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Gap gene properties of the pair-rule gene *runt* during *Drosophila* segmentation

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SUMMARY

The *Drosophila* Runt protein is a member of a new family of transcriptional regulators that have important roles in processes extending from pattern formation in insect embryos to leukemogenesis in humans. We used ectopic expression to investigate *runt*'s function in the pathway of *Drosophila* segmentation. Transient over-expression of *runt* under the control of a *Drosophila* heat-shock promoter caused stripe-specific defects in the expression patterns of the pair-rule genes *hairy* and *even-skipped* but had a more uniform effect on the secondary pair-rule gene *fushi tarazu*. Surprisingly, the expression of the gap segmentation genes, which are upstream of *runt* in the segmentation hierarchy was also altered in *hs/runt* embryos. A subset of these effects were interpreted as due to an antagonistic effect of *runt* on transcriptional activation by the maternal morphogen *bicoid*. In support of this, expression of synthetic reporter gene constructs containing oligomerized binding sites for the Bicoid protein was reduced in *hs/runt* embryos. Finally, genetic experiments demonstrated that regulation of gap gene expression by *runt* is a normal component of the regulatory program that generates the segmented body pattern of the *Drosophila* embryo.

Key words: *Drosophila*, segmentation, *runt*, *bicoid*, pair-rule gene, gap gene

INTRODUCTION

The protein encoded by the *Drosophila runt* gene is a member of a newly identified family of transcriptional regulators that participate in a variety of important developmental processes. Work on *runt* was initiated due to its vital role in generating the segmented body pattern during the early stages of embryogenesis (Nüsslein-Volhard and Wieschaus, 1980; Gergen and Wieschaus, 1985). Subsequently it was found that *runt* also participates in sex determination and nervous system development (Duffy and Gergen, 1991; Duffy et al., 1991). In both the segmentation and sex determination pathways *runt* affects the transcription of other genes. The nuclear localization of the Runt protein (Kania et al., 1990) provided an initial suggestion that it may be directly involved in regulating transcription in these pathways.

The recent characterization of cDNAs for a subunit of the mammalian transcription factor PEBP2/CBF (Ogawa et al., 1993b) supports the idea that the Runt protein directly regulates transcription. This factor binds to a conserved core site in the enhancers of mammalian type C retroviruses and the polyoma virus and has roles in regulating transcription in embryonal carcinoma cells as well as in hematopoiesis (for review, see Kagoshima et al., 1993). PEBP2/CBF is a heterodimer of two unrelated protein subunits. One of these, the α subunit is encoded by a small family of related genes, all of which share a significant region of homology with the *Drosophila runt* gene. This homology region, termed the Runt-domain, is a 128 amino acid region that is responsible for the sequence-specific DNA-binding properties of all of these proteins, including the *Drosophila* Runt protein (Kagoshima et al., 1993). This strongly suggests that regulation by Runt will involve its binding to DNA. Demonstration of this requires the identification of genes that are directly regulated by *runt* and localization of the target *cis*-regulatory sequences responsible for mediating this regulation.

The segmentation pathway in *Drosophila* has been subject to extensive genetic and molecular analysis, making this an excellent model system for investigating *runt*'s regulatory functions. Segmentation depends on a hierarchical pathway that in several sequential steps converts broad gradients of maternally provided spatial information into segmentally repeated patterns of gene expression. Regulation of gene transcription plays a central role in this hierarchy. Accordingly, as a first step maternal positional cues are used to regulate gap gene transcription and thereby generate their different broad and overlapping expression domains (Gaul and Jäckle, 1987; Tautz, 1988; Driever and Nüsslein-Volhard, 1989; Finkelstein and Perrimon, 1990; Hülskamp et al., 1990; Eldon and Pirotta, 1991; Kraut and Levine, 1991a; Pignoni et al., 1992). One clear example of how maternal information is used is provided by the maternal anterior system. The primary output of the anterior system is a gradient of Bicoid protein (Bcd), a homeobox transcription factor that activates transcription in anterior regions of the embryo. Concentration-dependent, and thus position-dependent activation can be achieved by varying

the number and affinity of Bcd-binding sites in the *cis*-regulatory regions of downstream target genes (Driever et al., 1989; Struhl et al., 1989; Ronchi et al., 1993).

The next step in the hierarchy involves the transfer of positional information from the gap genes to the pair-rule genes. The gap genes all appear to encode transcriptional regulatory proteins (Rosenberg et al., 1986; Tautz et al., 1987; Nauber et al., 1988; Dalton et al., 1989; Finkelstein et al., 1990; Pignoni et al., 1990; Capovilla et al., 1992). The phasing and overlaps in the expression domains of the gap proteins are used to establish of the expression patterns of the pair-rule genes. The transcriptional regulation that generates stripe 2 of the pair-rule gene *even-skipped* (*eve*) is a particularly elegant example of this. A discrete *cis*-element responsible for this stripe contains binding sites for four different transcription factors, the proteins encoded by the gap genes *hunchback* (*hb*), *Krüppel* (*Kr*) and *giant* (*gt*) as well as the maternal Bcd protein (Stanojevic et al., 1989). The stripe 2 element is activated by Bcd and Hb proteins and the stripe forms in a region of the embryo in which both are present. Conversely, the domains of Kr and Gt expression normally abut the posterior and anterior margins of *eve* stripe 2, respectively and act to define the boundaries of this stripe by repressing the activity of the stripe 2 element (Small et al., 1991, 1992; Stanojevic et al., 1991).

Runt is a pair-rule gene and is positioned downstream of the maternal and gap genes and upstream of the segment-polarity genes in the segmentation hierarchy. The identification of segment-polarity genes that are directly regulated by *runt* is complicated by the fact that the expression patterns of other pair-rule genes are altered in *runt* mutant embryos (Carroll and Scott, 1986; Frasch and Levine, 1987; Ingham and Gergen, 1988; Hooper et al., 1989; Gutjahr et al., 1993). Further, the identification of pair-rule targets of *runt* is complicated by the network of cross-regulatory interactions between these genes. For example, *runt* is interpreted as a positive regulator of the pair-rule gene *fushi tarazu* (*ftz*). However, the expression of the pair-rule genes *eve* and *hairy*, both of which are interpreted as negative regulators of *ftz* is also affected by *runt*. These previous observations are based on examining the consequences of reducing *runt* activity. Here we examine the consequences of over-expressing *runt* in order to identify transcriptional regulatory events that are most directly influenced by the presence or absence of the Runt protein.

The strategy that we used was to create transgenic *Drosophila* lines in which the expression of *runt* is under the control of the inducible *hsp70* promoter. Transient over-expression of *runt* in blastoderm stage embryos dramatically alters the transcriptional regulation of other pair-rule genes. The effects on *hairy*, *eve* and *runt* itself are stripe specific, whereas *ftz* is more uniformly affected. Somewhat surprisingly, we find that the expression of upstream gap genes is also affected by *runt*. Evidence is presented that a subset of the effects on the gap genes, as well as the repression of *hairy* stripe 1 and *eve* stripe 2 are due to antagonism of Bcd-dependent transcription. Genetic experiments indicate that *runt* plays a normal role in preventing *bcd*-dependent gene expression in the central regions of the embryo. The implications of these results on the hierarchical view of *Drosophila* pattern formation and on our understanding of the function of the Runt protein are discussed.

