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A Gradient of *bicoid* Protein in Drosophila Embryos

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Summary

The maternal gene *bicoid* (*bcd*) organizes anterior development in Drosophila. Its mRNA is localized at the anterior tip of the oocyte and early embryo. Antibodies raised against *bcd* fusion proteins recognize a 55–57 kd doublet band in Western blots of extracts of 0–4 hr old embryos. This protein is absent or reduced in embryonic extracts of nine of the 11 *bcd* alleles. The protein is concentrated in the nuclei of cleavage stage embryos. It cannot be detected in oocytes, indicating temporal control of *bcd* mRNA translation. The *bcd* protein is distributed in an exponential concentration gradient with a maximum at the anterior tip, reaching background levels in the posterior third of the embryo. The gradient is probably generated by diffusion from the local mRNA source and dispersed degradation.

Introduction

Gradients in development have been invoked as mechanisms for creating spatial diversity from seemingly uniform states since the beginning of this century (Morgan, 1901; Child, 1915). They were postulated on the basis of transplantation and isolation experiments performed in a number of embryonic systems, such as sea urchins (Runnström, 1929; Hörstadius, 1939), amphibians (Dalcq and Pasteel, 1938), and insects (Sander, 1959, 1976). In these experiments it appeared that the differentiation properties of the tissue along the embryonic axes changed in a quantitative rather than qualitative manner, and could best be explained by the gradual change of the concentration of a morphogenetic substance.

According to the concept of positional information (Wolpert, 1969), the concentration of a morphogen instructs cells within an embryonic field of their position. The cells then interprete this information by an appropriate program of differentiation. Several models have been proposed that describe the generation of stable gradients of morphogens which could specify subregions of the embryo in a concentration-dependent manner. The simplest gradient models use the property of diffusion to describe the distribution of the morphogen, starting with an initial asymmetry in the form of a local source (Lawrence, 1966; Stumpf, 1966; Crick, 1970; Lewis et al., 1977). In models involving autocatalysis and lateral inhibition, stable concentration gradients can result from very slight, random fluctuations (Gierer and Meinhardt, 1972). In several instances, localized entities have been found that are good candidates for sources of morphogen gradients (Sander, 1959; Kalthoff, 1979; Lehmann and Nüsslein-Volhard, 1986; Frohnhöfer and Nüsslein-Volhard, 1986; Weeks and Melton, 1987; and see below). The graded distribution of substances with biological activity has been demonstrated in the cases of the "head activator" in hydra (Schaller and Gierer, 1973; Schaller and Bodenmüller, 1981) and retinoic acid in chick limb buds (Maden, 1982; Thaller and Eichele, 1987), yet no morphogen gradient has been demonstrated in any early embryo. During Drosophila embryogenesis, the products of the genes *caudal* (*cad*) (Mlodzik et al., 1985; Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987) and *hunchback* (*hb*) (Tautz, 1988) are transiently distributed in shallow concentration gradients. However, the functions of these gradients are not known.

For the Drosophila embryo, evidence from experimental embryology (Frohnhöfer et al., 1986) as well as genetic analysis (Nüsslein-Volhard, 1979; Nüsslein-Volhard et al., 1987) indicates that the anteroposterior pattern is determined by two opposing gradients, with sources at the anterior and posterior egg poles, respectively (Lehmann and Nüsslein-Volhard, 1986; Frohnhöfer and Nüsslein-Volhard, 1986). Several lines of evidence indicate that the gene *bicoid* (*bcd*) is responsible for the anterior gradient. In embryos from bcd^- females, head and thorax are lacking and are replaced by a posterior telson. Transplantation of cytoplasm from the anterior tip of wild-type embryos into bcd^- embryos can restore a near-normal pattern as well as induce anterior structures at ectopic positions. The size and quality of the induced anterior structures depend on the amount (concentration) of the transplanted bcd^+ activity, which itself is determined by the number of wild-type bcd^+ gene copies in the donor female (Frohnhöfer and Nüsslein-Volhard, 1986). The *bcd* gene has been cloned and sequenced. It codes for an mRNA that is localized at the anterior tip of the oocyte and early embryo (Frigerio et al., 1986; Berleth et al., 1988).

A striking property of the bcd^+ activity is its long-range effect on neighboring regions. In bcd^- embryos not only are the structures normally formed at the site of mRNA localization deleted, but the anlagen of the entire anterior egg half are also lacking. Furthermore, the posterior anlagen are enlarged and spread toward the anterior (Frohnhöfer and Nüsslein-Volhard, 1986). In transplantation experiments using bcd^+ activity, the polarity and pattern of the embryo along more than half of its length can be changed (Frohnhöfer et al., 1987). These extraordinary features of the *bcd* gene can best be explained by invoking a gradient mechanism in which different concentrations of the *bcd* gene product determine the series of different structures along the anterior pattern (Frohnhöfer and Nüsslein-Volhard, 1986, 1987; Nüsslein-Volhard et al., 1987). Since the *bcd* mRNA is strictly localized at the anterior tip of the wild-type embryo, the RNA itself cannot fulfill the role of the anterior morphogen. The *bcd* protein, however, is a good candidate for the anterior gradient molecule. In addition, the presence of a homeobox in the cod-

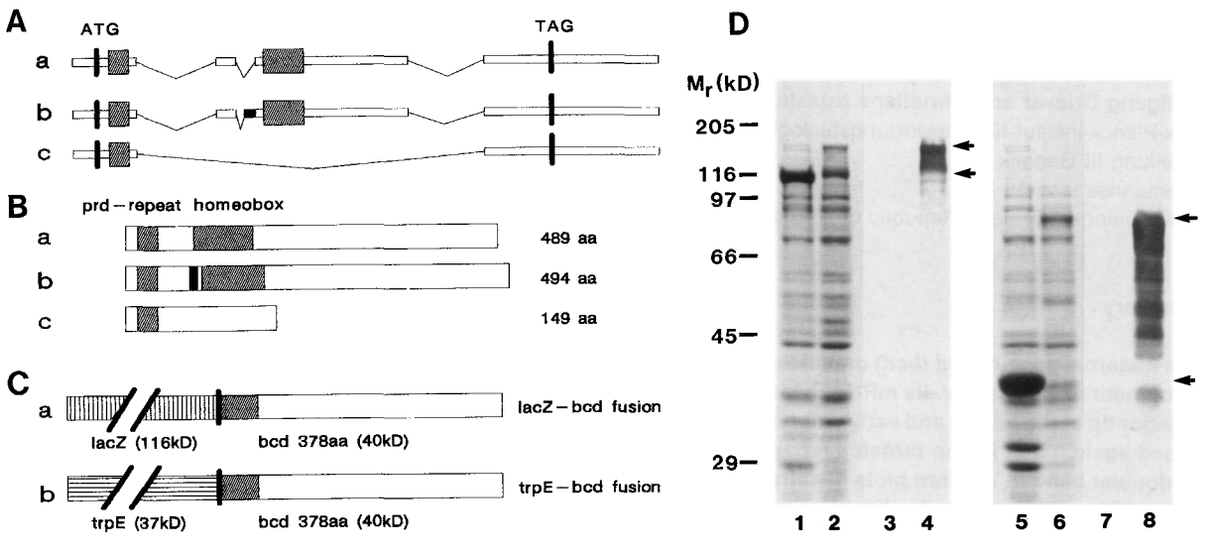

Figure 1. Structures of the *bcd* Transcripts and *bcd* Fusion Proteins, and Specificity of the Anti-*bcd* Antibody

(A) Structure of the most abundant, 2.6 kb *bcd* mRNA (a) and of the species with a shorter second intron (b). Junction of the splice donor site of the first intron and the splice acceptor site of the last intron results in a 1.6 kb mRNA (c; see also Berleth et al., 1988). (B) Putative proteins derived from the longest open reading frames in the above transcripts. The PRD repeat and the homeobox are indicated by shaded areas. Protein type b would contain five additional amino acids just in front of the homebox domain (black box). Type c protein (149 amino acids, 16.4 kd) lacks the homeobox. (C) Structures of the *bcd* fusion proteins: (a) LacZ–*bcd* fusion protein containing amino acids 112 to 489 of a type b protein (see Experimental Procedures); (b) TrpE–*bcd* fusion protein. (D) Specificity of anti-*bcd* antibodies. Coomassie blue–stained SDS-PAGE gels (lanes 1, 2, 5, 6) and Western blots (lanes 3, 4, 7, 8) showing total protein from induced bacteria containing the unfused *lacZ* gene (lanes 1 and 3), the *lacZ–bcd* fusion gene (lanes 2 and 4), the unfused *trpE* gene (lanes 5 and 7), or the *trpE–bcd* fusion gene (lanes 6 and 8). Bands in lanes 4 and 8 with lower apparent molecular weights than the *bcd* fusion proteins are due to breakdown products of unstable fusion proteins. Arrows indicate the sizes of the fusion and nonfusion proteins.

ing region of the *bcd* gene (Frigerio et al., 1986; Berleth et al., 1988) suggests that the protein may regulate the expression of zygotic genes.

