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Analysis of maternal effect mutant combinations elucidates regulation and function of the overlap of *hunchback* and *Krüppel* gene expression in the *Drosophila* blastoderm embryo

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Summary

The metameric organisation of the *Drosophila* embryo is generated early during development, due to the action of maternal effect and zygotic segmentation and homeotic genes. The gap genes participate in the complex process of pattern formation by providing a link between the maternal and the zygotic gene activities. Under the influence of maternal gene products they become expressed in distinct domains along the anteroposterior axis of the embryo; negative interactions between neighboring gap genes are thought to be involved in establishing the expression domains. The gap gene activities in turn are required for the correct patterning of the pair-rule genes; little is known, however, about the underlying mechanisms.

We have monitored the distribution of gap and pair-rule genes in wild-type embryos and in embryos in which the anteroposterior body pattern is greatly simplified due to combinations of maternal effect mutations (*staufen exuperantia*, *vasa exuperantia*, *bicoid oskar*, *bicoid oskar torsolike*, *vasa torso exuperantia*). We show that the domains of protein distribution of the gap genes *hunchback* and *Krüppel* overlap in wild-type embryos. Based on the analysis of the maternal mutant combinations, we suggest an explanation of how this overlap is generated. Furthermore, our data show that different constellations of gap gene activities provide different input for the pair-rule genes, and thus strongly suggest that the overlap of *hunchback* and *Krüppel* in wild-type is functional in the formation of the patterns of pair-rule genes.

Key words: *Drosophila*, pattern formation, segmentation, gap genes.

Introduction

The metameric organization of the *Drosophila* embryo is generated early during development. Maternal factors set the frame in which the zygotic segmentation and homeotic genes act. Due to the concerted action of the segmentation genes the appropriate number of metameric units, or segments, is established, and the homeotic selector genes control the diverse pathways by which each of the segments acquires a unique morphology. The gap genes participate in the complex process of pattern formation by providing a link between the maternal and the zygotic gene activities: the gap gene patterns are controlled by the maternal factors, and the gap gene activities in turn control the pattern of zygotic segmentation and homeotic genes (for review see Akam, 1987; Nüsslein-Volhard *et al.* 1987; Ingham, 1988).

Three groups of maternal effect genes organize the anteroposterior body pattern of the *Drosophila* embryo. Under their influence the gap genes establish distinct domains of expression along the anteroposterior axis: *hunchback (hb)* is expressed in the anterior of the embryo as a result of activation by the anterior maternal organizer *bicoid* (*bcd*) (Tautz, 1988; Driever and Nüsslein-Volhard, 1989). The *Krüppel (Kr)* domain is established in the middle of the embryo due to repression of an otherwise constitutive expression by all three maternal organizer activities (Gaul and Jäckle, 1987, 1989). Apart from the maternal instruction, negative interactions between neighboring gap genes are thought to be involved in the establishment of the expression domains of the gap genes (Meinhardt, 1986; Jäckle *et al.* 1986).

Gap gene activities are necessary for instructing the patterning of the pair-rule genes. Pair-rule genes are expressed in series of 7 (or 8) discrete stripes in wild-type embryos. In gap gene mutant embryos, the pat-

terns of the pair-rule genes show severe perturbations, indicating that the gap genes control pair-rule gene activity (Carroll and Scott, 1986; Frasch and Levine, 1987; Carroll *et al.* 1988). However, these defects are not suggestive of any straightforward interpretation, and it is not yet well understood how the few gap genes, with their comparatively broad, nonperiodic domains of expression manage to establish the intricate periodic pair-rule gene patterns.

We have monitored the distribution of gap and pair-rule genes in wild-type embryos and in embryos in which the anteroposterior body pattern is greatly simplified due to various combinations of maternal effect mutations (*staufen exuperantia* (*stau exu*), *vasa exuperantia* (*vas exu*), *bicoid oskar* (*bcd osk*), *bicoid oskar torsolike* (*bcd osk tsl*), *vasa torso exuperantia* (*vas tor exu*)); in such embryos the patterns of segmentation genes are likewise simplified. We show that the domains of *hb* and *Kr* protein distribution, rather than being adjacent, overlap in wild-type embryos. The analysis of maternal effect mutant combinations suggests that the overlap results from differential regulation of *hb* and *Kr* by the anterior maternal organizer *bcd*. Furthermore, we show that, in the maternal mutant combinations, the pair-rule genes pattern where the constellation of gap gene activities changes. The data also demonstrate that different constellations of gap gene activities provide qualitatively different input for the pair-rule genes, and thus strongly suggest that the overlap of *hb* and *Kr* is functionally relevant for the formation of the patterns of pair-rule genes.

Materials and methods

The mutant combinations bcd^{E1} osk^{166} tsl^{491} and bcd^{E2} osk^{166} have been described by Nüsslein-Volhard *et al.* (1987). The mutant combinations vas^{PD} exu^{PJ} and $stau^{HL}$ exu^{PJ} have been described by Schüpbach and Wieschaus (1986). For our study, however, we produced a different allele combination, $stau^{D3}$ exu^{PJ}, for only under such conditions does the 'bithoracic' phenotype become fully penetrant. The vas^{PD} tor^{WK} exu^{PJ} combination was kindly provided by Trudi Schüpbach.

To test which promoters are used for the expression of *hb* in *stau exu* and *vas exu* embryos, we crossed males carrying *hb* promoter–lacZ fusion genes to homozygous maternal effect mutant mothers and monitored the β-galactosidase (β-gal) distribution with anti-β-gal-antibodies. We used two different lines: One carries the upstream control elements of the 2.9 kb *hb* transcript necessary for the expression of the anterior *hb* domain, and has been shown to contain the *bcd* responsive elements (*hb* 1.2–lacZ; Schröder *et al.* 1988). The other carries upstream control elements of the 3.2 kb *hb* transcript, which is necessary for the early maternal expression and for two stripes of expression in the late blastoderm, at about 50 % and 15 % EL, respectively (Schröder, unpublished).

Embryos derived from homozygous mutant mothers (in the following, such embryos will be named according to the genotype of the mother for the sake of brevity) were dechorionated, permeabilized, and fixed using standard procedures. After washing and blocking in BBT (0.15 % crystalline bovine serum albumin, 10 mM-Tris–HCl, pH 7.5, 50 mM-NaCl, 40 mM-$MgCl_2$, 5 mM-$CaCl_2$, 20 mM-glucose, 50 mM-sucrose, 0.1 % Tween 20) they were treated with primary antibodies overnight. (Anti-*hb* antibodies, anti-*fushi tarazu (ftz)*, and anti-*engrailed* (*en*) antibodies were kindly provided by D. Tautz, H. Krause, and N. Patel, respectively.) The bound antibodies were detected with biotinylated secondary antibodies and stained with Vectastain ABC-kit (Vectorlabs) using diaminobenzidine following the manufacturer's instructions except that 0.03 % $CoCl_2$ were added to the staining solution. In order to compare expression levels under mutant conditions with those in wild-type, we added wild-type embryos to the batches of mutant embryos and processed them together. For the analysis of the wild-type patterns of *hb* and *Kr*, we also used the Vectastain 'Elite'kit and 0.02–0.1 % $NiCl_2$, which gave improved results.

