Transcriptional regulation of the *Drosophila* homeotic gene *teashirt* by the homeodomain protein Fushi tarazu

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Abstract

The *Drosophila* melanogaster gene *teashirt* (*tsh*) is essential for segment identity of the embryonic thorax and abdomen. A deletion 3′ to the *tsh* transcription unit causes the loss of *tsh* early expression in the even-numbered parasegments, and the corresponding larval cuticular patterns are disrupted. *tsh* function in the odd-numbered parasegments in these mutants is normal by both criteria. The in vivo activities of genomic fragments from the deleted region were tested in transgenic embryos. A 2.0 kb enhancer from the 3′ region acts mainly in the even-numbered parasegments and is dependent on *fushi tarazu* (*ftz*) activity, which encodes a homeodomain protein required for the development of even-numbered parasegments. *Ftz* protein binds in vitro to four distinct sequences in a 220 bp sub-fragment; these and neighboring sequences are conserved in the equivalent enhancer isolated from *Drosophila virilis*. Tsh protein produced under the control of the 220 bp enhancer partially rescues a null *tsh* mutation, with its strongest effect in the even-numbered parasegments. Mutation of the *Ftz* binding sites partially abrogates the capacity for rescue. These results suggest a composite mechanism for regulation of *tsh*, with different activators such as *ftz* contributing to the overall pattern of expression of this key regulator. © 1997 Elsevier Science Ireland Ltd.

Keywords: Drosophila; Fushi tarazu; Regulation; Teashirt

1. Introduction

The homeotic genes (Hox genes) are required to establish and maintain segmental identity in organisms including many vertebrates and invertebrates. In *Drosophila*, the Hox genes lie within the Antennapedia and Bithorax complexes (Lewis, 1978; Sanchez-Herrero et al., 1985; Kaufman et al., 1990). The proteins encoded by these genes share a common sequence of 60 amino acids, called the homeodomain (HD) (McGinnis et al., 1984; Scott and Weiner, 1984), which is required for DNA binding activity (Desplan et al., 1985; Müller et al., 1988). Hox genes are expressed from early embryogenesis to adulthood in specific domains (restricted to one or a group of segments) along the antero-posterior axis of the animal.

Other homeotic genes have been described, lying outside the Hox clusters and structurally different from the conventional Hox proteins (Jürgens, 1988; Jürgens and Weigel, 1988). One of them is the *teashirt* (*tsh*) gene which encodes a zinc finger protein (Fasano et al., 1991). By contrast to the classical Hox genes which are functional in restricted domains, *tsh* is expressed and required in a broader region corresponding to the trunk (thorax and abdomen) (Fasano et al., 1991). Previous genetic analyses have demonstrated that *tsh* cooperates with the other homeotic proteins from the Antennapedia and Bithorax complexes to promote global trunk identity versus head development. Furthermore it has been shown that *tsh* is specifically required to determine
the identity of the prothoracic segment (Röder et al., 1992; De Zulueta et al., 1994).

The spatial expression patterns of the Hox genes are initiated early in embryogenesis by sets of factors (Duncan, 1986; Ingham and Martinez-Arias, 1986; White and Lehmann, 1986; Reinitz and Levine, 1990) which are transiently expressed in embryos. In later stages, these restricted patterns are maintained by different, but non-exclusive, mechanisms such as auto-regulation, cross-regulatory interactions between Hox proteins and transcriptional regulation by the Trithorax and Polycomb group of genes. It has been shown, previously, that tsh expression is regulated during embryogenesis by Hox genes in ectoderm and mesoderm derivatives (Röder et al., 1992; Mathies et al., 1994). Moreover, some of these homeodomain transcription factors, including Antennapedia (Antp), Ultrabithorax (Ubx) and abdominal-A (abd-A), bind directly to a specific enhancer in the tsh regulatory region (McCormick et al., 1995).

Expression analysis of the endogenous tsh transcript, or a lacZ transgene driven by this specific tsh enhancer, in homeotic mutant embryos, indicate that Hox proteins are not required for initiation of the tsh expression but rather modulate and maintain the expression pattern in a segment-specific manner. Genetic evidence suggests that tsh is activated and restricted in the trunk of early embryos by a combination of maternal and segmentation genes (Röder, unpublished data). Apparently, none of the tested maternal, gap or pair rule mutants in isolation are capable of completely abolishing tsh transcription, indicating that these genes may act together throughout the embryo to pre-determine the trunk-specific tsh expression domain.

Extensive genetic and molecular studies have demonstrated that the expression pattern of the Hox genes is established by maternal and segmentation gene products acting as transcriptional activators or repressors in order to delimit expression boundaries (Riley et al., 1987; Carroll et al., 1988; Irish et al., 1989; Jack and McGinnis, 1990; Tenharmsel et al., 1993; Macias et al., 1994; Mann, 1994; Casares and Sanchez-Herrero, 1995). However, little evidence is available for a direct in vivo interaction of these regulatory proteins with Hox control elements (Müller and Bienz, 1992). Among these transcriptional factors, the protein encoded by the fushi tarazu (ftz) pair rule gene plays an essential role in activating the transcription of some homeotic genes (Ingham and Martinez-Arias, 1986) within even-numbered parasegments (ps). The transcription of Ubx is directly activated by Ftz protein through DNA binding sites located in specific cis-regulatory regions (Qian et al., 1991, 1993; Zhang and Bienz, 1992; Müller and Bienz, 1992). However, no published data clearly demonstrate such a direct regulation of Ftz to other Hox enhancers in vivo.

