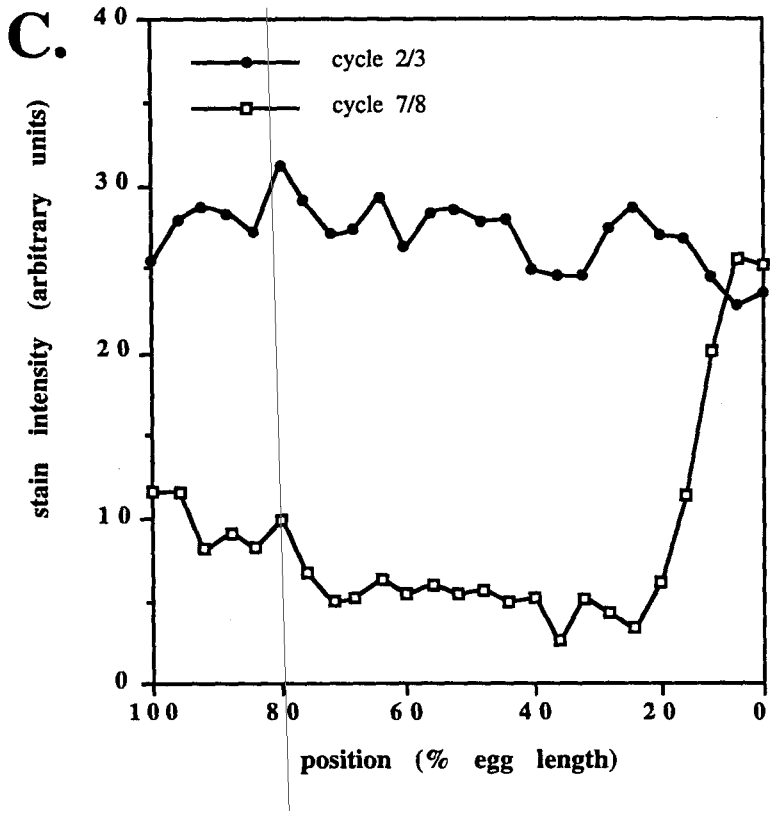
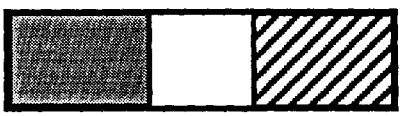

C.
40
30
20
10
0
cycle 2/3
cycle 7/8
stain intensity (arbitrary units)
100
80
60
40
20
0
position (% egg length)

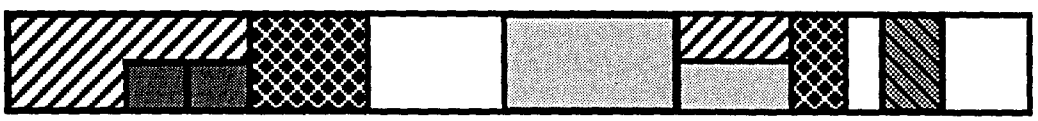
Figure 6. Cis-acting tags in the 3'UTRs of posterior localized RNAs. To date, the analysis of cis-acting tags required for posterior RNA localization has identified the 3'UTR as necessary and, in many cases, also sufficient. The 3'UTRs of these RNAs are shown. (A) Redundant elements within the 406 nt *Hsp83* 3'UTR are sufficient to confer protection from degradation at the posterior.[84] Cis-acting degradation tags are found in the 5' part of the transcript (not shown). (B) The 1207 nt *orb* 3'UTR possesses separable cis-acting elements required for transport into the oocyte, posterior localization and anterior localization.[71] (C) 728 nt of the 1043 nt *oskar* 3'UTR are sufficient to confer the complex pattern of localization of its RNA. Separable elements that are necessary for transport into the oocyte, proper translocation from the anterior to the posterior and posterior localization have been identified.[83] Sequences have also been identified that confer translational control.[85] (D) The full length 776 nt *CyclinB* 3'UTR has two small (94 nt and 87 nt) elements that are necessary for its posterior localization separated by a 39 nt element that is necessary for conferring translational control.[79]

A. *Hsp83*

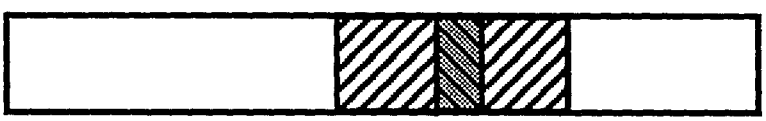


B. *orb*

C. *oskar*



D. *Cyclin B*



oocyte transport

posterior localization

anterior localization

translational control

100 nt

Figure 7. Hierarchy of posterior polar plasm assembly. Analysis of RNA and protein distribution patterns in mutant backgrounds has suggested a hierarchy of gene functions during posterior polar plasm assembly. See text for details.

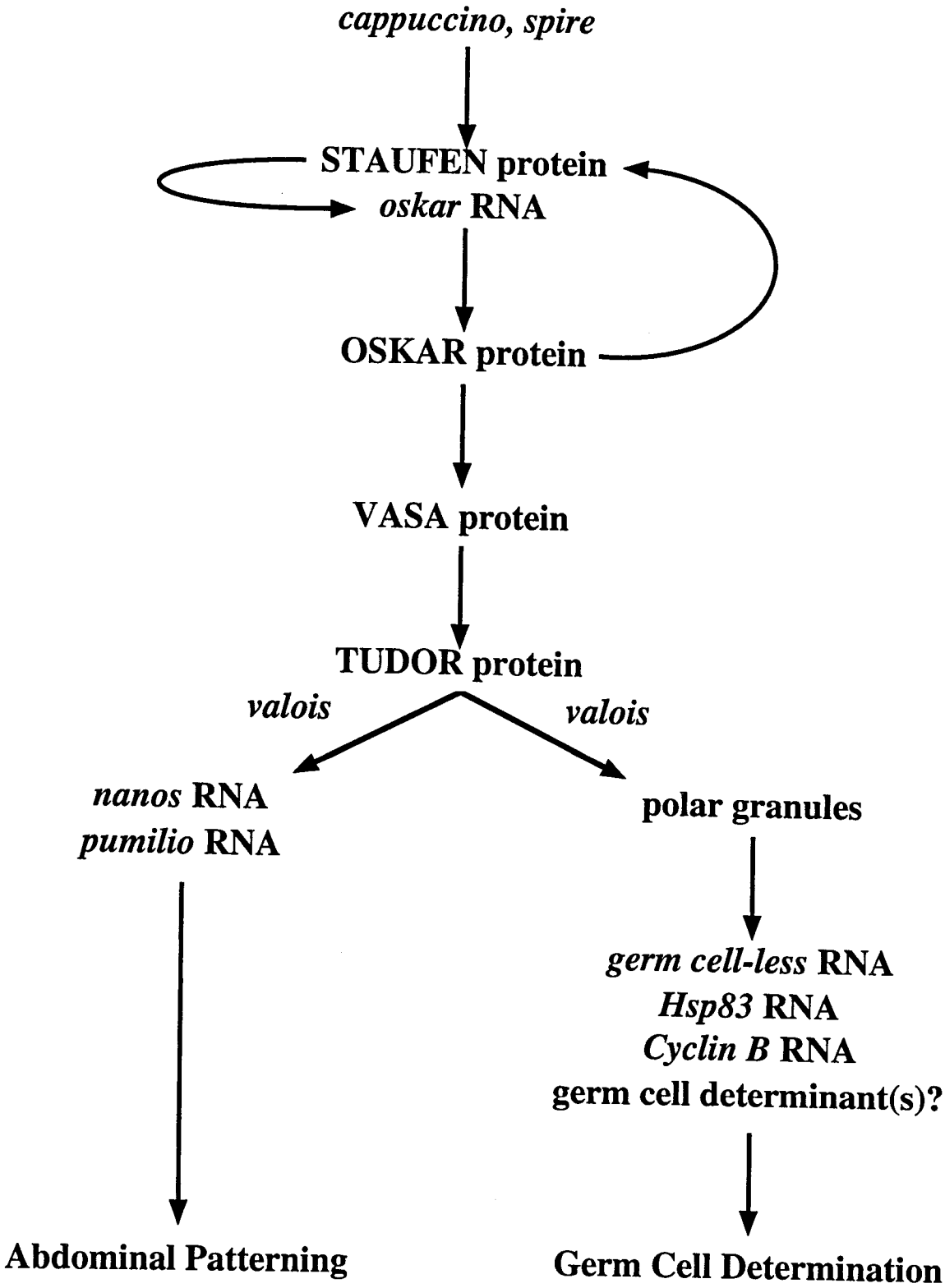
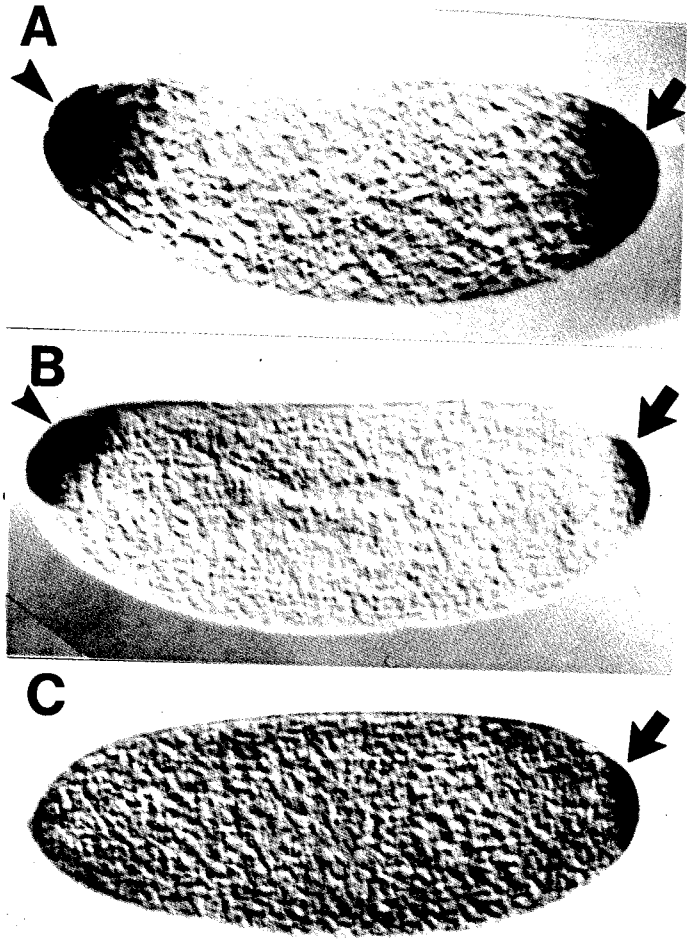

cappuccino, spire
STAUFEN protein
oskar RNA
OSKAR protein
VASA protein
TUDOR protein
valois
valois
nanos RNA
pumilio RNA
polar granules
germ cell-less RNA
Hsp83 RNA
Cyclin B RNA
germ cell determinant(s)?
Abdominal Patterning
Germ Cell Determination

Figure 8. Distribution of posterior localized RNAs in *oskar-bicoid* 3'UTR transgenic embryos. The RNA distributions of three posterior localized RNAs in embryos derived from mothers bearing an *oskar-bicoid* 3'UTR transgene are shown. These embryos have *oskar* RNA ectopically localized to the anterior in addition to endogenous *oskar* RNA localized to the posterior. Such embryos ectopically assemble polar granules, posterior polar plasm and pole cells at the anterior as well as forming mirror image duplications of the abdomen.[55] (A) *Hsp83* RNA is localized to the posterior and ectopically to the anterior (cf. Fig. 2.5 B). (B) RNA of the abdomen patterning gene, *nanos*, is also ectopically localized at the anterior. (C) In contrast, *mitochondrial 16S large rRNA* does not show a significant ectopic enrichment at the anterior of *oskar-bicoid* 3'UTR embryos, but rather shows a wildtype distribution. Anterior is to the left and posterior is to the right. Photographs in (B) and (C) courtesy of K.L. Whittaker.


A
B
C

CHAPTER 2

This paper appeared in Mol. Cell. Biol. **13**,3773-3781 (1993).

My contributions included performing the *osk-bcd* 3'UTR *in situs* and data analysis.

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 novel mechanism involving 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 posterior 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 the posterior polar plasm and the polar granules - _cappuccino_, _oskar_, _spire_, _staufen_, _tudor_, _valois_ and _vasa_. Mislocalization of _oskar_ RNA to the anterior pole, which has been shown to result in induction of germ cells at the anterior, leads to anterior protection of maternal _Hsp83_ RNA. These results suggest 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 (12, 13, 58, 59), and RNA encoding a *Drosophila* adducin homolog (11). 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 (14, 15, 18, 22-24, 28, 30, 35-37, 43, 45, 54, 57, 63). 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 (27, 46-49). 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 with spatially restricted functions in the *Drosophila* embryo have been identified in genetic screens (5, 17, 39, 41, 42, 50, 55, 56). However, it has been estimated that only one third of the transcription units of the *Drosophila* genome have been genetically defined (4). To isolate additional maternal molecules with spatially restricted functions, 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 (10). Apart from studying their possible developmental role, we expected that identification of localized RNAs would be useful in the analysis of RNA localization mechanisms *per se* (9, 10). Here we report a detailed analysis of the spatial localization of one of the posterior-localized RNAs we identified in this screen. It is encoded by the *Hsp83* gene, the sole *Drosophila* homolog of the mammalian *Hsp90* gene family (2, 10, 21).

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 (33, 65, 66). Despite the extensive biochemical studies of the *Hsp90* family of cytoplasmically-active regulatory molecular chaperones in mammals, yeast and *Drosophila*, little is known about their developmental regulation and functions (reviewed in 44). While mutational analyses of the *Saccharomyces cerevisiae Hsp82* genes have shown that *Hsp82* is an essential protein in yeast (3), no *Drosophila Hsp83* mutations have been identified (64).

Here, we show that maternal *Drosophila Hsp83* RNA is concentrated at the posterior pole of the early embryo via a novel RNA localization mechanism involving a combination of generalized degradation throughout the embryo and local protection of *Hsp83* RNA at the posterior pole. Analyses of the distribution of *Hsp83* RNA in embryos produced by mutants lacking polar granules, as well as in embryos that have a key polar plasm component - *oskar* - mislocalized anteriorly, reveal that *Hsp83* RNA 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 is described in detail elsewhere (10). Briefly, RNA was purified from anterior or posterior poles cut off frozen embryos and was used in the construction of directionally cloned cDNA libraries in λEXLX vectors (53). The entire anterior- and posterior-libraries were converted from phage to plasmid libraries by

Cre-*loxP* automatic plasmid subcloning (53), 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, 21). The *Drosophila Hsp83* gene was cloned over thirteen years ago (26), and both its heat-inducible and its developmentally regulated expression have been studied in some detail (33, 65, 66). 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.** Whole mount RNA tissue *in situ* hybridization was based on the method of Tautz and Pfeifle (61). 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 (61). Embryos were fixed as described (61), 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 (61). Ovaries and embryos were mounted in JB4 plastic mountant for microscopy (Polysciences).

Temporal And Quantitative Analysis Of *Hsp83* Localization In The Early Embryo. 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. In order to quantify the intensity of the *in situ* hybridization signals, the color reaction was stopped early in order to understain the embryos thus ensuring that the signal was not saturated. Images of whole mount embryos 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 measurements and production of pseudocolor images representing the concentration distribution of *Hsp83* transcripts were carried out using NIH Image public domain software (Version 4.2; written by J. Ayers and G. Fletcher, available via anonymous FTP from sumex-aim.stanford.edu). Measurements were then made of the average gray-scale values for pixels in twenty-five equisized areas along the midline of the antero-posterior axis (normalized for egg length). The equivalent values from a background image were subtracted. Five embryos from each stage were analyzed in this way, and the mean gray-scale values for each of the twenty-five areas were used to plot the relative intensity of *Hsp83 in situ* signal along the antero-posterior axis at two stages of early embryogenesis (Fig. 2). This method is similar to that used by St. Johnston et al. (58) and Driever and Nusslein-Volhard (13)

Fly Strains. Mutant embryos were obtained from females homozygous for *osk*^{166} (39), *capu*^{HK}, *spir*^{RP} (50), *nos*^{L7}, *pum*^{680} (41), *exu*^{PJ}, *vas*^{PD}, *stau*^{HL}, *vls*^{RB}, *tud*^{WC8} (55), and *bcd*^{E1} (17). 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} *pp e/TM3* (40). Embryos with *oskar* RNA mislocalized to the anterior pole were obtained from females heterozygous for *osk-bcd3'UTR* as described in (15). The *c83Z.-880* strain of flies germline transformed with a *Hsp83-lacZ* fusion gene was provided by H. Xiao and J. Lis (Cornell University) (65).

RESULTS

***Hsp83* RNA Is Expressed In A Dynamic Fashion During Oogenesis.** Oogenesis in *Drosophila* can be subdivided into distinct stages that occur in an ordered spatial and temporal array in the individual ovarioles that comprise each ovary (31). At the anterior tip of the ovariole is the germarium, in which germline stem cells divide asymmetrically to give rise to blast cells that will contribute the germline components of the ovarian follicles. These undergo a series of four mitotic divisions with incomplete cytokinesis, forming an interconnected 16-cell germline cyst. One of the cells forms the oocyte and the remaining fifteen the nurse cells. The germarium is subdivided into three regions (31) (Fig. 1). The follicle grows in size and matures in the vitellarium, in a series of 14 stages (Fig. 1; stage 1 is equivalent to germarial stage 3) (31). Most of the synthesis of RNA and and protein occurs in the nurse cells and these molecules are then transported into the oocyte. In addition, yolk is transported to the oocyte in the hemolymph, and is

taken up by pinocytosis, contributing significantly to the increase in oocyte volume from stage 8 onwards.

Hsp83 RNA expression is absent in region 1 of the germarium and is first detected in germarial regions 2 and 3 in all 16 cells of the germline cyst (Fig. 1A). In stage 1 through 5 egg chambers, *Hsp83* expression continues in all 16 cells of the nurse cell-oocyte complex (Fig. 1A). 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. During the first three hours of *Drosophila* embryogenesis, the fertilized zygotic nucleus undergoes a series of divisions in the absence of cytokinesis, forming a syncytial embryo containing roughly 6,000 nuclei (6). Migration of nuclei to the periphery of the embryo commences about an hour after fertilization, with nuclei first reaching the posterior pole after about eighty minutes. These nuclei contribute to the pole cells, which bud from the posterior and are the primordial germ cells. The remaining nuclei continue to divide in the cortical cytoplasm and, roughly three hours after fertilization, contribute to the somatic blastoderm cells that form by a process of co-ordinated membrane invagination (6). Subsequently, gastrulation-related cell movements and shape changes convert the two-dimensional sheet of blastoderm cells into a three-dimensional embryo with distinct tissue layers and organs.

Maternal *Hsp83* RNA is distributed throughout the early embryo from nuclear division cycles 1 through 5 (Fig. 1E). During cleavage cycles 6 to 8, *Hsp83* RNA is most highly concentrated at the posterior pole, forming a decreasing concentration gradient in the posterior half of the embryo (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, 66, this study).

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 (Fig. 2). An alternative - translocation of the generally-distributed *Hsp83* RNA to the posterior pole - is excluded by the fact that 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 (Fig. 2). In support of our quantitative analysis of *Hsp83* RNA by tissue *in situ* hybridization (Fig. 2), Northern blot analysis shows that there is less *Hsp83* RNA in 2-4 hr embryos than in 0-2 hr embryos (data not shown). Embryos homozygous for deletions of *Hsp83* still show posterior localization of *Hsp83* RNA (Fig. 5A), 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. Sequences required for posterior protection of the *Hsp83* RNA map 3' to the translation initiation site, since a *Hsp83-lacZ* fusion RNA (see below) is no longer protected at the posterior pole of early embryos (Fig. 6).

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. 5A). 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 in other tissues.

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 components of the posterior polar plasm. Maternal *Hsp83* RNA is distributed normally in early cleavage stage embryos produced by seven mutants that affect the integrity of the posterior polar plasm, polar granules and pole cell formation (*cappuccino*, *spire*, *oskar*, *vasa*, *staufen*, *valois*, and *tudor*) (examples are presented in Fig. 3A,C,E). 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. 3B,D,F). In contrast, in mutants that affect components of the polar plasm required for abdominal patterning but not for polar granule integrity or pole cell formation (*nanos* and *pumilio*), the posterior localization of *Hsp83* RNA is normal (the *nanos* results are shown in Fig. 3I-J). The loss of maternal *Hsp83* RNA from the posterior of embryos lacking posterior polar plasm suggests that it is a component of the polar plasm, and that it is protected from degradation by other components of the polar plasm, possibly the polar granules themselves.

Further evidence in support of this possibility comes from an analysis of the effects of mislocalization of the *oskar* RNA on the distribution pattern of maternal *Hsp83* RNA.

Mislocalization of *oskar* RNA to the anterior pole of the early embryo via the *bicoid* 3'UTR has been shown to result in the induction of germ cells anteriorly, a process dependent on the *vasa* and *tudor* gene products (15). We examined the dynamics of maternal *Hsp83* RNA degradation and protection in *oskar-bicoid 3'UTR* embryos. Maternal *Hsp83* RNA is uniformly distributed in early *oskar-bicoid 3'UTR* embryos (Fig. 4A); however, it is protected from degradation at *both* poles between nuclear cycles 6 and 8 (Fig. 4B). While protection of maternal *Hsp83* RNA is now bipolar, the spatial pattern of anterior-protected *Hsp83* RNA resembles that of *bicoid* RNA (Fig. 4B), a result consistent with the fact that ectopic anterior *oskar* RNA is distributed in a *bicoid*-like pattern (15). As at the posterior pole, this maternal *Hsp83* RNA is taken up into the ectopic anterior pole cells; however, a significantly higher concentration of *Hsp83* RNA remains in the anterior somatic cells underlying the ectopic pole cells than in the posterior somatic cells (Fig. 4C, D).

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. 5A). The anterior determinant, *bicoid*, a homeodomain protein present in the early embryo in an anterior to posterior gradient (12, 13), 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. 5B). This suggests that the *Hsp83* gene is transcriptionally activated in response to *bicoid*. Zygotic expression of *Hsp83* is not activated at the anterior of embryos that have *oskar* RNA mislocalized anteriorly (Fig. 4C), consistent with the expected absence of *bicoid* protein from these embryos (15).

Several gene products have been shown to localize the *bicoid* RNA to the anterior pole of the oocyte and early embryo (1, 58). 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 (1, 13, 58). In such embryos, there is a marked posterior shift in the boundary of *Hsp83* expression (Fig. 5C), consistent with the conclusion that anterior zygotic *Hsp83* expression is dependent on the *bicoid* protein.

bicoid is known to directly regulate zygotic expression of the *hunchback* gene in the anterior half of the embryo (13). 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. In addition, we have shown that mutations in *torso*, *buttonhead*, *empty spiracles*, *orthodenticle* and *giant*, all of which function in programing head development and/or the development of the termini (reviewed in 7, 16), are not required for activation of zygotic *Hsp83* transcription in the anterior third of the embryo (data not shown).

Since there is normal temporal and spatial activation of a germline transformed *Hsp83-lacZ* fusion gene (*c83Z.-880*) in the anterior third of the embryo (Fig. 6), *cis*-regulatory sequences sufficient for *bicoid* activation of zygotic *Hsp83* transcription map to a 2.7 kb region that includes 880 base pairs of DNA 5' to the *Hsp83* transcription initiation site, its first exon and its intron fused to the *E. coli lacZ* gene (65). Detailed *in vivo* and *in vitro* analyses of sequences required for *Hsp83* activation by *bicoid* are in progress and will be reported elsewhere (S.R.H. and H.D.L., unpublished).

DISCUSSION

While it has been known for some time that the *Hsp83* gene is expressed during normal development in the absence of heat shock (33, 65, 66), we have shown that transcription of *Hsp83* is dynamically regulated during oogenesis and embryogenesis. Maternal *Hsp83* RNA is localized to the posterior pole of the early embryo by a novel mechanism involving a combination of generalized RNA degradation and local protection by components of the posterior polar plasm. Zygotic transcription of *Hsp83* is restricted to the anterior third of the embryo and is absolutely dependent on *bicoid*, the anterior determinant. Here we discuss our results, their implications for understanding the mechanisms of RNA localization, and the possible developmental functions of *Hsp83*.

