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Effects of ectopic expression of *caudal* during *Drosophila* development

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Summary

The effects of heat-shock-induced ectopic expression of the homeobox gene *caudal* (*cad*) at all stages of *Drosophila* development have been examined. Presence of *cad* protein (CAD) at the anterior end of cellular blastoderm embryos was found to disrupt head development and segmentation, due to alteration of the expression of segmentation genes such as *fushi tarazu* and *engrailed*, as well as repression of head-determining genes such as *Deformed*. These results support the conclusion that, while CAD is probably required to activate transcription of *fushi tarazu* in the posterior half of the embryo, it should not be expressed in the anterior half prior to gastrulation, and thus suggest a role for the CAD gradient. Ectopic expression of CAD at later stages of development has no obvious effects on embryogenesis or imaginal disc development, suggesting that the homeotic genes of the Antennapedia and Bithorax Complexes are almost completely epistatic to *caudal*.

Key words: *caudal, fushi tarazu,* ectopic expression, *Drosophila* development, segmentation.

Introduction

The genetic analysis of development is facilitated by the ability to observe the effects of loss and gain of function of gene products, traditionally through the study of mutations. Recently, the genetic analysis of development has been supplemented by the technique of controlled ectopic expression of cloned genes. For example, the ubiquitous expression of the segmentation gene *fushi (ftz)* during early development has been used to study the establishment of metameric stability (Struhl, 1985; Ish-Horowicz *et al.* 1989), while we have provided a detailed study of the homeotic transformations caused by ectopic overexpression of the homeotic gene Antennapedia (Gibson and Gehring, 1988). Here, we report the use of heat-shock-induced ectopic expression of the *caudal* gene to address the problem of the role of a molecular gradient in the establishment of positional information during early *Drosophila* embryogenesis.

The *caudal* gene (Mlodzik *et al.* 1985; Hoey *et al.* 1986; MacDonald and Struhl, 1986) was first isolated by cross-hybridisation to the homeobox sequences present in a number of genes of the Antennapedia and Bithorax Complexes of *D. melanogaster* (McGinnis *et al.* 1984). These genes are thought to control segmental identity by virtue of their function as *trans* -acting regulators of transcription (Gehring, 1987; Krasnow *et al.* 1989; Scott *et al.* 1989). Maternal and zygotic *cad* messenger RNAs derive from two different promoters, but share a common coding sequence which encodes a single protein species (Mlodzik and Gehring, 1987*a*).

The earliest detectable expression of CAD protein (CAD) is after the fifth or sixth nuclear division (MacDonald and Struhl, 1986). During the syncitial blastoderm stage, CAD comes to be expressed in a gradient receding from the posterior pole. The formation of this gradient is dependent on the presence of the *bicoid* gene product, which is thought to repress *cad* translation in the anterior of the embryo (Mlodzik and Gehring, 1987*b*). Protein derived from maternal transcripts persists in the pole cells throughout gastrulation (Mlodzik and Gehring, 1987*a*), and zygotic *cad* message is detected in the third instar larval gonads. Thus, *cad* is also expressed in the germ line of *Drosophila*, the only homeobox gene known to be expressed there so far.

As the early gradient fades away, zygotic transcription appears to be initiated in a single stripe of 4–5 cells between 13 % and 19 % egg length, measured from the posterior pole. Protein continues to be expressed in these cells throughout germ band extension. Later on, expression is also found in the genital imaginal disc (Mlodzik and Gehring, 1987*a*). Such expression patterns, by comparison with those of the homeobox genes of the Antennapedia and Bithorax Complexes, are consistent with a role for *cad* in specifying segmental identity of the most posterior structures of the epidermis (A10), including the anal pads and the telson, as well as portions of the posterior gut and the malpighian tubules.

The generation of *cad* mutants by MacDonald and Struhl (1986) confirmed that *cad* plays a role in the promotion of posterior segmental development, although rather than leading to unequivocal homeotic transformations, loss of zygotic *cad* appears to result in deletion of particular posterior cuticular structures. The maternal function can be partially rescued by paternally supplied gene products. Nevertheless, *cad* is involved in promotion of the segmentation process, since in the absence of all *cad* activity in embryos, most of the abdominal segments are missing or fused. The phenotype of *cad* and its effects on the embryonic fate map suggest that *cad* probably does not encode a morphogen responsible for determination and positional information along the anterior–posterior axis. In order to further examine the role of *caudal*, we have studied the effects of ectopic overexpression of the gene at all stages of *Drosophila* development.

