Analysis of function of the pair-rule genes hairy, even-skipped and fushi tarazu in mosaic Drosophila embryos

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Summary
We report the first attempt of its kind to study genetic interactions using young Drosophila embryos that are mosaic for wildtype and mutant cells. Using nuclear transplantation we make mosaic embryos in which a patch of cells lacks a particular segmentation gene, A. With antibodies, we then look at the expression of another gene that is known to be downstream of gene A, with respect to the cells in the patch. We have examples of patches of hairy cells (where we monitor the effect on fushi tarazu (ftz) expression), even-skipped (monitoring ftz) and ftz (monitoring engrailed and Ultrabithorax). Our main finding is that the dependence of engrailed expression on the ftz gene is strictly cell-autonomous. This result goes some way towards explaining the dependence of Ultrabithorax expression on ftz, a dependence we show to be locally cell-autonomous within parts of parasegments 6 and 8 but non autonomous within parasegment 7.

Key words: segmentation genes, mosaic analysis, nuclear transplantation, cell autonomy, fushi tarazu.

Introduction
Nüsslein-Volhard and Wieschaus's identification and classification of Drosophila segmentation genes (1980) has inspired much molecular and developmental genetics. Although the amino acid sequences of many of the gene products, as well as the epistatic relations between the genes, are now described, we still lack a clear understanding of what each gene does and how it does it. In many current papers, a gene A is considered to 'regulate' another gene B, if mutants in A cause alteration in the pattern of expression of B. Certainly, if it is also true that mutants in B have no effect on the expression of gene A, one can draw the limited conclusion that gene A begins to act earlier and is 'upstream' of gene B. However, the effect of gene A's product on gene B's expression need not be direct. Interactions between a gene product and another gene (direct or indirect) may occur within the confines of a single cell, or may utilise growth factors or morphogens such that neighbouring or remote cells are involved. In the former case, the effect of removing one gene on the expression of another will be cell-autonomous in mosaics (Stern, 1968). In the latter case, the mutant effects may spread beyond the cells from which the gene is removed, or, alternatively, the surrounding wildtype cells may compensate for, or 'rescue', the mutant cell. Mosaic analysis has, in the past, proved an incisive approach to investigate genetic interactions, but methods to make and study mosaics in early Drosophila embryos have not yet been fully developed. This paper presents a new attempt. We take four examples of genetic interactions that occur in the early Drosophila embryo and analyse them in mosaics. They are: hairy (h) and even-skipped (eve), both of which are upstream of fushi tarazu (ftz) (Carroll and Scott, 1986; Frasch and Levine, 1987), and ftz, which is upstream of both engrailed (en) (Carroll and Scott, 1986; Harding et al. 1986; Howard and Ingham, 1986) and Ultrabithorax (Ubx) (Duncan, 1986; Ingham and Martinez-Arias, 1986). We call the four experiments h+ monitor ftz, eve- monitor ftz, ftz- monitor en and ftz- monitor Ubx. Our results suggest that of these four examples, only the dependence of en expression on ftz is straightforward and cell-autonomous, the other interactions being more complex.

Materials and methods
Nuclear transplantation
The methods were as described in Lawrence and Johnston (1984; 1986a,b), except that host eggs were collected and aged for 15 min and at 25°C before being dechorionated and dried — this procedure increased survival. When injected eggs reached the desired stage of development, the glue that stuck them to the coverslip was dissolved in heptane. The free embryos were then transferred to heptane saturated with 4% formaldehyde in Pipes buffer, pH 6.9 for 60 min, devitellinised by hand in the buffer, transferred to 4% formaldehyde in Pipes for 20 min, washed in water and transferred to methanol. These embryos were then taken through double staining for anti-
bodies in which one antigen was stained with DAB (brown), and another with DAB plus cobalt and nickel ions (grey). Embryos were examined under the dissecting microscope and selected ones were dissected with a needle and mounted under coverslips in Araldite (see Lawrence et al. 1987 and Lawrence and Johnston, 1989 for methods).

Experimental design
For all mosaic experiments, one ideally needs a gratuitous cell marker, but for young embryos, none yet exists. We have compromised by using either β-galactosidase (βgal) under the control of ftz and/or eve promoters, which give stripes of βgal expression in the young extended germ band stage, or by using the protein product of one of the genes of interest.