In this paper we show that the *bcd* gene product is a 55 kd protein translated soon after egg deposition. It is distributed in a steep concentration gradient whose maximum is at the anterior egg pole. We measured the distribution of *bcd* protein in wild-type embryos and found an exponential decay in protein concentration, a distribution pattern which can be readily explained by models involving a local source, diffusion, and dispersed decay. The protein is detectable at up to 30% egg length (where 0% is the posterior pole), providing an explanation for the long-range effects observed in *bcd*$^-$ embryos. The accompanying paper (Driever and Nüsslein-Volhard, 1988) provides evidence for the hypothesis that the positions of the embryonic anlagen in the anterior half of the embryo are determined by the *bcd* product in a concentration-dependent manner.

Results

Identification and Characterization of *bcd* Protein in Drosophila Embryonic Extracts

Schematic structures of *bcd* transcripts and their longest open reading frames (Frigerio et al., 1986; Berleth et al. 1988) are shown in Figures 1A and 1B. To analyze the *bcd* product in Drosophila, we raised rabbit polyclonal antibodies against a LacZ–*bcd* fusion protein containing the carboxy-terminal 378 amino acids of the longest open reading frame from the most abundant transcript (Figure 1C). Antibodies specific for the *bcd* gene product were purified by affinity chromatography on a TrpE–*bcd* fusion protein column (Figures 1C and 1D; see also Experimental Procedures). We also raised monoclonal antibodies against LacZ–*bcd* and TrpE–*bcd* fusion proteins (see Experimental Procedures).

We extracted protein from staged embryos, separated them by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed Western blots (Figure 2) by probing with monoclonal anti-*bcd* antibodies. The immunostain detected protein bands which exhibit a maximum in staining intensity between 2 and 4 hr after egg deposition. These proteins were not detectable in ovaries or at later stages of development. They correspond to an apparent doublet of proteins with molecular masses between 55 and 57 kd. The same proteins are recognized by rabbit polyclonal antibodies (data not shown). Other protein bands are due to immunological cross-reactivity of the second antibody, as judged from the control lane processed without first antibody (Figure 2).

The electrophoretic mobility of the immunoreactive bands (55 and 57 kd) is in good agreement with the predicted molecular mass of the *bcd* protein (53.9 kd). Slightly higher apparent molecular weights than calculated have been reported for proteins extremely rich in proline content, such as *fushi tarazu* (Carroll and Scott, 1986) and *Krüppel* (Gaul et al., 1987). The *bcd* protein con-

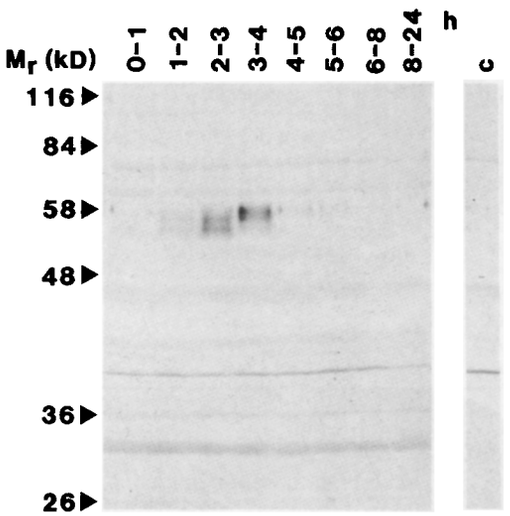

Figure 2. Developmental Profile of the *bcd* Protein in Drosophila Embryonic Extracts

Proteins from staged embryos were separated by SDS-PAGE and transferred to nitrocellulose. The Western blot was incubated with monoclonal anti-*bcd* antibodies. Ages of the embryos are indicated at the top in hours after egg deposition (21°C). Apparent molecular masses of marker proteins in kd are indicated at left. Lane c shows a blot incubated with second antibody only.

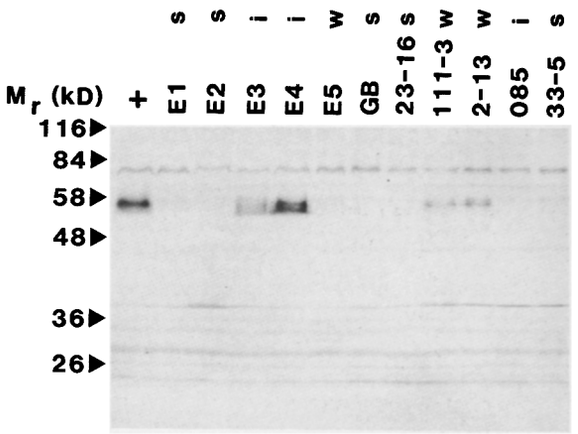

Figure 3. Detection of *bcd* Protein in *bcd* Mutants

Embryonic extracts from 2–3 hr old mutant embryos were separated by SDS-PAGE. The Western blot was subsequently developed as in Figure 2. The maternal genotypes (*bcd* alleles) of the embryos are indicated above the lanes; all females were heterozygous for *Df(3R)LIN*. Maternal *bcd* alleles are strong (s), intermediate (i), or weak (w), as indicated (Frohnhöfer and Nüsslein-Volhard, 1986). Apparent molecular masses of marker proteins in kd are indicated at left.

tains 10% proline. The 57 kd band might be a protein derived from the minor splicing variant (cDNA type b, Figure 1), although the five additional noncharged amino acids (545 daltons) would not be expected to result in a 2–3 kd rise in molecular mass. We were not able to detect *bicoid* protein with antibodies against a peptide of nine amino acids including the five appearing in cDNA b (unpublished data). It is more likely that the appearance of two bands is due to a posttranslational modification such as phosphorylation, which would be expected to produce a considerable change in electrophoretic mobility. Similar banding patterns for other developmentally regulated proteins expressed in Drosophila tissue culture have been found to be due to phosphorylations (e.g., *Ultrabithorax*; L. Gavis and D. Hogness, personal communication). In the case of *bcd*, the pattern of protein species changes during early development in that the protein forms with higher apparent molecular weight appear at later stages. The time at which the 55 and 57 kd proteins are detectable at their highest abundance (2–4 hr after egg deposition) has been shown to correspond to the temperature-sensitive period of the bcd^{E3} allele (Frohnhöfer and Nüsslein-Volhard, 1987).

To confirm that the 55 kd protein we have identified is the *bcd* gene product, we used monoclonal antibodies to probe protein extracts from embryos derived from females hemizygous for each of 11 ethyl methanesulfonate–induced *bcd* alleles (Figure 3). The immunologically detectable protein pattern was altered only in the 55–57 kd range, and none of the strong alleles (E1, E2, GB, 23-16, 33-5) gave rise to a protein in the *bcd* size range. However, the sensitivity of the method may preclude the detection of a severely truncated or very unstable protein with the antibody. Among the hypomorphic *bcd* alleles the pattern was diverse. Two of the intermediate alleles (E3, E4) showed normal or almost normal *bcd* protein levels. The *bcd* protein was reduced in the weak mutants 2-13 and 111-3 and could not be detected at all in two additional alleles, one of which shows only a very weak mutant phenotype (E5; Frohnhöfer and Nüsslein-Volhard, 1986).

