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Seeing Is Believing: The Bicoid Morphogen Gradient Matures

Review

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Although *Cell* has a long history of publishing some of the most significant advances in developmental biology, the back to back papers by Driever and Nüsslein-Volhard on the role of the Bicoid gradient in patterning the *Drosophila* embryo stand out as the first molecular demonstration of two of the longest standing concepts of the field, namely localized cytoplasmic determinants and morphogen gradients. Here we discuss the impact of this ground-breaking work and review recent results on *bicoid* mRNA localization and the dual role of Bicoid as a transcription and translation factor.

The idea of localized cytoplasmic determinants originates from the early 1900s, when experimental embryologists, such as Conklin and Wilson, proposed that the eggs of a number of organisms contain specialized regions of cytoplasm that direct the cells that inherit them to adopt a particular fate. For example, Conklin noticed that Ascidian eggs contain a yellow crescent that segregates into the muscle lineage and a gray crescent that partitions into the notochord, suggesting that these two regions contain factors that determine muscle and notochord cell fate, while Wilson showed that the asymmetric inheritance of the polar lobe of molluscan embryos determines which cells give rise to mesoderm (Conklin, 1905a, 1905b; Wilson, 1904). Indeed, the existence of such cytoplasmic determinants had first been conclusively demonstrated in 1974 by Illmensee and Mahowald, who proved that the pole plasm at the posterior of the *Drosophila* egg contains the germline determinants by showing that it induced the formation of ectopic or pole cells (primordial germ cells) when transplanted to the anterior of the egg (Illmensee and Mahowald, 1974). The nature of these cytoplasmic determinants was still a mystery, however, as the molecules responsible had not been identified.

The concept of morphogen gradients also dates back to the beginning of the last century, when it was observed that particular regions of eggs and embryos have long-range effects on the patterning of the adjacent tissue. For example, Thomas Hunt Morgan postulated that gradients of "formative substances" underlie many regeneration phenomena (Morgan, 1901), while experiments on sea urchins by Boveri and Hörstadius suggested that the pluteus larva was patterned by opposing animal and vegetal gradients (Boveri, 1901; Hörstadius, 1935). These early models were rather vague, however, and the gradients were often assumed to be gradients of metabolic activity (Child, 1941). The concept was refined by Crick, who calculated that a molecule produced from a localized source could diffuse to form a gradient over a field of about 50 cells in a few hours (Crick, 1970). In conjunction with Wolpert's French flag model of positional information (Wolpert, 1969), this led to the idea that a diffusion gradient could generate a long-range pattern if the cells respond to different threshold concentrations of the morphogen in distinct ways. At about the same time, results in several systems provided better evidence for the existence of such morphogen gradients. For example, Klaus Sander's elegant experiments on the eggs of the leaf hopper, *Euscelis*, indicated that the posterior cytoplasm is the source of a "morphogenetic gradient" that specifies position along the anterior-posterior axis (Sander, 1976). This cytoplasm is conveniently marked by endosymbiotic bacteria, and this allowed Sander to move the cytoplasm to more anterior regions of the egg, where it induces the formation of a mirror image pattern of abdominal segments flanked by more anterior thoracic structures. Another system that was most simply explained by a gradient model was the anterior-posterior axis of chick wing bud, where the results of grafting experiments suggested that the zone of polarizing activity at the posterior margin of the bud is the source of a morphogen that specifies the different digits at distinct threshold concentrations (Saunders, 1972; Tickle et al., 1975). These models remained controversial, however, largely because there was no direct evidence for such morphogens, despite many attempts to identify the molecules involved. As Lewis Wolpert stated, " One is acutely conscious of the absence of the physiological and molecular basis of positional information. But unless the right questions are asked, one has little hope of finding out how genetic information is interpreted in terms of spatial patterns."

When Christiane Nüsslein-Volhard started working on *Drosophila* in Walter Gehring's laboratory, she decided that the elusive molecules involved in patterning might be more easily identified by a genetic approach rather than a biochemical one, and she started analyzing maternal-effect mutations that affect axis formation in the embryo. She identified the first allele of *dorsal*, which abolishes all dorsal-ventral polarity in the embryo, and characterized the phenotype of *bicaudal*, in which the head and thorax are replaced by a second mirror image abdomen and telson (Nüsslein-Volhard, 1977; Nüsslein-Volhard et al., 1980). Promising though these mutants seemed, she and Eric Wischaus realized that it was going to be very difficult to understand a complex process such as axis formation without a more systematic approach. They therefore set the question of maternal signals to one side for a while and performed saturation mutageneses for zygotic mutants that affect the pat-

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terning of the embryonic cuticle in their joint lab at the EMBL in Heidelberg (Nüsslein-Volhard and Wieschaus, 1980). These screens, for which they were awarded the Nobel prize, revealed that the anterior-posterior axis of the embryo is patterned by a hierarchy of gap genes, pair rule genes, and segment polarity genes. Subsequent molecular analysis revealed that all of the gap and pair rule genes encode transcription factors that are expressed in patterns that roughly correspond to the regions of the embryo that are missing in each mutant (Akam, 1987). For example, the gap gene *hunchback* is transcribed in the anterior 50% of the embryo and is required for the development of a large contiguous region spanning the posterior head and the entire thorax, whereas the pair rule gene, *even-skipped*, comes on in seven stripes that predict where the even numbered segments will form (Frasch et al., 1987; Tautz et al., 1987).