A Novel Mechanism Is Used To Localize Maternal *Hsp83* RNA To The Posterior Pole Of The Early Embryo. It was hypothesized previously that RNAs could be localized in cells by a mechanism involving a combination of generalized degradation and local protection (19). However, to date there have been no examples of such a mechanism. Here we have shown that 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 by components of the posterior polar plasm. High levels of *Hsp83* RNA are distributed throughout the mature oocyte. An hour after fertilization, it is degraded rapidly throughout the embryo except at the posterior pole. Degradation of *Hsp83* RNA occurs rapidly and uniformly in the anterior half of the embryo but it is polarized in the posterior half of the embryo, resulting in production of a transient antero-posterior gradient of *Hsp83* RNA. Posterior localization of maternal *Hsp83* RNA thus represents the first example of a degradation-protection mechanism for RNA localization.

Posterior Protection Of Maternal *Hsp83* RNA Is Accomplished By Components Of The Posterior Polar Plasm. We examined the effects on posterior protection of *Hsp83* RNA, of seven loss-of-function mutations that disrupt the posterior

polar plasm, polar granules and the ability to form germ cells. All of these result in degradation of *Hsp83* RNA throughout the embryo without any posterior protection. Reciprocally, ectopic anterior localization of *oskar* RNA, which results in anterior assembly of components of the posterior polar plasm and formation of germ cells at the anterior pole (15), results in ectopic anterior protection of maternal *Hsp83* RNA and its uptake into the ectopic germ cells. *nanos* and *pumilio*, which disrupt components of the posterior polar plasm required for abdominal development but not for germ cell formation, have no effect on posterior protection of *Hsp83* RNA. Taken together, these results show that protection of *Hsp83* RNA is accomplished by components of the posterior polar plasm that function in polar granule formation and in germ cell specification. It remains to be determined what gene products are involved in the degradation of the *Hsp83* RNA throughout the remainder of the embryo.

***Hsp83* And The Germline.** Not only is *Hsp83* RNA a component of the the posterior polar plasm, but it is present in the pole cells and their germline progeny throughout embryogenesis (this study), larval development (65, 66) and in the adult (with two exceptions - see below) (65, 66, this study). Interestingly, the mouse *Hsp86* gene, a close relative of *Drosophila Hsp83* (25, 52), is expressed in germ cells (8, 20, 38) as might be a human *Hsp85* gene (34), suggesting that members of the *Hsp90* gene family might serve similar functions in germ cells of diverse animal species (33).

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. 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 (31, 60), suggesting that the choice between oocyte and nurse cell fate is initiated in region 1 of the germarium (31, 60). 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 (51). If *Hsp83* protein is absent from the germline during these stages, our results would suggest that *Hsp83* must be eliminated in order to enable these cell fate decisions to occur. 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 role of the *Hsp83* gene in the germline as well as in other tissues.

Zygotic *Hsp83* Transcription Is Regulated By The Anterior Determinant, *bicoid*. We have shown that zygotic activation of the *Hsp83* gene occurs in the anterior third of the early embryo and is absolutely dependent on *bicoid*, the anterior determinant. Absence of functional *bicoid* protein eliminates zygotic activation of *Hsp83* in the anterior third of the embryo. In contrast, extension of the *bicoid* protein distribution more posteriorly than in wildtype embryos, results in an expanded domain of zygotic *Hsp83* activation. None of an additional six genes that control anterior and/or terminal development (*torso*, *hunchback*, *giant*, *empty spiracles*, *orthodenticle*, *buttonhead*) are required for anterior activation of *Hsp83* transcription, suggesting that the *Hsp83* gene might be activated directly by the *bicoid* homeodomain protein. *Hsp83* is the first example of a gene encoding a cytoplasmically active regulatory protein that is spatially activated in response to the anterior genetic hierarchy in *Drosophila*.

Functional Significance Of Dynamic *Hsp83* Expression During Development. 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 44). 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 important 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. Two potential partner proteins have been shown to be generally expressed throughout most of the early embryo (29, 32, 62). One is the ecdysone receptor (*EcR*), which is present throughout the embryo (32). 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 (29, 62), raising the possibility that *Hsp83* regulates *src29A* activity in the anterior third of the embryo. Dynamic spatial and temporal regulation of *Hsp83* might, thus, 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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FIGURES

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 begins 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.

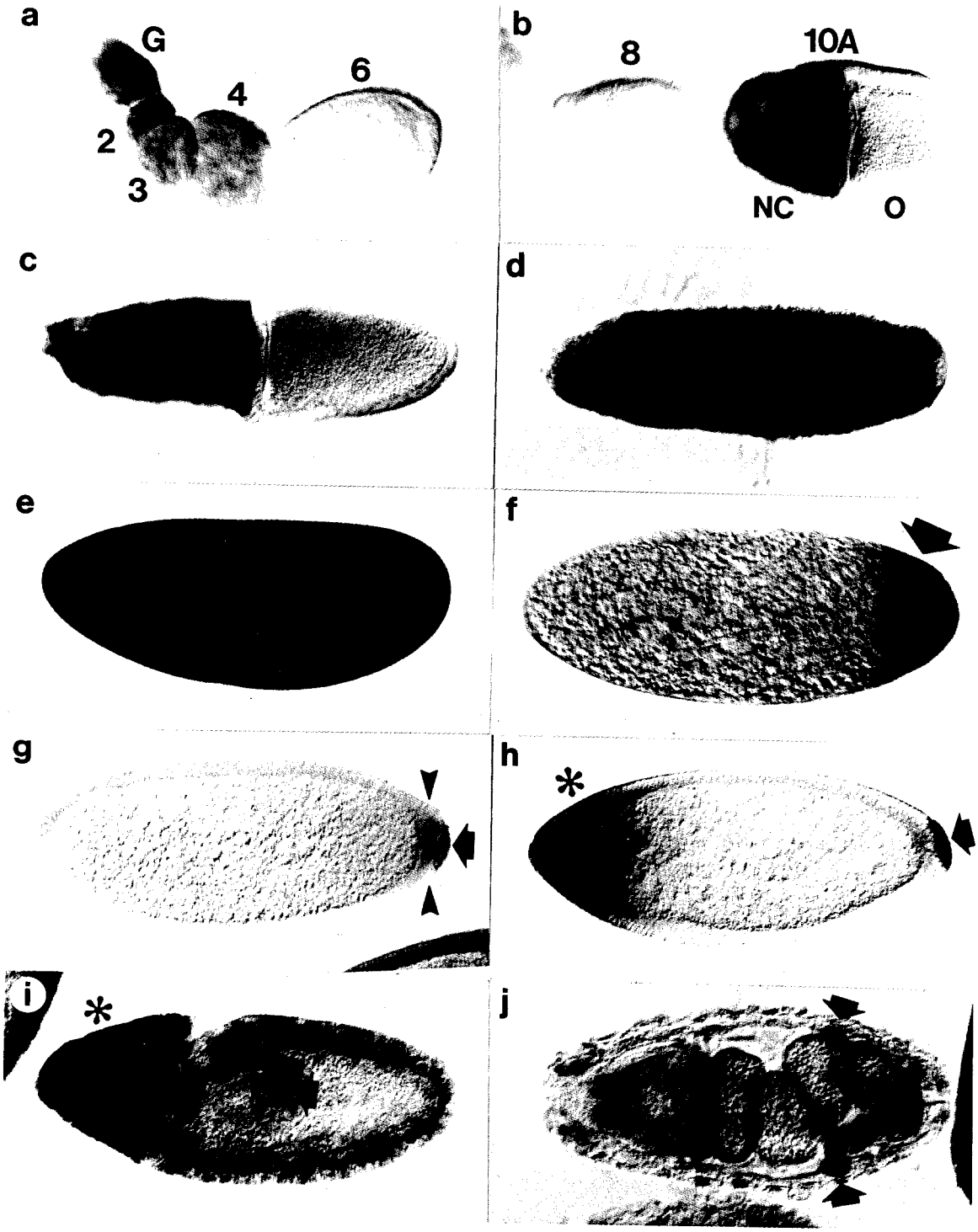
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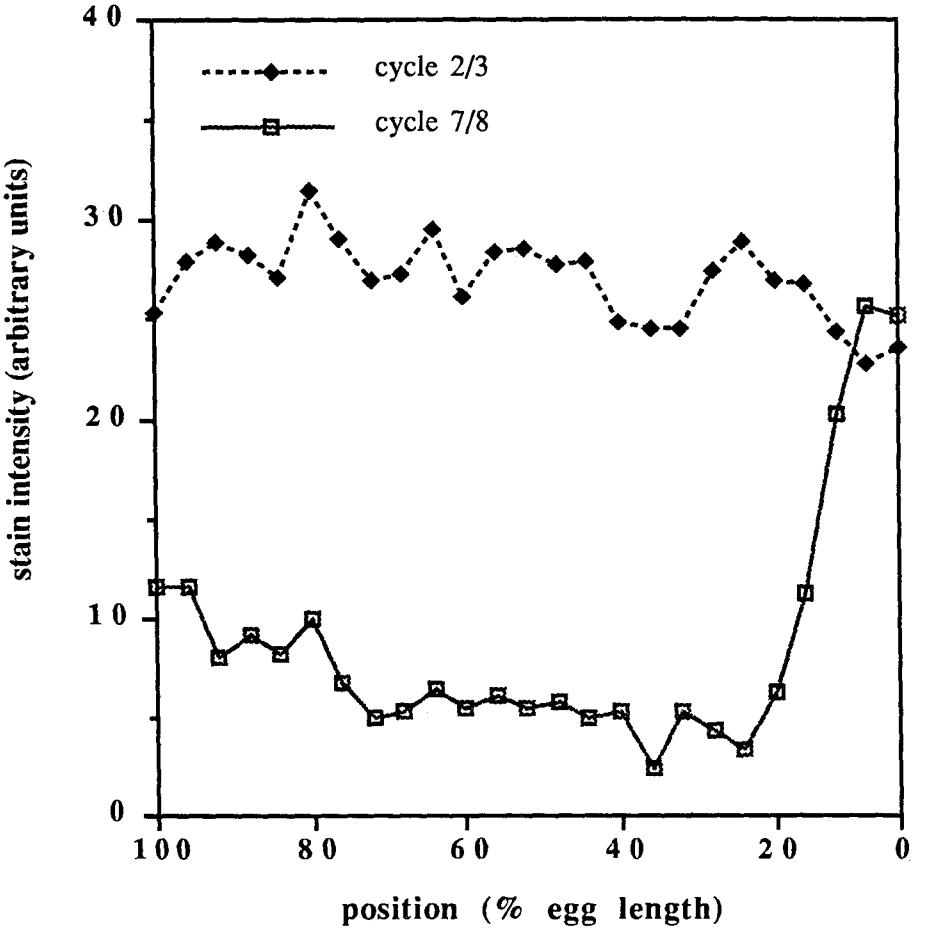
FIG. 2. Quantitative analysis of the distribution of *Hsp83* RNA in wildtype embryos. The distribution of *Hsp83* transcripts is shown at nuclear division cycle 2/3 (dotted line) and at cycle 7/8 (solid line). While the amount of *Hsp83* RNA at the posterior pole (0-5% egg length) remains constant, the *Hsp83* RNA throughout the remainder of the embryo is degraded. Each point represents the mean value obtained from five embryos; standard deviations varied between 1.5 and 12.0 units. See Materials and Methods for details about the methods used in the production of these plots.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cycle 2/3
cycle 7/8
stain intensity (arbitrary units)
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60
40
20
0
position (% egg length)

FIG. 3. Distribution of *Hsp 83* RNA in embryos produced by females homozygous for representative mutations that eliminate components of the posterior polar plasm and disrupt the integrity of the polar granules and the ability to form pole cells (A - F), or that eliminate components of the polar plasm without affecting the integrity of the polar granules or the ability to form pole cells (G - H). These are *spire* (A) and (B); *oskar* (C) and (D); *tudor* (E) and (F); and *nanos* (G) and (H). Maternal *Hsp83* RNA is distributed normally in the early cleavage stage embryos in all mutants (A, C, E, G). 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). In mutants in which the polar granules are present, (e.g. *nanos*) protection of maternal *Hsp83* RNA still occurs (arrow) (H). Note that, in all cases, the anterior zygotic expression of *Hsp83* is normal at this stage (asterisks). The apparent lower level of anterior RNA in *nanos* (H) is a consequence of slight under-development of the *in situ* staining reaction. In addition to the anterior expression, zygotic *Hsp83* transcripts can be detected at 15-25% egg length in the embryos produced by *cappuccino* mothers (data not shown). For all embryos, anterior is to the left and dorsal is up.

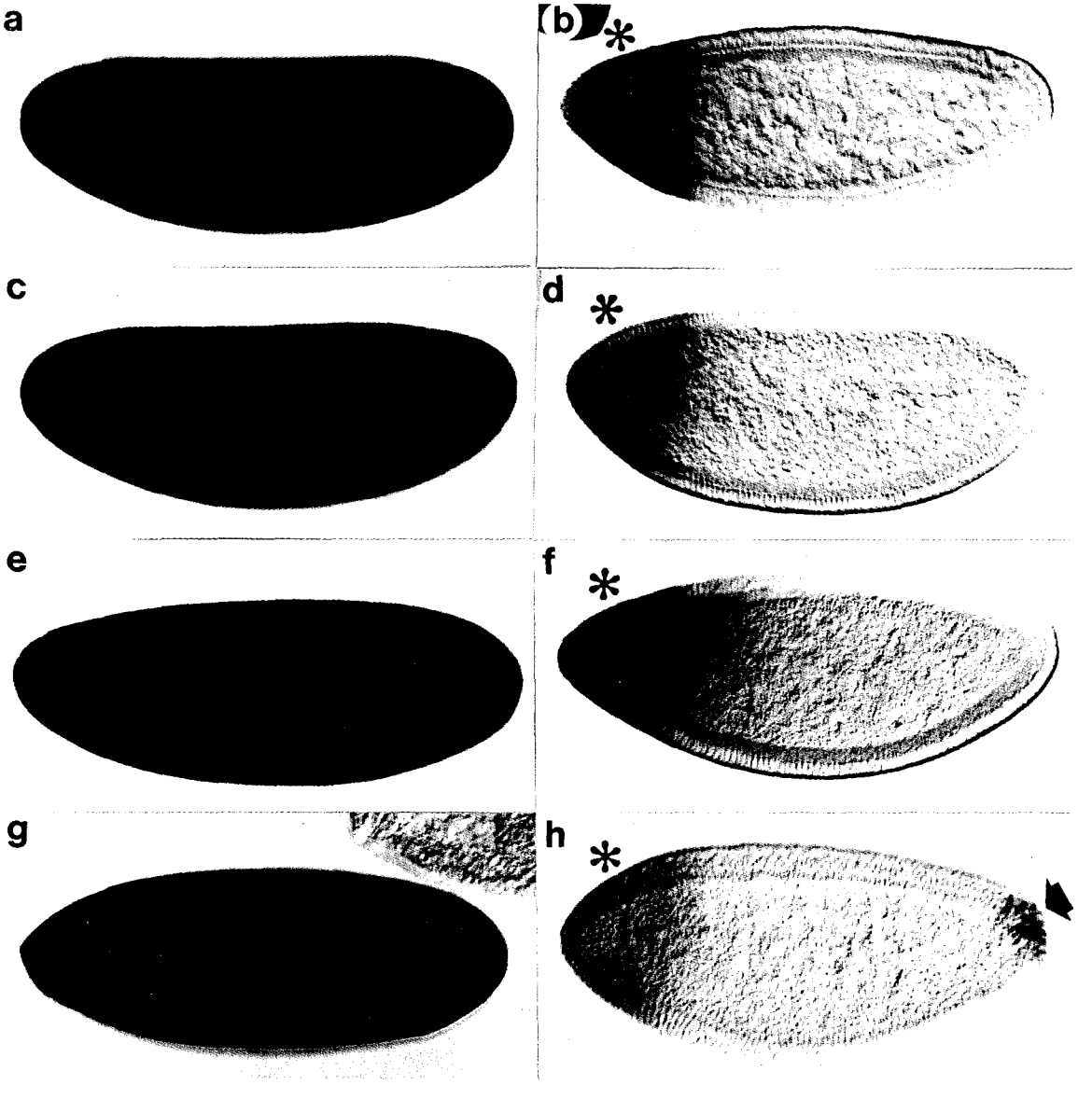
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FIG. 4. Mislocalization of *oskar* RNA to the anterior embryonic pole results in anterior protection of maternal *Hsp83* RNA. (A) Early cleavage stage embryo exhibiting a high, uniform concentration of maternal *Hsp83* RNA (*cf.* Fig. 1E). (B) Nuclear cycle 7 embryo in which *Hsp83* RNA is now protected at both the anterior (arrowhead) and the posterior (arrow) poles (*cf.* Fig. 1F). Note that the anterior protection pattern differs from that at the posterior pole and resembles that of *bicoid* RNA; this is because the mislocalized *oskar-bicoid3'UTR* RNA is localized in a *bicoid*-like pattern (15). (C) Cellular blastoderm stage embryo showing pole cells containing *Hsp83* RNA at both poles (*cf.* Fig. 1H). In addition, there is substantially more ectopically protected *Hsp83* RNA in the underlying anterior somatic cells than the posterior ones. Note that there is no zygotic expression of *Hsp83* in the anterior of the embryo (*cf.* Fig. 1H). (D) Gastrulating embryo showing *Hsp83* RNA in the normal and ectpoic pole cells, as well as persistent *Hsp83* RNA in the underlying anterior somatic cells. Orientation of the embryos is as in Fig. 1.

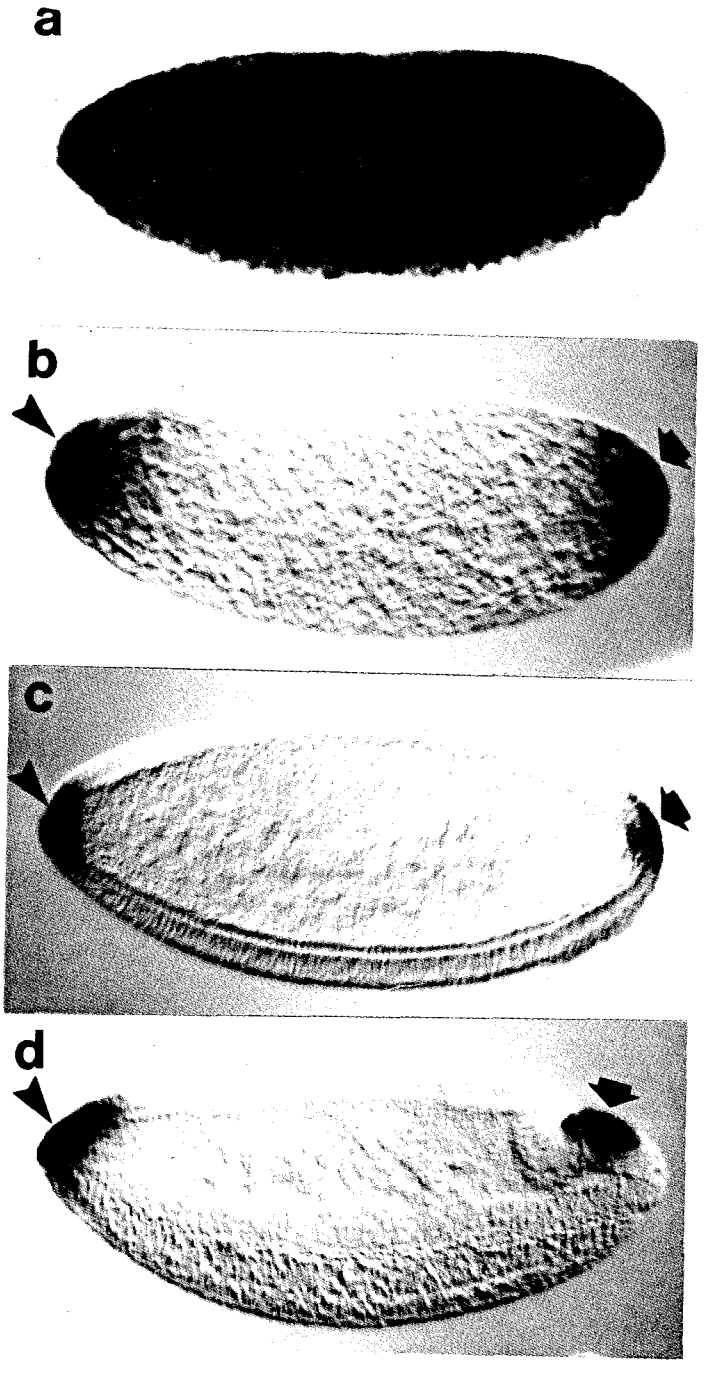
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FIG. 5. Regulation of zygotic *Hsp83* transcription by *bicoid*. (A) A cellular blastoderm stage embryo that is homozygous for a deficiency that removes the *Hsp83* gene [*Df(3L)HR370*]. The maternal *Hsp83* RNA can be seen in the pole cells (arrow), but anterior zygotic expression is absent (cf. Fig. 1H). 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).

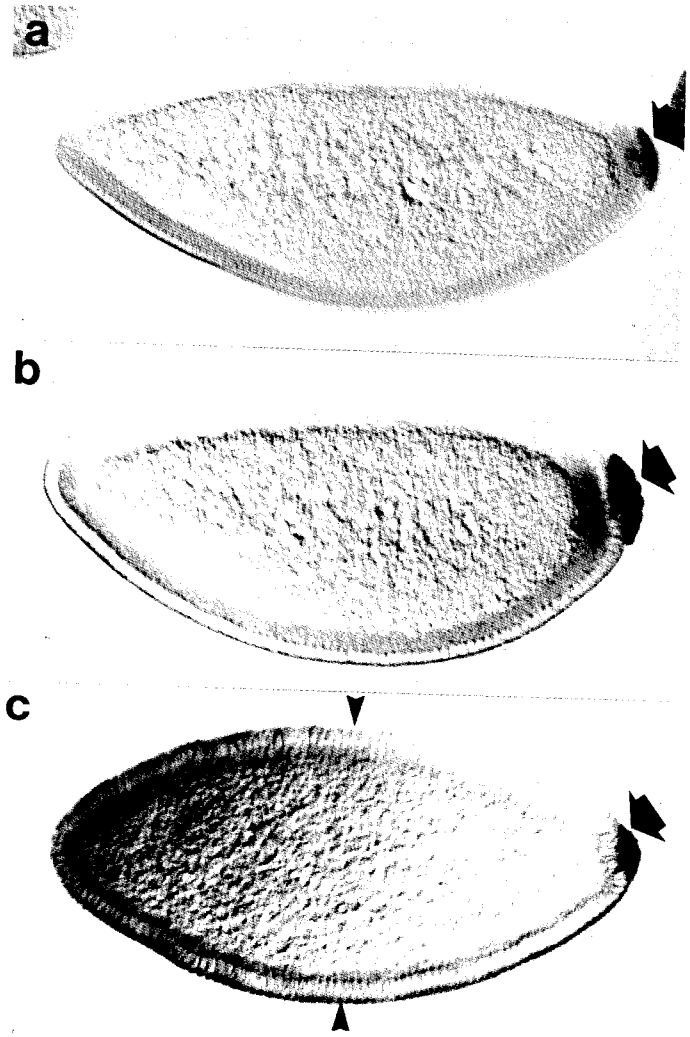
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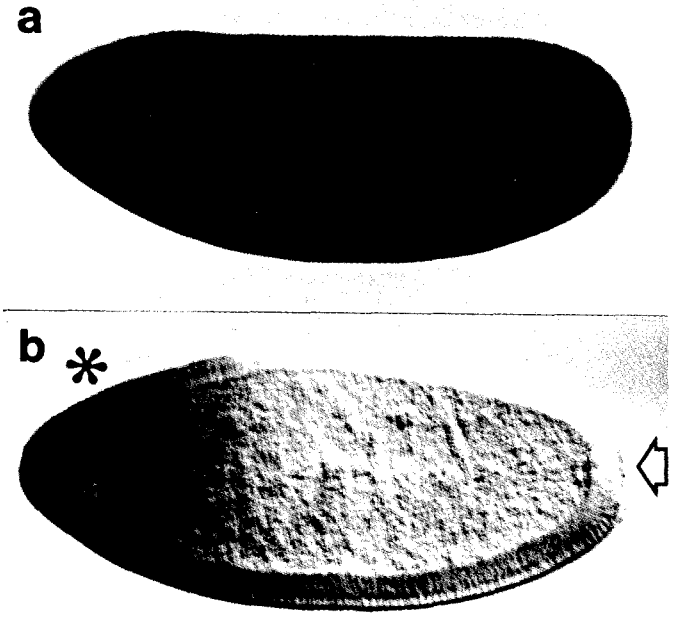
FIG. 6. Localization and expression of *Hsp83-lacZ* fusion transcripts. The embryos were derived from a female germline transformed with *c83Z.-880* (65), a construct that carries 880 bp of sequence 5' to the *Hsp83* transcription start site, the first exon and the intron, fused 300 base pairs downstream of the translation start codon to the *E. coli lacZ* gene. The *Hsp83-lacZ* fusion transcripts were visualized using ß-galactosidase probe. (A) Early cleavage stage embryo. The fusion RNA is uniformly distributed at a high concentration throughout the embryo. (B) Syncytial blastoderm embryo, exhibiting fusion transcripts in the anterior (asterisk) but no posteriorly protected maternal RNA (unfilled arrow) (*cf.* Fig. 1G). Thus the construct contains sequences sufficient for *bicoid*-dependent zygotic activation, but lacks sequences that tag the *Hsp83* RNA for posterior protection. Orientation is as in Fig. 1.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CHAPTER 3

This chapter will be submitted for publication in

Molecular and Cellular Biology.

