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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 Wiener, 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 anteroposterior 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

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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; Ten-Harmsel 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 Ingles, 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; Thali 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-

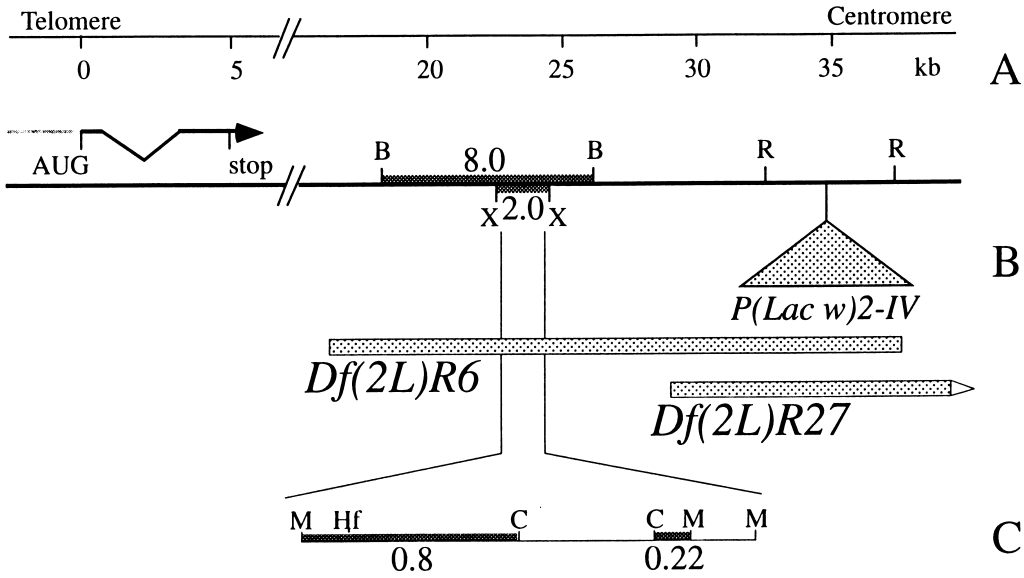


Fig. 1. Molecular map of the *tsh* gene. (A) The *tsh* cDNA and genomic maps (Fasano et al., 1991) are shown: R: *Eco*RI, B: *Bam*HI, X: *Xho*I. (B) The *P(Lac w)2-IV* enhancer trap insertion (Bier et al., 1989) is located about 20 kb downstream from the coding region in a 4.8 kb *Eco*R1 fragment. The mutations *Df(2L)R6* and *Df(2L)R27* have been induced by imperfect excision of the *P(Lac w)2-IV* insertion. *Df(2L)R6* deletes approximately 20 kb and *Df(2L)R27* overlaps it proximally. (C) Enlargement of part of the 2.0 kb XhoI fragment: *Msp*I: M, *Cla*I: C, *Hinf*I: Hf. The subfragments tested for in vivo enhancer activity are darkly shaded. All fragment sizes represent kbs. The 1300 bp fragment described by McCormick et al. (1995) is indicated by the line at the bottom of the figure.

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 even-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.

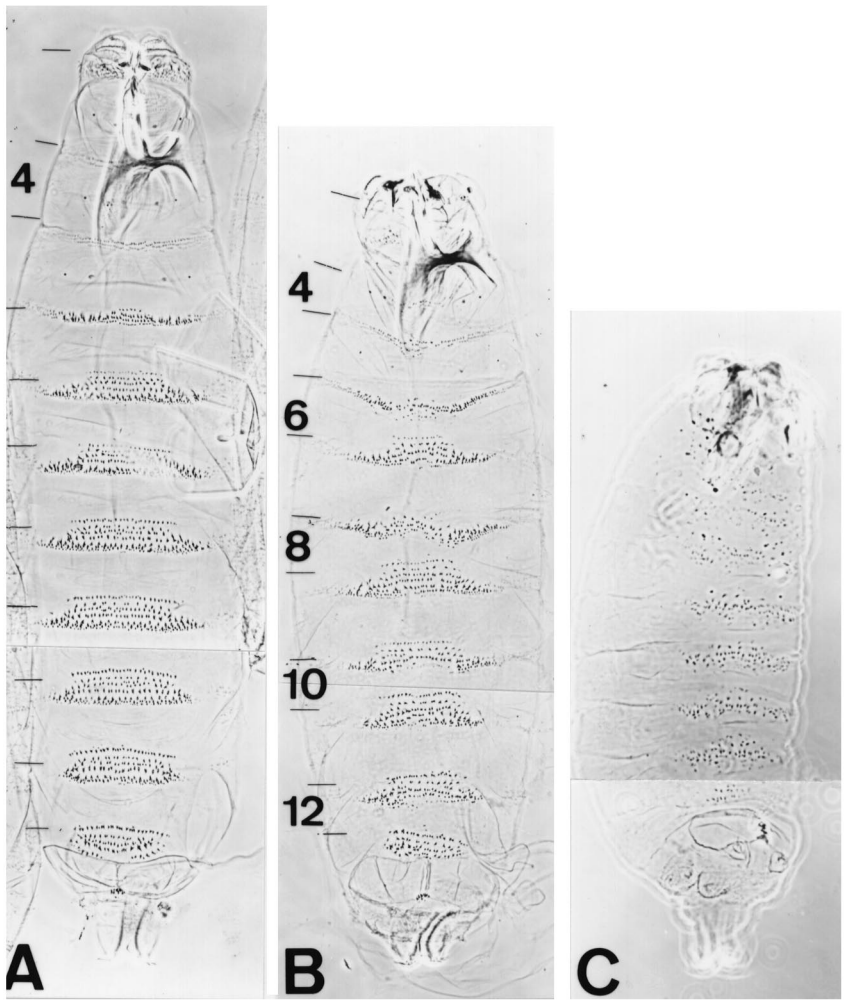


Fig. 2. Ventral cuticular phenotypes of a *Df(2L)R6* and tsh^{8} null homozygotes compared to wild type (A). Parasegments are indicated. In tsh^{8} larva (C), all of the trunk metameres are reduced in size compared to wild type. For *Df(2L)R6* (B), the divisions corresponding to even-numbered ps show a reduction in size compared to wild type; note that the organization of even-numbered denticle rows is abnormal. Ps 4, 6, 8 and 12 are more frequently affected than ps 10. Metameres from odd-numbered ps appear normal.

