

The myogenic regulatory gene *Mef2* is a direct target for transcriptional activation by Twist during *Drosophila* myogenesis

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MEF2 is a MADS-box transcription factor required for muscle development in *Drosophila*. Here, we show that the bHLH transcription factor Twist directly regulates *Mef2* expression in adult somatic muscle precursor cells via a 175-bp enhancer located 2245 bp upstream of the transcriptional start site. Within this element, a single evolutionarily conserved E box is essential for enhancer activity. Twist protein can bind to this E box to activate *Mef2* transcription, and ectopic expression of *twist* results in ectopic activation of the wild-type 175-bp enhancer. By use of a temperature-sensitive mutant of *twist*, we show that activation of *Mef2* transcription via this enhancer by Twist is required for normal adult muscle development, and reduction in Twist function results in phenotypes similar to those observed previously in *Mef2* mutant adults. The 175-bp enhancer is also active in the embryonic mesoderm, indicating that this enhancer functions at multiple times during development, and its function is dependent on the same conserved E box. In embryos, a reduction in Twist function also strongly reduced *Mef2* expression. These findings define a novel transcriptional pathway required for skeletal muscle development and identify Twist as an essential and direct regulator of *Mef2* expression in the somatic mesoderm.

[Key Words: Twist; MEF2; *Drosophila*; muscle development; ad epithelial cells; imaginal discs]

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Studies in *Drosophila* and vertebrates have shown that MADS-box proteins of the myocyte enhancer factor-2 (MEF2) family and basic helix-loop-helix (bHLH) factors act in collaboration to direct differentiation of skeletal muscle (for review, see Molkenin and Olson 1996). *Mef2* genes from fruit flies and vertebrates are expressed in precursors of all the muscle lineages early in development, and expression persists as the descendants of these cells differentiate (Edmondson et al. 1994; Lilly et al. 1994; Nguyen et al. 1994). The single *Drosophila Mef2* gene is required for differentiation of skeletal, cardiac, and visceral muscles (Bour et al. 1995; Lilly et al. 1995; Ranganayakulu et al. 1995), and in mice, targeted inactivation of *mef2c* results in defects in heart morphogenesis and differentiation (Lin et al. 1997).

Understanding how these regulatory genes are themselves regulated is a central issue in the field. Because *Mef2* genes are expressed in all myogenic lineages over a broad developmental time, multiple separate processes likely regulate *Mef2* expression. Analyses of the *Drosophila Mef2* 5'-flanking region revealed a number of

large regulatory regions spanning several kilobases (Lilly et al. 1995; Schulz et al. 1996) that, together, give rise to the complete pattern of *Mef2* expression. No direct regulators of any *Mef2* genes in somatic muscle, however, have been identified.

The bHLH protein Twist plays an important role in somatic muscle development in *Drosophila*. *twist* is expressed in the somatic mesoderm at high levels prior to differentiation (Dunin-Borkowski et al. 1995) and is required for differentiation of the somatic muscles (Baylies and Bate 1996). Although Twist activates a number of genes early in embryogenesis (Ip et al. 1992a,b), and *tin-man* is a target of Twist in the visceral mesoderm (Lee et al. 1997), no Twist targets have been identified in the somatic lineage.

During the larval stage, *twist* and *Mef2* are expressed in cells that give rise to adult somatic muscles (Bate et al. 1991; Ranganayakulu et al. 1995). These cells are specified in the embryo and are identifiable at the end of embryogenesis by their expression of high levels of *twist*. *twist* expression is maintained in proliferating adult muscle precursor cells throughout the larval and early pupal stages (Bate et al. 1991). The thoracic myoblasts are coincident with the ad epithelial cells (Poodry and Schneiderman 1970) that give rise to the adult thoracic

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musculature (Reed et al. 1975; Fernandes et al. 1991). As the myoblasts migrate from the discs and begin to fuse and differentiate, *twist* expression is lost (Fernandes et al. 1991). How either *twist* or *Mef2* are regulated in the ad epithelial cells and the functional significance of this expression is unknown.

In this paper, we have analyzed *Mef2* regulation in the precursors of the adult somatic muscles. Ad epithelial cell expression of *Mef2* is controlled by a 175-bp enhancer element 2245-bp upstream of the transcriptional start site. A single evolutionarily conserved E box is essential for enhancer activity, and Twist binds this site to activate *Mef2* transcription directly. The enhancer is also active in embryonic somatic myoblasts prior to differentiation, and its activity during embryogenesis is dependent on the same E box required for later expression. To test the role of the enhancer in vivo, we used a temperature-sensitive *twist* mutant. Reduction in Twist function during the larval stage causes a loss of *Mef2* expression in the adult muscle precursor cells, which results in adult muscle phenotypes similar to those observed for *Mef2* hypomorphic mutants. Reduction in Twist function during the embryonic stage also affects MEF2 accumulation. These results show a central role for Twist in the development of the somatic mesoderm and identify Twist as the first known regulator of *Mef2* expression in the somatic muscle lineage.

Results

Identification of the enhancer required for *Mef2* expression in adult muscle precursors

To identify the 5' regulatory sequences responsible for *Mef2* expression in adult muscle precursor cells, transgenic lines carrying different *Mef2* enhancer/promoter fragments fused to *lacZ* were tested for β -galactosidase activity in the ad epithelial cells of the wing imaginal disc. Schulz et al. (1996) reported that a 4-kb *EcoRI* fragment from -3564 to +521 is sufficient for expression in ad epithelial cells (see Fig. 1A, top) as well as in other compartments of the embryonic mesoderm (Lilly et al. 1995). We have further scanned *EcoRI* fragments spanning 12 kb upstream and 10 kb downstream of the transcriptional start site for mesodermal enhancers. The 4-kb fragment was the only region capable of driving *lacZ* expression in the wing disc ad epithelial cells (data not shown).

The 4-kb fragment was sequenced, and smaller constructs were tested for activity in ad epithelial cells (summarized in Fig. 1A). The ad epithelial cell enhancer was initially localized between the *SmaI* site at -2884 and the *DraI* site at -1674. A fragment spanning this region (-2887 to -1574) was able to activate transcription of an heterologous promoter in wing imaginal discs.

To further localize the ad epithelial cell *Mef2* enhancer, deletion constructs within this region were generated and tested for expression in transgenic animals. These constructs identified a 185-bp region from -2431 to -2247, which was required for ad epithelial cell expression of *lacZ* (shaded region in Fig. 1A).

In a second approach, the *Mef2* gene from *Drosophila virilis* was cloned, and a 3.9-kb region upstream of the transcription start site (from -4248 to -358) was sequenced and tested for activity in transgenic larval discs. This fragment drove expression of *lacZ* in *Drosophila melanogaster* imaginal discs (data not shown), indicating that the ad epithelial cell enhancer was present in this DNA, and that its regulation was conserved between these distantly related species. To identify elements that might be important for expression, the sequence of the 185-bp region from *D. melanogaster* was compared with the sequence of the 3.9-kb *Drosophila virilis* fragment. A block of 100 bp within the *D. melanogaster* 185-bp element was conserved in sequence and position from the transcriptional start site when compared with the *D. virilis* sequence (Fig. 1B). This suggested that the ad epithelial cell enhancer element might lie within these 100 bp.

The 185 bp of *D. melanogaster* sequence (from -2431 to -2247) contains three E-box sequences (5'-CANNTG-3'), which are potential binding sites for bHLH proteins (Murre et al. 1989). Of these E boxes, two are conserved in position in *D. virilis* (Fig. 1B). One of these, E2, has a different core sequence, whereas the other, E1, is identical in core and flanking sequences. Note that the E1 sequence (5'-CACATGTG-3') contains two overlapping E boxes. The third E box at the 5' end of the 185-bp region, is not conserved in *D. virilis*.