Cis-acting Sequences Program *Hsp83* RNA Distribution in the Early *Drosophila* Embryo: Maternal Transcript Localization to the Posterior Pole by a Novel Mechanism and BICOID-Activated Zygotic Transcription in the Anterior

Susan R. Halsell, William W. Fisher and Howard D. Lipshitz

ABSTRACT

The *Hsp83* gene encodes the sole *Drosophila* homolog of the mammalian *Hsp90* family of molecular chaperones. Genetic analysis of *Hsp83* indicates that it modulates the function of receptor tyrosine kinases during embryogenesis (23) and eye development (16). Previously, we showed that *Hsp83* is expressed in a dynamic fashion during *Drosophila* embryogenesis (22). Maternally transcribed *Hsp83* is localized to the posterior pole of the early embryo by a novel mechanism of generalized degradation and localized protection. This RNA is taken up into the primordial germ cells (pole cells) when they bud, and *Hsp83* RNA is present in the pole cells throughout embryogenesis. At late stage 4/early stage 5 of embryogenesis, BICOID controls the expression of *Hsp83* in the anterior third of the embryo. We have utilized *lacZ*-tagged transgenes to map the *cis*-regulatory elements required for the posterior protection of the maternal transcripts, for zygotic expression in the anterior of the embryo and to determine when zygotic *Hsp83* transcription commences in the germline. Sequences within the 3'UTR are sufficient to protect the maternal mRNA from degradation at the posterior pole through interactions with components of the posterior polar plasm. Sequences mapping within the intron are necessary for zygotic expression in response to the BICOID homeodomain protein; *in vitro*, BICOID binds to a sequence located in this region. Thus, *Hsp83* is likely to be a direct downstream target of BICOID. A later, independent phase of expression in the anterior occurs. Zygotic transcription of *Hsp83* in the germline commences at stage 11 of embryogenesis (5 to 7 hours after fertilization). These studies form the basis for a detailed molecular genetic analysis of *Hsp83* functions during early embryogenesis.

INTRODUCTION

Embryogenesis requires the extensive interplay of regulated gene expression and signal transduction cascades. An understanding of the cytoplasmic regulation of these processes is emerging (reviewed in 84). The mammalian *Hsp90* family of molecular chaperones have been implicated in regulating the activity of many cytoplasmic molecules, including steroid hormone receptors, *src* family tyrosine kinases, eIF-2α, protein kinase C, casein kinases, actin and tubulin (reviewed in 67). Recent studies show that *Hsp90* function is required for activation of steroid hormone receptors (6, 81), $pp60^{v\text{-}src}$ (118) and *raf* (113). Mutational analysis of *Hsp83*, the sole *Drosophila* homolog of *Hsp90*(5, 40), indicates that *Hsp83* functions in receptor tyrosine kinase signal transduction cascades initiated by *sevenless* during eye development (16) and by *torso* during embryogenesis (23), L. Jiang, A. Bashirullah and H.D. Lipshitz, in preparation).

In addition to stress-induced up-regulation of *Hsp83* transcription, *Hsp83* is expressed at high levels under non-stressed conditions during *Drosophila* development (22, 117, 120). The oogenic and embryonic expression of *Hsp83* is highly dynamic and occurs in many different cell types, including the germline progenitors known as pole cells (22). *Hsp90* homologs are also expressed in the germline of mice (14, 39, 63) and possibly humans (58). A recent report shows that in *C. elegans* maternally transcribed RNAs homologous to *Hsp90* are present in the early embryo; these are detected throughout embryogenesis and are enriched in the cells that give rise to the germline (88). These observations suggest that expression of *Hsp90* during gametogenesis and embryogenesis is well-conserved. *Drosophila* provides a unique opportunity to assay the function of *Hsp83* during oogenesis and embryogenesis because, unlike other organisms, it is present as a single copy gene (5, 40), mutations have been isolated (16, 89), and the oogenic and embryonic expression pattern has been examined (this report, 22).

Our interest in *Hsp83* expression originated from the molecular isolation and identification of *Hsp83* as encoding a posterior localized maternal RNA (21). Maternally transcribed *Hsp83* RNA provides an opportunity to examine a novel mechanism of RNA localization, accomplished by a combination of generalized degradation and localized protection of the transcripts at the posterior pole of the embryo. A number of RNAs are localized to the posterior pole of the *Drosophila* oocyte and embryo (reviewed in 42). One of the first RNAs localized to the posterior pole of the oocyte is encoded by *oskar* (28, 55). *oskar* function is critical for organizing the posterior polar plasm of the oocyte and embryo (28, 29, 55, 64, 91). The posterior plasm is a morphologically distinct region of the cytoplasm (48) which includes organelles known as polar granules (72-75). Functional posterior polar plasm is required for the formation of the pole cells and for abdominal patterning (34, 36, 49-51, 64, 66, 80). Besides *oskar*, additional maternal-effect loci required for the assembly of the posterior polar plasm include *cappuccino*, *spire*, *vasa*, *tudor*, *valois* and *staufen* (7, 76, 87). Mutations in any of these genes eliminate the posterior localization of maternal *Hsp83* RNA (22) as well as the localization of *nanos* RNA (110), which encodes the posterior morphogen required for abdominal pattern formation (66, 111) and the localization of the presumptive germ cell determinants (reviewed in 42).

At the anterior of the embryo, the homeodomain protein, BICOID, functions as the morphogen required for head and thoracic pattern formation (reviewed in 94). Its function is mediated, in part, by the activation of *hunchback* (24-26, 86, 97, 100), *orthodenticle* (31) and *empty spiracles* (19, 109) transcription and the repression of *Krüppel* transcription (45) in the anterior of the early embryo. BICOID binding sites in the *hunchback* (25, 27) and *Krüppel* (45) promoters have been identified. *Hsp83* expression in the anterior of the early embryo also requires BICOID function (22). In addition to BICOID, *torso* and *hunchback* function are required for proper regulation of transcription in the anterior. Studies show that *torso* mediates a retraction in the BICOID-activated

transcription of *hunchback* and *orthodenticle* in the anterior terminus of the embryo (26, 31, 99) and that HUNCHBACK acts synergistically with BICOID to activate transcription of *lacZ* transgenes and *orthodenticle*, *empty spiracles* and *buttonhead* (90). The head gap genes, *orthodenticle* (31, 32, 115), *empty spiracles* (19, 53, 109), *buttonhead* (115, 116) and *sloppy paired* (37, 38) encode transcription factors and are expressed and function in overlapping domains in the anterior. Therefore, it has been proposed that these genes act in a combinatorial manner to direct head pattern formation (13, 37).

Here, we examine the regulation of expression and localization of *Hsp83* RNA in the early *Drosophila* embryo. We have utilized *lacZ* tagged reporter constructs and germline transformation to map the *cis*-regulatory sequences required for protection of the maternal RNA at the posterior pole and zygotic activation of expression in the anterior of the embryo. Our results indicate that redundant sequences within the 3'UTR of *Hsp83* RNA are sufficient to mediate protection of the maternal transcripts from degradation at the posterior pole of the embryo. In addition, our results suggest that there are sequences within the 3'UTR that are necessary for stabilizing the RNA in the oocyte. Zygotic expression of *Hsp83* in the pole cells is first detected in stage 11 embryos. We have also examined the regulation of zygotic expression in the anterior of the embryo. We have mapped the *cis*-acting region through which BICOID activates the transcription of *Hsp83* in the anterior and show that BICOID can bind this region *in vitro*. Finally, analysis of the *Hsp83-lacZ* fusion construct expression patterns reveals that there is a later, separable phase of *Hsp83* expression in the anterior of the embryo.

MATERIALS AND METHODS

Whole Mount RNA *In Situ* Hybridization

RNA *in situ* hybridization of whole mount ovaries and embryos was performed as described previously, with minor modifications (22, 101). Ovaries were dissected from

well-fed adult females in PBS and were immediately fixed for 25 minutes in 10% formaldehyde (EM grade, Ted Pella Inc.), 50 mM EGTA, 10% DMSO in PBS. After fixation, the ovaries were washed with PTW (PBS + 0.1% Tween 20) and then gently rubbed between 2 microscope slides in order to break apart the ovarioles and devitellinize and dechorionate the late stage oocytes. The fixed ovaries were stored in PTW. Embryos were collected, dechorionated for 2 minutes in 50% bleach, washed extensively with H_20 and fixed for 20 minutes in 1:1 heptane:fixative (10% formaldehyde, 50 mM EGTA in PBS). Fixed embryos were devitellinized by removing the fixative and adding 2 volumes of methanol, shaking for 2 minutes and removing the devitellinized embryos at the bottom of the methanol phase. The embryos were washed extensively with methanol. Post-fixation, proteinase K digestion and refixation were essentially as described previously (101), except, in the case of the embryos, a 3 hour incubation in xylene preceded the first post-fixation. Digoxigenin-labeled antisense RNA probes were made using the Megascript RNA transcription kit (Ambion Inc.) according to the manufacturer's instructions and included digoxigenin-labeled UTP (Boehringer Mannheim). The RNA probes were subjected to limited alkaline hydrolysis by incubation in an equal volume of 100 mM $NaCO_3$ pH 10.2 for 1 hour at 60°C followed by ethanol precipitation. Pre-hybridization and hybridization were carried out at 55°C and detection of the probe was as previously described (101). Ovaries and embryos were mounted either in JB4 plastic mountant (Polysciences) or in 90% glycerol in PBS.

Construction Of *Hsp83-lacZ* Transgenes For Germline Transformation

A PCR based subcloning method was employed to make *lacZ* tagged transgenes with 5'-*Hsp83* sequences and/or 3'UTR sequences. For the 5'-*Hsp83* constructs, primers were designed to contain either a Not I restriction site or a Bgl II restriction site (Table 1). For the *Hsp83* 3'UTR constructs, primer pairs with either Aat II and Hind III restriction

sites or Hind III and Kpn I restriction sites were designed (Table 2). Standard protocols were used for all subsequent subcloning steps and CsCl plasmid purification (85).

In all cases, the PCR conditions for generating the subcloned fragment were as follows. 100 µl reactions were set up with 10 pg of linearized template (see below), 50 µm each of dATP, dCTP, dGTP and dTTP, 50 pmoles of each primer, 40 mM KCl, 1.5 mM $MgCl_2$, 60 mM Tris pH 8.5 and 2.5 units of AmpliTaq polymerase (Perkin Elmer). For some primer pairs, 6 mM $MgCl_2$ was used. Amplification was carried out in a thermocycler under the following conditions: 1 cycle at 94°C (2 minutes), 55°C (1.5 minutes and 72°C (1.5 minutes) followed by 25 cycles at 94°C (0.5 minutes), 55 °C (1.5 minutes) and 72°C (1.5 minutes). Following amplification, the PCR products were phenol extracted, restriction digested and phenol extracted again. The fragments were then ligated into the appropriate CaSpeR based, P-element transformation vectors (see below).

The 5'-*Hsp83-lacZ* constructs were made as follows. The template for the PCR reactions was either pB83 or pBint. pB83 is a *Hsp83* genomic/cDNA chimera subcloned into Bluescript and was made by inserting a 5' genomic BamH I-Sac 1 fragment isolated from clone 301.1 (47) next to a 3' cDNA Sac I-Apa I fragment (21). pB83 includes 879 bp of 5' upstream sequence, the 5'UTR (exon 1), the intron, the complete ORF (exon 2) and the 3'UTR of *Hsp83*. pBint was made as follows. First, a full-length cDNA clone, isolated from a 0-4 hour embryonic cDNA library (10), was digested with BsiW I (which cuts within exon 1) and EcoR V (which cuts within exon 2), and the fragment was gel purified. Next, clone -879.1564 (a genomic BamH I-Hind II fragment subcloned in Bluescript) was digested with BsiW I and Hind II. The fragment containing the vector and *Hsp83* sequence between BamH I and BsiW I was gel purified and ligated to the BsiW I-EcoR V cDNA fragment. Thus, pBint is a *Hsp83* genomic/cDNA chimera that lacks the intron but includes 879 bp of 5' upstream sequence, the 5'UTR and the first 288 codons of the ORF.

Once the 5' *Hsp83* fragments were amplified and restricted, they were subcloned into Not I-Bgl II digested CaSpeR vectors, either cßg2 (in the cases in which the *Hsp83* fragment included the endogenous promoter) or chs43.1 (in the cases in which the fragment lacked a promoter). cßg2 was derived from CaSpeR ßgal (104) and has a newly introduced BamH I-Not I-Bgl II polylinker. The polylinker was inserted by ligating annealed oligos (cßg2.5': 5'-AGGATCCGCGGCCGCAGATCT-3' and cßg2.3': 5'-GATCAGATCTGCGGCCGCGGATCCTTGCA-3') into CaSpeR ßgal that had been restricted with Pst I and BamH I. To generate chs43.1, a Bgl II cloning site was introduced downstream of the Not I cloning site in CaSpeR hs43 ßgal (105) by ligating annealed oligos (hs43.1.5': 5'-GATCCAAGCTTAGATCTC-3' and hs43.1.3': 5'-TCGAGAGATCTAAGCTTG-3') into the vector after it had been digested with BamH I and Xho I. These fusion constructs contain the full length ßgal gene. After subcloning, the *Hsp83-lacZ* plasmids were isolated by CsCl purification.

The following approach was taken to generate a series of *lacZ-Hsp83* 3'UTR constructs which include the 5'-*Hsp83* enhancer sequences and promoter. Previous reports (56) suggested that a full length *lacZ* gene might not be completely transcribed *in vivo*; if so, the prematurely terminated transcripts would not contain the *Hsp83* 3'UTR. Therefore, we inserted the *Hsp83* 3'UTR fragments downstream of a truncated *lacZ* tag. We accomplished this by constructing pB83Z, a Bluescript subclone that contains all of the *Hsp83* 5' upstream region, the first exon, the intron, and the first 111 codons of the open reading frame fused to 603 bp of *lacZ* sequence. At the 3' end of the *lacZ* sequence are Aat II, Hind III and Kpn I cloning sites for inserting the *Hsp83* 3'UTR fragments. pB83Z was constructed as follows. The unique Aat II cloning site was introduced into Bluescript by ligating annealed oligos (Aat II.5': 5'-GATCCAACCCGGGTAGACGTCATCTGCA-3' and Aat II.3': 5'-GATGACGTCTACCCGGGTTG-3') to BamH I-Pst I digested Bluescript. The cßg2 *lacZ* tagged clone, $^{-}$873.$^{+}$1621, was digested with Not I and Aat II. The Not I-Aat II fragment containing the *Hsp83* and *lacZ* sequences was subcloned into

Not I-Aat II digested Bluescript to generate pB83Z. Individual *Hsp83* 3'UTR PCR fragments were subcloned into appropriately digested pB83Z.

Using PCR, 5' fragments of the 3'UTR were amplified that were flanked with Aat II and Hind III restriction sites while 3' fragments of the 3'UTR were flanked with Hind III and Kpn I restriction sites (Table 2). The template for the PCR reaction was Eco9 (provided by T. Cutforth and G. Rubin), an 8 kb EcoR I fragment subcloned in Bluescript that contains the full length *Hsp83* transcription unit. The position of the Hind III restriction site within the amplified fragments allowed us to generate 5', 3' and internal deletions within the 3'UTR. For the 5' fragments of the 3'UTR that lack the *Hsp83* poly-adenylation site, the *Hsp70* 3'UTR was subcloned just downstream of the *Hsp83* 3'UTR sequence as a Hind III-Kpn I fragment (the "hs" series). The *Hsp70* 3'UTR was also cloned independent of any *Hsp83* 3'UTR sequence. The *Hsp70* fragment was generated by PCR from a CaSpeR-hs template (105) using the primers 70.5': 5'-GCCGGCAAGCTTCTAAGGCCAAAGAGTC-3' and 70.3': 5'-CGGACGGGTACCTGCAAGTTGACTTTAA-3'. 3'UTR constructs with internal deletions (the "Δ" series) were generated by first subcloning an Aat II-Hind III 5' fragment into pB83Z and subsequently adding a Hind III-Kpn I 3' fragment. Effectively, the internal *Hsp83* sequences are replaced with a 6 bp Hind III site. After subcloning into pB83Z, a Not I-Kpn I fragment was isolated and subcloned into Not I-Kpn I digested CaSpeR 4 (105). The plasmids were isolated by CsCl purification.

Germline Transformation

Germline transformation was carried out using standard procedures (83). w^{1118} embryos were coinjected with 500 μg/ml of the *Hsp83-lacZ* construct and 100 μg/ml pHSπ helper plasmid (96) . All lines were homozygosed or balanced over *CyO* or *TM3*.

Sequence Analysis

BestFit and FindPatterns were used to search the *Hsp83* sequence for repeated sequence elements, and MFold (121) was used to predict the secondary structure of the *Hsp83* 3'UTR. These programs are part of the University of Wisconsin GCG Package, Version 7.2, run at the Caltech Biology Division Sequence Analysis Facility)

Mobility Gel Shift Assay

Crude bacterial extracts were prepared as previously described (46, 108), from *E.coli* BL21(DE3) transformed with pAR Bcd HD ori (provided by C. Desplan), a T7 expression vector that encodes the BICOID homeodomain. After induction (98), the bacteria were pelleted and resuspended in 1/400 volume Buffer Z + 4 M guanidine-HCl (Buffer Z: 100 mM KCl, 25 mM Hepes pH 7.8, 1 mM DTT, 20% glycerol, 0.1% Triton X-100, 1 mM PMSF, 0.1 mM benzamidine, 1 mM EDTA). The cells were digested with 0.5 mg/ml lysozyme for 30 minutes at 4°C, sonicated and then centrifuged. The supernatant was dialyzed against Buffer Z + 1 M guanidine-HCl, then against Buffer Z alone.

The oligonucleotides used in the assay were synthesized with 5' CTAG overhangs and purified using Poly-Pak columns (Glen Research). The annealed double-stranded oligonucleotides were made blunt by end-filling with Klenow DNA polymerase in the presence of dATP, dCTP, dGTP and dTTP. Probe was generated by substituting [α-^{32}P]-dATP for dATP. The synthesized oligonucleotide pairs included a putative BICOID consensus binding site within the *Hsp83* gene (83bcd5.5': 5'-CTAGTGTAAATCCAA-3' and 83bcd5.3': 5'-CTAGTTGGATTTACA-3'), a high affinity BICOID binding site (bcd.5': 5'-CTAGTCTAATCCCA-3' and bcd.3': 5'-CTAGTGGGATTAGA-3') (25, 27) and a mutated BICOID site which no longer binds Bicoid *in vitro* (mutant bcd.5': 5'-

CTAGTCTAATTGCA-3' and mutant bcd.3': 5'-CTAGTGCAATTAGA-3') (N. Dostatni, pers. comm.).

Binding reactions were performed as follows. 20 µl reactions containing appropriately diluted bacterial extract in 10 mM Tris pH 7.5, 5% glycerol, 37.5 mM KCl, 4 mM spermine, 4 mM spermidine, 0.1% NP-40 and 50 µg/ml salmon sperm DNA were incubated on ice for 10 minutes prior to the addition of 0.15 ng (50,000 cpm) of labeled DNA in the presence or absence of cold competitor DNA. The reactions were incubated for an additional 10 minutes at room temperature. The entire sample was loaded onto an 8% acrylamide/bis-acrylamide (29:1) gel buffered in 0.25 X TBE and was run at 15 V/cm for 1 hour and 15 minutes. After drying, the gel was exposed to a PhosphorImager Screen.

Fly Stocks

Oregon-R and w^{1118} stocks are described in (68). *bicoid* (*bcd*) mutant embryos were collected from homozygous bcd^{E1} (35) mothers. The dosage of wild-type *bicoid* was varied by collecting embryos derived from mothers that were bcd^{E1}/*TM3* (1X bcd^{+}), Oregon-R (2X *bcd*), or heterozygous (4X bcd^{+}) or homozygous (6X bcd^{+}) for BB9 +16 (97), which carries two cloned copies of the $bicoid^{+}$ gene on the second chromosome in addition to the wild-type $bicoid^{+}$ gene on the third chromosome. Embryos deficient for zygotic *hunchback* (*hb*)were obtained by crossing *Df(3R)*hb^{PTX15} pp *e/TM3* parents (65). Embryos homozygous mutant for the zygotic head genes *buttonhead* (*btd*), *orthodenticle* (*otd*) or *empty spiracles* (*ems*) were obtained from stocks with btd^{X681} (115) or otd^{YH} (115) in trans to a *FM7, ftz-lacZ* balancer chromosome (54) or with ems^{7D99} (53) in trans to a *TM3, ftz-lacZ* balancer chromosome (prepared by S. Smolik and obtained from E.B. Lewis). The presence of the *ftz-lacZ* reporter construct on the balancer chromosome allowed the unequivocal identification of homozygous mutant embryos when whole mount RNA *in situs* included a *lacZ* probe. In order to test the effect of these mutations on the expression of the *Hsp83 -lacZ* head stripe, embryos were collected from a cross between

males bearing a homozygous -873.-155 transgene on the second chromosome and either btd^{X681}/*FM7, ftz-lacZ* or otd^{YH}/*FM7, ftz-lacZ* virgin females. In the case of ems^{7D99}, embryos were collected from a w^{1118}; -873.-155; ems^{7D99}/*TM3, ftz-lacZ* stock.

RESULTS

Distribution Of *Hsp83* RNA During Early *DROSOPHILA* Embryogenesis

Drosophila embryogenesis is subdivided into 17 stages and takes 20 hours to complete at 25°C (11). The first 13 nuclear divisions of embryogenesis occur within a syncytium, and these nuclei migrate to the periphery of the embryo, thus forming the syncytial blastoderm (stages 1-4, 0-2 hours 50 minutes). We have shown previously that *Hsp83* RNA is expressed in a highly dynamic manner during both *Drosophila* oogenesis and embryogenesis (22). We have also shown that *Hsp83* RNA present in the early embryo is contributed maternally and zygotically.

Maternal *Hsp83* RNA is localized to the posterior pole of the *Drosophila* embryo by a unique mechanism of generalized degradation and localized protection (22). For approximately the first 45 minutes of embryogenesis (nuclear cycles 1-5), maternal *Hsp83* is uniformly distributed (Fig. 1A). *Hsp83* RNA represents approximately 1% of the poly(A) RNA in the embryo at this stage (120). Then, over the next 20 minutes (nuclear cycles 6-8), the maternal transcripts are rapidly degraded throughout the embryo except at the posterior pole (Fig. 1B). Protection of maternal *Hsp83* transcripts from degradation at the posterior pole depends upon the function of *cappuccino*, *spire*, *staufen*, *spire*, *oskar*, *vasa*, *tudor* and *valois* (22), genes that are also required for the formation of the posterior polar plasm, polar granule integrity and pole cell formation (42, 61). The posteriorly protected maternal *Hsp83* RNA is taken up into the pole cells upon their formation (Fig 1C). *Hsp83* RNA is detected in the germ line cells throughout the remainder of

embryogenesis and in the gonads of larvae and adults (22, 117, 120, L. Jiang, A. Bashirullah, and H.D. Lipshitz, unpublished results).