The *bcd* Protein Distribution in Early Embryos

We stained whole mount preparations of early embryos with anti-*bcd* antibody (Figure 4). Soon after egg deposition the *bcd* protein first becomes detectable (Figure 4A) at the anterior tip of the egg, at or near the site of localization of the *bcd* mRNA (Berleth et al., 1988). At the time of pole cell formation (Figure 4B), *bcd* protein is already distributed in a concentration gradient with a maximum at the anterior tip. The basic shape of this gradient appears to be stable during the syncytial blastoderm stage, with the posterior limit of detection at about 30% egg length (Figures 4C and 4D). The total level of *bcd* protein appears to increase slightly until the onset of cellularization. It decreases slowly during cellularization and then more rapidly during gastrulation (Figures 4E and 4F). Traces of *bcd* protein can still be detected in the nuclei at the end of germ band elongation (data not shown). There is no indication of dorsoventral asymmetry in *bcd* protein distribution during early development.

Although *bcd* protein is present in the cytoplasm, its predominant location is in the nuclei, where it is strongly concentrated. Nuclear localization is expected for a homeobox-containing, putative DNA-binding protein. Yolk nuclei as well as peripheral nuclei are stained. In contrast to the *cad* protein (Macdonald and Struhl, 1986) and *Krüppel* protein (Gaul et al., 1987), the *bcd* protein is not associated with distinct subnuclear structures during inter-

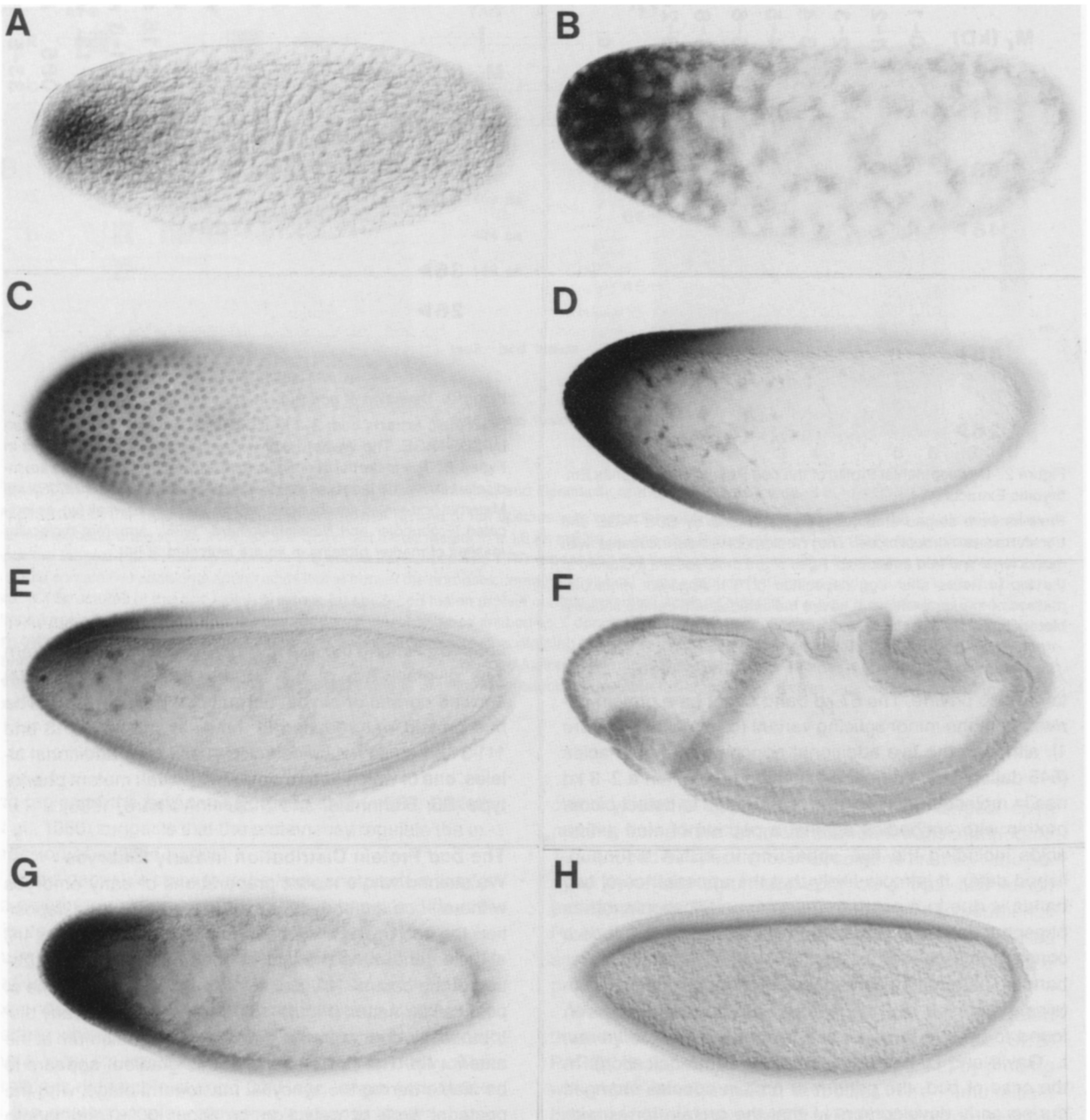

Figure 4. *bcd* Protein in Whole Mounts of Wild-Type Embryos

Wild-type embryos of different ages were stained for *bcd* protein as described in Experimental Procedures. Whole mount preparations were photographed using Nomarski optics. (A) Embryo shortly after egg deposition (stage 1). (B) Pole cell formation (stage 3). (C) Syncytial blastoderm (stage 4c), focused on the surface. (D) Syncytial blastoderm, focused on the plane of the pole cells. (E) Cellular blastoderm (stage 5b). (F) Beginning of germ band extension (stage 7). (G) Embryo of stage 4 during mitosis. Note the lack of staining of nuclear structures. (H) Embryo of a bcd^{E1}/bcd^{E1} female. In all cases anterior is at left, dorsal at top. Staging is according to Campos-Ortega and Hartenstein (1985).

phase. During mitosis the staining disappears completely from nuclear structures, and for a short time the embryos display a protein gradient in which no cellular substructures are preferentially stained (Figure 4G).

To determine the onset of *bcd* translation, we stained frozen sections of ovaries for the *bcd* protein. *bcd* protein was not detectable at any stage during oogenesis (data not shown). Since the *bcd* mRNA is already present during oogenesis (Frigerio et al., 1986; Berleth et al., 1988), this indicates that *bcd* mRNA translation is blocked during oogenesis. In unfertilized eggs establishment of the *bcd* protein gradient proceeds in a normal fashion. However, at about 2 to 4 hr after egg deposition, levels of *bcd* protein exceed the ones in fertilized eggs (see Figure 6E). This

Table 1. *bcd* Protein in *bcd* Mutant Embryos

Allele	Strength	*bcd* Protein on Western Blots	Immunostaining in Whole Mount Embryos
E1	Strong	Absent	Absent
E2	Strong	Absent	Absent
GB	Strong	Absent	Absent
335	Strong	Absent	Absent
23-16	Strong	Absent	Absent
E3	Intermediate	Present	Normal
E4	Intermediate	Present	Normal
085	Intermediate	Absent	Normal
2-13	Weak	Reduced	Reduced
E5	Weak	Absent	Reduced
111-3	Weak	Reduced	Reduced

suggests that either the transcript or the protein is more stable in unfertilized eggs. The finding of a higher than normal *bcd* protein level in unfertilized eggs explains the results of transplantation experiments. When unfertilized eggs were used as donors, the bcd^+ activity was found to be both more concentrated and more stable than in fertilized eggs (Frohnhöfer and Nüsslein-Volhard, 1986). We conclude that the event of fertilization is not required for the onset of *bcd* translation. Similar results have been obtained for translation of the maternal *cad* transcript (Macdonald and Struhl, 1986).

