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

Michael A. Welte, 1,3 Ian Duncan, 2 and Susan Lindquist 1,4

1Howard Hughes Medical Institute and Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637 USA; 2Department of Biology, Washington University, St. Louis, Missouri 63130 USA

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.

[Key Words: Heat shock; thermotolerance; phenocopy; ftz; proteolysis]

Received May 12, 1995; revised version accepted August 4, 1995.

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-
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
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 \textit{ftz\textsuperscript{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.

\textit{Krüppel} mutants are the strongest known enhancers of \textit{ftz\textsuperscript{Ual}} (Duncan 1986). We therefore asked if mutations in the \textit{Krüppel} gene also alter the penetrance of the heat-induced phenocopy. Oregon-R females were crossed to males carrying a \textit{Kr\textsuperscript{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 \textit{Kr\textsuperscript{2}} chromosome greatly increased the frequency of the heat-induced phenocopy [Table 1]. Control experiments confirmed that this \textit{Kr\textsuperscript{2}} chromosome increased the frequency of transformations due to the \textit{ftz\textsuperscript{Ual}} mutation. These enhancements were specific for A1 transformations; \textit{Kr} mutations did not raise the penetrance of segmentation defects in the abdomen either after heat shock [data not shown] or in the \textit{ftz\textsuperscript{Ual}} mutants (Duncan 1986). Thus the effects of \textit{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 \textit{ftz\textsuperscript{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-\textit{A} (\textit{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 \textit{ftz\textsuperscript{Ual}} embryos, \textit{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 \textit{Krüppel} mutation to increase penetrance and facilitate study of \textit{abd-A} expression. Embryos from a cross between \textit{Kr\textsuperscript{2}} heterozygous males and Oregon-R females (see above) were heat-shocked during cellularization, allowed to continue development, and fixed after they had completed germ-band retraction (Fig. 2). Double staining for engrailed (gray)—to demarcate posterior compartments—and \textit{abd-A} (brown) revealed that many of the heat-treated embryos showed patchy \textit{abd-A} staining in PS6 after germ-band retraction. Ectopic expression of \textit{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 \textit{abd-A} seen in the absence of heat shock.

In crosses involving \textit{Kr\textsuperscript{2}}, heat shock caused a reduction in the size of PS5 in some embryos (Fig. 2B, arrow). Also, the engraved 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 \textit{Kr\textsuperscript{2}} and \textit{ftz\textsuperscript{Ual}}. Thus, once again, heat shock at blastoderm and \textit{ftz\textsuperscript{Ual}} had identical consequences.

A curious feature of \textit{abd-A} expression in the \textit{ftz\textsuperscript{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 \textit{Kr\textsuperscript{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 \textit{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 \textit{abd-A} misexpression increased to 34%. The embryos still acquired ectopic \textit{abd-A} staining only during germ-band retraction [Table 2]. Thus, like the \textit{ftz\textsuperscript{Ual}} mutation, heat shock does not alter \textit{abd-A} expression immediately, but only after other developmental events have ensued. Although we have not attempted to follow the heat-induced misexpression of \textit{abd-A} through larval and pupal stages to map its precise relationship to the adult segmentation changes, it is likely that—as in the \textit{ftz\textsuperscript{Ual}} mutants—this misexpression is an early marker for the homeotic transformation (Kellerman et al. 1990). The almost identical adult phenotypes, \textit{abd-A} expression patterns, and \textit{Kr\textsuperscript{2}} interaction suggest that heat shock and the \textit{ftz\textsuperscript{Ual}} mutants cause homeotic transformations by a similar mechanism.

\textit{How might heat shock cause \textit{ftz} overexpression?}

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

| Heat shock | Flies heterozygous for \textit{Kr2} | Wild-type flies |
|-----------|---------------------------------|----------------|
|           | 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 |

2242 GENES & DEVELOPMENT
ftz protein [Kellerman et al. 1990]. If heat shock and the ftz\textsuperscript{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\textsuperscript{Ual} protein at 25°C. Cells transformed either with the wild-type ftz sequence or a ftz\textsuperscript{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                                             |

The 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...
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

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].