The ftz gene encodes a HD protein that acts as a transcriptional activator (Jaynes and O’Farrell, 1988; Fitzpatrick and Inglès, 1989; Han et al., 1989; Winslow et al., 1989; Ohkuma et al., 1990; Schier and Gehring, 1992). In vitro, it binds to a consensus TCAATTAAAT sequence (Desplan et al., 1988), which is also recognized by other HD-containing proteins (Müller et al., 1988; Hoey and Levine, 1988; Thaller et al., 1988; Jaynes and O’Farrell, 1988; Han et al., 1989; Ohkuma et al., 1990). How do similar proteins, which recognize the same or similar targets in vitro, have distinct functions in vivo? Several mechanisms (reviewed in Affolter et al., 1990; Hayashi and Scott, 1990) have been proposed including: distinct amino acid, and therefore distinct target sequences (Hanes and Brent, 1989, 1991; Treisman et al., 1989; Percival-Smith et al., 1990; Schier and Gehring, 1992; Furukubo-Tokunaga et al., 1992); competition for common target sequences depending on differential binding affinities and/or local concentrations (Gonzalez-Reyes et al., 1990; Lamka et al., 1992; Zeng et al., 1993; Capovilla et al., 1994); unique spatio-temporal expression patterns following cross regulation (reviewed in Akam, 1987; Ingham, 1988) and interactions with other regulatory proteins (Stern and Herr, 1991; Smith and Johnson, 1992; Copeland et al., 1996).

Here we describe the regulation of tsh by the pair-rule segmentation ftz gene in the embryo. We have obtained from a genetic screen a tsh mutant, Df(2L)R6, that lacks a large cis-regulatory region 3’ to the gene, which is characterized by a cuticular pair rule-like phenotype partially reminiscent of the ftz loss of function mutant phenotype. This mutation affects the early embryonic expression pattern of tsh, since it is no longer expressed in the even-numbered parasegments, which are delimited by the ftz activity. We show that this tsh deficiency deletes a cis-regulatory element responding to Ftz and our data strongly suggest that Ftz directly controls, in vivo, embryonic tsh expression in the trunk in even-numbered parasegments.

2. Results

2.1. An early embryonic regulatory element from the tsh gene

Little is known concerning the cis-regulatory domains of the tsh gene and the factors required for their modulation. In order to gain insight into regulation, we used enhancer trap insertions to induce new mutations in the tsh gene. The P(Lac w)2-IV insertion is homozygous, viable and located about 30 kb downstream of the tsh coding region; two imperfect excision events derived from this insert, giving Df(2L)R6 and Df(2L)R27 (Fig. 1B), are particularly informative with respect to early regulation of tsh.

Wild-type larval cuticle patterns consist of ventral denticle belts localized in three thoracic and eight abdominal (or trunk) segments (Fig. 2A), which derive from embryonic parasegments (ps) 3–13. Normal denticle belts are approximately equidistant from each other, separated by naked cuticle. Null alleles of tsh reduce the size of all trunk segments (Fig. 2C) (Fasano et al., 1991). Homozygous or hemi-
zygous Df(2L)R6 embryos exhibit a weak tsh\(^{-}\) phenotype, with segment size reduced only in alternate trunk segments, i.e. the mesothorax, the first abdominal segment and so on (Fig. 2B). By contrast, Df(2L)R27 mutation disrupts only the development of the prothorax (data not shown).

Southern blots show that Df(2L)R6 deletes about 20 kb of genomic DNA, 3’ to the tsh coding region. Df(2L)R27 deletes a region that partially overlaps the proximal part of the Df(2L)R6 deficiency (Fig. 1B). By comparing the two mutations, we conclude that the Df(2L)R6 pair-rule-like phenotype is caused by the absence of genomic DNA covering approximately 12 kb (Fig. 1B).

Transcription of the tsh gene was compared in wild-type (Fasano et al., 1991) and homozygous Df(2L)R6 embryos. In Df(2L)R6 embryos, transcripts are initially detected in a narrower region only 4–5 cell diameters wide compared to the 16 seen in the central part of wild type blastoderm embryos (Fig. 3A,B). During gastrulation, normal tsh transcript is expressed in ps 3–13 (Fig. 3C,E,G). In deletion homozygotes, transcription is also restricted to the trunk region but is expressed in a 6-striped pattern (Fig. 3D,F,H).