To determine if this small region was sufficient for ad epithelial cell enhancer activity, a 175-bp fragment from -2421 to -2247 was generated from the wild-type sequence of *D. melanogaster*. This sequence contained E1 and E2, but lacked the third E box because it was not conserved in *D. virilis*. In transgenic *D. melanogaster*, this region was capable of driving *lacZ* expression in the wing discs (Fig. 2A) in a pattern that recapitulated the expression observed for the largest -3564 to +521 construct. β -galactosidase activity was also detected in the haltere and leg imaginal discs, and groups of cells closely apposed to the abdominal body wall musculature, indicating that this enhancer is active in most, if not all, of the adult muscle precursor cells (data not shown).

The two E boxes were mutated singly and in combination to determine if these conserved sequence elements were required for activity of the 175-bp enhancer. Mutation of the most conserved E box, E1, ablated enhancer activity in the imaginal discs, whereas mutation of E2 had no effect upon expression (Fig. 2B,C). Mutation of both E boxes eliminated the activity of the enhancer (Fig. 2D). These results showed that only the most conserved E box (E1) was essential for enhancer activity.

An additional 30-bp construct (from -2308 to -2279) that contained both of the E boxes together with flanking sequence did not drive expression of *lacZ* in transgenic animals (data not shown), indicating that the E boxes alone are not sufficient for enhancer activity.

twist and *Mef2* are coexpressed in ad epithelial cells of the wing disc

To determine if *twist* and *Mef2* are expressed in overlap-

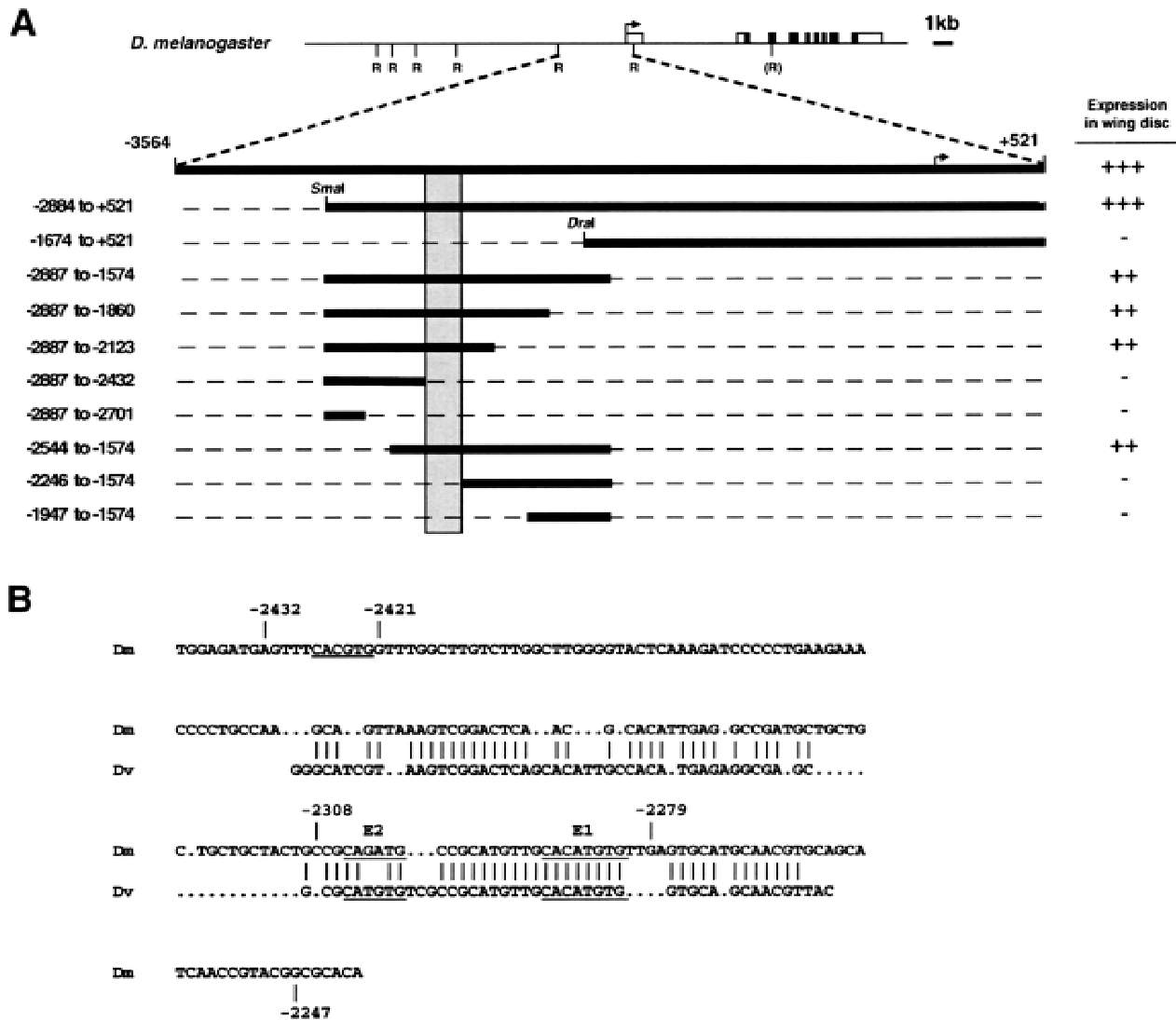


Figure 1. Characterization of the *Mef2* adephthelial cell enhancer. (A) The top line shows the genomic organization of the *D. melanogaster Mef2* locus. Solid and open boxes represent coding and noncoding transcribed regions, respectively. Vertical ticks labeled R show the locations of *EcoRI* restriction sites (R in parentheses represents a synthetic site; see Schulz et al. 1996). Each of these *EcoRI* fragments, cloned upstream of the *lacZ* gene, was tested for expression in the wing disc adephthelial cells. Only the fragment from -3564 to +521 showed expression. Below is an expansion of this ~4-kb region, within which the indicated fragments were tested. Summaries of their expression in wing imaginal discs are shown at right. The shaded bar denotes a 185-bp region that is required for expression in adephthelial cells. (B) Sequence similarity between *D. melanogaster* and *D. virilis* in the 185-bp region. Strong similarity is observed over a 100-bp segment. The *D. virilis* sequence shown is from -2448 to -2359. (Dm) *D. melanogaster* sequence; (Dv) *D. virilis* sequence. Three E boxes are underlined, and E1 and E2 are indicated.

ping groups of cells, imaginal discs were reacted with antibodies raised against either Twist or MEF2 protein. Separately labeled discs were then embedded in paraffin and sectioned. Figure 3A shows a whole disc reacted with anti-MEF2 and sections are shown in Figure 3B and C. MEF2 was detected in a specific group of cells near the proximal end of the disc. Most, if not all, of the cells in this region were labeled. Sections of imaginal discs stained with an anti-Twist antibody showed a very similar distribution of signal in most, or all, of these same cells (Fig. 3D). These data indicate that Twist and MEF2

accumulate in overlapping groups of cells, which are the precursors of the adult thoracic muscles (Bate et al. 1991; Fernandes et al. 1991). Although there may be some cells in which only one of the two proteins accumulates, the majority of adephthelial cells express both proteins.