Results (part I)

Overlap of the protein domains of hb *and* Kr *in wild-type*

Due to the maternal transcription of the *hb* gene, *hb* RNA is already present in freshly laid eggs. *hb* protein is first detected prior to pole-cell formation, showing a graded distribution along the anteroposterior axis with high levels in the anterior 1/3 and decreasing levels in the posterior 2/3 (Tautz *et al.* 1987). The zygotic expression of *hb* begins at syncytial blastoderm (stages 11/12), with *hb* protein present in the anterior half of the embryo. At the onset of cellularization, a second domain of expression has emerged posteriorly, forming a cap at the posterior pole (Fig. 1A). As cellularization proceeds, high levels of protein accumulate both in the anterior and in the posterior domain (Fig. 1B). At the end of cellular blastoderm, the anterior domain recedes from the anterior tip and becomes inhomogenous in that two stripes of stronger staining appear within the domain. In the posterior, a stripe replaces the cap (Fig. 1C) (see also Tautz, 1988). The borders of the *hb* domains are not sharp at any time. Fig. 1E shows a glancing view of the posterior margin of the anterior *hb* domain at the middle of stage 14: the nuclear staining reaches background level only gradually, over a range of about 5 cells.

Kr protein is first detectable at syncytial blastoderm (stage 12) in the middle region of the embryo. At the onset of cellularization, low levels of *Kr* protein are detectable in a band covering about 60–33 % EL (Fig. 1F). The accumulation of *Kr* protein within this band strongly increases as cellularization proceeds (Fig. 1G). The more sensitive staining technique thus reveals that the *Kr* protein domain is broader than we were able to detect earlier (we reported 54–39 % in Gaul and Jäckle (1987)). By the end of cellularization, this central *Kr* protein domain has narrowed, and two additional domains of *Kr* expression have been established, a 'cap' at the posterior pole, and a stripe at about 85 % EL (Fig. 1H). The *Kr* domain also never shows sharp borders. Rather, the protein staining is most intense in the center of the domain and fades out gradually toward the margins (Fig. 1K) (see also Gaul *et al.* 1987). The fading out of both *hb* and *Kr* staining at the margins makes it difficult to define the exact limits of the protein domains; they depend considerably on the sensitivity of detection.

The protein domains of *hb* (in the anterior) and *Kr* are not juxtaposed, but overlap substantially. This can be demonstrated by aligning matched embryos of which one is stained for *hb* and the other for *Kr* (see Fig. 1D,I). Judging from such superpositions, the overlap comprises about 8–12 cells at the middle of stage 14, depending on how strongly the antibody staining has been 'pushed' (see Materials and methods). The region of overlap is not homogenous with respect to *hb* and *Kr* protein concentration levels. The fact that both *hb* and

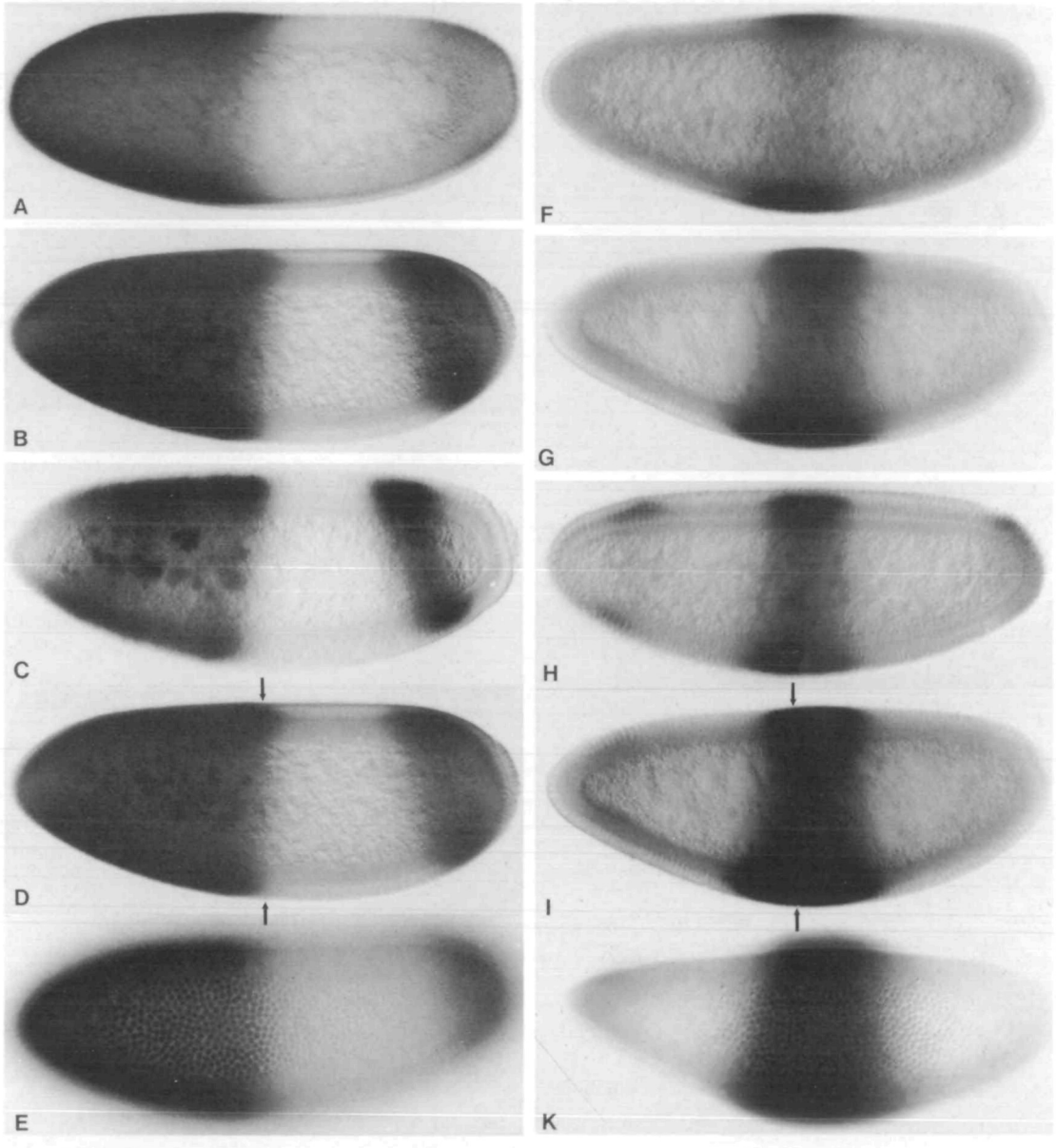
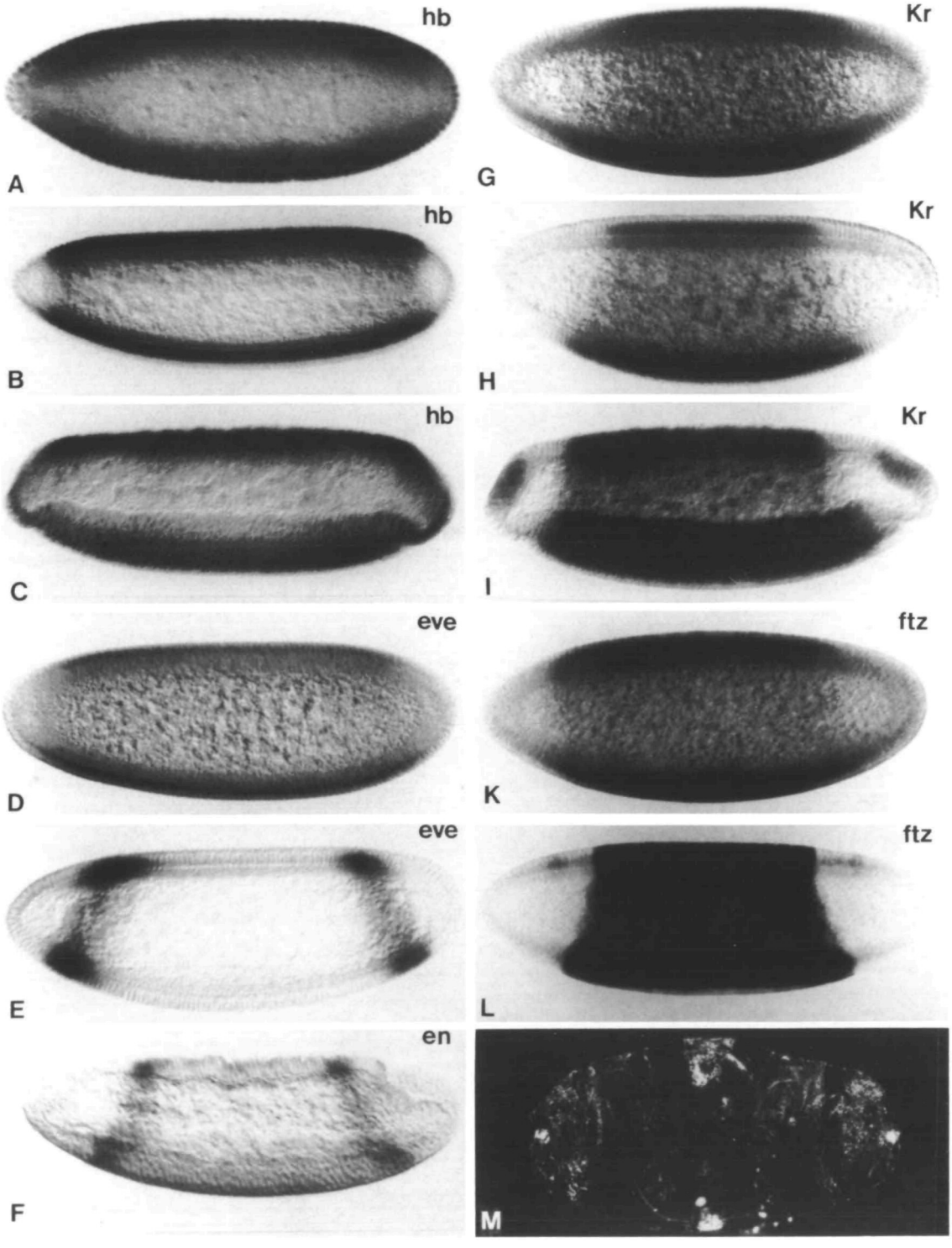