The precise location of the tsh stripes in Df(2L)R6 embryos was determined by comparing the location of Fushi tarazu (Ftz) or Invected (Inv) proteins with that of tsh messages in the same embryos. Ftz and Inv are, respectively, markers for even-numbered ps (Lawrence and Johnston, 1989) and anterior region of all parasegments (Coleman et al., 1987) in early embryos (before segment transition). The main band of tsh message in late blastoderm mutant embryos overlaps and extends anteriorly to the Inv stripe localized in ps 4 (Fig. 4A). Lower levels of tsh transcripts are localized posteriorly to this Inv stripe in ps 5. Soon afterwards the tsh transcript distribution pattern in Df(2L)R6 embryos is complementary to that of Ftz (Fig. 4B) except for the first tsh stripe, which overlaps Ftz protein in ps 4. Since Ftz is located in evenly-numbered ps, tsh transcripts in Df(2L)R6 homozygotes are largely restricted to the odd-numbered ps from ps3 to ps13 at the blastoderm stage.

In gastrulating Df(2L)R6 embryos, tsh is still expressed in the odd-numbered ps and additionally in the posterior compartments of even-numbered ps (Fig. 4C). Finally, at the extended germ band stage, tsh is expressed in all compartments of the trunk (data not shown). Together the results suggest that the Df(2L)R6 mutation deletes an element responsible for the activation of tsh transcription in the even-numbered ps of the trunk in early embryos and seems to delay temporal activation of tsh in these ps. We assume that the Df(2L)R6 mutant cuticular phenotype (Fig. 2B) is a consequence of the down-regulation of tsh expression in early stages of development (Fig. 4), since pattern deletions derive from even-numbered ps in this mutant.

2.2. tsh transcription depends on ftz activity

Genetic data have shown that a set of maternal, gap and pair rule genes is necessary to control the expression of tsh in blastoderm stage embryos (Röder, unpublished data). As tsh transcription is never completely abolished in mutants for these genes, it seems that the establishment of the tsh expression boundaries is controlled by the combined activities of maternal and segmentation genes.
The phenotype of Df(2L)R6 mutant embryos probably reflects the lack of control of tsh transcription exerted by one or several genes of the pair-rule class that are essential for the development of even-numbered ps. The pair-rule gene ftz is a likely candidate since it is specifically required to delimit the even-numbered ps (Wakimoto and Kaufman, 1981; Hafen et al., 1984; Carroll and Scott, 1985; Lawrence et al., 1987).

To test whether Ftz is a putative regulator, tsh transcription was examined in ftz^- embryos. Compared to the wild type at the blastoderm stage (Fig. 3A), the anterior boundary of tsh expression pattern seems normal in ftz^- homozygotes. However, tsh messages are less abundant or missing from the posterior domain in mutant embryos (Fig. 5A). This distribution, restricted to the first stripe of tsh expression, closely resembles that observed in Df(2L)R6 embryos at the same stage (Figs. 3B and 4A). Note that at this stage the number of cells in a ftz mutant embryo is normal, so loss of expression is due to a change in regulation and not a loss of cells. The absence of ftz activity therefore partially abolishes tsh transcription during early embryogenesis.

The embryonic pattern of tsh transcription was examined in embryos where the Ftz protein is ubiquitously expressed under the control of a heat shock promoter (phsftz) (Struhl, 1985; Krause et al., 1988). Following overproduction of Ftz protein for a 20-min period at 2–4 h of development, tsh transcription was analyzed at different embryonic stages. In blastoderm stage embryos, tsh transcripts are still detected within ps3 to 13, but in place of a transient 5-striped pattern (Fig. 3C), they are distributed in two large domains separated by a band of non-labelled cells (Fig. 5B) showing that ectopic Ftz can affect tsh expression. Later (Fig. 5C,D), expression of the phsftz transgene induces ectopic tsh expression in the tail region of stage 11 embryos. Together these experiments support the idea that ftz behaves as an activator of tsh transcription.
2.3. \textit{In vivo activity of genomic DNA from the Df(2L)R6 region}

To test whether the \textit{Df(2L)R6} mutation deletes enhancer elements responsible for \textit{tsh} expression in the even-numbered ps, different genomic fragments from the deleted region were fused to a minimal promoter driving \textit{lacZ} expression and introduced into flies to test for \textit{in vivo} enhancer activity. \textit{LacZ} transcripts under the control of an 8.0 kb \textit{Bam} HI fragment (p8.0lacZ; Fig. 1) are detected from the late blastoderm in a pattern of 5 stripes. Using double labeling with an anti-Inv antibody, we found that \textit{lacZ} mRNA is largely restricted to the even-numbered ps (Fig. 6) during early gastrulation. This pattern is complementary to the \textit{tsh} transcription pattern observed in \textit{Df(2L)R6} embryos (Fig. 4B) at this stage. This result indicates that the 8.0 kb genomic fragment includes regulatory elements required for the activation of \textit{tsh} transcription in even-numbered ps. At the germ band extension stage, this enhancer drives \textit{lacZ} expression in ps 3 to ps 13 in a similar way to the endogenous \textit{tsh} transcript, suggesting that, latter, the 8.0 kb enhancer contributes to the activation of \textit{tsh} expression in both even
and odd-parasegments in the trunk region during later stages (data not shown). No other early acting embryonic enhancer has been found in 35kb of genomic region surrounding the tsh coding region (Coré, unpublished data).

