A new homeobox-containing gene, msh-2, is transiently expressed early during mesoderm formation of Drosophila

ROLF BODMER*, LILY Y. JAN and YUH NUNG JAN

Howard Hughes Medical Institute and Departments of Physiology and Biochemistry, University of California, San Francisco, CA 94143, USA

* Present address: Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA

Summary

Many homeobox-containing genes of Drosophila regulate pathways of differentiation. These proteins probably function as promoter- or enhancer-selective transcription factors. We have isolated a new homeobox-containing gene, msh-2, by means of the polymerase chain reactions (PCR) using redundant primers. msh-2 is specifically expressed in mesodermal primordia during a short time period early in development. It first appears at blastoderm stage just before the ventral invagination of the mesoderm and shortly after twist, a gene required for mesoderm formation, is expressed. During germband elongation all the mesodermal cells in the segmented part of the embryo express msh-2, but soon afterwards msh-2 becomes restricted to the dorsal mesoderm, which includes the primordia for the visceral musculature and the heart. Prior to muscle differentiation, msh-2 expression ceases, except for two rows of cells that will be included in the dorsal vessel. Embryos that are deficient for the chromosomal region, 93C-F, which includes the msh-2 gene, show normal mesoderm invagination and dorsal spreading. However, later in development no visceral muscle and dorsal vessel differentiation can be detected, but some skeletal muscles do form, albeit abnormally. msh-2 expression, except for a patch in the head, is dependent on twist function. On the other hand, snail, another mesoderm determinant, does not appear to be required for msh-2 initiation, but is necessary for the maintenance of msh-2 expression after germband elongation. H2.0, a homeobox-containing gene specifically expressed in visceral mesoderm, is not transcribed in the mesoderm in 93C-F deficiency embryos. These results suggest that msh-2 may have a regulatory function in myogenesis and may be required for visceral mesoderm differentiation. msh-2 may act immediately after or downstream of twist.

Key words: mesoderm, homeodomain, myogenesis, differentiation, visceral muscles, heart, Drosophila.

Introduction

Many genes have been isolated that are involved in generating the metameric pattern in the ectoderm of Drosophila embryos (e.g. Nüsslein-Volhard and Wieschaus, 1980; Scott and Carroll, 1987; Ingham, 1988), but very little is known about genes that govern mesodermal pattern formation. In contrast to the ectoderm, where most cells maintain their positions relative to each other, mesodermal cell mixing occurs even across segment boundaries in Drosophila and mesoderm differentiation does not appear to be determined by lineage (Beer et al. 1987). Nevertheless, the final muscle pattern is organized in a highly stereotyped fashion and each muscle can be individually identified (Crossley, 1978). What are the mechanisms by which the mesodermal cell mass differentiates into this precise arrangement? How are individual or groups of mesodermal cells specified or subdivided to assume different cell fates? Observations in other insects revealed that muscle-organizing cells or pioneers exist that are involved in the assembly or differentiation of individual muscles during normal development (reviewed by Jellies, 1990). The genes, however, that regulate cell fates of muscle precursors and events that determine the precise muscle patterns are unexplored. Even the cellular or genetic basis for the distinction of major mesodermal subdivisions, e.g. somatic mesoderm, which forms the body wall muscles, versus visceral mesoderm, which forms the musculature around the gut, is not known.

A number of genes that are involved in establishing the dorsal-ventral polarity in Drosophila are also required for mesoderm formation early in development (for review see Levine, 1988). Two of those genes are zygotic, twist (twi) and snail (sna), and they are necessary for the initial mesoderm determination and are crucial for the mesoderm invagination at the beginning of gastrulation (Simpson, 1983; Nüsslein-Volhard et al. 1984). Mutations in these genes fail to
differentiate any muscles. twi and sna are expressed in the mesodermal anlagen at cellular blastoderm stage before the beginning of ventral furrow formation (Thissie et al. 1987, 1988; K. Arora and Nüsslein-Volhard, personal communication). The initiation of expression of twi and sna is independent of each other. Both genes are expressed roughly at the same time and in the same region of the blastoderm. They require each other for maintained expression (K. Arora and C. Nüsslein-Volhard, personal communication).

twi and sna are likely to be transcription factors (Thissie et al. 1988; Boulay et al. 1987). twi contains a helix–loop–helix (HLH) and sna a zinc-finger motif. Recent progress in elucidating the mechanism of myogenesis in vertebrates revealed a family of related genes that are involved in committing a cell to become a myoblast (Tapscott et al. 1988; Braun et al. 1989; Rhodes and Konieczny, 1989; Wright et al. 1989; Benezra et al. 1990). These genes all contain the HLH motif of DNA-binding proteins (Lassar et al. 1989; Davis et al. 1990; Benezra et al. 1990). The involvement of HLH genes in mesoderm specification thus appears to be general among vertebrates and invertebrates.

