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Zygotic *caudal* regulation by *hunchback* and its role in abdominal segment formation of the *Drosophila* embryo

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SUMMARY

***caudal* (*cad*) is a maternally and zygotically expressed gene in *Drosophila* whereby the two phases of expression can functionally replace each other. The zygotic expression forms an abdominal and a posterior domain, whereby only the posterior domain has so far been studied with respect to its regulation and function. We show here that the abdominal *cad* domain is regulated by the *hunchback* (*hb*) gradient through repression at high concentrations and activation at low concentrations of HB protein. To study the function of the abdominal *cad* domain in the absence of redundant interactions, we have utilized an experimental system in which the embryo lacks the normal *bicoid* (*bcd*) and *hb* expression. An artificial *hb* gradient is then introduced into such embryos, which results in an induction of an ectopic zygotic *cad* domain in the more anterior region. Employing this system, we show that the *cad* domain functions by activating the expression of the abdominal gap genes *knirps* (*kni*) and *giant* (*gt*). We conclude that *cad* is the so far missing region-specific activator of abdominal segmentation genes.**

Key words: *Drosophila* segmentation, *caudal*, abdominal gap genes, *hunchback* gradient

INTRODUCTION

Drosophila early development proceeds in a hierarchical manner in which the genes of one level of the hierarchy interact with each other and then regulate the genes of the next level in the hierarchy (St. Johnston and Nüsslein-Volhard, 1992; Pankratz and Jäckle, 1993). Several of these regulatory circuits are redundantly controlled either by different genes, or by different phases of expression of the same gene. The so far best studied example in this respect is the gap gene *hb*, which is expressed maternally and zygotically. The two phases of expression are under separate control of the anterior and posterior maternal systems (Tautz, 1988) and have partially redundant functions. The maternal expression of *hb* results in a protein gradient in the abdominal region of the embryo, which serves to regulate other gap genes in a concentration-dependent manner (Hülskamp et al., 1990; Struhl et al., 1992). However, essentially the same gradient function can be provided by the zygotic expression alone and the maternal expression is therefore dispensable under appropriate experimental conditions (Lehmann and Nüsslein-Volhard, 1987). Furthermore, some of the functions of the *hb* gradient are also provided by the *bcd* gradient (Hülskamp et al., 1990; Hoch et al., 1991; Struhl et al., 1992) and there is, in fact, a synergism between *bcd* and *hb* with respect to certain regulatory functions (Simpson-Brose et al., 1994). However, the *hb* gradient is also capable of regulating its target genes independently of *bcd* (Schulz and Tautz, 1994).

cad, as *hb*, has a maternal and a zygotic phase of expression. The maternal RNA is at first homogeneously distributed, but is under translational control from the anterior maternal system (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987a,b). The maternally derived CAD protein becomes thus distributed in a posterior-anterior concentration gradient. Zygotic *cad* expression at the blastoderm stage is often described as occurring only in a small posterior domain, which forms a 3- to 4-cell wide stripe. However, Macdonald and Struhl (1986) noticed that embryos devoid of maternal *cad* expression show an abdominal expression of *cad*, which is usually obscured by the maternal CAD protein gradient. We show here that this domain can be more easily visualized by whole-mount in situ hybridization. This allows direct study of its regulation by other genes, without the necessity to remove the maternal expression of *cad*.

Embryos maternally mutant for *cad* are viable, though they show occasional abdominal segmentation defects. Embryos zygotically mutant for *cad* are not viable, though this is not because of segmentation defects, but due to later functions of *cad* (Macdonald and Struhl, 1986). Only embryos lacking both maternal and zygotic expression of *cad* show severe segmentation defects (Macdonald and Struhl, 1986). This indicates that, as with *hb*, the maternal and zygotic expression of *cad* are redundant at least with respect to the segmentation function. Nonetheless, it is evident that *cad* does play an important role in the segmentation of the embryo, though its place in the genetic hierarchy was so far unclear.

We show here that the abdominal *cad* expression domain is under the control of the *hb* gradient. It is activated at low con-

centrations of *hb* and repressed at high concentrations. We show also that the abdominal *cad* domain itself is then required to activate the gap genes *kni* and *gt*.

