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Autonomous determination of anterior structures in the early *Drosophila* embryo by the *bicoid* morphogen

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Summary

A small number of maternal effect genes determine anterior–posterior pattern in the *Drosophila* embryo. Embryos from females mutant for the maternal gene *bicoid* lack head and thorax. *bcd* mRNA becomes localized to the anterior tip of the egg during oogenesis and is the source for the morphogen gradient of bcd protein.

Here we show that *in vitro* transcribed *bicoid* mRNA that has its own leader sequences substituted by the *Xenopus* β-globin 5′ untranslated sequences is translated more efficiently than *bicoid* mRNA with the natural 5′ mRNA leader when tested *in vitro* and in *Drosophila* Schneider cells. When injected into *bicoid* mutant embryos, only the *bcd* mRNA with the β-globin leader sequence, substituted for the natural leader, is able to induce anterior development. We used P-transformation to show that sequences in the 5′ leader are neither necessary for localization of the transcript nor for the translational block of the *bcd* mRNA during oogenesis.

For our injection experiments, we used only one of the identified splicing forms of *bcd* mRNA. The bcd protein species derived from this mRNA is able to induce anterior development at any position along the anterior–posterior axis. Thus *bicoid* protein can induce development of head and thorax independent of any other specifically localized morphogenetic factor. Our findings further support the notion that the concentration gradient of *bcd* protein, and not the existence of different forms of *bcd* protein, is responsible for specifying subregions of the embryo.

Key words: bicoid, morphogen, Drosophila embryogenesis, translational control, maternal gene.

Introduction

Development of the anterior half of the *Drosophila* embryo depends on the maternal gene *bicoid* (*bcd*) (Nüsslein-Volhard *et al.* 1987). Embryos from females that are homozygous mutant for strong *bcd* alleles lack head and thorax, and instead develop posterior terminal structures (the telson) at the anterior (Fig. 5) (Frohnhöfer and Nüsslein-Volhard, 1986). Cytoplasmic transplantation experiments revealed the presence of an activity located at the anterior tip of embryos from wild-type but not from *bcd* mutant females. This activity, when transplanted to any position along the anterior–posterior axis of a recipient embryo, was able to induce anterior development and suppress the formation of posterior structures at the site of injection (Frohnhöfer and Nüsslein-Volhard, 1986). However, these experiments could not distinguish whether *bcd* is the only anterior determinant in the *Drosophila* embryo, as other activities might have been cotransplanted.

The *bcd* gene is transcribed maternally and *bcd* mRNAs become localized at the anterior tip of the egg during oogenesis (Frigerio *et al.* 1986; Berleth *et al.* 1988). At least four distinct phases of *bcd* mRNA localization can be distinguished during oogenesis (St. Johnston *et al.* 1989). Between stages 6 and 9 of oogenesis (staging according to King, 1970), *bcd* mRNA accumulates in a ring at the anterior end of the oocyte. In stage 9–10a follicles, *bcd* mRNA also localizes to the apical regions of the nurse cells, but as the nurse cells contract during stage 10b–11, all the *bcd* mRNA becomes localized to the cortex at the anterior end of the oocyte. Finally, between stage 12 of oogenesis and egg deposition, *bcd* mRNA becomes localized to a spherical region of the egg that occupies a slightly dorsal position at the anterior pole. The gene products of the maternal genes *exuperantia* (Schüpbach and Wieschaus, 1986), *swallow* (Gans *et al.* 1975), and *staufen* (Schüpbach and Wieschaus, 1986) are involved in the process of *bcd* mRNA localization (Berleth *et al.* 1988; St. Johnston *et al.* 1989). Macdonald and Struhl (1988) demonstrated that sequences in the 3′ untranslated region of the *bcd* mRNA are responsible for a

proper RNA localization. During oogenesis, no bcd protein is detectable (Driever and Nüsslein-Volhard, 1988*a*). This finding and the lack of rescue activity of nurse cell cytoplasm when transplanted into embryos from bcd^{E1} females indicate that *bcd* transcripts are translationally regulated during oogenesis. Translational control has also been proposed for other maternal transcripts in *Drosophila* (e.g. *caudal*: Macdonald and Struhl, 1986).

The analysis of *bcd* cDNAs revealed the existence of several differentially spliced forms of *bcd* mRNA (Berleth *et al.* 1988; see Fig. 1A). Type a cDNAs appear to be most abundant. Type b splice products use an alternate splice acceptor site for the third exon and thus have 5 additional codons inserted just in front of the homeo domain. A corresponding protein could not be detected immunologically in embryonic extracts (Driever and Nüsslein-Volhard, 1988*a*). A third minor splice variant, type c, fused the first with the fourth exon. A specific biological role for individual splice products has not been investigated so far.

Translation of *bcd* protein begins after egg deposition; by the blastoderm stage, the protein is distributed in a concentration gradient along the anterior–posterior axis with a maximum at the anterior tip and extending over about two thirds of the syncytial embryo (Driever and Nüsslein-Volhard, 1988*a*). Genetic experiments allowed the modification of both the shape and height of the bcd protein gradient, and the resulting changes in the blastoderm fate map indicate that bcd protein concentration determines position in the anterior half of the embryo. For example, increasing the dosage of bcd^{+} in the mother led to an increased level of bcd protein in the embryo and a concomitant enlargement of anterior primordia, detectable as a posteriorward shift of the cephalic furrow (Driever and Nüsslein-Volhard, 1988*b*).

The bcd protein contains a homeo domain and has been shown to act as a regulator of at least one zygotic target gene, the gap gene *hunchback* (*hb*) (Tautz *et al.* 1987; Tautz, 1988; Schröder *et al.* 1988). Transcription of *hb* in the anterior half of the embryo is activated by binding of bcd protein to sequence elements in the upstream region of the zygotic *hb* promoter (Driever and Nüsslein-Volhard, 1989; Driever *et al.* 1989*a*,*b*; Struhl *et al.* 1989). Other zygotic target genes might bind *bcd* protein with different affinities (Driever *et al.* 1989*a*), thus converting the information supplied by the *bcd* protein gradient into distinct domains of zygotic gene expression.