A carefully timed series of whole mount *in situs* reveals that zygotic expression of *Hsp83* initiates after the nuclei migrate to the periphery at the posterior pole of the embryo (stage 3, 1 hour 5 minutes-1 hour 20 minutes) (Fig. 1C). These nuclei bud as the first cells of the embryo and form the germ line progenitors, known as pole cells. *Hsp83* RNA is distributed throughout stage 3 and early stage 4 embryos, but this RNA pattern then disappears in later stage 4 embryos (1 hour 20 minutes-2 hours 10 minutes) (Fig. 1D). A second pattern of zygotic transcription of *Hsp83* is then detected in the anterior third (69-100% EL, where 100% represents the anterior) of late stage 4/early stage 5 embryos (2 hours 10 minutes-2 hours 50 minutes) just as 6000 somatic cells begin to form simultaneously (Fig. 1E). This anterior expression persists during gastrulation and germband extension (stages 6-9, 2 hours 50 minutes-4 hours 20 minutes), but *Hsp83* RNA is no longer detected in the anteriormost region of the embryo at stage 10 (4 hours 20 minutes-5 hours 20 minutes) (Fig. 1G). We have examined the mechanisms by which the maternal RNA is restricted to the posterior pole and by which *Hsp83* is expressed zygotically in the anterior of the early embryo.

Cis-Acting Sequences Within the *Hsp83* 3'UTR Confer Protection Of Maternal Transcripts From Degradation At The Posterior Pole Of The Early Embryo

We have mapped the *cis*-acting sequences that confer protection of the maternal *Hsp83* transcripts at the posterior pole of the early embryo. Previous studies have shown that sequences downstream of position +1621 (codon 111 in the ORF) are necessary for the protection of the maternally encoded *Hsp83* RNA at the posterior pole (22). In order to map the sequences that are sufficient for this protection *in vivo*, *lacZ-Hsp83* 3'UTR transgenes have been made and introduced into flies by P-element mediated germ line

transformation (Fig. 2). The transgenes include 5'-*Hsp83* enhancer sequences tagged with *lacZ* and are followed by either a full length or truncated *Hsp83* 3'UTR. Whole mount RNA *in situ* hybridizations were performed with *lacZ* probes on at least 2 independent lines for each construct. Stage 5 embryos were scored, using the anterior zygotic expression of the transgene as an internal control for quantifying the *lacZ* RNA levels within the pole cells relative to the zygotic levels (Fig. 3A cf. Fig. 1E).

In all cases but one, embryos bearing *lacZ -Hsp83* 3'UTR constructs exhibit high levels of maternal RNA in the early embryo (Fig. 4E). The exception is the 351.715 construct in which maternally transcribed RNA is never detected in the embryo (Fig. 4F). The 351.715 transgene only differs from the other constructs in that it lacks the first 350 nt of the 3'UTR. Maternal *Hsp83* transcripts present in the embryo are first transcribed during stage 9 of oogenesis, approximately 16 hours prior to the onset of embryogenesis (22, 57). Whole mount RNA *in situs* on ovaries dissected from females bearing the 1.407 and 351.715 constructs were performed. In both cases, high levels of *lacZ* RNA are transcribed in the nurse cells of stage 9 and 10 follicles (Fig 4A, B), and this RNA is transported into the oocyte at stages 10-11. Presumably, 351.715 lacks 3'UTR sequence(s) that are necessary for stabilizing the transcript during the remainder of oogenesis.

Embryos with constructs that include the full length *Hsp83* 3'UTR (1.407 and 1.715) have wildtype levels of maternal transcripts within the pole cells (Fig. 2, 3A). The hs control construct, which replaces the *Hsp83* 3'UTR with the *Hsp70* 3'UTR, shows no pole cell staining (Fig. 2, 3B). Thus, the *Hsp83* 3'UTR is sufficient for conferring protection from degradation on the maternally transcribed RNA.

In order to map which sequences within the 3'UTR are necessary for this protection, a series of 5', 3' and internal deletions within the 3'UTR have been examined (Fig. 2). The 3'UTR 5' deletion constructs actually extend into the genomic DNA, 308 bp downstream of the end of the RNA (+715), thus ensuring that the transcripts are properly

processed at the 3' end. Deletions in the 3' deletion series (the "hs" series) remove the *Hsp83* polyadenylation site, therefore, the *Hsp70* 3'UTR was added to insure the proper termination and polyadenylation of the transcripts. This analysis shows that there is redundancy within the *Hsp83* 3'UTR in conferring the protection. When the 3' deletion series is examined, it is clear that 1.350hs, 1.300hs and 1.252 hs possess sequence(s) that confer protection of the *Hsp83* transcript at wildtype levels (Fig. 2). 1.200hs possesses a minimal sequence that is sufficient for protecting wildtype levels of RNA in the pole cells (Fig. 2, 3C). In the 5' deletion series, 253.715 also shows wildtype levels of RNA at the posterior (Fig. 2, 3D). Thus, there are redundant elements within the *Hsp83* 3'UTR, such that the first 200 nt and the final 155 nt of the *Hsp83* 3'UTR are each sufficient for conferring protection of the maternal RNA. Redundant localization elements have also been identified within the 3'UTRs of other posterior localized RNAs, including *oskar* (56) and *orb* (60).

Further truncations in the first 200 nt of the 3'UTR sequence do not completely abolish the protection of the transcript at the posterior. When embryos bear the 1.50hs transgene, RNA is still detected in the pole cells, although the levels are greatly reduced as compared to the zygotic staining seen within the same embryo (Fig. 2). Collections of embryos bearing the 1.100hs and 1.150hs constructs can be subdivided into two subsets based on their staining patterns in the pole cells relative to the intensity of staining in the anterior: (i) apparently wildtype levels of RNA in the pole cells or (ii) at greatly reduced levels (Figs. 2, 3E, F). This bimodal staining pattern is seen amongst embryos derived from individual lines of such constructs and is seen consistently in all lines examined. Similar results are seen in the 5' deletion construct, 301.715 (Fig. 2). Therefore, these constructs bear elements that confer protection but their number and/or spacing within the RNA is sufficiently altered so that the association of the RNA with components of the posterior polar plasm is eliminated in some embryos. The reduced levels of RNA in the pole cells of these embryos may represent adventitious uptake of residual maternal RNA or

perhaps reflect a low level of zygotic transcription in the pole cells. Zygotic transcription, however, is unlikely to contribute significantly to levels of RNA seen in the pole cells at this stage (see below) (22).

A series of internal deletion constructs, deleting as few as 50 nt and as many as 200 nt within the 3'UTR, have also been examined (Fig. 2). The internal deletions within the 3'UTR generally do not affect the ability of the transcript to be protected at the posterior pole (Fig. 2). This is consistent with each internal deletion construct bearing either the first 200 nt or final 154 nt of the 3'UTR, either of which is sufficient for protecting the transcript at wildtype levels. However, anomalous results are seen for three of the twelve constructs. Embryos bearing constructs 252Δ351 and 50Δ253 exhibit a bimodal staining pattern (Fig. 2), despite the presence of sequences that, in isolation, protect the RNA at wildtype levels. In the case of 200Δ351, all embryos display a reduced staining pattern within the pole cells, thus the ability to protect wildtype levels of the transcripts has been lost.

Computer sequence analysis using the BestFit program reveals no obvious repeated sequence motifs within the *Hsp83* 3'UTR. The secondary structure of the *Hsp83* 3'UTR was predicted using the MFold program (121). Unlike the *bicoid* RNA 3'UTR, which has an extensive predicted secondary structure of many long, overlapping stems (30, 69, 71), no extensive secondary structure is predicted within the *Hsp83* 3'UTR. Instead, a series of many separate small stems is predicted. This is consistent with the *in vivo* data which shows that the *Hsp83* 3'UTR can be subdivided into separate elements without disrupting its protection function. Therefore, the components of the posterior polar plasm may recognize an overall organization of sequence elements, possibly within the context of the secondary structure, of the *Hsp83* 3'UTR.

Zygotic Transcription Of *Hsp83* In The Pole Cells Initiates In Stage 11 Embryos

Hsp83 RNA is detected in the pole cells and gonad of the embryo (Fig. 1) (22) and in the gonad of larvae and adults (117, 120). In the early embryo (stages 1-2), *Hsp83* RNA is clearly maternally supplied (Fig. 1A, B). High levels of RNA persist throughout embryogenesis within the pole cells. Previously, we deleted the *Hsp83* gene and showed that zygotic transcription does not significantly contribute to the pool of RNA detected in the pole cells of stages 3-5 embryos (22). In order to determine if and when zygotic transcription occurs in the pole cells of the embryo, wildtype virgin females were crossed with males bearing the 1.715 construct. Whole mount RNA *in situs* were performed with *lacZ* probes; thus the only transcripts detected are transcribed from the paternally supplied transgene. Zygotically transcribed RNA in the pole cells is first detected at very low levels in stage 11 embryos (Fig. 5A, B). This stage corresponds to the period in which pole cells leave the posterior midgut pocket and begin their migration to the gonad (44, 62, 92, 112). Low levels of the RNA are detected in stages 12-14, and high levels of zygotically transcribed RNA are detected in the pole cells beginning at stage 15. The RNA is detected throughout the remainder of embryogenesis (Fig. 5C). These experiments do not address whether or not the maternal RNA is degraded during this period. However, high levels of endogenous *Hsp83* RNA are detected in stage 11-17 embryos (Fig. 1G cf. 5A), therefore, it is probable that maternally transcribed transcripts are stable at least through Stage 14.

BICOID Controls the Anterior Expression of *Hsp83* in Early Embryos

Hsp83 is expressed zygotically in the anterior third of late stage 4 embryos (Fig. 1E), and this domain of expression falls within the region of the embryo that is under the control of the anterior morphogen, BICOID. *bicoid* encodes a homeodomain protein (3, 33). Its RNA is maternally localized to the anterior of the oocyte, and this localization

persists in the early embryo (3, 33). BICOID is translated from the localized RNA source, diffuses away from the source and establishes a concentration gradient (24, 26). BICOID transcriptionally activates the zygotic expression of several genes in the anterior of the early embryo, including *hunchback* (24, 86, 97, 99), *orthodenticle* (31) and *empty spiracles* (19, 109). Previously, we showed that elimination of *bicoid* function results in a failure to activate the expression of *Hsp83* in the anterior of the embryo (Fig. 6A) (22).

We extended this result in the following experiments. First, it has been shown that the spatial domain in which the *hunchback* gene is zygotically expressed is directly dependent upon the concentration of BICOID (24, 97). By altering the number copies of functional *bicoid* genes that the embryo carries, the posterior boundary of *hunchback* expression is shifted. When the copy number of the *bicoid* gene is varied from one to six, the expression domain of *Hsp83* is also shifted (Fig. 6). Wildtype embryos carrying the two endogenous copies of *bicoid*$^+$ express *Hsp83* from 69-100% EL (Fig. 6C). The posterior boundary of *Hsp83* expression shifts to the anterior (81% EL, Fig. 6B) when only a single dose of *bicoid*$^+$ is present, and shifts more posteriorly when the *bicoid*$^+$ dosage is increased to four copies (67% EL) or six copies (60% EL, Fig. 6D). Thus, BICOID, either directly or indirectly, activates the transcription of *Hsp83* in the anterior of late stage 4/early stage 5 embryos.

Since BICOID activates the zygotic transcription of *hunchback* (25), a zinc finger transcription factor (100), it is possible that zygotic HUNCHBACK, rather than BICOID, directly activates the transcription of *Hsp83*. *Hunchback* is expressed zygotically in the anterior of the embryo during the period in which *Hsp83* is expressed (100). We tested this possibility by examining the distribution of *Hsp83* RNA in embryos that lack zygotically transcribed *hunchback*. Anterior expression of *Hsp83* is not affected in embryos that lack zygotically encoded *hunchback* (Fig. 6E). *hunchback* is also expressed maternally, and its protein distribution in the early embryo is similar to that seen for the zygotically encoded protein (65, 99, 100) This experiment does not address whether

maternal *hunchback* function affects *Hsp83* expression. Nevertheless, these results suggest that the activation of the anterior expression of *Hsp83* by BICOID may be direct. This is confirmed by the following *in vivo* expression and *in vitro* binding experiments.

Identification of Cis-Acting Sequences Which Regulate Expression of *Hsp83* in the Anterior Third of Late Syncytial Blastoderm Embryos

A series of 5'-*Hsp83-lacZ* fusion constructs were made in order to map the *cis*-regulatory sequences that are required for zygotic expression of the *Hsp83* gene (Fig. 7). After germline transformation, whole mount RNA *in situ* hybridizations were performed on independent lines for each construct. For ease of reading, embryos will be referred to simply by the name of the 5'-*Hsp83-lacZ* construct they bear. The first number in the construct name refers to the 5' end of the *Hsp83* fragment being tested and the second number refers to the 3' end of the fragment.

Examination of the expression pattern of the 5'-*Hsp83-lacZ* transgenes reveals that the BICOID- dependent *Hsp83* expression pattern in the anterior third of the embryo can be reproduced by these transgenes. Constructs $^{-}$873.$^{+}$1621 and $^{-}$873.$^{+}$1295 are sufficient for expression of the transgene in the anterior third of stage 5 embryos (Fig. 7, 8A, C). Constructs $^{-}$171.$^{+}$1621, $^{-}$89.$^{+}$1621, $^{-}$66.$^{+}$1621 and $^{+}$18.$^{+}$1621 have 5' deletions in the *Hsp83* sequence, and all direct *lacZ* expression in the anterior third (Fig. 7). When the intron sequence is deleted in the pBint construct, anterior expression is eliminated (Fig. 7, 8D). Expression in the anterior third of the embryo is also eliminated in the $^{-}$873.$^{+}$1009 construct (Fig. 7, 8E). All of the other constructs tested fail to express *lacZ* RNA in the anterior third of the embryo (Fig. 7). Thus, sequence(s) between $^{+}$1010 and $^{+}$1288 are necessary for the expression of *Hsp83* in the anterior third of early embryos.

Since expression of the endogenous *Hsp83* gene is dependent upon BICOID, we examined these *Hsp83* sequences for possible BICOID binding sites. By comparing previously identified Bicoid binding sites identified in the *hunchback* (25, 27) and *Krüppel*

(45) promoters, six potential BICOID binding sites were identified in *Hsp83* (Fig. 9) between positions -879 and +1621 (designated as 83Bcd1 through 83Bcd6) (Fig. 7, 9). These sites represent low or moderate affinity BICOID binding sites (27). Transgenes bearing only low affinity sites require a higher concentration of BICOID for their transcriptional activation (86, 100). Consistent with this finding, *Hsp83* is expressed only in the anterior third of the embryo where the concentration of BICOID is higher (Fig. 1E, 6C).

Based on the *in vivo* analysis of the 5'-*Hsp83-lacZ* transgene expression patterns, a single BICOID binding site, 83Bcd5, appears to be the site necessary for the transcriptional activation by BICOID. Despite the presence of 3 BICOID binding sites upstream of the transcription start (83Bcd1 through 83Bcd3) and 1 site within exon 2 (83Bcd6), the pBint transgene is not expressed in the anterior third of the embryo (Fig. 8D). However, constructs, such as +18.+1621, which lack the 83Bcd1 through 83Bcd3 sites, are still expressed in the anterior pattern. Thus, the 83Bcd4 through 83Bcd6 sites are sufficient to confer this expression pattern. -873.+1295 lacks the 83Bcd6 site, and it is expressed in the anterior (Fig. 8C). Elimination of 83Bcd5, positioned between +1273 and +1282, in construct -873.+1009 completely eliminates expression in the anterior third of the embryo. Thus the fragment bearing 83Bcd5 is necessary for the BICOID-activated transcription. Since this site has not been tested singly in any of the constructs, it is possible that this site alone is not sufficient for the BICOID-induced activation, but may act synergistically with the other binding sites. The *bicoid* requirement for expression of *Hsp83* and the necessity of the 83Bcd5 site suggests that BICOID may bind directly to the *Hsp83* gene and transcriptionally activate *Hsp83* expression.

BICOID Binds *Hsp83* Sequences *In Vitro*

We confirmed that BICOID protein can bind to 83Bcd5 DNA by performing an *in vitro* mobility gel shift assay (Fig. 10). Bacterially expressed BICOID homeodomain was

allowed to react with a radioactively labeled double stranded oligonucleotide that corresponds to the potential *Hsp83* Bicoid binding site, 83Bcd5. Under these binding conditions, BICOID binds to the *Hsp83* sequence (Fig. 10). The binding of BICOID to *Hsp83* is specific, as shown by performing the binding reaction in the presence of unlabeled double-stranded oligonucleotide competitors. The specific competitor used was a high affinity BICOID binding site (25, 27). The nonspecific competitor used has a single nucleotide change in the high affinity site which renders it incapable of binding BICOID (N. Dostatni and C. Desplan, pers. comm.). A 64-fold molar excess of the specific competitor is sufficient to titrate the BICOID protein and eliminate the shifted 83Bcd5/BICOID complex (Fig. 10). On the other hand, the nonspecific competitor does not completely abolish the formation of the 83Bcd5/BICOID complex until it is present in 1024-fold molar excess (Fig. 10). Taken together, the genetic and *in vitro* protein/DNA binding results suggest that BICOID activation of *Hsp83* expression is direct.

Expression of the 5'-*Hsp83-lacZ* Transgenes Reproduce Many but Not All Aspects of the Wildtype *Hsp83* Expression Pattern

Although constructs have been identified that direct expression of the *lacZ* reporter construct in the anterior third of the early embryo, they do not perfectly reproduce the endogenous expression pattern of *Hsp83*. *Hsp83* is first expressed anteriorly in late stage 4 embryos (Fig. 1E). The RNA is detectable throughout the anterior of the embryo until Stage 10, when the RNA is no longer detected in the anterior-most region of the embryo (Fig. 1G). By contrast, 5'-*Hsp83-lacZ* fusion RNA levels start to decrease in the anteriormost region of the $^{-}$873.$^{+}$1621 embryos during stage 6 (Fig. 8B). This suggests that additional enhancer sequences are missing in these constructs or that there are differences in the stability of wildtype *Hsp83* RNA and the *Hsp83-lacZ* fusion RNA. Since the 5' neighboring transcription unit is only 880 bp upstream of the *Hsp83* transcription site (Fig. 2A) (5), it is possible that *Hsp83* enhancer sequences lie in the

genomic DNA downstream of *Hsp83*. Therefore, an additional *Hsp83-lacZ* fusion construct, 1.T2, was made and tested. 1.T2 differs from construct -873.+1621 in that the SV40 terminator sequence present in -873.+1621 has been replaced with a fragment that includes the full length *Hsp83* 3'UTR (beginning at position 1) and extending 950 bp into the 63B-T2 transcription unit (79). 1.T2 directs expression in the anterior third of the embryo, and as in the case of constructs such as -873.+1621, a slight reduction in the anteriormost region of stage 6 embryos is observed (data not shown). It is possible that additional enhancer sequences lie between +1621 and the 3'UTR, but this seems unlikely since this sequence represents the ORF. Therefore, the premature resolution of the anterior expression in these embryos probably does not reflect a lack of *Hsp83* enhancer sequences but differences in the stability of the endogenous *Hsp83* and 5'-*Hsp83-lacZ* fusion transcripts.

There Is a Later, Separable Phase of *Hsp83* Expression in the Anterior of Early Embryos

Analysis of the *Hsp83-lacZ* fusion constructs possessing 5' upstream sequences reveals that there is a second phase of anterior expression that commences in cellular blastoderm embryos. This expression occurs independent of the earlier expression, and *cis*-regulatory sequences mediating this expression can be separated from the sequences necessary for the earlier expression. In stage 5 embryos, this expression pattern manifests itself as a stripe of RNA expression between 70 and 84% EL dorsally and 77 and 90% EL ventrally (Fig. 11). -873.-155 is not expressed in the anterior third of late stage 4 embryos (Fig. 11A) but is expressed as a stripe in stage 5 embryos (Fig. 11B). Constructs -873.-628 and -664.-415 are each sufficient to confer this head stripe expression (Fig. 11C, D). Thus, the *cis*-regulatory sequences sufficient for this expression map either as redundant elements within the two non-overlapping sequences of 245 bp and 249 bp respectively or map to the 14 bp sequence that is shared by the two constructs.

Presumably, this second phase of anterior expression is not apparent in the case of the endogenous *Hsp83* RNA because of the persistence of the *Hsp83* RNA transcribed throughout the anterior during the syncytial blastoderm stage.

In addition to maternally encoded BICOID, several transcription factors, including BUTTONHEAD, the *Drosophila* Sp1 homolog (116), EMPTY SPIRACLES, a homeodomain protein (19, 109) and ORTHODENTICLE, a homeodomain protein (31, 32) are transcribed zygotically in the anterior of late stage 4 embryos . By stage 5, these genes are expressed in a series of overlapping, circumferential rings. *buttonnhead* is expressed between 65% and 77% EL (116), *empty spiracles* is expressed between 69% and 75% EL dorsally and 73% and 88% EL ventrally (19), and *orthodenticle* is expressed between 70% and 90% EL (31, 32). Because the expression domains of these genes overlap the domain of expression directed by the 5'-*Hsp83-lacZ* transgenes, we tested the effects of mutations in each of these genes on the expression of the endogenous *Hsp83* gene. Mutations in each of these genes have no effect on the expression of *Hsp83* (data not shown). However, if the earlier BICOID-activated *Hsp83* expression is stable, then its persistence might mask any effects that mutations in these genes have on the stage 5-specific *Hsp83* expression.

In order to circumvent this possible masking effect, whole mount RNA *in situ* hybridizations were performed with a *lacZ* probe on mutant embryos that bore the -873.-155 transgene because this construct does not exhibit the earlier expression throughout the anterior. The mutant alleles were balanced over chromosomes marked with a *fushi tarazu-lacZ* (*ftz-lacZ*) transgene. This allowed unequivocal identification of the homozygous mutant embryos because they do not express the seven stripe *ftz* pattern. No alterations are seen in the expression of the head stripe in -873.-155 embryos singly mutant for each of the amorphic alleles, btd^{X681}, ems^{7D99} or otd^{YH} (data not shown).

DISCUSSION

Hsp83 RNA is expressed in a highly dynamic manner during *Drosophila* oogenesis and embryogenesis under non-stressed conditions (22). Previously, it was shown that a sequence required for the stress inducible expression of *Hsp83*, the heat shock consensus sequence, lies in the 5' upstream region of the gene (117). In this report, we have identified *cis*-regulatory sequences that are required for the localized protection of maternally transcribed *Hsp83* RNA at the posterior pole of the early embryo and for the zygotic transcription of the *Hsp83* gene in the anterior third of syncytial and cellular blastoderm embryos under non-stressed conditions (Fig. 12). The 3'UTR of *Hsp83* RNA is sufficient for the protection of maternal transcripts from degradation. There are redundant elements within the 3'UTR, mapping to the first 200 or the last 155 nt of the 3'UTR, which are each sufficient to confer this protection (Fig. 12). Zygotic transcription occurs in the anterior third of late stage 4/early stage 5 embryos (22). This expression is under the control of BICOID (22, this report). *Cis*-regulatory sequences sufficient and necessary for this expression reside within the intron (Fig. 12). In addition, sequence(s) within the 5' upstream region between -873 and -415 have been identified that are sufficient to induce expression of an anterior stripe of expression in stage 5 embryos (Fig. 12).

Localization Of Maternal *Hsp83* Transcripts By Localized Protection From Degradation

As with other RNAs that are localized to the posterior pole of the *Drosophila* oocyte and embryo, including *Cyclin B*, *germ cell-less*, *orb*, *oskar* and *nanos*, the *Hsp83* 3'UTR is required for localization of the RNA to the posterior pole (reviewed in 42, 93). *Hsp83* RNA localization, however, differs temporally and mechanistically from these RNAs. First, *Hsp83* RNA is not localized to the posterior pole until early embryogenesis, while the other posteriorly localized RNAs become localized during oogenesis. Second, the

localization mechanisms differ significantly. *oskar* RNA appears to localize to the posterior pole of stage 9 oocytes by a directed transport, possibly along microtubules (12, 102, 103). *Cyclin B*, *germ cell-less* and *nanos* RNAs are localized to the posterior pole of post-stage 10 oocytes (17, 18, 52, 110, 111), possibly by interacting with binding sites at the posterior pole that are established by *oskar* function (93). *Hsp83* RNA, on the other hand, is localized to the posterior pole by a mechanism of generalized degradation and localized protection. The temporal and mechanistic differences in *Hsp83* RNA localization may reflect different requirements for the *Hsp83* function in the embryo as opposed to the other RNAs. For example, NANOS is required for abdominal patterning in the embryo (64, 66), and it is critical that NANOS be localized to the appropriate site of action at the posterior (reviewed in 15, 20, 42). Since *Hsp83* is a cytoplasmic regulatory molecular chaperone, it may initially be required throughout the early embryo to regulate proteins that function during this period. The unlocalized *Hsp83* RNA may be translated throughout the early embryo. However, in older embryos, spatially restricted expression of *Hsp83* may limit its functions to specific cells, such as the pole cells.