Note that only one quarter of the donor eggs will be of the mutant genotype; others will be wildtype for the gene of interest.

h+ monitor ftz
Donors: eve-βgal; ftz-βgal (chromosome 2); ftz-βgal (chromosome 3).
Hosts: σ h22 Ki/TM3

The ftz-βgal transformants are courtesy of Hiromi et al. (1985), and the eve-βgal chromosome came from MacDonald (see Lawrence et al. 1987). One quarter of the embryos from this cross, when stained with anti-ftz antibody, showed the typical hairy phenotype — that is, instead of the typical ftz stripes, almost continuous ftz expression (Carroll and Scott, 1986; Lawrence and Johnston, 1989).

After nuclear transplantation, some embryos were fixed as young extended germ bands and treated with anti-βgal antibody. Mosaics were found and one is shown in Fig. 1. Most embryos were collected at stage 5(3)—6 (late blastoderm — early gastrula; see Lawrence and Johnston, 1989 for descriptions of stage 5 subdivisions, and Campos-Ortega and Hartenstein (1985) and Wieschaus and Nüsslein-Volhard (1986) for descriptions of the main stages) and treated with anti-ftz antibody (brown with DAB alone). Twenty two embryos that showed the hairy phenotype were selected and treated with anti-h antibody (grey due to DAB plus metal ions). Three mosaics with patches of cells that contained cells positive for h antigen were found.

This experiment was also done with the h+ embryos as donors and the h+ but β-gal-carrying stock as hosts. Two putative mosaics were found; these were identified by the criteria that the patches consisted of spreading or fused ftz stripes, and the cells in the patches failed to stain for h.

eve" monitor ftz
Donors: σ eve-βgal; eve113/+ σ eve-βgal; Df eve 1.27/CyO
Hosts: wildtype

One quarter of embryos from this cross gave the expected cuticle phenotype — a lawn of denticles with little sign of metamorphosis (Nüsslein-Volhard et al. 1985).

These embryos were stained for ftz (brown) and eve (grey). Mosaics of interest were detected as embryos with patches that did not express eve antigen. 172 embryos were studied in detail, of these four were too old to express eve, 40 were developing poorly or not at all, 115 showed no eve" patches, 10 were mosaics containing eve" territory, and three were entirely derived from eve" donor nuclei.

ftz" monitor en and ftz" monitor Ubx
Donors: σ Ki ftzW29/TM3 σ DfSchftz"/TM3
Hosts: ftz-βgal (chromosome 2); ftz-βgal (chromosome 3) or, in some experiments, eve-βgal; ftz-βgal(2); ftz-βgal(3).

ftz" monitor en
These embryos were double-stained, brown for βgal that stains the cytoplasm of host but not donor cells, and grey for en that marks the nuclei of all posterior compartment cells. Two classes of mosaics were expected: controls, in which both donor and host cells would be ftz" , and experimentals, where donor cells would be ftz" . Control mosaics were identified because they bore patches that lacked βgal stain, but en expression was normal (Fig. 7). Experimental mosaics were recognised by the following criteria: the boundary between βgal-expression and non-expressing cells was clearly demarcated, and the patch showed alterations in en expression. (In embryos that are entirely ftz" , the en stripes corresponding to the even-numbered parasegments are lacking; Howard and Ingham, 1986).

In ftz" monitor en, of 178 embryos studied, 30 were developing poorly, 122 showed no patches, 18 were too weakly stained for βgal, two were ftz" control mosaics and six were ftz" experimental mosaics.

ftz" monitor Ubx
Here, the same cross was used as with ftz" monitor en, and control and experimental mosaics were distinguished only on the grounds that control mosaics show normal Ubx expression in the area lacking in βgal, while experimental mosaics show altered Ubx expression.

In ftz" monitor Ubx, of 180 embryos studied, 32 were developing poorly, 133 showed no patches, 10 were ftz" control mosaics and 10 were ftz" experimental mosaics.

Antibodies
Anti-hairy antibody was kindly provided by Manfred Frasch (see Frasch et al. 1987), anti-en antibody by Kevin Coleman (see Patel et al. 1989) and anti-Ubx antibody by Michael Wilcox (see White and Wilcox, 1984).