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.

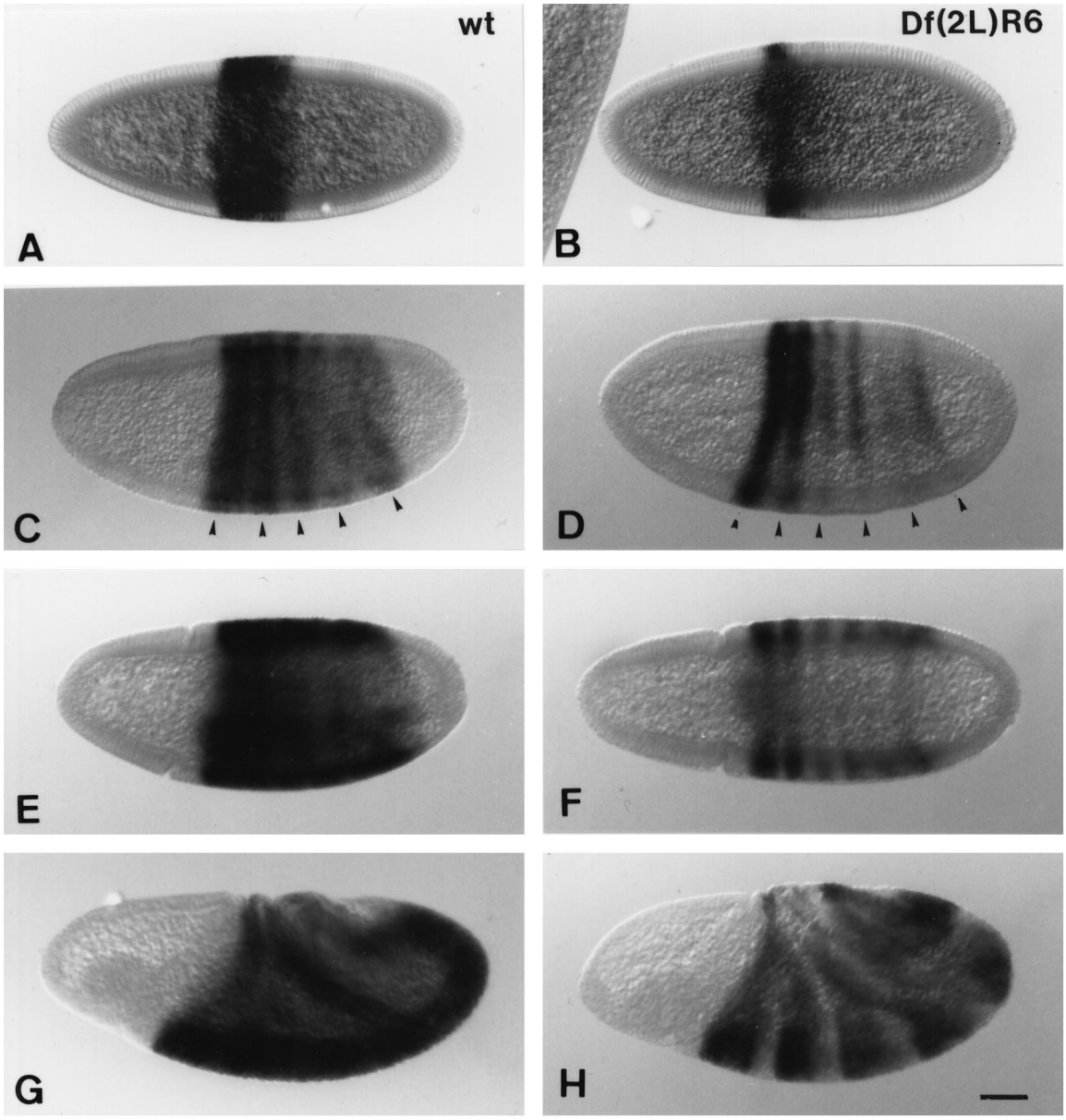


Fig. 3. Expression of *tsh* transcripts in wild type (A, C, E and G) and *Df(2L)R6* (B, D, F and H) homozygotes during early embryogenesis. *tsh* expression was detected by in situ hybridization to whole-mount embryos with a digoxygenin labelled probe. Anterior to the left and ventral side down. At the cellular blastoderm stage, the broad central domain of *tsh* expression is reduced in the deletion embryo (B) compared to wild type (A). In wild type embryos, *tsh* transcripts become more uniformly distributed throughout the trunk (C, E and G) during gastrulation. In mutant embryos, a pattern of six stripes appears at the beginning of gastrulation (arrow heads, D), which is maintained during later stages (F and H). The level of *tsh* transcription is stronger in the first two stripes (D); later (H) all the stripes display similar intensities. For stages see Campos-Ortega and Hartenstein (1985). Bars represent 50 μm for all figures.