In this paper, we address the following main questions. Is *bcd* sufficient to induce anterior development in the *Drosophila* embryo or is there a need for additional localized morphogenetic factors? Is a single bcd protein species sufficient to induce anterior development or is this function performed by several different forms of the protein, arising from the various spliced forms of *bcd* mRNA that have been identified? What might be the basis for the translational control of *bcd* mRNA during oogenesis?

Materials and methods

Plasmids and in vitro *transcription*

bcd mRNAs were transcribed from three different transcription vectors; the additional vector sequences at the 5′ end are: pGem 1 vector (Promega) 58 bases, pGem 3 vector (Promega) 62 bases and Bluescript vector (Stratagene) 73 bases. The constructs are described by Berleth *et al.* (1988) and Driever and Nüsslein-Volhard (1989). Transcripts from all three vectors had similar translation rates (data not shown). *bcd* TN3 transcripts were generated using a pSP 64 vector (Melton *et al.* 1984), and include additional 12 bases of vector sequences. The construct bcdTN3 was generated by inserting the NdeI (end repaired with Klenow polymerase) – *Eco*RV fragment from pARbcdNB and the *Eco*RV – *Eco*RI fragment from c53.46.6c (both described in Driever and Nüsslein-Volhard, 1989) into the *NcoI* (blunt ended with mung bean nuclease) – *Eco*RI digested pSPBP4 vector, a pSP 64 derivative containing a slightly modified *Xenopus* β-globin mRNA leader fragment (Siegel and Walter, 1988). The pSPBP4 vector is based on the pSP64T vector (D.A. Melton, Harvard University).

In vitro transcription was performed according to a protocol slightly modified from D.A. Melton *et al.* (1984). Template plasmids were linearized with *Eco*RI, extracted with phenol, precipitated and dissolved at $0.5\,\mu g\,\mu l^{-1}$ in TE (10 mM Tris–HCl pH 7.5; 2 mM EDTA). Setup of the transcription reaction: 5.5 μl diethyl pyrocarbonate (DEPC) treated distilled water; 6 μl 5× salts (200 mM Tris–HCl pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine); 3 μl 0.1 M dithiothreitol; 2 μl human placental ribonuclease inhibitor (Promega 10–20 units μl^{-1}); 6 μl capped NTP mix 5× (2.5 mM each ATP, UTP, CTP, 2.5 mM G(5′)ppp(5′)G, 0.5 mM GTP); 6 μl linearized DNA in TE $0.5\,\mu g\,\mu l^{-1}$; 1.5 μl Sp6 RNA Polymerase (10–20 units μl^{-1}). Transcription was performed for 60 min at 40°C, then 1.5 μl 10 mM GTP were added and incubation continued for 15 min. The RNA was extracted with phenol and chloroform, precipitated with ethanol in the presence of 0.3 M NaOAc, the pellet washed with cold 70 % ethanol, dried and dissolved in 20 μl DEPC-treated water (typical yield $1.5\,\mu g\,\mu l^{-1}$).

In vitro *translation and expression in* Drosophila *Schneider cells*

The mRNAs were translated *in vitro* using reticulocyte lysate or wheat germ extract (Amersham) according to the manufacturers' protocol. The relative efficiencies of *in vitro* translation were determined by cutting out the bcd protein bands from SDS–PAGE like the one shown in Fig. 2 and measuring the incorporated radioactivity by liquid scintillation counting. Transient expression in *Drosophila* Schneider cells, the preparation of extracts and the immunoblot analysis were as described (Driever and Nüsslein-Volhard, 1989). We constructed the plasmids pMetbcdTN3 and pMetbcdEE by inserting the *Hin*dIII Fragment from pbcdTN3 and, respectively, the *Eco*RI fragment from c53.46.6c, into the pRmHa-3 metallothionein promoter expression vector (Bunch *et al.* 1988).

Injection into embryos

The RNA was injected into pre-polecell-stage embryos at various dilutions (using DEPC-treated water) according to standard procedures (Frohnhöfer and Nüsslein-Volhard, 1986). The biological activity of the injected mRNA was scored by analyzing the cuticular phenotypes of the injected animals after 48 h of development at 18°C. Photography was

performed using dark-field or phase-contrast optics.

P-Transformation and immunohistochemical analysis

The *Hin*dIII–*Eco*RI fragment with the globin leader, the *bcd* ORF and the 3′ untranslated region of the *bcd* transcript was cloned into the P-vector pCaSpeRbcd*Bgl*II, which was kindly provided by David Stein, Tübingen. This vector, a pCaSpeR derivative (Pirrotta, 1988) carries the 2 kb maternal *bcd* promoter fragment from the *Bam*HI site to the *Pst*I site at position 1244 and the genomic fragment from 4292 to the *Eco*RI site at 5.9 kb (pPbcdTN3); numbering of the genomic sequence according to Berleth *et al.* 1988. P transformation was as previously described (Driever *et al.* 1989*a*). Immunohistochemistry using monoclonal anti-*bcd* protein antibody (Driever and Nüsslein-Volhard, 1988*a*) was performed using the Vectastain ABC elite kit peroxidase (Vector Laboratories). Ovaries were dissected in BSS (Chan and Gehring, 1971), frozen in liquid nitrogen and melted again while adding twofold concentrated SDS/mercaptoethanol sample buffer with 8 M urea and sonicating. Extracts from embryos were prepared using the same procedure.

Results

Injection of bcd *mRNA into embryos from* bcd^{E1} *mutant females*

Injection of poly(A)$^+$ mRNA isolated from young wild-type embryos into early embryos from mutant females was shown to rescue the mutant phenotype of several maternal effect genes that regulate pattern along the dorsoventral axis (Anderson and Nüsslein-Volhard, 1984). This approach has not been successful for rescue of the *bcd* mutant phenotype (Berleth, 1989). Similar to experiments perfomed with the maternal gene *easter* (Chasan and Anderson, 1989), we tried to substitute the *bcd* activity by injection of *in vitro* synthesized *bcd* mRNA into the anterior tip of embryos from females homozygous for the strong *bcd*^{E1} allele. We were not able to induce the formation of any anterior wild-type cuticular structures upon injection of the mRNAs at a wide range of concentrations (0.1 to 5 $\mu g\,\mu l^{-1}$) (Berleth, 1989; Table 1 A and data not shown). The *bcd* cDNA of the most abundant splice type a (Fig. 1A) that we used

Table 1. *Frequency of the induction of anterior structures by the injection of* bcd *mRNAs*