MATERIALS AND METHODS

Drosophila strains

The *hs/runt* transgene was constructed in the CaSper P-element transformation vector (Pirotta, 1988). Site directed mutagenesis was used to create a *Bam*HI recognition site 9 basepairs upstream of the initiator ATG for the runt protein (Zoller and Smith, 1984). The sequence GGATACGAG***ATG*** was altered at the two underlined positions to the sequence GAATTCGAG***ATG***. The initiator ATG for the *runt* open-reading frame (ORF) is italicized. Site directed mutagenesis was done on a composite cDNA insert (ED, nucleotides 30 to 2492 in Kania et al., 1990) subcloned into the *Eco*RI site of M13mp19. The integrity of clones containing the desired *Bam*HI site was verified by DNA sequence analysis, and then the 2.248 kb *Bam*HI + *Eco*RI fragment containing the entire *runt* ORF was subcloned into the *Eco*RI site of the plasmid vector BMKS (Stratagene) in a three way ligation with a 450 basepair *Eco*RI + *Bam*HI fragment base containing the *Drosophila HSP70* promoter (courtesy of V. Pirotta, University of Geneva). A portion of the *runt* cDNA insert extending towards the 3′ end of the mRNA from an *Sph*I site at nucleotide 1625 in the map of Kania et al. (1990) to a *Hin*dIII site in the flanking polylinker of BMKS was then replaced by an overlapping 1.8 kb *Sph*I fragment of *runt* genomic DNA. The resulting construct thus contains the heat-shock promoter with 207 nucleotides of *hsp70* 5′ untranslated leader, 8 nucleotides of the modified *runt* 5′ untranslated leader, the entire *runt* ORF and 3′ untranslated trailer, followed by 1 kb of 3′ flanking *runt* genomic DNA. This entire fusion gene was removed as an *Eco*RI + *Kpn*I fragment and cloned into *Eco*RI + *Bam*HI digested CaSper P-element transformation vector. The *Kpn*I and *Bam*HI sites were both filled in with T4 DNA polymerase, thus recreating a *Bam*HI site in the final construct. In the final construct, the *white* and *hs/runt* genes are divergently transcribed. Germ-line transformants were obtained by injecting CaSper:*hs/runt* DNA at a concentration of 1 mg/ml with 100 mg/ml of the P-transposase helper plasmid pπ25.7 wc (Karess and Rubin, 1984). Of 596 embryos injected with these DNAs, 85 successfully completed embryogenesis and of these 19 developed to adult stages. One transformant line, $hs/runt^{a}$ was obtained from the fertile surviving G_o adults. Other inserts of the *hs/runt* transgene were obtained by mobilizing the *hs/runt* transgene in this line with the Δ2-3 transposon (Robertson et al., 1988). Three different *hs/runt* lines were tested for their effects on the expression of *ftz*, *eve* and *gt*. They gave similar results after the standard heat-shock treatment.

The following stocks were used as sources for different segmentation mutations. *gt*: *Df(1)62g18/ FM6*; *Kr*: *y; Dp (1;2)B89,* y^{+} Kr^{-}*/SM1*; *runt*: *y w f runt*^{LB5}*/FM7c/*y^{+} *Ymal*^{102} (Lindsley and Zimm, 1992). The strain used as the wild-type control for the effects of heat-shock treatment was the parental *y* w^{67c23} strain used as the host for the establishment of the *hs/runt* line.

We used *Drosophila* lines containing the following reporter gene constructs: For *hairy* stripes 1 and 5, h^{KB}*/lacZ* (Pankratz et al., 1990); for *eve* stripes 2, 3 and 7, *eve*$^{5.2}$*/lacZ* (Harding et al., 1989; Stanojevic et al., 1991); for the *eve* autoregulatory, or pair-rule element, *eve*^{4E}*/lacZ* (Jiang et al., 1991); for *ftz*, *ftz/lacC* (Hiromi et al., 1985); for a *bcd*-dependent reporter, *HSBG [bcd]* (Ronchi et al., 1993), for a *bcd* + *hb*-dependent reporter, *HSBG [hb+bcd]* (J. Treisman and C. Desplan, Rockefeller University).

Embryo manipulation

Embryos were collected on apple juice plates for periods of up to 4 hours at 25°C. Embryos were washed off plates and collected on a nytex net mounted on the end of polypropylene cylinder. In order to insure uniform and efficient heat-shock treatment, the density of embryos was controlled such that they formed a monolayer on the net. Heat-shock treatments were carried out by resting the nets containing the embryos on the top of prewarmed blocks in a 37°C water bath for 20 minutes. After heat-shock treatment, the embryos in the net were

removed to room temperature, aged for another 30-40 minutes, and then processed for in situ hybridization. The in situ hybridization protocol used the non-radioactive digoxigenin-based system (Boehringer-Mannheim) essentially as described in Klingler and Gergen (1993) with the exception that the two phase fixation solution contained: 0.1 ml 1 M EGTA, 0.4 ml 37% formaldehyde, 3.5 ml 1× PBS, and 6.0 ml heptane.

Embryos collected and processed in this manner represent a mixture of developmental stages. The text refers to the stage of the embryos at the time of fixation, i.e. approximately 50 to 60 minutes subsequent to the time at which they were initially collected for heat-shock treatment. The expression patterns described are representative of the majority of the embryos of a particular developmental stage. All heat-shock experiments have been repeated on at least three separate occasions with similar results. Less severe, as well as more severe alterations are sometimes observed in other embryos, presumably due to subtle differences in their developmental stage and/or in their heat-shock response.

Antisense RNA probes for in situ hybridization were as follows:

Gene	Plasmid (Source)	Linearized with	RNA polymerase
bcd	pBS:bcd (C. Tsai)	*Kpn*I	T7
eve	p48-X1.4 (P. Macdonald)	*Xho*1	SP6
runt	pB:ED#17 (M. Kania)	*Hin*dIII	T7
runt (5′ leader)	pB:ED-5′(C. Tsai)	*Kpn*I	T7
hairy	pT:hairy Δ1 (D. Ish-Horowicz)	*Hin*dIII	T7
ftz	pT:ftz (D. Ish-Horowicz)	*Hin*dIII	T7
paired	pB:prd (C. Tsai)	*Not*I	T3
Kr	pT:Kr (H. Jäckle)	*Xba*I	T7
kni	pcJ15 (U. Nauber)	*Sal*I	T7
hb	pcJ3 (J. Posakony)	*Hin*dIII	T7
gt	pB:gt (P. Gergen)	*Eco*RI	T3
otd	(R. Finkelstein)	*Kpn*I	T3
ems	pc:ems (B. McGinnis)	*Sac*I	T7
lacZ	pXEBG (B. Butler)	*Eco*RI	T7

Immunohistochemical detection of the Bcd and Runt proteins was done as in Kania et al. (1990). The anti-Runt antibody was a cocktail of four different monoclonal antibodies, each at a final concentration of 50 mg/ml. The anti-Bcd antibody was prepared from supernatants of monoclonal line 733.3 obtained from D. Jay (Harvard University). Ammonium sulfate precipitation was used to purify IgG fractions from this supernatant. These concentrated antibody preparations were used at a final concentration of 100-250 mg/ml after pre-absorbing them against aged (>4 hours) *Drosophila* embryos.

RESULTS

Ectopic *runt* expression alters pair-rule gene transcription

To investigate the regulation of segmentation gene expression by *runt*, we generated transgenic lines of *Drosophila* where *runt* expression is under control of the inducible *hsp70* promoter. Heat-shock treatment leads to a high level of runt mRNA and protein accumulation in all somatic cells of blastoderm stage embryos homozygous for the *hs/runt* transgene (data not shown). The failure of the *hs/runt* transgene to be expressed in pole cells is not understood, but is similar to results obtained with other *hs* transgenes (e.g. Capovilla et al., 1992). Whole mount in situ hybridization was used to examine the RNA expression patterns of other segmentation genes and thus determine the regulatory consequences of this ectopic expression. As shown below, there are several dramatic pattern alterations in *hs/runt* embryos. The severity of these alterations is influenced by the duration and degree of the heat shock. All of the experiments described below used a 20 minute heat shock at 37°C, followed by a 30 to 40 minute recovery period before the embryos were processed for in situ hybridization. This protocol gives clear and reproducible effects in *hs/runt* embryos. Importantly, this treatment does not alter the patterns in genetically wild-type embryos that are processed identically. A second important factor for interpreting these experiments is the developmental stage of the affected embryos. In the text below, the developmental stage is described based on the morphology at the time of fixation. As all experiments followed the same protocol, this method allows for accurate comparison of embryos from different experiments. Finally, the expression patterns described below are representative of the majority of embryos at a particular developmental stage.

As expected, the pair-rule expression patterns of *ftz*, *eve* and *hairy* are altered in *hs/runt* embryos. From analyses of expression patterns in *runt* mutant embryos, we anticipated that *eve* and *hairy* would be repressed, and that *ftz* would be activated by *hs/runt* treatment. Aspects of the *eve* and *hairy* patterns are repressed in *hs/runt* embryos, but unexpectedly these effects are markedly stripe specific. For *hairy*, the most notable difference is the repression of stripes 1 and 6 (Fig. 1B). Stripes 2 and 5 are also reduced in intensity. In contrast to these repressive effects, *hairy* stripes 3 and 4 appear to become more intense in *hs/runt* embryos. In the case of *eve*, the most dramatic alteration is the elimination of stripe 2 (Fig. 1F). The intensity of *eve* stripes 4 and 7 is also consistently reduced and stripes 5 and 6 are not well resolved in *hs/runt* embryos. These stripe-specific effects of *hs/runt* on *eve* and *hairy* are most apparent at the mid-blastoderm stage when the pair-rule patterns are normally well formed, but similar effects are also observed on both younger and older blastoderm stage embryos.