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<sup>U1al</sup> mutations.

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<sup>U1al</sup> 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<sup>U1al</sup> 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<sup>U1al</sup> 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...
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 ftzUal mutation for the same deficiency and duplication [Duncan 1986].

Next, embryos carrying the ftzUal 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 ftzUal, 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, ftzUal 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 ftzUal 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 (%) | A1 transformation (%) |
| flies scored |                  | flies scored     |
|-------------|-----------------|-----------------|
| 1           | 0               | 21              |
| 2           | 0               | 11              |
| 3           | 0               | 4               |
| 4           | 1               | 11              |
| X           | 0.4             | 11.3            |

Embryos of the two different genotypes were siblings heat-shocked together for 15 min during cellularization [at 37°C].
Crucial lesion in a heat-induced phenocopy

Table 4. Phenocopy induction in a ftz\textsuperscript{Ual} background

| Treatment          | ftz\textsuperscript{Ual}/ftz\textsuperscript{O} | ftz\textsuperscript{Ual}/ftz\textsuperscript{+} |
|-------------------|---------------------------------|---------------------------------|
|                   | 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                         |
| X heat shock      | 22.9 35.1                        | 3.3 286                          |

Embryos of the two different genotypes were siblings heat-shocked together for 15 min during cellularization. The ftz null allele used was ftz\textsuperscript{600} [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 aphabet 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 during heat shock. 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; Luscher 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.

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

Table 5. Parallels between ftz\textsuperscript{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 \rightarrow A3 homeotic transformation is patchy homeotic transformation is not completely penetrant segmentation defects favor A4 |
| Genetics:  | Krüppel mutation increases penetrance penetration depends on ftz dosage Polycomb mutation increases penetrance* Bithorax complex: deletion shows higher penetrance than duplication* |

*These parallels were found by comparing previous characterizations of the heat shock-induced defect [Dura and Santamaria 1983] and of the ftz\textsuperscript{Ual} mutations [Duncan 1986].
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 reconstitute 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 cooperatively 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 ftzG/Ld phenocopy penetrance were explored with the following strains (Lindsey and Zimm 1992): Df(3R)Sc, pF/ Dp(3;3)D1, ftzLdG pLV/ftzG, pF; ftzG/sf, pF; ftzLdG/ftzLdG, ftzG, w106 ed e/TM3, Sb; cn bw sp Kr2/SM1. For most experiments involving Kr2, the latter strain was crossed to w1118; Sco/Cyo, S alleles. To provide a balance 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 ftzLdG 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 water bath 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 mem-

branes 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 DMftz1 (Kellerman et al. 1990), anti-abd-A antibody DMabdB.1 (Kellerman et al. 1990), anti-engrailed antibody 4D9 (Patel et al. 1989), and anti-vea antibody 2B8 (Patel et al. 1994).

Plasmid constructions

Plasmid HF1 is a modified version of pVZ1 (Henikoff and Eghededarzadeh 1987) carrying the Xbal–BamHI fragment of Struhl's hsp70–ftz fusion gene (Struhl 1985). The sal–BglII fragment spanning the junction between the two exons was replaced with the corresponding sal–BglII fragment from either the genomic wild-type clone ftzG (Hiromi et al. 1985) or from the genomic mutant clone ftzG, w106 ed e/TM3, Sb; cn bw sp Kr2/SM1. For most experiments involving Kr2, the latter strain was crossed to w1118; Sco/Cyo, S alleles to provide a balance 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 ftzLdG 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 water bath 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 DMftz1 (Kellerman et al. 1990), anti-abd-A antibody DMabdB.1 (Kellerman et al. 1990), anti-engrailed antibody 4D9 (Patel et al. 1989), and anti-vea antibody 2B8 (Patel et al. 1994).