Genes that are downstream of the initial mesoderm specification events have not yet been identified. A few genes that act later in muscle differentiation, however, have been found. They are differentially regulated in various muscle tissues, e.g. β3-tubulin gene (Gasch et al. 1989) or myosin heavy-chain (O’Donnell et al. 1989). Recently, a few genes that are expressed in mesodermal cells early in development have been isolated because of their sequence similarity to known genes (Barad et al. 1988; Côté et al. 1987, see Fig. 6e,f; Bopp et al. 1989; B. Jacq and W.J. Gehring, personal communication).

We report here the identification of msh-2, a mesoderm-specific homeobox-containing gene. msh-2 expression pattern is limited to the mesoderm, especially to the dorsally migrating sheath of cells that gives rise to the visceral mesoderm. Genetic evidence is consistent with the hypothesis that msh-2 acts directly downstream of the early mesodermal determinant twi. msh-2 may serve as a link between twi and genes that are expressed later in development such as H2.0.

Materials and methods

Polymerase chain reaction

The PCR protocol was adapted from Mack and Sninsky (1988). The primers used in this study were derived from the cut homeobox (Blochlinger et al. 1988). In order to allow for the degeneracy of the genetic code, primer 1 (P1) was 512-fold redundant and primer 2 (P2) 2048-fold redundant. The sequence of P1 was 5’CGAATTCTGGTCTCTTCTCGXGAXGA(G)/CA and primer 2 was 5’GAAATTCA(A/G)/XCGCATXCGXGTGA(G/A)TTXTG(A/G)ACCA derived from the RVLFSSEQ protein sequence of the cut homeodomain and P2 was 5’GAAATTCA(A/G)/XCGCATXCGXGTGA(G/A)TTXTG(A/G)ACCA derived from the WFHNHRML sequence. The primers also contained EcoRI sites at the 5’ends. P2 contained additional redundancy in the third base position of the first histidine to allow also for glutamine which is more common in that position of homeodomains. The template DNA was isolated from embryos that were homozygous mutant for the cut deficiency Df(1)ct60A (Johnson and Judd, 1979; Bodmer et al. 1987) in order to avoid preferential amplification of the cut homeobox.

For this purpose, about 500 mutant embryos were hand-selected based on their spiracle phenotype (Bodmer et al. 1987). Between 10 and 50 ng of DNA was used together with 1 mM final concentration of each primer in a 60 μl reaction volume. The other components of the reaction mixture were the same as described in Mack and Sninsky (1988). In an amplification with PCR, 30 cycles were carried out in a thermo-cycler (Elmer-Cetus) using the following low-stringency conditions for hybridization of the primers: 1 min denaturation step at 94°C, 2 min hybridization step at 40°C and 30s elongation step at 72°C. After amplification 1/3 of the reaction was run on a 4% agarose gel (3% Nusieve and 1% GTG Sekam agarose, FMC BioProducts). The predominant band of about 160 bp was cut out of the gel, the DNA was purified (gene clean, Bio 101), subcloned into a bluescript vector (Stratagene) and sequenced (Sanger et al. 1977).

Isolation of cDNA and Northern analysis

Screening of a cDNA library (Nolan et al. 1986) was carried out as described (Blochlinger et al. 1988). A 7 kb insert was sequenced (Sanger et al. 1977). The sequence and its translation is shown in Fig. 1. Northern analysis was carried out as described in Schwarz et al. (1988). A 32P-labelled probe was derived from the double-stranded insert of the sequenced msh-2 cDNA clone using random primers.

Whole mount tissue in situ hybridization

In order to visualize the tissue distribution of transcripts, embryos were incubated as whole-mount preparations with a nonradioactive probe made from the msh-2 cDNA or the H2.0 cDNA (pg2.7, Barad et al. 1988) using digoxigenin and an immuno-enzymatic detection kit (genius, Boehringer) according to the protocol of Tautz and Pfeifle (1989).