Fig. 1. Protein expression of *hb* and *Kr* in wild-type embryos. Orientation is anterior to the left and dorsal up. (A–C) *hb* expression. At early stage 14 (A), *hb* is expressed in the anterior half of the embryo, as well as in a 'cap' at the posterior pole. At the middle of stage 14 (B), the accumulation of protein has increased in both domains, the posterior domain becomes non-homogenous in that the terminalmost portion of the domain shows lower levels of expression. At late stage 14 (C), the anterior domain recedes from the tip and the posterior 'cap' is replaced by a stripe. (D,E) Embryo at the middle of stage 14 in different focal planes. (D) Sagittal optical section. The arrows mark 50 % EL. Compare with I, which shows a *Kr* staining of an embryo of about the same age. (E) Focus on the periphery of the embryo, in the region of the posterior border of the anterior *hb* domain. The *hb* staining fades out gradually toward the posterior margin, over a range of about 5 nuclei. (F–K) *Kr* expression. At early stage 14 (F), a band of *Kr* staining is visible, covering about 60 to 33 % EL. The staining is quite weak at this stage, and barely above background level at the margins. By the middle of stage 14 (G), the accumulation of protein has strongly increased. At late stage 14 (H), two additional domains of *Kr* expression are visible, a 'cap' at the posterior pole, and a stripe at about 82 % EL. (I,K) Embryo at the middle of stage 14 in different focal planes. (I) Sagittal optical section. The arrows mark 50 % EL. (K) Focus on the periphery of the embryo, in the region of the *Kr* domain. The *Kr* staining fades out toward the margins, attenuating more gradually in the posterior than in the anterior.



Kr staining fade out toward the margins of the domains (Fig. 1E,K) implies that it is only in the center (approximately) of the entire band of overlap that *hb* and *Kr* product are both present in relatively high concentrations, while anteriorly high *hb* levels are accompanied by decreasing concentrations of *Kr*, and posteriorly high levels of *Kr* by ceasing levels of *hb*.

This co-distribution of *hb* and *Kr* protein in a band of at least 8 cells in width immediately raises two questions: (I) how does the overlap come about?, and (II) is this overlap functionally relevant for the process of segmentation, or, more specifically, does the simultaneous presence of *hb* and *Kr* have an effect on the expression state of the pair-rule genes?

Fig. 2. The effect of *stau exu* mutations on the expression patterns of *hb*, *Kr*, *eve*, *ftz* and the segment polarity gene *engrailed (en)*, as visualized by antibody staining, and on the cuticle phenotype. (A–C) *hb* expression at early (A), late (B) stage 14 and gastrulation (C). *hb* is expressed throughout the embryo. (D,E) *eve* expression in early (D) and late (E) stage 14. *eve* is initially (D) expressed almost throughout the embryo. At late stage 14, two broad *eve* stripes have formed close to the poles, with the center of the stripes lying at about 20 and 80 % EL, respectively (for description of the wild-type pattern of *eve*, see Frasch *et al.* (1987)). (F) *en* expression at gastrulation stage: two stripes are present at about 25 and 75 % EL, respectively. At later stages, two additional stripes of *en* expression are found in either pole region, which are probably associated with the telson (for description of the *en* wild-type pattern, see DiNardo *et al.* (1985)). (G–I) *Kr* expression at early (G), late (H) stage 14 and gastrulation (I). *Kr* is expressed in a broad domain covering most of the embryo (about 20–80 % EL). The represssion at the poles is due to the action of terminal organizer genes (Gaul and Jäckle, 1989). High levels of expression persist until after gastrulation. In gastrulating embryos (I) the posterior domain of *Kr* expression is duplicated anteriorly. (K,L) *ftz* expression in early (K) and late (L) stage 14. At late stage 14, *ftz* is expressed in a broad domain in the middle region (about 25–75 % EL), in addition two partial stripes form (at approximately 15 and 85 % EL), which may be associated with the telson (for description of the *ftz* wild-type pattern, see Carroll and Scott (1985)). (M) Cuticle phenotype, dark-field photograph. Two telsons develop at either end of the embryo. In the middle, segments of thoracic identity form (for a detailed description of the phenotype, see Schüpbach and Wieschaus (1986)).