Integrity of the posterior polar plasm and the presence of the polar granules is a requirement for the protection of the maternal *Hsp83* transcripts at the posterior (22) Presumably, the *Hsp83* 3'UTR interacts with components of the posterior polar plasm. The 5' and 3' portions of the *Hsp83* 3'UTR are sufficient for protection at the posterior pole. Computer sequence analysis using the BestFit program reveals no obvious repeated sequence motifs within the *Hsp83* 3'UTR. The lack of repeated sequences within the redundant elements is similar to the lack of obvious repeated sequence motifs within the redundant localization elements of the *oskar* 3'UTR (56). *bicoid* RNA is also localized during oogenesis, but it differs mechanistically from *oskar* RNA localization. Localization of *bicoid* RNA to the anterior of the oocyte is a multi-step process that depends on a 53 nt sequence element, BLE1, and the overall secondary structure of the 3'UTR (30, 69-71). 630 nt of the *bicoid* 3'UTR is absolutely required for the localization of *bicoid* RNA to the

anterior of the oocyte. This region of the *bicoid* RNA is predicted to fold into an extensive, multi-stemmed secondary structure. Alterations in 3 of the predicted stems eliminates the retention of *bicoid* RNA in the anterior cytoplasm of the early embryo (30)

The *Hsp83* 3'UTR is only 407 nt in length. This, coupled with the fact that either the 5' 200 nt and 3' 155 nt of the 3'UTR are capable of conferring protection, makes it unlikely that a single, extensive secondary structure would be present. In fact, sequence analysis using the MFold program predicts that a series of small stems form in the *Hsp83* 3'UTR. Therefore, this prediction is consistent with the *in vivo* results observed with the *lacZ-Hsp83* 3'UTR transgenes. Embryos bearing the 252Δ351, 200Δ351 and 50Δ253 fusion constructs do not show the expected wildtype protection of the fusion transcripts. This result, coupled with the predicted secondary structure, suggests that an appropriate combination of sequence elements and secondary structure may be required for interaction of the *Hsp83* 3'UTR with the posterior polar plasm. These internal deletions may disrupt this balance. This type of interaction has been suggested for *oskar* RNA, which contains redundant localization elements but exhibits neither obvious repeated sequence elements nor extensive secondary structure (56).

Maternal *Hsp83* transcripts are degraded everywhere except the posterior pole, and *Hsp83* RNA transcribed in stage 3 embryos is apparently degraded in stage 4 embryos, less than an hour after their transcription. This is in marked contrast to *Hsp83* RNA transcribed maternally, whose accumulated levels are stable for approximately 23 hours (16 hours during oogenesis, then in the pole cells until at least stage 11, 7 hours post-fertilization). Zygotically encoded RNA in the anterior, whose accumulated levels remain high for at least 2.5 hours. This suggests that *Hsp83* transcripts may be inherently stable but that there is a mechanism in place in stage 2-4 embryos for rapidly degrading *Hsp83* transcripts.

Generally, RNAs are thought to degrade in a deadenylation-dependent process (reviewed in (2). RNAs such as c-*fos* can be rapidly degraded, and this degradation has

been linked to the presence of an AU rich element (the ARE) within the 3'UTR which promotes the rapid shortening of the poly(A) tail (2). Computer sequence homology searches of the *Hsp83* 3'UTR do not reveal the presence of such sequence elements. However, the lack of maternally transcribed RNA in the 351.715 embryos suggests that there is an RNA stabilizing element(s) within the *Hsp83* 3'UTR. Other mechanisms in which RNA degradation occurs independent of deadenylation are emerging (reviewed in 2). Endonucleolytic cleavage of mammalian transferrin receptor RNA (4) and *Xenopus Xlhbox2B* RNA (8, 9) proceeds their rapid degradation. The endonucleolytic activity of the *Xenopus* oocyte has been partially purified, and a similar activity has also been identified in the *Drosophila* embryo (9). In addition to the endonucleolytic activity, a sequence-specific degradation inhibitory factor has also been identified (9). Binding of the iron response-element binding protein to the transferrin receptor mRNA has been suggested to inhibit its endonucleolytic cleavage (4). Therefore, in stage 2-4 embryos, there may be factors which specifically target *Hsp83* RNA for degradation throughout the embryo but components within the posterior polar plasm may inhibit the degradation. This protection might result via the association of the *Hsp83* 3'UTR with the posterior polar plasm to mask the RNA and protect it from degradation. Alternatively, there may be specific degradation inhibitory factors localized in the posterior polar plasm. Nevertheless, the importance of the posterior polar plasm in conferring the protection from degradation is clear since posterior group mutations which eliminate the posterior polar plasm fail to protect *Hsp83* transcripts, and ectopic localization of polar plasm to the anterior in *oskar-bicoid* 3'UTR embryos is sufficient to protect *Hsp83* RNA at the anterior (22). Possibly, this activity is associated with the polar granules which are sequestered to the pole cells upon their formation in stage 3 embryos (73, 74). This may explain why there is no localized protection activity seen for the zygotic transcripts in stage 3-4 embryos.

Initiation of Zygotic Hsp83 Expression

Zygotic *Hsp83* expression is first detected uniformly in stage 3 embryos. This expression is lost in stage 4 embryos. We could not map the regulatory elements required for this uniform expression because the 5'-*Hsp83-lacZ* constructs do not express *lacZ* in this particular expression pattern. Embryos bearing these constructs also lack maternally transcribed *lacZ* RNA (data not shown). This is in marked contrast to the *lacZ-Hsp83* 3'UTR constructs which have high levels of maternally encoded fusion RNA in the early embryo and zygotically express the RNA uniformly in stage 3 embryos (data not shown). The only exception is the 351.715 construct which lacks maternal RNA in the early embryo. In this case, the fusion RNA is transcribed normally during oogenesis, but it is not stably maintained in later stage oocytes. The lack of *Hsp83-lacZ* RNA in stage 3 embryos may reflect incomplete transcription of the full-length *lacZ* tag or differences in the stability of the fusion RNAs in which the *Hsp83* 3'UTR is replaced with SV40 terminator sequences.

Zygotic Expression In The Anterior Of The Early Embryo

Hsp83 is expressed in the anterior third of late stage 4/early stage 5 embryos. *bicoid* function is required for this expression. The presence of BICOID consensus binding sites within the *Hsp83* gene, the ability of the BICOID to bind one of these sites *in vitro* and the necessity of this binding site for the *in vivo* expression of a 5'-*Hsp83-lacZ* transgene suggests that the transcriptional activation of *Hsp83* by BICOID is direct. The expression of *Hsp83* in the anterior differs from the BICOID-regulated expression pattern of *hunchback* and *orthodenticle* (25, 31, 82, 86). Zygotic *hunchback* and *orthodenticle* expression in the anterior undergoes a *torso*-dependent repression in the anteriormost region of stage 5 embryos (26, 31, 82, 99) Repression of the *Hsp83* expression pattern in the anteriormost region of the embryo occurs later, in stage 10 embryos. It is thought that *hunchback* and *orthodenticle* are expressed throughout the anterior of Stage 4 embryos, but

that BICOID-activated transcription continues in stage 5 embryos (82). In the terminal region of the embryo, however, BICOID is phosphorylated under the control of the *torso* tyrosine kinase receptor pathway, and this phosphorylation presumably renders BICOID inactive in the anteriormost region of the embryo (82). In order for the repression of *hunchback* and *orthodenticle* transcription to be visualized, their RNAs must have relatively short half-lives. *Hsp83* RNA may have a significantly longer half-life than these two RNAs. Therefore, the RNA first expressed under BICOID control may persist in stage 5 embryos and not be subject to this *torso*-mediated repression. This is likely to be the case because *Hsp83-lacZ* RNA expressed in response to BICOID show reduced levels in the anteriormost region of stage 6 embryos, approximately 90 minutes before the loss seen for the endogenous *Hsp83* RNA. These reporter transcripts may have a sufficiently short half-life to allow visualization of the same type of repression seen in the expression patterns of *hunchback* and *orthodenticle* .

Zygotically expressed HUNCHBACK is dispensable for the expression of *Hsp83* in the anterior of the embryo. Maternally transcribed *hunchback*, however, may be required in addition to BICOID for the expression of *Hsp83*. Recently, it was shown that HUNCHBACK acts synergistically with BICOID to drive gene expression throughout the anterior of the early embryo, and that maternally encoded HUNCHBACK can perform this function in the absence of the zygotically encoded HUNCHBACK (90). Artificial promoters which possess only high affinity BICOID binding sites upstream of a basal promoter and a reporter tag are not expressed in as broad an expression domain as would be predicted from their effect on the intact *hunchback* promoter (43, 82). In addition the expression patterns of the BICOID-regulated genes *orthodenticle*, *empty spiracles* and *buttonhead* are altered in the embryos that lack maternal and zygotic *hunchback* function, despite the fact that lack of zygotic *hunchback* function alone does not affect their expression (90). This is consistent with the lack of defects in the *Hsp83* expression pattern in zygotic *hunchback* mutants. In addition, only 6 potential BICOID binding sites

are present throughout the entire *Hsp83* sequence studied in this report, and *in vivo* analysis reveals that as few as three of these sites are sufficient for the anterior expression of *Hsp83* . Each of the sites apparently represents low or perhaps intermediate affinity binding sites for BICOID (25, 27, 45). Consistent with the lower affinity of these sites, *Hsp83* is not expressed in as broad a domain in the anterior as is zygotically transcribed *hunchback*, which possesses a series of high affinity BICOID binding sites (25, 27, 99, 100). Finally, computer sequence analysis shows that potential HUNCHBACK binding sites (95, 107) are present throughout the *Hsp83* sequences studied here (data not shown). Taken together, these results suggest that *Hsp83* expression throughout the anterior of the late stage 4/early stage 5 embryos may require HUNCHBACK in addition to BICOID.

Analysis of the 5'-*Hsp83-lacZ* fusion constructs reveals that there is an additional phase of *Hsp83* expression in the anterior of cellular blastoderm embryos that is separable spatially and temporally from that seen earlier throughout the anterior third of the embryo. This expression manifests itself as a stripe. In addition, the *cis*-regulatory sequences sufficient for this expression map separately from those required for expression throughout the anterior. This stripe of expression resembles that seen for the anterior gap genes *buttonhead* (116), *empty spiracles* (19) and *orthodenticle* (31, 32). Each of these genes encode putative transcription factors (19, 31, 32, 109, 116), and any one could, therefore, control the stripe expression pattern of the -873.-155 transgene. However, mutations in each of these genes singly do not alter either the expression pattern of endogenous *Hsp83* or that of the -873.-155 construct. It is possible that another gene product may control the expression of *Hsp83* within this domain. For example, the *sloppy paired* locus encodes a protein with homology to the *fork head* domain (38), a putative DNA binding domain identified in the *fork head* gene (114) and the rat HNF-3 family of transcription factors (59), and it is expressed in the anterior of early syncytial blastoderm embryos (37, 38). This pattern resolves into a stripe of expression extending from 70-81% EL dorsally and 71-87% EL ventrally in late syncytial blastoderm embryos (37). Alternatively, instead of

acting individually, these genes may act together to regulate *Hsp83* expression. Such a combinatorial model for specifying head development has been proposed (13). Based on the overlapping expression domains of these head gap genes (37), the head stripe expression of *Hsp83* may be under the combined control of *sloppy paired*, *orthodenticle* and/or *empty spiracles*. As such, the head stripe of *Hsp83* expression may represent an ideal opportunity to test the combinatorial model of head development.

Hsp83 Function In The Early Embryo

Hsp83 is the *Drosophila* homolog of the *Hsp90* family of mammalian regulatory molecular chaperones (5, 40). Recently, mutations in the *Drosophila Hsp83* gene have been described (16). The mutations were isolated in a genetic screen for enhancers of *sevenless* (89), a gene that encodes a receptor tyrosine kinase necessary for proper eye development (41, 106). This suggests that *Hsp83* may modulate the tyrosine kinase signaling cascade during eye development. During embryogenesis, the receptor tyrosine kinase encoded by *torso* initiates a signal transduction cascade necessary for specifying embryonic pattern (reviewed in 119). Mutations in *Hsp83* maternally suppress dominant gain-of-function *torso* alleles (23, L. Jiang, A. Bashirullah, H.D. Lipshitz in preparation). One of the targets of this signal transduction pathway, *l(1)pole hole*, the *Drosophila raf* homolog (77, 78), is required for *torso* function (1). Biochemically, *Hsp90* has been shown to interact with *raf* and to potentiate its activity (113). These results suggest that one function of *Hsp83* in the early embryo may be regulation of *D-raf* activity in the *torso*-dependent signal cascade. Should this be the case, this may explain the initial uniform distribution of *Hsp83* RNA in the early embryo followed by its rapid degradation.

Localization of Hsp83 RNA to the pole cells and zygotic expression of *Hsp83* later in embryogenesis may reflect a requirement for *Hsp83* in other developmental processes. Recent reports show that the *C. elegans Hsp90* homolog is also maternally expressed. It is present in the soma and enriched in the germline progenitors of the embryo (88). This

suggests that *Hsp83* function may reflect a well-conserved mechanism for regulating developmentally required molecules in the cytoplasm at particular stages in specific cell types. *Hsp83* is present as a single copy gene in *Drosophila* (5, 40), thus making *Drosophila* an ideal genetic system for examining *Hsp90* function during embryogenesis.

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TABLES

Table 1. The PCR primers used for amplifying the *Hsp83* fragments used in the construction of the 5'-*Hsp83-lacZ* fusion constructs are shown. The first number used in the primer names (e.g. -873) refers to the location of the first nucleotide within the *Hsp83* sequence, relative to the transcription start site. The primers are comprised of a 6 or 8 nt leader, followed by a restriction enzyme site (underlined and highlighted) and then the *Hsp83* sequence. The restriction enzyme sites have been underlined and highlighted. The forward primers (the ".5" series) contain a Not I restriction site, and the reverse primers (the ".3" series) contain a Bgl II restriction site.

Table 1

Primers Used to Generate the*Hsp83* 5' Subcloning Fragments

Forward Primers	Sequence (5'-3')
-873.5	GAA TAA CTG CGG CCG CTA ACG GGA ACT TGA AG
-644.5	GAA TAA CTG CGG CCG CTA CTT GAC TGG GCT TG
-171.5	GAA TAA CTG CGG CCG CGA ATT CGC CCG CAC AG
-89.5	GAA TAA CTG CGG CCG CGC ATC CAG AAG CCT C
-66.5	GAA TAA CTG CGG CCG CTC TAG AGA CTT CCA GTT C
+18.5	GAA TAA CTG CGG CCG CCG TAC GGT GTG CGT CG

Reverse Primers	Sequence (5'-3')
-628.3	CGC CTA AGA TCT ACA AGC CCA GTC AAG
-415.3	CGC CTA AGA TCT CTC CAC CAG ACT ATC
-155.3	CTT GCT AGA TCT CCT GTG CGG GCG AAT TC
-48.3	CGC CTA AGA TCT CGA ACT TGG AAG TCT CTA G
+34.3	CTT GCT AGA TCT ACG ACG CAC ACC GTA CG
+333.3	CTT GCT AGA TCT TTG CAC GTT GCC ACC TC
+740.3	CTT GCT AGA TCT TGT GCG TAT GCG GCA TG
+1009.3	CGC GGC AGA TCT TAT GCA AAT TGG GTC GG
+1295.3	CTT GCT AGA TCT CTG GCA TCT GCA ATG G
+1621.3	CTT GCT AGA TCT CTG CAG AGC CTC CAT G

Table 2. The PCR primers used for amplifying the *Hsp83* 3'UTR fragments used in the construction of the *Hsp83* 3'UTR-*lacZ* fusion constructs are shown. The first number used in each primer name (e.g. 1) refers to the location of the first nucleotide within the 406 nt *Hsp83* 3'UTR sequence, such that nucleotide 1 is equivalent to nucleotide 3431 of the genomic sequence (5), Genbank Accession XO3810). The primers include a 6 nt leader, a restriction site (underlined and highlighted) followed by the *Hsp83* sequence. The restriction sites are as follows: Aat II for the ".5A" series, Hind III for the ".5H" and ".3H" series and Kpn I for the ".3K" series of primers. The T2 sequence was provided by T. Cutforth and G. Rubin (pers. comm.).

Table 2

Primers Used to Generate the *Hsp83* 3'UTR Subcloning Fragments

Forward Primers	Sequence (5'-3')
1.5A	GTA GCT **GAC GTC** GCG ACC AGT CGA AAC
51.5H	GCC GGC **AAG CTT** ATA CAC AAT TTA CTT G
101.5H	GCC GGC **AAG CTT** GAG TTA AAT TTT GTA TTC
151.5H	GTA GCT **AAG CTT** CAT ACG CTT AAC TC
201.5H	GTA GCT **AAG CTT** CTC ACA GAA CAT TC
253.5H	GCC GGC **AAG CCA** AGT AAT TTA TG
301.5H	GTA GCT **AAG CTT** GTA GAG CTA TAT AAA G
351.5H	GTA GCT **AAG CTT** CCG ATC GAT GAT AAA C

Reverse Primers	Sequence (5'-3')
50.3H	CGG ACG **AAG CTT** GTG AAT GCG AGT GAT AG
100.3H	GTA GCT **AAG CTT** GGC CGT AGT AAA CTC
150.3H	CGG ACG **AAG CTT** TCT GTC GCT TAT AAC G
200.3H	CGG ACG **AAG CTT** AAC CTA ACC ATT TAA CG
252.3H	CGG ACG **AAG CTT** ATT CTA AAT TAT AAG
300.3H	GTA GCT **AAG CTT** CAA CTA GGG CGC GTA TG
350.3H	CGG ACG **AAG CTT** GTC ATC AAA CCT TAA T
406.3H	CGG ACG **AAG CTT** AGA TTT TTA AAA CAC
715.3H	CGG ACG **AAG CTT** CAT ACA AAT CCT TGC
406.3K	CGG ACG **GGT ACC** AGA TTT TTA AAA CAC
715.3K	CGG ACG **GGT ACC** CAT ACA AAT CCT TGC
T2.K	AC **GGT ACC** GCA AAG ACG AGT AG

FIGURES

Figure 1. Whole mount *in situ* analysis of *Hsp83* RNA distribution in wildtype embryos. (A) Maternally encoded *Hsp83* RNA is distributed uniformly throughout the embryo between nuclear cycles 1 through 5. (B) Between nuclear cycles 6 and 8, the maternal RNA is localized to the posterior pole by a unique mechanism of generalized degradation and localized protection (22). (C) In pole bud (stage 3) embryos, maternal RNA is taken up into the pole cells as they form (arrowhead; (22). Zygotically transcribed *Hsp83* RNA is also detected throughout the somatic region of the embryo. (D) During stage 4, the uniform zygotic RNA is no longer detected. High levels of RNA are detected in the pole cells and such levels are seen in the pole cells throughout embryogenesis (arrowheads). (E) Zygotic transcription commences in the anterior third (69% to 100% EL) of late stage4/early stage 5 embryos. (F) In stage 6 embryos, the RNA is still detected throughout the anterior. (G) In stage 10 embryos, *Hsp83* RNA is no longer detected in the anteriormost region of the embryo (asterisk). In addition, expression is seen in the anterior and posterior midgut and procephalic and ventral neuroblasts. (H) *Hsp83* RNA is detected in the gonads of stage 16 embryos. In addition RNA is detected in the gut and oenocytes. Embryos are oriented with the anterior to the left and dorsal up, except for (H) which is oriented dorsal side up. This convention will be followed throughout the paper.