In order to define a putative Ftz-response element, we dissected the 8.0 kb regulatory region further (Fig. 1). Transformant lines with the same vector were established with a 2.0 kb XhoI subfragment (p2.0lacZ) of the 8.0 kb one.
2.4. ftz$^+$ activity is necessary for in vivo activity of the 2.0 kb tsh enhancer

To ask whether the putative enhancer could contain Ftz
response elements, we crossed the p2.0lacZ lines to ftz
mutants. In situ hybridization with a lacZ probe to
p2.0lacZ ftz embryos (Fig. 7B,D,F) shows that the striped
pattern of the reporter gene at the blastoderm stage is com-
pletely abolished in the absence of ftz$^+$ activity in the trunk
domain (compare with wild type embryos in Fig. 7A,C,E).
In older embryos, when the germ band is extending, the odd-
umbered ps alone exhibit β-gal activity (compare Fig.
7E,F).

In phsftz, ectopic expression of Ftz induces additional
transcription of lacZ in ps 14, in embryos carrying the
p2.0LacZ construct (Fig. 7G). LacZ expression is also stron-
ger and more frequent in ps 0 and 2 compared to non-heat
shock controls.

These data reveal that the 2.0 kb fragment contains one or
several Ftz-dependent enhancers required to control the in
vivo transcription of tsh in embryos.

2.5. In vitro binding of Ftz protein to the 2.0 kb tsh
regulatory domain

To test if the Ftz protein recognizes the putative 3' tsh
regulatory element in vitro, full-length Ftz protein and poly-
clonal anti-Ftz antibody (Krause et al., 1988) were used to
immunoprecipitate XhoI digested fragments comprising the
Df(2L)R6 deletion. Four fragments of 2.0, 3.6, 4.0 and 4.9
kb were tested; only the 2.0 kb XhoI fragment described
above (Fig. 1C) shows specific binding of the Ftz protein,
especially within a 1.5 kb Hinfl sub-fragment (Fig. 8A). The
2.0 kb XhoI fragment was then cut by Clal and MspI and
using gel shift assays, two subfragments of 220 (Fig. 8B)
and 800 bps (data not shown) were specifically bound to the
Ftz protein. Within the Ftz homeodomain (HD) peptide we
observed differential shifts with increasing amounts of pro-
tein on the 220 bp fragment (Fig. 8B, lanes 5–8), corre-
sponding to different protein/DNA complexes. The HD
seems to bind to one site with high affinity and to three
other sites with weaker affinity, as judged from the sequen-
tial appearance of retarded bands with increasing amounts
of protein. The specificity of the binding is confirmed by
competition assays using increasing levels of unlabelled 220
bp fragments as a specific probe (Fig. 8, lanes 12–14) and
poly(dIdC) as a non-specific competitor (Fig. 8, lanes 9–
11). Paradoxically, reaction with the full-length protein
reveals only one shifted band. The full-length protein may
bind to one high affinity site or to all the sites with the same
affinity, and once bound may exclude other Ftz molecules
from binding.

We concentrated on the 220 bp fragment for DNase I
protection studies with the Ftz HD peptide. The nucleotide
sequence of the 220 bp reveals 3 ATTA motifs (Fig. 8D),
the core consensus sequence recognized by many homeo-
domain proteins (Beachy et al., 1988; Desplan et al., 1988; Hoey and Levine, 1988; Müller et al., 1988; Driever et al., 1989). Purified Ftz HD protects four sites (Fig. 8C,D). Three of the protected regions overlap with the ATTA sequences. None of the four sites shares sequence homology outside the ATTA with any published Ftz binding site (Desplan et al., 1988; Pick et al., 1990; Müller and Bienz, 1992; Schier and Gehring, 1992).

2.6. A Drosophila virilis sequence homologous to the 220 bp fragment

DNA corresponding to the tsh gene, including encoding sequence and regulatory regions was cloned from the Drosophila virilis strain by cross homology. Comparative analysis of the 220 bp regulatory domain reveals that the virilis and melanogaster derived sequences are highly conserved, notably within the regions flanking the four putative Ftz-binding sites (Fig. 8D) reinforcing the functional relevance of this enhancer.

2.7. Partial rescue of tsh mutants by the 220 bp enhancer driving a tsh cDNA

We made a lacZ transgene under the control of the 220 bp fragment. Several different lines carrying this transgene gave similar results irrespective of the number (up to 3) of insertions used. LacZ expression can be detected at the blastoderm stage but never in more than 50% of embryos. When detected, lacZ is always expressed in ftz-like stripes (Fig. 9A,B), often with the second stripe appearing later than the others (Fig. 9A, arrow). At later stages, lacZ is detected in all segments in the trunk (Fig. 9C). We attempted to analyze the effects of loss or gain of ftz function on these transgenes. Due to the non-penetrant and weak nature of the lacZ expression, we were unable to determine with certitude whether ftz modifies the activity of this enhancer. However, the striped pattern observed and the in vitro experiments suggest that ftz may regulate this enhancer.

To bypass the problem of weak expression of the 220 bp regulatory element, we attempted to analyze its function in vivo. The 220 bp enhancer was incorporated into a minigene driving tsh expression from its own promoter (see Section 4). Two different transgenic lines were tested for their ability to rescue tsh null embryos.

