The basis for a heat-induced developmental defect: defining crucial lesions

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Because lethal heat shocks perturb a multitude of cellular processes, the primary lesions responsible for death from heat stress remain to be defined. In *Drosophila*, sublethal heat treatments produce developmental anomalies that frequently mimic the effects of known mutations and are hence referred to as phenocopies. Mutations subject to phenocopy mimicry provide signposts to those biological processes most sensitive to heat and most important for the function and survival of the organism as a whole. We have analyzed a particular developmental defect inducible in early embryos of *Drosophila melanogaster*. By molecular, phenotypic, and genetic criteria, we have found extensive parallels between this phenocopy and certain dominant mutations in the segmentation gene *fushi tarazu* (*ftz*). Our analysis of this phenocopy indicates that the crucial lesion is interference with proper turnover of *ftz* protein, resulting in *ftz* overexpression. Our results provide a novel explanation for a heat-induced developmental defect. Perturbations in relative amounts of important regulatory proteins may be a common mechanism by which heat-shock phenocopies arise.

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Lethal temperatures inhibit respiration, block amino-acid and ion transport, impair membrane function, reduce DNA synthesis, and inhibit the repair of DNA damage. In many cells, intermediate filaments collapse and actin fibers rearrange. Gene expression is dramatically changed. For example, in *Drosophila*, general transcription is turned off, splicing ceases, RNA turnover is altered, and the translation machinery switches specificity. It is not clear which of these perturbations are the causes, and which are the consequences, of lethal lesions induced by elevated temperatures (Nover 1991; Laszlo 1992; Parsell and Lindquist 1993).

The complexity of these changes makes identification of the primary lesions in dying cells very difficult. This problem might be simplified by the analysis of sublethal heat shocks that cause specific developmental anomalies (Lindquist 1986; Petersen and Mitchell 1991). Such anomalies have been observed in a wide variety of organisms from plants and microorganisms to insects, mollusks, and vertebrates (Nover 1991; Zimmerman and Cohill 1991). Sublethal heat shocks cause remarkably specific perturbations that are induced only during very narrow sensitive periods. For example, 36-hr *Drosophila* pupae exposed to 40.8°C for 35 min develop into adults with characteristic bristle defects on the posterior dorsal thorax: Normally straight bristles now form angles (Mitchell and Lipps 1978; Mitchell and Petersen 1982). At 42 hr, the same treatment produces bristles with spear-like tips, while at 23 hr it causes the absence of a specific vein in the wing. This high degree of specificity suggests that at a given time in development a limited set of processes is particularly susceptible to heat damage; in some cases, there may even be a single most vulnerable target.

In *Drosophila*, many heat-induced defects mimic the effects of known mutations and are known as phenocopies (Goldschmidt 1935a,b). These mutations provide a potential means of identifying the pathways that are most sensitive to heat and that must be protected to guarantee proper function and survival of the organism as a whole. Phenocopies focus the search for critical lesions, but the problem remains complex. To date, no heat-induced developmental defect has been defined in molecular terms (for reviews, see Petersen 1990; Zimmerman and Cohill 1991), although a variety of explanations have been proposed. For example, heat shock may delete structures by killing a set of especially sensitive cells (Edwards et al. 1974), or cause defects by reducing the expression of a critical gene below a threshold level (Petersen and Young 1989).

In an attempt to elucidate one crucial lesion, we have focused on a phenocopy inducible in early *Drosophila* embryos. A brief heat pulse administered during cellu-

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larization induces homeotic transformations in the adult abdomen that resemble those caused by *Ultra-abdominal* (*Uab*) alleles, dominant mutations in the bithorax complex (Santamaria 1979). The narrow sensitive period for phenocopy induction suggests that heat shock does not perturb bithorax-complex genes directly, but rather one of their regulators (Santamaria 1979; Dura and Santamaria 1983). In fact, the heat-induced homeotic transformation differs from that of *Uab* flies and more precisely mimics that caused by certain dominant alleles of the segmentation gene *fushi tarazu* (*ftz*) (Duncan 1986). These alleles (ftz^{Ual}, for Ultra-abdominal-like) encode ftz proteins that have increased half-lives relative to wild type and accumulate to abnormally high levels during embryogenesis (Kellerman et al. 1990). This excess accumulation appears to be responsible for the homeotic transformations and other defects in the ftz^{Ual} mutants (Duncan 1986; Kellerman et al. 1990). *ftz* is a regulator for bithorax-complex genes (Duncan 1986; Ingham and Martinez-Arias 1986; Ish-Horowicz et al. 1989), and its major period of expression correlates with the sensitive period for phenocopy induction (Santamaria 1979), suggesting that the heat-induced defect may be due to perturbations in *ftz* expression (Duncan 1986).

Here we report that the heat-induced phenocopy mimics the ftz^{Ual} mutants, not only in adult phenotype, but also by several molecular and genetic criteria. Our analysis provides evidence that the crucial lesion in this particular phenocopy is interference with proper ftz turnover, causing *ftz* overexpression.

Results

How closely does the heat-induced defect resemble ftz^{Ual}?

We first reexamined the heat-induced anomalies in the adult abdomen. Wild-type embryos were heat-shocked during cellularization for 15 min at 37°C. Surviving adults showed a wide variety of abdominal abnormalities (Fig. 1). As previously described, the single most frequent defects were homeotic transformations of portions of the first abdominal segment (A1) to a more posterior segment (Fig. 1B, solid arrowhead). As shown by Duncan (1986), these transformed patches attain the identity of the third abdominal segment (A3). The ftz^{Ual} alleles cause this same transformation, a pattern not seen in any other mutant (Duncan 1986). In addition, and again in contrast to many other homeotic transformations, both ftz^{Ual} and heat shock typically do not transform the whole segment, but only patches of it.