Materials and methods

Generation of HTcad flies

The pHTcad plasmid was constructed as a derivative of pHT4 with the *rosy* gene as a visible eye-colour marker (Schneuwly *et al.* 1987). Genomic *cad* DNA from the *Cla*I restriction site 21 nucleotides upstream of the maternal transcription start site was fused to downstream sequences of the cDNA clone pSC33F (Mlodzik and Gehring, 1987*a*) at the *Xho*I site in the first exon. The insert fragment from the resulting plasmid, p33FG, was excised with *Cla*I and *Eco*RI, and blunt-ended with T4 DNA polymerase. After addition of *Kpn*I linker oligonucleotides, this fragment was inserted into the *Kpn*I site of pHT4 to yield pHTcad. The pSP65 plasmid was used as the vector for all intermediate subcloning steps. The flies were injected with CsCl gradient purified pHTcad DNA in the presence of pπ25.1wc as the source of transposase enzyme (Karess and Rubin, 1984). Homozygous or balanced lines were generated by crossing to appropriate balancer chromosomes.

A construct lacking most of the sequences of the leader of the maternal *cad* mRNA was constructed using a similar strategy as described above. Sequences upstream of the *Xho*I site were derived from a deletion clone generated for sequencing that was lacking most the leader (88 nucleotides upstream of the translation start codon were still present). However, injections of this construct (pHTcadA) never gave rise to a transformant line even though a larger than usual number of embryos were injected with two different preparations of plasmid DNA.

Heat shocks

Embryonic heat shocks were performed essentially as described in Gibson and Gehring (1988), except that the embryos were staged at the syncitial blastoderm stage (just as the periphery of the embryos started to clear) rather than the early cellular blastoderm stage (when the membranes start to form between nuclei). Such embryos are no more than two and one half hours old (Campos-Ortega and Hartenstein, 1985). Larval heat shocks were also administered as previously described, except that the heat shocks were delivered by submerging the glass fly tubes in up to three centimetres of water (such that the top of the food was clearly below the surrounding water level) at 37°C for 45 min. Typically, between three and five successive heat shocks were given at 4 h intervals (recovery at 25°C), starting from 68 h after egg laying. The cuticular structures were examined after mounting of embryos or adult heads in Hoyer's or Faure's medium respectively, also as described in Gibson and Gehring (1988).

Rescue of the hypomorphic cad^1 allele (MacDonald and Struhl, 1986) was achieved with the following crosses (cad^2 and cad^3 could not be rescued in this assay):

$$\frac{\text{CyO}}{\text{L}};\frac{\text{rf10}}{\text{TM3}}\times\frac{+}{+};\frac{\text{HTcad2}}{\text{TM3}}\qquad\frac{b\,pr\,cad^1}{\text{CyO}};\frac{+}{+}\times\frac{\text{CyO}}{\text{L}};\frac{\text{rf10}}{\text{TM3}}$$

$$♀\frac{\text{CyO}}{+};\frac{\text{HTcad2}}{\text{TM3}}\times\frac{b\,pr\,cad^1}{\text{L}};\frac{+}{\text{TM3}}♂$$

$$\frac{b\,pr\,cad^1}{\text{CyO}};\frac{\text{HTcad2}}{\text{TM3}}$$

The rf10 chromosome contains the markers ftz^{9H34}, ry^{506}, e^s. The final stock was maintained at 18°C: the lethal *b pr* cad^1 (*caudal* mutation) and HTcad2 (heat-shock *caudal*) chromosomes are balanced over the CyO and TM3 chromosomes, respectively. When grown at 25°C, we observed *b pr Sb* progeny of this cross, indicating that about 10 percent of homozygous cad^1 embryos had been rescued by the HTcad2 chromosome, by comparison with 1 percent homozygous *b pr* cad^1 survivors in an otherwise wild type background.