MATERIALS AND METHODS

The construction and the expression pattern of the *hb-bcd*3′UTR construct are described in Schulz and Tautz (1994). In short, the *hb-bcd*3′UTR construct carries the maternal *hb* promoter, the full *hb* coding sequence and part of the *bcd* 3′-end. The 3′-end was chosen such that it causes the anterior localization of the *hb* RNA, but does not include the nanos response elements (NRE; Wharton and Struhl, 1991). It is therefore not under the regulatory control of *nos*. HB protein is expressed as an anterior-posterior gradient from this construct which has a shape similar to that described for *bcd* (Driever and Nüsslein-Volhard, 1988). The *nos-bcd*3′UTR construct is described in Gavis and Lehmann (1992) and utilizes also the *bcd* 3′-end for anterior localization. The *hb-bcd*3′UTR construct has no phenotypic effects on wild-type flies, while the *nos-bcd*3′UTR construct acts as a dominant maternal sterile. However, it can be propagated paternally and can thus be used in certain mating schemes.

The mutant genotypes of the embryos could usually be assessed unequivocally on the basis of the expression patterns of the genes studied. This was in some cases facilitated by double labelling (see text). Finally, where necessary, we made sure that the staining patterns that were allocated to certain genotypes did occur in the expected frequencies predicted from the mating schemes.

cad^{2} and tsl^{691} alleles were used in the different crosses. The regulation of *cad* was further studied by in situ hybridization in nos^{L7}; bcd^{E1}, hb^{14F} and bcd^{E1}, tsl^{o35} alleles.

During the course of these and other experiments (Schulz, 1995), we noticed that the suppression of *bcd* in the embryos carrying the *nos-bcd*3′UTR construct is not complete. There is still a residual *bcd* activity at the most anterior tip of the embryos, which becomes apparent in the combinations with *tsl*. Since the repression effect of the terminal system on the gap genes is removed under these conditions, a weak activating effect of the residual *bcd* activity on *hb* becomes effective and a small anterior *hb* domain, accompanied by a small *Kr* and *cad* domain, is expressed (data not shown). Thus, the respective experiments with the *tsl* combinations were all done in a double mutant combination with *bcd*, fully to exclude potentially interfering effects from *bcd*.

Whole-mount in situ hybridizations were basically done as described in Tautz and Pfeifle (1989), but using the modifications for RNA probes described in Klingler and Gergen (1993).

RESULTS

Zygotic *cad* expression

Employing whole-mount in situ hybridization instead of antibody staining, it is possible to resolve the zygotic *cad* expression domain in the abdominal region of the embryo (Fig. 1A). Its first onset of expression appears to occur at stage 13, though this is difficult to determine exactly since the maternal *cad* RNA is still present in this region at earlier stages. However, given that the maternal and zygotic expression of *cad* can functionally replace each other (Macdonald and Struhl, 1986), it is possible that the expression appears already prior to stage 13. The domain is under the control of the anterior maternal system, since it shifts anteriorly in embryos zygotically mutant for *hb* (Fig. 1B) and maternally mutant for *bcd* (Fig. 1C). In contrast to the posterior *cad* domain (Mlodzik and