A variety of segmentation defects also occurred, at low frequency. In many cases, hemi-tergites were incompletely fused dorsally, or tergites were inappropriately fused with neighboring tergites. In rare cases, parts of a segment were replaced with mirror images of the remaining structures. The most striking segmentation defects, however, were partially or completely missing hemi-tergites. For example, the abdomen in Fig. 1C (open arrowhead) lacks about half of the tergite located in the fourth abdominal segment (A4). Such pattern deletions are also observed in ftz^{Ual} mutants, where they are concentrated in even-numbered abdominal segments, especially in A4 (Duncan 1986). Out of a total of

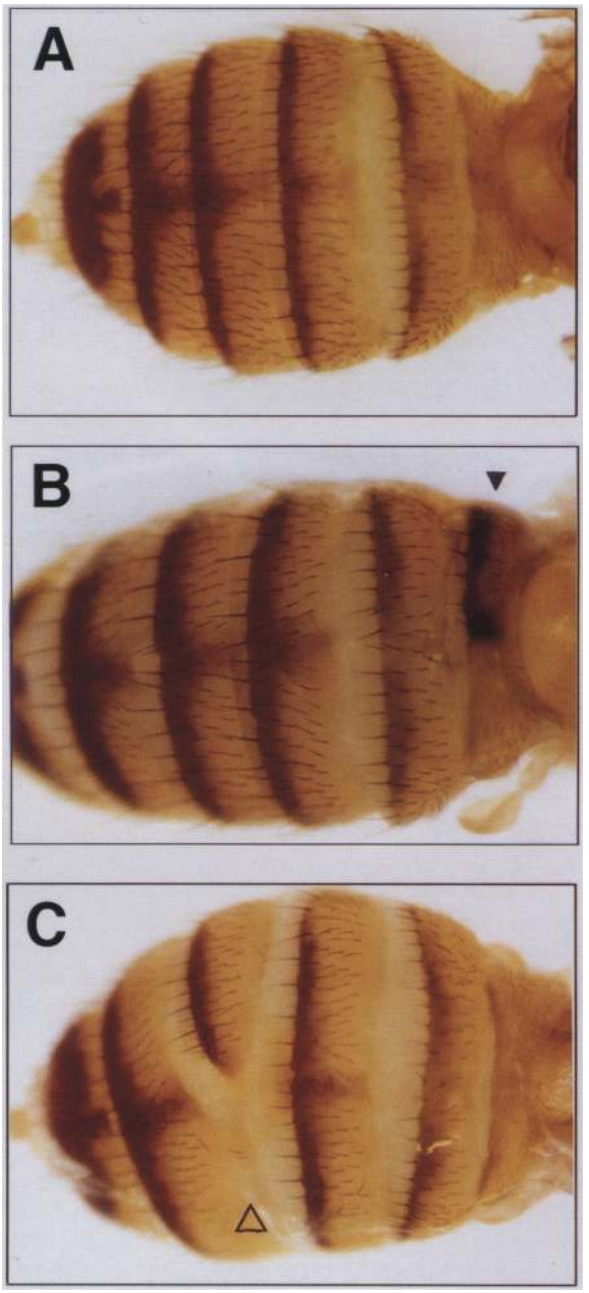

Figure 1. Heat-induced defects in the adult abdomen. Embryos were exposed to 37°C for 15 min during cellularization, as described in Materials and methods, and were then allowed to develop to adulthood. Various defects in the abdomen are marked by arrowheads. (*A*) No defect, wild-type pattern; (*B*) half of the first abdominal segment (solid arrowhead) shows pigmentation and bristles typical of more posterior segments; (*C*) one half of the tergite in the fourth abdominal segment (open arrowhead) is almost completely missing.

61 heat-induced deletions (observed in several different experiments), 46 occurred in even-numbered segments, most frequently in A4.

Yet another similarity between the phenocopy and ftz^{Ual} mutants is that the transformations occur only in a subset of the adults (Duncan 1986; Santamaria 1979; this paper). Such incomplete penetrance provides a sensitive background in which to measure the effects of other mutants and, thus, a means for identifying interacting genes.

Krüppel mutants are the strongest known enhancers of ftz^{Ual} (Duncan 1986). We therefore asked if mutations in the *Krüppel* gene also alter the penetrance of the heat-induced phenocopy. Oregon-R females were crossed to males carrying a Kr^2 mutation heterozygous with a balancer chromosome, and the progeny were exposed to a 15-min heat shock at the time of cellularization. Homeotic transformations in A1 were then scored in adults. In each of several different experiments at different temperatures, the Kr^2 chromosome greatly increased the frequency of the heat-induced phenocopy (Table 1). Control experiments confirmed that this Kr^2 chromosome increased the frequency of transformations due to the ftz^{Ual} mutation. These enhancements were specific for A1 transformations; *Kr* mutations did not raise the penetrance of segmentation defects in the abdomen either after heat shock (data not shown) or in the ftz^{Ual} mutants (Duncan 1986). Thus the effects of *Krüppel* are identical for the mutant and the heat-induced phenocopy.

Next we asked if heat shock perturbs homeotic selector gene expression in the same manner as the ftz^{Ual} mutation. In both cases, anterior A1, which is located in parasegment 6 (PS6), is transformed to anterior A3, located in parasegment 8 (PS8) (Duncan 1986). PS8 identity is defined by the bithorax-complex gene *abdominal-A* (*abd-A*) (Sánchez-Herrero et al. 1985), which normally is expressed in PS8 but not in the anterior compartment of PS6 (Kellerman et al. 1990). In ftz^{Ual} embryos, abd-A protein is ectopically expressed in the anterior compartment of PS6 (Kellerman et al. 1990). This misexpression is probably directly responsible for the switch in A1 segmental identity.

Because the phenocopy has low penetrance in wild-type flies, we employed the *Krüppel* mutation to increase penetrance and facilitate study of abd-A expression. Embryos from a cross between Kr^2 heterozygous males and Oregon-R females (see above) were heat-shocked during cellularization, allowed to continue development, and fixed after they had completed germband retraction (Fig. 2). Double staining for engrailed (gray)—to demarcate posterior compartments—and abd-A (brown) revealed that many of the heat-treated embryos showed patchy abd-A staining in PS6 after germ-band retraction. Ectopic expression of abd-A was also observed in wild-type embryos that had been heat-shocked at cellularization, albeit—as expected—at a lower frequency. In neither genotype was ectopic expression of abd-A seen in the absence of heat shock.