In addressing these questions, it was instructive to analyze the patterns of gap and pair-rule genes in a number of maternal effect mutant combinations: *stau exu*, *vas exu*, *bcd osk tsl*, *bcd osk*, *vas tor exu*. The phenotypes of these mutant combinations lack most of the wild-type pattern elements, and (except in the case of *vas tor exu*) the residual elements are arranged in mirror-image symmetry. In such embryos, the patterns of gap and pair-rule genes (and similarly of segment polarity and homeotic genes, cf. Gaul and Jäckle, 1989) become much simpler, and the reduction in the number of parameters facilitates the interpretation of the correlations between the relevant patterns.

The patterns of hb *and* Kr *expression in* stau exu *and* vas exu *embryos*

The maternal effect genes that are involved in the organization of the anteroposterior body pattern have been classified into three groups according to their mutant phenotypes: they are required for the organization of either (1) the anterior, (2) the posterior, or (3) the terminal regions of the embryo (Nüsslein-Volhard *et al.* 1987).

In the maternal effect mutant combinations *stau exu* and *vas exu*, the anterior pattern organizer *bcd* is affected by a reduction in the amount of product and a change in spatial distribution (*exu*); in addition, posterior organizers are defective (*stau*, *vas*, respectively). The combination of these mutations leads to a ubiquitous distribution of low amounts of *bcd* activity (Struhl *et al.* 1989; and see below). The terminal organizers, however, are intact in both mutant combinations.

The phenotype of *stau exu* embryos is characterized by a lack of head, gnathal and abdominal structures. At the poles of the embryo, two telsons of opposite orientation develop, while, in the middle, segments of thoracic identity form (Fig. 2M) (Schüpbach and Wieschaus, 1986). In contrast, the only cuticular structures formed in *vas exu* embryos are of gnathal origin (maxillary mouthhooks and cirri); besides, posterior midgut invaginations form at both ends of these embryos during gastrulation, indicating that posterior terminal structures do develop (Fig. 3H) (Schüpbach and Wieschaus, 1986).

These two mutant combinations exhibit different patterns of *hb* and *Kr* expression. In *stau exu* embryos, *hb* is expressed along the entire length of the egg, at levels we could not distinguish from wild-type (see Materials and methods) (Fig. 2A–C). The omnipresence of *hb* protein is caused by the ubiquitous presence of maternal *hb* transcript that results from the *stau* mutation (Tautz, 1988), as well as by ubiquitous activation of the zygotic transcription of *hb* by *bcd*. This could be demonstrated by the following experiment. It has been shown that the zygotic expression of *hb* in the anterior half of the wild-type embryo is due to the activation by *bcd* (Tautz, 1988). The regulatory sequence regions necessary for the normal expression of *hb* have been determined by using *hb*–promoter–lacZ fusion gene constructs in combination with germ-line transformations; they could be delimited to a region of approximately 300 bp upstream of the site of transcription initiation (Schröder *et al.* 1988). We crossed male flies carrying a *hb*–lacZ gene fusion construct containing the *bcd* responsive sequences to *stau exu* homozygous mothers (see Materials and methods) and found that β-gal is distributed uniformly throughout *stau exu* embryos (data not shown). We conclude that the ubiquitous zygotic expression of *hb* protein in *stau exu* embryos is due to activation by *bcd*; *bcd* therefore has to be present throughout the embryo.

Kr is expressed in a very broad domain in *stau exu* embryos that covers about 60 % of the egg, also at levels that we could not distinguish from wild-type (see Materials and methods) (Fig. 2G–I). *Kr* is not expressed, however, in either pole region, due to the presence of terminal organizers and their zygotic 'mediator(s)', which act to repress *Kr* expression there (Gaul and Jäckle, 1989). So we find that, in this mutant combination, *hb* and *Kr* protein are co-distributed within a very large region, encompassing about 60 % of the embryo.

In *vas exu* embryos, *hb* is expressed at wild-type levels in the entire embryo (again due to the ubiquitous presence of maternal *hb* transcript (*vas* mutation) and to the ubiquitous activation of zygotic *hb* expression by *bcd*, as confirmed by *vas exu hb*–promoter–lacZ crosses; see also Struhl *et al.* 1989) (Fig. 3A). However,

no expression of *Kr* is detected during blastoderm (Fig. 3E).

Discussion (part I)

What mechanism generates the overlap of the hb *and* Kr *domains?*

The broad co-distribution of *hb* and *Kr* product that is observed in *stau exu* embryos has two important implications. First, it has to be due to genuine co-expression, and therefore suggests that the relatively narrow overlap of the domains of *hb* and *Kr* that is observed in wild-type embryos similarly results from an overlap of expression territories rather than from diffusion. In fact, the region of co-distribution of *hb* and *Kr* in *stau exu* embryos can be regarded as a 'blow up' of the central portion of the wild-type overlap, i.e. that portion of the overlap that shows higher concentrations of both *hb* and *Kr* protein. Second, the co-expression of *hb* and *Kr* in *stau exu* embryos indicates that *hb* by itself is not sufficient to repress *Kr* expression (and *vice versa*). That *hb* cannot play a crucial role in *Kr* repression is also suggested by the comparison of *stau exu* and *vas exu* embryos: while the *hb* distribution is the same in both mutants, *Kr* is expressed in the former and not expressed in the latter. But what is it that causes