Direct transcriptional activation by Twist through a single E box

To determine if Twist could directly activate the 175-bp enhancer in vivo, *Drosophila* Schneider line 2 (SL2) cells

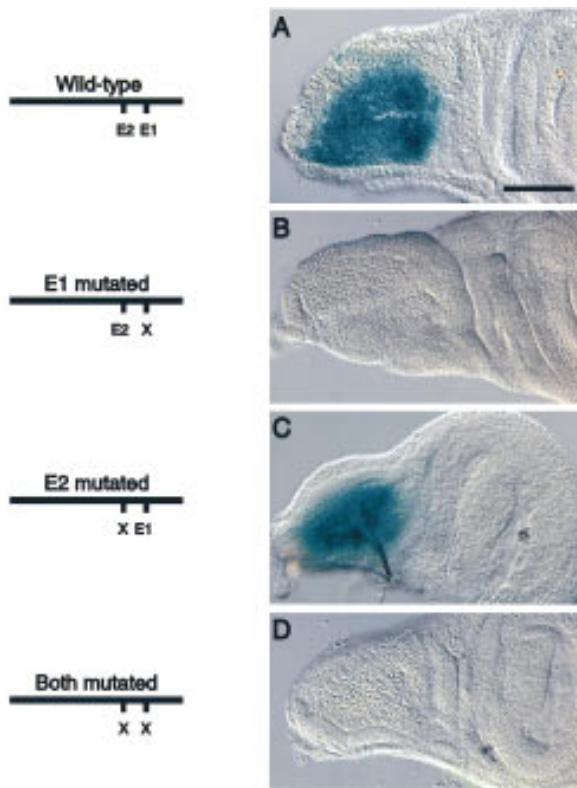


Figure 2. Expression of the 175-bp ad epithelial cell enhancer in wing imaginal discs depends on the E1 E box. Transgenic lines carrying enhancer-*lacZ* constructs were tested for β -galactosidase expression in wing imaginal discs. (A) Wild-type 175-bp enhancer drives high levels of expression in the ad epithelial cells; (B) enhancer with E1 mutated has no expression; (C) enhancer with E2 mutated drives *lacZ* expression at wild-type levels; (D) the enhancer with both E1 and E2 mutated has no activity. Bar, 50 μ m.

were cotransfected either with *twist* expression plasmid or with expression plasmid lacking a cDNA insert, along with the wild-type 175-bp enhancer-*lacZ* reporter and each of the mutant enhancer-*lacZ* constructs. Following transfection, cell lysates were assayed for β -galactosidase activity (Fig. 4A). Twist activated the wild-type enhancer several hundredfold. This activation occurred through the E1 E box, because mutation of that E box resulted in a complete loss of activity, whereas mutation of the E2 E box had no effect on enhancer activation.

Activation of the enhancer by another mesoderm-specific bHLH transcription factor, Nautilus [(Nau) Michelson et al. 1990; Paterson et al. 1991], was also tested. Under conditions where Twist strongly activated the enhancer, Nau-expressing lysates showed no activation over background, indicating that Nau was unable to activate transcription through this enhancer (Twist, 127 ± 33 -fold; vector alone: 1.00 ± 0.67 -fold; Nau, 0.78 ± 0.20 -fold).

To confirm that Twist was able to bind to the E1 sequence, electrophoretic mobility shift assays were performed with a radioactively labeled DNA probe corre-

sponding to the E1 E box and Twist protein generated by transcription and translation in vitro (Fig. 4B). Addition of Twist-containing lysate generated a band (labeled B in Fig. 4B) not detected in control lysate. Binding to this site was competed by addition of unlabeled E1 probe, but not by addition of unlabeled E1 mutant DNA nor wild-type E2 DNA. These results indicate that Twist binds specifically to the E1 E box.

Ectopic Twist activates the 175-bp enhancer

To further investigate the responsiveness of the 175-bp enhancer to Twist, *twist* expression was ectopically induced in embryos to determine whether this resulted in ectopic activation of the 175-bp enhancer. Males carrying a UAS-*twist* construct (Baylies and Bate 1996) and a construct expressing *GAL4* under the control of a heat-shock promoter (Brand and Perrimon 1990) were mated to females homozygous for different enhancer-*lacZ* constructs. Embryos were collected from this cross and following heat shock the induction of β -galactosidase reporter was determined.

Embryos carrying the wild-type 175-bp enhancer-*lacZ* construct showed expression of *lacZ* in the embryonic mesoderm in the absence of ectopic *twist* (Fig. 5A, also see below). After induction of *twist* expression, strong expression of the *lacZ* reporter was observed throughout the embryo (Fig. 5C). Embryos carrying the enhancer-*lacZ* in which E1 had been mutated showed no expres-

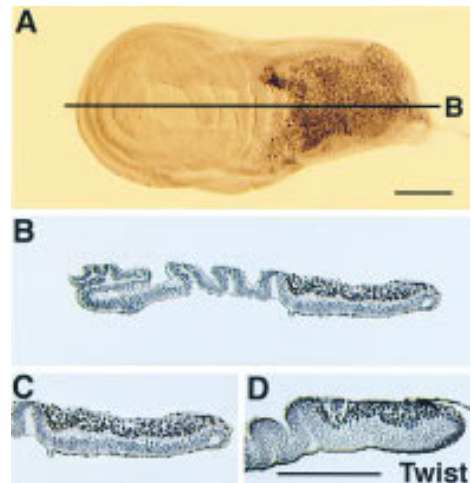


Figure 3. *Mef2* and *twist* are expressed in overlapping domains in wing imaginal discs. Third larval instar wing imaginal discs were reacted with either anti-MEF2 (A) or anti-Twist (not shown) polyclonal antibodies. Discs were then embedded in paraffin and sectioned to localize the immunoreactive cells. Anti-MEF2 staining was localized to the proximal end of the disc (B), and closer observation (C) revealed that it was expressed in most or all of the cells in this region. Twist protein accumulated in a very similar pattern (D), showing that these two proteins are predominantly coexpressed in the adult muscle precursor cells. Bar, 50 μ m.