(A) *Injection into the anterior of embryos from* bcd^{E1} *females*

	bcd mRNA		bcdTN3 mRNA
RNA concentration ($\mu g\,\mu l^{-1}$)	0.4	5	0.4
Number of embryos	75 (100)	62 (100)	64 (100)
Phenotype			
– anterior not developed	3	17	5
– *bcd*$^-$ mutant (Filzkörper present at the anterior)	72	45	4
– thoracic and gnathal structures induced	0	0	31
– head and thorax completely rescued	0	0	24

(B) *Injection into the posterior of embryos from wild-type females*

	bcdTN3 mRNA
RNA concentration ($\mu g\,\mu l^{-1}$)	1.7
Number of embryos	81 (200)
Phenotype (in the posterior half of the embryo)	
– telson (Filzkörper present, most often abdominal segmentation distorted)	29
– no telson and no anterior structures formed	3
– thoracic structures and parts of the telson formed	13
– thoracic and cephalic structures formed (abdomen reduced)	35
– complete head duplicated	1

(C) *Injection into the middle of embryos from* bcd^{E1} *mutant females*

	bcdTN3 mRNA
RNA concentration ($\mu g\,\mu l^{-1}$)	1.7
Number of embryos	38 (100)
Phenotype	
– *bcd*$^-$ mutant	2
– abdominal development distorted	7
– thoracic structures formed in the middle of the embryo	8
– thoracic and cephalic structures formed in the middle of the embryo (abdomen often completely suppressed)	21

Embryos were injected with *in vitro* transcribed mRNA at the indicated concentrations. The number of embryos indicates the total number of embryos injected in brackets (in sets of 100) and the number of those that developed cuticle and were scored for the phenotype. For details see Materials and methods section.

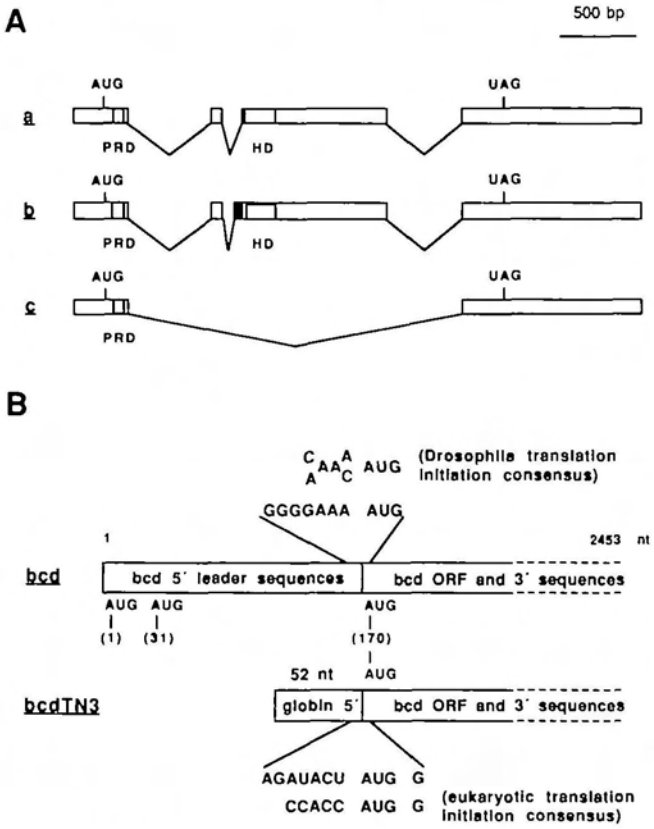

Fig. 1. Structure of *bcd* mRNAs. (A) Splicing patterns of the primary *bcd* transcript according to Berleth *et al.* 1988. AUG is the beginning and UAG the end of the largest open reading frame; PRD is the paired repeat, a histidine- and proline-rich sequence; HD is the *bcd* homeo domain. (B) Structure of the 5′ ends of bcd mRNA and bcdTN3 mRNA transcripts (globin 5′=*Xenopus* β-globin 5′ untranslated sequences). The figure also indicates the translation initiation sequences from the bcd mRNA and the heterologous construct in comparison with the translation initiation consensus sequences for eukaryotes and for *Drosophila* (Kozak, 1986; Cavener, 1987). In addition to the sequences shown, the *in vitro* transcripts include vector sequences (see Materials and methods).

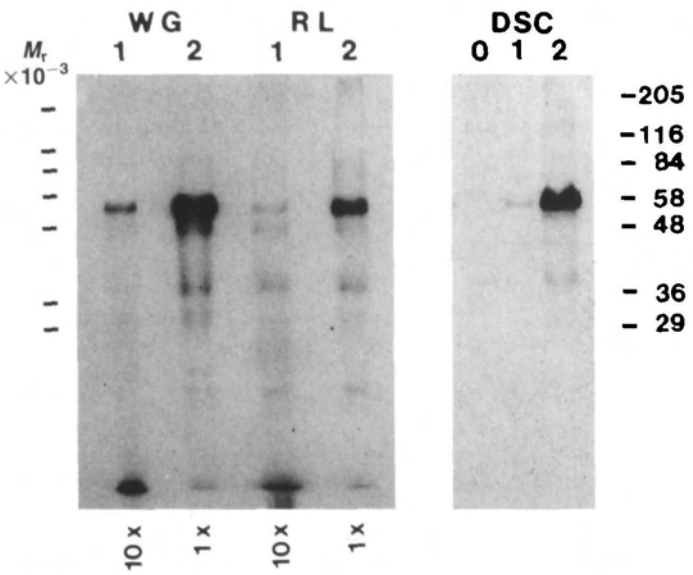

Fig. 2. Sequences in the 5′ non-translated leader regulate the efficiency of *bcd* mRNA translation. *In vitro* transcribed bcd mRNA (lanes 1) and bcdTN3 mRNA with the *bcd* 5′ untranslated region exchanged with the one from the *Xenopus* β-globin (lanes 2), were translated *in vitro* in a wheat germ extract (WG) and a rabbit reticulocyte lysate (RL) in the presence of [^{35}S]methionine. The products were displayed by SDS–PAGE and visualized by autoradiography. For the products of the bcd mRNA translations, ten times more material was loaded on the gel than for those of bcdTN3 mRNA. Both types of transcripts were also transiently expressed from the metallothionein promoter in *Drosophila* Schneider cells (DSC); cells were lysed and extracts analysed by immuno blotting: The lane designated 0 shows extracts from control cells. Relative molecular masses are indicated at the left and right side.

as a template for the transcription begins a few bases downstream of a TATA box, contains a polyadenylation signal and is at both ends only a few bases longer than a number of other cDNAs (Berleth *et al.* 1988). Thus we believe that our template is a full-length cDNA, though the transcription start site has not been mapped. All missense mutations in the cDNA with respect to the genomic sequence have been corrected (Driever and Nüsslein-Volhard, 1989).