In contrast to the above results, *ftz* is activated by *hs/runt* and in a much more uniform manner (Fig. 1J). This activating effect is readily apparent in mid-blastoderm stage embryos, but again similar effects are also observed in both older and younger embryos. Expression of *ftz* in the interstripe regions is de-repressed and there is an almost uniform, high level of *ftz* mRNA accumulation within an interval extending from 15% to 65% egg-length (EL, 0%=posterior pole). This spans from positions where the anterior margin of the first *ftz* stripe and the posterior margin of the seventh stripe would form in normal embryos. This increased expression is consistent with previous interpretations that *runt* acts as a positive regulator of *ftz*. Furthermore, as *hs/runt* does not lead to the uniform repression of *eve* and *hairy* within this region, *runt* must have regulatory effects on *ftz* that are independent of its effects on the expression of these other two pair-rule genes.

The above results demonstrate that *runt* acts as a regulator of pair-rule gene expression but do not show that this is due to transcriptional regulation. Previous work has identified *cis*-regulatory elements that participate in the transcriptional regulation of *ftz*, *eve* and *hairy* during segmentation. We examined the expression patterns of reporter gene constructs containing regulatory elements from these genes in order to determine if *hs/runt* treatment alters their transcriptional regulation. The *ftz/lacC* reporter gene contains three major regulatory elements from the *ftz* upstream region (Hiromi et al., 1985). This reporter gene is expressed in a characteristic 7-striped pattern in late

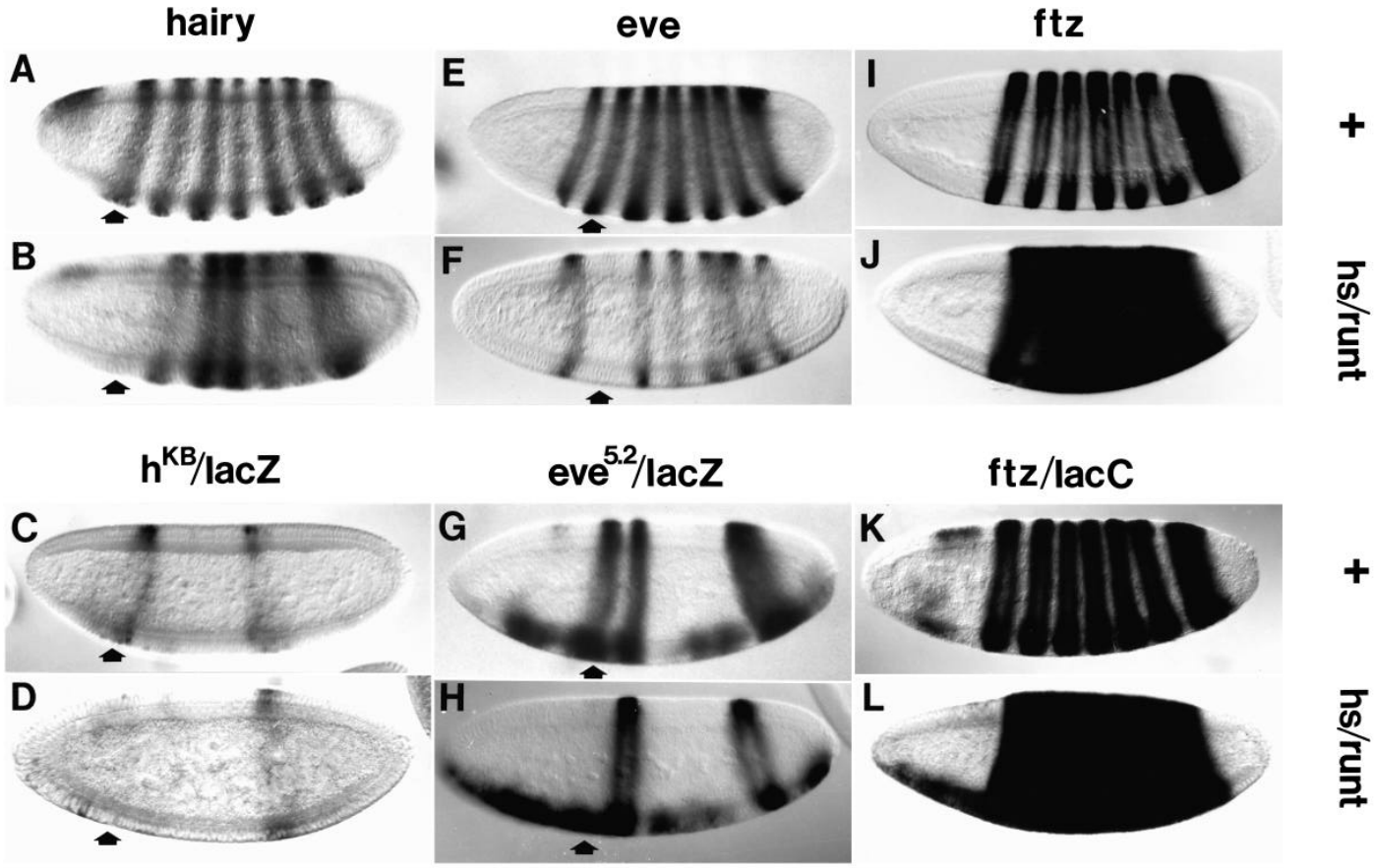

Fig. 1. Pair-rule gene expression in hs/runt embryos. The pattern of *hairy*, *eve* and *ftz* mRNA accumulation as visualized by in situ hybridization in normal (A,E,I) and *hs/runt* (B,F,J) embryos. These and all other embryos are shown with the anterior end to the left and ventral side down. (C-L) The results of in situ hybridization with a probe that detects *lacZ* mRNA accumulation. The wild-type expression patterns of *hairy*^{KB}*/lacZ*, *eve*$^{-5.2}$*/lacZ*, and *ftz/lacC* are shown in C, G and K, respectively. The expression in *hs/runt* embryos is shown in D, H and L. The position where *hairy* stripe 1 should form is indicated by the arrows in A-D. The arrows in E-H point to the position where *eve* stripe 2 would normally form. The *eve* and *ftz* reporter genes show expression in ventral regions that is due to sequences in the *rosy* P-element transformation vector. This artifactual expression is not significantly affected by *hs/runt*.

blastoderm stage embryos (Fig. 1K). In *hs/runt* embryos, this reporter gene shows a broad band of expression similar to that observed for the endogenous *ftz* gene (Fig. 1L). This result confirms that *runt* regulates *ftz* transcription, and indicates that *cis*-elements within the *ftz/lacC* reporter gene mediate this regulation. Further work is needed to determine if this is due to a direct interaction between the Runt protein and these *cis*-elements.

The *eve* upstream region contains several stripe-specific *cis*-elements that are regulated by the gap genes, as well as an autoregulatory element that can direct the formation of all 7 stripes, but only in the presence of wild-type *eve* function (Goto et al., 1989; Harding et al., 1989; Jiang et al., 1991; Small et al., 1991). Previous work has indicated that *runt* acts through the autoregulatory element to repress *eve* expression (Goto et al., 1989). However, the stripe-specific alterations that are observed in *hs/runt* embryos suggest that in this case *runt* may be acting through the stripe-specific elements. In fact, the expression of reporter genes containing the *eve* autoregulatory element was not significantly altered in our regularly treated *hs/runt* embryos (not shown). Conversely, a reporter gene that contains regulatory elements for *eve* stripes 2, 3 and 7 and that lacks the autoregulatory element is affected in a manner identical to the endogenous *eve* gene, i.e. stripe 2 is eliminated and stripe 7 is reduced in intensity (Fig. 1G,H). The fact that stripe 3 is not affected indicates that the repression of stripes 2 and 7 expression is not due to a generalized reduction in the transcription of the reporter gene construct in these embryos. The results obtained with reporter genes for *hairy* are similar to those obtained for *eve*. The *h*^{KB}*/lacZ* reporter gene contains *cis*-regulatory elements for *hairy* stripes 1 and 5 (Fig. 1C; Pankratz et al., 1990). In *hs/runt* embryos, the expression of this reporter gene in the region corresponding to *hairy* stripe 1 is eliminated and the expression of stripe 5 is reduced (Fig. 1D). The results obtained with these *eve* and *hairy* reporter gene constructs provide further evidence that ectopic *runt* expression alters the transcriptional regulation of pair-rule genes. These results also strongly suggest that stripe-specific *cis*-regulatory elements of *eve* and *hairy* respond to the regulatory effects of *runt*.