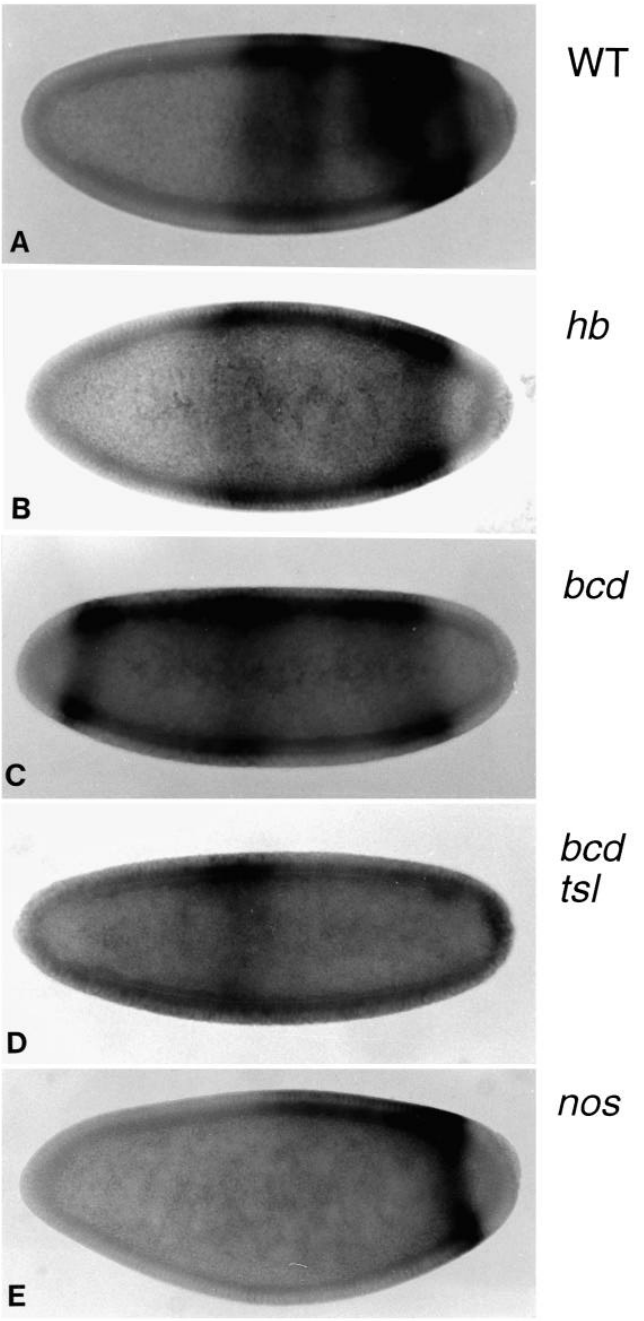

Fig. 1. Regulation of the abdominal *cad* domain. Whole-mount in situ hybridizations with the *cad* probe in wild type and in different types of mutant embryos. (A) Wild-type embryo at early stage 14. The zygotic domains become fully apparent at this stage. (B) Embryo zygotically mutant for *hb*. The anterior border of the abdominal *cad* domain has shifted anteriorly. (C) Embryo maternally mutant for *bcd*. The posterior *cad* domain is duplicated at the anterior end under these conditions and the abdominal domain is located centrally. Its anterior border is shifted to the same position as in *hb* mutant embryos, consistent with the fact that zygotic *hb* is not present in bcd^{-} embryos (Tautz, 1988). (D) Embryo maternally mutant for *bcd* and *tsl*. The posterior *cad* domain disappears (Mlodzik and Gehring, 1987b) and only the abdominal domain is retained. (E) Embryo maternally mutant for *nos*. Only the posterior *cad* domain is visible, while the abdominal domain has disappeared. Note, however, that a dorsal expression of *cad* remains which is of unknown function.

Gehring, 1987b), it is not under the control of the terminal system, as it is still present in embryos mutant for *tsl* (Fig. 2A) and embryos double mutant for *bcd* and *tsl* (Fig. 1D). Finally, the domain disappears in embryos maternally mutant for the posterior control gene *nos* (Fig. 1E), suggesting a negative regulatory effect of *hb* (see below). These embryos retain only the posterior domain and a dorsal expression in the abdominal region, which is of as yet unknown function.

hb control of the abdominal *cad* domain

To understand the regulation of the abdominal *cad* domain further, we have utilized a construct that leads to an artificial *hb* gradient in the anterior third of the embryo (the *hb-bcd*3′UTR construct; Schulz and Tautz, 1994). This construct can be crossed into different types of mutant backgrounds and permits study of the effects of the *hb* gradient in the absence of other potentially interfering genetic functions. To create embryos that are devoid of such interfering functions, in particular those of the normal maternal *hb* expression as well as of *bcd* expression, we have used another artificial construct. This construct places *nos* at the anterior pole of the embryo (the *nos-bcd*3′UTR construct; Gavis and Lehmann, 1992). *nos* functions by inhibiting the translation of both *bcd* and *hb* RNA (Wharton and Struhl, 1991; Gavis and Lehmann, 1992). This function is mediated by short elements in the untranslated 3′-end of the mRNAs of these two genes, the nanos response elements (NRE) (Wharton and Struhl, 1991). The NREs were excluded from both the *nos-bcd*3′UTR construct and the *hb-bcd*3′UTR construct making them insensitive to the translational regulation by *nos*.