bcd Protein Distribution in *bcd* Mutant Embryos

In whole mounts of embryos from flies carrying strong *bcd* mutant alleles, no *bcd* protein could be detected, confirming the data of the Western blot analysis (Figure 4H, Table 1). This might be due to nonsense mutations early in the open reading frame, greatly decreased protein stability, or ethyl methanesulfonate–induced small deletions (as has been shown in the case of the alleles bcd^{E1} and bcd^{E2}; see Berleth et al., 1988). In contrast to the results of the Western analysis, *bcd* protein can be detected in whole mount immunostainings of mutant embryos from flies carrying each of six hypomorphic alleles. In the case of the weak alleles the level is reduced, while it appears to be normal for the intermediate alleles. Thus the phenotypes observed for the weak alleles may be caused by reduced levels of active protein (due to greater instability or a lower rate of synthesis), while for the intermediate alleles normal levels of protein with reduced activity may prevail. The failure to detect a 55 kd band in the bcd^{085} mutant in Western blots (with a normal level in whole mount embryos) suggests a truncated protein that is not recognized by the monoclonal antibody.

The Shape of the *bcd* Protein Concentration Gradient

We measured the intensity of the *bcd* immunostain either directly in whole mount embryos by using a sensitive photodiode scanning about 3 μm in area, or by digitizing a high-resolution video image obtained directly from the microscope and processing it via an image-analyzing program (see Experimental Procedures). Both methods give similar results. Using these techniques we avoided many of the problems caused by the nonlinearity of signals in densitometric analyses of photographic prints. Under the applied conditions the signal of the horseradish peroxidase (HRP) reaction is proportional to the *bcd* protein concentration as tested by measuring immunostain intensities of serial dilutions of the *bcd* protein in dot blots on nitrocellulose (see Experimental Procedures). Figure 5 shows the intensity of immunostaining obtained for different amounts of fusion protein applied to nitrocellulose.

The intensity of *bcd* protein immunostain measured along a line from the anterior to the posterior of the embryo is displayed in Figure 6A, including the 2-fold standard deviation. The *bcd* protein is distributed in a sharp, nonlinear concentration gradient. There is a very steep decline in *bcd* protein concentration in the region close to the anterior pole, while at positions posterior to 50% egg length further decreases in concentration are barely detectable. The background level can be evaluated from Figure 6F, which shows *bcd* immunostain intensity in bcd^{E1} mutant embryos and control embryos. By visually comparing the range of staining intensities measured in the wild-type embryos with that obtained from serial dilutions of *bcd* protein on nitrocellulose (Figure 5), one can roughly estimate that the *bcd* protein concentration changes by 2 to 3 orders of magnitude within the anterior half of the embryo.

A plot of the logarithm of the concentration (Figure 6B) is near-linear over the largest portion of the egg length, indicating an exponential decay of the protein concentration toward the posterior. In the anteriormost 15% of the embryo, the slope is steeper. This is probably due to the fact that in this region the more intensely stained nuclei enter the plain of focus (see below). Furthermore, this is the region where the *bcd* mRNA, the source of the *bcd* protein, is located. From the graph we estimate that background levels are reached at about 30% egg length (Figures 6B and 6F).

The intensity of the anti-*bcd* immunostain was also determined along the dorsal and ventral periphery of the egg, at the positions of the nuclei in early nuclear cycle 14 embryos (Figure 6C). As expected for proteins localized in the nucleus, the *bcd* protein concentration in the nuclei is higher than in the surrounding cytoplasmic regions. Because of ambiguities in the positioning of the line of measurement and a strong influence of egg curva-

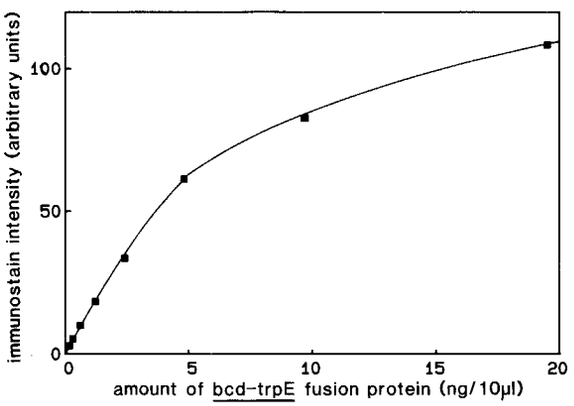

Figure 5. Determination of the Linear Range of the Immunostaining Reaction

For details, see Experimental Procedures.

ture on the measurement (underestimation of terminal values), we did not use this method of determining the protein distribution in subsequent analyses. At first sight both methods seem to yield principally different curves. However, taking into account the different biases in measuring the anteriormost values, the shapes of the graphs becomes quite similar apart from different absolute values.

To assess the influence of egg curvature and nuclear density on the measurements, we examined the stain intensity of an evenly distributed protein with both cytoplasmic and nuclear localization. Such a situation is provided in the distribution of the *cad* protein in bcd^{E1} mutant embryos during early nuclear cycle 14. Figure 6D shows the distribution of *cad* protein in mutant bcd^{E1} and in wild-type embryos during early nuclear cycle 14. In wild-type embryos, *cad* protein is distributed in an approximately linear gradient from 0% to 100% egg length with a high point at the posterior pole (Macdonald and Struhl, 1986); this gradient is less steep than the *bcd* protein gradient. In bcd^{E1} mutant embryos, the *cad* protein immunostain reveals a density profile that is even except for two small terminal peaks. These peaks are due to the fact that at terminal positions the more intensely stained nuclei are at the plane of focus, while they are out of focus between 10% and 90% egg length. This implies that the measurements of *bcd* protein distribution as displayed, for example, in Figures 6A and 6B are not significantly affected by the egg shape except at positions between 90% and 100% egg length.

The shape of the *bcd* protein gradient in unfertilized eggs (Figure 6E) is similar to that in fertilized eggs. The nonterminal position of the peak corresponds to the localization of *bcd* mRNA in very early fertilized embryos (Berleth et al., 1988). Between young (0–1 hr) and old (1–7 hr) unfertilized eggs, there is a distinct increase in *bcd* protein concentration.

Discussion

Establishment of the *bcd* Protein Gradient

The source of the *bcd* protein is the localized *bcd* mRNA. Comparison of the distributions of *bcd* mRNA and protein reveals a striking incongruity. *bcd* mRNA is localized at the anterior tip of the embryo (80%–100% egg length) and is barely detectable at positions posterior to 70% egg length. In contrast, *bcd* protein concentration reaches the limit of detection at about 30% egg length (Figure 6B).

A simple conceivable mechanism for the formation of the *bcd* protein concentration gradient is diffusion starting from the local mRNA source, and degradation throughout the embryo. An appropriate balance between the rates of *bcd* mRNA translation, of diffusion, and of dispersed proteolytic degradation would generate a relatively stable, nonlinear, graded distribution of the *bcd* protein. Such a model would require low stability of the *bcd* protein. The *bcd* protein contains several PEST sequences, which are present in proteins of short half-life and are thought to be signals for degradation (Rogers et al., 1986; Rechsteiner et al., 1987). The most significant PEST sequence spans from amino acid 170 to 203 (for the sequence, see Berleth et al., 1988). From the observation that the last traces of the protein can be detected after gastrulation whereas the mRNA disappears during cellularization, we estimate a half-life for the *bcd* protein of less than ½ hr.