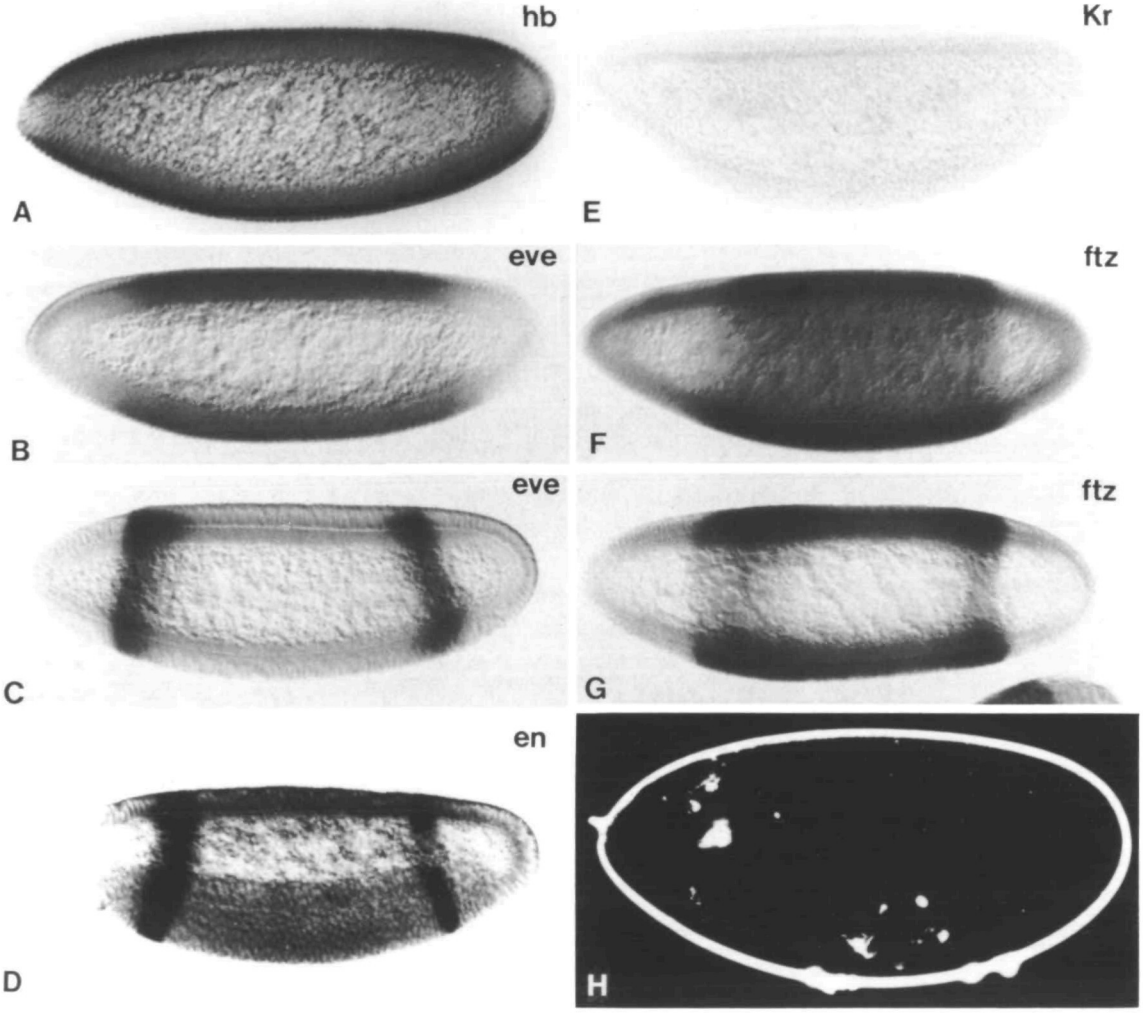


Fig. 3. The effect of *vas exu* mutations on the expression patterns of *hb*, *Kr*, *eve*, *ftz* and *en* as visualized by antibody staining, and on the cuticle phenotype. (A) *hb* expression in a mid-stage 14 embryo. *hb* is expressed throughout the egg. (B,C) *eve* expression in early (B) and late (C) stage 14. By late stage 14, two stripes close to the poles (at about 20 and 80 % EL) have formed. (D) *en* expression at gastrulation. Two stripes are present at about 25 and 75 % EL, later additional stripes form in the pole regions. (E) Late stage 14 embryo stained against *Kr* protein. Before the end of cellularization, no *Kr* expression could be detected. With the onset of gastrulation, *Kr* protein becomes visible in different regions of the embryo (data not shown). (F,G) *ftz* expression in early (F) and late (G) stage 14. At late stage 14, *ftz* is expressed in a broad domain in the middle region (about 20–75 % EL); note that the staining is weaker within the domain than at its margins. (H) Cuticle phenotype, dark-field photograph. Very few cuticular landmarks are discernible; we identify maxillary structures (cirri and mouthhooks) in 'mirror-image' orientation (for a detailed description, see Schüpbach and Wieschaus (1986)).

the difference between the two mutant combinations with respect to *Kr* expression?

The analysis of *bcd* mutant embryos indicates that *bcd* has a strong negative effect on *Kr* expression (Gaul and Jäckle, 1987). Moreover, the comparison between the patterns of *bcd* and *Kr* protein in wild-type embryos clearly shows that this negative effect must be concentration-dependant: *bcd*, showing a graded distribution with its highpoint at the anterior pole, is detectable at lower levels throughout the *Kr* domain (Driever and Nüsslein-Volhard, 1988*a*). Consequently, it could be a difference in the level of *bcd* activity (or in the activity of some *bcd*-dependant factor other than *hb*) that is responsible for the difference in the expression of *Kr* between *vas exu* and *stau exu* embryos.

The marked difference in the phenotypes of *vas exu* and *stau exu* embryos indeed supports this interpretation: As has been shown by several independant experiments, *bcd* acts as a morphogen that determines 'position' in the anterior half of the embryo. Increases or decreases in *bcd* protein levels in a given region of the embryo cause a corresponding shift of the anterior anlagen to the anterior or to the posterior, respectively (Frohnhöfer and Nüsslein-Volhard, 1987; Driever and Nüsslein-Volhard, 1988*b*). The cuticular structures formed in *vas exu* embryos derive from more anterior positions in the fate map than those formed in *stau exu* embryos: the middle region produces gnathal elements instead of thorax. Thus, since higher levels of *bcd* determine structures lying more anteriorly in the fate map, we can infer that the level of *bcd* activity is higher in *vas exu* embryos than it is in *stau exu* embryos.

The difference regarding the expression of *Kr* and *hb* between *stau exu* and *vas exu* embryos consequently suggests that the threshold level of *bcd* for repression of *Kr* is different from the one for activation of *hb*: in *stau exu* embryos, the level of *bcd* is too low to repress *Kr* expression, while in *vas exu* embryos, with higher levels of *bcd*, *Kr* expression is repressed. In contrast, *hb* is activated indiscriminately in both mutants. We conclude that the threshold level of *bcd* for *hb* activation is lower than that for *Kr* repression.

This difference in threshold levels provides an explanation for the overlap of the domains of *hb* and *Kr* that is observed in wild-type. In wild-type embryos, *bcd* shows a graded distribution with its highpoint at the anterior pole and an exponential decrease in concentration towards posterior (Driever and Nüsslein-Volhard, 1988*a*). Under such conditions, the difference in the threshold levels for *hb* activation on the one hand, and *Kr* repression on the other, causes a simultaneous expression of *Kr* and *hb* in a defined region: where the level of *bcd* is too low to repress *Kr* but still high enough to activate *hb*. Only where *bcd* levels are sufficiently high, will *Kr* be repressed.