Each wild-type hemi-thoracic segment bears a Keilin’s organ located ventro-laterally below two, more dorsal, black dot organs. These sensory structures derive from the anterior compartment of each segment with the exception of one (of the three) hair(s) from each Keilin organ which derives from the posterior compartment (Hama et al., 1990) (Fig. 10A). Thus the parasegmental origin of each sensory structure is known. In tsh<sup>22</sup> (null) embryos only the dorsally located black dot organs differentiate; the Keilin’s organs never develop (Fasano et al., 1991) (Fig. 10B). Weak rescue activity is observed in all tsh<sup>+</sup> embryos carrying the p220tsh minigenes presumably reflecting the late activity of the enhancer (Fig. 9C). Improved rescue was observed in the even-numbered ps in the thorax. Two criteria were used to measure this activity: the spacing between denticle belts (upper panel, Fig. 10E) and the presence of Keilin’s organs (lower panel, Fig. 10E) in the thoracic segments. Each pair of denticle belts is separated by naked cuticle which is drastically reduced in tsh null larvae (Figs. 1A,C and 10B).
Rescue is much more complete in the mesothorax compared to the metathorax as a consequence of tsh activity under the control of the 220 bp enhancer (Fig. 10C,D; column 2,E). In the best cases, two Keilin organ hairs are detected in the mesothorax and one in the metathorax (8 cases; Fig. 10D). In many cases the spacing between denticle belts is greater in the mesothorax compared to the metathorax (12 cases; Fig. 10C). Although the parasegmental origin of the observed Keilin organs is not known in these experiments, it seems likely that the mesothoracic organs derive from ps 4 and the metathoracic organs derive from ps 6, in keeping with the expectation that the 220 bp enhancer gives a higher level of Tsh activity in even-numbered ps compared to the odd-numbered ones.

2.8. In vivo mutational analysis of the Ftz binding sites from the 220 bp enhancer

We have tested the functional significance of the four Ftz binding sites by altering them to GGATTA which binds preferentially to the Bicoid (Bcd) HD protein and poorly to Ftz (see Section 4) (Pick et al., 1990). The altered enhancer was incorporated into a minigene and tested for rescuing ability. Rescue activity from this construct was weak but detectable (column 3 in Fig. 9E) showing that the construct produces active Tsh protein. For the establishment of Keilin's organs, rescue was more complete in the mesothorax compared to the metathorax with this construct (column 3 lower panel, Fig. 10E). Nevertheless, rescue from the
mutated enhancer is not as effective as with the minigene under the control of the wild-type enhancer (column 2 lower panel, Fig. 10E), emphasizing the fact that Ftz-binding sites are required for the activity of the 220 bp enhancer.

Together our data strongly suggest that Ftz protein acts as a direct activator of tsh expression in the even-numbered ps in the embryonic trunk region.

3. Discussion

The tsh gene is expressed in the trunk (thorax and abdomen) of Drosophila embryos, where it acts together with Hox genes to establish normal segmental identity (Fasano et al., 1991; Röder et al., 1992). We have identified a cis-regulatory region involved in the transcriptional control of tsh expression in even-numbered ps, which is dependent on ftz” gene function. We show that Ftz binds to a 220 bp enhancer from this cis-regulatory domain, which is necessary in vivo for activation of tsh transcription during embryogenesis. In vivo activity of the 220 bp tsh enhancer is partly reduced by mutation of the putative Ftz-binding sites suggesting that Ftz directly activates tsh transcription via this regulatory element. As ftz also activates early Hox gene expression (Ingham and Martinez-Arias, 1986), the coordinated activation of Hox and tsh expression is set up early.
lular blastoderm stage, could be activated differently in even-numbered and odd-numbered ps. In Df(2L)R6 mutant embryos, indeed, tsh transcripts are missing in even-numbered ps at blastoderm and gastrulation (Figs. 3 and 4) and a 2.0 kb fragment (and a 0.22 kb subfragment), included within the deletion, directs lacZ expression in vivo in these embryonic trunk ps (Figs. 7, and 9). Over a 35 kb long genomic region at the tsh locus, we have not detected any other enhancers capable in isolation of driving the expression of a lacZ transgene in early embryos (Coré, unpublished data). Especially, we have not isolated a regulatory element required for odd-numbered ps expression of the tsh gene in early embryos, but it is not excluded that such an element is located outside the 35 kb region. It is noteworthy that from the extended germ band stage, the 2.0 kb regulatory element is able to activate reporter gene transcription in both even and odd-parasegments (Fig. 7), suggesting that a second set of regulatory factors is required to control tsh expression until the end of embryogenesis. Antp and Ubx proteins, which contain homeodomains (HD) similar to Ftz, are likely candidates for this late activation process: loss or gain of function mutations alter β-gal activity driven by the 2.0 kb enhancer, and both the Antp and Ubx proteins recognize sequences in vitro within it (McCormick et al., 1995).