Translation efficiency of bcd *mRNA*

We tested the mRNAs by *in vitro* translation in a wheat germ and a reticulocyte lysate system and found that they were translated very inefficiently (Fig. 2, lanes WG 1 and RL 1). In addition, expression of the same cDNA in *Drosophila* Schneider cells from the metallothionein promoter resulted in low levels of bcd protein (Fig. 2, lane DSC 1). In an attempt to circumvent putative translational control mechanisms that would reduce the efficiency of translation, we exchanged the 5′ non-translated sequence of the abundant splice type a of *bcd* mRNA (Fig. 1) for that of the *Xenopus* β-globin mRNA (bcdTN3 expression construct). This sequence was chosen because it has neither upstream AUGs nor a strong secondary structure. Tests in all three translation systems showed that bcdTN3 mRNA with the globin leader is translated about 50 times more efficiently than the native *bcd* mRNAs (Fig. 2). From wheat germ translations, we recovered 41 (±3) times more activity for bcdTN3 mRNA than for bcd mRNA templates. Using reticulocyte lysates, the factor was 51 (±4; average of three determinations each).

Rescue of the bcd *mutant phenotype by the injection of bcdTN3 mRNA*

The development of anterior structures can be induced by the injection of *bcd*TN3 mRNA at appropriate concentrations into the anterior tip of embryos from *bcd*^{E1} mutant females (Table 1A, Fig. 4 and 5B; see also Driever *et al.* 1989*b*). By autoradiography and by *in situ* hybridization, we find that injected mRNAs maintain a high point at the site of injection for more than one hour (V.S. and D. Ferrandon, unpublished). When we analyse the distribution of bcd protein 90 min after

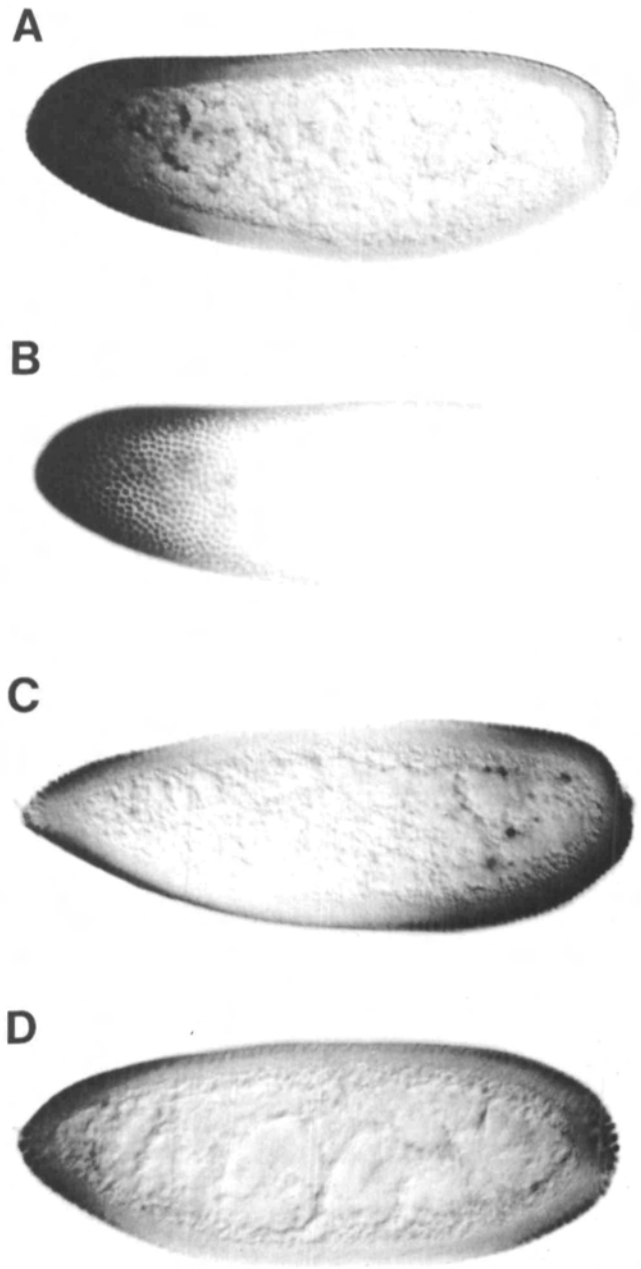

Fig. 3. bcd protein distribution in embryos injected with bcdTN3 mRNA. Embryos were injected with bcdTN3 mRNA before pole cell formation, developed for 90 min, fixed and immunostained for bcd protein as whole mounts. Anterior is left. The photographs show optical sections using Nomarski optics. (A and B) Embryo from a bcd^{E1} homozygous female, injected at the anterior pole. Plane of focus at the center (A) and the surface (B) of the same embryo. A nuclear gradient of bcd protein forms similar to the one detectable in wild-type embryos. (C and D) Embryos from wild-type females, injected at the posterior pole. The embryo in C was probably injected with a larger amount of bcdTN3 mRNA than the one in D. The staining at the anterior pole shows the wild-type bcd protein and might serve as a comparison.

injection, using a monoclonal antibody that detects only the full-length *bcd* protein derived from the injected mRNA in whole-mount immunostain reactions (Fig. 3 A and B), we detect the formation of a protein concentration gradient similar to the one in embryos from wild-type females.