2.3. In vivo activity of genomic DNA from the Df(2L)R6 region

To test whether the *Df(2L)R6* mutation deletes enhancer elements responsible for *tsh* expression in the even-numbered ps, different genomic fragments from the deleted region were fused to a minimal promoter driving *lacZ* expression and introduced into flies to test for in vivo enhancer activity. *LacZ* transcripts under the control of an 8.0 kb *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 *lacZ* mRNA is largely restricted to the even-numbered ps (Fig. 6) during early gastrulation. This pattern is complementary to the *tsh* transcription pattern observed in *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 *tsh* transcription in even-numbered ps. At the germ band extension stage, this enhancer drives *lacZ* expression in ps 3 to ps 13 in a similar way to the endogenous *tsh* transcript, suggesting that, latter, the 8.0 kb enhancer contributes to the activation of *tsh* expression in both even

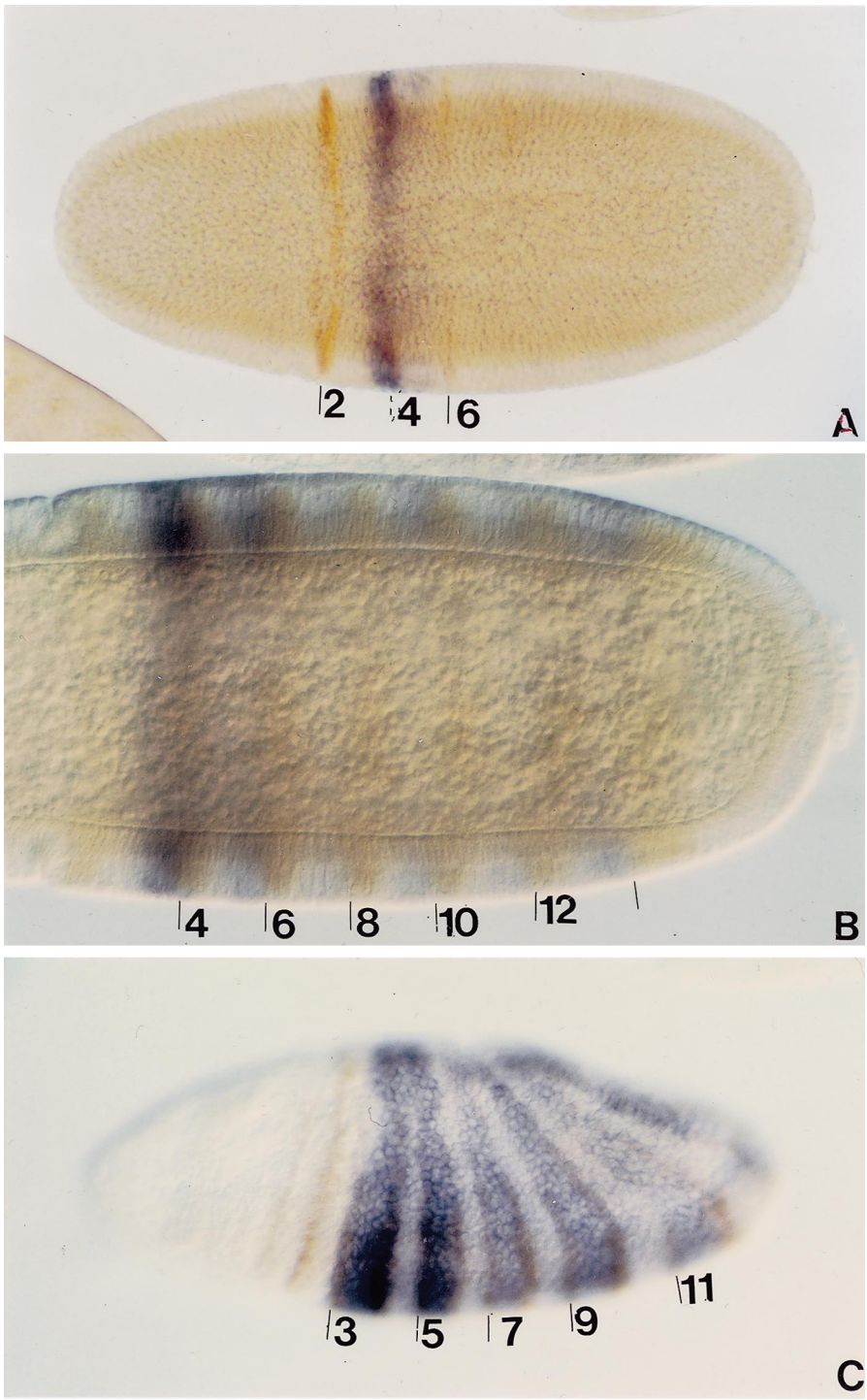


Fig. 4. Spatio-temporal localization of *tsh* transcripts in *Df(2L)R6* homozygous embryos with respect to Ftz (B) or Invected (A, C) proteins (both brown). (A) The predominant *tsh* band overlays the Inv stripe in the anterior part of ps4 and extends anteriorly to it (ps 3) in blastoderm embryos. Low levels of *tsh* transcripts are detected posteriorly in the rest of ps 4 and some of ps 5. (B) Apart from the first *tsh* stripe in ps 3, the Ftz protein located in even-numbered ps intercalates between the *tsh* stripes (blue) in stage 6 embryos. Note that at this stage, Ftz protein is narrowing in the posterior region of each stripe. Dorsally there appears to be an overlap between Ftz and *tsh*; this is due to a problem in the focal plane and therefore misleading. (C) During germ band extension, *tsh* transcripts are still detected in odd-numbered ps as well as in the posterior compartments of even-numbered ones. Note that all Inv staining in the trunk is dark brown due to the overlap of *tsh* transcripts and anti-Inv antibody, whereas when Inv is alone (e.g. anterior to ps 3) it appears as a light brown stripe.