In crosses involving Kr^2, heat shock caused a reduction in the size of PS5 in some embryos (Fig. 2B, arrow). Also, the engrailed stripes delineating PS5 were sometimes partially fused or contained large gaps. This phenomenon was also observed in the absence of heat shock in crosses involving Kr^2 and ftz^{Ual}. Thus, once again, heat shock at blastoderm and ftz^{Ual} had identical consequences.

A curious feature of abd-A expression in the ftz^{Ual} embryos is that it is normal before germ-band retraction. To determine if this is also a feature of the heat-shock phenocopy, embryos from a cross of Kr^2 heterozygotes and Oregon-R females were heat-shocked during blastoderm formation and fixed before, during, or after germ-band retraction. Whereas 24% of the embryos showed ectopic abd-A expression after germ-band retraction, hardly any of the embryos showed ectopic expression at the beginning of germ-band retraction (Table 2 and Fig 2C, arrowhead). With a more severe heat shock, the frequency of abd-A misexpression increased to 34%. The embryos still acquired ectopic abd-A staining only during germband retraction (Table 2). Thus, like the ftz^{Ual} mutation, heat shock does not alter abd-A expression immediately, but only after other developmental events have ensued. Although we have not attempted to follow the heat-induced misexpression of abd-A through larval and pupal stages to map its precise relationship to the adult segmentation changes, it is likely that—as in the ftz^{Ual} mutants—this misexpression is an early marker for the homeotic transformation (Kellerman et al. 1990). The almost identical adult phenotypes, abd-A expression patterns, and Kr^2 interaction suggest that heat shock and the ftz^{Ual} mutants cause homeotic transformations by a similar mechanism.

How might heat shock cause ftz *overexpression?*

The basic defect in the ftz^{Ual} mutants appears to be the stabilization, and consequent excess accumulation, of

Table 1. Krüppel *strongly enhances heat-induced A1 transformations*

	Flies heterozygous for Kr^2		Wild-type flies	
Heat shock	A1 transformation (%)	flies scored	A1 transformation (%)	flies scored
None	1.5	199	0	249
36°C	24	34	5	146
36.5°C	33	51	7	148
37°C	22	108	5	197

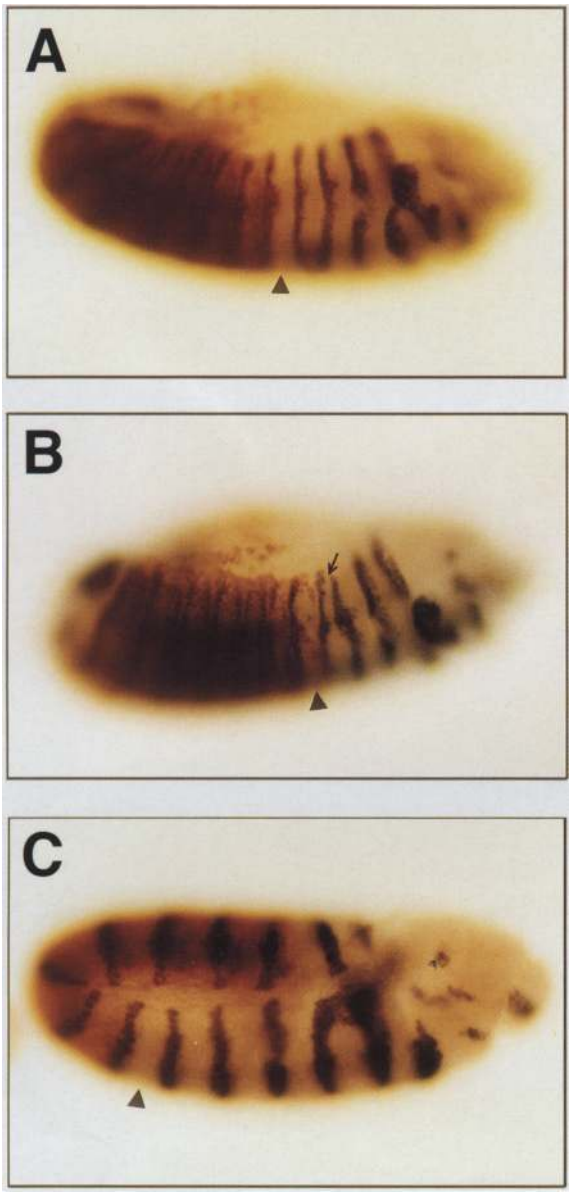

Figure 2. Ectopic expression of abd-A following heat shock. Embryos from a cross between Oregon-R females and males heterozygous for Kr^2 were either held at room temperature (*A*) or exposed to 36.5°C for 15 min during cellularization (*B*,*C*) and fixed at various times thereafter. All embryos are double-labeled for abd-A (brown) and engrailed (gray). The anterior compartment of PS6 is marked by arrowheads. (*A*) Non-heat-shocked embryo after germ-band retraction. No staining for abd-A in PS6. (*B*) Heat-shocked embryo after germ-band retraction. Patchy abd-A staining in PS6. (*C*) Heat-shocked embryo during germ-band retraction. No abd-A staining is yet detectable in PS6.

ftz protein (Kellerman et al. 1990). If heat shock and the ftz^{Ual} mutations cause transformations by the same mechanism, heat shock must somehow increase ftz protein levels. Since *ftz* does not contain any known heat-shock transcription or translation signals, a likely mechanism is the stabilization of the ftz protein or message. Because the ftz protein and message have unusually short half-lives in the embryo, about 6 and 7 min, respectively (Edgar et al. 1986; Kellerman et al. 1990), reduced turnover could substantially increase ftz levels. Heat shock is known to decrease the turnover of certain proteins in mammalian and *Drosophila* tissue-culture cells (Lüscher and Eisenman 1988; R. Petersen and S. Lindquist, pers. comm.) and in the developing *Drosophila* wing (Petersen and Young 1989). Decreased turnover of certain short-lived messages has also been reported [*hsp70* in *Drosophila* (Petersen and Lindquist 1988; Petersen and Lindquist 1989), and *c-fos* in mammalian tissue culture cells (Andrews et al. 1987)].