Immunohistochemical staining

All embryo fixations and antibody stainings were performed as described previously (Patel *et al.* 1987), with the following modification: the biotin–avidin HRP system was replaced with secondary HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies (BioRad). The primary antibodies used were as follows: rabbit anti-*Kr* (Gaul *et al.* 1987), rabbit anti-*hb* (Tautz, 1988), rabbit anti-*ftz* (Mlodzik *et al.* 1987; Krause *et al.* 1988), monoclonal anti-*en* (Patel *et al.* 1989), and rabbit anti-*Dfd* (Mahaffey *et al.* 1989).

Results

Using the same approach to that used to analyse the effects of ectopic expression of *Antp*, *cad* sequences derived from the zygotic cDNA clone 33F and genomic DNA (Mlodzik and Gehring, 1987*a*: see Methods) were placed under the transcriptional control of the *Drosophila hsp70* heat-shock promoter in the pHT4 vector described by Schneuwly *et al.* (1987). The resulting plasmid, pHTcad (Fig. 1 A), was then used to transform flies by P-element-mediated transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982), and three independent lines were established. Most of the experiments described below were performed with the HTcad3 line, which carries a homozygous viable insertion on the third chromosome, although similar results were obtained with the other two lines. We were unable to obtain transformants with a second construct, in which most of the untranslated leader of the maternal RNA was deleted (see Methods). As shown in Fig. 1 B, shortly after a 20 min heat shock at the syncitial blastoderm stage of development, the nuclei of all cells of HTcad3 embryos contain relatively high levels of CAD detected by whole-mount immunohistochemical staining. Since the protein appears to be evenly distributed, it is likely that enough

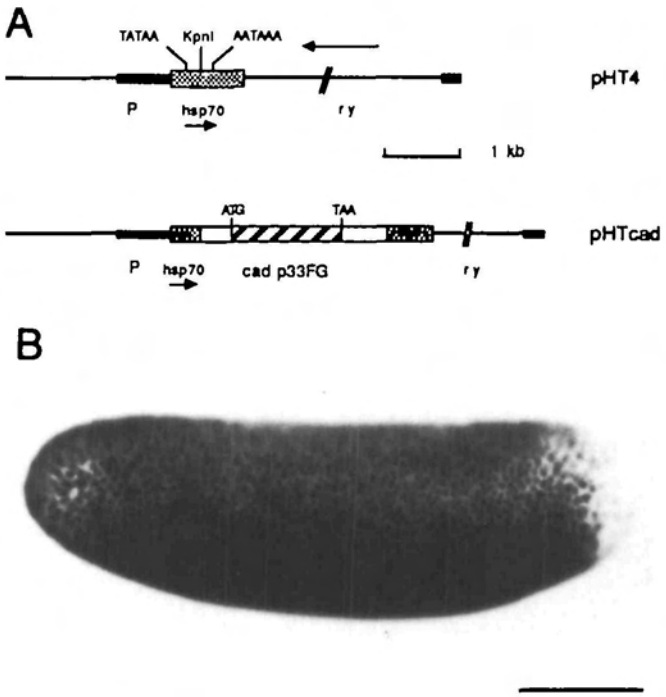

Fig. 1. Ectopic expression of *cad* during early embryogenesis. (A) Diagram of the pHTcad construct used to transform flies. *cad* coding sequences were inserted into the pHT4 vector (Schneuwly *et al.* 1987) between *hsp70* untranslated leader and trailer sequences, as described in the Methods section. (B) Immunohistochemical localization of CAD in the nuclei of all cells of a cellular blastoderm HTcad3 embryo after thirty minutes of recovery (25°C) from a twenty minute heat shock at 37°C. Due to the shape of the embryo and the plane of focus, the pole cells at the posterior end are not visible.

mRNA has been produced to overcome the block of translation at the anterior end of the embryo. We confirmed that the construct produces functional CAD by crossing the HTcad2 chromosome into flies homozygous for the hypomorphic cad^{1} allele (MacDonald and Struhl, 1986), and observing 10% rescue after growth at 25°C (see Methods for details).