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 *Xho*I subfragment (p2.0lacZ) of the 8.0 kb one.

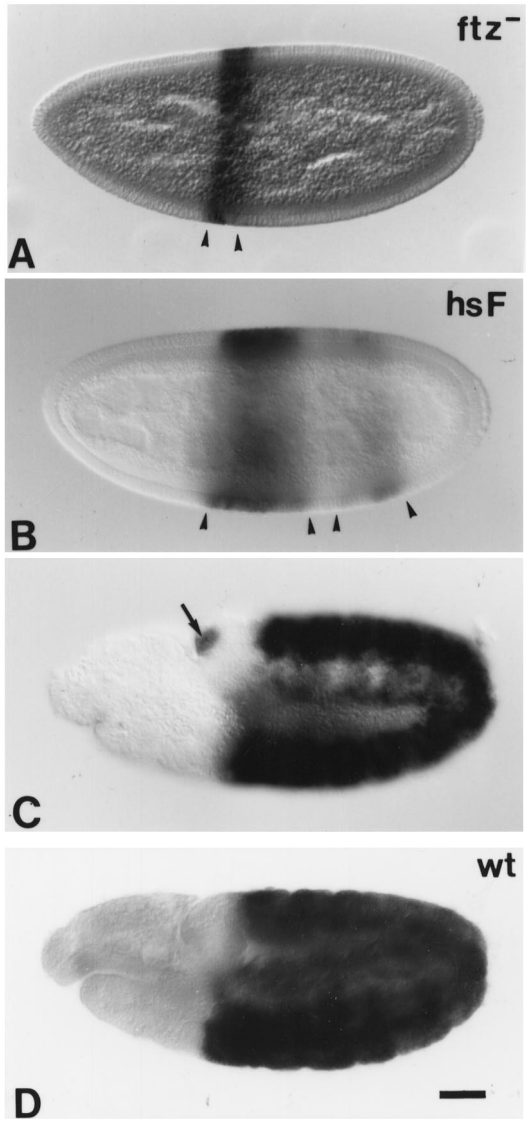


Fig. 5. *tsh* expression depends on ftz^{+} gene activity. (A) Homozygous ftz^{13} embryo at the blastoderm stage. *tsh* transcription (delimited by arrow heads) is repressed in the posterior part of the broad central domain (Fig. 3A) in a way similar to that in *Df(2L)R6* homozygotes (Fig. 3B). Following ectopic Ftz expression, two uniform domains of *tsh* expression are detected at the blastoderm stage (B) and ectopically in the tail at the extended germ band stage (C). Wild type controls never exhibit this ectopic expression in the tail (D).

LacZ expression is similar in both lines, although the smaller fragment exhibits a generally weaker pattern of expression at the blastoderm stage (Fig. 7A) and shows higher levels of *lacZ* in dorsal and lateral compared to ventral positions.

Ectopic expression is seen in the head of embryos carrying the *p8.0lacZ* and *p2.0lacZ* insertions at the blastoderm stage (Figs. 4A, 5A and 7A). The tested fragments presumably lack regulatory elements needed for the repression of *tsh* in the head and therefore restriction to the *tsh* expression domain.

2.4. $ftz^{\pm}$ 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 completely 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-numbered 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 stronger 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 polyclonal anti-Ftz antibody (Krause et al., 1988) were used to immunoprecipitate *Xho*I 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 *Xho*I fragment described above (Fig. 1C) shows specific binding of the Ftz protein, especially within a 1.5 kb *Hinf*I sub-fragment (Fig. 8A). The 2.0 kb *Xho*I fragment was then cut by *Cla*I and *Msp*I 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 protein on the 220 bp fragment (Fig. 8B, lanes 5–8), corresponding 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 sequential 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-

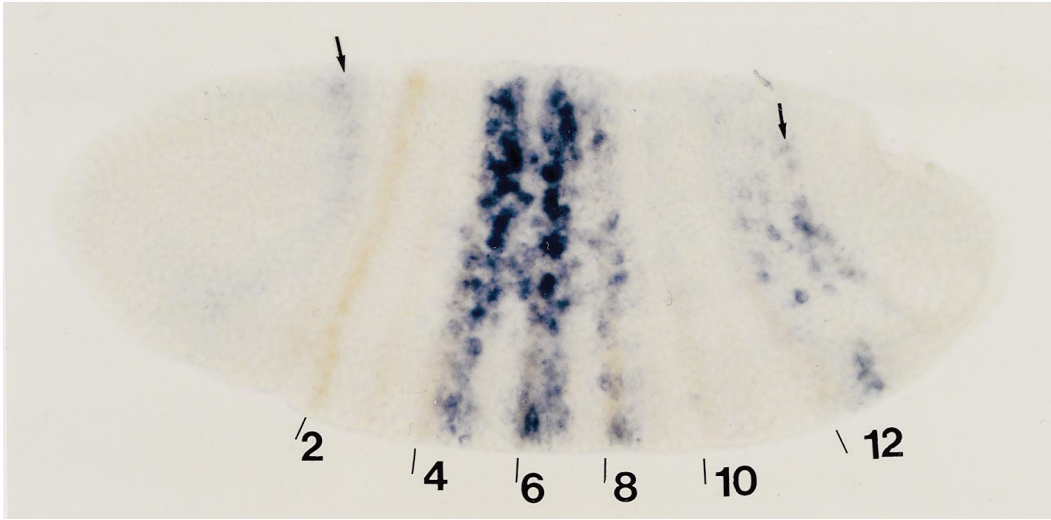


Fig. 6. The 8.0 kb enhancer initiates *LacZ* expression in even-numbered ps during early embryonic stages. *LacZ* transcripts (blue) are shown together with anti-Inv (brown) in a stage 6 embryo. *LacZ* transcripts are detected in a striped pattern in the trunk. At this stage, Inv labels mainly the (posterior compartments of) even-numbered ps. Transcripts overlay and extend posteriorly to the Inv stripes (indicated) in ps 4, 6 and 8 as well as weakly in ps 10 and 12. An additional stripe occurs in the head region of the embryo (anterior arrow) and dorsally, *LacZ* is detected in ps 13 and the posterior compartment of ps 14 (posterior arrow).