Nomenclature
The ftz and eve stripes are named according to the parasegments (ps) whose anterior margins they define. For example, ftz stripe ps2 is the most anterior ftz stripe, and eve stripe ps3 the second most anterior eve stripe.
Cell size
Some mosaics had large areas of cells of two different sizes (Fig. 3). For example, of the 10 mosaics including eve− cells, five bore patches of larger and smaller cells, and usually the border of the patch of cells of one size was coincident with the border between cells expressing eve, and cells not expressing the gene. In one exceptional case, the smaller cells included areas of both eve and eve+ cells. Zusman and Wieschaus (1987), who studied the blastoderm stages of gynandromorphs made by ring chromosome loss, noticed that the male tissue was made up of larger cells than the female, and suggested this might be due to some problem caused by the chromosome loss. These and our present results, plus the observation that, after nuclear transplantation, the proportion of gynandromorphs out of all mosaics is less than expected (Lawrence and Johnston, 1986b), suggest that gynandromorphs do not develop as well as single-sex mosaics, and that asynchrony of nuclear division is a symptom of some malaise. One possibility is that donor nuclei have already begun sex-specific changes, such as dosage compensation, by the time they are transplanted.

Results

h+ monitor ftz
In h− embryos the territory corresponding to ps2−14 is filled with ftz stripes that have almost fused together leaving occasional and variable interstripes (Carroll and Scott, 1986; Lawrence and Johnston, 1989). Mosaic patches of h− in a h+ host would therefore be expected to show increased ftz expression, and two putative mosaics were found in which adjacent ftz stripes are locally fused. Associated with this is a lack of h staining, which suggests that the patch is a genuine mosaic. However, the criteria are poor. Mosaic h+ patches in a h− host can be more positively determined, since they will be expected to show some cells that stain for h antigen. Three putative mosaics were identified from 22 h− host embryos. In Fig. 14 there is a patch of 18 cells that differ significantly from the background. At this stage (late 5(2)) the embryo has a large, almost continuous field of cells that stain strongly for ftz. The patch of cells contains 16 that appear not to stain for ftz at all and eight that stain for h. Two cells stain for both ftz and h. It is clear that the cells which do not stain for ftz are neighbouring or nearby the cells that express h—for nowhere else in this embryo are there any cells expressing h. The h-expressing cells must be from the donor but, because of the lack of an independent marker, we do not know which, if any, of the cells not expressing ftz are from the host.

eve− monitor ftz
In embryos lacking the eve gene, the ftz stripes are somewhat destabilised, frequently the first one (corresponding at its anterior limit to the anterior limit of ps2; Lawrence et al. 1987; Carroll et al. 1988a) is partially missing. The stripes, unlike those in the wildtype, are symmetric and lack sharp boundaries at both anterior and posterior margins (Lawrence and Johnson, 1989).

In one mosaic at late gastrulation (stage 7; Fig. 2), much of one ventral half is eve− and the other eve+; this allows a direct comparison of stripes across the midline. It is clear that the ftz stripes are much wider on the eve− side, and this is largely due to an anteriorwards extension of the anterior margin of the stripes. At either extreme of the patch, the eve+ cells reappear, and the stripes slip back into left–right registration. Exactly the same pattern is found in the mesoderm, which underlies the ectoderm and can be studied independently. This mosaic cannot be studied usefully in great detail because of the lack of a gratuitous cell marker—it cannot always be assumed the lack of eve antigen in a cell means that that cell is eve−. Nevertheless it is clear that there is an eve− patch and within this patch the behaviour of the stripes is largely autonomous.

Another mosaic at early gastrulation (stage 6) allows a similar conclusion, but points to more local effects. At this stage, in the wildtype, the ftz stripes have sharply demarcated anterior edges and very few, if any, weakly stained cells at those edges (Lawrence and Johnson, 1989). Fig. 4 shows that the eve-expressing cells are confined to the anterior part of the embryo, and none occur posterior to parasegment 6. Almost the whole of the eve stripe ps3 is present and the ftz stripe ps4 is narrower and its anterior margin is relatively sharp. However, the eve stripe ps5 is only partially present and, where eve cells are missing, the anterior margin of the ftz stripe of ps6 bears weakly stained cells (Fig. 4). The stripe does not show any large anterior shift in this region. This result indicates that eve is required locally to sharpen the ftz stripe. Other parts of the same mosaic support the same interpretation.