Early ectopic cad *expression disrupts head development and segmentation*

The protein gradient was disrupted by ectopic overexpression of HT*cad* as early as possible in development. Zygotic transcription is not detectable in *Drosophila* until two hours after fertilization, at the syncitial blastoderm stage. Embryos of this age can be staged by dechorionating a collection of two hour old embryos (maintained at 25°C), submerging them in water, and selecting those in which the periphery of the embryo just commences to clear when observed under a dissecting microscope (see Campos-Ortega and Hartenstein, 1986 for a detailed description of early embryogenesis). If syncitial blastoderm HTcad3 embryos are subjected to a 20 min heat shock at 37°C, and allowed to complete embryogenesis at 25°C, two effects on development are apparent: a partial failure of head involution, and a significant disruption of segmentation (Fig. 2).

By contrast with the ectopic expression of Antennapedia protein (Gibson and Gehring, 1988), the failure of head involution after ectopic expression of Caudal protein is not accompanied by any obvious signs of homeotic transformation of segmental identity. The defect involves a malformation of the embryonic head skeleton as well as a displacement of the mouthparts relative to one another, but no supernumerary denticles or structures characteristic of more posterior body parts have been detected, even after multiple heat shocks delivered during the first seven hours of embryogenesis. Nor have we been able to detect any consistent or significant malformation of the posterior embryonic structures (see Jürgens, 1987) in response to ectopic expression of CAD. Although occasional thickening of the telson is observed, such an effect can also be induced by ectopic expression of a variety of other constructs (unpublished data).

Segmentation defects were manifested as fusions of one or more denticle belts in 45% of HTcad3 embryos heat shocked at the syncitial blastoderm stage (n=89, derived from four independent experiments, each with similar percentages of defects). A typical defect is shown in Fig. 2 B, while a strongly affected embryo in Fig. 2 C illustrates the point that all of the fusions observed involved fusions of T1/T2, T3/A1, A2/A3, A4/A5 and/or A6/A7. Minor defects usually involving A3 and A4 can be seen in up to 20% of wild-type embryos heat-shocked at the same stage of development (G.G., unpublished observations). Our interpretation of the segmental nature of the regions deleted in affected embryos is shown in Fig. 3. The defects are too variable and incomplete to allow a precise determination of which cells are deleted. Comparison with the findings of Struhl (1985) and Ish-Horowicz and Gyurkovics (1988) suggests that ectopically expressed CAD causes similar defects to those caused by ectopic expression of the pair-rule segmentation gene *fushi tarazu*. At the segmental level, it clearly does not have effects complementary to removal of the maternal *cad* contribution. However, globally the defects are most frequent at the opposite end of the embryo as those seen in loss-of-function mutant embryos. A similar range of segmentation defects has also been observed after ectopic expression of the segment polarity gene *engrailed* (Poole and Kornberg, 1988), although by contrast Engrailed apparently causes segmental fusions more frequently in the posterior than in the anterior portions of the embryo.

Effects of ectopic expression of cad *on the embryonic fate map*

We have used antibodies against three proteins to monitor changes in the embryonic fate map in response to ectopic expression of CAD in syncitial blastoderm embryos. The wild-type protein staining patterns of *fushi tarazu* (FTZ) at the cellular blastoderm stage, and *engrailed* (EN) and *Deformed* (DFD) at germ band extension are shown in Fig. 4A, C, and E, respectively.