Gap gene expression patterns are altered in *hs/runt* embryos

The establishment of the *hairy* and *eve* expression patterns

depends on the regulation of stripe-specific *cis*-regulatory elements by maternal and gap gene regulatory products (Pankratz et al., 1990; Riddihough and Ish-Horowicz, 1991; Small et al., 1991, 1992; Stanojevic et al., 1991). One explanation for the stripe-specific effects described above is that *hs/runt* interferes with the expression or function of the gap genes. To address this possibility, we examined gap gene expression patterns in *hs/runt* embryos. Five different gap genes, *Krüppel* (*Kr*), *knirps* (*kni*), *giant* (*gt*), *hunchback* (*hb*), and *tailless* (*tll*) contribute to the regulation of pair-rule gene expression within the segmented region of the embryo (hereafter referred to as the presegmental region). The expression patterns of all of these, except for *tll* (not shown), are clearly altered in *hs/runt* embryos (Fig. 2). Both positive and negative effects on gap gene regulation are observed, depending on the particular domain of expression and its position along the anteroposterior axis. In order to interpret these effects, it is important to take into account the differences in the regulatory cues that are used to generate gap gene expression domains in different regions of the embryo. Thus, the following description considers the gap gene expression patterns in *hs/runt* embryos on a region by region basis.

The stimulation of the central domain of *Kr* expression is the major positive regulatory effect observed within the presegmental region of the embryo. In wild-type embryos that have just completed the 13th and final syncitial nuclear division cycle, there is a central domain of *Kr* expression extending from 45 to 60% EL (Fig. 2A). In *hs/runt* embryos of this same developmental stage, this centrally located expression domain is expanded in both the anterior and posterior directions (Fig. 2B). The boundaries of this *Kr* domain normally are defined by repression from the maternal anterior and posterior systems (Gaul and Jäckle, 1987). The expansion of *Kr*'s central domain suggests that repression of *Kr* by these two maternal systems is antagonized in *hs/runt* embryos.

The enlarged central domain of *Kr* expression was of particular interest because it seemed likely that it could account for the repression of *eve* stripe 2. *Kr* is normally expressed posterior to this stripe and acts to define its posterior boundary by repressing stripe 2 element-dependent transcription (Small et al., 1991). The enlarged *Kr* domain observed in *hs/runt* embryos extends from the posterior margin of *eve* stripe 1 to the anterior margin of *eve* stripe 6 and thus spans the region in which *eve* stripe 2 should form. This alteration could readily explain the repression of *eve* stripe 2 in *hs/runt* embryos. However, in *hs/runt* embryos that were also mutant for *Kr*, *eve* stripe 2 is still eliminated (Fig. 3B). Thus the anterior expansion of *Kr* does not account for the specific elimination of this stripe in *hs/runt* embryos.

The gap genes *kni*, *gt*, *hb* and *tll* all have expression domains that are posterior to the central *Kr* domain and that fall within the presegmental region of the embryo (Fig. 2C,E,G). The posterior domain of *kni* is normally juxtaposed and posterior to the central *Kr* domain. In early blastoderm stage *hs/runt* embryos, the level of *kni* expression within this domain is reduced in intensity (Fig. 2D). A similar, but stronger effect is observed on *gt*. The posterior expression domain of *gt* is essen-

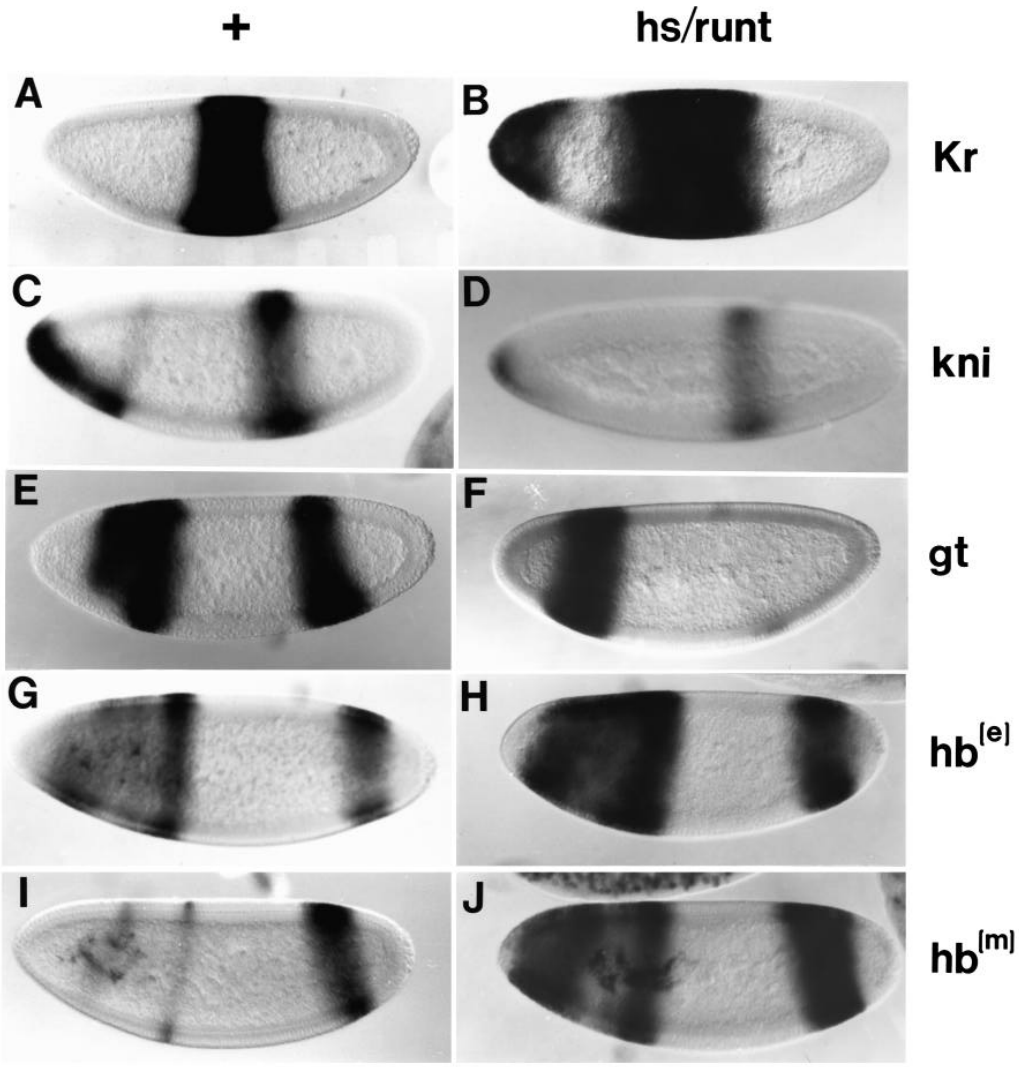

Fig. 2. Gap gene expression patterns in *hs/runt* embryos. Expression of mRNAs from the gap genes *Kr*, *kni*, and *gt* in early blastoderm stage wild-type (A,C,E) and *hs/runt* embryos (B,D,F). The pattern of *hb* expression is shown for both early blastoderm ($hb^{[e]}$) and mid-blastoderm stages ($hb^{[m]}$) of wild-type (G,I) and *hs/runt* (H,J) embryos.

tially eliminated in early blastoderm stage *hs/runt* embryos (Fig. 2F). This aspect of *gt* expression is subject to repression by *Kr* (Kraut and Levine, 1991b; Eldon and Pirotta, 1991), and thus could be indirectly due to the posterior expansion of *Kr* that occurs in *hs/runt* embryos. However, the repression of *gt*'s posterior domain occurs in *hs/runt* embryos that are also mutant for *Kr* (Fig. 3D). Thus the negative effect of *runt* on *gt* is not due solely to *runt*'s positive effects on *Kr*. The more posterior stripes of *hb* and *tll* are not significantly affected in *hs/runt* embryos, although a slight anterior expansion of *hb* is sometimes observed. It is notable that the differences in the sensitivity of these different domains of gap gene expression to *hs/runt* correlate with the primary regulatory cues used in their establishment. The posterior stripes of *hb* and *tll* depend on the activity of the maternal terminal system, and are relatively unaffected by *hs/runt*. Conversely, the posterior domains of *kni* and *gt*, which are negatively affected by *hs/runt*, are activated by the maternal posterior system. The repression of these two domains suggests that ectopic *runt* expression antagonizes the activity of the maternal posterior system. This putative antagonism is consistent with the effects of *hs/runt* on *Kr* that were described above.