In the syncytial Drosophila embryo, no cell boundaries limit the diffusion of a protein the size of the *bcd* protein. Diffusion constants for proteins in the cytoplasm have been calculated to be in the range of $0.3–1.0 \times 10^{-8}$ cm^2/sec (Mastro et al., 1984; Wojcieszyn et al., 1981). These estimates derive from fluorescence recovery after spot photobleaching of fluorescent conjugates as well as from electron spin resonance of spin labels. The diffusion constants are reported to be relatively independent of the molecular weights of the proteins (Kreis et al., 1982; Mastro et al., 1984). Diffusion constants of this order of magnitude are capable of generating a gradient over a 200 µm distance, starting at a local source, within about 1 hr. The calculated time corresponds well to the time between the onset of *bcd* translation after egg deposition and the earliest time when the mature-shaped gradient can be detected in the egg of 500 µm length.

These calculations show that the *bcd* protein gradient may simply be generated by diffusion and nonspecific proteolytic activities. However, more complex processes involving specific interactions with other compounds cannot be excluded. For example, active wave-like contractions of the cytoplasm observed during each cleavage cycle (Foe and Alberts, 1983) may influence the formation and shape of the gradient. Furthermore, the degradation of *bcd* protein may (but need not) involve specific proteases. Secondary modifications of the protein might interfere with its stability and thus influence the shape of the gradient.

Translational Control of *bcd*

Although *bcd* transcription starts early in oogenesis and the mRNA is localized long before egg deposition, we could not detect *bcd* protein in oocytes. This implies that *bcd* mRNA translation either is blocked during oogenesis or might require translational activation as a part of the general activation of the maternal program of the oocyte (Mahowald et al., 1983). Translation begins, independent of fertilization, soon after egg deposition. For *bcd* mRNA

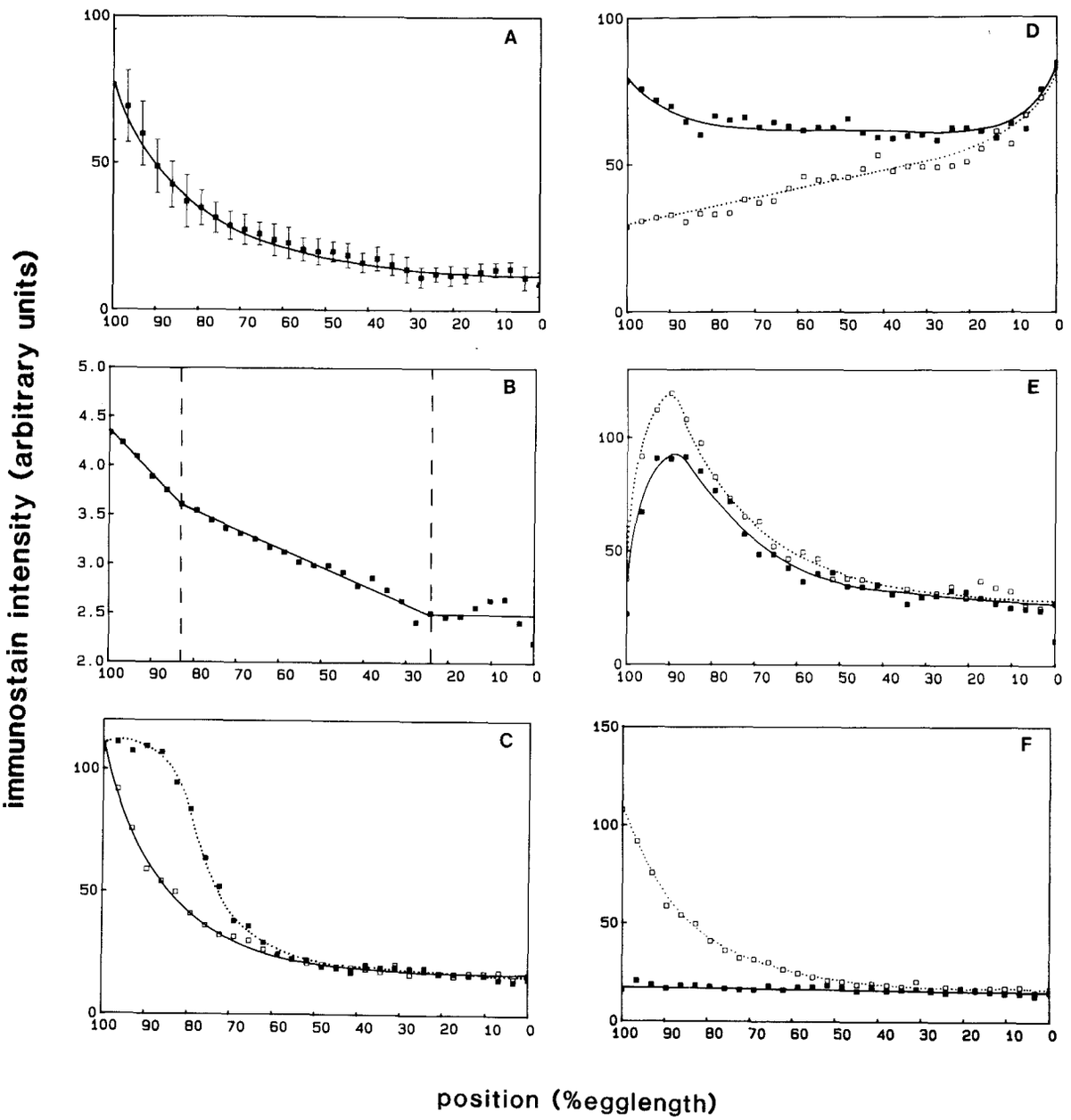

Figure 6. Quantitation of Immunostain Intensity in Whole Mount Preparations of Embryos

Video images were obtained from whole mount preparations and were analyzed by digitizing images as described in Experimental Procedures. (A) Anti-*bcd* protein immunostain intensity measured along the anteroposterior axis in wild-type early nuclear cycle 14 embryos. Data from ten embryos were averaged and are displayed with 2-fold standard deviations. (B) Natural logarithms of measurements in (A). (C) Anti-*bcd* immunostain intensity measured along the anteroposterior axis (open squares) versus along the line of nuclei at the dorsal or ventral periphery (filled squares) during early nuclear cycle 14. (D) Expression of *cad* protein in wild-type (open squares) and bcd^{E1} mutant embryos (filled squares) as revealed from anti-*cad* whole mount immunostainings of early nuclear cycle 14 embryos. (E) *bcd* protein distribution in 0–1 hr (filled squares) and 1–7 hr (open squares) unfertilized eggs. Staining was done in two different batches. Posteriormost values were assumed to be background levels and were used for relating the two separate stainings. (F) Immunostain intensity in an embryo from a bcd^{E1}/bcd^{E1} female (closed squares). Open squares show normal *bcd* protein distribution in osk^- embryos stained in the same batch. osk^- embryos have the same *bcd* protein distribution as wild-type embryos (Driever and Nüsslein-Volhard, 1988).

the mechanism of translational control might involve components that trap the *bcd* mRNA at the anterior of the oocyte and early embryo. On the other hand, the *cad* message, which is also translated only after egg deposition, is evenly distributed throughout the egg (Macdonald and Struhl, 1986). The biological significance of the translational control is not entirely obvious. Since the *bcd* protein concentration increases during the early hours of embryogenesis, the final shape of the gradient might ultimately depend on the total time of *bcd* mRNA translation. Alter-

natively, together with other maternal messages, the *bcd* mRNA may be subject to a more general translational control mechanism, allowing for a concerted action of several gene products in early development.