The 2.0 kb element studied here lacks some repressor or silencer elements since expression of the lacZ reporter gene is not limited to the trunk, the normal domain of tsh expression (Fasano et al., 1991). LacZ, under the influence of the 0.22, 2.0 or 8.0 kb fragments, is expressed in a head domain during the cellular blastoderm and gastrulation stages (Figs. 5, 7 and 9) and occasionally in two gnathal ps at the elongated germ band stage. The Df(2L)R6 mutant does not express tsh messages within the head region (Fig. 3) suggesting that other parts of the gene play crucial roles for restricting tsh transcription to the trunk of the developing embryo.

3.2. tsh transcription is dependent on ftz gene activity

tsh transcription is activated early in embryogenesis, at the cellular blastoderm stage, when the parasegmental boundaries are being laid down in the embryo (Martinez-Arias and Lawrence, 1985; Lawrence et al., 1987). The metamized embryo is made up of even- and odd-numbered ps controlled, in part, by the activities of the pair-rule genes ftz and even-skipped (eve) respectively (Lawrence et al., 1987; Lawrence and Johnston, 1989). Several roles for ftz function have been proposed: first, it activates engrailed in order to delimit the sharp anterior boundary of the even-numbered ps; second, it is required to turn on the homeotic products within specific even-numbered ps (Ingham and Martinez-Arias, 1986; Ish-Horowicz et al., 1989; Qian et al., 1991; Müller and Bienz, 1992); third, it activates its own transcription (Pick et al., 1990; Schier and Gehring, 1992); fourth, pair rule gene function has been implicated in

3.1. Activation and repression of tsh transcription

During the first stages of embryonic development, the tsh expression pattern is very dynamic. A striped pattern progressively sets up during cellular blastoderm stage to result at gastrulation in a homogenous distribution of tsh messages within the whole presumptive trunk region. Genetic analysis has shown that the establishment of the tsh expression domain is achieved by a combination of maternal, gap and pair rule genes activities (Röder and Kerridge, unpublished data). Maternal and segmentation genes act either as repressors or activators of tsh transcription in order to delimit the boundaries of tsh expression domains at blastoderm stage. The tsh expression pattern is altered in all the mutants that have been tested but it is never completely abolishe, suggesting a high level of complexity in the control of the initiation of tsh transcription.

The present data reveal that tsh transcription, at the cel-

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the regulation of proneural genes (Skeath et al., 1992) and finally, pair-rule genes, including ftz, are essential for cell movement during germ band elongation (Irvine and Wieschaus, 1994). tsh transcription is also under Ftz control, in the even-numbered ps of the trunk (Figs. 5, and 9). The effect of ftz mutations on the 2.0 kb tsh enhancer is independent of the homeotic gene products because the blastoderm expression pattern of tsh transcripts is not affected by homeotic mutations (Röder et al., 1992) and ubiquitous expression of Ftz does not promote expression of homeotic products (Ubx, Antp) in the tail region of the embryo where tsh is ectopically expressed (Fig. 5E) (Coré, unpublished). The altered expression of tsh in ftz null mutant embryos reveals that as early as the blastoderm stage part of tsh expression depends on ftz activity suggesting that Ftz could act as a direct activator of tsh transcription and that no other early embryonic gene is able to complement the ftz deficiency at this stage. In contrast, the over-
production of Ftz does not lead to ubiquitous expression of tsh throughout the whole embryo although we can detect restricted ectopic activation of tsh. Perhaps Ftz may require cofactors to activate tsh outside the trunk region or repressor activities prevail in these domains. At the blastoderm stage tsh expression is altered in phsftz embryos (Fig. 5B,C) showing that at this stage Ftz affects tsh activation in the trunk region. A band of non-labelled cells in the trunk may reflect the activity of repressor factors that may interfere with the overexpression of Ftz. It has been demonstrated that the expression of other bona fide Ftz target genes like Ubx, abd-A, engrailed (en) or a ftz-lacZ transgene are weakly altered in embryos expressing ubiquitous and high levels of Ftz protein, corresponding to broader stripes in the case of the ftz-lacZ transgene and en or to ectopic expression occurring, respectively, in ps 5 and ps 14 for Ubx and abd-A (Ish-Horowicz and Gyrkovics, 1988; Ish-Horowicz et al., 1989; Macias et al., 1994). According to the authors, overproduction of Ftz seems to have no effect on Ubx and abd-A expression until extension of the germ band. Finally, two of these genes, ftz and Ubx, have been demonstrated to be direct in vivo targets of Ftz protein (Müller and Bienz, 1992; Schier and Gehring, 1992; Schier and Gehring, 1993). These data show that the Ftz protein is not always sufficient by itself to induce transcriptional activation of its target genes throughout the whole embryo.

3.3. Direct or indirect regulation of tsh by the Ftz homeodomain protein?

In vitro studies reveal that Ftz protein specifically binds to the regulatory domain responsible for even-numbered expression of tsh transcription (Fig. 8). This result, and the rapid response of the regulatory element to altered ftz gene activity (Figs. 7A,B), suggest that Ftz acts as a direct activator of tsh transcription.