While this beautiful analysis provided the basis for understanding how the embryo is subdivided into segments, it left a major question unanswered, which was how the gap genes are turned on in the first place. Since these genes come on when zygotic transcription begins during the syncytial blastoderm stage, they cannot be regulated by other upstream zygotic genes, strongly suggesting that they are under the control of localized maternal signals already present in the egg. The idea that the key positional cues are provided maternally was further reinforced by some elegant cytoplasmic transplantation experiments by Hans-Georg Frohnhöfer and Ruth Lehmann, who were the first graduate students to join Christiane Nüsslein-Volhard in her new laboratory in Tübingen (Frohnhöfer et al., 1986). They set out to ask whether the egg contained localized cytoplasmic determinants by pricking it in various places and allowing about 5% of the cytoplasm to leak out. Although removal of cytoplasm from most regions had a negligible effect on patterning, anterior pricking gave rise to embryos in which the head and thorax were replaced by a duplication of the posterior telson, whereas posterior pricking resulted in embryos that failed to form an abdomen or pole cells. Furthermore, transplantation of the anterior cytoplasm into other regions of the embryo induced the formation of head structures at the site of injection, with thoracic structures on either side (Frohnhöfer and Nüsslein-Volhard, 1986). Thus, the A-P axis of *Drosophila* appeared to be specified by localized cytoplasmic determinants at the anterior and posterior of the egg, and the anterior determinant had some of the expected properties of a morphogen because it induced head structures at high concentrations and thoracic structures at lower ones.

Because these determinants must be deposited in the egg during oogenesis, these results switched attention back to the mother's contribution to axis formation, and the Nüsslein-Volhard group in Tübingen and Eric Wieschaus and Trudi Schüpbach in Princeton embarked on a second set of saturation screens, this time for maternal-effect mutants, i.e., mutations that cause no obvious defects in the homozygous mutant flies themselves but that disrupt the patterning of the embryos laid by mutant mothers (Nüsslein-Volhard et al., 1987; Schüpbach and Wieschaus, 1986). These screens identified a large number of maternal genes, but most of these could be classified into four groups of genes whose mutants produce similar or identical phenotypes (Nüsslein-Volhard et al., 1987). This contrasted with the results of the zygotic screens, which revealed a large number of different phenotypes, and suggested that the genes in each maternal class act together to produce a single spatial cue in the embryo. More importantly, mutants in the anterior and posterior groups gave the same phenotypes as pricking the corresponding pole of the egg. This led to the prediction that the genes in each of these groups direct the production of a localized determinant and that they might therefore provide a molecular handle to identify what these mysterious activities actually were.

Although it is not directly relevant to the Bicoid papers, it is worth mentioning that the other two classes of maternal genes, the terminal and dorsal groups, were even more surprising since they were not predicted by the cytoplasmic transplantation experiments and indicated that some of the maternal information is provided to the egg in another form. It later transpired that the terminal genes generate an extracellular signal at the two ends of the egg that acts through the Torso receptor to induce the formation of the most terminal structures, the acron and telson, while the dorsal group gene products constitute a second signaling pathway that specifies ventral fates (St Johnston and Nüsslein-Volhard, 1992). Indeed, the discovery of the terminal group explained the strange behavior of the telson. Even though the telson is the most posterior structure in the embryo, it is not affected by the removal of the posterior cytoplasm, but a duplicated telson forms at the anterior when the anterior cytoplasm is removed. The analysis of the double mutants showed that the terminal pathway specifies the two ends of the embryo independently of the anterior and posterior systems, but the anterior determinant directs this region to form acron instead of telson (Nüsslein-Volhard et al., 1987).

Hans-Georg Frohnhöfer was given the job of analyzing the maternal genes involved in anterior patterning for his PhD thesis and performed a beautiful series of experiments that showed that *bicoid* is the key gene in this group (Frohnhöfer and Nüsslein-Volhard, 1986, 1987). (1) Strong *bicoid* alleles produce a complete loss of head and thorax and the formation of a duplicated anterior telson, whereas mutants in the other genes in this group, *exuperantia* and *swallow*, cause only a partial loss of the head and an expansion of the thorax. (2) *bicoid* mutants can be completely rescued by the injection of wild-type anterior cytoplasm, suggesting that they lack the anterior determinant, but are still able to respond to it normally if it is supplied by transplantation. (3) The size of the presumptive head region is proportional to the *bicoid* gene dosage in the mother since the cephalic furrow is shifted posteriorly in embryos from females that carry extra copies of *bicoid* and is shifted anteriorly in embryos from *bicoid* heterozygous mothers. This indicates that the concentration of the anterior determinant depends on the amount of *bicoid* gene product. (4) *exuperantia* and *swallow* mutants still contain some anterior determinant activity, but it is no longer localized correctly since the removal of the anterior cytoplasm does not make the phenotype any worse. This led to the proposal that these genes are required for the correct