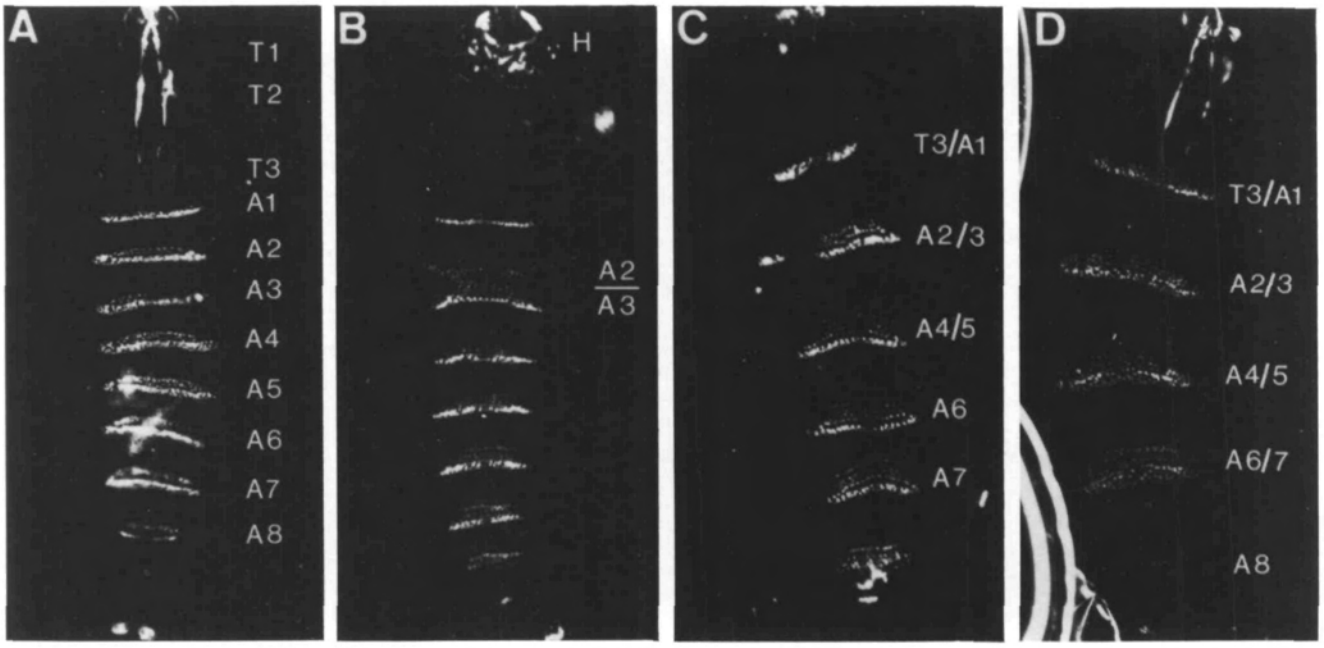

Fig. 2. Segmentation defects caused by ectopic expression of CAD at syncitial blastoderm stage. Dark-field micrographs of cuticle preparations illustrate the segmentation defects caused by a twenty minute induction of CAD by heat shock at 37°C at the syncitial blastoderm stage of development. (A) Non-heat-shock (wild-type) control. (B) HTcad3 embryo showing head defects and malformation of thoracic segments. (C) Strongly malformed HTcad3 embryo showing head defects, fusion of the setal belts of segments T1/T2, T3/A1, A2/A3, A4/A5, as well as the partial fusion of A6 and A7. (D) Strongly malformed heat-shock-*ftz* embryo for comparison.

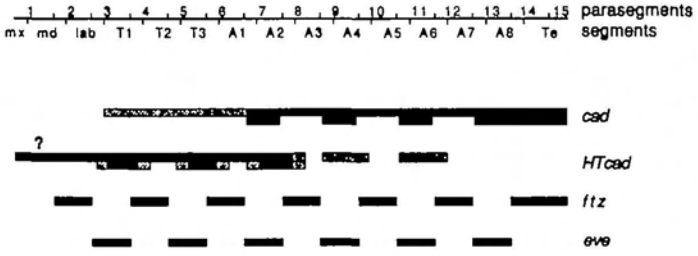

Fig. 3. Summary of segmentation defects caused by ectopic expression of CAD. The bottom four lines summarize the nature of the deletions observed in embryonic cuticles relative to segments and parasegments. Solid bars indicate regions always effected, hatched bars those often disrupted. *cad*: deletions associated with loss of both zygotic and maternal *cad* gene function (MacDonald and Struhl, 1986). *HTcad*: deletions associated with ectopic expression of CAD (this report). *ftz*: deletions associated with loss of *fushi tarazu* gene function (Wakimoto and Kaufman, 1981). *eve*: deletions associated with loss of *even skipped* gene function (Nüsslein-Volhard *et al.* 1985), which are similar to deletions caused by ectopic expression of Ftz (see Struhl, 1985 and Ish-Horowicz and Gyurkovics, 1988).