Molecular Mechanisms of *bcd* Function

The presence of a homeobox domain (McGinnis et al., 1984; Laughon and Scott, 1984; Desplan et al., 1985) in *bcd* suggests that the *bcd* protein binds specifically to DNA and thereby regulates the transcription of zygotic target genes. The predominant nuclear location of the *bcd* protein in early embryos supports this hypothesis. There is no precedent for how the protein might act in regulating different target genes in a concentration-dependent manner during early development. The concentration of *bcd* protein in the nuclei, from which it must be released and taken up again during each nuclear cycle, changes from the anteriormost position to 50% egg length by 2 or 3 orders of magnitude in an exponential fashion. This large range of concentration would allow differential binding, in a concentration-dependent manner, to promoter sites with low, intermediate, or high affinity for the *bcd* protein. Multiple binding sites for the *bcd* protein might exist in a promoter region, and a monomer–oligomer equilibrium of *bcd* protein could lead to differential binding behaviors. Dimer formation of DNA-binding proteins has been shown in prokaryotes as well as eukaryotes (e.g., GCN4 in yeast; Hope and Struhl, 1987). The properties of the *bcd* protein therefore might provide a molecular basis for a transition of the continuously changing *bcd* concentration into a series of discrete states of activated genes.

Gradients in Early Development

During the last decades a number of models explaining the formation and interpretation of morphogenetic gradients have been proposed (Lawrence, 1966; Wolpert, 1969; Crick, 1970; Gierer and Meinhardt, 1972; Lewis et al., 1977; Meinhardt, 1977, 1982, 1986; MacWilliams, 1978). According to our results, it is likely that the *bcd* protein gradient is generated simply by diffusion from a local source and dispersed degradation. So far there is no evidence for more elaborate regulatory mechanisms such as autocatalysis as proposed by Gierer and Meinhardt (1972). In Meinhardt's (1977, 1986) models for early insect development, only one maternally derived gradient with a high point at the posterior end determines four cardinal (central) and two marginal (terminal) zones in a concentration-dependent manner. The borders between any two cardinal regions were proposed to act as organizing zones for the initiation of the first periodic pattern, the double segments. In a similar fashion, for the anterior pattern the *bcd* protein gradient might regulate some of the earliest zygotically transcribed genes, the gap genes, each of which is necessary for the development of broad regions along the anteroposterior axis (Nüsslein-Volhard and Wieschaus, 1980). The possible influences of *bcd* on the expression of the gap genes are discussed in detail by Driever and Nüsslein-Volhard (1988).

In addition to controlling the transcription of gap genes, *bcd* might interact with other maternal gene products. The *cad* gene is expressed both maternally and zygotically (Macdonald and Struhl, 1986). In wild-type embryos the *cad* protein derived from the maternal transcript shows a graded distribution at early nuclear cycle 14, but is homogeneously distributed in the very early stages of embryonic development. In bcd^- embryos at early nuclear cycle 14, *cad* protein derived from maternal mRNA is evenly distributed (Figure 6D). These results suggest that the *bcd* protein negatively regulates *cad* translation or *cad* transcript stability directly or indirectly, thereby generating the *cad* protein gradient. The fact that in the wild type *cad* protein gradient formation precedes *cad* RNA degradation favors an involvement of *bcd* in translational control. The effects of *bcd* on the abdominal region might therefore also result from its interaction with the *cad* gradient. Similar to *cad*, the gene *hb* (in addition to its zygotic expression) gives rise to maternal transcripts and protein that forms a gradient, but in this case with an anterior maximum and due to interaction with posterior activity (Tautz, 1988). Both the maternal *cad* and maternal *hb* gradients have in common that their genetic deletion does not result in a mutant embryonic phenotype; in their absence embryonic development is largely normal (Macdonald and Struhl, 1986; Lehmann and Nüsslein-Volhard, 1987). Therefore *cad* and *hb* may have more general functions (e.g., in stabilizing transcriptional events during early development) rather than exerting a discrete morphogenetic function.

In contrast, in the case of the *bcd* protein gradient the region that is covered by the gradient and the one in which phenotypic effects can be detected in mutant embryos correlate very well. Furthermore, the *bcd* mRNA and protein distribution can explain in detail the results of transplantation experiments (Frohnhöfer and Nüsslein-Volhard, 1986, 1987; Nüsslein-Volhard et al., 1987). The accompanying paper (Driever and Nüsslein-Volhard, 1988) presents extensive evidence that the *bcd* protein gradient determines anterior pattern in the Drosophila embryo.

Experimental Procedures

Fly Strains

The wild-type stock was Oregon R. The *bcd* alleles and *Df(3R)LIN*, bcd^-, have been described (Frohnhöfer and Nüsslein-Volhard, 1986). All mutant chromosomes carried suitable visible markers. Flies were grown and eggs collected under standard conditions (Nüsslein-Volhard et al., 1984). Staging of embryos was according to Campos-Ortega and Hartenstein (1985).

DNA Techniques

The *bcd* cDNA clones have been described previously (Frigerio et al., 1986; Berleth et al., 1988). The *lacZ–bcd* fusion gene was obtained by cloning the 1898 bp PstI–HindIII fragment coding for 77% of the largest open reading frame into the PstI and HindIII sites of pUR291 (Rüther and Müller-Hill, 1985). To obtain the *trpE–bcd* fusion, the 1910 bp BamHI–HindIII fragment containing the *bcd* sequences was isolated from the *lacZ–bcd* fusion and cloned into the BamHI and HindIII sites of pATH11, thereby extending the *trpE* open reading frame (Körner, unpublished; Klämbt and Schmidt, 1986). Cloning was carried out in JM83 host strains as described in Maniatis et al. (1982). The *lacZ–bcd* fusion construct (pUR*bcd*) was transformed into E. coli 71-18 hosts, and the *trpE–bcd* fusion construct (pATH*bcd*) was transformed into an E. coli C-600 host.

Production and Purification of Polyclonal Antibodies

The LacZ–*bcd* fusion protein was used as the antigen. The TrpE–*bcd* fusion was used for purification of the antibody. This strategy proved to be efficient in producing antibodies with little or no cross-reactivity to the carrier protein (see Klämbt and Schmidt, 1986; Gaul et al., 1987).

For preparation of LacZ–*bcd* fusion protein, bacteria were grown in L broth (50 μg/ml ampicillin) to an OD_{600} of approximately 0.4 and were subsequently induced for 2 hr by addition of 1 mM isopropyl thiogalactoside. Isolation of the LacZ–*bcd* protein was according to a protocol modified from Rio et al. (1986). Bacteria from 2 liter induced cultures were thawed on ice in 50 ml of buffer A (50 mM Tris [pH 7.9], 200 mM NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM leupeptin), and lysozyme was added (final concentration 0.2 mg/ml). After a 20 min incubation on ice, Triton X-100 was added to 1%; after a further 10 min on ice, NP-40 was added to 0.5%. After an additional incubation for 10 min, the suspension was sonicated (three times for 30 sec; Branson Sonifier with microtip, setting 4) on ice. Ten milliliters of the lysate was layered on top of a 10 ml sucrose cushion (40% sucrose, 10 mM Tris [pH 7.5], 1 mM EDTA, 200 mM NaCl) and centrifuged (30 min; 15000 × g, 4°C). The pellet was resuspended in 5 ml of PBS, and 20 ml of extraction buffer B (8 M urea, 0.5 M NaCl, 0.5 M Tris–HCl [pH 7.9], 1 mM EDTA, 30 mM β-mercaptoethanol, 1 μM pepstatin, 1 μM leupeptin) was added. The suspension was dissolved by extensive vortexing and then dialyzed against dialysis buffer C (50 mM Tris [pH 7.9], 0.5 M NaCl, 10% glycerol, 1 mM phenylmethysulfonyl fluoride). Precipitated material was sedimented, and supernatant and precipitate were analyzed by SDS-PAGE. About 80% of the LacZ–*bcd* fusion protein was in the pellet, and it was approximately 20% pure. LacZ–*bcd* fusion protein was dissolved in sample buffer (Laemmli, 1974) and subjected to preparative SDS-PAGE. Protein bands were visualized by incubating the gel with 0.25 M KCl in H_2O. The LacZ–*bcd* fusion protein band was cut out. Gel slices were pressed through very fine metal sieves and suspended in 2 vols of complete Freund's adjuvant (incomplete for booster injections). Rabbits were injected at multiple subcutaneous and intramuscular sites, boosted after 4 weeks, and after an additional 2 weeks bled on a weekly schedule. Two out of four rabbits produced high titers of antibodies against the *bcd* part of the fusion protein.