Plasmid constructions

Plasmid HF1 is a modified version of pVZ1 (Henikoff and Eghededarzadeh 1987) carrying the Xbal–BamHI fragment of Struhl’s hsp70–ftz fusion gene (Struhl 1985). The sal–BglII fragment spanning the junction between the two exons was replaced with the corresponding sal–BglII fragment from either the genomic wild-type clone ftzG (Hiromi et al. 1985) or from the genomic mutant clone ftzG, w106 ed e/TM3, Sb; cn bw sp Kr2/SM1. For most experiments involving Kr2, the latter strain was crossed to w1118; Sco/Cyo, S alleles to provide a balance with more easily scored markers.

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 co-transformed with pcoNeo (Rio and Rubin 1985) at a ratio of 4:3 or 1:3. Stably transformed lines were maintained by suppl}
ftz protein levels

Cells carrying ftz constructs were gently pelleted and resuspended in fresh media at a concentration of 10⁷ 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) and staining with Coomassie blue to check for equal loading. The bound antibody was detected with ECL reagents (Amersham) and staining with Coomassie blue to check for equal loading. 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 transfectants and all of several different constructs tested.

Acknowledgments

We thank G. Struhl for the original hsp70–ftz fusion construct, the Bowling Green Stock Center for fly strains, N. Patel for antibodies, and D. Duncan for expert technical advice on embryonic fixation and staining. We thank J. Feder and Z. Wang for comments on the manuscript. This work was supported by the Howard Hughes Medical Institute. M.A.W. was supported by a Howard Hughes Predoctoral Fellowship.

The publication costs of this article were defrayed in part by the advertisement in accordance with 18 USC section 1734 solely to indicate this fact.

References

Andrews, G.K., M.A. Harding, J.P. Calvet, and E.D. Adamson. 1987. The heat shock response in HeLa cells is accompanied by elevated expression of the c-fos proto-oncogene. Mol. Cell. Biol. 7:3452–3458.

Ashburner, M. 1989. Drosophila: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Dearolf, C.R., J. Topol, and C.S. Parker. 1989. Transcriptional control of Drosophila fushi tarazu zebra stripe expression. Genes & Dev. 3: 384–398.

Craig, E.A. and C.A. Gross. 1991. Is hsp70 the cellular thermometer? Trends Biochem. Sci. 16: 135–140.

DiDomenico, B.J., G.E. Bugaisky, and S. Lindquist. 1982. The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. Cell 31: 593–603.

Duncan, I. 1986. Control of bithorax complex functions by the segmentation gene fushi tarazu of D. melanogaster. Cell 47: 297–309.

Dura, J.-M. and P. Santamaria. 1983. Heat shock-induced phenotypes: cis-regulation of the bithorax complex in Drosophila melanogaster. Mol. Gen. Genet. 189: 235–239.

Edgar, B.A., M.P. Weir, G. Schubiger, and T. Kornberg. 1986. Repression and turnover pattern of fushi tarazu RNA in the early Drosophila embryo. Cell 47: 747–754.

Edwards, M.J. 1967. Congenital defects in guinea pigs following induced hyperthermia during gestation. Arch. Pathol. 84: 42–48.

Edwards, M.J., R. Mulley, S. Ring, and R.A. Wanner. 1974. Mitotic cell death and delay of mitotic activity in guinea-pig embryos following brief maternal hyperthermia. J. Embryol. Exp. Morphol. 32: 593–602.

Finley, D., E. Ožkaynak, and A. Varshavsky. 1987. The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. Cell 48: 1035–1046.

Frasch, M. and M. Levine. 1987. Complementary patterns of even-skipped and fushi tarazu expression involve their differential regulation by a common set of segmentation genes in Drosophila. Genes & Dev. 1: 981–995.

Goldschmidt, R. 1935a. Gen und Ausseneigenschaft [Untersuchungen an Drosophila] I. Z. Indukt. Abstamm. Vererbungsl. 69: 38–69.