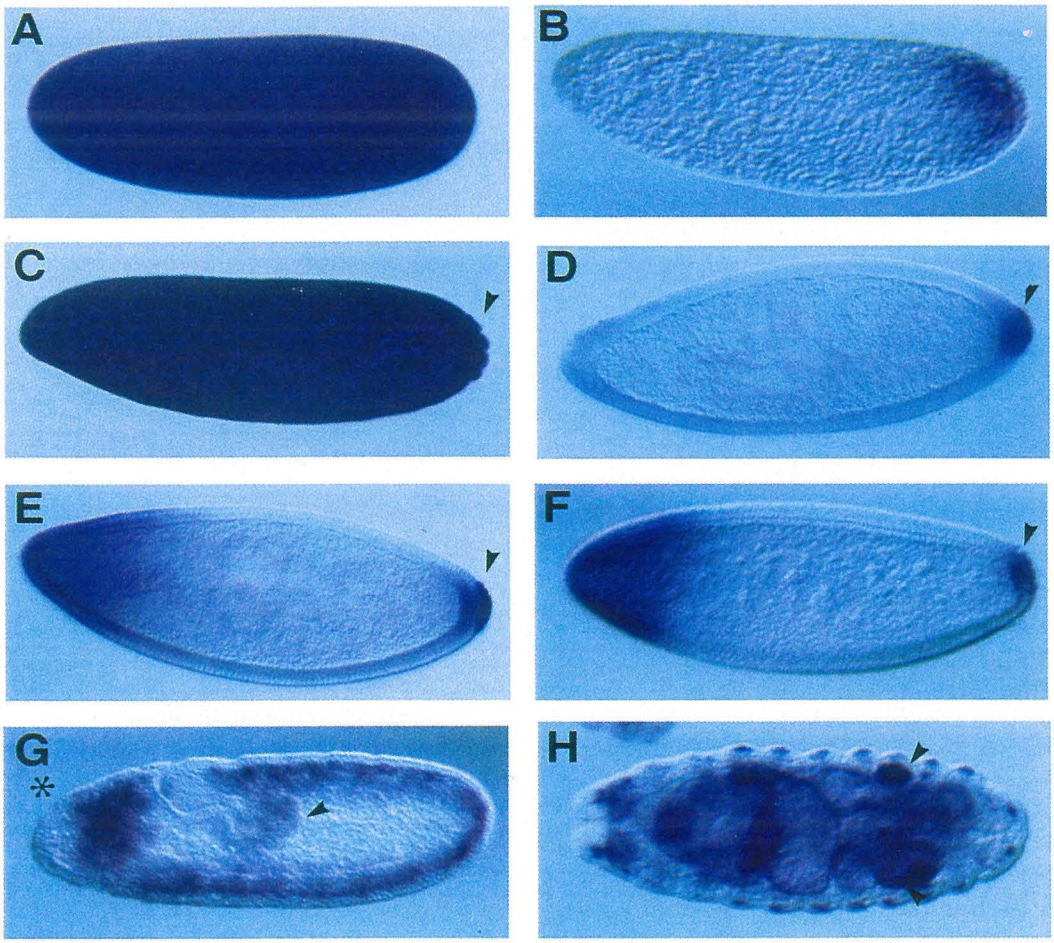
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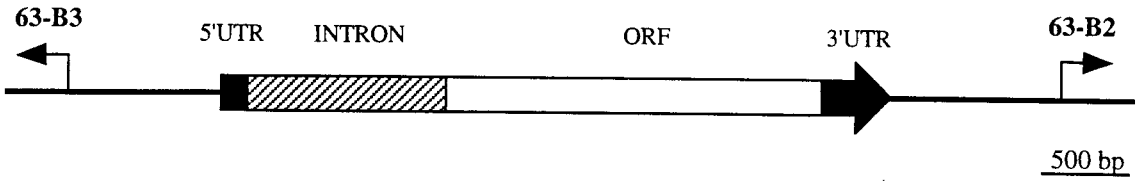
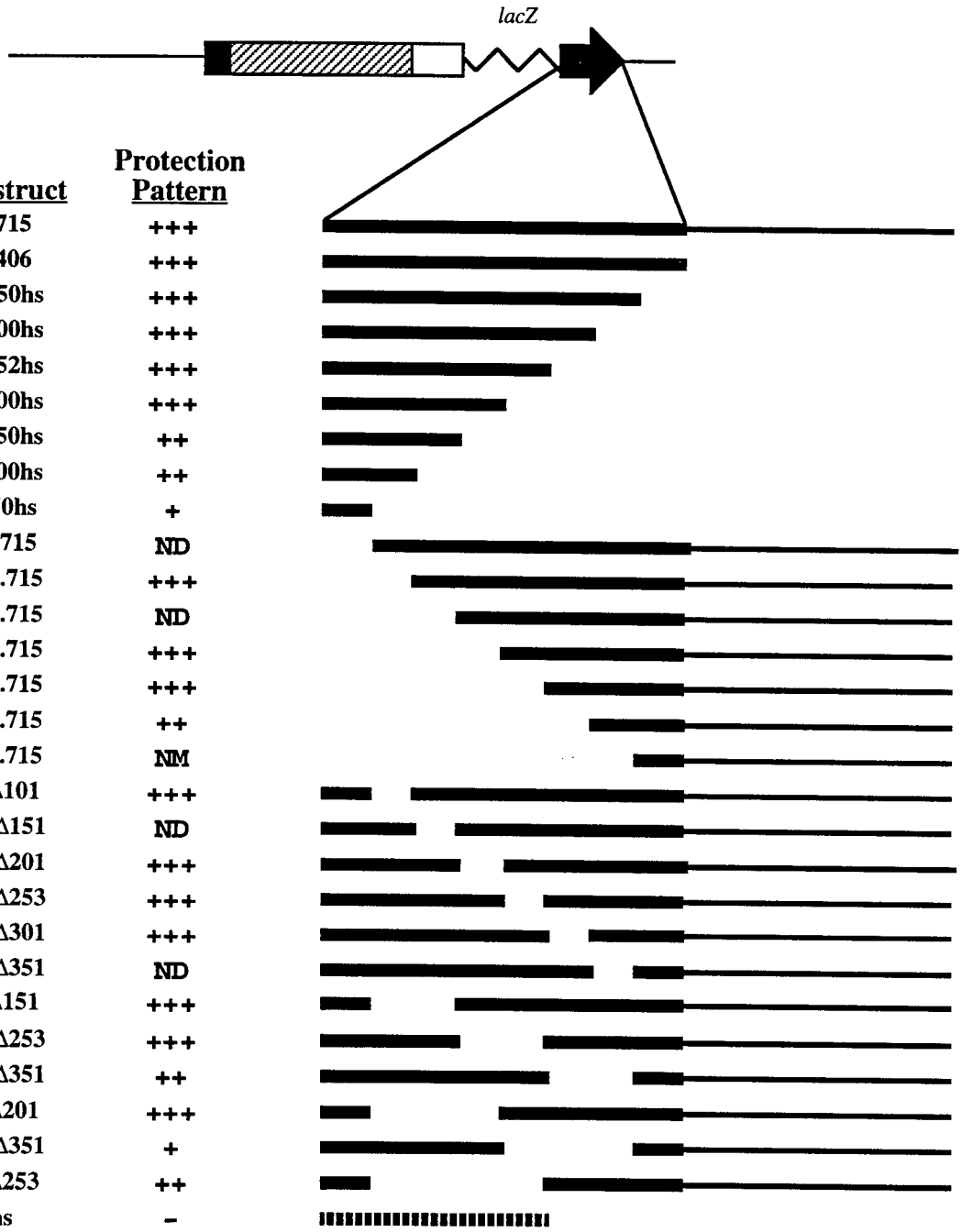
Figure 2. Genomic organization of the *Hsp83* gene and map of the *lacZ-Hsp83* 3'UTR transgenes. (A) The *Hsp83* gene is composed of 2 exons and a single intron (5, 40). Exon 1 is 149 bp long and encodes the 5'UTR of the mRNA, the intron is 1139 bp and exon 2 comprises the 717 codon ORF followed by the 407 bp 3'UTR. Transcription unit 63B-T3 flanks the *Hsp83* gene 880 bp upstream of the *Hsp83* transcription start site (5), and another transcription unit, 63B-T2 flanks it approximately 950 bp downstream ((79), S. Halsell, unpublished observation). (B) *lacZ-Hsp83* 3'UTR transgenes were constructed to map the *cis*-acting sequences required for localized protection of maternally transcribed RNA from degradation. Transcription of the transgenes is driven by *Hsp83* sequences between $^{-}$873 and $^{+}$1621, fused upstream of a 603 bp *lacZ* tag and followed by the 407 nt *Hsp83* 3'UTR (1.407) or the *Hsp70* 3'UTR (hs). 3', 5' and internal deletions within the *Hsp83* 3'UTR were generated. Genomic sequences downstream of the 3'UTR (thin line) were included in the 5' deletion constructs, and the 3' deletion constructs included the *Hsp70* 3'UTR downstream of the deletion to ensure proper poly-adenylation of the transcripts. The transgenes were introduced into flies by germline transformation. Whole mount RNA *in situs* were performed with *lacZ* probes, and the intensity of staining within the pole cells was scored relative to the anterior zygotic staining of stage 5 embryos. (+++): wildtype levels in pole cells; (++): bimodal staining pattern; wildtype and reduced levels seen in pole cells; (+): reduced levels detected; (-): no RNA detected; (NM): no maternal RNA present in the embryo; (ND): not done.

A. *Hsp83*



B. *lacZ-Hsp83* 3'UTR Transgenes



Construct	Protection Pattern
1.715	+++
1.406	+++
1.350hs	+++
1.300hs	+++
1.252hs	+++
1.200hs	+++
1.150hs	++
1.100hs	++
1.50hs	+
51.715	ND
101.715	+++
151.715	ND
201.715	+++
253.715	+++
301.715	++
351.715	NM
50Δ101	+++
100Δ151	ND
150Δ201	+++
200Δ253	+++
252Δ301	+++
300Δ351	ND
50Δ151	+++
150Δ253	+++
252Δ351	++
50Δ201	+++
200Δ351	+
50Δ253	++
hs	-

Figure 3. Protection of *lacZ-Hsp83* 3'UTR RNA at the posterior of the embryo. (A) Wildtype levels of RNA are detected in the pole cells of 1.407 embryos (black arrowhead). (B) Embryos bearing the hs construct fail to protect maternal RNA at the posterior and no RNA is detected in the pole cells (white arrowhead). Embryos bearing either (C) 1.200hs or (D) 253.715 transgenes have wildtype levels of RNA in the pole cells (black arrowheads). A bimodal staining pattern is seen in embryos carrying the 1.100hs transgene (E, F). Pole cell staining at (E) wildtype levels (black arrowhead) and (F) reduced levels (gray arrowhead) is detected.

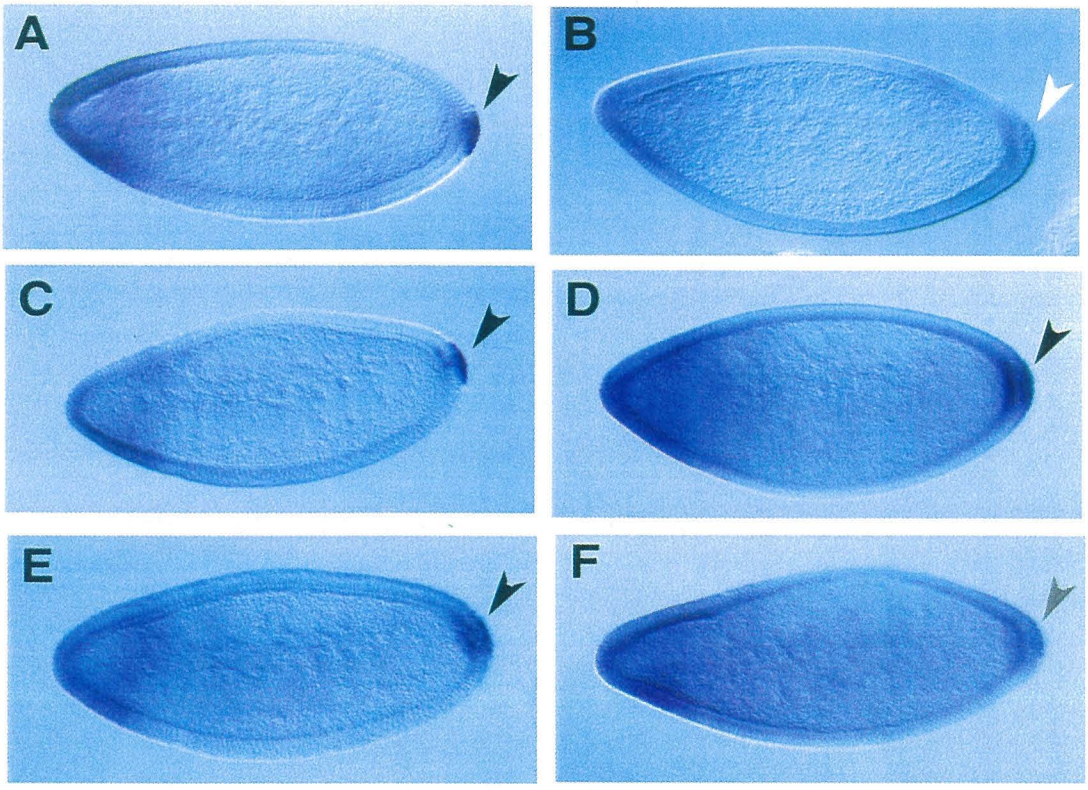

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Figure 4. Transcription of the *lacZ-Hsp83* 3'UTR transgenes during oogenesis. Ovaries were dissected from females bearing either a 1.715 or the 351.715 transgene, and whole mount RNA *in situs* were performed with *lacZ* probes. The transcription and RNA distribution of the 1.715 transgene resembles the endogenous *Hsp83* expression pattern (22). (A) In stage 10b follicles, the 1.715 RNA transcribed in the nurse cells is transported into the oocyte, (C) stage 12 oocytes exhibit a high uniform distribution of the RNA and (E) early embryos have a high uniform levels of the RNA. (B) In contrast, the 351.715 transgene is transcribed in the nurse cells and transported into the oocyte at stage 10b as in wildtype, but in stage 12 follicles (D), the detectable levels of the RNA is low relative to wildtype and the 1.715 transgene. (F) No maternally transcribed 351.715 RNA is detected in the early embryo. (A-D) Nurse cells are oriented to the left, the oocyte to the right.

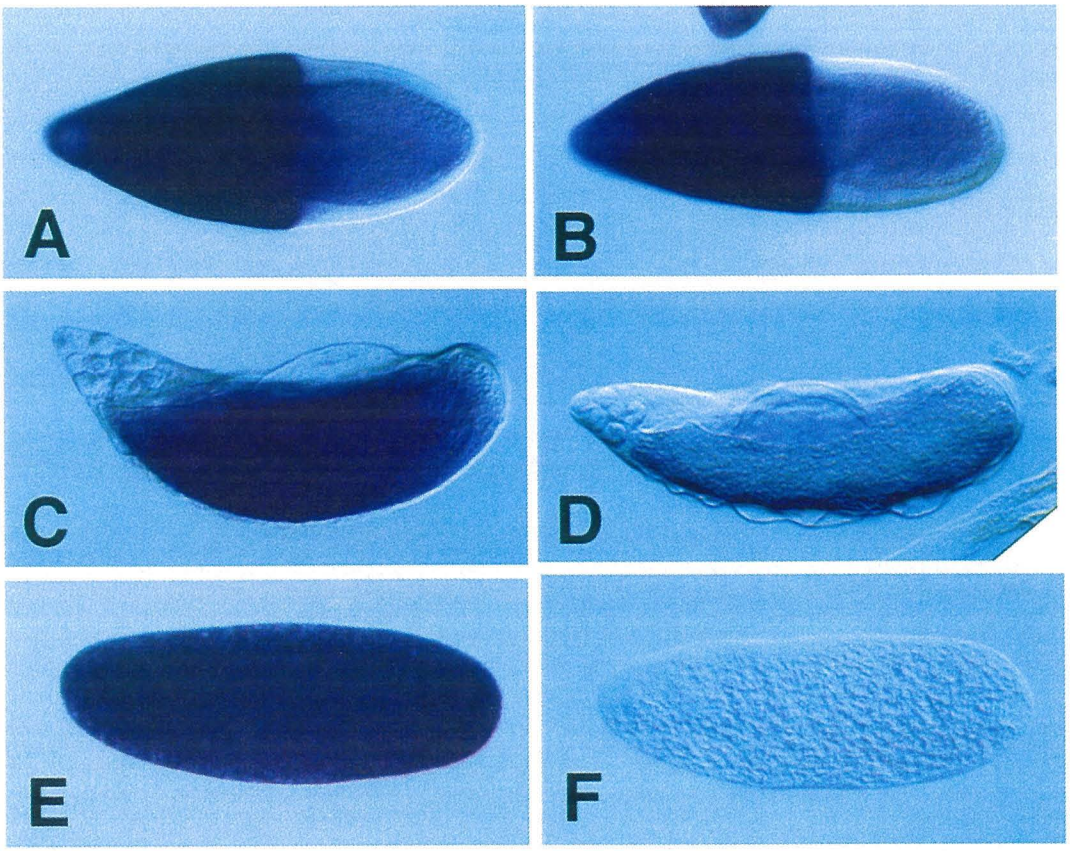
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Figure 5. Zygotic transcription in the pole cells commences at stage 11 of embryogenesis. Zygotic transcription in the pole cells was detected by mating males carrying the 1.715 transgene with Oregon-R females, followed by whole mount RNA *in situs* using *lacZ* probe. (A) Zygotic transcription is first detected at low levels in the pole cells of stage 11 embryos (arrowhead). (B) A closeup of the embryo in (A) is shown. The pole cells (arrowheads) are beginning to migrate out of the posterior midgut invagination. (C) In stage 16 embryos, high levels of zygotically transcribed RNA are detected in the pole cells within the gonad (arrowhead, dorsal view).

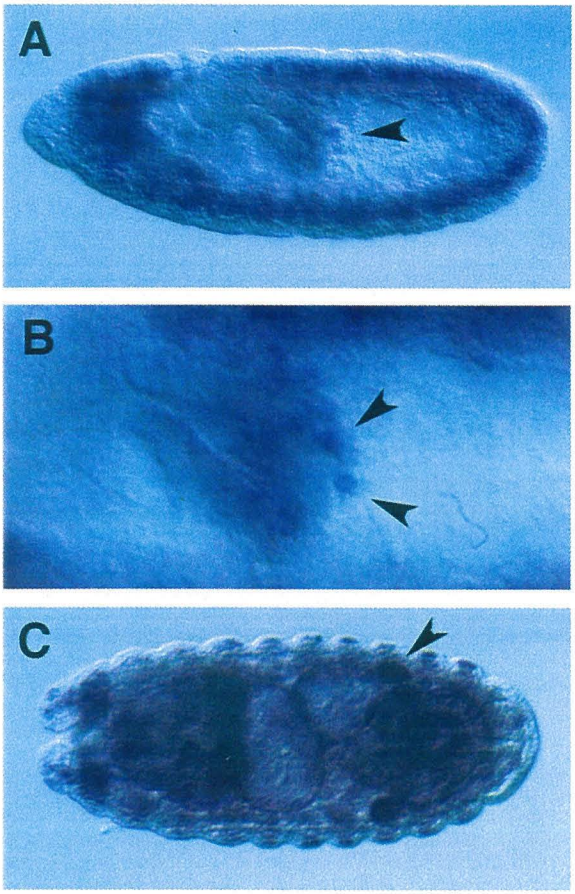
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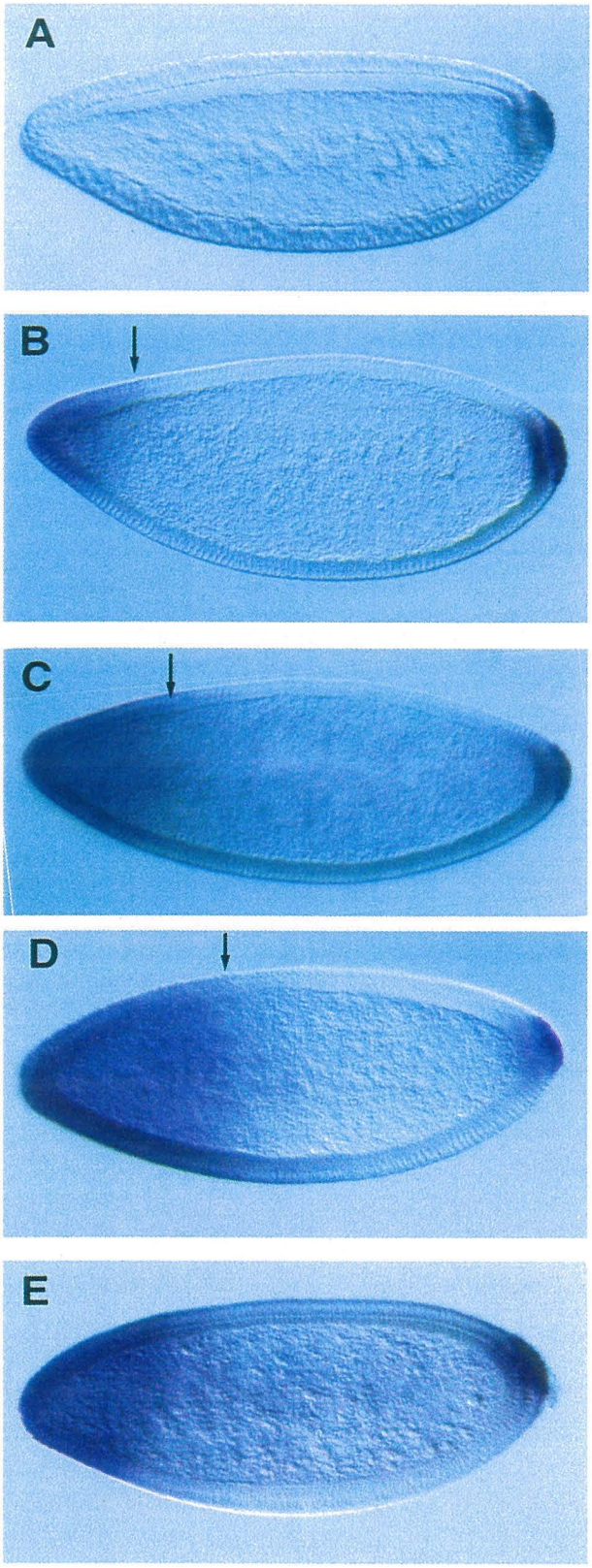
Figure 6: BICOID controls the expression of *Hsp83* in the anterior of late stage 4/early stage 5 embryos. Whole mount RNA *in situs* were performed using *Hsp83* probes. (A) Embryos derived from *bicoid*^{el} mutant mothers do not express *Hsp83* in the anterior of the early embryo. Varying the dosage of *bicoid*$^{+}$ in the embryo shifts the posterior boundary of *Hsp83* expression (see materials and methods for genotypes). The posterior boundary of expression (arrows) shifts from (B) 81% EL, single dose of *bicoid*$^{+}$, to (C) 69% EL, two doses of *bicoid*$^{+}$ to (D) 60% EL when six doses *bicoid*$^{+}$ are present. (E) *Hsp83* is expressed in the wildtype anterior pattern in embryos deficient for zygotically expressed HUNCHBACK.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D
E

Figure 7. 5'-*Hsp83-lacZ* transgenes used to map the *cis*-acting sequences required for zygotic expression in the anterior of the early embryo. *Hsp83* sequence between $^{-}$873 and $^{+}$1621, including the 5' upstream region, the first exon, the intron and the first 111 codons of the ORF were fused to a full-length *lacZ* tag (construct $^{-}$873.$^{+}$1621) and terminated with SV40 sequences (not drawn to scale). The six potential BICOID binding sites are indicated (83Bcd1 through 83Bcd6; see Figure 9). Transgenes with deletions within this *Hsp83* sequence were tested for their ability to direct expression in the anterior of the embryo. Expression was seen either in the "Anterior Third" of the embryo commencing at late stage4/early stage 5 or as a "Stripe" between 70 and 84% EL dorsally and 77 and 90% EL ventrally in stage 5 embryos.

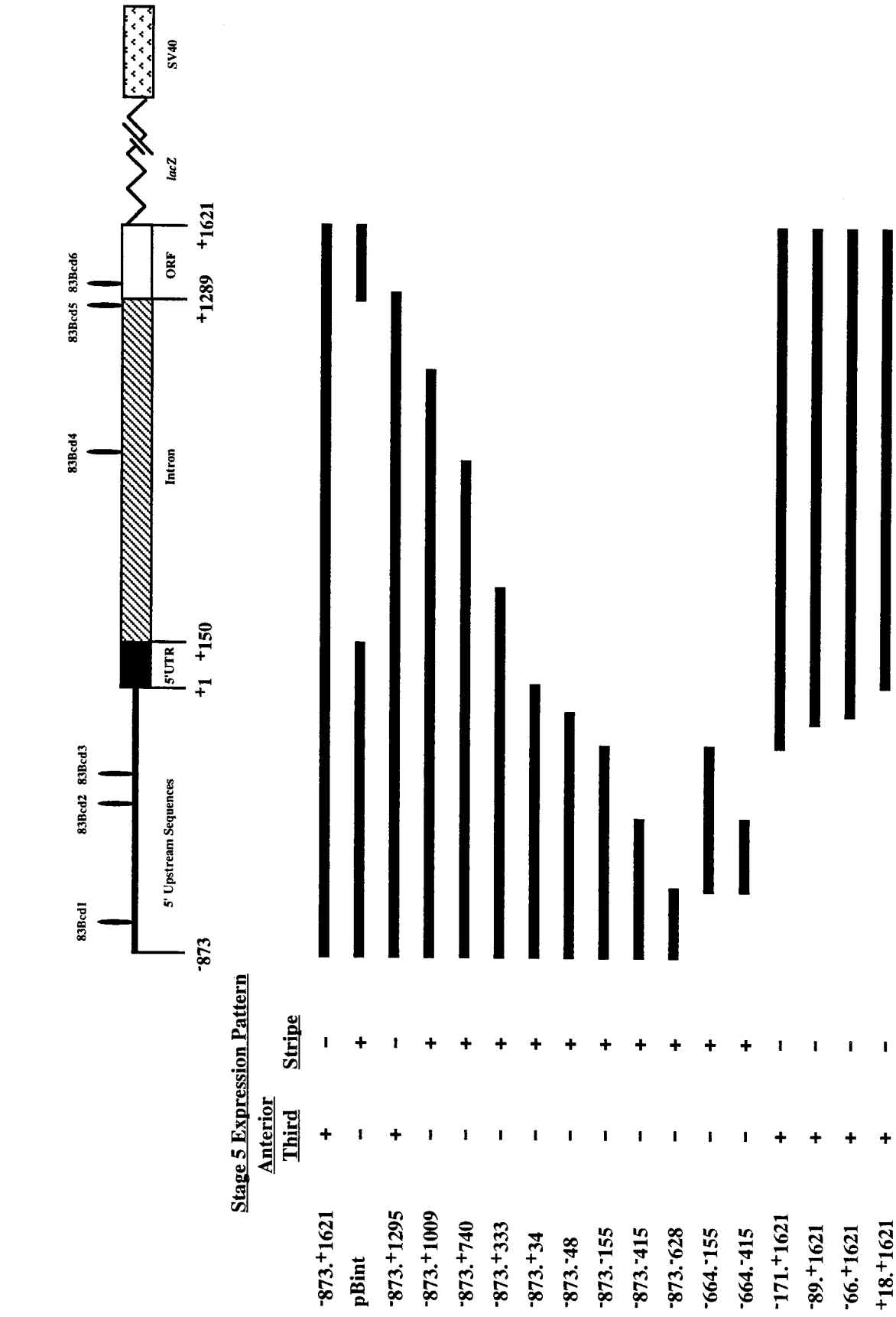

83Bcd1
83Bcd2
83Bcd3
83Bcd4
83Bcd5
83Bcd6
5' Upstream Sequences
5'UTR
Intron
ORF
lacZ
SV40
-873
+1
+150
+1289
+1621
Stage 5 Expression Pattern
Anterior
Third
Stripe
-873.+1621 + -
pBint - +
-873.+1295 + -
-873.+1009 - +
-873.+740 - +
-873.+333 - +
-873.+34 - +
-873.-48 - +
-873.-155 - +
-873.-415 - +
-873.-628 - +
-664.-155 - +
-664.-415 - +
-171.+1621 + -
-89.+1621 + -
-66.+1621 + -
+18.+1621 + -

Figure 8. Whole mount RNA *in situs* of 5'-*Hsp83-lacZ* transgenic embryos. *lacZ* probes were used to detect the expression pattern of 5'-*Hsp83-lacZ* transgenes in the early embryo. RNA is not detected in the pole cells because maternally transcribed RNA is never detected in embryos bearing transgenes terminated with the SV40 terminator sequence (data not shown). Transgenes (A) $^{-}$873.$^{+}$1621 and (C) $^{-}$873.$^{+}$1295 are expressed in the anterior third of late stage 4/early stage 5 embryos. Embryos carrying the (D) pBint and (E) $^{-}$873.$^{+}$1009 transgenes do not express in the anterior third of the embryo. A reduction in the level of RNA detected in the anteriormost region (asterisk) of stage 6 $^{-}$873.$^{+}$1621 embryos (B) is seen relative to of *Hsp83* in wildtype stage 6 embryos (Fig. 1F).