One might conjecture that eve and ftz compete for territory, and the sharpening of the anterior margins of the stripes depends simply and directly on that process. However, the interpretation of one other mosaic at stage 5(3) (Figs 5, 6) argues that this is not correct. In this mosaic, the presumed donor territory is eve+ and has larger cells than the host and, because none of the large cells, wherever they are located, expresses eve, it may be legitimate to use cell size as a marker in this case. The larger cells express ftz normally. Fig. 6 shows that immediately anterior to ftz stripe ps12 (which mainly consists of larger donor cells) there are four cells which do not express either ftz or eve. On a straightforward competition model, one would expect that, since they have never expressed eve and are adjacent to the ftz stripe, they should come to express ftz, but this is not the case.

ftz− monitor en
A control mosaic is illustrated in Fig. 7. It shows that the donor and host cells can be easily distinguished wherever βgal is expressed. A large experimental mosaic is shown in Figs 8, 9 and 15; note that an area of tissue that lacks βgal extends from ps8 backwards on the right side. It is marked by a swath of cells that lack both βgal and en expression in the even-numbered parasegments, but show normal en expression in the odd-numbered ones. There are several boundaries between host and donor cells that are sharply demarcated by the βgal staining—in the even-
numbered parasegments, wherever the βgal staining is missing, so is the en. In the wildtype, only a few rows of cells in the anterior part of the parasegment express en and this is also found in the wildtype patches (see the two en-expressing cells in the dorsolateral part of ps10, Fig. 9). The effect of ftz− patches appears completely cell-autonomous on en.

Figs 10 and 16 illustrate another example of a patch of mutant tissue just failing to reach the midline in ps10, and as expected therefore, the underlying mesoderm is all wildtype and expresses βgal. These examples show the precise cell-by-cell dependence of en on ftz expression. The three cells indicated as (a) in Fig. 16 are βgal-expressing, and therefore genetically ftz−; nevertheless, en cannot be detected in them. This suggests a border between ftz+ and ftz− cells is insufficient to induce en expression; probably a certain level of ftz product is needed, and this normally occurs at or very near the anterior border of the parasegment.

Where patches of ftz− cells do extend to the mesoderm, we see the same autonomous dependence of en on ftz−.

ftz− monitor Ubx

In ftz− embryos, the pattern of Ubx expression is considerably altered (Ingham and Martinez-Arias, 1986; Martinez-Arias and White, 1988) and this is true of patches of ftz− tissue. Some mosaic areas are shown in Figs 11–13.

Fig. 11 is a very young extended germ band (late stage 8) and shows weak Ubx expression mostly in ps6 and in the anterior part of ps7. The staining of Ubx is too weak and heterogeneous to decide whether the lack of Ubx staining in the ftz− territory is precisely cell-autonomous, but there is clearly a local dependence of Ubx expression on ftz – in the territory lacking βgal there is very little detectable Ubx expression, while across the ventral midline where βgal stripes can be seen, Ubx expression is stronger.

One mosaic at stage 10 shows strong expression of Ubx (Figs 12, 13 and 17). The border between host and donor tissue can only be plotted where βgal is stained, and this is in about the anterior third of all parasegments, which roughly coincides with the posterior compartments. In Fig. 17 we see a patch of territory that lacks βgal extending from ps5–8. In ps6 there is a cell autonomous drop in Ubx staining in mutant territory, while in ps7 and the anterior region of ps8, an increase. The dotted line marks the boundary between mutant and wildtype territory; a boundary that coincides precisely with a change in expression of Ubx in the anterior parts of ps6 and 8. In ps7, Ubx is expressed more strongly in the anterior part where ftz is missing, but this is not cell-autonomous; about one line of cells is negative for βgal (and is therefore ftz−) but in these cells Ubx expression is very low (as in the ftz+ territory) (Figs 13 and 17).

The other mosaics give further snippets of information: for example, in one mosaic in the mesoderm, the ftz− patch is associated with lowered levels of Ubx expression in both ps6 and ps7, although the staining did not allow an assessment of autonomy.

Discussion

Using mosaic Drosophila embryos, we have looked for cell autonomy in the dependence of ftz expression on h+ and eve+, en expression on ftz+ and Ubx expression on ftz+.

In patches of cells that lack hairy, we see a tendency for ftz to be ectopically expressed and, in patches of h+ cells in a h− background, there is a reduction in the number of cells expressing ftz. The pattern of ftz expression does not suggest any simple model. For example, Ish-Horowicz and Pinchin (1987) have proposed that h might repress the transcription of ftz. If this model is applied to one mosaic (Fig. 14), all the cells not expressing ftz might be expected to express h, which is not the case. There are even two cells that express both h and ftz. Instead, it seems that a small number of cells expressing h have inactivated ftz in a larger number, which suggests non-autonomy; although other explanations are possible (Carroll et al. 1988b). In any case, the simple model is not supported by the stripes of expression of ftz and h in the wildtype where some cells express both genes even at gastrulation (Carroll et al. 1988b; our unpublished observations).
In the absence of eve+, the ftz stripes, which normally delineate the anterior borders of the even-numbered parasegments (Lawrence et al., 1987), lack sharp anterior boundaries (Lawrence and Johnston, 1989) and fail to activate en (Howard and Ingham, 1986). We show here that the action of eve+, in sharpening the anterior boundaries of ftz, is a local one, although it may not be strictly cell-autonomous. For example, in a region that completely lacks eve+, the ftz stripes remain broad and are extended anteriorly when compared with wildtype areas of the same mosaic. However, when eve+ is missing from a small number of cells adjacent to the anterior boundary of the ftz stripe, these cells do not necessarily express ftz. Local action of eve+ is required to sharpen the stripes; small patches of eve+ cells are associated with nearby ftz stripes that fail to sharpen at their anterior edges.