Hershko, A. and A. Ciechanover. 1992. The ubiquitin system for protein degradation. Annu. Rev. Biochem. 61: 761–807.

Howard Hughes Medical Institute. M.A.W. was supported by a Howard Hughes Predoctoral Fellowship.

The publication costs of this article were defrayed in part by the advertisement in accordance with 18 USC section 1734 solely to indicate this fact.
induction in Drosophila. Cell 15: 907–918.
Mitchell, H.K. and N.S. Petersen. 1982. Developmental abnormalities in Drosophila induced by heat shock. Dev. Genet. 3: 91–102.
Mitchell, H.K. and N.S. Petersen. 1985. The recessive phenotype of forked can be uncovered by heat shock in Drosophila. Dev. Genet. 6: 93–100.
Novo, L. 1991. Heat Shock Response [ed. L. Novo], CRC Press, Boca Raton, FL.
Parsell, D.A. and S. Lindquist. 1993. The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27: 437–496.
Patel, N.H., E. Martin-Blanco, K.G. Coleman, S.J. Poole, M.C. Ellis, T.B. Kornberg, and C.S. Goodman. 1989. Expression of engrailed proteins in arthropods, annelids, and chordates. Cell 58: 955–968.
Patel, N.H., B.G. Condron, and K. Zinn. 1994. Pair-rule expression patterns of even-skipped are found in both short- and long-germ beetles. Nature 367: 429–434.
Petersen, N.S. 1990. Effects of heat and chemical stress on development. Adv. Genet. 28: 275–296.
Petersen, N.S. and H.K. Mitchell. 1987. The induction of a multiple wing hair phenocopy by heat shock in mutant heterozygotes. Dev. Biol. 121: 335–341.
Petersen, N.S. and P. Young. 1989. Effects of heat shock on protein processing and turnover in developing Drosophila wings. Dev. Genet. 10: 11–15.
Petersen, N.S. and H.K. Mitchell. 1991. Environmentally induced development defects in Drosophila. Results Probl. Cell Differ. 7: 29–43.
Petersen, R. and S. Lindquist. 1988. The Drosophila hsp70 message is rapidly degraded at normal temperatures and stabilized by heat shock. Gene 72: 161–168.
Petersen, R.B. and S. Lindquist. 1989. Regulation of HSP70 synthesis by messenger RNA degradation. Cell Regul. 1: 135–149.
Rio, D.C. and G.M. Rubin. 1985. Transformation of cultured Drosophila melanogaster cells with a dominant selectable marker. Mol. Cell. Biol. 5: 1833–1838.
Sánchez-Herrero, E., I. Vernós, R. Marco, and G. Morata. 1985. Genetic organization of Drosophila bithorax complex. Nature 313: 108–113.
Santamaria, P. 1979. Heat shock induced phenocopies of dominant mutants of the bithorax complex in Drosophila melanogaster. Mol. Gen. Genet. 172: 161–163.
Struhl, G. 1985. Near-reciprocal phenotypes caused by inactivation or indiscriminate expression of the Drosophila segmentation gene ftz. Nature 318: 677–680.
Sulik, K.K., C.S. Cook, and W.S. Webster. 1988. Teratogens and craniofacial malformations: Relationships to cell death. Dev. Suppl. 103: 213–231.
Zimmerman, J.L. and P.R. Cohill. 1991. Heat shock and thermotolerance in plant and animal embryogenesis. New Biol. 3: 641–650.
The basis for a heat-induced developmental defect: defining crucial lesions.

M A Welte, I Duncan and S Lindquist

*Genes Dev.* 1995, 9:
Access the most recent version at doi:10.1101/gad.9.18.2240

References
This article cites 45 articles, 9 of which can be accessed free at:
http://genesdev.cshlp.org/content/9/18/2240.full.html#ref-list-1

License

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.