Injection of *bcd*TN3 mRNA at a concentration of $0.4\,\mu g\,\mu l^{-1}$ suppressed the formation of telson structures at the anterior in nearly all of the injected embryos, and induced the development of all the anterior structures present in wild-type larvae (acron, head and thorax) in about 30% of these embryos (Table 1 A). About 10% of the larvae hatched, and some developed into adults. Embryos that failed to hatch frequently exhibited fused abdominal segments (A3, A4 and A5 were affected in most cases). Since similar phenotypes can be observed in embryos from females that carry 6 or 8 functional copies of the *bcd* gene in their genome (Berleth, 1989; our unpublished data), we interpret these defects as being caused by the injection of excessive amounts of *bcd*TN3 mRNA into the embryo. We propose that the defects in abdominal segmentation we obtain are an indirect effect: increased levels of bcd protein lead to a posteriorward extension of the *hb* protein gradient and thereby might inhibit *knirps* expression, which is necessary for abdomen formation (Hülskamp *et al.* 1989; Irish *et al.* 1989; Struhl, 1989).

Our findings support the model in which bcd protein concentration directly determines position; an elevated level of mRNA and thus protein results in an enlargement of anterior and a compression of posterior pattern, giving rise to defects in abdominal segmentation. Indeed, during gastrulation in some cases we observed that the cephalic furrow was shifted back as far as 40% egg length (the normal position of the cephalic furrow is at 65% egg length; 0% is at the posterior pole; data not shown).

Induction of anterior development at ectopic positions

Confirming and extending the results from cytoplasmic transplantations (Frohnhöfer and Nüsslein-Volhard, 1986), we found that *in vitro* transcribed bcdTN3 mRNA can induce anterior development at any ectopic position along the anterior–posterior axis (Fig. 5). Injection into the posterior pole of embryos from bcd^{-} (not shown) or wild-type females (Table 1B and Fig. 5E) suppresses posterior development and induces the formation of head and thoracic structures at the posterior end. Injection of bcdTN3 mRNA at $1.7\,\mu g\,\mu l^{-1}$ into the posterior pole in one case resulted in the development of a complete second head and thorax (Fig. 6). Fig. 3 demonstrates that bcd protein gradients similar to the one in the anterior of wild-type embryos are formed upon the injection of the RNA. The requirement for a 4 to 5 times greater amount of bcd mRNA for the induction of a head at the posterior as opposed to the anterior end of the embryo may be due to the negative effect of the activity of posterior group genes on *bcd* mRNA stability (Frohnhöfer and Nüsslein-Volhard, 1986; Wharton and Struhl, 1989). The

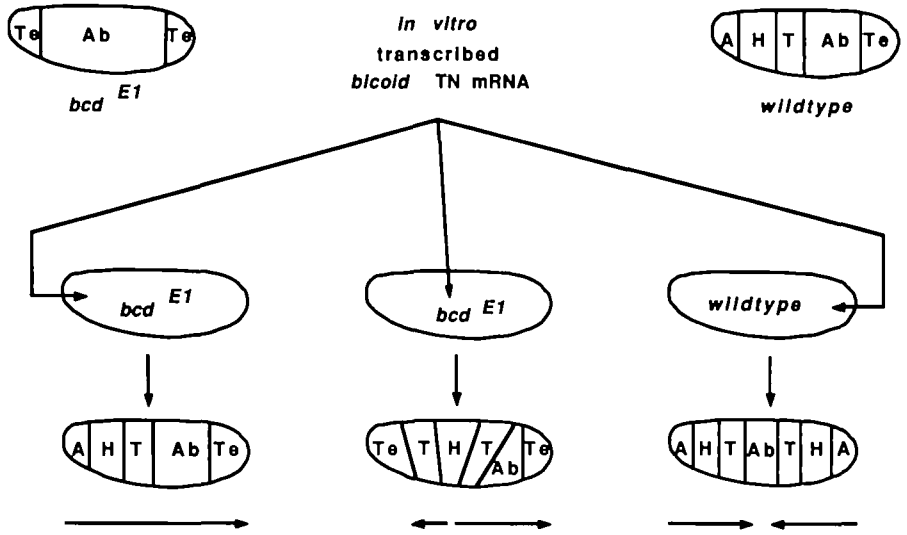

Fig. 4. Schematic description of the mRNA injection experiments. Shown are the experimental setup (middle row) and the induced changes on the blastoderm fate map as revealed by the analyses of the cuticular patterns (bottom row; arrows indicate the polarity of the embryo, from anterior to posterior). When injecting into the middle of the embryo, a local response (e.g. predominantly at the dorsal side) is common. At the top, the blastoderm fate maps of the recipient embryos (from *bcd* ^{E1} mutant females or wild-type, respectively) are drawn (modified from Frohnhöfer and Nüsslein-Volhard, 1986). Embryos are oriented with anterior to the left, dorsal at the top.

Abbreviations: A=Acron, Ab=Abdomen, H=Head, T=Thorax, Te=Telson.

formation of duplicated abdominal segments with mirror-image symmetry (A1 in Fig. 5E) appears to be an indirect organizing influence of *bcd* on abdominal pattern formation, as *bcd* on its own is not able to induce abdominal structures (Frohnhöfer and Nüsslein-Volhard, 1986; Lehmann and Frohnhöfer, 1989).

Injection into the middle of embryos from *bcd*$^-$ females results in the formation of gnathal and thoracic structures at the site of injection (Table 1C, Fig. 5 C and D, results summarized in Fig. 4): we were able to identify pro-, meso- and metathoracic structures, antennal and maxillary sense organs, mouth hooks, cirri and other sclerotic structures that could not be assigned unambiguously. The structures usually appear pairwise and are arranged in a bilateral symmetric way transverse to the anterior–posterior axis of the embryo. None of the involuted parts of a wild-type head or acronal structures were identified. This result is consistent with data obtained from the analysis of maternal genes that affect terminal development in the embryo. The formation of acronal structures requires, in addition to *bcd*, the *torso* gene group, whose activity is restricted to the termini of the embryo (Nüsslein-Volhard *et al.* 1987; Klingler *et al.* 1988).