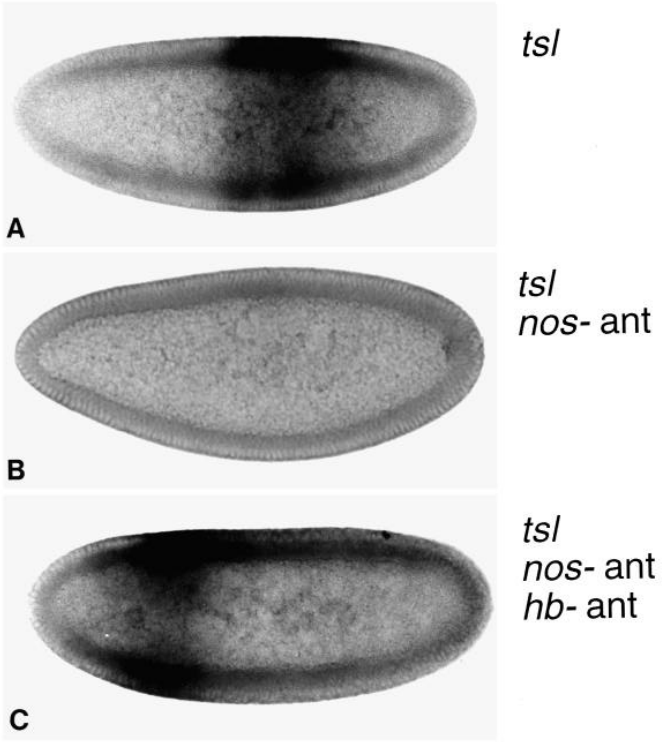

Fig. 2. Regulation of the abdominal *cad* domain by the *hb* gradient. Whole-mount in situ hybridizations with the *cad* probe in different mutant backgrounds. (A) Embryo maternally mutant for *tsl*. Only the normal abdominal *cad* domain is visible. (B) Embryo maternally mutant for *bcd*, *tsl* and carrying the *nos-bcd*3′UTR construct (*nos*-ant) at the anterior pole. This embryo is devoid of all known anterior-posterior pattern information, since, in addition to the lack of *bcd* and of the terminal system, the *nos-bcd*3′UTR construct suppresses also the function of maternal *hb* (Gavis and Lehmann, 1992). No zygotic *cad* expression occurs under these conditions. (C) Embryo of the same genotype as in B, but carrying in addition the *hb-bcd*3′UTR construct (*hb*-ant) that provides an artifical anterior HB protein gradient (Schulz and Tautz, 1994). A zygotic *cad* domain is again established, albeit at a much more anterior position, in accordance with the more anterior position of the *hb* gradient.

When the *nos-bcd*3′UTR construct is crossed into embryos that are maternally mutant for *tsl*, one can create embryos that are devoid of all known regulatory activities that are required for anterior-posterior segmentation. The abdominal *cad* domain is indeed absent in these embryos (Fig. 2B). If one now introduces the *hb-bcd*3′UTR construct into such an embryo, one finds that a *cad* domain appears again, albeit at a much more anterior position (Fig. 2C), consistent with the fact that the *hb* gradient is now located more anteriorly (Schulz and Tautz, 1994). This result indicates that *hb* acts both as an activator and as a repressor on this *cad* domain. The activation effect is evident from the fact that the *cad* domain depends on the presence of *hb* in this background. The repression effect can be inferred from the fact that *cad* is not expressed in the most anterior portion of these embryos, where *hb* is present at high concentrations. Furthermore, repression of the abdominal *cad* domain by *hb* can also be inferred from the results in *nos* mutant background described above (Fig. 1E). Under these conditions, the maternal HB protein is present at relatively high levels in the abdominal region of the embryo and can therefore repress its target genes in this region (Tautz, 1988; Wang and Lehmann, 1991).