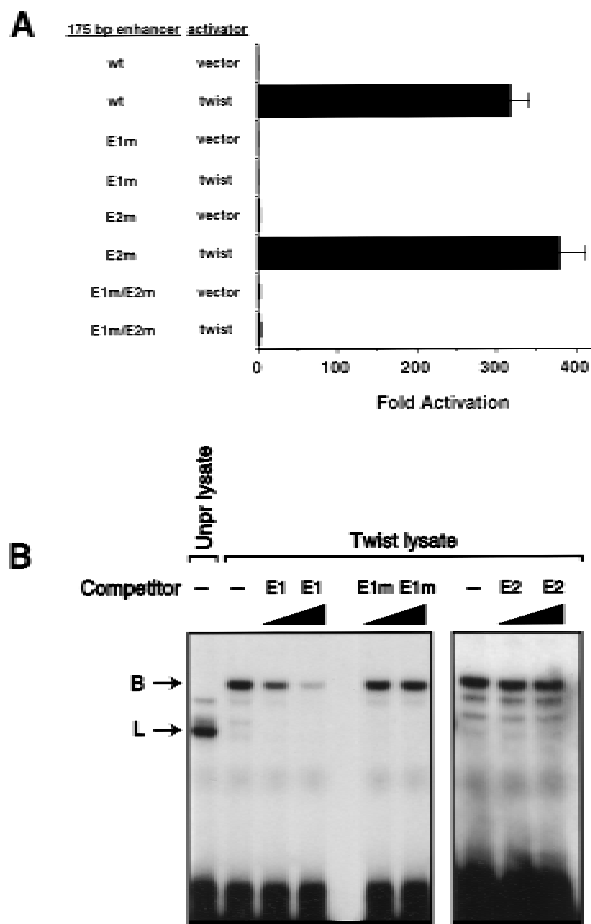


Figure 4. Twist activates the 175-bp enhancer in *Drosophila* SL2 cells. (A) SL2 cells were transfected with the indicated β -galactosidase reporter plasmids and either *twist* expression vector or expression vector without a cDNA insert. The data represent the mean values obtained in four independent transfections and are expressed as the fold-activation obtained in each sample over the activity generated by the wild-type enhancer construct in the absence of *twist* expression plasmid. Error bars represent the standard error of the means for the four experiments. The mean level of β -galactosidase activity produced by the wild-type enhancer construct in the absence of Twist was 3.4×10^{-6} units and in the presence of Twist was 1.1×10^{-3} units (one unit is defined as the amount of enzyme that will convert >95% of the β -D-galactose from 1 nmole of Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc-AMC in a 10- μ l reaction in 1 hr at 37°C and at pH 4.5). (B) Twist binding to the E1 E box. An oligonucleotide corresponding to the E1 E box was used as a probe in an electrophoretic mobility shift assay by use of in vitro-translated Twist. Unprogrammed lysate was included in a parallel lane. A 50- or 300-fold excess of unlabeled E1, unlabeled E1 mutant (E1m), or unlabeled E2 oligonucleotides were used as competitors. Competition by the wild-type E1 oligonucleotide but neither the E1 mutant nor E2 indicates a specific interaction between Twist and E1. B, probe bound with Twist; L, complex detected in unprogrammed lysate.

sion of *lacZ* in the absence (Fig. 5B) or presence (Fig. 5D) of ectopic Twist, indicating the single Twist-binding site in the enhancer was required for Twist responsiveness.

Taken together, the results presented in Figures 2, 3, 4, and 5 strongly support the conclusion that Twist directly activates transcription of *Mef2* in the adult muscle precursor cells of the wing imaginal discs. The activity of the 175-bp enhancer is absolutely dependent on the E1 E box, and in vivo and in vitro experiments show conclusively that Twist can interact with this sequence and activate transcription of reporter genes.

Twist activation of *Mef2* is required for normal adult myogenesis

To determine whether *twist* is required for adult somatic myogenesis and if the effects are mediated by *Mef2*, a temperature-sensitive heteroallelic combination of two different *twist* alleles, *twi*^{RY50} and *twi*^{V50}, was employed (Thisse et al. 1987). The RY50 and V50 alleles were crossed into a genetically marked background to permit identification of RY50/V50 mutant larvae (see Materials and Methods). Crosses were set up at the permissive temperature (18°C), and embryos were incubated at this temperature until hatching. Newly hatched mutant larvae were collected and subjected to two different incubation temperatures: To reduce Twist function for the rest of development, larvae were shifted to the restrictive temperature (30°C); as a control, mutants were maintained at 18°C for the rest of their development.

To determine the effects of reducing Twist function upon *Mef2* expression in the adult muscle precursor cells, the expression of both *Mef2* and a *Mef2-lacZ* transgenic construct was analyzed. Reduction in Twist function resulted in a loss of *Mef2* expression in the ad epithelial cells (Fig. 6A, top). Two other genes known to be expressed specifically in the ad epithelial cells of the imaginal discs are the fibroblast growth factor receptor-1 homolog *heartless* (*htl*; Emori and Saigo 1993) and the homeobox gene *cut* (Blochlinger et al. 1990). Expression of these genes was unaffected by reducing Twist function (Fig. 6A), indicating that ad epithelial cells had formed and proliferated normally.

The expression of a *Mef2-lacZ* construct was also sensitive to reduction in Twist function (Fig. 6B): The transgene was active at the permissive temperature (18°C) but inactive at the restrictive temperature (30°C). This further shows that the ad epithelial cell enhancer examined is responsive to the levels of Twist function as is the endogenous *Mef2* gene.

Of the *twist* mutant larvae shifted to the restrictive temperature, 63% ($n = 150$) developed to adults, compared with 82% ($n = 150$) of control *y w* treated in the same manner. This indicates that there is a postembryonic requirement for Twist for normal viability. In addition, several of the resulting adults showed defects in wing positioning. This phenotype is common among flies in which the patterning or function of the indirect flight muscles is defective (see, e.g., Deak 1977; Mogami and Hotta 1981; for review, see Bernstein et al. 1993). *twist* mutants raised at the permissive temperature showed good viability (94%, $n = 250$) and no wing position abnormalities.

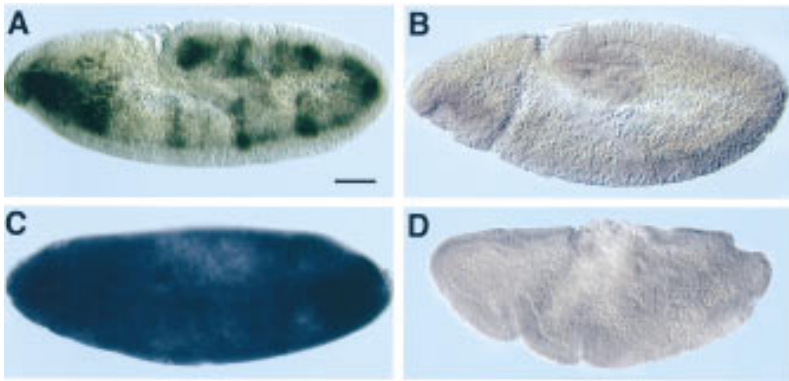


Figure 5. Twist activates the 175-bp enhancer in embryos. Embryos carrying either the wild-type 175-bp enhancer-*lacZ* construct (A,C) or the E1 mutated construct (B,D) were stained for β -galactosidase protein in the absence (A,B) or presence (C,D) of ectopically expressed *twist*. The 175-bp enhancer was active in wild-type embryos (A; see also Fig. 11), and activity was induced throughout the embryo after ubiquitous expression of *twist* (C). The E1 mutated enhancer was inactive in wild-type embryos (B) and was not induced by ectopic Twist (D), although the morphology of this embryo was affected. Bar, 100 μ m.

The adult musculature of wild type and *RY50/V50* raised at the permissive or restrictive temperature was analyzed (Fig. 7, A–C). Within the thorax, there are two columns of dorsal longitudinal indirect flight muscles (DLMs), shown in cross section in panels A–C. In wild-type flies, there are six DLM fibers in each half thorax (panel A, numbered). There was a reduction to five fibers, however, in the *twist* mutants raised at the permissive temperature (panel B), and to three fibers in the mutants raised at the restrictive temperature (panel C). Analysis of a number of individuals showed that these DLM defects were highly reproducible (summarized in Fig. 7, D–F). Patterning defects were also observed in other thoracic and abdominal muscles including the tergal depressor of the trochanter muscles and the lateral transverse muscles of the abdomen (data not shown). These results show that *twist* is required for normal development of somatic muscle in the adult, and correlate with observations that *twist* is expressed in the precursor myoblasts of the adult somatic muscles (Bate et al. 1991; Currie and Bate 1991). The mild defects that are observed in *RY50/V50* raised at the permissive temperature (five DLM fibers per hemithorax) indicate that even at this temperature, the mutant Twist protein is functionally compromised.

Previously, Ranganayakulu et al. (1995) showed that *Mef2* function is required for normal patterning of the DLMs, and the phenotypes observed here when Twist function is reduced are very similar to those observed previously. Because antibody staining showed that reduction in Twist function resulted in a loss of MEF2, it can be concluded that the patterning defects observed in this study are likely caused by a loss of MEF2 expression when Twist function is reduced.

Embryonic expression of the 175-bp enhancer

Because *twist* and *Mef2* are also coexpressed at various times during embryogenesis (Lilly et al. 1994; Nguyen et al. 1994; Taylor et al. 1995), lines containing the 175-bp enhancer-*lacZ* construct were tested for *lacZ* expression in embryos. Early in embryogenesis, both *twist* and *Mef2* are expressed at high levels in the developing mesoderm, however, there was only weak expression from the 175-

bp enhancer at this stage and this was not uniform in the mesoderm (Fig. 8A). This indicates that at this stage Twist alone cannot activate the 175-bp enhancer, and it is likely that the genomic region governing the earliest expression of *Mef2* is either outside of this enhancer or overlaps it.