We cannot offer a simple interpretation of this, but it does suggest that the dependence of ftz on eve is not a direct competition for space. It is already clear that the processes leading to the formation of eve and ftz stripes, which depend on many upstream genes including hairy and runt (Carroll and Scott, 1986; Frasch and Levine, 1987) and to their sharpening, are complex.

We show that the dependence of en on ftz+ is cell-autonomous and this fact supports arguments made elsewhere (Howard and Ingham, 1986; Lawrence et al., 1987) and show that there is an interaction within single cells. The autonomy does not show the action is direct, only that it is independent of intercellular signalling. In the wildtype gastrula, the en protein is formed in cells near to the sharp and stable anterior edge of the ftz stripe. The results are consistent with the hypothesis that en is activated where ftz itself, or some other molecule directly dependent on ftz, reaches a threshold concentration (Howard and Ingham, 1986; Lawrence et al., 1987; Lawrence and Johnston, 1989).

The dependence of Ubx on ftz is expected to be complex, and the results confirm this. Given that en depends in a cell-autonomous fashion on ftz, and given that Ubx is usually down regulated by en (Carroll et al., 1988a; Martinez-Arias and White, 1988) and abdA (Struhl and White, 1985), some of the effects of the ftz- patches can be interpreted. For example, Figs 12, 13 and 17 show that, in the anterior part of ps8, ftz- cells express more Ubx product than the neighbouring ftz+ cells. In the ftz- cells, we know from the previous mosaics that en will be missing, and for this reason Ubx expression would be expected to increase as observed. In ps7 and ps8, where the abdA gene is expressed, the situation is even more complex: if abdA were suppressed, directly or indirectly by the lack of ftz, Ubx expression might then increase (Struhl and White, 1985). It is still an open question whether, in vivo, the Ubx gene is directly regulated by the ftz protein, a
hypothesis that has been considered (Duncan, 1986; Ingham and Martinez-Arias, 1986; Han et al. 1989; Winslow et al. 1989). There are a number of problems with the hypothesis; one is the lack of spatial correlation between $\textit{Ubx}$ and $\textit{ftz}$ expression in the wildtype. For example, at the time that $\textit{Ubx}$ RNA can first be detected by $\textit{in situ}$ (gastrulation; see Ingham and Martinez-Arias, 1986) $\textit{ftz}$ protein is already expressed in only the anterior $2/3$ of the parasegment (Lawrence and Johnston, 1989), yet $\textit{Ubx}$ is most strongly expressed in the posterior region. $\textit{Ubx}$ expression in the odd-numbered parasegments is also strong where $\textit{ftz}$ is not expressed at all. It seems that $\textit{ftz}$'s role in establishing metameric units and $\textit{en}$ expression helps determine the pattern of $\textit{Ubx}$ but is not necessary for $\textit{Ubx}$ expression per se; accordingly, $\textit{Ubx}$ expression in $\textit{ftz}$- embryos is abnormal but strong (Martinez-Arias and White, 1988; see also Tremml and Bienz, 1989). A direct effect of $\textit{ftz}$ on $\textit{Ubx}$ is neither demanded nor ruled out by our results: the cell autonomy we observe in the anterior parts of ps6 and 8 could depend on the action of $\textit{ftz}$ on $\textit{en}$, and the non-autonomy seen in ps7 could obviously be indirect.

Prospects

In order to take this approach further, we need a good cell-autonomous marker that is expressed in early embryos and could be used to distinguish donor from host cells. Ideally, such a marker would not depend on antibody labelling. Labelled antibodies could then be used to identify the genotype of the donor cells and to determine the response of host and donor cells with respect to another gene. Lack of cell autonomy would then be strong evidence that the two genes did not interact directly, while autonomy would be consistent with such a direct link.

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References


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