There are multiple effects of *hs/runt* expression in regions anterior to the central *Kr* domain. The gap gene *hb* is expressed just anterior to *Kr*. In fact, there are two phases in the transcriptional regulation of *hb* in the anterior end of the embryo, both of which result in expression domains that border on the central *Kr* domain. The first phase is the activation of the proximal *hb* promoter during nuclear division cycle 11 (Schröder et al., 1989). This promoter is activated in direct response to the maternal *bcd* gradient (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989), and produces a broad band of mRNA accumulation extending from 100% EL almost to the middle of the embryo. There is no obvious difference in this aspect of *hb* expression in *hs/runt* embryos. In contrast to this, there are significant changes in the *hb* pattern in *hs/runt* embryos that have completed the 13th nuclear division. At this stage in wild-type embryos the distal *hb* promoter is activated and generates two stripes of expression (Fig. 2G). In *hs/runt* embryos the anterior *hb* stripe is broadened and shifted anteriorly (from 56% to 60% EL, Fig. 2H). There is also increased expression of *hb* in regions anterior to this stripe, especially in older blastoderm stage *hs/runt* embryos (Fig. 2J). Presumably the broadened anterior stripe of *hb* expression is due to mis-regulation of the distal *hb* promoter. The ectopic expression in more anterior regions could also be due to mis-regulation of this promoter, or may reflect a de-repression of the proximal *hb* promoter in the anterior-most regions of late blastoderm stage *hs/runt* embryos.

The anterior expression of *gt* is also affected in *hs/runt* embryos. In normal embryos the initial band of anterior *gt* expression extending from 80 to 60% EL splits into two stripes during the early blastoderm stage. The posterior of these two lies just anterior to the *hb* stripe and represents the most anterior domain of gap gene expression within the presegmental region of the embryo. This *gt* stripe is uniformly expressed around the dorsoventral circumference of the embryo (Fig. 2E). The other, more anterior stripe of *gt* lies outside of the presegmental region in an area that will give rise to the head. A distinctive characteristic of this stripe is its reduced expression in the ventral regions of the embryo. In early blastoderm stage *hs/runt* embryos, these two stripes are replaced by a single broad stripe with uniform expression on the dorsoventral axis (Fig. 2F). The posterior margin of this stripe (68% EL) is anterior to the posterior limits of *gt* expression in this region of wild-type embryos (62% EL). This anterior shift is similar to that described above for the anterior stripe of *hb* expression. One interpretation of the changes in these *hb* and *gt* domains is that *hs/runt* treatment is causing an anterior expansion of the presegmental region at the expense of the more anterior, precephalic region. Note that the previously described anterior expansion of *Kr*'s central domain in *hs/runt* embryos is also consistent with this interpretation.

There are several other significant changes in gap gene expression patterns in the precephalic region of *hs/runt* embryos. *Kr* normally is not expressed in this region in early blastoderm stage embryos. In *hs/runt* embryos, there is a broad cap of *Kr* expression at the anterior end (Fig. 2B). The effect on *kni* is reciprocal to this. In wild-type embryos there is a broad patch of ventral expression that extends from the anterior pole to 75% EL (Fig. 2C), and at this position a circumferential stripe of expression forms. In *hs/runt* embryos this complex

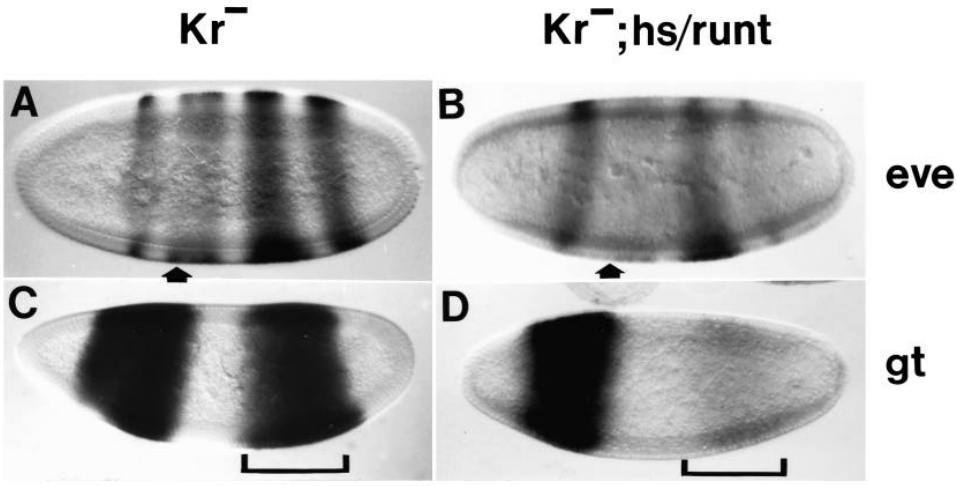

Fig. 3. Elimination of *eve* stripe 2 does not require *Kr* activity. The top two panels show *eve* mRNA accumulation gene in two different embryos mutant for the gap gene *Kr*. The embryo in A is wild-type for *runt*, the embryo in B is a *hs/runt* embryo. The arrow indicates the position of *eve* stripe 2. Note that this stripe fuses with stripe 3 in embryos mutant for *Kr*. The expression of the gap gene *gt* in Kr^{-} and Kr^{-};*hs/runt* embryos is shown in C and D, respectively. The posterior *gt* domain is indicated with a bracket. Although this domain is expanded in *Kr* mutant embryos (compare 3C to the wild-type *gt* pattern in Fig. 2E), it is still eliminated in *Kr* mutant *hs/runt* embryos. The *Kr* mutant embryos in these experiments are homozygous for a chromosome that is deleted for *Kr*. Double hybridization with an in situ probe that detects *Kr* mRNA was used to unambiguously identify these mutant embryos.

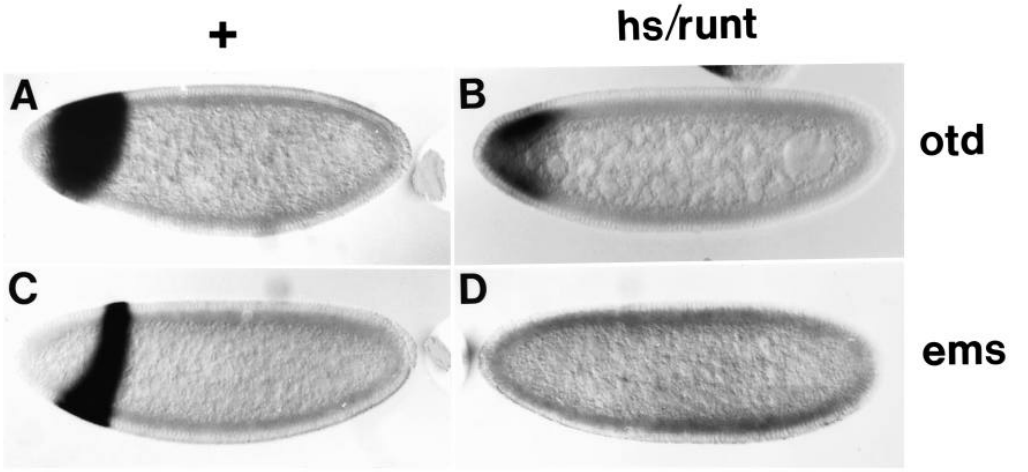

Fig. 4. *Hs/runt* represses head gap gene expression. Early blastoderm stage embryos hybridized with probes for the head gap genes *otd* (A,B) and *ems* (C,D). The embryos on the left show the patterns in normal embryos. Expression of these genes in *hs/runt* embryos is shown by the two embryos on the right.

pattern is replaced by a small cap of low-level expression that is restricted to the anterior pole (Fig. 2D). These several observations strongly suggest that *hs/runt* treatment interferes with the regulatory program that normally operates in the head region of the embryo. In order to investigate this further, we examined the effect of *hs/runt* treatment on *empty spiracles* (*ems*) and *orthodenticle* (*otd*), two genes that have been classified as head gap genes based on their early roles in anterior pattern formation (Cohen and Jürgens, 1990). The expression of both of these genes is altered in early blastoderm stage *hs/runt* embryos. The band of *otd* expression that normally extends from 75 to 95% EL is replaced by an anteriorly shifted domain with a reduced level of expression (Fig. 4A,B). In contrast, the expression of *ems* is almost completely suppressed in the anterior end of *hs/runt* embryos (Fig. 4D). These results provide strong evidence that ectopic *runt* expression disrupts the normal regulation of gap gene expression in the head of the *Drosophila* embryo.

runt interferes with *bcd*-dependent gene expression

Proper expression of gap genes in the anterior end of the embryo is dependent on the maternal anterior system. The principle output of this system is a morphogenetic gradient of Bcd protein with the highest levels at the anterior pole. Several of the results presented above suggested that *runt* antagonizes *bcd* activity. First, the anterior expansion of *Kr*'s central domain as well as the shift in the anterior band of *gt* expression in *hs/runt* embryos are similar to effects observed when *bcd* is reduced (Gaul and Jäckle, 1987; Eldon and Pirotta, 1991; Kraut and Levine, 1991a). Similarly, the anterior expression domains of the head-gap genes *ems* and *otd*, both of which are negatively affected by *runt*, are also both *bcd* dependent (Dalton et al., 1989; Finkelstein and Perrimon, 1990). Further evidence comes from the effects of *hs/runt* on the more anterior pair-rule stripes. The proper expression of *eve* stripe 2 depends on activation by *bcd* and *hb* and repression by *gt* and *Kr* (Small et al., 1992). As described above, the elimination of this stripe in *hs/runt* embryos is not due to the expanded expression of *Kr*. This stripe is also eliminated in *hs/runt* embryos that are mutant for *gt* (not shown). Thus the elimination of *eve* stripe 2 is presumably due to a regulatory interaction between *runt* and the activators of this stripe. In this context, it is notable that *hairy* stripe 1, which is eliminated in *hs/runt* embryos, is also *bcd* dependent (Riddihough and Ish-Horowicz, 1991). In fact, in addition to *bcd*, *runt* is the only other segmentation gene shown to regulate this stripe. Taken together, these observations strongly suggested that interference with *bcd*-dependent regulation could account for many of the defects in the anterior ends of *hs/runt* embryos.