The activity of the 175-bp enhancer increased during embryogenesis until stage 12, where it was expressed in segmentally repeating groups of cells corresponding to the unfused and undifferentiated myoblasts of the larval somatic muscles (Fig. 8B, arrows). These cells were identified as somatic myoblasts because some lines with high levels of expression had β -galactosidase protein that persisted during differentiation. At this stage, *twist* and *Mef2* are coexpressed in many of these cells (Taylor et al. 1995). By stage 15, levels of β -galactosidase had declined in the differentiated somatic muscles, although protein persisted in some of the muscle fibers of the head and thorax (Fig. 8C).

The expression of *lacZ* in lines carrying the 175-bp enhancer with the E2 E box mutated was the same as for the wild-type enhancer, whereas both the E1 mutant enhancer and the double-mutant enhancer were inactive throughout embryogenesis. These results indicate that the 175-bp enhancer is active in a subset of mesodermal cells during embryogenesis, and that the activity of this element likely depends upon a bHLH factor.

Because ectopic Twist was able to activate *Mef2* expression in embryos and because *twist* and *Mef2* are coexpressed in somatic myoblasts by stage 12 (Taylor et al. 1995), the expression of *Mef2* in the *twist* temperature-sensitive mutant background was studied. When *twist* was inactivated by temperature shift after gastrulation, there was a severe reduction in the level of MEF2 protein in the mutant embryos (Fig. 8E) as compared to heterozygous siblings (Fig. 8D).

Discussion

Members of the MEF2 family of transcription factors play central roles in muscle cell differentiation. In the absence of MEF2 function, myoblasts are properly specified in the embryo, but do not activate terminal muscle differentiation genes (Bour et al. 1995; Lilly et al. 1995;

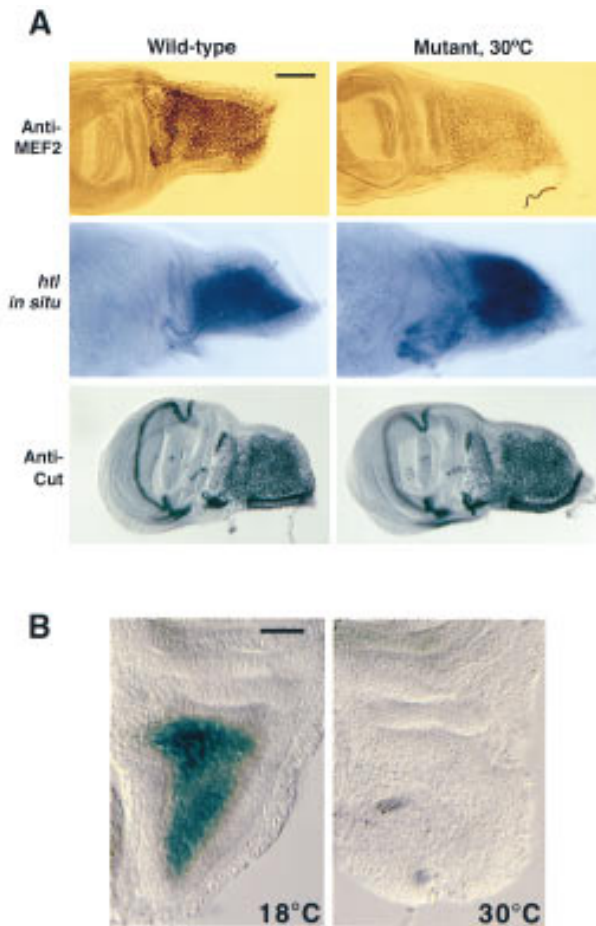


Figure 6. Expression of ad epithelial cell markers in wild-type and *RY50/V50 twist* mutants. (A) (Left) Wild type. (Right) *RY50/V50* raised at the restrictive temperature. (Top) Immunostaining by an anti-MEF2 polyclonal antibody. (Center) In situ hybridization with a digoxigenin-labeled antisense probe for the *Drosophila* fibroblast growth factor receptor homolog -1, *htl*. (Bottom) Immunostaining by an anti-Cut monoclonal antibody. Note that MEF2 accumulation is greatly reduced, yet the other markers are expressed at normal levels. Bar, 50 μ m. (B) A ~3.4-kb *Mef2* enhancer/promoter fragment is sensitive to the levels of *twist* expression. Transgenic flies carrying the -2884 to +521 fragment cloned upstream of *lacZ* were crossed into the *twist* mutant backgrounds. *RY50/V50* mutants raised at either the permissive (left, 18°C) or restrictive (right, 30°C) temperatures were analyzed for β -galactosidase activity in wing imaginal discs. Transgene expression was only observed at 18°C, indicating that Twist function is required for enhancer activity. Bar, 25 μ m.

Ranganayakulu et al. 1995; Lin et al. 1997). Whereas an understanding of the mechanism for transcriptional activation by MEF2 proteins is emerging, little is known of the pathways that control *Mef2* gene expression in different muscle cell types.

In this study, we have identified a novel enhancer that is responsible for *Mef2* expression in somatic myoblasts prior to differentiation at two different developmental stages. A single conserved E box is essential for enhancer

activity, and we show that the bHLH transcription factor Twist is capable of binding to this E box to activate transcription both in tissue culture and when ectopically expressed during embryogenesis. Furthermore, expression of the endogenous *Mef2* gene is strongly reduced when Twist function is reduced, showing that one of the functions of Twist is to directly activate transcription of *Mef2*.

twist expression after gastrulation has been shown to be necessary for differentiation of the embryonic somatic mesoderm (Baylies and Bate 1996). On the basis of our results, the mutant phenotypes observed when Twist function is reduced could be caused by a loss of *Mef2* expression in somatic myoblasts, although it is possible that somatic myoblasts do not develop normally when Twist function is reduced prior to this time. In adult muscle precursor cells, a reduction in Twist function in the larva clearly results in a loss of enhancer activity and of *Mef2* expression in the imaginal myoblasts. These observations show an essential function in vivo for the 175-bp enhancer in the development of the skeletal musculature.

The 175-bp enhancer is one in a series of regulatory modules that control *Mef2* expression during development of the somatic mesoderm. Early in embryogenesis, expression of *twist* and *Mef2* are high throughout the mesoderm, yet the activity of the 175-bp enhancer is low and is not uniformly distributed. This indicates that sequences either outside the 175-bp enhancer or overlapping with it are required for normal *Mef2* expression at this time, and that factors in addition to Twist may be required for enhancer function. It is likely that *Mef2* is regulated by Twist at this stage (Taylor et al. 1995), but whether this activation is direct is unknown.

Later, as skeletal muscle cells differentiate, *Mef2* expression persists in the somatic mesoderm (Bour et al. 1995; Lilly et al. 1995), despite a decline in both Twist levels (Dunin-Borkowski et al. 1995) and activity of the 175-bp enhancer. This indicates that *Mef2* is regulated by factors other than Twist through a separate enhancer at this time. Enhancer activity responsible for *Mef2* expression in differentiated muscles has been identified (Lilly et al. 1995; Schulz et al. 1996), however, the location and regulation of these enhancers has not been studied. Finally, a separate enhancer element has been recently defined that regulates *Mef2* expression in some muscle founder cells in the embryo (R.A. Schulz, unpubl.; B. Zhao, R. Cripps, E. Olson, unpubl.). These observations underscore the complexity of regulatory mechanisms that contribute to *Mef2* expression.