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^{8} (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^{-} 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).

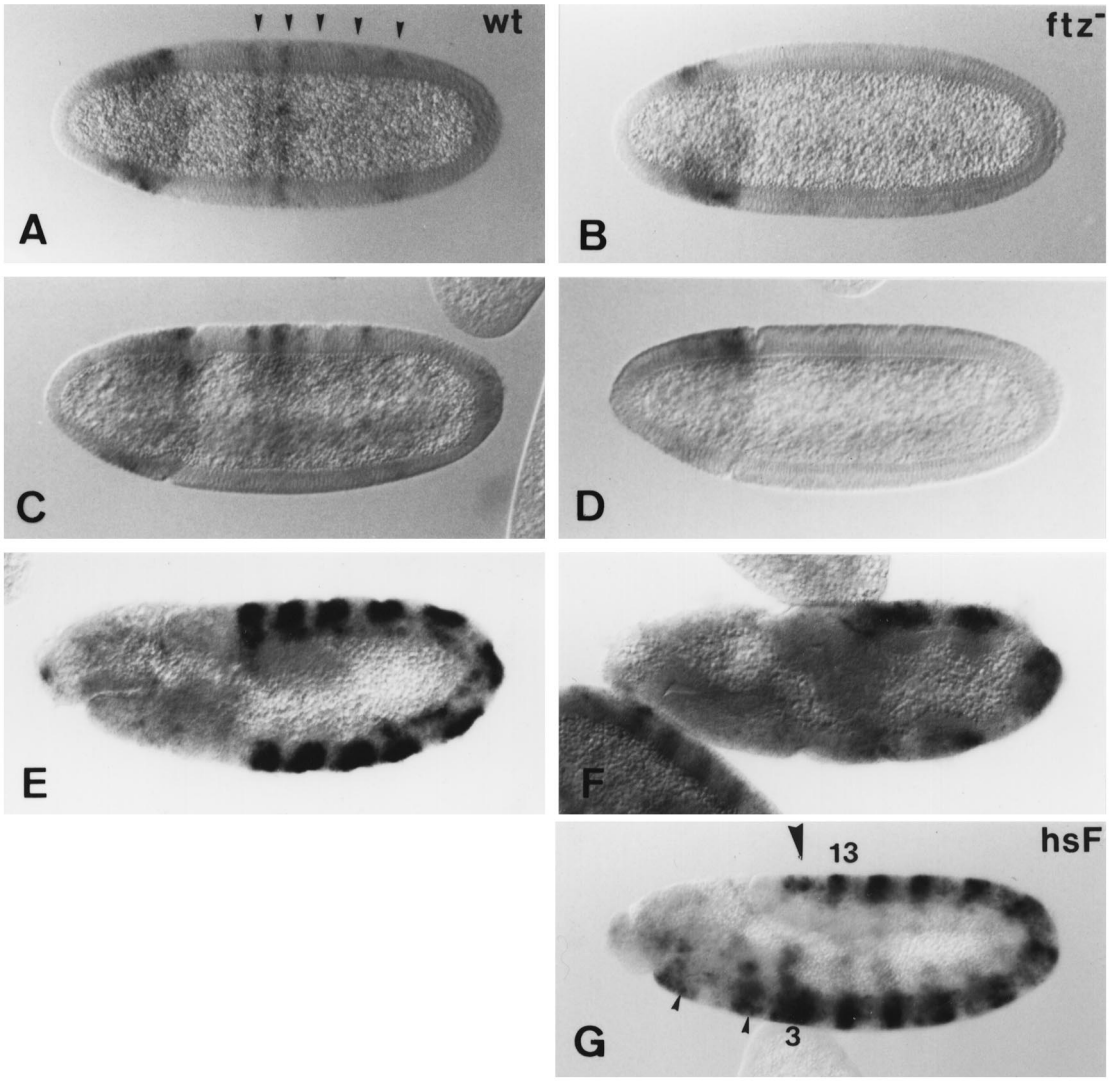


Fig. 7. The 2.0 kb enhancer responds to altered *ftz* gene activity. *LacZ* transcripts in the p2.0LacZ transformant are depicted in wild type (A, C and E), ftz^{13} (B, D and F) and heat shocked *phsftz* (G) embryos. In the absence of ftz^{+} product, *LacZ* transcripts are not detected in the trunk (B and D). Note that ectopic expression is not altered in the head (A, B, C and D) providing an internal control. During germ band extension, *LacZ* transcripts are detected in the odd-numbered ps (F) independently of *ftz* control, whereas in wild type, *LacZ* transcripts are found in both even and odd-numbered ps of the trunk (E). Ubiquitous expression of the Ftz protein promotes ectopic expression of the *LacZ* transcripts in the tail region of embryos carrying the p2.0LacZ construct (G). In the absence of heat shock treatment *LacZ* is sometimes weakly activated in ps 0 and 2 (data not shown); the expression of the transgene in these two regions (G; small arrow heads) is enhanced by ubiquitous expression of Ftz.