Several lines of evidence indicate that the Bcd protein is directly responsible for activating transcription of genes expressed in the anterior regions of *Drosophila* embryos. Two convincing examples are the activation of *eve* stripe 2 (Small et al., 1991, 1992), and the activation of the proximal *hb* promoter (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989). Interestingly, the former is extremely sensitive to *hs/runt* inhibition whereas the latter is not. One significant difference between these is that the activation of *hb* occurs during the syncitial nuclear division cycles whereas *eve* stripe 2 is most strongly expressed subsequent to the final syncitial nuclear division cycle during the blastoderm stage. This difference in stage of expression could account for the differential sensitivity to *runt*. Alternatively, these different responses may be due to differences in the proximal *hb* promoter and the *eve* stripe 2 element. In order to investigate this, we examined the effects of *hs/runt* on two different reporter genes with well-defined *cis*-regulatory control regions. The first, *HSBG[bcd]*, contains three copies of the bcd recognition sequence upstream of a minimal promoter. This reporter gene produces an anterior cap of *lacZ* mRNA expression in early blastoderm stage embryos (Fig. 5A). This expression is *bcd* dependent (Ronchi et al., 1993). We also examined the expression of a related reporter gene, *HSBG[bcd+hb]*, that contains three copies of the recognition sequence for the hb protein in addition to the three bicoid-binding sites (J. Treisman and C. Desplan, personal communication). In normal embryos, the expression of this gene is significantly stronger and extends more posteriorly than the expression of *HSBG[bcd]* (Fig. 5C). The expression of both reporter genes is affected in early blastoderm stage *hs/runt* embryos. The expression of *HSBG[bcd]* is almost completely eliminated (Fig. 5B), whereas the expression pattern of *HSBG[bcd+hb]* is both reduced and anteriorly shifted in *hs/runt* embryos (Fig. 5D). These results provide strong evidence that *bcd*-dependent transcriptional activation is antagonized in blastoderm stage *hs/runt* embryos. Both of these reporter genes are initially activated during the

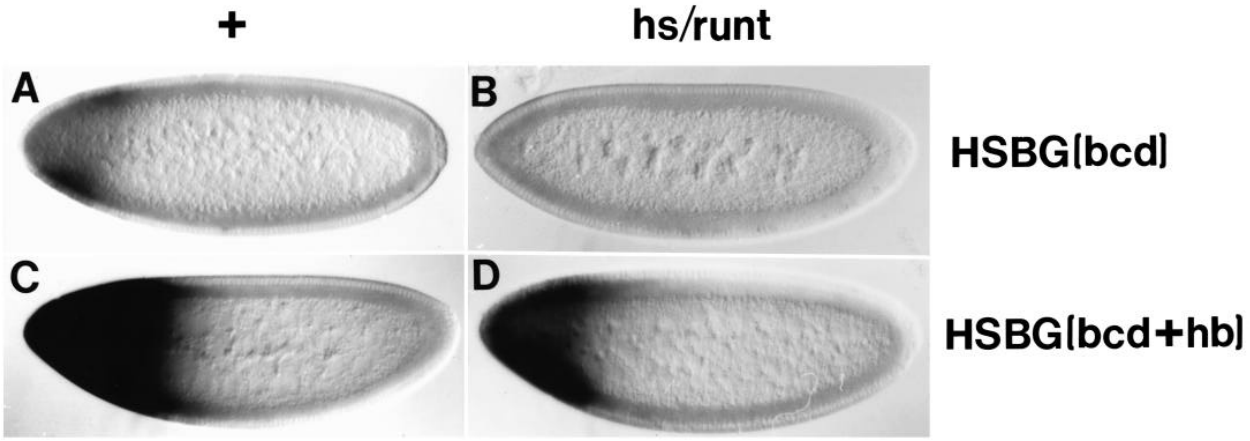

Fig. 5. Ectopic *runt* expression antagonizes *bcd*-dependent transcription. Expression of the *lacZ* mRNA from the *HSBG[bcd]* reporter gene is shown in A and B. The embryo in A is a wild-type embryo that was heat-shocked, the embryo in B is a *hs/runt* embryo. The lower two panels show the stronger expression of the *HSBG[bcd+hb]* reporter gene in control (C) and *hs/runt* embryos (D).

syncitial stages prior to formation of the blastoderm. As *HSBG[bcd]* is only expressed at a very low level during these earlier stages, it is difficult to determine whether this expression is repressed by *hs/runt* treatment. The stronger expression of the *HSBG[bcd+hb]* reporter may be slightly reduced in preblastoderm *hs/runt* embryos, but not as significantly as at later stages (not shown). This suggests that the effect of *runt* on *bcd*-dependent transcription is dependent on the developmental stage of the embryo.

The *bcd* mRNA is synthesized and then localized to the anterior pole of the oocyte during oogenesis (Frigerio et al., 1986; Berleth et al., 1988). The negative effects on *bcd*-dependent expression in *hs/runt* embryos are due to ectopic expression of a transgene in the early blastoderm stage. Therefore this effect cannot be due to an alteration in *bcd* transcription. No significant difference was observed in the *bcd* mRNA and protein expression patterns in *hs/runt* embryos (data not shown). These results indicate that *hs/runt* treatment is not affecting the synthesis, stability or localization of the Bcd protein. They further suggest that *runt* is responsible, either directly or indirectly, for interfering with the ability of the Bcd protein to activate transcription during the blastoderm stage of embryogenesis.

Gap-gene properties of *runt* during normal embryogenesis

The above observations are based on ectopic expression of *runt*. Many of these effects are observed in early blastoderm stage embryos (prior to nuclear elongation). Based on the experimental protocol used (see Materials and Methods), these embryos were either in the 12th or 13th syncitial nuclear divisions at the time *runt* expression was induced by heat-shock treatment. In wild-type embryos during these stages, *runt* has not yet developed its characteristic pair-rule expression pattern, but instead is expressed in a central broad field (Klingler and Gergen, 1993). Therefore we speculated that many of the effects observed in early blastoderm stage embryos might reflect regulatory functions normally provided by *runt*'s early broad field.

Based on the ectopic expression experiments, one obvious prediction is that there would be overexpression of *bcd*-dependent genes in *runt* mutant embryos. For example, the posterior margin of the anterior domain of *hb* expression is established at a point on the *bcd* gradient where the level of activity drops below the threshold value required for activation of the *hb* promoter (Driever et al., 1989). In the simplest model, this *hb* expression domain would extend more posteriorly in the absence of *runt* activity. However, this is not observed (data not shown). The anterior expression domain of *hb* is also subject to repression by *Kr*, which is normally expressed just posterior to and abutting this domain (Jäckle et al., 1986). Therefore *Kr* could be responsible for preventing the posterior expansion of *hb* in *runt* mutant embryos. We tested this possibility by examining *hb* expression in embryos mutant for both *runt* and *Kr*. There is posterior expansion of the anterior *hb* domain in these double mutant embryos (Fig. 6E). This effect is stronger than the slight posterior expansion of *hb* observed in embryos mutant only for *Kr* (Fig. 6C). Notably, the posterior margin of this expanded domain is more diffuse in the double mutant embryos than in either single mutant, and continues to extend more posteriorly as the embryos progress to later stages. In fact, the anterior and posterior domains of *hb* become fused on the dorsal side of double mutant embryos by the completion of cellularization. This result suggests that formation and maintenance of the normally sharp border of *hb*'s anterior expression domain is due to repression by both *runt* and *Kr*. There is also an effect on *hb*'s posterior pattern. This domain expands anteriorly towards the central region of these double mutant embryos. However, even more striking is the significant expansion of the posterior domain of *gt* into the central regions of *runt, Kr* double mutant embryos (Fig. 6F). Again, these effects are much more pronounced than the changes that are observed in embryos that are mutant for only *runt* or *Kr*. These results provide strong evidence that *runt* plays a role in suppressing the expression of these two gap genes in the central regions of the embryo. These alterations are observed in early to mid-blastoderm stage embryos, i.e. prior to the establishment of *runt*'s characteristic pair-rule expression pattern. Therefore the earlier expression and activity of *runt* is involved in the