We found several Ftz binding sites in the 2 kb element and four specific sites were characterized in vivo and in vitro in the 220 bp enhancer. Three possess the ATTA core sequence (Fig. 8), now well documented as being that recognized by the HD (reviewed in Scott et al., 1989; Affolter et al., 1990; Treisman et al., 1992); no other consensus nucleotide sequence outside this invariant one is obvious either between or compared to other published Ftz bound sequences (Pick et al., 1990; Müller and Bienz, 1992; Schier and Gehring, 1992). A fourth binding site (Fig. 8) has a TTAT sequence which could represent a variant core motif. Similar non-ATTA Ftz in vitro binding sequences were found in the PBX (Müller and Bienz, 1992), and the BRE (Qian et al., 1993) enhancers of Ubx and the AE enhancer of fzt (Schier and Gehring, 1992).

To assess the functional significance of these Ftz binding elements we have produced a minigene to direct tsh expression from its own promoter combined to the 220 bp enhancer in a wild type or a mutated form in order to test the ability to rescue a null tsh mutation. We show that the mutated Ftz binding sites are able to partially rescue the tsh phenotype but at a very weak level compared to the wild type minigene demonstrating that these Ftz binding elements are necessary for the activity of the 220 bp regulatory element. As we have introduced subtle changes in the Ftz recognition sequences, we cannot exclude that, in vivo, nucleotides lying outside the ATTA core motif could be critical for Ftz activity.

The 220 bp enhancer has been sequenced in Drosophila virilis (Fig. 8) and is remarkably conserved in comparison to that of D. melanogaster (separated from each other for about 60 million years). Conservation is not restricted to the ATTA core sequences; neighboring sequences are also conserved between the two species, implicating that other binding proteins acting with Ftz might be critical for enhancer activity. For example, the HD protein Paired (Prd) interacts with, and is essential for the activity of, Ftz in vivo (Copleand et al., 1996). The alteration of the ATTA motifs and neighboring bases of the tsh 220 bp enhancer may perturb the action of other essential regulatory proteins, such as Prd, rendering the enhancer inactive.

It is clear that the 220 bp enhancer is insufficient for full Ftz dependent activity. The larger 2 kb fragment exhibits stronger in vivo activity and other sites in this fragment also
bind to Ftz in vitro. These observations indicate that Ftz acts on many sites in the 2.0 kb regulatory domain to activate in combination tsh transcription.

In conclusion, ftz+ gene activity is critical for the activation of tsh transcription at the blastoderm stage in the even-numbered parasegments and is likely to be direct as has been suggested for the regulation of both segmentation and homeotic target genes. The requirements for Tsh in determining trunk segmental identity, where it shares common properties with certain Hox proteins, as well as its unique function for the normal size of trunk segments (Röder et al., 1992), make it crucial that its expression be precisely regulated in space and time.

4. Experimental procedures

4.1. Mutant stocks, larval cuticle preparations and heat shocks

The Df(2L)R6 and Df(2L)R27 mutations were induced by mobilizing a P element transposon insert in tsh;P(LacW)2-IV (Bier et al., 1989). Female w/w;P(LacW)2-IV were crossed to Sp/CyO; rySbP(ry' Δ2–3)/TM6B males. About 500 w;P(Lac w)2-IV/CyO; rySbP(ry' Δ2–3)/+ males were individually crossed to w/w;CyO/Sc o females. Single male wCy+ flies, resulting from jumps of the P(LacW)2-IV insertion, were tested for complementation with w/w tsh+/CyO females. Other mutant chromosomes are described in Lindsley and Zimm (1992).

The ftz13 null allele and the phsftz transformant AA1 line were used (Krause et al., 1988). AA1 embryos were heat shocked between 2 and 4 h AEL for 20 min at 36°C. Embryos were allowed to recover for 3.5 h at 25°C before fixation and in situ hybridization. Cuticles of first instar larvae were prepared as described in Röder et al. (1992).

4.2. pLacZ constructs and germ line transformation

The p8.0 LacZ plasmid was constructed by ligating an 8.0 kb BamHI fragment from the 3' regulatory region of teashirt (Fig. 1A) into the unique BamHI site of the pCaSpeRβgal vector (Thummel et al., 1988) in both orientations. The 2.0 kb XhoI sub-fragment (of the 8.0 kb one) was cloned in the XhoI site of the C4PLZ vector (from S. Crew's laboratory) for the p2.0 LacZ lines. Transformants with smaller blunted 220 and 300 bp subfragments of the 2.0 kb one, were sub-cloned into the StuI restriction site of the C4PLZ vector yielding the p0.22LacZ and p0.8LacZ lines. P-element mediated germ line transformation were performed as described previously (Rubin and Spradling, 1982) by injecting yDf(1)w67c2 embryos, w+ transformants were localized to individual chromosomes and balanced with CyO or TM3. For all LacZ constructs, at least three independent transformants were tested for LacZ activity.