Segmentation defects seen in the mature embryonic cuticle are reflected in an altered distribution of FTZ staining within half an hour of induction of CAD. Fig. 4B shows the most frequently observed FTZ distribution: stripes 2, 3, and 4 are abnormally spaced and reduced (indicated with open arrowheads). Occasionally one of these stripes is completely missing (data not shown). In addition, in most heat-shocked HTcad3 embryos, the posterior three stripes appear to stain more intensely than the anterior ones. These results are in direct contrast to the effects of loss of maternal and zygotic *cad* function, which causes the anterior FTZ stripes to broaden and the posterior FTZ stripes to become severely reduced (MacDonald and Struhl, 1986).

The expression of EN and DFD protein is strongly altered at the germ band extension stage in 35–40 % of HTcad3 embryos heat shocked at the syncitial blastoderm (Fig. 4D and F). The modification and reduction of staining of EN and DFD respectively provides strong evidence that the effects of CAD on the head are primarily due to disruption of head segmentation and the repression of head-determining gene activity, rather than to *cad* activity *per se*. In most embryos, the segmental repeat pattern of 14 stripes of EN-expressing cells (DiNardo *et al.* 1985) can still be seen, but some stripes are often reduced and irregularly spaced. Most often, the EN stripes in the head, thoracic and anterior abdominal anlagen appear changed in intensity and spacing (Fig. 4D). However, occasionally *en* expression is also affected in more posterior regions of the embryo (e.g. see most posterior reduced EN stripe in Fig. 4D). There is little sign of an increase in width of each EN stripe similar to that seen after ectopic expression of FTZ (Ish-Horowicz *et al.* 1989), suggesting that the segmental defects observed in the cuticle may arise as a result of a more general mechanism involving slight disruption of the expression of a number of segmentation genes. The extent of reduction of DFD staining is