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

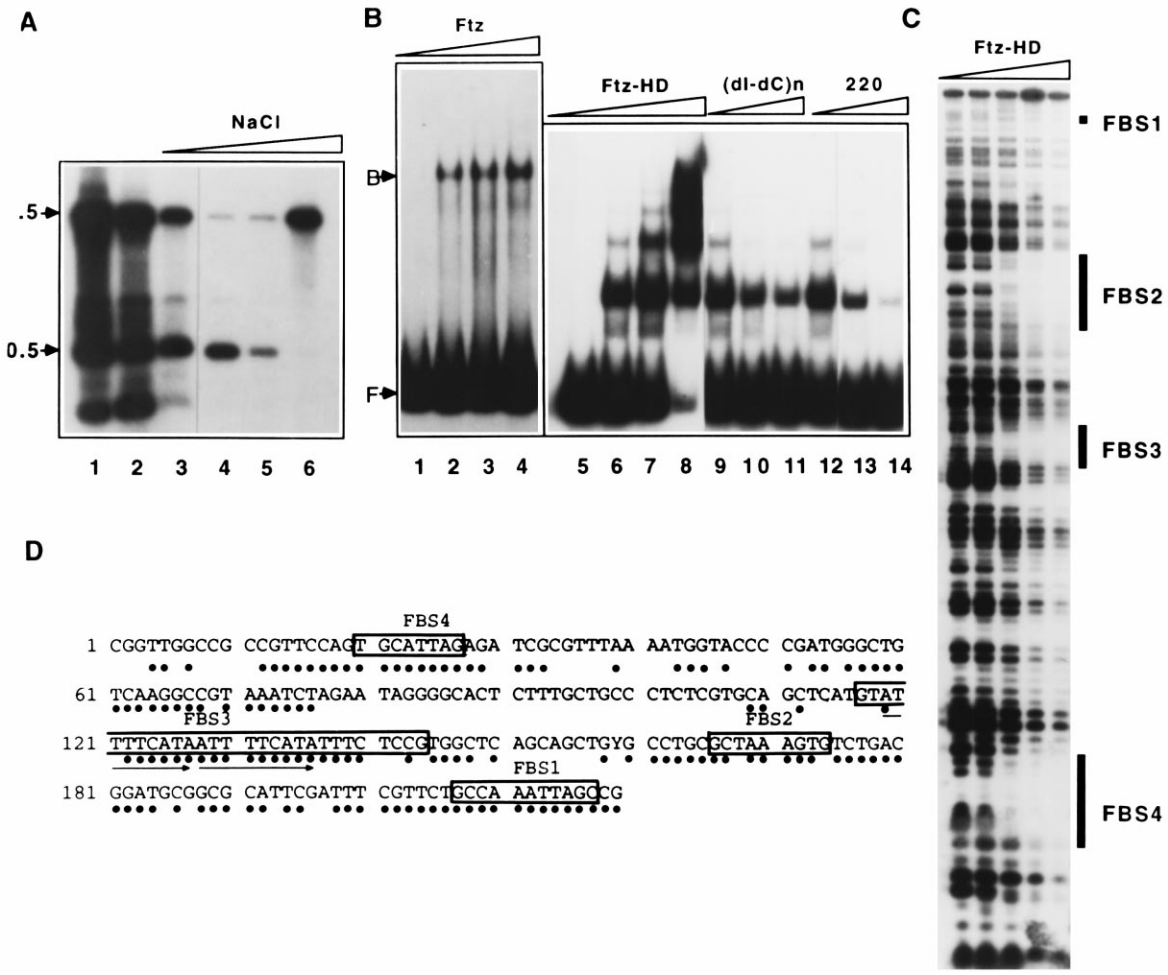


Fig. 8. Ftz protein *in vitro* binds to the 2.0 kb *Xho*I fragment from the 3′ *tsh* regulatory region. (A) Immunoprecipitation of *Hinf*I digests of the 2.0 kb *Xho*I fragment with full length Ftz protein. Lane1, total probe before immunoprecipitation; lane 2, flow through; lanes 3–6, fractions of DNA probe after elution with 0.2, 0.3, 0.4 and 1 M NaCl respectively. Ftz specifically binds to the 1.5 kb fragment which is eluted by 1M NaCl. (B) Gel shift assay with the 220 bp *Cla*I-*Msp*I fragment. Lanes 1 and 5: no protein. Lanes 2–4: 0.5, 1 and 10ng of full length Ftz protein. Lanes 6–8: 0.5, 1 and 10 ng of Ftz HD-glutathione transferase peptide. Increasing amounts of Ftz HD peptide allow the formation of several complexes. The specificity of binding of the Ftz HD (1 ng) on distinct sequences are revealed following non-specific (lanes 9–11 are 1X, 10X and 100X excess of poly(dI.dC)) or specific (lanes 12–14 are 1X, 5X and 10X excess of unlabelled 220 pb fragment) competition. F, free probe; B, bound probe. (C) Ftz protein binding sites in the 220 bp fragment revealed by footprinting analysis. 2 ng of labelled fragment was incubated with increasing amounts of purified Ftz HD. Lane 1, no protein; lane 2, 10 ng; lane 3, 50 ng; lane 4, 250 ng; lane 5, 500 ng of protein added. The nucleotide sequences protected by Ftz HD are indicated by vertical bars. (D). Nucleotide sequence from the 220bp fragment orientated 5′ to 3′. Ftz binding sites (FBS1–4) are shown in boxes. The two arrows indicate the presence of a direct tandem repeat of 9 contiguous nucleotides spanning the ATTA core of FBS3. The black dots indicate the nucleotides which are conserved within the homologous *Drosophila virilis* sequence.

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.