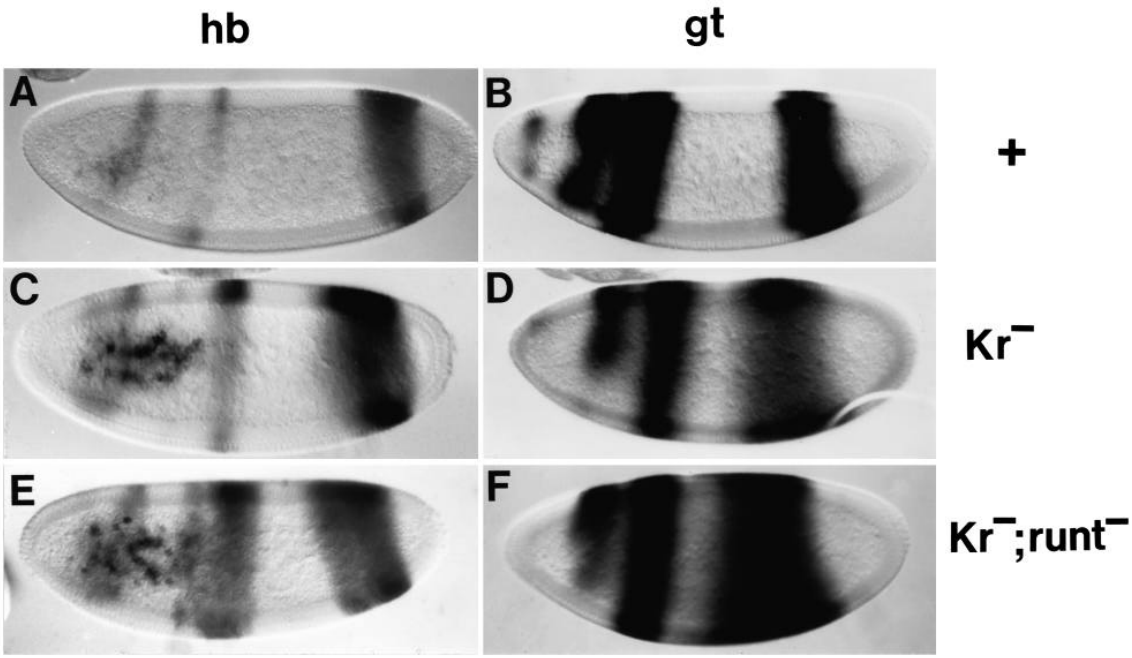

Fig. 6. Effects of *runt* mutations on gap gene expression patterns. The top two panels show the pattern of *hb* (A) and *gt* (B) mRNA accumulation in early blastoderm stage wild-type embryos. (C,D) The patterns in embryos mutant for *Kr*. These embryos were identified as in Fig. 3 above. The expression of these two gap genes in embryos mutant for both *Kr* and *runt* is shown in E and F. These double mutant embryos were identified using transcript null alleles for both *Kr* and *runt* and then hybridizing with probes for both of these genes as well as for either *hb* (E) or *gt* (F).

position-specific regulation of gap gene expression in the *Drosophila* embryo.

DISCUSSION

runt has gap-like functions in segmentation

A surprising finding of this work is the regulation of gap gene expression by *runt*, a pair-rule gene downstream of the gap genes in the conventional view of the segmentation hierarchy. Many of the effects that we have described result from the expression of a *hs/runt* transgene. As pointed out by others (Parkhurst and Ish-Horowicz, 1991; Jarman et al., 1993), it is important to use caution when interpreting the results of ectopic expression experiments such as these. However, the alteration of *gt* and *hb* expression patterns in embryos where *runt* is eliminated indicates that the regulation of gap genes by *runt* is a component of the normal regulatory circuitry of embryonic pattern formation. The effects on *gt* and *hb* are observed in stages preceding the establishment of *runt*'s pair-rule expression pattern. Further evidence that *runt* is active prior to its expression as a pair-rule gene comes from work on sex determination. *Drosophila* females mutant for *runt* fail to express the *Sex-lethal* gene within a broad domain that roughly corresponds to the presegmental region of the embryo (Duffy and Gergen, 1991). The gap in the *Sex-lethal* expression pattern is also observed in early blastoderm stage embryos, prior to the establishment of *runt*'s pair-rule expression pattern. There is expression of *runt* mRNA within a central broad field of the embryo during the syncitial nuclear division cycles that precede blastoderm formation (Klingler and Gergen, 1993). This expression correlates extremely well, both spatially and temporally with the early requirements for *runt* in sex determination and gap gene regulation.

Our results indicate that *runt* and *Kr* cooperate to prevent expression of the gap genes *gt* and *hb* in the central regions of the embryo. Another similarity between *runt* and *Kr* is that the primary regulation involved in generating their central broad domains of expression involves pole-region-dependent repression (Gaul and Jäckle, 1987; Klingler and Gergen, 1993). For example, in embryos from *bcd* mutant mothers, the early *runt* and *Kr* domains are both expanded significantly towards the anterior pole. Thus, at one level *bcd* is acting to repress the expression of *runt* and *Kr*. Yet, in those regions where they are able to escape from this repression they appear to antagonize the effects of *bcd* (Fig. 7). A similar situation exists at the posterior end of the embryo, but in this case the interactions involve two of the maternal systems. The posterior margins of the central *Kr* and *runt* domains are defined by the maternal posterior and terminal systems, respectively (Gaul and Jäckle, 1987; Klingler and Gergen, 1993). Similarly, the posterior domains of *gt* and *hb*, both of which expand towards the center of *runt*, *Kr* double mutant embryos, are also activated by the maternal posterior and terminal systems, respectively (Kraut and Levine, 1991a; Eldon and Pirotta, 1991; Tautz, 1988). Thus the underlying principle is similar to that at the anterior end. In regions where *runt* and *Kr* escape from repression, they appear to antagonize the action of their repressors. A net result of such reciprocal negative regulatory interactions is a sharpening of the positional information provided by the different maternal systems at specific boundary points in the embryo. Thus, *runt*'s gap activity seems to help define the central region

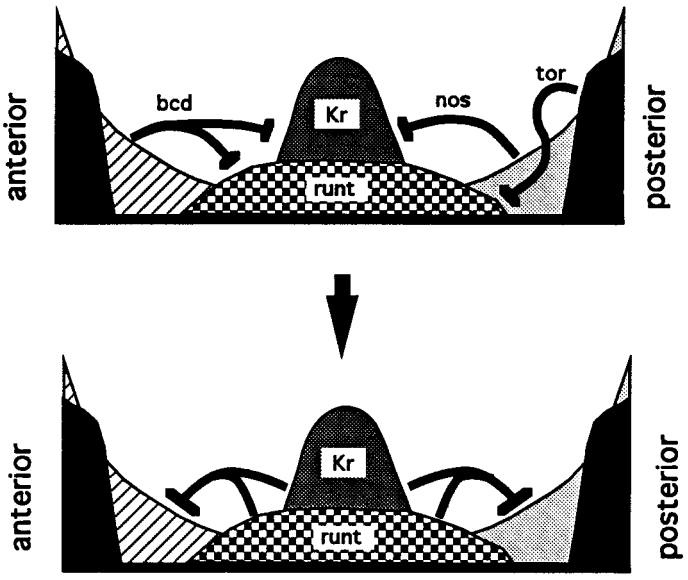

Fig. 7. Segmentation function of *runt*'s early broad field of expression. A summarized view of the antagonistic regulatory interactions that *runt* and *Kr* have with the maternal morphogenetic gradients that emanate from the anterior and posterior poles of the embryo is shown. Maternal positional information plays primarily a negative role in regulating both the early broad field of *runt* expression and the central domain of *Kr* expression. The activity of the maternal anterior system is thought to be entirely reflected in the concentration gradient of Bcd protein that forms in preblastoderm stage embryos. We indicate *bcd* as a repressor of both *Kr* and *runt* based on the dramatic anterior expansion of their expression in embryos lacking *bcd* activity. Similarly, a gradient of *nanos* (*nos*) activity is thought to be the central output of the posterior maternal system. One difference indicated in the figure is that *runt*'s early broad field is not affected by posterior group mutations, whereas the central *Kr* domain is. As indicated, it is the terminal system, which acts through the *torso* (*tor*) tyrosine kinase receptor protein, that sets the posterior boundary of *runt*'s early broad field. The lower panel shows our interpretation of the negative regulatory effects that *runt* and *Kr* have on the activities of the maternal anterior and posterior systems.

of the blastoderm embryo and sharpen the distinction between this and the flanking anterior and posterior regions.