4.3. In situ hybridization and immunostaining of embryos

The probes used for RNA in situ hybridization to whole mount embryos (Tautz and Pfeifle, 1989) were a 2.5 kb EcoRI fragment from teashirt cDNA (Fasano et al., 1991) and a 4.5 kb HindIII LacZ fragment derived from pCaSpeRβgal plasmid. Double labeling on whole-mount embryos by in situ hybridization and immunostaining is that described by Dougan and DiNardo (1992). For immunostaining, the monoclonal anti-Invekt antibody or a polyclonal anti-Ftz antibody together with a HRP conjugated secondary antibody (Vector) were used. The embryos were mounted in 80% glycerol and observed with Nomarski optics.

Mutant tsh or ftz embryos were distinguished since they had no β-gal activity from P element insertions carried by the CyO, pbbLacZ or ptzfLacZ balancer chromosomes.

4.4. Immunoprecipitation and gel shift assays

The Ftz full-length protein, was produced in E. coli as inclusion bodies and purified on DNA cellulose (Sigma) (Krause et al., 1988). The Ftz homeodomain peptide, cloned in a pGex vector as a fusion with glutathione transferase, was purified on glutathione beads after induction in bacteria. All the DNA fragments tested were end-labelled with αP32 by filling in restriction sites. For immunoprecipitation, binding reactions were performed in 25 μl of binding buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.25 mM EDTA, 1 mM DTT, 10% glycerol) with Ftz protein, 5 μg herring sperm DNA and 2 ng of labelled HinfI digests for 30 min on ice. The complexes were incubated with 3 μl of anti-Ftz polyclonal antibody (Krause et al., 1988) and precipitated with Protein A- sepharose (Sigma). The pellets were washed with 0.2 M NaCl and then were exposed to increasing ionic strengths from 0.3 M to 1 M NaCl. The NaCl fractions containing immunoprecipitated fragments were resolved on 2% agarose gels.

For gel shift assays, reactions were performed in 20 μl of binding buffer (10 mM Hepes pH 8.0, 0.1 mM EDTA, 30 mM KCl, 0.1 mg/ml BSA, 2 mM DTT, 10% glycerol) with different concentrations of Ftz protein and 0.2 ng of DNA probe for 30 min on ice. The complexes were analyzed on 5% non-denaturing polyacrylamide gels.

4.5. DNase I footprinting assays, isolation of Drosophila virilis homologue and DNA sequencing

DNase I footprinting reactions were performed in 25 μl of buffer (10 mM Tris pH 7.5, 30 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.1 mM DTT, poly(dI:dC)) with Ftz homeodomain, and 2 ng of αP32 labelled probe for 15 min on ice. DNase I (0.006 units) was added in 50 μl of 10 mM MgCl2, 5 mM CaCl2 and the digestion was allowed to proceed for 1 min at room temperature. The reaction was stopped with 100 μl of 200 mM NaCl, 20 mM EDTA, 1% SDS. After
4.6. In vitro mutagenesis of Ftz binding sites

To generate mutated Ftz HD binding sites, four mutant oligos were prepared to the four different in vitro Ftz binding sites (FBS) together with novel unique restriction sites to recognize mutant constructs. Novel restriction sites are underlined and as indicated. Altered residues are in bold type (see Fig. 8D):

FBS1: CGATTTCGTTCCCGCGCGATTAGCGGATC (novel SacII site)
FBS2: GCCAGGCGTAATCCCTCTGACGGATG (novel HaeII site)
FBS3: TATTTTCATAATCCCAAATTTTCTCCGT (novel SspI site)
FBS4: GCCGGCCGTTCATGGGATTAGAGATCGC (novel NcoI site)

Single-stranded DNA from the wild-type 220 bp fragment was annealed to single mutant oligonucleotide sequences and the second strand synthesized. Mutant sequences were selected for their ability to cleave with the new restriction enzyme site co-introduced into the mutant oligos. These steps were performed sequentially for each mutant oligonucleotide sequence. At the final step the 220 bp fragment was sequenced to verify the altered residues.

4.7. Construction of tsh minigenes and examination of larval cuticles

A construct in pCaSper4 (Thummel and Pirrotta, 1991) was made comprising two upstream genomic fragments from the tsh walk, the 1.3 and 2.3 kb EcoRI fragments, linked to a 3.1 kb region comprising the entire coding sequence from the tsh gene (Fasano et al., 1991). In the same construct we introduced the 220 bp (Ftz-type) or mutant (Bcd-type) 220 bp fragments upstream of the genomic fragments in the same vector and in the same orientation. These three minigene constructs were inserted separately into the genome by P-element mediated transformation (see above). For each case two or more transformants were examined for activity in a tsh homozygous mutant background. All tsh mutant larvae could be unambiguously identified due to the incomplete rescue of the mutant phenotype by the minigenes. Cuticles were examined under the microscope; the size of the naked cuticle separating the denticle belts was scored and compared in the meso- and meta-thoraces. The presence of Kellin’s organs was also compared in meso- and meta-thoraces in the same larvae, for each minigene in the absence of endogenous tsh activity. When a Kellin organ of a particular animal was improved in one segment compared to its neighbor, the organ in this hemisegment was scored as such. The 1.3 and 2.3 kb genomic DNA drives tsh expression in ps 13 during retraction of the germ band (data not shown), showing that this 3.6 kb genomic fragment has a natural tsh promoter.

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