Phenotypic effects

Placing of the *hb-bcd*3′UTR construct into embryos carrying the *nos-bcd*3′UTR construct leads to a full restoration of the abdominal segment pattern (Schulz and Tautz, 1994; Fig. 3). We have previously shown that this is due to proper regulation of the more posterior gap genes by the artificial *hb* gradient (Schulz and Tautz, 1994). Interestingly, the removal of zygotic *cad* from these embryos results in a severe disruption of the abdominal segmentation pattern (Fig. 3C). This indicates that the zygotic *cad* domain has a direct role in abdominal segment formation, at least under these conditions. We note that the maternal *cad* expression is still present in these embryos. However, in contrast to the normal situation (Macdonald and Struhl, 1986), it is apparently not capable of rescuing the lack of the zygotic *cad* expression. This incongruence might be due to the fact that *bcd* is also not present in the *nos-bcd*3′UTR construct embryos. Thus it seems possible that *bcd* might also contribute to the specific *cad* functions in the abdomen. There is indeed molecular evidence for such a synergistic interaction between *cad* and *bcd* (H. Jäckle, personal communication). However, a direct test by analysing the phenotype of embryos that are maternally mutant for *bcd* and zygotically mutant for *cad* does not support this inference, since the *bcd* phenotype is not enhanced in the absence of *cad* (results not shown). However, the two situations are not directly comparable, since the maternal *hb* gradient that is present in *bcd* mutant embryos provides a different concentration range and thus a somewhat different set of regulatory interactions to the artificial *hb* gradient in the embryos carrying the *nos-bcd*3′UTR construct.

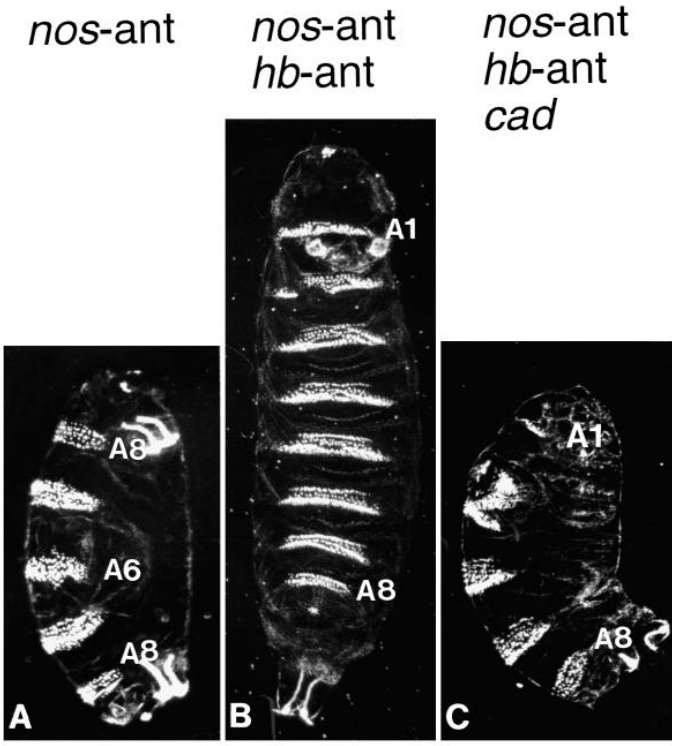

Fig. 3. Dependence of abdominal segmentation on zygotic *cad* expression. Cuticle preparations of first instar larvae of different mutant genotypes. (A) Larva carrying the *nos-bcd*3′UTR construct. These larvae develop a mirror-symmetric pattern of posterior denticle belts, due to the absence of *bcd* and maternal *hb* (Gavis and Lehmann, 1992; Hülskamp et al., 1990). (B) Larva carrying both the *nos-bcd*3′UTR construct and the *hb-bcd*3′UTR construct. The abdominal segment pattern is rescued by the *hb-bcd*3′UTR construct (Schulz and Tautz, 1994). (C) Larva carrying both the *nos-bcd*3′UTR construct and the *hb-bcd*3′UTR construct, but zygotically mutant for *cad*. Only the posterior part of the abdominal pattern is established, while the anterior abdominal region is not properly developed. Interestingly, a denticle belt that is probably A1 forms at the anterior end, indicating that only the abdominal gap genes *kni* and *gt* are affected in this situation.