Ectopic expression of Twist in the embryo can induce reporter gene expression through the 175-bp enhancer, yet early in embryogenesis, when Twist is present uniformly in the mesoderm, the enhancer was not fully active. A possible explanation for this discrepancy is that Twist is over-expressed under the control of the *hs-GAL4* driver. Perhaps high levels of Twist are sufficient to activate the enhancer throughout the embryo. In the mesoderm, lower levels of Twist alone cannot support enhancer activity.

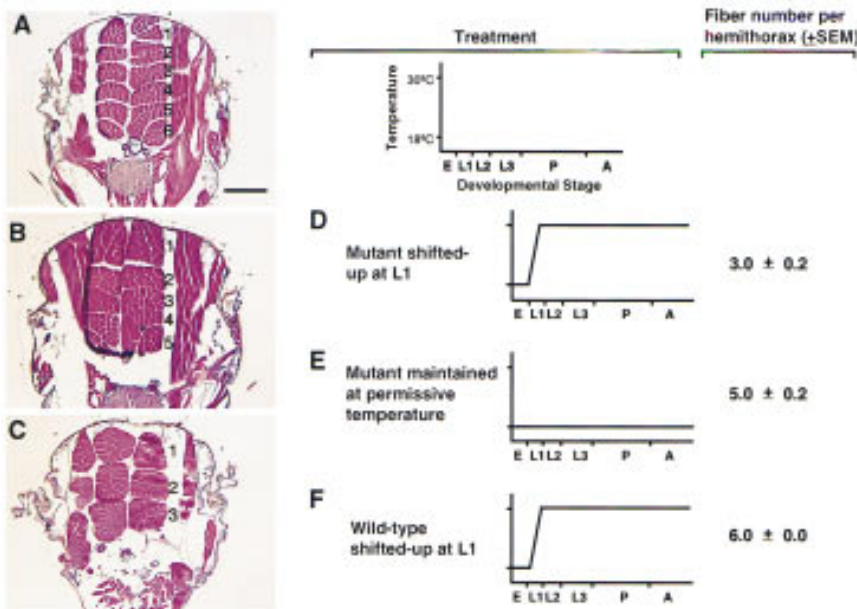


Figure 7. Adult muscle phenotypes resulting from reduced *twist* expression. (A–C) vertical transverse paraffin sections of thoraces to visualize the two rows of dorsal longitudinal indirect flight muscles (muscles are numbered 1–6 where present). (A) wild-type; (B) *RY50/V50* raised at 18°C; (C) *RY50/V50* raised at 30°C. Bar, 100 μ m. (D–F) summary of temperature shifts performed and fiber defects observed; fiber numbers represent the average number of DLM fibers per hemithorax. (D) *RY50/V50* raised at 30°C after embryogenesis; (E) *RY50/V50* raised at 18°C; (F) wild-type raised at 30°C after embryogenesis. (S.E.M.) Standard error of the mean. Developmental stages: (E) embryonic stage; (L1–3) larval instars; (P) pupal stage; (A) adult stage.

A myogenic regulatory function for twist

The requirement for *Drosophila twist* in somatic muscle development contrasts with studies of the murine homolog M-Twist, which inhibits muscle cell differentiation in culture (Hebrok et al. 1994; Spicer et al. 1996).

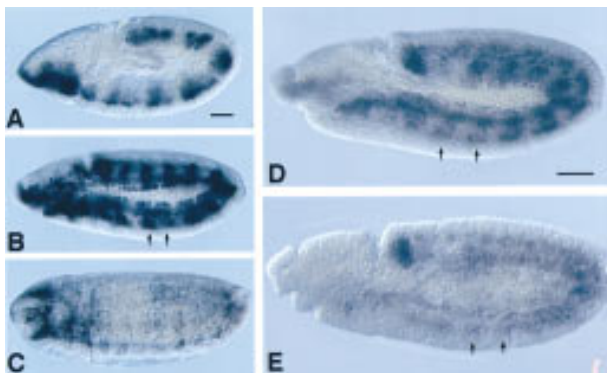


Figure 8. Expression of the 175-bp enhancer during embryogenesis. (A) At stage 9, the enhancer was only weakly active in the developing mesoderm and was segmentally modulated. (B) at late stage 11 the enhancer was active strongly in segmentally repeating groups of cells that are the precursors of the larval somatic muscles (arrows). (C) By stage 15, enhancer activity was waning. (D–E) Accumulation of MEF2 in the presence (D) or absence (E) of Twist function after gastrulation. (D) MEF2 accumulation in a late stage 11 *twist* mutant heterozygous embryo, showing normal expression in somatic muscle myoblasts (arrows). (E) MEF2 accumulation in a sibling temperature-sensitive *twist* mutant in which Twist function had been reduced after gastrulation. There is a general reduction in *Mef2* expression in the somatic myoblasts (arrows), as well as in the precursors of the pharyngeal muscles (out of focus at anterior end). Bar, 100 μ m.

Thus, it has been unclear if Twist acts as an activator or a repressor in the myogenic lineage. Here, we show that *Drosophila* Twist is a direct and positive regulator of *Mef2* expression, correlating with its requirement for muscle development in *Drosophila* and establishing it as a potent transcriptional activator in these cells. These results are consistent with other roles for Twist both early during mesoderm formation, where Twist activates *snail*, (Ip et al. 1992a) *rhomboid* (Ip et al. 1992b) and possibly other genes (Lai et al. 1991; Leptin 1991; Shishido et al. 1993), and later in the activation of *tinman* in the visceral mesoderm (Lee et al. 1997).

Twist function after gastrulation is important for the division of the mesoderm into somatic, cardiac, and visceral muscle lineages (Baylies and Bate 1996). Whereas our results show a role of Twist in the activation of *Mef2* expression in somatic myoblasts, Twist must be involved in the activation or repression of additional genes to mediate these other functions. The identity of these genes is not yet known.

The role of twist in adult muscle development

One proposed function of *twist* during the larval stage might be to maintain adult muscle precursor cells in an undifferentiated state to control the proliferation of these cells prior to differentiation (Bate 1993). A reduction in *twist* function during the larval stage, however, does not result in major defects in the ad epithelial cells. These cells exhibit many of the characteristics of wild-type cells, including expression of *htl* and *cut*, and the nuclear localization of Cut (Fig. 6) reveals that ad epithelial cells apparently formed and proliferated normally. Thus, Twist must play a more subtle role in the development of the adult muscle cells.

We have shown that when Twist function is reduced during the larval stage, *Mef2* is not expressed in the adult muscle precursor cells, resulting in abnormal patterning of the adult somatic muscles, most strikingly in the DLMs. Hypomorphic *Mef2* mutant adults show very similar defects (Ranganayakulu et al. 1995), indicating that this phenotype, resulting from loss of *twist* expression, likely occurs through a requirement to maintain normal *Mef2* levels.

It is striking that reducing Twist function during the embryonic stage has a severe effect on development of the musculature (Baylies and Bate 1996), yet, a reduction during the larval and pupal stages has a more minor effect on adult myogenesis. The basis for this difference in *twist* function at different stages is unclear. Perhaps the major function of *twist* in adult myogenesis occurs during the embryonic stage, at the same time as the critical role of *twist* in embryonic muscle development. Alternatively, it might be that adult myogenesis is initiated independently of *twist*, and *twist* expression in the ad epithelial cells is only required for muscle patterning.