This picture of the regulation of *hb* and *Kr* in the anterior of the embryo does not take into account interactions between the two gap genes themselves. Yet, as has been described earlier, the *Kr* domain extends anteriorly in hb^- embryos, indicating that *hb* does have a negative influence on *Kr* expression (Jäckle *et al.* 1986; Harding and Levine, 1988); and the *hb* domain extends posteriorly in Kr^- embryos (Jäckle *et al.* 1986). The latter effect seems to be rather weak and occurs too late to be relevant for the establishment of the anterior (blastoderm) *hb* domain. The effect of *hb* on *Kr*, however, while not as strong as the effect of *bcd* on *Kr*, is quite pronounced and visible as soon as the *Kr* domain can be detected with antibody staining. Therefore, *hb* has to play a certain role in the control of *Kr* expression in the anterior of the embryo, even if it cannot repress *Kr* expression by itself, as is indicated by the co-expression of *hb* and *Kr* in *stau exu* embryos. We see two possible mechanisms by which *hb* could be involved in *Kr* repression along with *bcd*:

(i) *bcd* acts as a repressor of *Kr*, but needs help from *hb* as a co-factor at lower levels of concentration. It is only at higher levels that *bcd* can repress *Kr* by itself completely. Such a mechanism implies that *bcd* alone is not a 'good' repressor for *Kr*, that *hb* acts as an 'enhancer', and that it can do so only if *bcd* is already present at a certain threshold level.

(ii) Another possibility is that *hb* is only indirectly involved in *Kr* control. One idea employs the fact that the transcription factor that *hb* shows homology to, *Xenopus* transcription factor IIIA, binds not only to DNA but also to RNA (Tautz *et al.* 1987; Klug and Rhodes, 1987). Thus, *hb* might be involved in positive post-transcriptional regulation of *bcd*. Under such conditions, *bcd* would be the only repressor of *Kr*, and an effective one. The lack of *hb* would cause less *bcd* protein to be produced, and the concentrations necessary for *Kr* repression would only be reached at a more anterior position.

Results (part II)

Gap gene and pair-rule gene expression in maternal mutant combinations

We still have to address the question whether the co-expression of *hb* and *Kr* in wild-type embryos is functional. The examination of the correlation between the gap gene and the pair-rule gene patterns in the maternal mutant combinations characterized above suggests an answer to this question, and it also provides some more general information about the way the gap genes control pair-rule gene activity.

To determine the constellations of gap gene activities in the maternal mutant combinations, we monitored the expression of *hb* and *Kr*. *knirps (kni)*, the gap gene expressed in the posterior of wild-type embryos (Nauber *et al.* 1988), is eliminated in all cases. Due to the mutations in posterior organizer genes (*osk*, *vas*, *stau*, respectively) maternal *hb* protein is expressed uniformly throughout the egg; this in turn causes *kni* expression to be repressed (Hülskamp *et al.* 1989). The distribution of terminal organizers (and of the zygotic gene activities that depend on them and mediate their effect), on the other hand, can be inferred from their negative effect on *Kr* expression (Gaul and Jäckle,

1989), as well as from the phenotypes of the maternal mutant combinations. The main mediator of terminal organizer activity is the gap gene *tailless* (*tll*) (Klingler *et al.* 1988); however, apart from *tll* at least one more zygotic factor, *huckebein,* is involved in the organization of the terminal region. This factor apparently acts in the terminalmost portion of the embryo to suppress segmentation gene expression (Weigel, unpublished observation). To monitor pair-rule gene expression, we used *ftz* and *even skipped (eve)* antibodies; in wild-type, *ftz* and *eve* are initially expressed in the entire region that is to be segmented; later, their patterns resolve into series of stripes that are roughly complementary (Carroll and Scott, 1985; Frasch *et al.* 1987).

In *bcd osk tsl* embryos, all three maternal organizers are defective, and no cuticle pattern is formed (Nüsslein-Volhard *et al.* 1987). In such embryos, *Kr* is the only zygotically active gap gene and is expressed throughout the embryo (Fig. 4A). No patterns of pair-rule genes form: *ftz* and *eve* are uniformly expressed in the entire embryo (Fig. 4B,C). Thus we find that, if there is no gap gene pattern; that is, if there is no change in the state of expression of gap genes along the anteroposterior axis, no pair-rule gene patterns are formed, either. This result proves that zygotic gene patterns cannot form *de novo* in the *Drosophila* blastoderm, but rather depend on a prior pattern. In the absence of patterned instruction from maternal organizers, the gap genes do not pattern, and this causes a subsequent failure of pair-rule genes to resolve spatial patterns.

In contrast, in *bcd osk* embryos, in which anterior and posterior organizers are defective, while terminal organizers are intact, patterns do form. The cuticle phenotype of *bcd osk* embryos shows telsons at both ends and naked cuticle in the middle (Nüsslein-Volhard *et al.* 1987). In these embryos, *Kr* is expressed in the middle region of the embryo only (Fig. 4E); the repression of *Kr* expression at the poles is due to the presence of terminal organizers and their zygotic 'targets' there (Gaul and Jäckle, 1989). Zygotic *hb* expression is found in the two terminal regions, due to activation by terminal organizers (data not shown). This constellation of gap gene expression is correlated with the following pair-rule gene patterns: *eve* 'tries' to produce two peripheral stripes, but the expression of *eve* persists in the middle and the stripes do not resolve (Fig. 4F). Meanwhile, *ftz* forms a broad band in the central region and one peripheral stripe on either side (Fig. 4G). Thus, unlike in wild-type, *ftz* and *eve* are co-expressed in the middle region of *bcd osk* embryos. (Frasch *et al.* (1988) similarly found a broad co-expression of *eve* and *ftz* in *hb kni* double mutants.) We observe that, in the central region, the uniform expression of *Kr* is accompanied by a uniform pair-rule gene expression; and patterning of the pair-rule genes occurs where the input from the gap genes changes, namely around the borders of the *Kr* domain.

In *stau exu* embryos, as we described above, *hb* and *Kr* are co-expressed in a broad middle region encompassing about 60 % of the embryo (Fig. 2A–C, G–I). In the pole regions of *stau exu* embryos, *hb* and the zygotic terminal genes are present; the latter is implied by the phenotype and by the repression of *Kr* expression at the termini. In these embryos, *eve* forms two stripes in the terminal region (Fig. 2E). The distribution of the *ftz* protein is roughly complementary – a very broad domain is established in the central region of the embryo as well as two narrow stripes near the poles (Fig. 2L; see also Winslow *et al.* 1988). Thus, in the large region where both *Kr* and *hb* are expressed, no further minima or maxima of *ftz* or *eve* expression form. It is in the vicinity of the borders of the *Kr* domain that their expression patterns resolve. The *ftz* pattern closely resembles the *Kr* pattern, whereas the patterns of *eve* and *Kr* are approximately complementary.

In *vas exu* embryos, as we described earlier, *Kr* is absent, while *hb* is ubiquitously expressed (Fig. 3A,E). From the phenotype we can infer that maternal terminal organizer activity, and, correspondingly, activity of their zygotic 'mediator(s)', is present in the pole regions of these embryos: proctodeal invaginations form at both ends. In such embryos, like in *stau exu* embryos, the initially uniform expression of *eve* gradually resolves into two peripheral stripes (Fig. 3B,C), while the *ftz* expression forms a single broad domain in the middle of the embryo (Fig. 3F,G). However, the two mutant combinations differ in that the peripheral stripes of *ftz* expression that are observed in *stau exu* embryos do not form in *vas exu* embryos. Moreover, as cellularization proceeds, the broad central domain of *ftz* expression becomes non-homogenous in *vas exu* embryos, showing a loss of intensity in the middle, while stripes of stronger expression are maintained at the margins (see also Winslow *et al.* 1988).