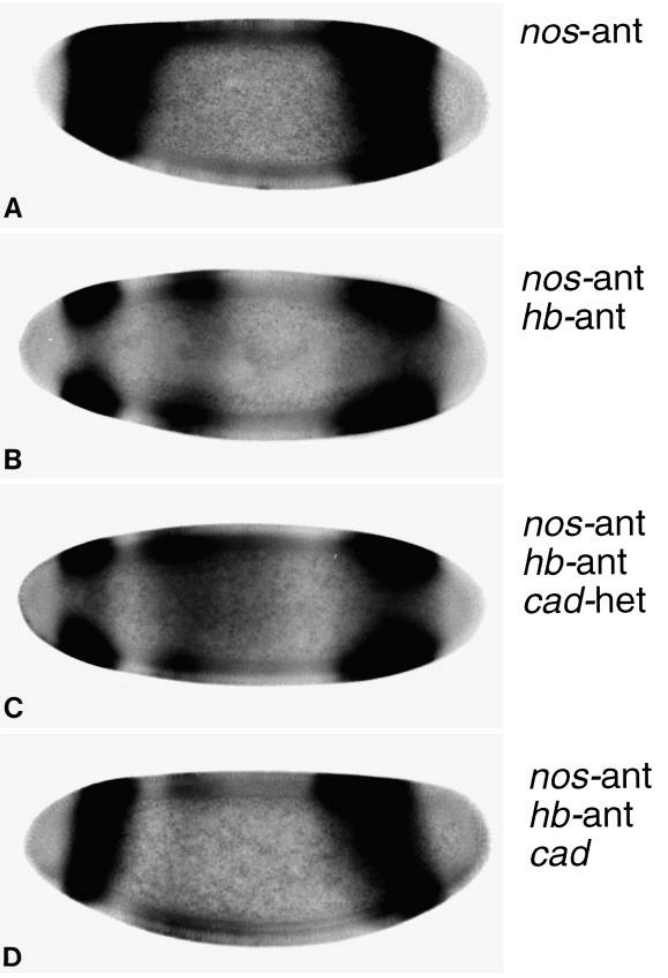

Fig. 4. Regulation of *kni* by zygotic *cad*. Whole-mount double in situ hybridizations with *kni* and *hb* probes. All embryos carry the *nos-bcd*3′UTR construct and the embryos in B-D in addition the *hb-bcd*3′UTR construct. Zygotic *hb* expression in these embryos is seen as a duplicated posterior domain, due to the absence of *bcd* (Tautz, 1988; Gavis and Lehmann, 1992). In embryos carrying the *hb-bcd*3′UTR construct, the anterior duplicated domain forms somewhat later and is narrower. This helps to identify the proper genotypes (note that the maternal *hb* RNA from the *hb-bcd*3′UTR construct is already degraded at this developmental stage). Furthermore, the *hb* staining serves as an internal control for the intensity of the *kni* staining. (A) Embryo carrying only the *nos-bcd*3′UTR construct. *kni* is only faintly expressed in these embryos (Gavis and Lehmann, 1992). (B) Embryo carrying the *nos* and the *hb-bcd*3′UTR construct. The zygotic *kni* domain becomes strongly established due to an enhancement by *Kr* in these embryos (Pankratz et al., 1989; Schulz and Tautz, 1994). (C) Embryo with the same genotype as in B, but heterozygous for zygotic *cad*. The posterior border of the *kni* domain becomes fuzzy and extends towards posterior, while the staining intensity in the anterior is not changed. (D) Embryo with the same genotype as in B but lacking zygotic *cad* expression. The *kni* domain becomes generally weaker and extends towards posterior. The weak expression of *kni* under these conditions is presumably due to activation by the maternal *cad* and residual enhancement by *Kr*.

This effect was also noted previously with respect to the rescue function of the *hb-bcd*3′UTR construct in the *bcd*$^{-}$ background (Schulz and Tautz, 1994).

cad as activator for *kni* and *gt*

To track the molecular basis of the phenotypic defects seen in the above experiments, we have analysed the expression patterns of *kni* and *gt* in the respective mutant backgrounds. To facilitate the identification of the genotypes and to provide internal standards for quantitative comparisons, we have done simultaneous in situ hybridizations with two different probes. In the case of *kni*, we have used *hb* and, in the case of *gt*, we have used *tll* as a second probe.