Antibody staining

Whole-mount embryos were stained with antibodies as described (Bodmer et al. 1987). The muscle-specific antibody 6D5 (Bier et al. 1990) was used at 1:15 dilution, affinity-purified anti-HRP antibodies at 1:500 (Jan and Jan, 1982; Bodmer and Jan, 1987), preabsorbed anti-Twist antibodies (Roth et al. 1990) at 1:1000 and anti-β-galactosidase antibodies (Vector Labs) at 1:1000 (Bier et al. 1989).

Drosophila stocks

The deficiencies for the 93E region, Df(3R)eBS2, rsd/TM3 (93C-F) and Df(3R)e-N19/TM2 (93B-94A), were obtained from the Bloomington Drosophila stock center (Lindsley and Zimm, 1987). The deficiencies were crossed to a TM3 Balancer chromosome which contains a P-element insertion that expresses β-galactosidase (β-gal) (obtained from D. Hogness). This balancer was used as a marker to distinguish the embryos that were homozygous for the deficiencies. twi1D96a and sna1H431 were obtained from C. Nüsslein-Volhard; both are strong mutations (K. Arora and C. Nüsslein-Volhard, personal communication).

Results

Identification of the msh-2 gene

In order to find new homeobox-containing genes, we used PCR amplification. The degenerate oligonucleotide primers were derived from conserved homeobox regions, based on the homeobox sequence from the
**Drosophila** gene **cut.** Genomic DNA from *Drosophila* embryos that were deficient for the cut gene was used as template DNA for the amplification. A 1.7 kb cDNA containing the amplified DNA fragment was subsequently isolated and the sequence revealed an open reading frame of 412 amino acids starting with the first methionine in the open reading frame (Fig. 1). Another potential start site is 45 amino acids downstream of this methionine; it also conforms with the consensus translational start (C/AAAA/C, Cavener, 1987, overlined in Fig. 1). Northern analysis showed expression of an approximately 1.7 kb transcript between 3 and 9h of embryonic development (Fig. 2). This transcript is also expressed at low levels between 9 and 12 h. Because of the comparable size of the cDNA and the transcript, it is likely that the sequenced cDNA is full length.

**Sequence analysis of msh-2 reveals a homeodomain which is most closely related to msh, another mesoderm-specific homeobox-containing gene (Fig. 3; Gehring, 1987, and personal communication, cytological location: 99B) and to two genes isolated by Kim and Nirenberg (1989). The sequence of the msh-2 homeodomain has recently been reported and localizes to the chromosomal location 93E1-3 (Kim and Nirenberg, 1989).**

**Expression pattern of msh-2**

In order to establish the expression pattern of this gene during development, whole-mount embryos were hybridized with a non-radioactive, digoxygenin-labelled probe derived from the sequenced cDNA (see Materials and methods). The temporal pattern of expression of msh-2 is shown in Fig. 1.
pression was compared with the pattern of a protein encoded by twi (Fig. 4), which is required for mesoderm development and is the earliest zygotic gene product known that is mesoderm-specific (Thisse et al. 1988).

In Fig. 2, developmental Northern analysis of msh-2. Poly(A)* RNA blots were kindly provided by T. Jongens. The whole 1.7 kb insert of a cDNA clone, including the homeobox region, was used as a probe. The 1.7 kb transcript is first detected in 3–6 h embryos. No maternal component can be detected in RNA from ovaries (ov). The transcript in 6–9 h and 9–12 h embryos may represent a doublet. An actin probe was used to control for relative amounts of RNA loaded in each lane. The lane with RNA from 3–6 h embryos apparently contains less RNA than the 6–9 h lane and most likely accounts for the less-intense band in the 3–6 h lane as compared to the 6–9 h lane.

The first expression of msh-2 is seen in late blastoderm just prior to the ventral invagination of the mesoderm at about 2 h 45 min after egg laying (at 25°C) (Fig. 4A,B). The onset of msh-2 expression is therefore 20 to 30 min later than the appearance of Twist protein (Thisse et al. 1988). The extent of msh-2 expression along the ventral longitudinal axis coincides with Twist in the segmented part of the embryo (Fig. 4A–C). The strip of msh-2-expressing cells is about 14–16 cells wide (Fig. 4B), which is slightly less than the number of Twist-containing cells at that stage (data not shown). msh-2 RNA is absent in the cephalic region except for a group of cells at the anterior tip of the embryo (Fig. 4A). The msh-2 domain in the head extends more dorsally than the Twist protein pattern. At the posterior tip, Twist-expressing cells are more numerous. These patterns become more pronounced after gastrulation has begun (arrows in Fig. 4E,F).