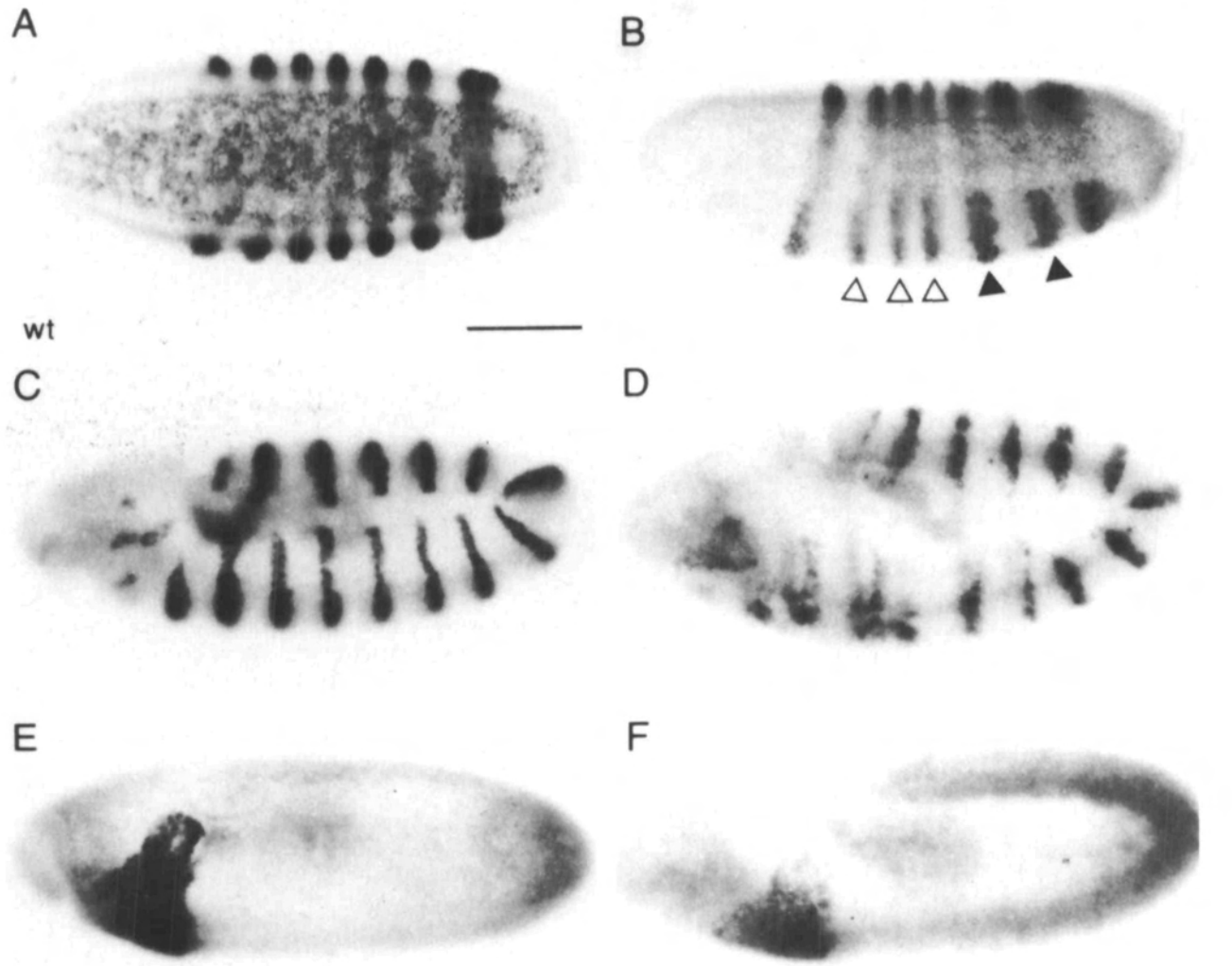

Fig. 4. Effects of ectopic expression of CAD on the embryonic fate map. Immunohistochemically stained embryos at the cellular blastoderm stage (A, B), or germ-band extension stage (C–F) show the pattern of expression of FTZ (A, B), EN (C, D) or DFD (E, F) protein. (A, C, E) Wild-type embryos, (B, D, F) HTcad3 embryos which were given a twenty minute heat shock at 37°C at the syncitial blastoderm stage. B shows the relative intensification of staining in the posterior *ftz* stripes (dark arrowheads), as well as disruption of the spacing of the anterior *ftz* stripes (open arrowheads). In some embryos, one of the stripes 2 to 4 was missing. (D) *en* expression in heat-shocked HTcad3 embryos. Note the malformation and spacing of EN stripes in the anterior part of the germband and the head region. The most posterior EN stripe is also reduced. However, this posterior defect is seen in less than 10% of the embryos analyzed. (F) *Dfd* staining in HTcad3 embryo. Reduction of *Dfd* expression in cell number and intensity is detectable in almost all embryos analyzed. Panel F shows a typical example.

quite variable, but seems to be approximately equivalent in all cells given that DFD is normally expressed most strongly in the ventral cells of the maxillary and mandibular segments (Jack *et al.* 1988; Mahaffey *et al.* 1989). No alterations in the patterns of expression of the zygotic gap genes, *Krüppel* and *hunchback*, were seen in response to early induction of *cad*.

Ectopic cad expression during later development

Since the known homeotic selector genes *Antp*, *Sex combs reduced*, *Dfd*, and *Ultrabithorax* (Gibson and Gehring, 1988; Gibson *et al.* 1990; Kuziora and McGinnis, 1988; Mann and Hogness, 1990) all produce their strongest homeotic transformations when ectopically expressed between five and seven hours after fertilization, the effects of ectopic CAD expression were also examined during later stages of development. However, no increased defects were observed. Indeed, after germ band extension, no effects on embryonic development were seen at all, and just prior to this stage, heat shocks caused only very minor disruptions of head involution in HTcad3 embryos (data not shown). Consequently, there does not appear to be a transformation of head parts to telson, as might have been expected if *cad* acting alone during a short period of

development is capable of promoting posterior segmental identity.

The effect of ectopic expression of CAD on imaginal disc development has also been examined, since the *cad* gene is expressed in the genital disc during larval development. Although the delivery of three successive heat shocks at four hour intervals is lethal to up to 50% of HTcad3 early third instar larvae, no reproducible adult cuticular defects have been observed. Since the dead animals fail to progress beyond the white prepupal stage, we believe the lethality is due to defective larval development rather than imaginal disc transformations. It is noteworthy that such reduced survival is associated with the ectopic expression of almost all of the homeodomain-containing proteins we have examined and also for the eye-specific homeodomain protein *rough* (Kimmel *et al.* 1990). Surviving adults are also fertile, arguing that development of the genital disc is not seriously disrupted by over-expression of *cad*.