Testing the model in a tissue-culture system

ftz is expressed at very low levels in the embryo, and the pattern of its expression changes dramatically over short periods of development (Edgar et al. 1986; Krause et al. 1988). To determine directly if heat shock affects the turnover of ftz protein, we therefore established a tissue-culture test system. Because constitutive expression of ftz, a potent transcription factor, was expected to be toxic, the inducible *hsp70* promoter was employed to drive *ftz* expression (Struhl 1985). This promoter is induced by temperatures as low as 33°C, allowing us to use very mild conditions to induce *ftz*, followed by higher temperatures to assess the effects of the heat shock on ftz turnover.

First, we asked if tissue-culture cells would reproduce faithfully the difference in turnover between the wild-type ftz protein and the ftz^{Ual} protein at 25°C. Cells transformed either with the wild-type *ftz* sequence or a ftz^{Ual} sequence were induced with a mild heat treatment and returned to 25°C. One sample was harvested immediately (I). Cycloheximide was added to prevent further protein synthesis, and remaining samples were harvested at various times thereafter (Fig. 3; the band marked with the arrow is unrelated to ftz and is detected only with certain batches of the secondary antibody, providing a convenient internal control for equal loading). The wild-type ftz protein (Fig. 3A) was degraded much more rapidly than the mutant protein (Fig. 3B,C). Thus, although ftz is turned over more slowly in tissue-culture cells than in embryos, the difference between the wild-type and the mutant protein was reproduced (Kellerman et al. 1990). In addition, ftz protein correctly localized to the nucleus in the tissue-culture cells and showed subnuclear points of very high concentration as it does in embryos (immunofluorescent staining, not shown). Thus the tissue-culture cells mimic embryos in many aspects of ftz metabolism and provide a valid system to assess the effects of heat shock on ftz turnover.

When cells carrying the wild-type *ftz* construct were shifted from 25°C to 37°C, ftz turnover was blocked (data not shown). To determine if even a brief heat treatment could have a lasting effect on ftz protein turnover, cells were maintained at 25°C (Fig. 4A) or heat-shocked at 39°C for 15 min and then returned to 25°C (Fig. 4B). (The

Table 2. *Heat-shocked embryos acquire ectopic abd-A expression during germ-band retraction*

Heat treatment during cellularization	Age at scoring	Number of embryos scored	Percent embryos with ectopic abd-A expression (%)
None	after germ-band retraction	116	0
36.5°C/15 min	after germ-band retraction	446	24
36.5°C/15 min	before/early germ-band retraction	176	1
33°C/15 min plus 36.5°C/15 min	after germ-band retraction	352	34
33°C/15 min plus 36.5°C/15 min	during germ-band retraction	250	28
33°C/15 min plus 36.5°C/15 min	before/early germ-band retraction	173	2

secondary antibody employed in these experiments did not cross-react with the unidentified band, and equal loading was demonstrated by Coomassie blue staining, not shown). Tissue-culture cells are much more resistant to heat than embryos, surviving incubation at 39°C for at least 2 hr. Yet even this short 15-min heat shock caused a dramatic stabilization of ftz protein.

On longer exposures of such blots (Fig. 4C,D), a ladder of bands appeared above the major ftz band, but only in heat-shocked samples. Such ladders are the hallmark of proteins tagged with multi-chain ubiquitins and destined for proteolysis (Hershko and Ciechanover 1992). If these bands do indeed represent ubiquitin conjugates, these results suggest that ubiquitin-mediated proteolysis of ftz is blocked by heat shock.

We also examined the effect of heat shock on *ftz* mRNA turnover. Different results were obtained in different experiments. In two of five experiments, no significant changes in *ftz* message turnover were observed

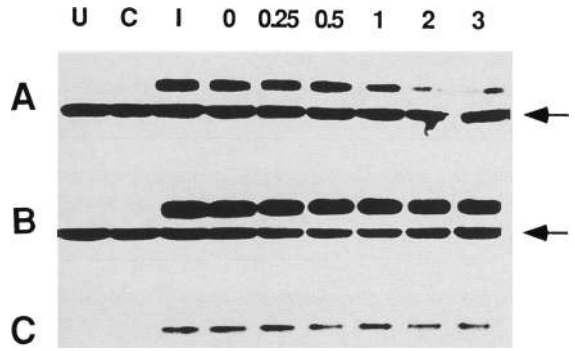

Figure 3. Turnover of wild-type and mutant ftz protein in tissue culture at 25°C. S2 cells stably transformed with various constructs expressing ftz from the *hsp70* promoter were either maintained at 25°C (U) or induced to express ftz protein—either in the presence (C) or absence of cycloheximide (1 μg/ml)—by a mild-heat treatment (33°C for 30 min) followed by a recovery at 25°C. Proteins were isolated either at the end of the induction period (I) or at the indicated time (in hr) after addition of cycloheximide to prevent further protein synthesis (0–3). Proteins were electrophoretically separated, and after transfer to membranes, ftz protein was detected using the ftz-specific antibody DMftz.1. The secondary antibody used in this experiment cross-reacted with a non-specific band (arrow) that serves as a loading control. (*A*) Wild-type ftz protein; (*B*) ftz^{Ual2} protein; (*C*) shorter exposure of the ftz region of the same blot shown in *B*.