During the first phase of major mesodermal cell movements, which comprise ventral invagination of the mesodermal primordia and germband extension, most mesodermal cells posterior to the cephalic furrow express msh-2. This expression pattern coincides with that of Twist protein (Fig. 4D–I). On the other hand, Twist is also expressed in the anterior and possibly some of the posterior midgut primordia (Fig. 4F; Thisse et al. 1988), whereas msh-2 is not expressed in the midgut primordia (Fig. 4E). The period of general msh-2 expression throughout the mesoderm lasts until about 5 h of development.

A second phase of mesodermal migration starts at about 4 h of development. The mesodermal mass starts to spread dorsally in a single layer of cells, which is seen with anti-Twist antibodies as well as a msh-2 probe (Fig. 4H–I). As the cell layer reaches the dorsal margin (which is located in the middle region of the embryo in a side view of the extended germband) the ventrally located mesodermal cell mass ceases msh-2 expression after about 5 h of embryogenesis, but the dorsal cells continue expression for another one to two hours (Fig. 4J,K,M). In contrast, Twist protein is still present in the ventral muscle primordia at this time (Fig. 4L). At these stages, cephalic msh-2 expression surrounds...
the stomodeum (Fig. 4J). The dorsal, msh-2-positive mesoderm cells are part of the primordia for the visceral musculature. Expression in the dorsal mesodermal ribbon becomes further reduced to patches of cells (Fig. 4M) adjacent to the gut in the middle of each segment, slightly dorsal to the tracheal pits. The somatic and visceral mesoderm could not be distinguished morphologically before expression in these primordia ceased. Twist protein expression is also diminished when msh-2 RNA becomes reduced to the dorsal mesoderm (Thisse et al. 1988).

At about 7 h of development, when only patches of presumably visceral primordia express msh-2, a row of cells at the dorsal-most margin below the surface starts expressing msh-2 (Fig. 4M,N). These cells are the only ones that continue to express msh-2 beyond the germband-extended stage. They continue expressing msh-2 until cuticle is secreted, which blocks antibody access to whole-mount embryos. At these late stages msh-2 expressing cells can be identified as part of the dorsal vessel (Fig. 4O). These observations indicate that the dorsal row of msh-2-expressing cells during germband-extended stage is part of the heart primordia. Since msh-2 is mesoderm-specific, these findings raise the question of whether the primordial heart cells originate from the mesoderm mass that invaginates ventrally or locally from the dorsal region. The earliest expression in the heart primordia is very weak (arrowheads in Fig. 4M) and gets stronger later (Fig. 4N,O). This could be because of new initiation of msh-2 in dorsally derived cells or persistence in ventrally derived mesodermal cells. Possible dorsal sources for the heart cells are amnioserosa cells or dorsal ectoderm. Mesodermal cells that were transplanted in a previous study to the anlage of the amnioserosa or the dorsal ectoderm were not incorporated into the heart but rather into somatic and visceral muscles (Beer et al. 1987). These experiments favor the dorsal origin of the heart cells. On the other hand, it was observed that embryos that are mutant for twi or sna do not exhibit msh-2 expression in the dorsal region (see below). This suggests a ventral mesodermal origin for these cells or at least a dependence on ventral mesoderm. Further experiments are needed to clarify the origin of the heart.

msh-2 expression in mutants defective in gastrulation
Because msh-2 expression starts as early as the blastoderm stage and continues to be expressed in the mesoderm, we wondered if genes that are required for mesoderm formation and ventral invagination are necessary for the expression of msh-2. For this purpose, we examined mutant embryos of twi and sna (Nüsslein-Volhard et al. 1984). Both genes are expressed in the mesodermal anlagen at the blastoderm stage prior to msh-2 (Thisse et al. 1988; K. Arora and C. Nüsslein-Volhard, personal communication).

In twi mutant embryos, msh-2 is not expressed at any time during embryonic development, except for the cephalic patch (Fig. 5A,C). As mentioned above expression in dorsal heart primordia is also abolished in twi, even in 14 h old embryos (data not shown). This finding suggests that all of the msh-2 expression posterior to the cephalic furrow depends on the function of twi. Therefore, twi must be upstream of msh-2. msh-2 expression begins very shortly after Twist protein is detected. This close temporal spacing between Twist protein and msh-2 RNA appearance suggests that msh-2 is directly regulated by twi. In the head, msh-2 does not seem to be regulated by twi.