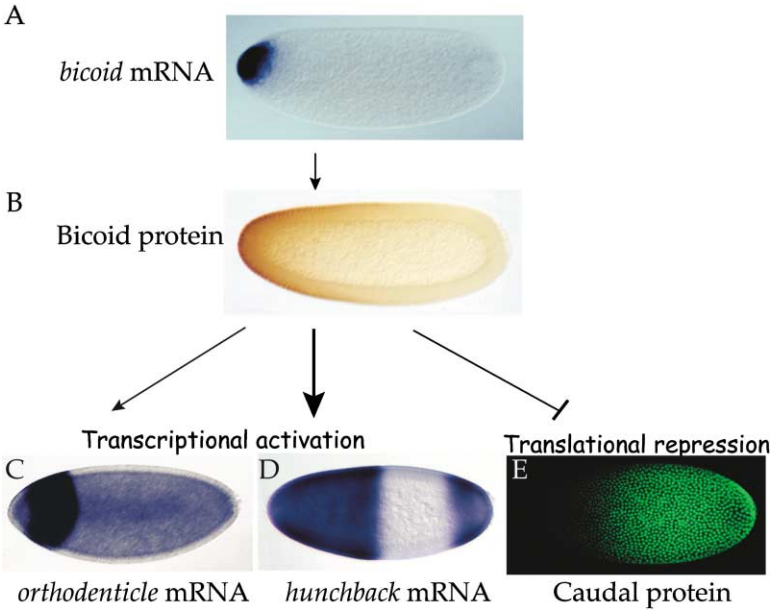

Figure 1. The Bicoid Morphogen Gradient

(A) *bicoid* mRNA is tightly localized to the anterior cytoplasm of the egg.

(B) Bicoid protein is translated after fertilization and diffuses posteriorly to form a morphogen gradient that extends over 60% of the length of the embryo.

(C) High concentrations of Bicoid activate the transcription of *orthodenticle* in the anterior of the embryo (image courtesy of Julien Royet).

(D) *hunchback* transcription is activated at a lower threshold concentration of Bicoid, and it is therefore expressed throughout the anterior half of the embryo. (Note that the posterior stripe of *hunchback* is under separate regulation from the terminal system.)

(E) Bicoid represses the translation of *caudal* mRNA to produce a posterior to anterior gradient of Caudal protein (image courtesy of Rolando Rivera-Pomar).

localization of the *bicoid*-dependent activity to the anterior pole.

The *bicoid* locus was identified soon afterwards, through its homology with the pair-rule gene *paired*, and Hans-Georg's predictions were spectacularly confirmed (Berleth et al., 1988; Frigerio et al., 1986). In situ hybridizations revealed that *bicoid* mRNA is strictly localized to the anterior cytoplasm of the freshly laid egg, in exactly the position one would expect for the localized anterior determinant from the pricking experiments (Figure 1A). Furthermore, both *exuperantia* and *swallow* mutants disrupt the anterior localization of the mRNA during oogenesis and give a uniform or shallow gradient of mRNA in the freshly laid egg. Thus, *bicoid* mRNA fulfilled all of the criteria for the first localized cytoplasmic determinant. It is worth mentioning, however, that the final proof of this requires the demonstration that the mRNA is not only necessary, but sufficient to induce anterior structures, and this only came in 1990, when Wolfgang Driever, Vivan Siegel (the former Editor of this journal), and Christiane Nüsslein-Volhard showed that in vitro transcribed *bicoid* mRNA can induce head structures, wherever it is injected in the egg (Driever et al., 1990).

Although these results proved that *bicoid* mRNA is the localized anterior determinant, they did not reveal how it exerted its long-range effects on patterning. In one model, Bicoid would simply specify the anterior regions of the head, and the intermediate positional values of the posterior head and thorax would arise from downstream interactions between this anterior region and most posterior ones, which are specified independently. Alternatively, Bicoid protein could act as a morphogen by diffusing away from its anterior source to form a gradient that specifies the various regions of the head and thorax at different threshold concentrations. The two papers reprinted in the supplement to this issue of *Cell* provide the definitive answer to this question and demonstrate that Bicoid protein forms a morphogen gradient (Driever and Nüsslein-Volhard, 1988a, 1988b). The key was the development of very clean polyclonal antibodies against Bicoid protein, which reveal that it forms an exponential gradient that extends from an anterior high point over more than 50% of the embryo (Figure 1B). More importantly, the examination of the Bicoid distribution in various mutants shows that the shape of the gradient correlates perfectly with the final cuticle pattern. For example, *exuperantia* mutant embryos have a shallow gradient with a much lower anterior maximum, and the resulting larvae lack anterior head structures and have an expanded thorax. Thus, the anteriormost structures are not required for the formation of the thorax, which is specified directly by intermediate concentrations of Bicoid. This rules out the relay model in which Bicoid acts only at high concentrations to define the most anterior positional values, which then induce the more posterior regions through downstream inductive interactions. The direct visualization of the Bicoid gradient and the demonstration that Bicoid acts autonomously at different thresholds to pattern the anterior of the embryo therefore ended over 80 years of speculation over whether morphogen gradients exist and the nature of the molecules involved. This work represents the crowning achievement of one of the most impressive series of experiments in the history of developmental biology and vindicates Nüsslein-Volhard's vision that the genetic approach was the right way to address the question of pattern formation.