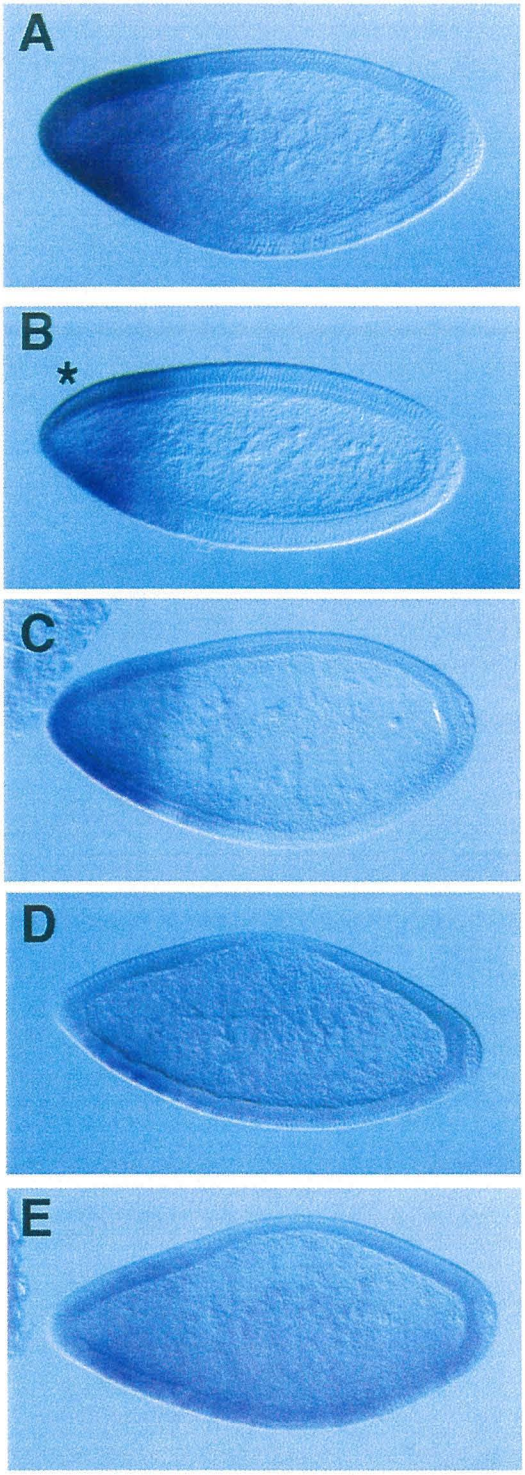

A
B
*
C
D
E

Figure 9. Six BICOID consensus binding sites are detected within *Hsp83*. Comparisons of the *Hsp83* sequence between -873 and +1621 reveal six potential BICOID binding sites. 83Bcd1 through 83Bcd4 resemble the X1 and X2 weak BICOID binding sites and 83Bcd6 resembles the X3 weak binding site found in the *hunchback* promoter (25, 27). Site 83Bcd6 resembles the moderate BICOID binding site found within the *Krüppel* promoter (45). The position of each site within the *Hsp83* sequence is indicated.

Site	Position	Sequence
83Bcd1	$^{-}$780 to $^{-}$772	TATA-AGCAA
83Bcd2	$^{-}$378 to $^{-}$370	CATA-AGCTG
83Bcd3	$^{-}$254 to $^{-}$246	TTTA-AGCAA
83Bcd4	$^{+}$785 to $^{+}$793	AGTA-AGCAA
83Bcd5	$^{+}$1273 to $^{+}$1282	TGTAAATCCA
83Bcd6	$^{+}$1348 to $^{+}$1357	GATC-ATCAA

Figure 10. *In vitro* mobility gel shift assay. The ability of the BICOID homeodomain to bind to radioactively labeled 83Bcd5 was tested *in vitro*, in the presence of increasing molar excess of nonradioactive (A) specific competitor (a high affinity BICOID binding site) or (B) non-specific competitor (a mutated BICOID binding site). The protein/DNA complex is detected as a band migrating with reduced mobility (arrowheads). The presence of BICOID is necessary to shift the mobility of the DNA (- vs. + protein). (A) The BICOID/83Bcd5 complex fails to form when a 64-fold molar excess of specific competitor is included in the binding reaction. (B) In contrast, a 1024-fold molar excess of non-specific competitor is required to eliminate the BICOID/83Bcd5 complex.

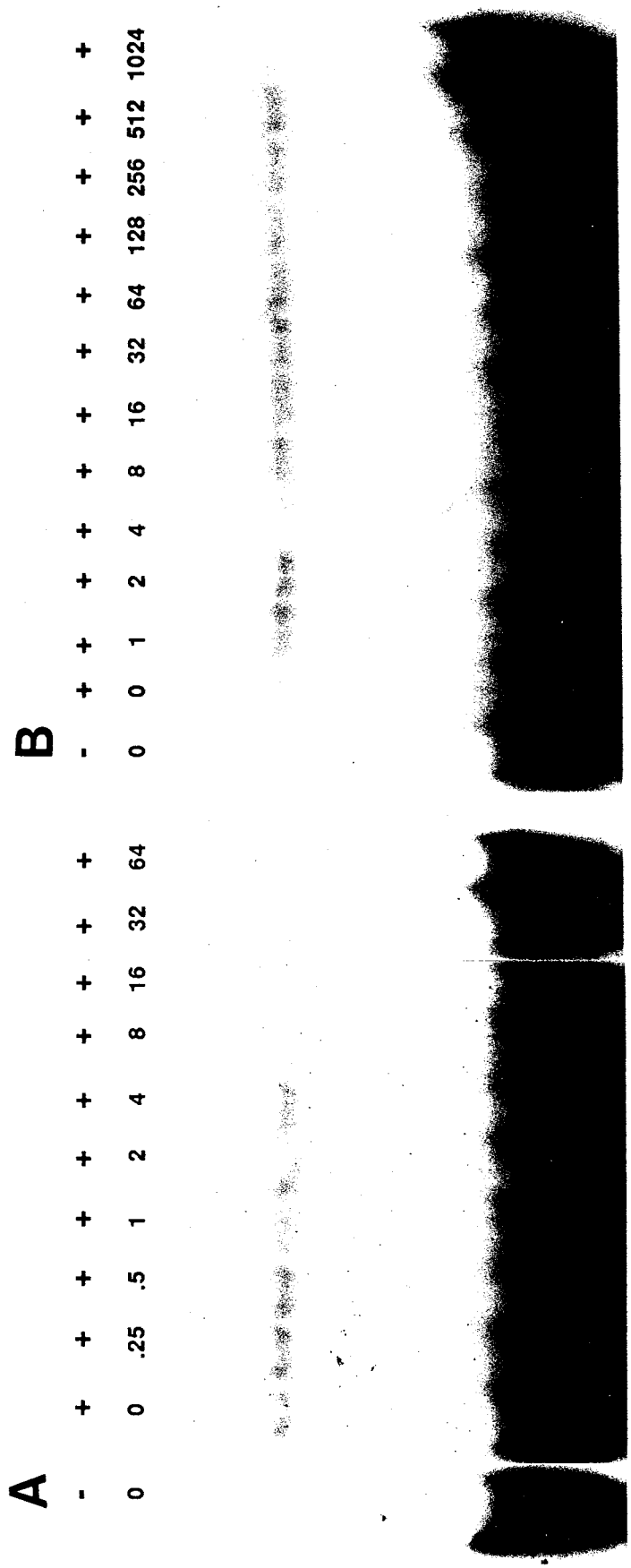

A
- + + + + + + + + + + +
0 0 .25 .5 1 2 4 8 16 32 64
B
- + + + + + + + + + + +
0 0 1 2 4 8 16 32 64 128 256 512 1024

Figure 11. A second, later expression in the anterior is revealed by the 5'-*Hsp83-lacZ* transgenes. Transgenes that fail to express in the anterior third of the late stage4/early stage 5 embryo (A) are expressed in a stripe in stage 5 embryos (B-D). (A) In late stage4/early stage 5 -873.-155 embryos, RNA is not detected in the anterior third (compare to Fig. 8A, C). (B) The -873.-155 transgene is expressed in a stripe pattern (asterisk) in stage 5 embryos. The same expression pattern is detected in (C) -873.-628 and (D) -664.-415 transgenic embryos.

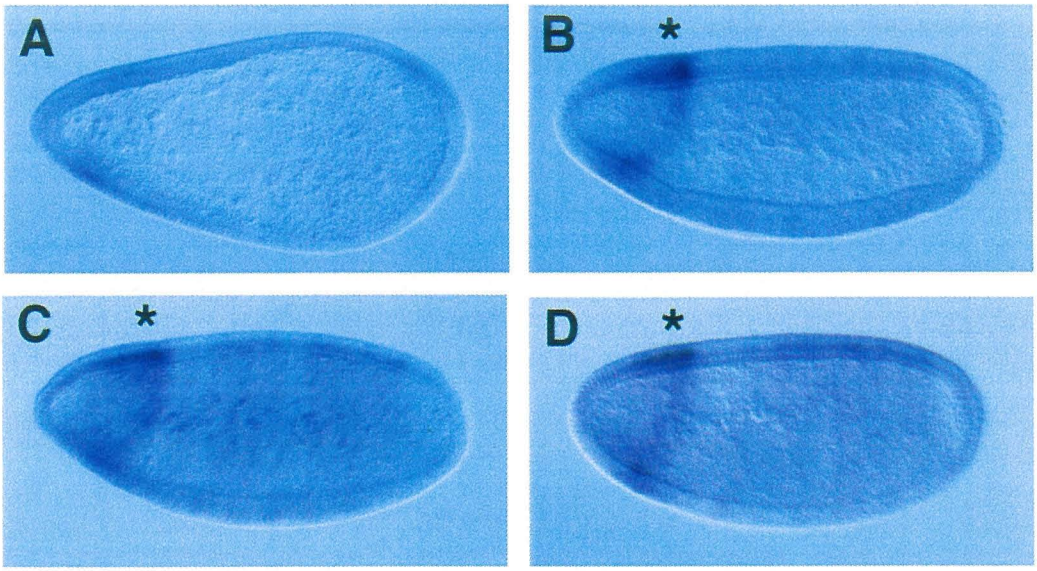
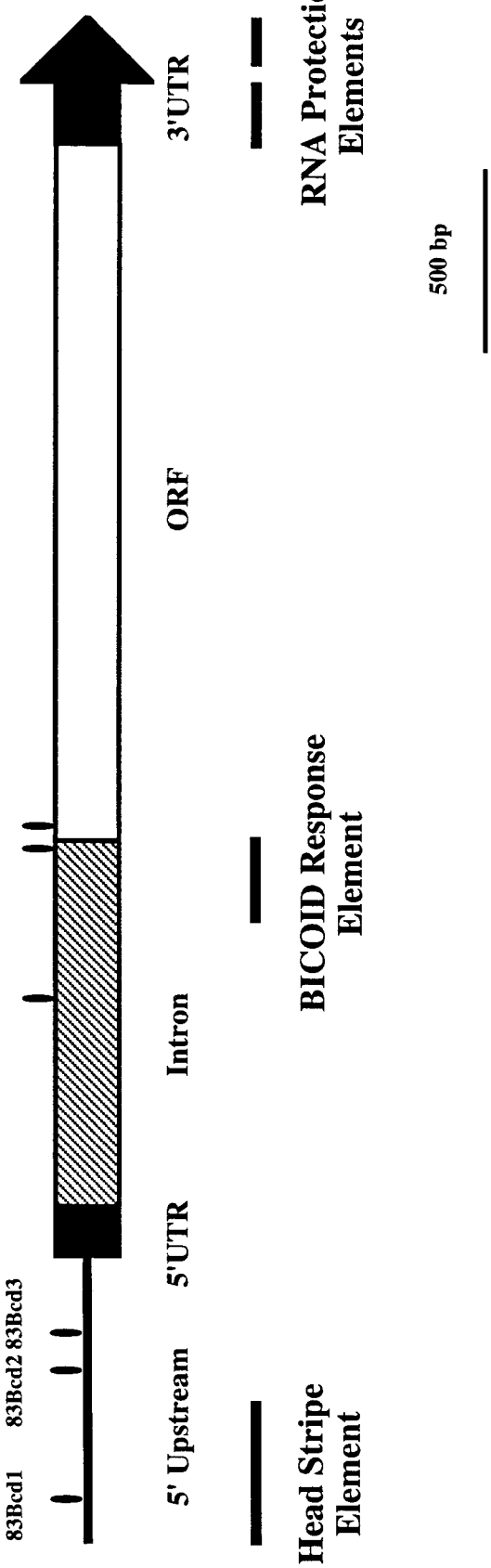

A
B
*
C
*
D
*

Figure 12. *Cis*-acting sequences required for *Hsp83* RNA distribution in the early embryo. *Cis*-acting sequences sufficient for the protection of the maternal RNA from degradation, the BICOID-activated transcription in the anterior third of the embryo and the expression of *Hsp83* as a stripe in the anterior have been mapped. See text for details.


83Bcd1
83Bcd2
83Bcd3
83Bcd4
83Bcd5
83Bcd6
5' Upstream
5'UTR
Intron
ORF
3'UTR
Head Stripe Element
BICOID Response Element
RNA Protection Elements
500 bp

APPENDIX

This paper appeared in Science **243**, 1062-1066 (1989).

My contributions included the genetic dosage experiments.

RECIPROCAL EFFECTS OF HYPER- AND HYPOACTIVITY MUTATIONS IN THE *DROSOPHILA* PATTERN GENE *torso*

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ABSTRACT

In *Drosophila*, five "terminal" polarity genes must be active in females to produce embryos with normal anterior and posterior ends. Hypoactivity mutations in one such gene, *torso*, result in the loss of the most posterior domain of *fushi tarazu* expression and the terminal cuticular structures. In contrast, a *torso* hyperactivity mutation causes the loss of central *fushi tarazu* expression and central cuticular structures. Cytoplasmic leakage, transplantation and temperature-shift experiments suggest that the latter effect is caused by abnormal persistence of the *torso* product in the central region of the embryo during early development. Thus, the amount and timing of *torso* activity is key to distinguishing the central and terminal regions of the embryo. Mutations in the *tailless* terminal gene act as dominant maternal suppressors of the hyperactive *torso* allele, indicating that the *torso* product acts through, or in concert with, the *tailless* product.

RESULTS AND DISCUSSION

Three classes of maternal effect pattern genes specify the anterior-posterior axis of the *Drosophila* embryo (*1,2*). When females carry "anterior" mutations, their progeny lack acronal, gnathal, and thoracic structures. Loss of the abdominal region (and the germ cells) occurs in offspring of the "posterior" or grandchildless-*knirps* class mutants. The "terminal", or *torso*-like, genes comprise six loci that are involved in specifying the asegmental anterior (acron) and posterior (telson) structures of the embryo (Fig. 1, A and B) (*1-4*). Five of these are maternal effect genes [*torso* (*1*), *trunk* (*1*), *torsolike* (*5*), *fs(1)Nasrat* (*3*), *fs(1)polehole* (*3*)] and one is zygotic in action [*tailless* (*4*)]. Here we focus on the mechanism of *torso* (*tor*) gene action. We show that hyper- and hypoactivity mutations in *tor* lead to reciprocal pattern defects. We investigate the basis for these effects and discuss their implications for the role of *tor* in establishing embryonic pattern.

Mothers homozygous for the *spliced*^{RL3} mutation (*6*) produce embryos which lack thoracic and abdominal structures and possess only acron and telson (Fig. 1E, Table 1B). The *spliced* mutation is semi-dominant: at 25^{o}C heterozygous females produce offspring lacking up to three abdominal segments, with the second and third abdominal segments showing the greatest sensitivity (Table 1D). The *spliced* phenotype is complementary to that produced in progeny of females homozygous for hypoactivity mutations in the terminal class genes (compare Fig. 1B to Fig. 1, C through E) (*1-4*). We have used chromosomal deficiencies and a duplication for the *tor* region to demonstrate that the *spliced* mutation maps to the same cytological region as *tor* (43C3-43E7) (*1*), and that it is a hypermorphic (*7*) (hyperactivity) *tor* allele (Table 1). Specifically, when a *spliced* homozygous or heterozygous mother carries an extra dose of the wildtype *tor*$^{+}$ gene, the embryos exhibit a more extreme phenotype, with a consistent loss of an additional one to two abdominal segments (compare Table 1, A with B; C with D). In contrast, reduction of the number of copies of the *spliced* gene in *spliced* hemizygous mothers rescues the central region defects, resulting in the presence of the wildtype number of abdominal segments (compare Table 1, B and G). Further, hypoactivity mutations in the *tor* gene also rescue the abdominal defects of *spliced* individuals (compare Table 1, D and E,F). These genetic tests are consistent with the conclusion that *spliced* is a hyperactivity allele of *tor*, which we, therefore, designate *tor*^{splc} (*8*). While hypoactivity of *tor* causes a loss of the acron and telson and respecification of these regions to form central structures (*1*), hyperactivity of *tor* instead has no effect on the termini and causes loss of the thorax and abdomen (*9*). The *tor*^{splc} allele is heat sensitive. This fact enabled us to observe a progressive deletion of central embryonic regions due to increased *tor* expression in embryos from *tor*^{splc} homozygous mothers (Fig. 1, C through E). The cuticular defects correlated well with alterations in the expression of the zygotic pair-rule segmentation gene, *fushi tarazu* (*ftz*), an earlier, molecular marker of embryonic pattern (Fig. 2, A through D) (*10-13*). In wildtype embryos *ftz* is expressed early in embryogenesis in a pattern of seven stripes (Fig. 2A) (*10-*

12). In embryos (from tor^{splc} females) raised at low temperatures (18 to 19°C), zero to three of the abdominal segments are missing, with A2 and A3 exhibiting the greatest sensitivity (Fig. 1C, Table 1B). This correlates with a reduction, or complete absence, of *ftz* stripe 4 (Fig. 2B). At intermediate temperatures (21-23°C) most of the eight abdominal segments are absent whereas acronal, gnathal, and thoracic structures develop normally anteriorly, and A7, A8 and the telson develop normally posteriorly (Fig. 1D, Table 1B). At these temperatures, there is a compression and fusion of *ftz* stripes 2, 3 and 5 with a concomitant broadening of stripe 7 (Fig. 2C). At high temperatures (25°C) the progeny of homozygous mothers develop as bags of cuticle with acronal and gnathal structures, such as mouth hooks and pharynx, anteriorly and a telson with filzkörper posteriorly (Fig. 1E, Table 1B). Only *ftz* stripe 1 and the broad posterior stripe remain in these embryos (Fig. 2D). About 25% of the embryos that are allowed to develop at 25°C show no activation of *ftz* expression. The embryonic fate map of embryos from tor^{splc} females, reflected by the pattern of *ftz* expression, thus suggests that the absence of stripes 2-5 likely corresponds to loss of segments T1 through A5. The presence of stripe 1 and the most posterior stripe (the expanded and fused stripes 6 and 7) are consistent with the presence of acronal, gnathal and telson structures. The alterations in *ftz* expression and the loss of central structures caused by hyperactivity of *tor* contrasts with the loss of *ftz* stripe 7 and terminal structures caused by hypoactivity alleles of *tor* (Fig. 1B, Fig. 2G) (*11, 14*).

Taken together, these data suggest that the *tor* gene product possesses dual functions in early embryogenesis: to promote the development of the acron and telson in the terminal regions of the embryo, and to suppress the development of central structures (thorax and abdomen) at the termini. This explains why hyperactivity of *tor* has no effect on the termini of the embryo while causing loss of the central structures.

To test whether the loss of thoracic and abdominal structures in progeny of tor^{splc} mothers is a direct consequence of active *tor* gene product in the central part of the embryo, cytoplasmic leakage and transplantation experiments were carried out (Table 2, Fig. 2, E

through H) (*15*). We assayed directly for the rescue of the pattern of *ftz* expression at a time corresponding to the germ band extended stage, four to five hours after leakage or transplantation. Leakage of cytoplasm from the central region of early tor^{splc} embryos [stage 1 to 2, 0 to 80 minutes after fertilization (*16*)] does not rescue the abnormalities in *ftz* expression (Table 2, Fig. 2E). However, leakage from slightly older embryos [stage 3 to 4, 80 to 150 minutes after fertilization (*16*)] results in restoration of up to four stripes of *ftz* expression in the central region of 15% of the embryos (Table 2, Fig. 2F). This result is consistent with a model which proposes that *tor* activity is normally present in the central region during stages 1 to 2 (hence leakage of excess *tor* product has no effect) but must disappear from this region by stages 3 to 4 for normal central development to ensue. The tor^{splc} mutation could result in the production of either a constitutively active *tor* product or a *tor* product with increased stability (see below), resulting in the presence of *tor* activity centrally at stages 3 to 4. Removal of the *tor* product by leakage from the central region at these stages thus rescues the central defect in embryos from tor^{splc} females.

To confirm that *tor* activity is present centrally at stages 3 to 4 in embryos produced by tor^{splc} mothers, but not in wildtype embryos, we transplanted cytoplasm from the central region into the posterior pole of embryos from tor^{-} mothers. We assayed for the reappearance of the seventh *ftz* stripe that is missing in tor^{-} embryos (*11*). Cytoplasm from the tor^{splc} embryos rescued the seventh stripe in 23% of the tor^{-} embryos (Table 2, Fig. 2H), but cytoplasm from wildtype embryos was unable to exert any rescuing effect (Table 2, Fig. 2G). These data are consistent with the suggestion that the loss of central structures in embryos from tor^{splc} females is a consequence of abnormal persistence of *tor* activity in the central region beyond stages 1 to 2.

The temperature sensitivity of the tor^{splc} mutation allowed us to carry out temperature shift experiments to determine, by an independent method, when the tor^{splc} gene product acts to cause the loss of central embryonic structures (*17*). The temperature-sensitive period includes stages 3 and 4 (1 to 2 hours after fertilization at 29°C, Fig. 3),

consistent with our hypothesis that it is the presence of *tor* product in the central region of progeny of tor^{splc} females at these stages, that results in the loss of central structures.

We consider two of the models that can explain the reciprocal phenotypic effects of hyper- and hypoactivity of the tor^{+} product, and our rescue of these defects by cytoplasmic leakage and transplantation. In one, the tor^{+} product is normally uniformly distributed in the egg or embryo but only activated in the termini, and the tor^{splc} defect causes constitutive activation throughout the embryo. Alternatively, the tor^{+} product is normally active throughout the embryo early in development (stages 1 to 2), but must subsequently disappear from the central region, remaining active only at the poles (stages 3 to 4) (*18*). If the tor^{splc} gene product is more stable than the wildtype product and/or is constitutively active, it could persist in the central region beyond stages 1 to 2, causing defects there. Duncan (*19*) has proposed a similar model to explain the effects of the hypermorphic ftz^{Ual} mutations. To examine possible regulatory interactions among terminal class genes, we determined whether a reduction in activity of other terminal genes might rescue the pattern defects caused by *tor* hyperactivity. Reduction of the dosage of the terminal gene, *fs(1)Nasrat* (*3*), from the normal two doses to a single dose, did not rescue the central embryonic defects in progeny of females that are homozygous for tor^{splc}. However, reducing the number of copies of the *tailless* (*tll*) terminal gene (*4*) had striking effects on the tor^{splc} phenotype. Firstly, reduction to a single dose of the wildtype tll^{+} gene in the mother (genotype: tor^{splc}/tor^{splc}; $tll^{1}/+$) was sufficient to substantially rescue the central region in her offspring (compare Table 1, B and Ji), indicating that loss-of-function mutations in *tll* can act as dominant maternal suppressors of tor^{splc}(*20*). This is the first evidence that the *tll* gene has a maternal function in addition to its zygotic function (*21*), analogous to the dual maternal-zygotic activity of the gap gene *hunchback* (*22*). More remarkably, when tor^{splc}/tor^{splc}; $tll^{1}/+$ mothers were mated to $tll^{1}/+$ fathers, there was complete rescue of the central region in the 25% of the embryos that were homozygous for the tll^{1} mutation (Fig. 1F, Table 1Jii), suggesting that a further reduction in *tll* gene

function zygotically could completely rescue the central defects caused by hyperactivity of the *tor* gene product. This provides direct evidence that the *tor* gene product acts through, or in concert with, *tll* to promote terminal development and repress central development, as was suggested previously (*4*).