In sna mutant embryos, msh-2 is initially expressed in cells that form the mesodermal anlagen (Fig. 5B). However, shortly after the germband has extended (4–5 h of development) msh-2 expression posterior to the cephalic furrow ceases (Fig. 5D). It appears that in sna embryos initiation of msh-2 expression posterior to the cephalic furrow does occur but fails to be maintained. Since twi is also transiently expressed in sna and its time course of expression is similar to that of msh-2 (K. Arora and C. Nüsslein-Volhard, personal communication), msh-2 expression posterior to the cephalic furrow in sna mutants may be entirely due to twi. On the other hand, msh-2 expression anterior to the cephalic furrow is enlarged and extends more ventrally in sna, and is not turned off at later stages (Fig. 5B,D). Therefore, msh-2 expression in the cephalic region must be under a different regulatory hierarchy than posterior to the cephalic furrow.

**Phenotype of embryos that are deficient for the 93C-F region**
Since msh-2 maps to the 93E region, we examined the phenotype of two deficiencies for this region of the chromosome (see Materials and methods). In mind that there are a number of genes including two other homeobox-containing genes (Kim and Nirenberg, 1989) that are removed in these deficiencies, we investigated possible muscle defects in mutant embryos. We wanted to see if muscle defects might correlate with the pattern of normal msh-2 expression and could potentially be due to the loss of msh-2 function. The deficiency phenotype was analyzed using a muscle-specific antibody and PlacW insertion lines that show β-gal expression in all or a subset of somatic and visceral muscles (Bier et al. 1989).

The antibody 6D5 (Bier et al. 1990) stains all visceral (Fig. 6A) and somatic muscles (Fig. 6C) (except for the heart) after they differentiate. Embryos deficient for the 93C-F region which includes the msh-2 gene do not show any labelling of visceral muscles around the gut with this antibody (Fig. 6B). In addition, inspection of these embryos under Nomarski optics revealed that the typical thin layer of visceral muscles around the gut is absent or at least severely reduced. The outline of the midgut and the hindgut can still be detected (arrows in Fig. 6B), but the normal constrictions of the gut do not appear (compare with Fig. 6A). In contrast, somatic muscles of the body wall do form in these deficiency embryos, although they are reduced and abnormal (Fig. 6D) compared with the normal muscle pattern (Fig. 6C). The pharyngeal muscle, which is located in the interior of the embryo, also forms in the deficiency.
embryos (arrowhead Fig. 6A,B). Development in these embryos must, therefore, progress far enough to produce differentiated muscle structures.

The possible failure of visceral muscle development in these deficiency embryos was further corroborated using a PlacW insertion that expresses β-gal specifically in visceral muscles during development. Expression of β-gal in the B6 2nd 30 line begins at a stage (8–9 h of development) when the visceral muscle primordia are in a conspicuous palisade arrangement (Fig. 6E), before they spread around the newly formed gut. In deficiency embryos, no staining can be detected in the locations of the visceral palisades or its derivatives (arrows in Fig. 6F). Therefore, even early in the development there are no signs of the visceral muscle formation.

The β-gal expression patterns of two other PlacW insertion lines were used to investigate dorsal vessel development in deficiency embryos. One line, A1 2nd 26, labels all the muscles including the heart; the other, C3 2nd 2, labels the ectodermal cells overlying the dorsal vessel (data not shown). A1 2nd 26 shows no labelling of visceral muscles or of heart structures in deficiency embryos at any time (data not shown). Even in well-differentiated late-stage embryos inspected under Nomarski optics, no characteristic heart structures can be observed. In contrast, the dorsal ectodermal cells overlying the heart (labelled in the C3 2nd 2 line) have a similar appearance in mutant and wild-type embryos (data not shown). These results indicate that the heart is specifically affected; other dorsal structures and dorsal closure appear normal. This finding, together with the lack of msh-2 expression in heart primordia in twi and sna mutant embryos (Fig. 5C,D), argues that the dorsal vessel derives from the ventrally invaginated mesoderm or at least is dependent on its formation.