In order to test whether the pair-rule gene patterning in *vas exu* embryos results from terminal organizer activity, we examined *vas tor exu* triple mutants, in which terminal organizer activity is eliminated in addition. In these embryos, *hb* is again expressed ubiquitously (Fig. 4I) and *Kr* expression is absent. But *eve* expression now forms a small cap at the anterior pole (Fig. 4K), and *ftz* shows a complementary distribution in being expressed in almost the entire egg, except for a small cap at the anterior pole (Fig. 4L). As is observed in *vas exu* embryos, the *ftz* domain in *vas tor exu* embryos becomes non-homogenous at late blastoderm stages, showing a loss of intensity in the posterior while a single stripe of stronger expression is maintained at the anterior margin of the domain (data not shown). These experiments show that, in the posterior of *vas exu* embryos, it is indeed the terminal organizers (and their zygotic mediators) that are responsible for the patterning of pair-rule genes. In the anterior, however, the situation is more complicated: the terminal organizer activity is involved in pattern formation, for the pair-rule gene pattern in the anterior of the triple mutant differs from the one in the double mutant. However, since the anterior and the posterior region in *vas tor exu* embryos are not identical, the terminal organizer activities cannot be the sole determinant of the patterning of the pair-rule genes.

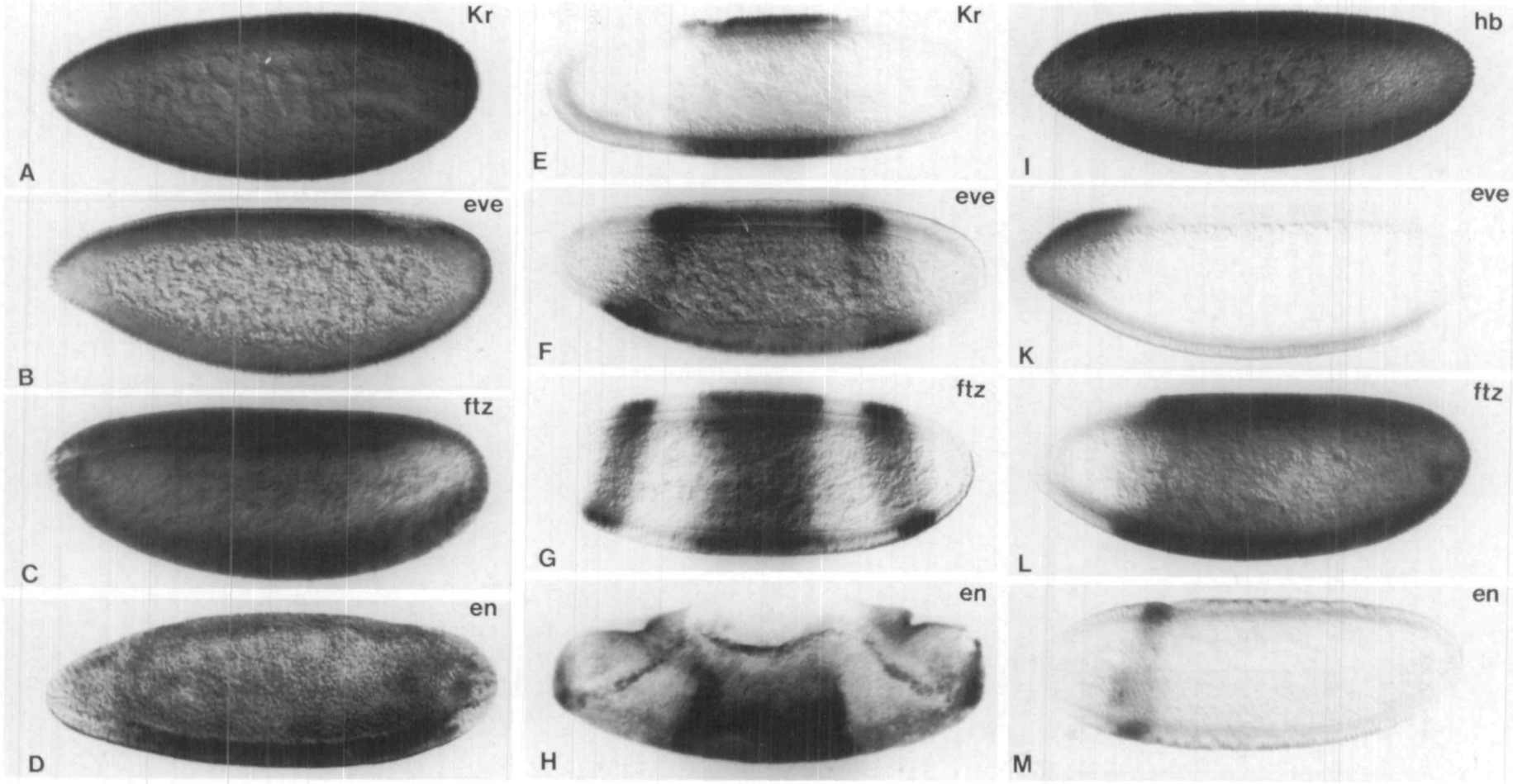


Fig. 4. The effect of *bcd osk tsl*, *bcd osk* and *vas tor exu* mutations on the expression of *Kr*, *hb*, *eve*, *ftz* and *en* as visualized by antibody staining. (A–D) *bcd osk tsl* embryos. All of the genes monitored show a ubiquitous expression. (A) *Kr* expression in a mid-stage 14 embryo. (B) *eve* expression in a mid-stage 14 embryo. (C) *ftz* expression in a late stage 14 embryo. (D) *en* expression in a gastrulating embryo. (E–H) *bcd osk* embryos. (E) *Kr* expression in a mid-stage 14 embryo, forming a broad domain of expression in the middle region (about 30–70 % EL). (F) *eve* expression in a late stage 14 embryo. *eve* expression forms a single, broad domain, which shows higher levels of expression at its margins. (G) *ftz* expression in a late stage 14 embryo. *ftz* expression forms a broad domain in the middle, flanked by one stripe on either side. (H) *en* expression in a gastrulating embryo, forming a broad domain in the middle region of the embryo, flanked by one stripe on either side. (I–M) *vas tor exu* embryos. (I) *hb* expression in a mid-stage 14 embryo. Like in *vas exu* embryos (see Fig. 3A), *hb* is expressed throughout the embryo. (K) *eve* expression in a mid-stage 14 embryo, forming a cap at the anterior pole. (L) *ftz* expression in a mid-stage 14 embryo, covering most of the embryo except for a cap at the anterior pole. Later, the expression becomes non-homogenous: a stripe of strong expression is maintained in the anterior, while expression fades out in the posterior (data not shown). (M) *en* expression in a gastrulating embryo. One single stripe forms close to the anterior pole of the embryo.