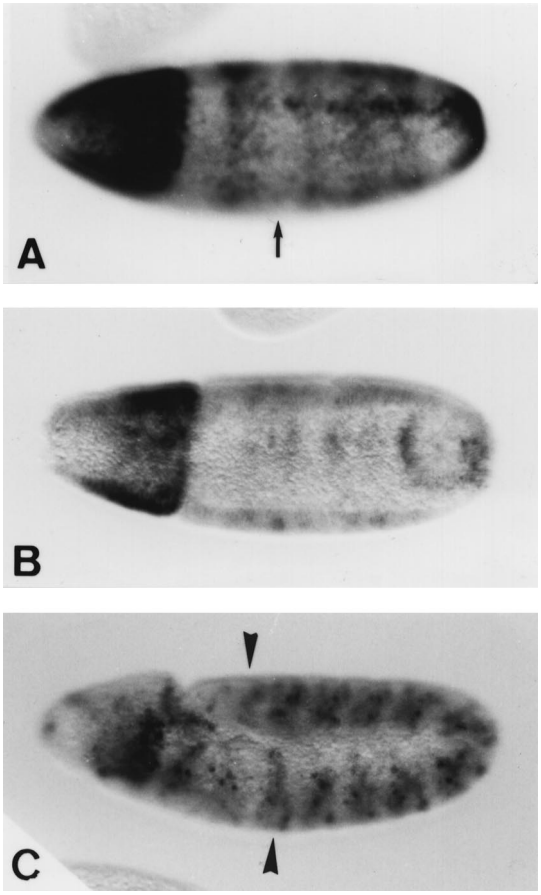


Fig. 9. *LacZ* expression directed by the 220 bp enhancer. *lacZ* transcript is expressed under the control of the 220 bp enhancer in blastoderm (A), gastrulating (B) and germ band extended (C) embryos. The transgene messages are detected by in situ hybridization with a *lacZ* probe after overstaining of the embryos overnight at 4°C. At blastoderm the *p220lacZ* is expressed either in 4 (A) or 5 stripes in the trunk region. At gastrulation, five stripes are now detected (B). At germ band extended stage, the transgene is expressed in all the trunk segments within the normal *tsh* expression domain (between arrow heads). At all stages, strong *lacZ* expression is detected in the head region.

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 abolished, suggesting a high level of complexity in the control of the initiation of *tsh* transcription.

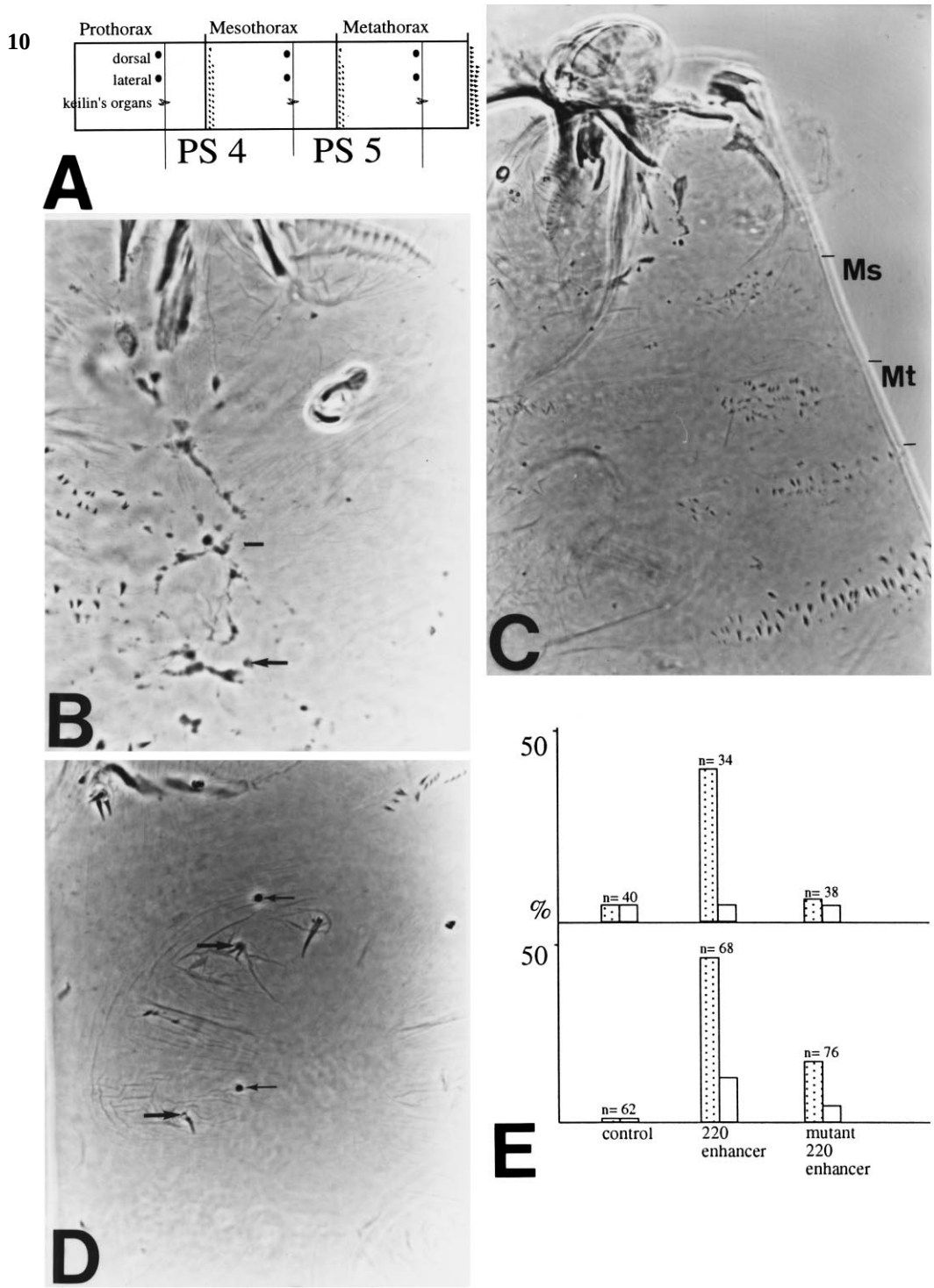
The present data reveal that *tsh* transcription, at the cellular blastoderm stage, could be activated differently in even-numbered and odd-numbered ps. In *Df(2L)R6* mutant embryos, indeed, *tsh* transcripts are missing in even-numbered ps at blastoderm and gastrulation (Figs. 3 and 4) and a 2.0 kb 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 metamerized 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