How the Bicoid Gradient Works

Although these two papers showed that Bicoid is a morphogen, they did not explain how the gradient is interpreted, nor how the thresholds are set to produce different responses at increasing Bicoid concentrations. However, the presence of a homeodomain in Bicoid suggested that it functions as a transcription factor, and the obvious targets were the gap genes that are expressed in the anterior part of the embryo. Indeed, it was demonstrated a year later that Bicoid regulates the

anterior expression of *hunchback* directly by binding to multiple sites in its upstream regulatory region (Driever and Nüsslein-Volhard, 1989a; Struhl et al., 1989). In vitro binding studies showed that three of these sites bind Bicoid with high affinity, whereas other sites that match the consensus binding sequence less well bind with lower affinity. Furthermore, constructs that contain multiple copies of the high-affinity sites upstream of a heterologous promoter are expressed in a large anterior domain that resembles that of *hunchback*, whereas similar constructs with low-affinity sites are expressed in a much smaller anterior region (Driever and Nüsslein-Volhard, 1989b). These experiments suggested a very simple model in which the thresholds for the response to Bicoid are determined by the affinity of the Bicoid binding sites in the upstream regions of its target genes. The high-affinity sites in *hunchback* can bind Bicoid at low concentrations and drive expression throughout most of the Bicoid gradient (Figure 1C). In contrast, another hypothetical target gene, called "gene X," with low-affinity sites would be expressed in a more restricted anterior domain, where Bicoid levels are high. This model was so convincing that it was almost ten years before it was actually put to the test by identifying gene X and analyzing its regulation. However, in 1998, Gao and Finkelstein demonstrated that Bicoid directly activates *orthodenticle* (*otd)* in an anterior domain that is about half the size of that of *hunchback* and found that this anterior expression depends on three low-affinity Bicoid binding sites in the *otd* regulatory region that match the consensus sequence at only six out of nine positions (Figure 1D) (Gao and Finkelstein, 1998).

As is often the case, this simple view of the Bicoid morphogen gradient has become more complicated as we have learned more about it. For example, it turns out that Bicoid is not sufficient to generate anterior pattern because it requires Hunchback as an essential cofactor. Although zygotic *hunchback* expression is under the direct control of Bicoid, it is also expressed during oogenesis to produce a maternal mRNA that is uniformly distributed in the egg (Schröder et al., 1988). This maternal RNA is translationally repressed in the posterior half of the embryo by the posterior determinant, Nanos, and this restricts the expression of maternal Hunchback protein to a very similar anterior domain to that of the zygotic protein under Bicoid control (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989). Furthermore, the removal of both maternal and zygotic Hunchback results in embryos that lack all anterior structures, even though they form a normal Bicoid gradient, and this phenotype arises because Bicoid target genes, such as *orthodenticle*, are no longer expressed (Lehmann and Nüsslein-Volhard, 1987; Simpson Brose et al., 1994). Thus, transcriptional activation by Bicoid depends on Hunchback, and this regulation is probably direct because the upstream regulatory regions of both *orthodenticle* and *hunchback* contain Hunchback binding sites. However, these results do not affect the role of Bicoid as a morphogen since Hunchback is present at uniform levels in the anterior of the embryo, and the domains of anterior gap gene expression are therefore defined by the Bicoid gradient alone.

Morphogens after Bicoid

The discovery of the Bicoid gradient made morphogens fashionable again, and over 700 papers have been published on this subject in the following 15 years. A year after the Bicoid papers, the dorsal-ventral axis of *Drosophila* was shown to be patterned by a gradient of another transcription factor, Dorsal (which is the product of the first maternal-effect gene that Nüsslein-Volhard identified) (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). Unlike Bicoid, which forms a gradient by diffusing through the cytoplasm of the syncytial blastoderm embryo, the Dorsal gradient is a gradient of nuclear localization and is under the control of an extracellular signal, cleaved Spätzle, which activates the Toll receptor to release Dorsal from a cytoplasmic anchor. Indeed, the shape of the Dorsal gradient seems to be largely determined by the extracellular distribution of Spätzle, which is inferred to form a gradient on the ventral surface of the egg (Morisato and Anderson, 1995).

Drosophila is unusual in that much of the early patterning occurs before cellularization, and this makes it possible for transcription factors to form gradients by diffusion. Indeed, the transcription factors encoded by the gap genes also appear to form morphogen gradients by diffusing away from their transcriptional domains. In most other systems, however, patterning occurs in fields of cells, and gradients of morphogens must therefore form extracellularly. In this respect, the control of the Dorsal gradient by Spätzle provides a more relevant paradigm, and all of the subsequent morphogens have proved to be secreted signaling molecules, such as DPP, Wingless, Spitz, and Hedgehog in *Drosophila*, Activin in *Xenopus*, and Sonic Hedgehog in the mammalian neural tube (Neumann and Cohen, 1997).

Although one might expect the mechanisms that establish the primary body axis to be highly conserved, *bicoid* homologs have only been found in closely related species of flies, and the gene is thought to have evolved quite recently in this lineage through the tandem duplication of the Hox3 gene, *zen*, which is present in only a single copy in the genome of the lower Diptera, such as the mosquito (Stauber et al., 1999). Recent work in the flour beetle, *Tribolium*, which also lacks *bicoid*, has shown that one of the *otd* homologs forms an anterior to posterior gradient of maternally encoded protein in the early embryo. Furthermore, double RNAi ablation of both maternal *otd* and *hunchback* mRNAs results in the loss of the entire head and thorax, a phenotype that resembles that of strong *bicoid* mutants in *Drosophila* (Schröder, 2003). Thus, the Bicoid gradient appears to have been a recent invention that has taken over the function of maternal Otd and Hb in more primitive insects.

Bicoid beyond Morphogens

Although Bicoid is an atypical morphogen recently invented by higher Diptera, it has become an important paradigm for two other areas of research: mRNA translational control and RNA localization.