Discussion (part II)

Correlations between gap gene and pair-rule gene patterns in the maternal mutant combinations

Our experiments show that the patterns of gap genes and the patterns of pair-rule genes are correlated. In all mutant combinations we find that, within an area of uniform gap gene activity, no alteration occurs in the constellation of pair-rule gene expression. In *bcd osk tsl* embryos, ubiquitous expression of *Kr* and lack of any other zygotic gap gene activity is correlated with ubiquitous expression of *ftz* and *eve*. In the middle regions of *bcd osk* and *stau exu* embryos, which are homogenous with respect to *hb* and *Kr* activity, *ftz* and *eve* are present or absent in a uniform manner. Conversely, in both mutant combinations, the pair-rule gene expression alters in a region where the gap gene input changes, namely around the borders of the *Kr* domains. Hence, it appears that unless some differential information is provided by the gap genes, the uniformity of pair-rule gene expression (or non-expression) is not broken down.

The results for *vas exu* embryos fit in with this analysis. The comparison between *vas exu* and *vas tor exu* embryos shows that in both the anterior and the posterior terminal regions of *vas exu* embryos terminal organizers (and their zygotic mediator(s)) are active in addition to *hb*, providing differential information for the patterning of the pair-rule genes. The residual pattern of pair-rule genes in *vas tor exu* embryos (as well as the slight asymmetry in the pair-rule gene patterns of *vas exu* embryos), however, shows that terminal organizer activities are not the sole determinant of pattern formation in the anterior of *vas exu* embryos. In fact, the difference between the anterior and the posterior may result from the slightly asymmetric distribution of *bcd* protein in such embryos, with higher levels of concentration in the anterior (Struhl *et al.* 1989).

The pair-rule gene patterns at the very termini of *vas exu, stau exu*, and *bcd osk* embryos cannot be explained specifically at present. As pointed out, it is certain that zygotic genes are active in the terminal regions of these embryos, but no probes are currently available, and we therefore know too little about their distribution to determine the relation to the pair-rule gene patterns.

If we now compare the middle regions of *bcd osk*, *stau exu*, and *vas exu* embryos, i.e. those regions that are homogenous with respect to the constellation of gap gene activities, we find that the difference between the three mutant combinations in the constellation of gap gene activities is reflected in a difference in the expression of pair-rule genes. The combination of *hb* and *Kr* expression in the middle region of *stau exu* embryos is correlated with the presence of *ftz* and the absence of *eve*. In *bcd osk* embryos only *Kr* is present, and a different constellation is generated at the pair-rule gene level: *ftz* is on, and *eve* is on at a low level. In *vas exu* embryos, presence of *hb* with absence of *Kr* is correlated with the absence of *eve* and the expression of *ftz* at lower levels. Thus, qualitatively different input from the gap genes leads to different states of expression of pair-rule genes. This means that it does make a difference for the expression of pair-rule genes what kind of gap gene expression a blastoderm cell experiences. In particular, we find that it makes a difference whether a blastoderm cell experiences the expression of *Kr* alone, of *hb* without *Kr*, or of *hb* and *Kr* at the same time. This suggests that the overlap of *hb* and *Kr* expression that is observed in wild-type embryos is indeed functional and encodes information required for the formation of the normal pair-rule gene patterns.

Possible mechanisms of control of pair-rule gene pattern formation by the gap genes

We have seen that qualitatively different states of gap gene expression are correlated with different states of pair-rule gene expression and that the co-expression of *hb* and *Kr* appears to be thus relevant. Based on this observation, one could conceive a combinatorial model of how the gap genes organize the formation of the pair-rule gene patterns in wild-type: qualitatively different states of gap gene expression along the anteroposterior axis of the embryo (... *hb*, *hb+Kr*, *Kr*, ...) would provide different input for the pair-rule genes and eventually 'translate' into different states of pair-rule gene expression. However, the information encoded by the gap genes would not suffice to produce the patterns of the pair-rule genes in this way: within the range of *Kr* expression, e.g. at least three stripes and two interstripes of *ftz* expression have to form in wild-type embryos, while at most three qualitatively defined 'states' of gap gene expression (*hb+Kr*, *Kr*, *Kr+kni*) would be there to provide the input. Even the overlap of *hb* and *Kr* expression in wild-type encompasses not only the second *ftz* stripe, but also at least part of both the second and the third *eve* stripe (Gaul, unpublished).

Of course, more genes might be involved in the generation of the pair-rule gene patterns (in particular, the gap gene *giant* has to be added to the scheme). But, as we have indicated above, the distribution of *hb* and *Kr* is graded toward the margins of their domains, and it seems very likely that this, too, is functionally relevant, i.e. that, for the generation of the pair-rule gene patterns, it not only matters which gap genes are expressed in a particular region of the embryo, but also at what level they are expressed. The very fact that the mutant alleles of gap genes differ in the strength of their effect on segmentation and that a graded phenotypic series from wild-type to loss-of-function phenotypes can be established shows that the requirement for gap gene activity is graded, and it therefore rules out simple binary input–output modes in the interaction of gap genes with pair-rule genes. Even under merely heterozygous conditions (at least for *hb*, *Kr*, and *kni*) the segment patterns of hatched larvae show abnormalities (Wieschaus *et al.* 1984; Lehmann, 1985); these effects are already visible in the pair-rule gene patterns at blastoderm (Frasch and Levine, 1987).

Hence, in addition to the qualitative aspect, i.e. the constellation of gap gene activities, the level of concentration of the gap gene activities appears to be func-

tional in the formation of the pair-rule gene patterns. The gap gene activities, in instructing the pair-rule genes, would thus act as oppositely directed short-range gradients that overlap partially (cf. Odell and Edgar, 1989). Within a band of overlap, accordingly, regions of different ratios of concentration of the two activities would have to be distinguished as having different effects on the pair-rule genes.

Conclusions

With a more sensitive staining technique, the recording of the wild-type distribution of *Kr* (and of *hb*) protein has been improved. The *Kr* protein domain is broader than reported earlier; it covers about 60–33 % EL. This also means that the domain of *Kr* gene expression is roughly co-extensive with the region of *Kr* requirement, i.e. the region that is affected in amorphic *Kr* mutants. The problem that arose from the earlier perception of an incongruity between the phenotypic gap and the domain of expression is thus eliminated.

The overlap of the *hb* and *Kr* protein domains in wild-type, and, in particular, our data on the mutant combinations *stau exu* and *vas exu* show that negative interactions between *hb* and *Kr* do not play the crucial role in the establishment of their domains. We favor the idea that the maternal anterior organizer *bcd* provides the decisive input both for the establishment of the anterior *hb* domain and for the determination of the anterior border of the *Kr* domain in wild-type embryos. The overlap of *hb* and *Kr* protein could easily be explained mechanistically, if, e.g. *bcd* had a somewhat lower affinity for binding to the *Kr* control region than for binding to the *hb* control region.

The overlap of the domains of *Kr* and *hb* that is observed in wild-type helps to picture how the few gap genes are able to instruct the patterning of the pair-rule genes. Co-expression of neighboring gap genes provides additional differential information for pair-rule gene pattern formation. However, this does not suffice, and there is evidence that gap gene concentration levels, and ratios between concentration levels, have to be taken into account in addition.

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