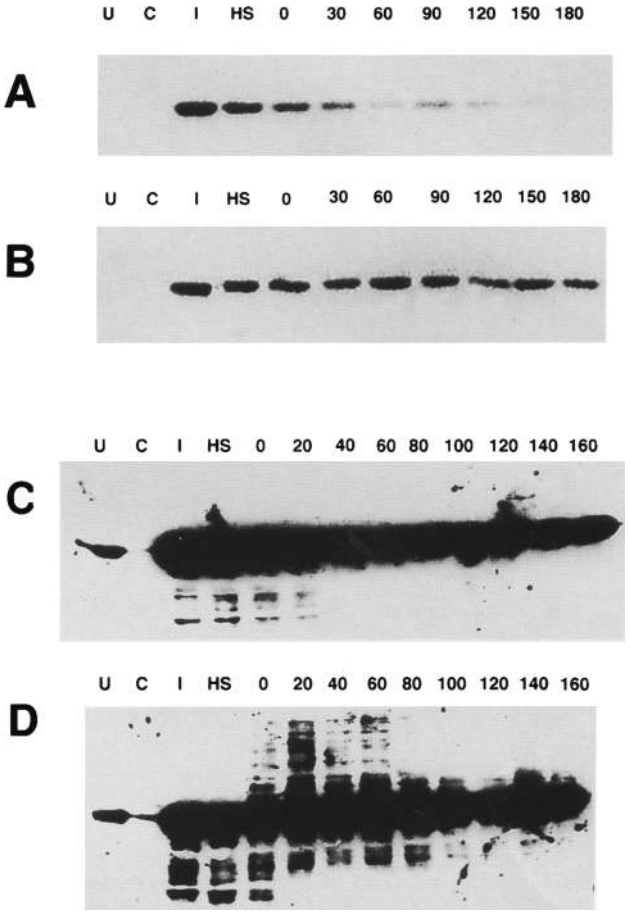

Figure 4. A brief heat pulse impairs ftz protein turnover. S2 cells stably transformed with the construct pHF3 were induced as for Fig. 3, then cycloheximide was added, and cells were exposed either to 25°C (*A,C*) or 39°C (*B,D*) for 15 min (HS marks the beginning of this heat shock). Afterwards, samples were returned to 25°C, and proteins were isolated at the indicated times (in min). Samples U, C, and I are as described for Fig. 3. (*A,B*) The drop in ftz intensity from sample I to sample 0 occurred during the time required to administer the drug to the remaining samples (<2 min). This fast turnover of a subpopulation of ftz protein was seen repeatedly, but its magnitude varied among experiments. In all cases, however, the turnover of the bulk of ftz protein was delayed after heat shock. (*C,D*) An experiment similar to that of *A* and *B*, with different time points and the blot exposed for 1.5 hr to reveal very faint signals. Shorter exposures revealed a stabilization of ftz protein similar to that of *A* and *B*.

after heat shock; in one, turnover of the *ftz* message was markedly reduced; in the remaining two experiments, turnover was completely blocked by the heat shock (data not shown). Although the effects of heat shock on mRNA turnover are more variable than on protein turnover, it seems likely that under some circumstances the stabilization of *ftz* mRNA makes a contribution to ftz overexpression after heat shock.

Evidence for ftz *overexpression in the embryo*

Next we asked if heat shock causes *ftz* overexpression in the embryo. Because measuring absolute levels of ftz in embryos is very difficult, we instead assessed ftz levels indirectly by determining the effect of heat shock on the expression pattern of *ftz* relative to that of the pair-rule gene *even-skipped* (*eve*). Early during cellularization, eve is expressed in stripes that are complementary to, and overlapping with, those of ftz. Nuclei that initially express both ftz and eve turn off the expression of both, resulting in evenly spaced, alternating ftz and eve stripes which are separated by rows of non-staining nuclei (Fig. 5A; Frasch and Levine 1987). Dosage experiments indicate that this process of stripe sharpening depends upon the mutual repression of *ftz* and *eve* (Kellerman et al. 1990; Mattson and Duncan 1990). Thus when the dosage of *eve* exceeds that of *ftz*, the zones of ftz and eve extinction are shifted so that sharpened eve stripes are broadened relative to ftz stripes. Reciprocal defects are seen when the dosage of ftz exceeds that of eve, or when ftz accumulates to excess due to stabilization by the ftz^{Ual} mutations.

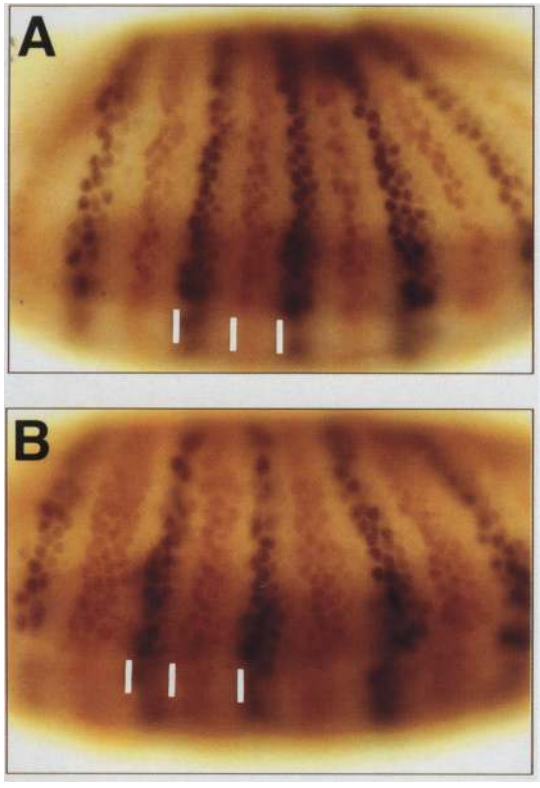

Figure 5. Altered ftz expression after heat shock in embryos. Wild-type embryos during early cellularization were either held at room-temperature (*A*) or exposed to 37°C for 15 min (*B*). After recovery at 25°C, embryos were fixed and double-labeled for ftz protein (orange) and eve protein (gray). Vertical bars mark the anterior edge of the stripes (the anterior end of the embryo is to the *left*).

In wild-type embryos, heat shock at early blastoderm caused stripe sharpening to be strongly biased in favor of ftz (Fig. 5). In non-heat shocked controls, ftz (orange) and eve (gray) were expressed in stripes of roughly similar width (Fig. 5A). In heat-treated embryos, however, ftz stripes became wider than eve stripes after stripe sharpening (Fig. 5B). As a consequence, the edges of ftz stripes were shifted anteriorly relative to the edges of eve stripes (Fig. 5A,B, white marks). This result indicates that ftz is overexpressed relative to eve following heat shock.

ftz and eve are important regulators of engrailed and thus control where parasegments form. Because engrailed is activated at the sharpened anterior edges of both ftz and eve stripes (Lawrence et al. 1987; Lawrence and Johnston 1989), engrailed stripes are evenly spaced in wild-type embryos (Fig. 6A), but show an alternating wide and narrow spacing in ftz^{Ual} embryos (Kellerman et al. 1990). A similar pattern of engrailed stripes was observed in wild-type embryos after heat shock at early blastoderm (Fig. 6B). Double staining for engrailed and ftz confirmed that the wide parasegments were those initiated by ftz (marked with arrowheads; the embryo in Fig. 6C has not yet reached high levels of engrailed expression and thus ftz staining is more readily apparent). Notably, when embryos were allowed to progress beyond stripe sharpening prior to heat shock, the spacing of engrailed stripes was not perturbed (data not shown). Thus, changes in engrailed spacing appear to result from an imbalance between ftz and eve during stripe sharpening and provide further evidence for ftz overexpression after heat shock.