Discussion

The observed partial rescue of the cad^1 mutation indicates that the heat-shock construct HTcad3 produces active protein which can be detected immunologically in all cells of the early embryo. However, none of the effects of ectopic CAD expression are indicative of homeotic transformations expected to arise as a result of CAD activity. Indeed, some caution must also be taken in the interpretation of the positive developmental defects caused by the protein, since most of the results reported here are also seen with derivatives of Antp which have deletions of large portions of the amino-terminus of that protein (Gibson *et al.* 1990). Nevertheless, we believe the results do allow us to address a number of questions regarding *cad* function.

The relatively mild consequences of destruction of the CAD gradient at the syncitial blastoderm by ubiquitous expression of the protein throughout the embryo argues against the importance of the early protein gradient in the establishment of positional information along the anterior–posterior axis. Rather, it is likely that the gradient is normally generated in order to prevent CAD from functioning in the head region during early development. Given that maternally supplied *cad* message is required early during development in the posterior region of the embryo (Mlodzik *et al.* 1985; MacDonald and Struhl, 1986), some mechanism has evolved to inhibit CAD production in the anterior region (Mlodzik and Gehring, 1987*b*). It is possible that this mechanism is translational repression by the bicoid protein (Mlodzik and Gehring, 1987*b*; W. Driever, pers. comm.), which is expressed in a gradient with the opposite orientation to CAD (Driever and Nüsslein-Volhard, 1988). This situation is directly comparable to the proposed role for the posterior organizing gene *nanos* in repression of translation of the gap gene *hunchback* in posterior segments (Hülskamp *et al.* 1989; Irish *et al.* 1989; Struhl, 1989). It is interesting to note that we were unable to obtain transformants with a construct lacking the leader of the maternal mRNA and thus some of the deleted sequences in this construct may be required for translational repression of *cad* RNA in the anterior regions of the embryo.

Our results are consistent with a role for CAD in the segmentation process. The loss of *cad* function in early embryos results in considerable segmentation defects in the posterior region of the embryo (MacDonald and Struhl, 1986) at least in part as a result of improper activation of the pair-rule segmentation gene *ftz*. Dearolf *et al.* (1989) have recently provided evidence that CAD indeed acts directly on the zebra element of the *ftz* promoter (Hiromi *et al.* 1985) to stimulate transcription in the posterior stripes. We have found that overexpression of CAD in posterior regions has only limited effects on segmentation, but that ectopic expression of CAD in anterior regions severely disrupts this process. Consequently, the CAD gradient is functionally necessary for the segmentation process, but almost certainly not in a strictly concentration-dependent manner.

The fact that no clear alterations of segmental identity were produced as a result of ectopic expression of CAD during early development provides a further example of the strict hierarchy of epistatic interactions which exists amongst the homeotic selector genes (Kuziora and McGinnis, 1989; Gibson and Gehring, 1988; Gibson *et al.* 1990; Gonzalez-Reyes *et al.* 1990; Mann and Hogness, 1990). Similarly, the finding that over-expression of CAD has almost no effect on imaginal disc development reflects that, particularly in the imaginal discs, states of determination are extremely stable. Remarkable as they are for their functional specificity and regulatory power, the homeotic selector genes are clearly restricted in their ability to act outside their normal domain of expression. If homeotic selector genes ultimately regulate the activity of so-called realizator genes, which are thought to include genes involved in cell growth, division, and cytodifferentiation (Garcia-Bellido, 1975), they do so in a highly regulated manner, depending on the cellular context within which they are expressed. The fact that homeotic selector proteins do not act universally strongly suggests that they require additional transcription factors to act. Two immediate challenges thus present themselves with respect to understanding how homeotic genes direct developmental determination: what is the nature of the additional factors, and what features of each homeodomain-containing protein allow them to interact differently.

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