The genetic pathway that generates the segmented body pattern in *Drosophila* has been organized into a hierarchy based on the spatial and temporal expression patterns of the segmentation genes and their genetic regulatory interactions. In a strict interpretation, a 'downstream' pair-rule gene *runt* regulating the expression of 'upstream' gap genes contradicts this hierarchical organization. However, in our interpretation, *runt*'s activity as a pair-rule gene is still downstream of the gap genes, and the gap-like regulatory function is due to the earlier broad field of *runt* expression. Therefore, this view does not contradict the hierarchical organization of segmentation genes. It does raise the possibility that alternative, 'non-classical' activities of other segmentation genes may play important roles in this developmental pathway. For example, evidence suggests that a gap-like activity is associated with early expression of the pair-rule gene *eve*. There is a broad band of *eve* expression that overlaps with and extends anterior of *runt*'s early broad field (Harding et al., 1986; our unpublished observations). This expression correlates well with the stripe-specific defects in the expression of several pair-rule genes that are observed in *eve* mutant embryos (Carroll and Scott, 1986; Ingham and Gergen, 1988). Importantly, these defects are observed at the earliest stages of stripe formation, prior to the stages when most other pair-rule gene regulatory interactions become apparent. It will be interesting to determine if this earlier activity of *eve* is similar to that of *runt* in its ability to influence gap gene expression. The striped patterns of other pair-rule genes are also preceded by broader, gap-like expression domains (Kilchherr et al., 1986; Grossniklaus et al., 1992). It remains an open question whether these are also functionally relevant, or merely reflect an early phase in the evolution of their expression patterns.

Pair-rule gene regulation by *runt*

The expression patterns of all of the pair-rule genes that have been examined to date are altered in *runt* mutant embryos (Carroll and Scott, 1986; Frasch and Levine, 1987; Ingham and Gergen, 1988; Hooper et al., 1989; Gutjahr et al., 1993). One aim of these ectopic expression experiments was to identify which of these regulatory effects are most direct. In addition to *eve*, *hairy* and *ftz*, we have also examined the expression of the endogenous *runt* gene and the *paired* (*prd*) gene in *hs/runt* embryos. The regulation of all of these genes is affected, thus confirming *runt*'s importance as a pair-rule regulator. Some insight on pair-rule regulatory circuitry is provided by the different responses of these genes to *runt*. Stripe-specific defects are observed for *eve*, *hairy* and *runt* in *hs/runt* embryos. These three genes have other features in common. They cross-regulate each others expression and are involved in regulating the expression of the other pair-rule genes (i.e. *ftz* and *prd*). Conversely, the initial pair-rule patterns of *eve*, *hairy* and *runt* are not altered in *ftz* and *prd* mutant embryos (Frasch and Levine, 1987, Hooper et al., 1989; Klingler and Gergen, 1993). Based on these regulatory interactions *eve*, *hairy* and *runt* have been classified as primary pair-rule genes, whereas *ftz* and *prd* have been described as secondary or tertiary pair-rule genes.

As primary pair-rule genes, *eve*, *hairy* and *runt* are thought to respond 'directly' to the positional cues provided by the gap genes. The identification of gap-responsive, stripe-specific elements in the *cis*-regulatory regions of each of these three genes strongly supports this view (Harding et al., 1989; Goto et al., 1989; Riddihough and Ish-Horowicz, 1991; M. Klingler, J. Soong and JPG unpublished observations). It seems likely that these stripe-specific *cis*-elements are involved in mediating the regulatory responses of the primary pair-rule genes to *hs/runt* treatment. The stripe-specific repression of reporter gene constructs containing *cis*-elements for *hairy* stripe 1 and *eve* stripe 2 in *hs/runt* embryos supports this. The *eve* and *hairy* patterns observed in *runt* mutant embryos provides a further suggestion that *runt* regulates these genes through their stripe-specific elements. In both cases there are non-uniform effects that can be described as stripe-specific and there are also abnormalities in stripe spacing (Frasch and Levine, 1987; Ingham and Gergen, 1988; Hooper et al., 1989). Finally, it is notable that *hs/runt* treatment fails to repress a reporter gene containing the *eve* autoregulatory element. This suggests that the altered regulation of this element in *runt*

mutants (Goto et al., 1989) is indirect and due to effects on the expression of other genes.

The stripe-specific regulation of the three primary pair-rule genes is qualitatively different from the more uniform response of the secondary pair-rule gene *ftz*. The pair-rule gene *prd* responds similarly to *ftz* and becomes uniformly expressed within the presegmental region of *hs/runt* embryos (C. Tsai, unpublished). Given the differences in their responses, the uniform activation of *ftz* and *prd* observed in *hs/runt* embryos cannot be due solely to *runt*'s effects on the expression of the primary pair-rule genes. These observations suggest that the most direct regulatory effects of *runt* on these secondary pair-rule genes involve *cis*-element(s) that normally contribute to regulating their expression throughout the presegmental region. Identification of these *cis*-elements and their mode of interaction with *runt* is important to our understanding of the segmentation pathway and should also provide further insight on the function of the Runt protein.

Function of the Runt protein

One effect of ectopic *runt* expression that we have characterized in detail is the antagonism of *bcd*-dependent activation, an effect obtained on synthetic promoter reporter gene constructs containing three copies of a Bcd recognition sequence. From our in vivo data, this antagonism is apparently not due to an effect on the synthesis or stability of the Bcd protein, but instead reflects interference with its function as a transcriptional activator. The Runt protein contains a novel type of DNA-binding motif referred to as the Runt-domain (Kagoshima et al., 1993). The effects of Runt could be readily accounted for if the protein were to recognize to the same binding site as the Bcd protein and thus prevent Bcd-dependent activation by a simple competition mechanism. The consensus binding site for the mammalian Runt-domain proteins is PuACCPuCA (Melnikova et al., 1993; K. Shigesada, personal communication). This is not similar to the consensus Bcd-binding-site (TCTAATCCC, Driever and Nüsslein-Volhard, 1989) that is present in the *HSBG[bcd]* reporter gene construct used in this study (Ronchi et al., 1993). The mammalian Runt-domain proteins as purified from cells are in a heterodimeric complex with an unrelated partner protein referred to as PEBP2/CBFβ (Ogawa et al., 1993a; Wang et al., 1993). This partner protein increases the DNA-binding affinity of the Runt-domain proteins, but does not appear to alter the sequence specificity (Melnikova et al., 1993; K. Shigesada, personal communication). The DNA-binding affinity of the *Drosophila* Runt protein is increased by addition of murine PEBP2/CBFβ. However, even under these conditions, we have not been able to demonstrate an in vitro interaction between Runt and the Bcd recognition sequence (C. Tsai, unpublished). Thus, although we cannot rule out the possibility that Runt may directly interact with the Bcd-recognition sequence in vivo, we do not favor this model.

A second simple model to explain this antagonism is that the Runt protein causes the Bcd activation domain to be masked, perhaps through a direct protein-protein interaction. Bcd acts as a transcriptional activator both in cultured *Drosophila* cells and in yeast (Driever and Nüsslein-Volhard, 1989; Driever et al., 1989; Struhl et al., 1989). In neither of these heterologous systems did the runt protein interfere with *bcd*-dependent activation (C. Tsai, S. Lin, J. Till and T. Chu, unpublished observations). Although there are many potential explanations for such negative results, they strongly suggest that other factors play a role in the antagonism of *bcd*-dependent activation by *runt*. In this context it is important to note that several of the other regulatory events observed in *hs/runt* embryos (increased expression of *Kr*, *ftz*) indicate that *runt* does not act solely by sequestering factors that are generally required for transcriptional activation.

As noted above, there are stage-specific differences in the effect of *hs/runt* on bcd-dependent expression. These differences are easily accounted for if the interaction between *runt* and *bcd* depends on other developmentally regulated factors. Several other observations suggest that the regulatory functions of *runt* are influenced by the developmental context. The relatively uniform expression of *ftz* in the presegmental region of *hs/runt* embryos provides evidence that *runt* may act to directly stimulate *ftz* transcription. However, the fact that this occurs only in the presegmental region indicates that other spatially regulated factors influence the response to *runt*. Future work should reveal whether *runt* is interacting with other activating factors that are present only in the presegmental region, or alternatively whether the activation by *runt* is not strong enough to overcome factors that suppress expression at the poles. The diverse specificity of *runt*'s many regulatory functions during *Drosophila* embryogenesis strongly suggest that interactions with other factors are central to its function. Several of the regulatory effects described here provide an excellent starting point for further investigating the mechanism of action of this novel transcriptional regulatory protein.

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