Is the bcd *mRNA leader involved in translational control during oogenesis?*

To investigate the influence of the 5′ sequences on translational control, we used a P-transformation vector that allows the expression of bcdTN3 mRNA during oogenesis. The CaSpeR-derived plasmid pPbcdTN3 carries a 2 kb *bcd* promoter fragment as well as *bcd* 3′ sequences and has the bcdTN3 cDNA sequences inserted at the *Pst*I site just a few basepairs downstream from the putative start site of transcription. We generated 12 transgenic lines in a w^1 stock, 4 of which carried insertions on the second chromosome. Those were crossed into $bcd^{E1}/TM3$ flies. Females homozygous for the strong allele bcd^{E1} that carry one pPbcdTN3 insertion give rise to progeny with a phenotype similar to that of weak *bcd* alleles (Fig. 7A; Frohnhöfer and Nüsslein-Volhard, 1986). The analysis of the expression pattern of *even-skipped* (Frasch *et al.* 1987) reveals that the gnathal and thoracic region is expanded and shifted slightly more anteriorwards than in embryos from mothers heterozygote for a deficiency (Fig. 7B; Frohnhöfer and Nüsslein-Volhard, 1987). Two copies of pPbcdTN3 in the germline rescue the bcd^{E1} mutant phenotype completely and give rise to fertile adults. We conclude that our construct provides slightly less bcd^+ activity to the embryo than a wild-type copy of the gene does. For the *in vivo* function of the gene, in our assay the 169 bp leader sequence seems not to contain any essential elements.

To test whether the observed translational control of *bcd* mRNA during oogenesis is due to sequence elements in the leader, we analysed ovaries from females carrying two pPbcdTN3 insertions for the presence of bcd protein. In whole mounts of ovaries, immunohistologically stained for bcd protein with a monoclonal antibody, we were not able to detect any specific, *bcd*-dependent nuclear staining in the nurse cells where the mRNA is synthesized and present at distinct apical patches during early stages of development (data not shown). Neither could we detect any specific staining in the oocyte. Though we used the same method of immunodetection that gives strong signals for bcd protein during syncytial blastoderm stages, we might have failed to detect very low levels of expression during oogenesis. Therefore, we prepared extracts for immunoblot analysis from ovaries of females homozygous for bcd^{E1} and pPbcdTN3 (Fig. 8). As a control, we analysed on the same Western blot extracts of blastoderm stage embryos from females of the genotypes pPbcdTN3/pPbcdTN3; bcd^{E1}/bcd^{E1}, pPbcdTN3/+; bcd^{E1}/bcd^{E1} and from a strain carrying 8 functional copies of the *bcd* gene, for which we also prepared ovarian extracts. We estimate that stages of

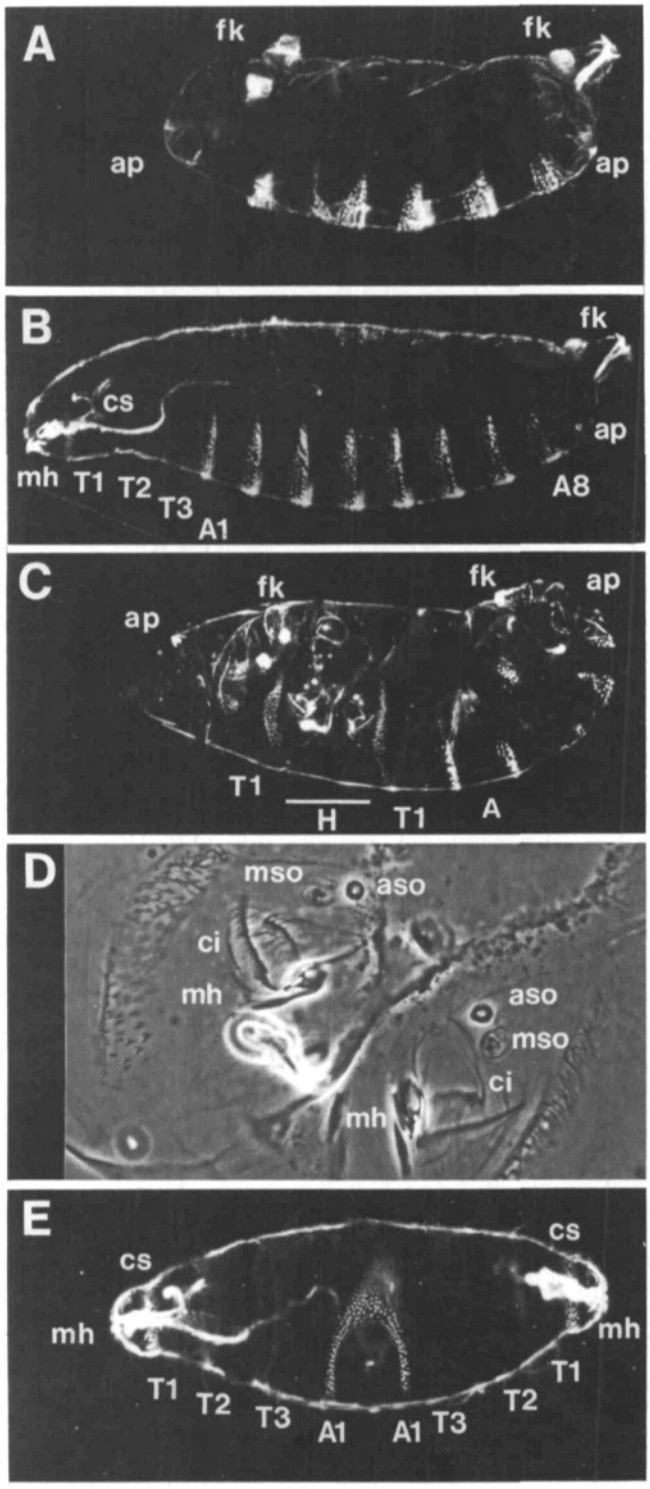

Fig. 5. Cuticular phenotypes of embryos injected at various positions with *in vitro* transcribed mRNAs coding for bcd protein. (A) Uninjected control embryo from females homozygous mutant for the strong bcd^{E1} allele. Instead of head and thorax, a duplication of posterior terminal structures is formed at the anterior. (B) Embryo from a bcd^{E1} mutant female injected at the anterior tip with bcdTN3 mRNA ($0.4\,\mu g\,\mu l^{-1}$). All the structures present in wild-type head and thorax are completely rescued. (C) Embryo from a bcd^{E1} mutant female injected at the middle of the embryo with bcdTN3 mRNA ($1.5\,\mu g\,\mu l^{-1}$). While posterior terminal structures still form at both termini, head structures are formed at the site of injection, surrounded by thoracic dentical belts and sense organs. (D) Closeup of the induced head region from the embryo shown in C. The bilateral symmetry of the induced head structures is typical for the arrangement of structures in such animals (see also Frohnhöfer and Nüsslein-Volhard, 1986). (E) Wild-type embryo injected at the posterior pole with bcdTN3 mRNA ($1.7\,\mu g\,\mu l^{-1}$). Head structures and thorax are induced in the posterior half of the embryo. All embryos were injected during early cleavages (stage 2, before pole cell formation). Pictures were taken using dark-field optics (except D, phase contrast optics). Anterior is always to the left, dorsal at top. A=Abdomen, A1=first abdominal segment, A8=eighth abdominal segment, ap=anal plate, aso=antennal sense organ, ci=cirri, fk=filzkörper, mh=mouth hooks, mso=maxillary sense organs, T1 to T3=first through third thoracic segment. The structures of the head are described by Jürgens *et al.* 1986.