Bicoid as a Translation Factor

Among the many hypotheses engendered by the Bicoid protein gradient was the proposal that Bicoid plays a role in translational control of *caudal* mRNA (Driever

and Nüsslein-Volhard, 1988a). *caudal* was previously identified as a maternally and zygotically expressed mRNA encoding a homeodomain protein (Mlodzik et al., 1985). Both the expression pattern and nature of Caudal had initially made it interesting as a potential patterning molecule. However, *caudal* mutants show only weak patterning defects and the gene would have been forgotten, had it not been for an interesting feature of *caudal:* although *caudal* mRNA is initially distributed uniformly throughout the embryo, Caudal protein forms a posterior to the anterior gradient during the syncytial stages of embryogenesis, before the onset of zygotic transcription (Figure 1E) (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987). This suggested that the Caudal protein gradient is formed by the spatial regulation of *caudal* mRNA translation, and the reciprocal gradient of Bicoid made it a strong candidate for such an activity. Indeed, the Caudal gradient does not form in *bicoid* mutant embryos, and the protein is found at high levels everywhere. This result led Driever and Nüsslein-Volhard to propose that Bicoid has a role, direct or indirect, in translational regulation of *caudal* (Driever and Nüsslein-Volhard, 1988a).

The curiosity that Bicoid was possibly a direct regulator of *caudal* mRNA translation prompted a series of experiments, both in cell culture assays and in vivo, which revealed that Bicoid regulates *caudal* mRNA translation via a segment of the 3′UTR of the mRNA (Rivera-Pomar et al., 1996; Dubnau and Struhl, 1996). Remarkably, not only does the Bicoid homeodomain bind the RNA directly to mediate repression (Rivera-Pomar et al., 1996), but the critical Lysine 9 in helix 3 of the homeodomain that confers its DNA binding specificity (Hanes and Brent, 1989; Treisman et al., 1989) is also essential for its RNA binding activity (Chan and Struhl, 1997). Amino acid 54 of the homeodomain and sequences adjacent to it are also required for translational repression, but not DNA binding, revealing that Bicoid interacts with DNA and RNA in different ways (Niessing et al., 1999; Niessing et al., 2000).

Bicoid has now become a paradigm for the study of translational control in development. In many organisms, the early stages of development are controlled by maternal mRNAs stored in the egg, and in the past decade, much progress has been made toward understanding the mechanisms underlying their translational silencing and activation (Wickens et al., 2000). *caudal* mRNA is one of these, and one question that applies to this mRNA as to many others is how the binding of a protein, such as Bicoid, to the 3′UTR of an mRNA can cause translational silencing. In a first step, Niessing et al. (1999) showed that Bicoid inhibits cap-dependent but not internal ribosome entry site (IRES)-dependent translation, suggesting inhibition of translation at the initiation step. Consistent with this, Bicoid was found to be associated with 7MeG-cap binding protein complexes in *Drosophila* extracts (Niessing et al., 2002). Cap-dependent translation requires assembly of a complex that recruits the small ribosomal subunit to mRNA (reviewed by Raught et al., 2000). This process is initiated by the binding of the 7MeG cap binding protein, eIF4e, which then recruits the translational scaffold protein eIF4G to the mRNA, allowing the subsequent binding of additional initiation factors and recruitment of the ribosomal subunits. Initiation of cap-dependent translation can be regulated through modulation of the crucial eIF4e:eIF4G interaction, by proteins mimicking the interaction of eIF4G with eIF4e. Further inspection revealed that Bicoid contains a short sequence with homology to the eIF4e binding motif, YxxxxK, of eIF4G. Mutation of this motif both prevents Bicoid's association with the 5′ cap complex and abolishes the ability of the protein to repress translation (Niessing et al., 2002). Hence, Bicoid appears to repress *caudal* translation by binding to its 3′UTR and preventing assembly of the 5′ cap complex on the mRNA and, thus, blocks translational initiation.

***bicoid* mRNA Localization and Cell Polarity**

Underlying the Bicoid gradient is *bicoid* mRNA, whose tight concentration at anterior of the embryo was a great step toward showing that *bicoid* is the localized anterior determinant (Berleth et al., 1988). mRNA localization itself was still quite a phenomenon; the possible role of maternal mRNAs in cytoplasmic determination had been discussed for some time, but only a handful of localized mRNAs had been observed, beginning in 1983, with Jeffery's demonstration by in situ hybridization that actin mRNA is localized in early ascidian embryos (Jeffery et al., 1983). In *Drosophila fushi-tarazu, hairy,* and *paired*, RNAs had recently been discovered to be cortically localized in embryos prior to cellularization (Hafen et al., 1984; Ingham et al., 1985; Kilchherr et al., 1986). Spectacular images showed Vg1 mRNA, encoding a transforming growth factor-β family member, localized at the vegetal cortex of *Xenopus* oocytes (Weeks and Melton, 1987). In addition to its original embryological implications, mRNA localization was put forth as an efficient mechanism to selectively deploy gene activities within cells (Lawrence and Singer, 1986).