In both ftz^{Ual} and heat-shocked wild-type embryos, the relative spacing of engrailed stripes normalizes during germ-band retraction. At the same time, ectopic stripes form, and gaps appear in the normal engrailed stripes (Fig. 6D; Kellerman et al. 1990, and data not shown). These defects provide yet another parallel between the ftz^{Ual} mutation and heat shock and foreshadow the segmentation defects observed in adults.

ftz *levels affect phenocopy penetrance*

Although our observations strongly suggest that heat shock causes *ftz* overexpression, they do not demonstrate that this overexpression is relevant to phenocopy induction. If the phenocopy is indeed due to elevated ftz levels, phenocopy penetrance should vary with *ftz* dosage. To test this prediction, Oregon-R females were crossed to males carrying a deletion of the *ftz* region on one chromosome and a duplication on the other (*Df(3R)Scr/Dp(3;3)D1*). The deficiency *Df(3R)Scr* removes not only *ftz*, but also the gene *Sex combs reduced*

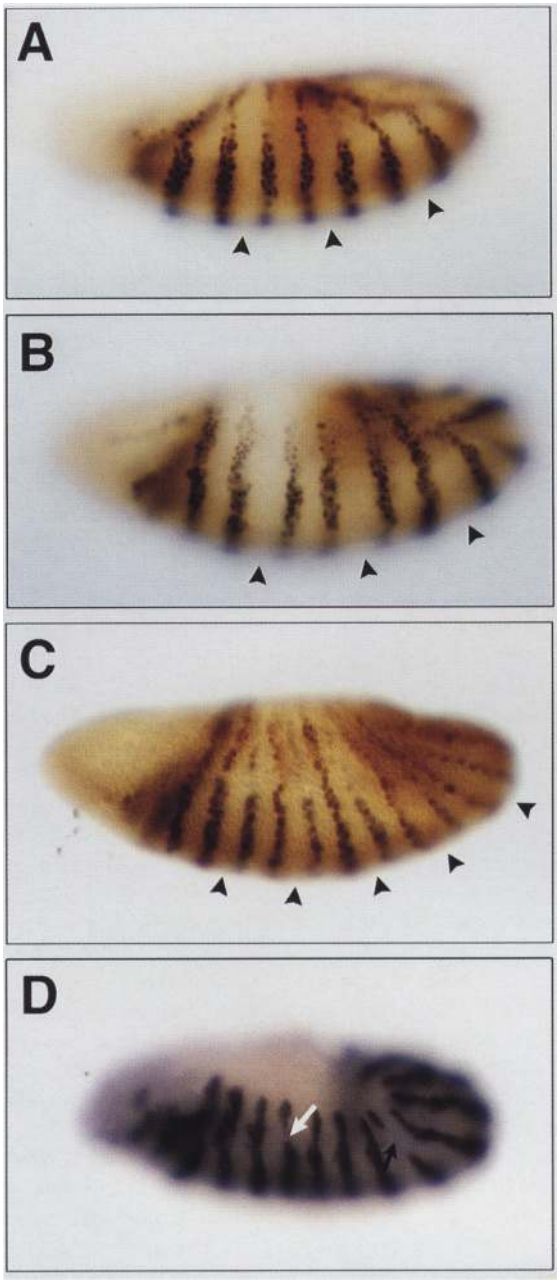

Figure 6. Heat shock alters the width of parasegments. Wild-type embryos during early cellularization were either held at room temperature (*A*) or exposed to 37°C for 15 min (*B,C,D*). After recovery at 25°C, embryos were fixed and labeled for engrailed protein (gray) and—for panels *A*, *B*, and *C*—also for ftz (orange). The even-numbered (ftz-expressing) parasegments are marked by arrowheads (a parasegment comprises the interstripe region and the engrailed stripe anterior to it). In the heat-shocked embryos early during germ-band extension (*B*, *C*), these are wider than the neighboring parasegments. Later, near the end of germ-band retraction (*D*), weakened engrailed stripes (arrowhead) and outright gaps (arrow) are observed.

(*Scr*). Male progeny heterozygous for this deficiency can therefore be recognized by their smaller sex combs while progeny heterozygous for the duplication have sex combs slightly larger than wild type.

The frequency at which heat shocks induced A1 transformations (Table 3) was much lower in flies carrying one copy of *ftz* (heterozygous for the deficiency) than in flies carrying three copies (heterozygous for the duplication). In similar experiments with wild-type flies, transformation frequencies were intermediate (Table 1). Thus, penetrance of the heat-induced defect depends on *ftz* dosage. Duncan has reported similar changes in the penetrance of the ftz^{Ual} mutation for the same deficiency and duplication (Duncan 1986).

Next, embryos carrying the ftz^{Ual1} mutation, in combination with either a wild-type or a null allele of *ftz*, were heat-shocked (Table 4). In flies where the only functional *ftz* gene was ftz^{Ual1}, heat shock resulted in an even higher increase in the penetrance of the transformation than in the flies carrying three copies of the wild-type gene (cf. Table 3). Addition of a wild-type *ftz* gene resulted in another modest increase. Thus, increased levels of *ftz* result in higher phenocopy penetrance, and *ftz* overexpression is directly related to phenocopy induction.

Discussion

By molecular, phenotypic, and genetic criteria, ftz^{Ual} mutations and heat shocks administered at the time of cellularization have remarkably similar consequences. These similarities extend from blastoderm formation through germ-band extension and germ-band retraction, and into the adult stage (summarized in Table 5). The extensive parallels indicate that the heat-induced defect is a phenocopy of the ftz^{Ual} mutation and suggest that it is caused by the same molecular lesion, overexpression of *ftz*. Direct support of this hypothesis was provided by three lines of analysis: First, heat shock blocks turnover of the ftz protein in tissue-culture cells. Second, the shift of parasegmental borders in heat-shocked embryos implies that *ftz* is overexpressed relative to *eve*. Third, and most convincing, the penetrance of the heat-induced transformation depends on the dosage of *ftz*.