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 Gyurkovics, 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 2.0 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 TTTA 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 *ftz* (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 (Copeland 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 2.0 kb fragment exhibits stronger in vivo activity and other sites in this fragment also

Fig. 10. In vivo activity of wild type and Ftz binding site-mutated 220 bp enhancers. (A) A schematic representation of the wild-type thorax showing segmental and parasegmental boundaries; anterior to the right. Dorsal and lateral black dot and Keilin's organs are indicated. (B–D) Cuticles tsh^{8} homozygotes; anterior at the top; C and D also carry two copies of a *tsh* minigene under control of the 220 bp Ftz-dependent enhancer. (B) The denticles are not widely spaced as in wild type and the Keilin's organs are absent. Note the presence of a network of black structures (arrows) in the ventro-lateral part of each segment. (C). Activity of the *tsh* minigene, even without the 220 bp enhancer (not shown), removes the black structures shown by *tsh* homozygotes (compare Fig. 2D with C and B with D). The spacing between mesothoracic (Ms) and metathoracic (Mt) denticle belts is larger than that between metathoracic and first abdominal denticle belts, when the minigene is under the control of the 220 bp enhancer. (D) The same genotype as in C. This ideal case shows a two-pronged Keilin organ in the mesothorax (upper large arrow) and a uni-pronged one in the metathorax (lower large arrow). The small arrows show the meso- (upper) and metathoracic (lower) black dot organs that are always found in tsh^{8} homozygotes (B) (Fasano et al., 1991). (E) Summary of the rescue activities of the *tsh* minigenes in tsh^{8} homozygotes. Results are expressed as a percentage of the total number (*n*) of larvae (upper panel) or hemithorax (lower panel) scored. The relative spacing between denticle belts (upper panel) or the relative degree of differentiation of Keilin organs (lower panel) is compared between the mesothorax and metathorax. When the spacing or the degree of differentiation of Keilin organs was greater in one segment compared to its neighbor, this was scored and expressed as a percentage of the total. Column 1: control larvae with a *tsh* cDNA under the control of 3.6 kb of genomic DNA from the *tsh* promoter. Compared to tsh^{8} homozygotes without this minigene, slight rescue activity is detected since the black structures seen in tsh^{8} homozygotes (B) are rescued and Keilin organs may differentiate. Column 2: larvae with the same minigene as column 1 but in addition possess the wild type 220 bp enhancer. Column 3: larvae with the same minigene as in column 2 but the four in vitro Ftz binding sites of the 220 bp enhancer have been mutated (see Section 4).

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/Sco* females. Single male *wCy*$^+$ flies, resulting from jumps of the *P(Lacw)2-IV* insertion, were tested for complementation with *w/w tsh*^{8}*/CyO* females. Other mutant chromosomes are described in Lindsley and Zimm (1992).

The *ftz*^{13} 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 p8.0LacZ plasmid was constructed by ligating an 8.0 kb *Bam*HI fragment from the 3′ regulatory region of *teashirt* (Fig. 1A) into the unique *Bam*HI site of the pCaSpeRβgal vector (Thummel et al., 1988) in both orientations. The 2.0 kb *Xho*I sub-fragment (of the 8.0 kb one) was cloned in the *Xho*I site of the C4PLZ vector (from S. Crew's laboratory) for the p2.0LacZ lines. Transformants with smaller blunted 220 and 800 bp subfragments of the 2.0 kb one, were subcloned into the *Stu*I restriction site of the C4PLZ vector yielding the p0.22LacZ and p0.8LacZ lines. P-element mediated germ line transformations 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 *Eco*RI fragment from *teashirt* cDNA (Fasano et al., 1991) and a 4.5 kb *Hind*III *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-Invected 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, phbLacZ* or *pftzLacZ* 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 αP^{32} by filling in restriction sites. For immunoprecipitation, binding reactions were performed in 25 μl of binding buffer (20 mM Tris pH7.5, 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 *Hin*fI 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 $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, poly(dI.dC.)) with Ftz homeodomain, and 2 ng of αP^{32} labelled probe for 15 min on ice. DNAse I (0.006 units) was added in 50 μl of 10 mM $MgCl_2$, 5 mM $CaCl_2$ 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

extraction with phenol:chloroform:isoamyl alcohol (25:24:1), the samples were ethanol precipitated and analyzed on an 8% polyacrylamide sequencing gel. *Drosophila virilis* DNA homologous to the 2.0 kb enhancer from *D. melanogaster* was isolated by homology. Chemical cleavage DNA sequencing reactions were performed according to Maxam and Gilbert (1980).

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: CGATTTCGTTC**C**GC**GG**GATTAGCCGATC
(novel SacII site)
FBS2: GCC**A**GCGCTAA**TCC**CTCTGACGGATG
(novel HaeII site)
FBS3: TATTTTCATAAT**CCC**A**A**TATTTCTCCGT
(novel SspI site)
FBS4: GCCGCCGTTCCA**TG**G**G**ATTAGAGATCGC
(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^{8} 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 Keilin'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 Keilin 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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