Embryos carrying only the *nos-bcd*3′UTR construct show a duplication of the posterior *hb* stripe at late blastoderm stage (Fig. 4A). *kni* is only very weakly expressed in this situation, since it requires the presence of *Kr* as an additional enhancer of expression (Pankratz et al., 1989). *Kr*, however, is absent in these embryos (Gavis and Lehmann, 1992). Bringing the *hb-bcd*3′UTR construct into this background restores *Kr* expression (Schulz and Tautz, 1994) and concomitantly establishes a *kni* domain (Fig. 4B). This domain becomes significantly weaker in embryos lacking zygotic *cad* expression (Fig. 4D), indicating that *cad* acts as an activator of *kni*.

gt is expressed in a broad central domain in the *nos-bcd*3′UTR construct embryos (Fig. 5A), but becomes somewhat repressed from the anterior region in the presence of the *hb-bcd*3′UTR construct (Fig. 5B). It was previously shown that *hb* acts as a concentration-dependent repressor of *gt* (Eldon and Pirotta, 1991; Kraut and Levine, 1991a; Struhl et al., 1992) and one would therefore have expected a much stronger effect on the *gt* expression, when the *hb* gradient is introduced. However, the fact that *gt* is still weakly present in the anterior region suggests that *hb* might have ectopically activated an activator of *gt* which then counteracts the repression effect of *hb* on *gt* (compare Schulz and Tautz, 1994). It becomes evident that this activator is *cad*, since genetic removal of zygotic *cad* expression removes the anterior *gt* expression (Fig. 5C).

Our data include some evidence that the activation effect of *cad* on *gt* may be dosage sensitive. Embryos heterozygous for zygotic *cad* show a weakening of the expression of *gt* in the anterior region (not shown). This reduction in *gt* expression becomes particularly evident when the embryos are stained with the *kni* probe, which is an indirect indicator of *gt* function. It was previously shown that high levels of *gt* act as a repressor on *kni* and thus set its posterior border (Eldon and Pirotta, 1991). The posterior border of the *kni* domain seen in Fig. 4B is controlled by this effect. Accordingly, weakening of *gt* expression by removing one zygotic copy of *cad* has an effect on this border. It shifts posteriorly and becomes fuzzy in embryos heterozygous for *cad* (Fig. 4C). In contrast, the anterior part of the *kni* domain appears to be expressed at the same level as with two copies of *cad* in the embryo (compare Fig. 4B). This indicates that the activation effect of *cad* on *kni* can be achieved with lower levels than the activation of *gt* by *cad*.

It should be noted that, although our experiments were designed to study the function of only the zygotic *cad* expression domain, it seems likely that the maternal expression of *cad*, which is still present in the embryos described above, has the same function. This could explain why both *kni* and *gt* are expressed even in the absence of zygotic *cad*.