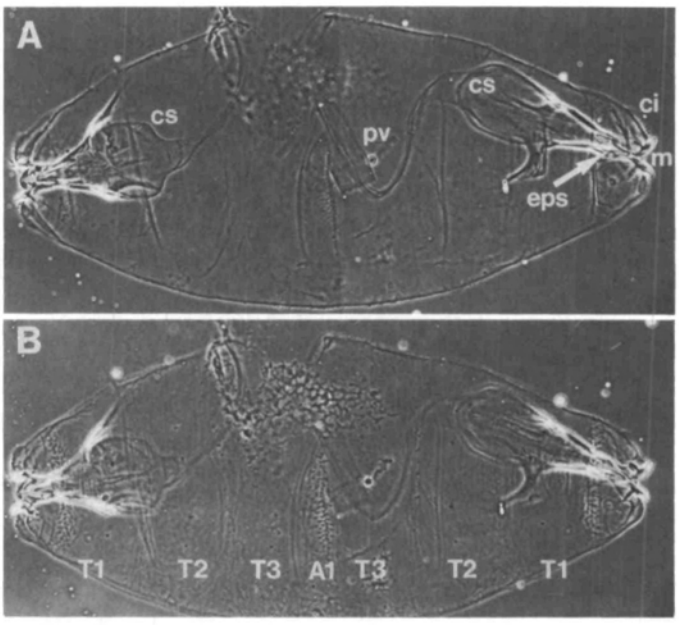

Fig. 6. Experimental induction of dicephalic embryos. A wild-type embryo, injected with bcdTN3 mRNA at the posterior pole, developed an exceptionally perfect mirror-image duplication of head and thorax. The only hint to the posterior origin of the right head is the size reduction of the labrum, which is hardly visible underneath the epistomal sclerite (eps) in (A). A1=first abdominal segment, T1 to T3=first through third thoracic segment, ci=cirri, m=mouth hooks, pv=proventriculus. Anterior is left. Phase-contrast pictures: (A) focus at central plane, (B) focus on ventral cuticle.

oogenesis where *bcd* mRNA is detectable contribute to more than 80% of the ovary of a well-fed female. In none of the ovarian extracts were we able to detect bcd protein, although we obtained strong signals for bcd protein from the embryonic extracts. Thus we conclude that, like wild-type *bcd* mRNA, bcdTN3 mRNA is not translated during oogenesis. The *bcd* mRNA leader sequence seems not to be required for translational control during oogenesis.

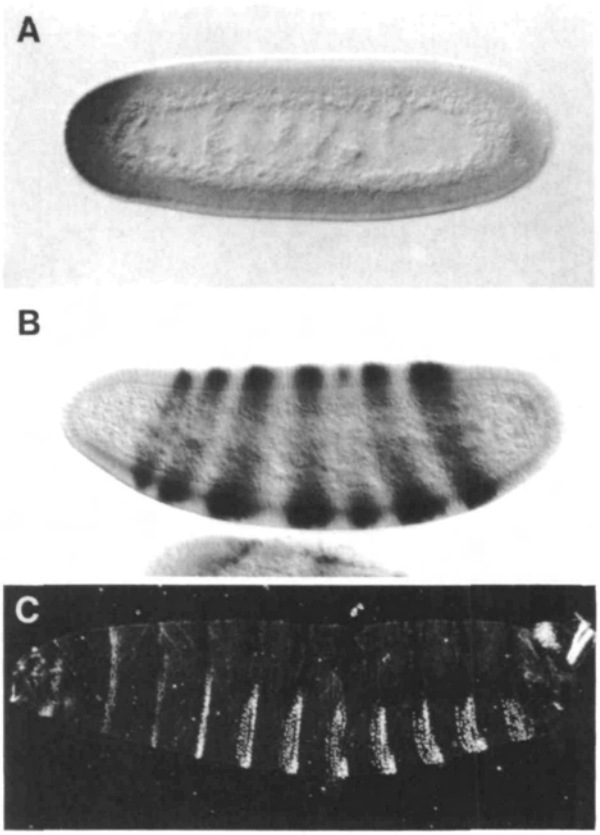

Fig. 7. Rescue of anterior structures in embryos from homozygous mutant bcd^{E1} females carrying a single pPbcdTN3 insertion. (A) Anti-bcd protein immunostain of a syncytial blastoderm embryo visualizing the formation of the bcd protein gradient translated from maternally transcribed bcdTN3 mRNA. (B) Anti-*eve* protein immunostain of a cellular blastoderm embryo. The segmented anlagen of head and thorax are restored but shifted further anterior and more expanded than in embryos from females carrying *Def (3R) lin*, including *bcd*. (C) Dark-field micrograph of the cuticle pattern. The development of gnathal and thoracic derivatives is completely rescued when compared to the bcd^{E1} mutant phenotype, a strong *bcd* allele. Fragments of the cephalopharyngeal sceleton, but no labral sclerites were found.

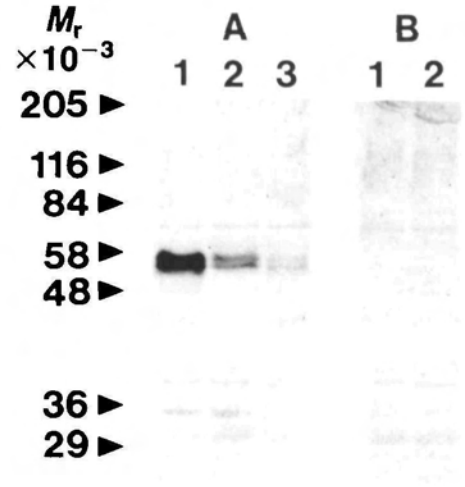

Fig. 8. Detection of pPbcdTN3-derived protein in embryos and oocytes. Protein extracts from 1–3 h old embryos (A) and whole ovaries (B) were prepared, separated by SDS–PAGE and analysed for the presence of bcd protein by immunoblot using monoclonal anti-bcd antibodies. The genotypes are: (1) a strain carrying 8 functional copies of the *bcd* gene, (2) pPbcdTN3/pPbcdTN3; bcd^{E1}/bcd^{E1}, and (3) pPbcdTN3/+; bcd^{E1}/bcd^{E1}.