It previously has been assumed that heat shock causes phenocopies either by killing subsets of cells outright or

Table 3. *Effect of* ftz *copy number on phenocopy penetrance*

	Df(3R)Scr/+		Dp(3;3)D1/+	
Sample	A1 transformation (%)	flies scored	A1 transformation (%)	flies scored
1	0	32	21	58
2	0	53	11	72
3	0	65	4	74
4	1	105	11	140
$\bar{X}$	0.4		11.3	

Embryos of the two different genotypes were siblings heat-shocked together for 15 min during cellularization (at 37°C).

Table 4. *Phenocopy induction in a* ftz^{Ual} *background*

	ftz^{Ual}/ftz^{O}		ftz^{Ual1}/ftz^{+}	
Treatment	A1 transformation (%)	flies scored	A1 transformation (%)	flies scored
No heat shock	2.8	286	7.7	300
36.5°C	25.7	70	42.6	61
37°C	22.2	284	33.3	264
$\bar{X}$ heat shock	22.9		35.1	

Embryos of the two different genotypes were siblings heat-shocked together for 15 min during cellularization. The *ftz* null allele used was ftz^{w20} (Wakimoto et al. 1984).

by reducing the amount of a specific target protein (Petersen 1990). Likely examples of the former phenomenon include reductions in organ size [e.g., microcephaly and acephaly in mammalian embryos after heat shock (Edwards 1967; Sulik et al. 1988)]. Likely examples of the latter include phenocopies of loss-of-function mutations that are enhanced when levels of the gene product are lowered genetically [e.g., *forked* and *multiple wing hairs* (Mitchell and Petersen 1985; Petersen and Mitchell 1987)].

Our hypothesis that heat shock causes a developmental defect by increasing the expression of a critical target gene is novel. Given the profound effects that heat shock has on gene regulation, however, it makes a great deal of sense. The transcription of normal cellular genes and the translation of normal messages is completely repressed in *Drosophila*, as cells devote their synthetic capacity to the production of heat-shock proteins. During recovery, the entire process must be reversed. Because individual gene products are parts of complex networks, it is critical that the normal balance of different products be preserved throughout. The stabilization of short-lived messages and proteins during heat shock should help. Without stabilization, short-lived products would all but vanish while new synthesis is suspended. Not surprisingly, many highly unstable proteins and messages are temporarily stabilized after heat shock (Andrews et al. 1987; Lüscher and Eisenman 1988; Petersen and Lindquist 1988; Petersen and Lindquist 1989; Petersen and Young 1989). Yet this stabilization introduces another problem: If degradation of these short-lived products is not restored with precisely the same kinetics as transcription and translation are restored, gene expression will be unbalanced.

Table 5. *Parallels between* ftz^{Ual} *mutations and heat shock at blastoderm*

Molecular:	spacing and width of *ftz* and *eve* stripes affected
	early *engrailed* pattern: alternating wide and narrow parasegments
	ectopic stripes and gaps in late *engrailed* pattern
	abd-A ectopically expressed in PS6 (anterior compartment)
	no ectopic *abd-A* expression before germ-band retraction
Adults:	homeotic transformation: A1 → A3
	homeotic transformation is patchy
	homeotic transformation is not completely penetrant
	segmentation defects favor A4
Genetics:	*Krüppel* mutation increases penetrance
	penetrance depends on *ftz* dosage
	Polycomb mutation increases penetrance[a]
	Bithorax complex: deletion shows higher penetrance than duplication[a]

[a]These parallels were found by comparing previous characterizations of the heat shock-induced defect (Dura and Santamaria 1983) and of the ftz^{Ual} mutations (Duncan 1986).

Two characteristics would seem to make *ftz* especially vulnerable to such imbalances. First, its protein and message are unusually short-lived, so that any imbalance between degradation and new synthesis will affect the expression of *ftz* more than that of most other genes. Second, ftz positively regulates its own expression (Hiromi and Gehring 1987; Dearolf et al. 1989). Thus, even slight overaccumulation of ftz could be amplified by the autoregulatory feedback loop, locking in an aberrant pattern. Curiously, *eve* has similar characteristics yet does not appear to be overexpressed (at least relative to *ftz*) after heat shock. Presumably, its regulation is in some subtle way more tightly controlled.

What molecular mechanisms might be responsible for the stabilization of ftz protein after heat shock? Elevated temperatures promote the denaturation and misfolding of proteins, producing a sudden increase in substrates for the quality control arm of the proteolytic machinery. Recent data indicate it is the ubiquitin pathway that is responsible for the degradation of these heat-damaged substrates (for review, see Parsell and Lindquist 1993), and it has been suggested that after heat shock this pathway is transiently overwhelmed by new substrates, delaying the turnover of short-lived proteins normally degraded by this pathway (Finley et al. 1987). Our data suggest that ftz is degraded via the ubiquitin pathway, providing a simple explanation for the accumulation of ftz after heat shock.

An appealing aspect of this model is that it provides a natural mechanism for coordinating different aspects of

gene regulation and limiting the imbalances that the massive, heat-induced changes in gene expression might otherwise cause. The degradation of short-lived proteins should resume at that point during recovery when most of the heat-damaged substrates have disappeared. This is also postulated to be the point when normal transcription and translation are restored and heat-shock protein synthesis is repressed. That is, heat-damaged transcription and translation factors will have been degraded or repaired, and the new pool of free heat-shock proteins will then be available to reinstitute the autoregulatory mechanisms that repress heat-shock protein synthesis (Craig and Gross 1991; Lindquist 1993). Thus not only would many different aspects of the heat-shock response be activated by the appearance of heat-damaged substrates, but the disappearance of these substrates through degradation and repair would coordinately restore normal gene regulation after heat shock.

In conclusion, our analysis strongly suggests that at least one heat-induced developmental defect is attributable to overexpression of a critical regulatory component. We propose that maintenance of the correct balance of regulatory proteins is one of the most heat-sensitive processes in the cell. As the roles of heat-shock proteins in protein folding and gene regulation become better defined, such information may provide avenues for ameliorating the toxic effects of heat.