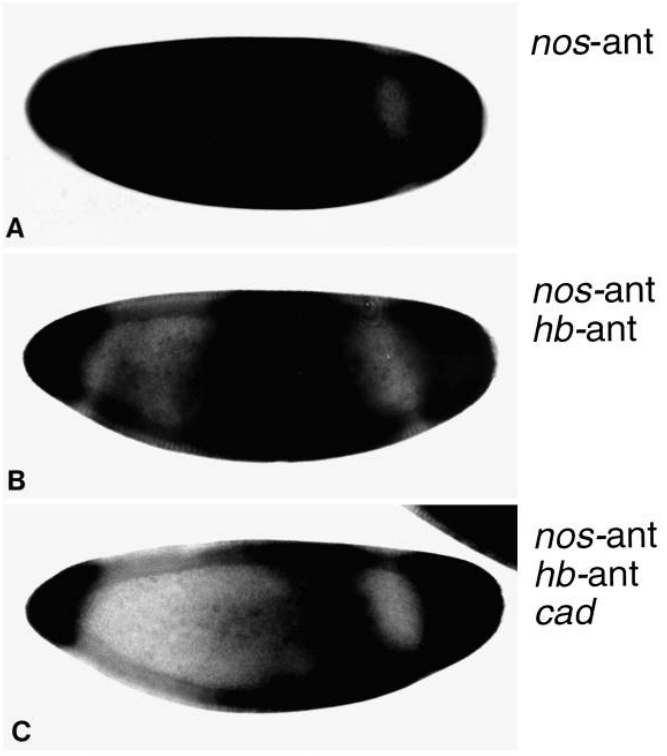

Fig. 5. Regulation of *gt* by zygotic *cad*. Whole-mount double in situ hybridizations with *gt* and *tll* probes. The *tll* staining serves as a marker in a similar way as described for *hb* in the legend of Fig. 4. (A) Embryo carrying the *nos-bcd*3′UTR construct. *gt* forms a broad central domain almost abutting the *tll* domains. (B) Embryo carrying both the *nos* and the *hb-bcd*3′UTR construct. *gt* becomes somewhat repressed in the anterior region, though it is still visible. The modulation seen is most likely due to an additional repression effect by *Kr*, which is activated in this region under the *hb* control (Schulz and Tautz, 1994) and which acts as an additional repressor on *gt* (Kraut and Levine, 1991b). (C) Embryo with the same genotype as in B, but lacking zygotic *cad* expression. *gt* expression is now completely absent from the anterior region. The expression in the posterior region is presumably due to activation by maternal *cad*.

DISCUSSION

We have established that *cad* acts very high up in the genetic segmentation gene hierarchy. We found that the maternal *hb* gradient regulates a zygotic *cad* domain and that one can manipulate this regulation such that the function of *cad* as an activator of the abdominal gap genes *kni* and *gt* becomes apparent. Previously, this function of *cad* was concealed by redundant regulatory effects, both of its own maternal expression, but also by partial compensation through the other maternal systems. In our experiments, we were able to remove the interference from these systems and have thus obtained a clearer picture of the functional role of *cad*.

Region-specific activators for the abdominal *kni* and *gt* expression domains have not been found previously. It was therefore thought that they are under the control of a general activator and that their expression domains are only delimited by repression effects (Kraut and Levine, 1991a,b; Eldon and Pirotta, 1991; Pankratz et al., 1992). The situation for *kni* is, however, more complicated, since *Kr* acts as an enhancer of *kni* expression (Pankratz et al., 1989). In contrast, the activator for the weak primary expression of *kni* could not be identified (Pankratz et al., 1992). Our results suggest now that the abdominal *cad* expression domain acts as a specific activator for both *kni* and *gt* and that this activation is region specific. It has previously been suggested that *cad* acts also as a specific activator for the pair-rule gene *fushi tarazu* in the abdominal region (Dearolf et al., 1989).

Our experiments deal only with the regulatory effects of the zygotic abdominal *cad* domain. However, it is likely that the same function is provided by the maternal expression. The activation of the posterior gap genes is required fairly early during development, at a time when the zygotic *cad* domain may not yet be fully established. However, *cad* is maternally present in the respective region, prior to the activation of the zygotic domain. It is therefore likely that the maternal CAD protein functions as the first activator of the abdominal gap genes and that the zygotic domain takes over this function when the maternal protein is degraded. This is probably the reason why the zygotic domain can fully replace the maternal *cad* function when it is absent (Macdonald and Struhl, 1986).

The morphogenetic capacities of the *hb* gradient are further

emphasized by our experiments. *hb* has previously been shown to be a concentration-dependent activator and repressor of *Kr* (Hülskamp et al., 1990; Struhl et al., 1992; Schulz and Tautz, 1994). The same finding can now be extended to the abdominal zygotic *cad* expression domain. Given that *hb* also acts as a concentration-dependent repressor on the anterior borders of *kni* and *gt* expression (Hülskamp et al., 1990; Struhl et al., 1992), we have to conclude that at least six different threshold concentrations are read from the *hb* gradient. Though we have shown that *hb* can autonomously achieve this regulation (Schulz and Tautz, 1994), it is also clear that the embryos are much more vulnerable to regulatory side effects when they are under the sole control of the *hb* gradient. It is therefore not surprising that most regulatory effects of the *hb* gradient are redundantly provided by other regulatory circuits. It appears that the embryo uses, on the one hand, the maximum of spatial information that is contained in a morphogenetic gradient, while, on the other hand, safeguards itself against a potential misinterpretation of this information by utilizing redundant pathways (Tautz, 1992). Our experimental system for studying the regulatory interactions between genes in the absence of other pattern-forming systems provides a solution to this redundancy problem. In principle, it should be possible to study the function of the other gap genes in a similar way to analyse the regulatory capacities of the morphogenetic gradients that they are supposed to provide (Hülskamp and Tautz, 1991; Pankratz and Jäckle, 1993).

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