Subsequent work has revealed that mRNA localization plays a central role in *Drosophila* axis formation. Not only is the anterior of the embryo defined by the localization of *bicoid* mRNA, but the localization of *oskar* mRNA to the posterior of the oocyte defines where the pole plasm forms, and thus where the abdomen and germ cells develop (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Kim-Ha et al., 1991). The pole plasm contains several other localized RNAs, including the noncoding RNA, *Pgc*, and *gcl* mRNA, both of which are required for pole cell formation, and the posterior determinant, *nanos* mRNA (Jongens et al., 1992; Nakamura et al., 1996; Wang and Lehmann, 1991). Like *bicoid*, *nanos* mRNA is translated after fertilization to form gradient of Nanos protein, in this case from posterior to anterior. Nanos then represses translation of maternal *hunchback* mRNA in conjunction with Pumilio (Barker et al., 1992; Murata and Wharton, 1995; Wharton, 1991). Thus, both the anterior and posterior determinants form gradients from the poles of the embryo that produce mirror image gradients of transcription factors by repressing the translation of unlocalized maternal mRNAs.

mRNA localization also plays an essential role much earlier in anterior-posterior axis formation. *gurken* mRNA localizes next to the oocyte nucleus at the posterior of the oocyte at stage 6, where it is translated to produce a TGFα-like protein that signals to the adjacent terminal follicle cells to induce them to adopt a posterior fate (González-Reyes et al., 1995; Roth et al., 1995). These cells subsequently signal back to the oocyte at

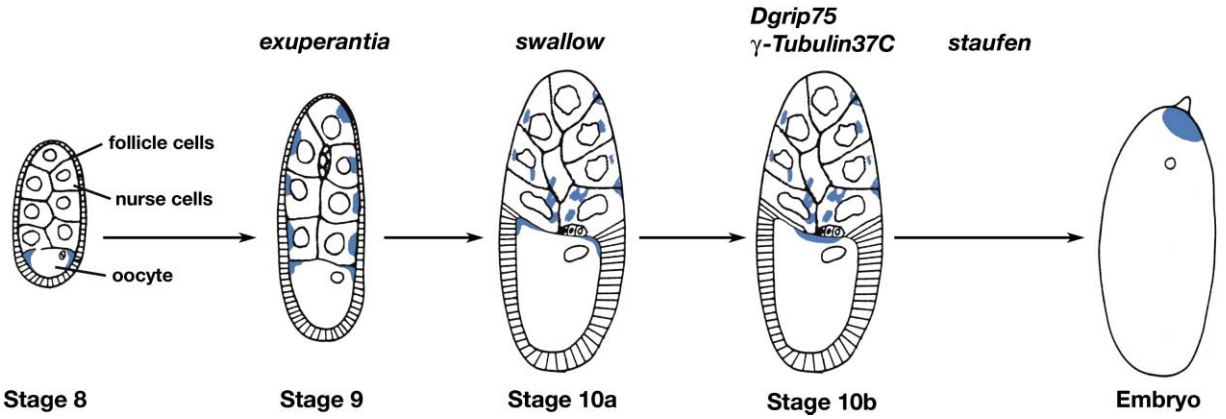

Figure 2. Steps in *bicoid* mRNA Localization

During oogenesis, *bicoid* mRNA is produced in the nurse cells and transported in a microtubule-dependent manner into the oocyte. During stages 8 and 9, *bicoid* mRNA is concentrated in a ring around the anterior cortex of the oocyte by a mechanism involving Exuperantia and microtubules. At stage 10a, *bicoid* mRNA is maintained at the anterior and begins to redistribute along the anterior cortex in a *swallow*-dependent manner. At stage 10b, in a process dependent on the centrosomal components γ-Tubulin37C and Dgrip75, *bicoid* mRNA becomes concentrated as a disk at the center of the oocyte anterior cortex. Maintenance of the mRNA in a cap at the anterior until embryogenesis requires Staufen. (Adapted from St Johnston et al. (1989) by E. Hjörleifsdóttir and G. Hjörleifsson.)

stage 7 to induce the anterior-posterior polarity that defines where *bicoid* and *oskar* mRNAs localize. This repolarization of the oocyte also triggers the movement of the oocyte nucleus and *gurken* mRNA to the dorsal/anterior corner of the oocyte, where Gurken signals for a second time to polarize the future dorsal-ventral axis of the embryo (Neuman-Silberberg and Schüpbach, 1993; Schüpbach, 1987).

Thus, the anterior-posterior and dorsal-ventral axes of *Drosophila* are defined by the localization of *bicoid*, *oskar*, and *gurken* mRNAs to three different positions within the same cell.

The ability to apply genetics and carry out phenotypic screens has made *Drosophila* an ideal organism for studying the mechanisms underlying mRNA localization. This is exemplified by *exuperantia* and *swallow,* whose mutation affects *bicoid* mRNA localization in the oocyte and the Bicoid gradient, causing head defects (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988b; Stephenson et al., 1988). Further analysis of these and other genes allowed the dissection of the *bicoid* mRNA localization into discrete steps, each requiring specific factors (Figure 2): Exuperantia for formation of a tight ring of *bicoid* mRNA around the oocyte anterior at stage 10a, Swallow for the anchoring of the mRNA at the anterior at stages 10b-11, and the double-stranded RNA binding protein Staufen for maintained localization of the mRNA at the anterior after stage 10 (Macdonald et al., 1991; St Johnston et al., 1991, 1989 Martin et al., 2003).