Bacteria carrying the *trpE–bcd* constructs were grown in M9 medium (Maniatis et al., 1982), supplemented with 5 g/l tryptophan-free Casamino acids (Difco), to an OD_{600} of 0.4. Fusion protein synthesis was induced by addition of 5 μg/ml indolylacrylic acid (Serva) for 4 hr. The fusion protein was isolated according to Rio et al. (1986). TrpE–*bcd* protein was dialyzed against 0.1 M HEPES (pH 7.5), 0.5 M NaCl (three times for 2 hr, once overnight), and precipitated material was sedimented 10000 × g, 10 min). The supernatant (1.4 mg/ml protein) was used to prepare an affinity column (Affigel 10/15, 3:1 ratio; Biorad) according to the manufacturer's protocol (14.5 mg of protein coupled to 10 ml of Affigel 10/15). The column was washed with elution buffer (4 M $MgCl_2$ in PBS) and equilibrated in PBS.

Ten milliliters of antiserum was diluted 1:1 with PBS, 300 mM NaCl, 0.1% Triton X-100, and loaded onto the column. The column was washed (PBS, 300 mM NaCl, 0.1% Triton X-100), and the antibody was eluted with 4 M $MgCl_2$ (pH 3.8). Fractions containing protein were pooled and dialyzed against PBS. To remove any residual anti-β-galactosidase antibodies, the antibodies were passed through a β-galactosidase–Affigel column.

Antibodies cross-reacting with Drosophila proteins other than *bcd* were absorbed by passing the antibodies over an Affigel column prepared with proteins extracted from 8–24 hr embryos. The antisera as well as affinity-purified antibodies was checked for activity against *bcd*-specific sequences as described by Gaul et al. (1987). The purity of the antibody preparation can be judged from Figure 1D.

Generation of Monoclonal Antibodies

Female BALB/cJ mice were injected intraperitoneally with about 50 μg of LacZ–*bcd* fusion protein (purified by SDS-PAGE as described above) in complete Freund's adjuvant (GIBCO). They were boosted several times with the LacZ–*bcd* fusion protein (in incomplete Freund's adjuvant) and finally two times intravenously with TrpE–*bcd* fusion protein (in PBS); the latter injections were at 3 days and at 12 hr before cell fusion. This scheme should especially stimulate proliferation of cells producing antibodies against the *bcd* part of the fusion proteins. Fusion with P3-NC1/1-Ag4-1 myeloma cells (Köhler et al., 1976) using polyethylene glycol, selection, screening, and subcloning were done according to standard procedures (Köhler and Milstein, 1976; Fazekas de St. Groth and Schneidegger, 1980; Saumweber, 1980).

Western Blot Analysis

Staged embryos were collected on agar plates, washed, dechorionated in 50% bleach, and washed again (10 mM Tris [pH 7.2], 300 mM NaCl, 0.5% Triton X-100). One hundred microliter samples of settled embryos were frozen in liquid nitrogen and thawed while being homogenized with 300 μl of 2× sample buffer, 8 M urea. After incubation for 5 min at 95°C, undissolved material was sedimented. Fifteen microliters of the supernatant was applied to each slot on a 3 mm thick 10% SDS–polyacrylamide gel. Following SDS-PAGE, protein was blotted to nitrocellulose; complete transfer at high field was obtained within 3 hr as checked by the transfer of prestained molecular weight markers (Sigma). Blots were incubated for 30 min in 5% low-fat dry milk in PBS, 0.1% Tween 80, and then overnight at 4°C with the first antibody in the same solution. Blots were washed four times for 15 min each and incubated with affinity-purified, alkaline phosphatase–coupled goat anti-rabbit antibodies (1:2500, Jackson Immunoresearch) for 3 hr at room temperature. After washing, staining was developed with bromochloroindolyl phosphate (5 mg/ml in dimethyl sulfoxide; 1 part) and nitro blue tetrazolium (1 mg/ml in H_2O; 10 parts) at pH 10.2 in 50 mM Na_2CO_3, 2 mM $MgCl_2$ (100 parts), for several hours (Leary et al. 1983).

Whole Mounts of Embryos

Immunological staining of whole mount embryos with biotinylated HRP–avidin complexes bound to biotinylated second antibody (Vector Laboratories, Avidin Biotin ABC system) was carried out as described by Macdonald and Struhl (1986) except that during the washes we added 100 mM NaCl to the solutions. Antibodies against *cad* protein were obtained from Paul Macdonald (Macdonald and Struhl, 1986).

Microphotographs were taken on Agfapan 25 (Agfa) with a Zeiss photomicroscope using Nomarski optics.

Measurement of Whole Mount Staining

With embryos used for quantitative analysis, the HRP staining reaction was done for a relatively short time (4 min, versus ca. 8 min for "qualitative" photography) to keep the HRP reaction within the linear range. To test the linearity of the ABC-HRP staining reaction within a given concentration range, we spotted serial dilutions of the TrpE–*bcd* fusion protein on nitrocellulose (150 pg to 10 μg in a volume of 10 μl) and performed an immunostaining reaction identical to the whole mount immunostaining. The nitrocellulose was dissolved in dimethyl sulfoxide between two glass plates and scanned in a densitometer (Joyce and Loebel). The ABC-HRP staining intensity was proportional to the antigen concentration from about 150 pg to 10 ng per 10 μl of immobilized protein. Comparison with stain intensities in whole mounts showed that the signal intensity in the embryos was well within the linear range posterior to 90% egg length.

Video pictures were obtained from early nuclear cycle 14, whole mount–stained embryos using a Zeiss photomicroscope with bright field optics and a Panasonic WV-1850/G video camera. The image was digitized on an IBM-AT equipped with an FG100 board (Imaging Technologies Inc.) and analyzed using Imagepro software (Media Cybernetics). First, a background picture was taken, and the immunostain image was corrected for background by the difference function supplied by the multiimage operation submenu. By this procedure we can exclude an influence of slightly inhomogeneous illumination or other technical factors on the image analysis.

The computer assigned gray values between 1 and 256 to the pixels (image points). We measured these gray values along an axis from the anterior to the posterior pole (except where stated otherwise) in a central plane of focus (pole cells in focus). Thirty equidistant points (correction for egg length) were measured and analyzed for five to ten embryos of each genotype. Mean values and standard deviations were calculated (see Figure 6A). The arbitrary units displayed on the y-axis of the graphs correspond to the gray values 1–256 assigned by the computer. Using a different approach of quantifying the staining (scanning the whole mounts with sensitive photodiodes through a micro-

scope; equipment supplied by T. Bonhoeffer and V. Braitenberg, Tübingen) gave approximately the same results.

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References

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M., and Nüsslein-Volhard, C. (1988). The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. EMBO J., in press.

Campos-Ortega, J. A., and Hartenstein, V. (1985). The Embryonic Development of *Drosophila melanogaster.* (Heidelberg: Springer-Verlag).

Carroll, S. B., and Scott, M. P. (1985). Localization of the *fushi tarazu* protein during Drosophila embryogenesis. Cell *43*, 47–57.

Child, C. M. (1915). Individuality in Organisms. (Chigaco: University of Chicago Press).

Crick, F. (1970). Diffusion in embryogenesis. Nature *225*, 420–422.

Dalcq, A., and Pasteels, J. (1938). Potential morphogénétique, regulation et "axial gradients" de Child. Bull. Acad. Roy. Méd. Belg. *6*, 261–308.

Desplan, C., Thies, J., and O'Farrell, P. H. (1985). The Drosophila developmental gene, *engrailed*, encodes a sequence-specific DNA binding activity. Nature *318*, 630–635.

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

Fazekas de St. Groth, S., and Schneidegger, D. (1980). Production of monoclonal antibodies: strategy and tactics. J. Immunol. Meth. *35*, 1–21.