We have shown that the *tor* gene product possesses both positive and negative functions in establishing the anterior-posterior embryonic pattern: it promotes terminal development and represses central development. Initially, it is likely to be present throughout the embryo, but its activity must be restricted to the termini early in development in order that the development of central structures not be repressed. The *tll* gene product mediates both the positive and negative *tor* functions. When *tll* is ectopically activated in the central region (as in tor^{splc} embryos) loss of the thorax and abdomen occurs. When functional *tll* product is eliminated from embryos produced by tor^{splc} mothers, the persistent *tor* product cannot exert its repressive effect in the central region and normal thoracic and abdominal development ensues. The absence of tll^{+} function from the termini of these centrally rescued embryos prevents the the positive action of *tor* in terminal development, resulting in the absence of terminal structures.

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6. The *spliced*^{RL3} allele was induced with ethyl methanesulfonate (EMS) and provided to us by T. Schüpbach. It maps meiotically to the same location as *torso* (T. Schupbach and E. Wieschaus, *Genetics* (in press)).
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8. Our cytoplasmic transplantation experiments are fully consistent with this conclusion (Fig. 2, G and H). Final, irrefutable proof that *spliced* is a *tor* allele lies outside the scope of the present work. Further evidence will come from reversion of the *spliced* allele resulting in reduced or null *tor* mutations. We are currently conducting reversion mutageneses of *tor*^{splc} using the point mutagen n-nitroso n-ethyl urea (ENU) in order to demonstrate that *tor*$^{-}$ revertants result. Direct evidence regarding the nature of the *spliced* allele will require molecular analysis of the *tor* gene, its mutant alleles, and its products.
9. Three doses of *tor*$^{+}$ do not result in an abnormal segmentation pattern (Table 1H). The *tor* locus is not haplo-insufficient since embryos from hemizygous mothers retain a normal segmentation pattern (Table 1I).

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13. The construct used comprises the upstream enhancer element and zebra stripe element of the *ftz* regulatory region (*12*) as well as the transcribed but untranslated *ftz* leader region, fused to the *E. coli lacZ* gene at codon number two of the *ftz* open reading frame. The code name of the construct is *EΔ-669*. It was constructed and inserted on the third chromosome via P element-mediated transformation by C. Dearolf, J. Topol and C. Parker (submitted for publication). Staining for ß-galactosidase activity was as in (*12*) with minor modifications.

14. In addition to deleting complementary regions of the fate map, hypoactive and hyperactive *tor* mutations have opposite effects on the spatial distribution of positional values along the embryonic anterior-posterior axis. While the remaining segmentation pattern in progeny of *tor* hypomorphs is expanded toward the anterior and posterior poles of the embryo, hypermorphic mothers produce embryos in which the *ftz* stripes corresponding to the central thoracic and abdominal region appear compressed toward the center, consistent with the loss of the central structures.

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18. In either case, since tor^{splc} behaves as a hypermorphic allele upon addition or removal of doses of the wildtype tor^{+} gene, the tor^{+} gene product must be present at stages 1 to 2 in the central region of embryos produced by wildtype mothers. If the tor^{+} gene product was not normally present centrally in wildtype, but was ectopically expressed there in progeny of tor^{splc} mothers, then tor^{splc} should behave as a neomorphic mutation rather than a hypermorphic one. The simplest molecular model explaining how addition or removal of the wildtype tor^{+} product causes a change in the central region of tor^{splc} embryos, proposes that auto-activation of tor^{+} normally occurs. Thus, the presence of constitutive or hyperstable tor^{splc} product centrally would increase the activity or stability of the tor^{+} gene product in this region.

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20. That this suppression is maternal is shown by the fact that we see no rescue of the central defect in embryos produced by tor^{splc}/tor^{splc};+/+ females mated to tll^{1}/+ males. This dominant maternal suppression of the tor^{splc} allele provides us with a selective screen (maternal fertility) for transposon tagging of *tor*, *tll* and any other locus that can be mutated to suppress tor^{splc}.

21. The previous test for maternal *tll* gene activity (*4*) addressed whether extra maternal doses of tll^{+} could rescue the mutant phenotype of tll^{-} homozygous zygotes. Since the results were negative, they could not exclude the possibility that *tll* is both maternally and zygotically active. Together with the present results, these data suggest that two maternal doses of tll^{+} might have been insufficient to raise the amount of activity in the zygote to a level capable of rescuing the tll^{-} zygotic phenotype. Alternatively, there may be functional differences between the maternal and zygotic *tll* activities.

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27. We thank T. Schüpbach for providing the *tor*^{splc} allele, for communicating unpublished data and for helpful discussions. We thank E.B. Lewis for providing *Dp(2;3)P32*, M. Ashburner for *Df(2R)CA58*, and J. Topol, C. Dearolf and C. Parker for providing the *ftz*-ß-gal *EΔ-669* transformed line. P. Sternberg, S. Lewis and D. Mathog provided critical comments on the manuscript. T.R.S. is supported by a junior postdoctoral fellowship from the American Cancer Society (California Division), S.R.H. by a predoctoral traineeship from the NIH, USPHS GM07616. This research was funded by USPHS research grant HD23099 to H.D.L.

TABLES

Table 1. Phenotypic and gene dosage analysis. The number of abdominal segments is expressed to the nearest half-segment. In all pairwise comparisons cited in the text, the differences are significant at better than or equal to the P=0.02% level by using the nonparametric Wilcoxon rank sum test (*24*). Rearrangement breakpoints in the *tor* region are as follows: *Dp(2;3)P32*: 41A - 44C/D (*25*). *Df(2R)CA58*: 43A3/4 - 43F8 (*26*). In (J), part [i] presents data for the tll^1/+ and +/+ heterozygous embryos (75%) and part [ii] for tll^1/tll^1 embryos (25%) that were recognized by the absence of termini.

TABLE 1

Female Genotype	Doses tor^{splc}	Doses tor^{+}	Temperature (°C)	No. of Abdominal Segments (+SD)	n
A. tor^{splc}/tor^{splc}; *Dp(2;3)P32/+*	2	1	25	0 (±0.0)	37
			22-23	0 (±1.0)	26
			18-19	5 (±1.5)	25
B. tor^{splc}/tor^{splc}	2	0	25	0 (±0.0)	34
			22-23	1.5(±1.5)	51
			18-19	6.5(±2.0)	22
C. tor^{splc}/+; *Dp(2;3)P32/+*	1	2	25	5 (±1.5)	30
			22-23	6.5(±1.0)	16
			18-19	7.5(±1.0)	13
D. tor^{splc}/+	1	1	25	7 (±1.5)	30
			22-23	8 (±0.0)	24
			18-19	8 (±0.5)	22
E. tor^{splc}/tor^{1}	1	0	25	8 (±0.0)	24
			22-23	8 (±0.0)	29
			18-19	8 (±0.0)	22
F. tor^{splc}/tor^{PM51}	1	0	25	8 (±0.0)	41
G. tor^{splc}/*Df(2R)CA58*	1	0	25	8 (±0.5)	28
			22-23	8 (±0.0)	24
			18-19	8 (±0.0)	18
H. +/+; *Dp(2;3)P32/+*	0	3	25	8 (±0.0)	25
I. +/*Df(2R)CA58*	0	1	25	8 (±0.0)	32
			18-19	8 (±0.0)	39
J. tor^{splc}/tor^{splc}; tll^{1}/+ [i]	2	0	25	2.5(±1.5)	35
			22-23	3 (±2.0)	56
[ii]	2	0	22-23	7 (±0.0)	19

Table 2. Results of cytoplasmic leakage and transplantation experiments. Leakage and transplantation were as described in (*15*). However, instead of analyzing the embryonic cuticle, the embryos were allowed to develop for 4 - 5 hours at 25^{o}C following treatment. They were then assayed for *ftz*-ß-galactosidase expression (*13*). n, number of stained embryos examined. -, not applicable. ND, not done. [1], stripe 1 was shifted anteriorly, as expected (*2,15*). [2], stripes 2 through 5 were partially or completely restored. [3], stripe 7 was partially or completely restored.

TABLE 2

Treatment	Host			Donor			n	Rescue
	Maternal Genotype	Developmental Stage	Position (% Egg Length)	Maternal Genotype	Developmental Stage	Position (% Egg Length)		
None	*tor*^{splc}	1,2	-	-	-	-	76	none
		3,4	-	-	-	-	77	none
Leakage	*tor*^{splc}	1,2	90-100	-	-	-	54	none[1]
		3,4	90-100	-	-	-	ND	ND
Leakage	*tor*^{splc}	1,2	40-60	-	-	-	77	none
		3,4	40-60	-	-	-	73	11 (15%)[2]
Leakage	*tor*^{splc}	1,2	0-10	-	-	-	130	none
		3,4	0-10	-	-	-	39	none
Transplant	*tor*^{PM51}	1,2	0-10	wildtype	3,4	40-60	29	none
Transplant	*tor*^{PM51}	1,2	0-10	*tor*^{splc}	3,4	40-60	30	7 (23%)[3]

FIGURES

Figure 1: Phenotypes of wildtype and mutant embryos shown in dark field. **(A)** Wildtype. For a detailed description of the cuticular pattern, see (*23*). M, mouthhooks; T1-3, thoracic segments; A1-8, abdominal segments; Te, telson; Fk, filzkörper. **(B)** Null *torso* phenotype (maternal genotype: tor^{1}/tor^{1}). Note the reduction in pharyngeal head skeleton anteriorly (arrow). Posteriorly, the embryo ends with a patch of denticles corresponding to A8 (arrow with star), and lacks the telson. See (*1*) for detailed description. **(C)** Weak tor^{splc} phenotype (maternal genotype tor^{splc}/tor^{splc}) seen in embryos that develop at 19°C. Note the reduction or loss of abdominal segments 2, 3 and 4. **(D)** Intermediate tor^{splc} phenotype [maternal genotype as in **(C)**] seen when embryos develop at 22.5°C. Note absence or reduction of abdominal segments 1 through 5. This embryo exhibits more segments than the average for the intermediate class. **(E)** Strong tor^{splc} phenotype [maternal genotype as in **(C)**] produced when embryos develop at 25°C. Note undifferentiated cuticle bag lacking T1 through A8 segments but possessing mouthhook material and uninvoluted pharynx (arrow) anteriorly and telson with filzkörper posteriorly. **(F)** Rescue of tor^{splc} phenotype by reduction of tll^{+} to single dose in mother (maternal genotype: tor^{splc}/tor^{splc}; $tll^{1}/+$) and to zero doses in the embryo (embryonic genotype: $tor^{splc}/+$; tll^{1}/tll^{1}). Note the absence of terminal structures [symbols as in **(B)**] but complete rescue of thorax and abdomen. Embryo developed at 22.5°C. In **(A-F)** anterior is towards the top of the page; in **(A, C, D** and **E)** ventral is to the left, and in **(B** and **F)** the entire view is of the ventral side. Methods were as in (*16*). For quantitative data and statistical analysis see Table 1.

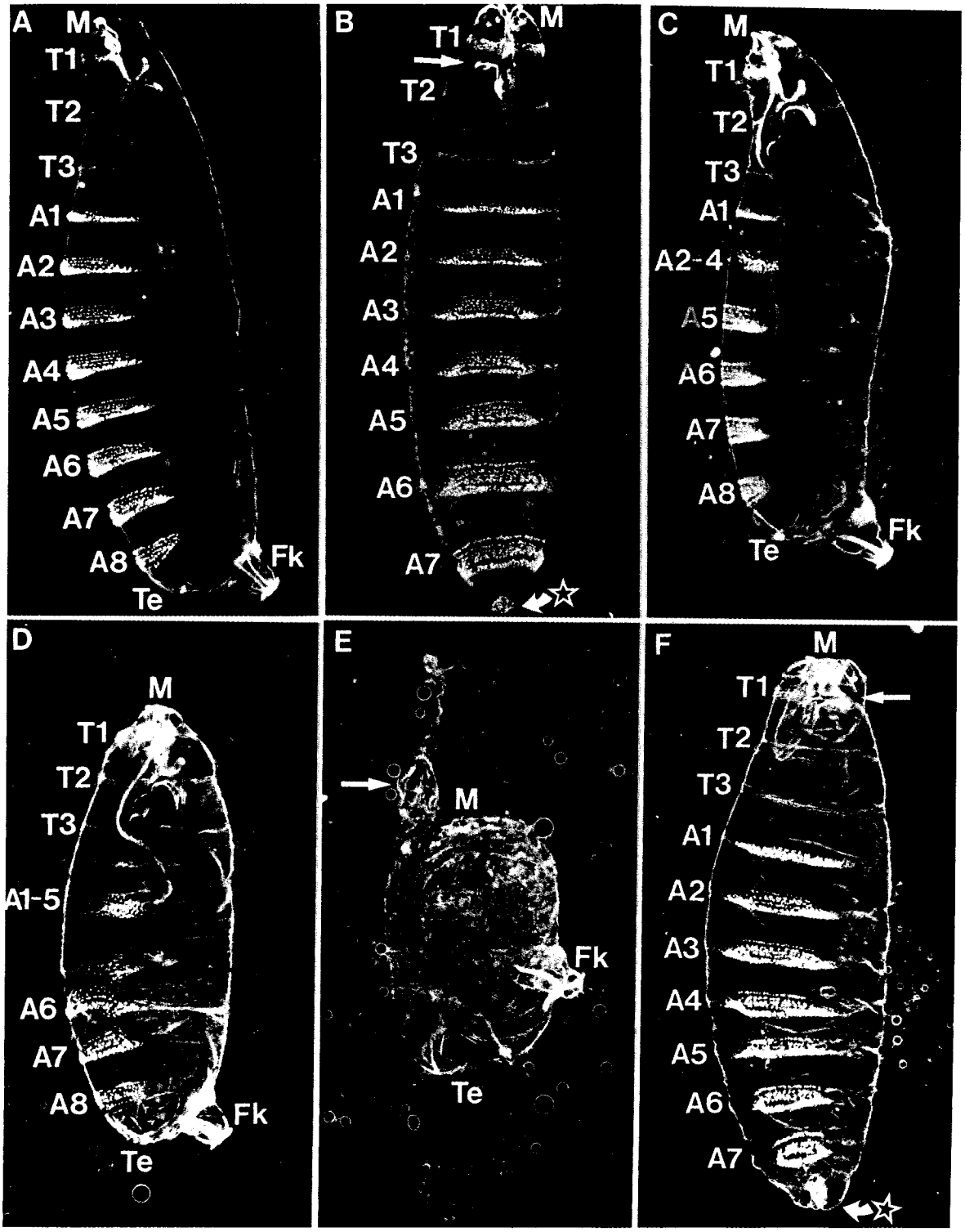
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Figure 2: Expression of a *ftz*-ß-gal construct (*13*) visualized with differential interference contrast optics in embryos produced by wildtype and tor^{splc} mothers, and in rescued embryos produced by mutant females. The *ftz* stripes are numbered from anterior to posterior. Embryos in **(A)** - **(G)** are at the germ band extended stage, while embryo in **(H)** has a retracted germband. **(A)** Wildtype. Seven stripes are present at the extended germ band stage, with the seventh stripe broader than the anterior six (*10-12*). **(B)** Embryo produced by homozygous tor^{splc} females. This embryo was raised at 19°C, and shows a loss of stripe 4 and a slight broadening of stripe 7. **(C)** Embryo raised at 22.5°C that shows compression of stripes 2, 3 and 5 (asterisk) and a broad posterior stained region, presumably due to the expansion of stripe 7 and its fusion with stripe 6. **(D)** Embryo raised at 25°C showing stripe 1, absence of stripes 2-5, and a wide posterior band of expression. **(E)** Control embryo for the cytoplasmic leakage experiment, raised as in **(D)**. The asterisk marks the remains of the compressed stripes 2, 3 and 5. **(F)** Embryo from which cytoplasm was leaked from between 40 and 60% egg length ventrally. Note the restoration of distinct stripes 2, 3, 4 and 5 in the central region, something never seen in unleaked controls. Crosses in **(B-F)** were tor^{splc}/tor^{splc}; *EΔ-669/EΔ-669* females to *EΔ-669/EΔ-669* males. Note that germ band extension is often incomplete in embryos produced by tor^{splc} females. **(G)** Embryo produced by tor^{PM51}/tor^{PM51}; *EΔ-669/EΔ-669* mother, into the posterior pole of which stage 4 wildtype central cytoplasm was injected. Stripe 7 is missing and stripes 4 through 6 are spaced further apart than in wildtype [compare **(A)**]. Germ band retraction does not occur normally in embryos produced by tor^{-} mothers. **(H)** Embryo from mother as in **(G)**, but into the posterior pole of which central cytoplasm from tor^{splc}/tor^{splc}; *EΔ-669/EΔ-669* stage 4 embryos was injected. Note the appearance of stripe 7, the correct spacing of the more anterior stripes, and the restoration of the ability to undergo normal germ band retraction. In **(A)** - **(D)**, **(G)** and **(H)** the view is a sagittal optical section with ventral to the left and anterior

towards the top of the page, in (**E**) and (**F**) the view is a horizontal optical section with anterior towards the top of the page.

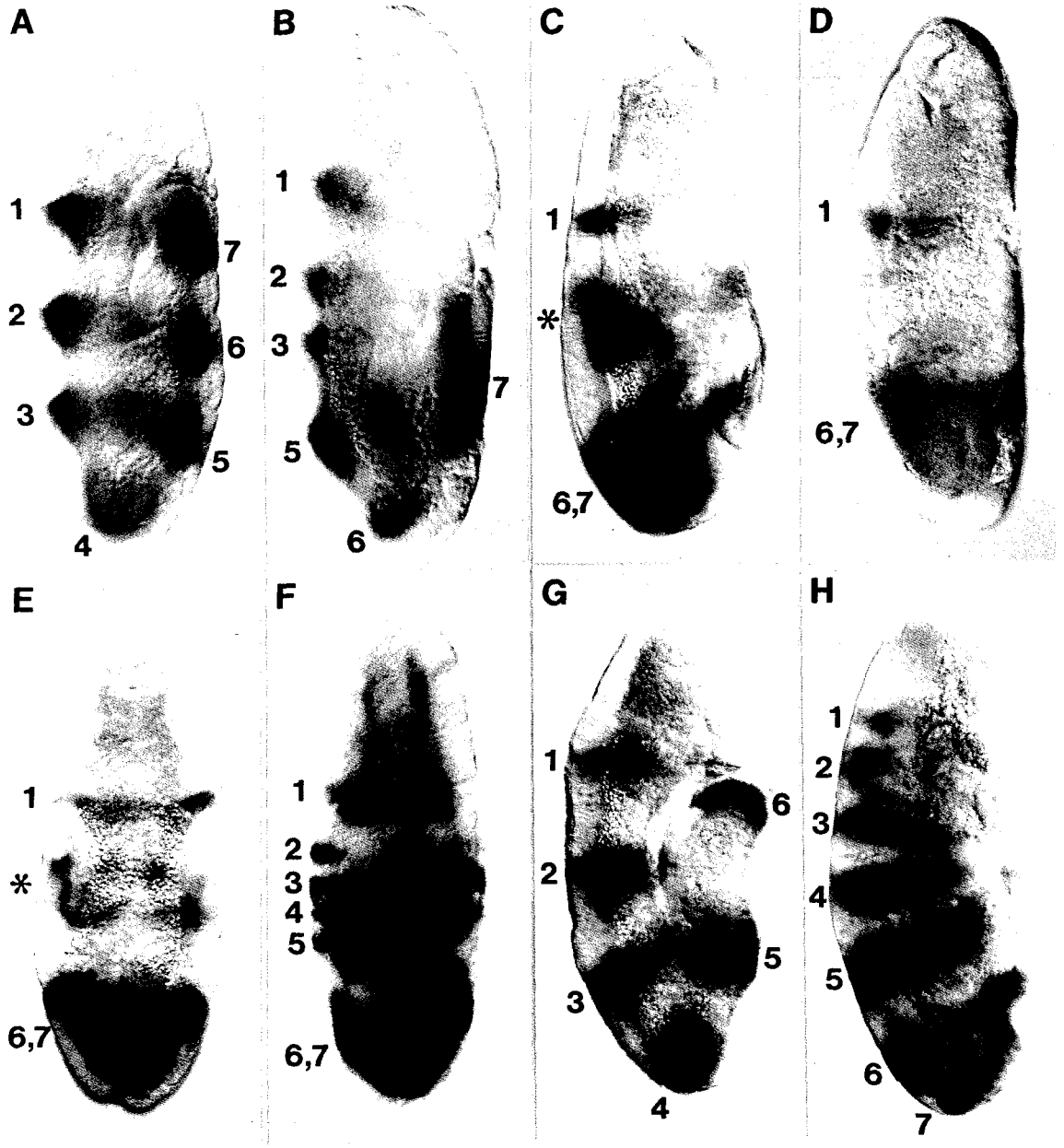
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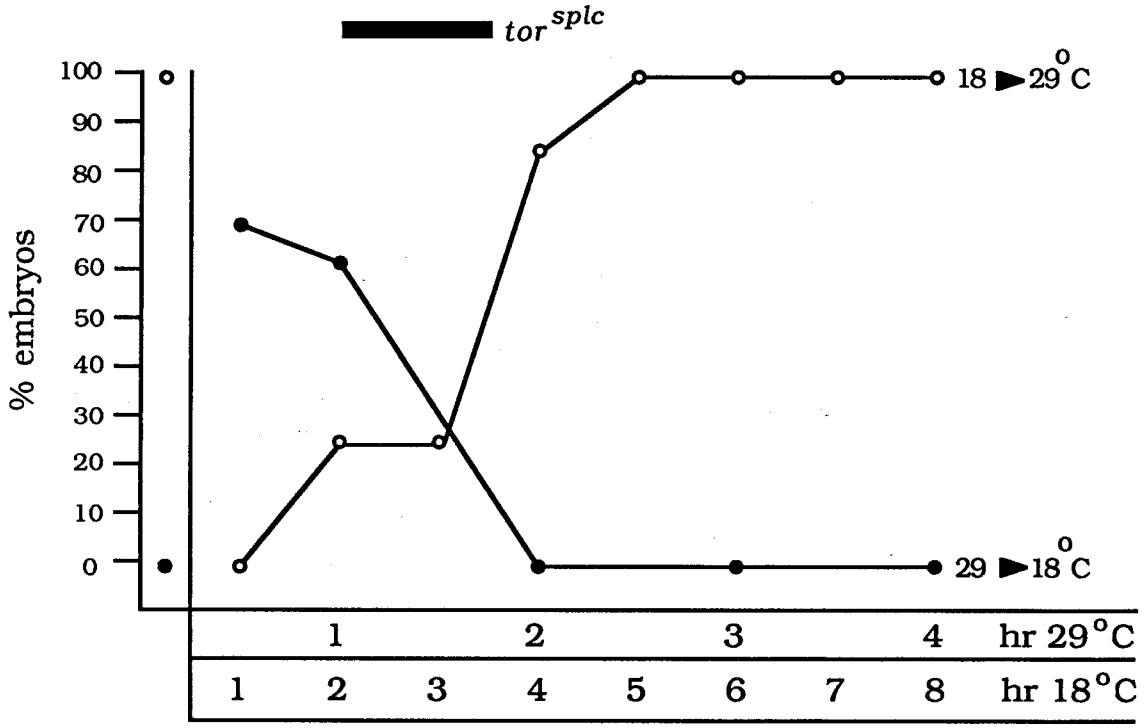
Figure 3: Temperature-sensitive period of tor^{splc}. Each point on the ordinate represents the percentage of the embryos (produced by homozygous tor^{splc} mothers) showing one or more abdominal segments. Open circles, shifts from 18 to 29°C, filled circles: shifts from 29 to 18°C. For each point, an average of 39 embryos was scored (Range, 15 to 80). Embryos were shifted at the age indicated on the abscissa. The control values for unshifted embryos that were allowed to develop continuously at 18 or 29°C are shown in the column to the left of the shift curves. Cellularization occurs at approximately 2 hours at 29°C (4 hours at 18°C). As indicated by the filled bar, the temperature sensitive period of tor^{splc} can be seen to largely span stages 3 and 4 (*16*). Experiments were performed as in (*1*).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