The DLM defects we observed can be explained in terms of DLM development during the pupal stage. DLMs develop when ad epithelial cells from the wing disc surround and associate with three pairs of larval body wall muscles, the larval oblique muscles (LOMs; Fernandes et al. 1991). Once the ad epithelial cells arrive, each LOM splits longitudinally to form templates for the six pairs of DLMs found in the adult. After splitting, adult myoblasts fuse with the LOMs and commence differentiation. Our results show that the DLM defects observed here and in *Mef2* mutants likely result from a lack of *Mef2* expression in the ad epithelial cells, because *twist* is expressed in the adult myoblasts but not in the LOMs used as templates for DLM development (Fernandes et al. 1991).

The most likely cause of the DLM patterning defect is a failure of the LOMs to undergo splitting. Absence of LOM splitting would result in the observed reduction of final fiber number from six per hemithorax in wild-type flies to three in the mutants. Intermediate numbers of DLMs may result from the splitting of some LOMs but not others.

Conservation of regulatory pathways controlling *Mef2* expression

Drosophila and vertebrates share the diversification of the mesoderm into skeletal, cardiac, and visceral muscle lineages, and there is conservation in both sequence and function in many of the major myogenic factors. It is, therefore, likely that there is also conservation in the mechanisms that regulate these factors during development. One enhancer required for *Mef2* expression in a subset of cardiac cells is regulated by Tinman (Gajewski et al. 1997), and it is tempting to speculate that the vertebrate *tinman*-like gene *Nkx2.5* might regulate cardiac expression of *mef2* genes in the mouse. Here, we have identified a second enhancer that is responsive to Twist and that is expressed in somatic myoblasts. It is likely

that *Drosophila Mef2* contains other enhancers that direct complex temporal and spatial expression patterns in other mesodermal compartments. Identification of these enhancers and the factors that regulate them should reveal the transcriptional networks governing *Mef2* expression in multiple lineages.

Materials and methods

Generation of constructs for promoter/enhancer analysis

Constructs containing *EcoRI* genomic fragments are described in Lilly et al. (1995) and Schulz et al. (1996). For analysis of enhancer activity in transgenic flies, constructs devoid of an endogenous start site were cloned into the CaSpeR-hsp-AUG- β -gal vector (CHAB, which contains an *hsp* promoter upstream of *lacZ*; Thummel and Pirrotta 1992), whereas those including the transcriptional start site of *Mef2* were cloned into a similar vector in which the heat shock promoter had been removed. Initial constructs were generated by use of appropriate restriction sites (see Fig. 1); all subsequent constructs were generated by PCR. PCR products were first cloned into the pCR2.1 vector (Invitrogen) and then cloned into CHAB by use of appropriate flanking sites.

Mutagenesis of the 175-bp enhancer was carried out by gene SOEing (Horton 1993). The E1 E box was changed from 5'-CATGTG-3' to 5'-CGGCCGTG-3' that ablates both overlapping E boxes, and the E2 E box was mutated from 5'-CAGATG-3' to 5'-GACGTC-3'. The smallest construct generated was a 30-bp fragment (-2306 to -2277); this was made by annealing two oligonucleotides to give the correct final sequence and this also generated 5' overhangs at each end to permit insertion into the *EcoRI* site of CHAB.

Sequencing of genomic DNA and of PCR-generated mutant constructs was carried out with an ABI 373 automated sequencer.

P-element-mediated germ-line transformation was carried out as described (Rubin and Spradling 1982). *y w* embryos were coinjected with vector DNA and $\Delta 2-3$ helper plasmid (Robertson et al. 1988). G_0 adults were crossed with *y w*, and transformants isolated in the G_1 generation. For each new construct generated, at least three independent lines were analyzed.

Cloning of the *D. virilis Mef2* gene

A *Drosophila virilis* genomic library made by J. Tamkun (University of California, Santa Cruz) and provided by S. Wasserman (University of Texas Southwestern Medical Center, Dallas) was screened with a probe made from the MADS and MEF2 regions of *D. melanogaster Mef2* cDNA. From the clones isolated in the primary screen, secondary screening was performed on duplicate filters: one duplicate was probed with the same probe as the primary; the other duplicate was hybridized with a probe spanning the transcriptional start site of *D. melanogaster Mef2* (from -140 to +440). Two independent clones hybridized with both probes. Sequencing both of these identified a region of strong sequence similarity to the transcriptional start site of *D. melanogaster Mef2* (90% homology over 52 bp, from -15 to +37 in *D. melanogaster*). Upstream of the start site there is ~4.2 kb of 5' genomic sequence to the bacteriophage arm. A 3.9-kb fragment extending from a *Sall* site in the bacteriophage arm to an *EcoRI* site upstream of the transcriptional start (-4248 to -358) was cloned into CHAB and used to generate transgenic lines.

Cell culture and transfections

For cotransfection assays, the *twist* expression plasmid pPac-

Twist (Shirokawa and Courey 1997), was provided by J. Shirokawa and A. Courey (University of California, Los Angeles), and the *nanu* expression plasmid was from B. Paterson [National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD]. Reporter constructs were the same as those used for *P*-element mediated germ-line transformation of flies, which contain the 175-bp enhancer (wild type or mutant), and a minimal promoter upstream of the *lacZ* gene. SL2 cells were maintained at 25°C in Schneider's *Drosophila* medium (GIBCO/BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS). Two hours prior to transfection, cells were seeded at ~80% confluence onto T25 flasks. Cells were transfected with the DOSPER reagent (Boehringer Mannheim). Six micrograms of DNA (3 µg of activator plasmid and 3 µg of reporter plasmid) were mixed with 18 µg of DOSPER in 2 ml of Dulbecco's modification of minimal essential medium (DMEM) without serum or antibiotics and incubated for 15 min at room temperature. Schneider's *Drosophila* medium was removed from the cells and the DMEM containing the DNA/lipid complex was added. After 5 hr, the DMEM was removed, 3 ml of fresh Schneider's *Drosophila* medium containing 10% FCS was added, and the cells incubated for 43 hr prior to harvesting.

β-Galactosidase assays

Transfected cells were harvested by scraping the attached cells into the culture medium and centrifuging all adherent and non-adherent SL2 cells. Cell pellets were resuspended in 3 ml of PBS and centrifuged again. The cells were then resuspended in 0.1 ml of 0.1 M sodium phosphate (pH 7.0) and lysed by sonication. Debris was pelleted by centrifugation at 12,000g at 4°C for 15 min, and the supernatant was removed. The cell lysates were quantitated for total protein (Lowry et al. 1951), and 50 µg of lysate from each transfection was assayed for β-galactosidase activity as described previously (Sambrook et al. 1989). To allow for direct comparison of samples with dramatically different levels of β-galactosidase activity, reactions containing two 10-fold dilutions of extract from each sample were also conducted with 5 and 0.5 µg of lysate from each transfected sample. The total amount of cellular lysate in each reaction was held constant at 50 µg by adding the appropriate amount of untransfected SL2 cell lysate to each sample. Serial dilutions of purified β-galactosidase enzyme (Boehringer Mannheim) were also analyzed by mixing with 50 µg of lysate from untransfected SL2 cells to allow for direct comparison of the number of units of enzyme present in each transfected sample. Reactions were conducted at 37°C for 2 hr, then stopped by the addition of 1 M sodium carbonate. Samples were measured for absorbance at 420 nm to determine the amount of β-galactosidase activity in each sample. Only samples in the linear range of the assay (absorbance readings of 0.05 to 1.0) were used for comparison.