Materials and methods

Fly strains

Flies were raised on standard cornmeal–agar media. Unless otherwise noted, the Oregon-R wild-type strain was used. Effects on ftz^{Ual} phenocopy penetrance were explored with the following strains (Lindsley and Zimm 1992): *Df(3R) Scr*, p^p/*Dp(3;3)D1*; ftz^{Ual1} p^p/ftz^{Ual1} p^p; ftz^{Ual3}/ftz^{Ual3}; ftz^{w20} *red e/TM3, Sb*; *cn bw sp* Kr^2/*SM1*. For most experiments involving Kr^2, the latter strain was crossed to w^{1118};*Sco/CyO*, S^2 *cn bw* to provide a balancer with more easily scored markers.

Blastoderm heat shocks

Embryos were collected for either 10 or 30 min and aged at 25°C until they had reached approximately the end of the syncytial blastoderm stage. To ensure that the majority of the embryos would be in the sensitive window for ftz^{Ual} phenocopy induction (Santamaria 1979), embryos were dechorionated in 50% bleach and inspected under a microscope. For experiments to test the influence of different genotypes, embryos were collected for 30 min and the point when the majority of the embryos had reached cellularization was determined by observation with a dissecting microscope. For the experiments assessing changes in ftz and eve expression, higher precision in timing was achieved by collecting embryos for only 10 min, and inspecting a subset periodically under a compound microscope. Obvious changes in the width of parasegments were observed if the membranes had reached about 1/2 to 3/4 of the length of the nuclei. Embryos were heat-shocked by floating them on a temperature-controlled waterbath at 37°C for 15 min unless otherwise noted. If adult phenotypes were to be assessed, embryos were transferred to food vials and allowed to develop. If embryos were to be fixed, they were either put on filter membranes and incubated in a humid chamber (for long-term recovery) or were floated on water at room temperature (for short recoveries, 2 hr and less).

Scoring heat-induced defects

To assess heat-induced changes in adult flies, the dorsal side of their abdomen was inspected under a dissecting microscope. Transformation of the first abdominal segment was judged as described previously (Santamaria 1979; Dura and Santamaria 1983).

After embryos had been stained for abd-A and engrailed protein (see below), we examined if abd-A expression extended into the anterior compartment of PS6. Embryos were scored as positive for ectopic abd-A expression only if this expression clearly extended into PS6 or was noncontiguous with the engrailed stripe. Because not the whole surface of all embryos could clearly be scored this way, the frequencies of ectopic abd-A expression given in Table 2 represent lower estimates.

Antibody labeling

All embryos were fixed, reacted with antibodies, and mounted as described (Kellerman et al. 1990). Embryos were photographed under differential interference contrast or bright-field optics on an Olympus BH-2 microscope using Kodak Gold Plus 100 film.

The primary antibodies used were anti-ftz antibody DMftz.1 (Kellerman et al. 1990), anti-abd-A antibody DMabd-A.1 (Kellerman et al. 1990), anti-engrailed antibody 4D9 (Patel et al. 1989), and anti-eve antibody 2B8 (Patel et al. 1994).

Plasmid constructions

Plasmid HF1 is a modified version of pVZ1 (Henikoff and Eghtedarzadeh 1987) carrying the *Xba*I–*Bam*HI fragment of Struhl's *hsp70–ftz* fusion gene (Struhl 1985). The *Sal*I–*Bgl*II fragment spanning the junction between the two exons was replaced with the corresponding *Sal*I–*Bgl*II fragment from either the genomic wild-type clone ftzG (Hiromi et al. 1985) or from the genomic mutant clone $ftz^{Ual2P \rightarrow H}$/pEMBL to create otherwise identical constructs encoding either the wild-type ftz protein (pHF2) or the mutant ftz^{Ual2} protein (pHFU). These constructs were used to compare the stability of wild-type and mutant protein in tissue culture. Another wild-type construct (pHF3) with additional 3′ sequences from the *ftz* gene was utilized in most experiments on ftz protein stability after heat shock. Here, the *Bgl*II fragment of ftzG carrying 3′ sequences was used to replace the corresponding *Bgl*II–*Bam*HI fragments in pHF1.

Tissue culture and transformation

Cells of Schneider's *Drosophila melanogaster* line 2 (S2 cells) were cultured and transformed as described (DiDomenico et al. 1982; Ashburner 1989). Plasmids carrying various *ftz* constructs were cotransformed with pcopneo (Rio and Rubin 1985) at a ratio of 4:3 or 1:3. Stably transformed lines were maintained by supplementing the media with 1 mg/ml of G418 (Geneticin, Gibco). Cells were grown in the absence of the drug for at least 1 week prior to the experiments to avoid drug effects.

After prolonged passage, some lines accelerated the turnover of ftz protein, presumably because even the low level of constitutive ftz expression at 25°C was detrimental.

ftz protein levels

Cells carrying *ftz* constructs were gently pelleted and resuspended in fresh media at a concentration of 10^7 cells/ml. ftz expression was induced with a 30-min incubation at 33°C. After a recovery (15–30 min) at 25°C, cycloheximide was added at a final concentration of 1 μg/ml. To demonstrate that the drug had efficiently suppressed new protein synthesis, each experiment included a control in which cycloheximide was administered before the inducing heat treatment. In all cases, this prevented the induction of ftz protein synthesis. To isolate proteins, samples were put on ice and washed briefly with 3 ml of ice-cold PBS (containing 1 mM PMSF), and proteins were precipitated and electrophoresed as described (DiDomenico et al. 1982). After transfer of proteins to Immobilon membranes (Amersham) and staining with Coomassie blue to check for equal loading, the blots were reacted with antibody DMftz.1, and bound antibody was detected with ECL reagents (Amersham) using a secondary antibody and horseradish peroxidase coupled to protein A. The exact kinetics of ftz protein turnover was somewhat variable among experiments, so only samples treated in parallel were compared. Stabilization of ftz protein by elevated temperature was observed with different transformants and all of several different constructs tested.

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The basis for a heat-induced developmental defect: defining crucial lesions.

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