Discussion

Translational control of bcd *mRNA*

Previous investigations have suggested that *bcd* mRNA is subject to translational control. Though the *bcd* mRNA is already present in late previtellogenic follicles (Frigerio *et al.* 1986; Berleth *et al.* 1988), bcd protein has not been detected during any stage of oogenesis (Driever and Nüsslein-Volhard, 1988*a*). Thus *bcd* mRNA appears not to be efficiently translated before egg deposition. Further, cytoplasm from mature stage 14 oocytes but not from earlier stages of oogenesis, when the *bcd* mRNA is already detectable, has the ability to rescue the *bcd* mutant phenotype when transplanted into embryos from bcd^{-} mutant females (Sander and Lehmann, 1988).

Selective translational repression of maternal transcripts during oogenesis has been reported in several systems, and different transcripts seem to be regulated by different mechanisms. For example, in *Xenopus* oocytes some transcripts are bound by specific proteins and are thus inaccessible to the translational machinery, a phenomenon known as maternal masking (Raff, 1980; Richter and Smith, 1984). In other cases, the translational machinery itself (e.g. eIF-4F) is altered, either in activity or availability (Audet *et al.* 1987; Huang *et al.* 1987). Finally it has been shown that polyadenylation of mRNAs can affect their translatability (Vassalli *et al.* 1989; Fox *et al.* 1989).

When we analysed the translation efficiency of bcd mRNA and bcdTN3 mRNA in test systems from plant, vertebrate and insect we obtained similar results. Thus we suspected that the primary structure of the bcd mRNA is responsible for the low efficiency of translation. A small open reading frame of 14 codons (including one additional in frame AUG) is located at the 5′ end of the mRNA. Such minicistrons have been proposed to be involved in translational regulation (Kozak, 1984; Mueller and Hinnebusch, 1986). In addition, secondary structure prediction programs (Zucker and Stiegler, 1981) suggest that the bcd mRNA leader might contain GC-rich stem-loop structures just upstream of the start AUG that might be responsible for the inefficient translation (Pelletier and Sonnenberg, 1985). Several alternative structures can be proposed that have the GC-rich region from nucleotide 149

to 155 (sometimes extending till nucleotide 161) base paired with other parts of the molecule (e.g. the region from nucleotide 172 to 185; the AUG starts at nucleotide 170). We think it unlikely that the higher translation rate of the *bcd* TN3 mRNA may simply be caused by a better translation initiation context, as the natural *bcd* mRNA has the greater homology to the *Drosophila* translation initiation consensus (Cavener, 1987).

Replacement of the *bcd* upstream leader sequence with that of β-globin did not relieve its translational repression during oogenesis. This finding argues against an important role of the leader for the translational block. Which part of the bcd mRNA confers translational control still remains to be elucidated. It is possible that the translational block is closely linked to the different phases of bcd mRNA localization during oogenesis and early embryogenesis (St. Johnston *et al.* 1989).

bcd *protein determines anterior development independent of any other localized, anterior specific morphogenic activity*

Though a large body of information on the function of *bcd* during early embryogenesis has accumulated (Frohnhöfer and Nüsslein-Volhard, 1986; Driever and Nüsslein-Volhard, 1988*a*,*b*; Driever *et al.* 1989*a*,*b*; Struhl *et al.* 1989), some major questions concerning the function of *bcd* as the morphogen for anterior development had remained. First, it is unclear whether the presence of different spliced forms of the *bcd* transcript is functionally significant. For example, the different transcripts could code for functionally distinct proteins which would then be responsible for subdividing the embryo into domains. Our data argue against this, since they demonstrate that a single *bcd* protein species derived from one splice form of *bcd* mRNA (Type a in Fig. 1) can exert all morphogenetic functions of the *bcd* gene. The minor splice variants may result from aberrant processing of the mRNA and may not fulfill a specific function during embryonic development.

Second, we could not previously exclude the presence of other, so far unidentified localized anterior activities in the egg, which might cooperate with, or modify *bcd* activity, possibly in a region-specific manner. The transplantation experiments performed by Frohnhöfer and Nüsslein-Volhard (1986) demonstrate that the localized *bcd* activity is necessary for the induction of anterior development, but they can not prove that it is sufficient, as other anterior activities might have been cotransplanted.

The induction of anterior structures by the injections of *in vitro* synthesized *bcd* mRNAs at ectopic positions reveals that *bcd* is the only localized morphogenic maternal factor specific for anterior development. Independent of any other localized activity, *bcd* can induce the formation of gnathal structures and thorax at any position along the anterior–posterior axis. These findings rule out that bcd protein might be subject to region-specific (anterior) modifications in order to generate differentially active bcd protein species. We were able to induce acronal structures at the termini of the embryo only, consistent with previous data (Nüsslein-Volhard *et al.* 1987; Klingler *et al.* 1988) demonstrating that terminal structures depend on the activity of the *torso* group of maternal effect genes. The presence of *bcd* activity in the terminal region results in the development of anterior terminal structures (the acron) instead of posterior terminal structures (the telson).

Our analysis strongly supports the idea that *bcd* acts as a morphogen, specifying subregions of the embryo in a concentration-dependent manner (Driever and Nüsslein-Volhard, 1988*b*). There is no evidence indicating the existence of other, anterior-specific maternal morphogens; indeed all the identified maternal genes affecting anterior development appear to act by modifying *bcd* mRNA localization or stability and thus *bcd* protein distribution (*exuperantia*, *swallow*, and *staufen*: Berleth *et al.* 1988; Driever and Nüsslein-Volhard, 1988*b*; St Johnston *et al.* 1989; *bicaudal*: Wharton and Struhl, 1989), rather than presenting a parallel maternal input into anterior pattern formation.

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