In vitro DNA binding assay

For in vitro transcription and translation of Twist, a 1.6-kb *NdeI* to *EcoRI* cDNA fragment containing the *twist* coding region was excised from the pAR 3040 plasmid (Ip et al. 1992b; kindly provided by M. Levine, University of California, Berkeley) and cloned into the unique *NdeI*-*EcoRI* sites of pCITE-2a (Novagen). Twist protein was generated from this construct with the TnT transcription and translation kit (Promega Corp.). To generate radiolabeled E1 probe DNA, two oligonucleotides of the sequence 5'-GGATGTTGCACATGTGTTGAGT-3' and 5'-GGACTCAACACATGTGCAACAT-3' were annealed. After annealing, the 5' GG overhangs were filled in with Klenow enzyme (New England Biolabs) and [α -³²P]dCTP. Probes were

purified on a G25 spin column (Boehringer Mannheim), and 50,000–120,000 cpm were used in each assay. For competition experiments, wild-type E1 oligonucleotide, mutant E1 oligonucleotide, or wild-type E2 oligonucleotide was included in the reactions at 50× and 300× concentrations. The E1 mutant oligonucleotides had the sequences 5'-GGATGTTGCGGCCGT-GTTGAG T-3' and 5'-GGACTCAACACGGCCGCAACA T-3' before annealing. The sequence of the mutated E box in this fragment is the same as that in the analyses of enhancer function in transgenic flies and in cotransfection assays. The E2 oligonucleotides had the sequences 5'-GGACTGCCGCAGAT-GCCGCATG-3' and 5'-GGCATGCGGCATCTGCGGCAGT-3'. Each gel shift reaction consisted of probe, 1 µg of poly[d(I-C)]; 3–4 µl of TnT lysate and 1 µl of a 10× buffer consisting of 400 mM KCl, 150 mM HEPES at pH 7.9, 10 mM EDTA, 5 mM DTT, 50% glycerol. After incubation at room temperature for 20 min, reactions were electrophoresed on a 5% (wt/vol) polyacrylamide gel at 22 mA. Dried gels were subjected to autoradiography.

Histochemistry, immunohistochemistry, and in situ hybridization

Embryos were stained with antibodies as described by Patel et al. (1987). Imaginal discs for immunohistochemistry were dissected from larvae in PBS, fixed for 30 min on ice in 4% paraformaldehyde in PBS, washed in PBS and then incubated in 0.2% (vol/vol) Triton X-100 in PBS at room temperature for 20 min. Discs were then treated for antibody staining as for embryos. The anti-MEF2 antibody was from B. Paterson (NCI, NIH, Bethesda, MD; Lilly et al. 1995) and was used at a concentration of 1:1000. The anti-Twist polyclonal antibody was from C. Thisse and B. Thisse (University of Strasbourg, France) and was used at 1:500. These antibodies were detected by use of a peroxidase-linked goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) at 1:1000. The anti-β-galactosidase monoclonal antibody (Promega) was used at 1:300, and the anti-Cut monoclonal antibody was used at 1:150. These antibodies were detected with the Vectastain Elite Staining Kit (Vector Laboratories, CA). The anti-Cut antibody, developed by I. Rebay, G. Dailey, K. Lopardo, and G. Rubin, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, the University of Iowa, Iowa City, under contract from the National Institute of Child Health and Development (NICHD). Stained discs were prepared for sectioning as described by Lyons et al. (1990).

Imaginal disc in situ hybridization was performed essentially as described by Tautz and Pfeiffle (1989) and O'Neill and Bier (1994). Briefly, imaginal discs were dissected from larvae and fixed in 4% formaldehyde, 0.5% Triton X-100 in PBS on ice for 30 min. After washing in 0.5% Tween-20 in PBS (PBTw), discs were treated with 12.5 µg/ml proteinase K in PBTw for 2 min. at room temperature, and then quenched in 2 mg/ml glycine in PBTw. Discs then were postfixed in 4% formaldehyde, 0.2% glutaraldehyde in PBTw for 20 min at room temperature. Remaining discs were then washed and subjected to prehybridization and hybridization as described. A digoxigenin (Boehringer Mannheim) labeled antisense riboprobe for the *htl* gene was generated from a plasmid provided by A. Michelson (Harvard University, MA). The riboprobe was detected by use of an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:1000), and BM purple AP substrate for the color reaction (Boehringer Mannheim).

Imaginal discs for X-gal staining were dissected from larvae, fixed for 20 minutes in 4% (vol/vol) formaldehyde in 1× PBS on

ice, washed in 1× PBS and then stained in an X-gal solution at 37°C as described (Ashburner 1989).

Fly culture and temperature shifts

Flies were grown on standard culture medium at the indicated temperatures. All genetic symbols are described in Lindsley and Zimm (1992). The stocks *SM1/twi^{RY50} bw* and *SM1/twi^{V50} bw sp*; *st e* were provided by R. Reuter, (Universität zu Köln, Germany). Only the heteroallelic combination of these two alleles is temperature sensitive; neither allele alone is temperature sensitive (Thisse et al. 1987).

For larval temperature-shift experiments, crosses were set up in cages where flies laid eggs on agar plates darkened with grape juice. These plates were incubated at 18°C, and mutant larvae were collected from the plates shortly after hatching in groups of 50, and placed in well-yeasted vials for subsequent culture. The permissive temperature in these experiments was 18 ± 1.0°C. The restrictive temperature was 30 ± 1.0°C. For embryonic temperature-shift experiments, eggs were collected over a 2-hr period at 18°C and aged at the permissive temperature until after gastrulation. The incubation temperature was then shifted to 30°C by floating the agar plates in a water bath for the remainder of the experiment.

Identification of twist mutants

To separate the *twist* mutant larvae from nonmutant siblings at the larval stage, each mutant was crossed into a genetic background that was *y w* on the X chromosome and in which the mutants were balanced over a *CyO* balancer chromosome (designated *CyO, y⁺*) onto which the *y⁺* gene had been introduced (see Mardahl et al. 1993). From the cross of *y w; CyO, y⁺/twi^{RY50} bw × y w; CyO, y⁺/twi^{V50} bw sp* heteroallelic mutant first instar larvae could be identified on the basis of the color of their mouth hooks: *y⁻* individuals have yellow mouth parts, compared with the black mouthparts and dark denticle belts of *y⁺* individuals.

To identify *twist* mutant offspring during the embryonic stage, stocks were balanced over an *SM6b* chromosome that carries an *even-skipped-lacZ* transgene. Mutant offspring were identified on the basis of their lack of reaction with anti-β-galactosidase antibody compared with siblings.

Analysis of adult phenotypes

For sectioning adult thoraces, the heads and abdomens were removed from 1- to 3-day-old adult flies. The thoraces were then fixed and embedded in paraffin by use of standard procedures (Lyons et al. 1990). Sections of 7–10 μm were stained with hematoxylin for 30 sec and eosin for 30 sec, then dehydrated and mounted in Permount. To quantify the muscle phenotype observed in the *twist* mutant adults, thoraces were dissected as described by Peckham et al. (1990) until the point at which thoraces were longitudinally bisected. Half-thoraces were observed under the dissecting microscope, and the number of DLM fibers was counted in each half-thorax. By convention, the average DLM count for flies in any class was determined by averaging the number of fibers in the right hemithorax, except where the right hemithorax was damaged, in which case the fiber number was taken from the left hemithorax for that individual fly. We have not seen any consistent left-right bias in any of the flies that were dissected. At least nine different adults were tested for each experiment.

Ectopic expression of twist

Ectopic expression of *twist* in embryos was under the control of a heat shock promoter via the GAL4 system (Brand and Perrimon 1993; Taylor et al. 1995). *hs-GAL4* was from the Bloomington Drosophila Stock Center, and *UAS-twi* was provided by M. Baylies (Memorial Sloane-Kettering Cancer Center, New York, NY). These lines were used to generate *UAS-twi; hs-GAL4/TM2, Ubx*. Males of this genotype were crossed to females homozygous for enhancer-*lacZ* constructs, and progeny subjected to heat shock and immunohistochemistry (Taylor et al. 1995).

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