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

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

Frohnhöfer, H. G. (1987). Maternale Gene und die Anlage des anteroposterioren Musters in *Drosophila* Embryonen. Ph.D. thesis, Eberhard-Karls-Universität, Tübingen.

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

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

Frohnhöfer, H. G., Lehmann, R., and Nüsslein-Volhard, C. (1986). Manipulating the anterior posterior pattern of the *Drosophila* embryo. J. Embryol. Exp. Morphol. *97*, 169–179.

Gaul, U., Seifert, E., Schuh, R., and Jäckle, H. (1987). Analysis of *Krüppel* protein distribution during early Drosophila development reveals posttranscriptional regulation. Cell *50*, 639–647.

Gierer, A., and Meinhardt, H. (1972). A theory of biological pattern formation. Kybernetik *12*, 30–39.

Hörstadius, S. (1939). The mechanics of sea urchin development. Biol. Rev. *14*, 132–179.

Hope, I. A., and Struhl, K. (1987). GCN4, a eucaryotic transcriptional activator protein, binds as a dimer to target DNA. EMBO J. *6*, 2781–2784.

Kalthoff, K. (1979). Analysis of a morphogenetic determinant in an insect embryo (Smittia spec. Chironomidae, Diptera). Symp. Soc. Dev. Biol. *37*, 97–126.

Klämbt, C., and Schmidt, O. (1986). Developmental expression and tissue distribution of the *lethal (2) giant larvae* protein of *Drosophila melanogaster.* EMBO J. *5*, 2955–2961.

Köhler, G., and Milstein, C. (1976). Derivation of specific antibody-producing tissue culture and tumor cell lines by cell fusion. Eur. J. Immunol. *6*, 511–519.

Köhler, G., Howe, S. C., and Milstein, C. (1976). Fusion between immunoglobulin secreting and non secreting myeloma cell lines. Eur. J. Immunol. *4*, 292–295.

Kreis, T. E., Geiger, B., and Schlessinger, J. (1982). Mobility of microinjected rhodamine actin within living chicken gizzard cells determined by fluorescence photobleaching recovery. Cell *29*, 835–845.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Laughon, A., and Scott, M. P. (1984). Sequence of a *Drosophila* segmentation gene: protein structure homology with DNA binding proteins. Nature *310*, 25–30.

Lawrence, P. A. (1966). Gradients in the insect segment: the orientation of hairs in the milkweed bug *Oncopeltus farsciatus*. J. Exp. Biol. *44*, 607–620.

Leary, J. J. (1983). Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. Proc. Natl. Acad. Sci. USA *80*, 4045–4049.

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

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

Lewis, J. H., Slack, J. M. W., and Wolpert, L. (1977). Thresholds in development. J. Theor. Biol. *65*, 579–590.

Macdonald, P. M., and Struhl, G. (1986). A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. Nature *324*, 537–545.

MacWilliams, H. K. (1978). A model of gradient interpretation based on morphogen binding. J. Theor. Biol. *72*, 385–411.

Maden, M. (1982). Vitamin A and pattern formation in the regenerating limb. Nature *295*, 672–675.

Mahowald, A. P., Goralski, T. J., and Caulton, J. H. (1983). In vitro activation of *Drosophila* eggs. Dev. Biol. *98*, 437–445.

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

Mastro, A. M., Babich, M. A., Taylor, W. D., and Keith, A. D. (1984). Diffusion of a small molecule in the cytoplasm of mammalian cells. Proc. Natl. Acad. Sci. USA *81*, 3414–3418.

McGinnis, W., Levine, M. S., Hafen, E., Kuroiwa, A., and Gehring, W. J. (1984). A conserved DNA sequence in homeotic genes of *Drosophila Antennapedia* and *bithorax* complexes. Nature *308*, 423–433.

Meinhardt, H. (1977). A model for pattern formation in insect embryogenesis. J. Cell Sci. *23*, 117–139.

Meinhardt, H. (1982). Models of Biological Pattern Formation. (London: Academic Press).

Meinhardt, H. (1986). Hierarchical inductions of cell states: a model for segmentation in Drosophila. J. Cell Sci. (Suppl.) *4*, 357–387.

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

Mlodzik, M., Fjose, A., and Gehring, W. J. (1985). Isolation of *caudal*, a *Drosophila* homeobox containing gene with maternal expression,

whose transcripts form a concentration gradient at the preblastoderm stage. EMBO J. *4*, 2961–2969.

Morgan, T. H. (1901). Regeneration. (New York: Macmillan).

Nüsslein-Volhard, C. (1979). Maternal effect mutations that alter the spatial coordinates of the embryo of *Drosophila melanogaster*. In Determinants of Spatial Organization, I. Konigsberg and S. Subtelney, eds. (New York: Academic Press), pp. 185–211

Nüsslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. Nature *287*, 795–801.

Nüsslein-Volhard, C., Wieschaus, E., and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. Wilhelm Roux's Arch. Dev. Biol. *183*, 267–282.

Nüsslein-Volhard, C., Frohnhöfer, H. G., and Lehmann, R. (1987). Determination of anteroposterior polarity in *Drosophila*. Science *238*, 1675–1681.

Rechsteiner, M., Rogers, S., and Rote, K. (1987). Protein structure and intracellular stability. Trends Biochem. Sci. *12*, 390–394.

Rio, D. C., Laski, F. A., and Rubin, G. M. (1986). Identification and immunochemical analysis of biologically active Drosophila P element transposase. Cell *44*, 21–32.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science *234*, 364–368.

Runnström, J. (1929). Über Selbstdifferenzierung und Induktion beim Seeigelkeim. Wilhelm Roux's Arch. Entwicklungsmech. Org. *117*, 123–145.

Rüther, U., and Müller-Hill, B. (1983). Easy identification of cDNA clones. EMBO J. *2*, 1791–1794.

Sander, K. (1959). Analyse des ooplasmatischen Reaktionssystems von *Eucelis plebejus* Fall. (Cicadina) durch Isolieren und Kombinieren in Eiteilen I: Die Differenzierungsleistungen vorderer und hinterer Eiteile. Wilhelm Roux's Arch. Entwicklungsmech. Org. *151*, 430–497.

Sander, K. (1960). Analyse des ooplasmatischen Reaktionssystems von *Euscelis plebejus* Fall. (Cicadina) durch Isolieren und Kombinieren von Eiteilen II: Die Differenzierungsleistungen nach Verlagerung von Hinterpolmaterial. Wilhelm Roux's Arch. Entwicklungsmech. Org. *151*, 660–707.

Sander, K. (1976). Specification of the basic body pattern in insect embryogenesis. Adv. Insect Physiol. *12*, 125–238.

Saumweber, H. (1980). Monoklonale Antikörper gegen Kernproteine von *Drosophila melanogaster*. Ph.D. thesis, Eberhard-Karls-Universität, Tübingen.

Schaller, H. C., and Bodenmüller, H. (1981). Isolation and amino acid sequence of a morphogenetic peptide from hydra. Proc. Natl. Acad. Sci. USA *78*, 7000–7004.

Schaller, H. C., and Gierer, A. (1973). Distributions of the head-activating substance in hydra and its localization in membraneous particles in nerve cells. J. Embryol. Exp. Morph. *29*, 39–52.

Stumpf, H. (1966). Über gefälleabhängige Bildungen des Insektensegments. J. Insect Physiol. *12*, 601–617.

Tautz, D. (1988). Regulation of the *Drosophila segmentation gene hunchback* by two maternal morphogenetic centres. Nature *332*, 281–284.

Thaller, C., and Eichele, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. Nature *327*, 625–628.

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

Wojcieszyn, J. W., Schlegel, R. A., Wu, E.-S., and Jacobson, K. A. (1981). Diffusion of injected macromolecules within the cytoplasm of living cells. Proc. Natl. Acad. Sci. USA *78*, 4407–4410.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. J. Theor. Biol. *25*, 1–47.