The polarized distribution of maternal mRNAs such as *bicoid* is highly suggestive of an underlying polarization of the cell and of the cytoskeleton within it. A first test of the hypothesis that the cytoskeleton plays a role in *bicoid* mRNA localization involved the use of cytoskeletal inhibitors (Pokrywka and Stephenson, 1991). These, and later genetics experiments (González-Reyes et al., 1995; Lane and Kalderon, 1994; Roth et al., 1995), revealed the crucial role of microtubules and of microtubule polarity in *bicoid* mRNA localization. The oocyte microtubule cytoskeleton is dynamic and undergoes dramatic reorganization at several points during oogenesis. The correlation between microtubule organization and distribution of the localized maternal mRNAs led to the simple proposal that the mRNAs are imported into the oocyte by minus-ended motors moving on microtubules emanating from a microtubule-organizing center at the posterior pole (Theurkauf et al., 1992). Once in the oocyte, and upon reorganization of the microtubule cytoskeleton, *bicoid* mRNA selectively partitions to the anterior oocyte cortex, where microtubule minus ends are enriched. The fact that *bicoid* mRNA localizes to both the anterior and posterior poles of *gurken* and *PKA* mutant oocytes, in which the microtubule cytoskeleton is mispolarized (González-Reyes et al., 1995; Lane and Kalderon, 1994; Roth et al., 1995), suggested that *bicoid* mRNA movement is mediated by microtubule minus end-directed motors and that anterior localization of *bicoid* is the result of an active process, rather than by simple trapping upon entry of the mRNA into the oocyte. Although the existence and nature of an association of *bicoid* mRNA with a microtubule motor still remain to be demonstrated, the analysis of *exuperantia*, *swallow*, and new mutants isolated in screens specifically aimed at understanding *bicoid* mRNA localization have revealed particularly interesting roles of these proteins and of the oocyte microtubules in this process.

After its import into the oocyte, *bicoid* mRNA localizes as a ring around the anterior cortex. In a second step, the ring of *bicoid* mRNA evolves into a disc or cap, at the center of the anterior pole, where it remains until embryogenesis. *exuperantia* mutants fail to form the initial ring of *bicoid* mRNA, whereas *swallow* mutants fail to transform the ring into a disc. The dual role of Exuperantia in *bicoid* mRNA localization has been most clearly revealed by a remarkable set of experiments in which fluorescently labeled *bicoid* mRNA was injected into wild-type and *exuperantia* mutant egg chambers. When synthetic, fluorescent *bicoid* RNA was injected

into oocytes, the RNA localized in a microtubule-dependent manner all around the oocyte cortex, with the exception of the posterior pole (Cha et al., 2001; Glotzer et al., 1997). In contrast, when injected into *exuperantia* mutant oocytes, the RNA failed to move to the cortex and remained cytoplasmic. Thus, although Exuperantia is required for cortical localization in the oocyte, it is not sufficient to direct *bicoid* mRNA to the anterior (Cha et al., 2001). This result left open two possibilities: (1) that, as originally proposed, *bicoid* mRNA is transported from the nurse cells into the oocyte, where it is trapped upon entry at the anterior (Berleth et al., 1988) or (2) that nurse cell factors, absent in the oocyte cytoplasm, cooperate with Exuperantia to specifically localize *bicoid* mRNA to the oocyte anterior. This question was put to the test in a two-step injection strategy, where the RNA was first injected into a nurse cell, was rapidly withdrawn, and then was reinjected into an oocyte. Remarkably, *bicoid* mRNA that had been passaged through a wild-type nurse cell now was specifically targeted to the anterior, regardless of whether the oocyte was wild-type or an *exuperantia* mutant. In contrast, injection of the RNA into *exuperantia* mutant nurse cells did not render the RNA competent to localize at the oocyte anterior. Thus, in the nurse cells, Exuperantia recruits additional factors that specifically target the mRNA to the anterior of the oocyte (Cha et al., 2001). At this point, it appears that Exuperantia has two roles in *bicoid* mRNA localization: Exuperantia is necessary for the microtubule-dependent localization of *bicoid* RNA to the oocyte cortex in general and also is required in the nurse cells to render the *bicoid* mRNA localization complex competent to discriminate between lateral and anterior microtubules. A profound implication of these results is that the oocyte contains qualitatively different populations of microtubules and that *bicoid* mRNA can distinguish between them. The analysis of the movement of *gurken* mRNA has led to a very similar conclusion (MacDougall et al., 2003). Live imaging of injected *gurken* mRNA indicates that it is localized in two dynein-dependent steps: it first moves to the minus ends of microtubules at the anterior of the oocyte and then associates with a different population of microtubules that are nucleated around the nucleus to move to the dorsal anterior corner.

Further support for the existence of distinct populations of microtubules in the oocyte comes from the analysis of *swallow* and of two genes encoding centrosomal proteins: a maternal-specific γ-tubulin (γ-tub37C) and γ-tubulin ring complex protein 75 (Dgrip75). Swallow localizes to the oocyte anterior at stage 10 and is required for movement of *bicoid* mRNA from a ring to a disc and for its maintenance at the anterior pole after this stage (Schnorrer et al., 2000). A qualitative change in the microtubules occurs at the time of the *bicoid* mRNA ring-to-disc transition: γ-*tub37C* and *Dgrip75* become enriched at the anterior, and the distribution of the minus end reporter Nod:β-gal evolves from a ring to a disc shape (Schnorrer et al., 2002). This strongly suggests that a distinct microtubule-organizing center forms at the anterior at this stage, causing the assembly of microtubules with different properties from those in the rest of the oocyte. Swallow distribution is not affected in the γ-*tub37C* and *Dgrip75* mutants, and this, together with the fact that Swallow is detected in a biochemical complex with dynein light chain and the centrosomal proteins, suggests that upon anterior localization, it may be anchored at the cortex, independent of microtubules, whose structure or dynamics it might regulate. The fact that in γ-*tub37C* and *Dgrip75* mutants, *bicoid* localization is dramatically affected, while other microtubule-dependent processes are not, further supports the existence of different subpopulations of microtubules in the oocyte. Finally, the fact that the ring-to-disc transition begins normally in the γ-*tub37C* and *Dgrip75* mutants but eventually fails, such that *bicoid* mRNA particles spread throughout the oocyte, suggests that rather than simple anchoring, continuous transport of *bicoid* mRNA may be required for maintenance of the RNA at the anterior pole (Schnorrer et al., 2002). Thus, the analysis of *bicoid* mRNA localization has revealed exciting new aspects of microtubule dynamics in cell polarity.

RNAs have the capacity to adopt very complex secondary and tertiary structures and rarely remain single stranded. These structures are likely to play an important role in mRNA localization, and of all localized mRNAs, the role of sequence and structure in *bicoid* mRNA localization has been most extensively explored. Both phylogenetic and mutational analyses suggest that the *bicoid* mRNA localization element in the 3′UTR consists of five stem loops that together contain the signals necessary and sufficient for correct targeting of *bicoid* mRNA from its site of synthesis in the nurse cells to its final destination at the anterior of the oocyte and to stabilize the mRNA (Macdonald et al., 1993, 1990; Macdonald and Struhl, 1988; Mancebo et al., 2001; Seeger and Kaufman, 1990). Base-pairing interactions within this predicted structure are essential for *bicoid* localization, suggesting the importance of tertiary structure in formation of a localization complex. For example, the interaction of Staufen with *bicoid* mRNA requires three of these double-stranded stems, but does not depend on their sequence, consistent with the presence of multiple dsRNA binding domains in Staufen (Ferrandon et al., 1994). Furthermore, the loop at the end stem III can base-pair with a bulge in the middle of this stem to mediate the oligomerization of the mRNA, and mutations that prevent this disrupt the ability of the RNA to recruit Staufen and to form aggregates that move in a microtubule-dependent manner, indicating that quaternary structure plays a role in localization (Ferrandon et al., 1997; Wagner et al., 2001). Mutational analysis of *bicoid* has also revealed that different steps in the localization process are mediated by partially redundant elements that act at different stages (Macdonald and Kerr, 1997, 1998). Finally, a signal in the *bicoid* 3′UTR mediates degradation of the mRNA at cellularization (Surdej and Jacobs-Lorena, 1998).

As redundancy is a plague for genetic analysis, biochemistry has also been applied toward the identification of proteins involved in *bicoid* mRNA localization. This has led to the purification of a large protein complex that assembles specifically on a minimal version of the *bicoid* localization signal and is sensitive to inactivating mutations in the RNA signal (Arn et al., 2003). An important conclusion of this arduous work is that recognition of the *bicoid* mRNA localization signal is mediated by

multiple low-affinity interactions between proteins and the mRNA, providing a plausible explanation for the difficulty in identifying individual *bicoid* RNA binding proteins whose specific depletion causes a failure in localization. Taken together, these analyses have revealed the incredible complexity of *bicoid* mRNA and of what it takes to assemble a functional mRNA localization complex. Finally, the existence and nature of the association of *bicoid* mRNA with a microtubule motor, while extremely probable, remain to be demonstrated.

Bicoid as a Model for Morphogen Gradients

The question of how gradients are established and stably maintained across a developing field was a central issue at the time of the Bicoid papers and remains so today. Inasmuch as modeling of morphogens was an active field in the past, knowledge of the molecules and circuits involved has brought the field into a new age, where the organism can be readily manipulated and predictions tested. There is increasing evidence that the shape of the gradients formed by secreted morphogens can be regulated by multiple feedback mechanisms that act on the secretion, range, and degradation of the morphogen. With this in mind, a numerical screen was performed for robust networks that generate a stable steady-state gradient that is resistant to fluctuations in expression levels or the size of the tissue (Eldar et al., 2003). Contrary to the commonly held view that morphogen gradients are created simply by diffusion of the molecule away from the source coupled to a uniform rate of degradation, this study reached the striking conclusion that to ensure stability over distance and time, a morphogen must decay rapidly at the source, but much more slowly over the rest of the field. This prediction was then tested and appears to hold true for the Hedgehog and Wingless gradients in the *Drosophila* wing disc, which, depending on the reciprocal relationship between the specific morphogen and its receptor, undergo increased morphogen degradation at the source (Hedgehog) or stabilization at a distance (Wingless). In the latter case, an additional prediction was that Wingless stabilization is an active process (e.g., inhibition of the proteolytic machinery), rather than a process of passive protection. Clearly, Bicoid is not a typical morphogen, due to the fact that it exerts its effect within a syncytium of rapidly dividing nuclei. Nevertheless, its simplicity makes it ideal for modeling how the robustness of biological systems is achieved. Measurements of the Bicoid gradient have revealed that it is highly variable from embryo to embryo, while the domain of expression of its target gene *hunchback* is very reproducible and only minimally sensitive to genetic perturbation (Houchmandzadeh et al., 2002). So far, only certain alleles of *staufen* appear to affect the high reproducibility of the limits of *hunchback* expression, independent of any effect on Bicoid expression. What role *staufen* might play in robustness of *hunchback* expression domain is mysterious. Clearly Bicoid continues to be interesting and will keep us occupied and fascinated for the foreseeable future.

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