A possible explanation for the lack of visceral musculature and heart development in 93C-F deficiencies would be that the mesoderm does not spread dorsally after invagination in these mutant embryos. This would probably result in the absence of the visceral mesoderm and the heart primordia that derive from or depend on the dorsal mesoderm. If this were the case, Twist protein would be expected to be absent from below the dorsal region of the ectoderm in deficiency embryos. Labelling of such embryos with anti-Twist antibodies, however, revealed a normal pattern of dorsally migrated mesodermal cells (arrows in Fig. 7B). Therefore, the deficiency phenotype is probably not a disruption of major morphogenic movements of mesodermal cells. More likely, the cells that normally constitute the primordia for the viscera and heart muscles are present at the right locations but fail to be specified correctly, and therefore, do not express any differentiated characteristics of these muscle types.

We conclude from the phenotypic analysis of 93C-F deficiencies, which include the msh-2 gene, that the abnormal somatic and visceral muscle development in deficiency embryos correlates with the msh-2 expression pattern in these tissues. Whereas, the somatic muscles are abnormal, the visceral muscles appear to be absent altogether. Interestingly, transcription of msh-2 is also regulated differently in the somatic versus visceral mesoderm: msh-2 expression ceases in the
Fig. 6. Muscle phenotype of deficiencies that delete the msh-2 gene. The micrographs on the left are wild-type and those on the right are homozygous Df(3R)e-N19 embryos. A to D are mid-embryonic stage embryos stained with 6D5, a muscle-specific antibody that stains differentiated somatic (C) as well as visceral muscles (arrows in A). Deficiency embryos do not show any muscle differentiation or antibody staining around the gut (arrows in B) including the hindgut and the foregut. Antibody penetration problems in these mutant embryos can be ruled out since the internal pharyngeal muscle also stains in the mutant (arrowhead in A and B). It is interesting to note that the typical constrictions (A) of the gut do not occur normally in these embryos. On the other hand, some skeletal muscles do form although abnormally (arrowheads in D). There are other abnormalities in these embryos: for example, the nervous system is morphologically abnormal (data not shown). E and F shows the staining pattern due to the P{lacW} transformant line B6 2nd 30 (Bier et al. 1989) in 8–9 h old embryos visualized with an anti-β-gal antibody. This line specifically labels the visceral musculature and its palisade primordia (arrows in E). A deficiency embryo of a similar stage does not exhibit any staining around the gut, especially where the visceral palisades should be located (arrows in F, compare also with Fig. 7C), or any other visceral structures at any time in development. This embryo is shown in a top view as an optical section. This line also stains a small subset of CNS cells in wild-type as well as in mutant embryos.

Fig. 7. Expression of H2.0 RNA and Twist protein in embryos deficient for the msh-2 gene. A and C show the wild-type H2.0 expression in 6 h (lateral view) and 9-10 h (top view) embryos, respectively. In A the visceral primordia show H2.0 expression. Note the similarity to the msh-2 expression pattern in embryos of this stage (compare with Fig. 4K). In C the visceral palisades align along the midgut (arrows) as well as mesoderm unrelated, ectodermal patches in between segments (arrowheads) express H2.0. D shows the H2.0 expression in a 9–10 h Df(3R)e-N19 embryo (lateral view). H2.0 is not contained within this deficiency (Barad et al. 1988). In these deficiency embryos (D), no H2.0 is expressed around the gut (arrows), only the ectodermal patches remain positive (arrowheads). B shows a 6–7 h Df(3R)eBS2 embryo stained with anti-Twist and anti-β-gal antibodies, the latter antibody distinguishes the mutant from the non-mutant embryos (see Materials and methods). The morphogenic movements of the mesoderm (dorsal mesoderm is indicated by arrows) and the Twist-protein distribution appears unaltered (compare with Fig. 4I).

presumably somatic primordia after dorsal migration, but persists in the presumptive visceral and heart primordia (Fig. 4K,N). Differential RNA stability could also have a function in this process. Since the mesodermal movements appear to be normal in deficiency embryos, we speculate that a possible function of msh-2 may be in determination of mesodermal cell fates.

H2.0 may be a downstream target of msh-2
In a search for other homeobox-containing genes, Barad et al. (1988) found a gene, H2.0, which is primarily expressed in the visceral primordia of the mesoderm (Fig. 7A). H2.0 expression persists throughout visceral development (Fig. 7C, Barad et al. 1988). The onset of H2.0 expression coincides with msh-2 expression precisely at the time when msh-2 RNA becomes restricted to the dorsal region of the mesoderm (compare Fig. 7A with Fig. 4K). Both msh-2 and H2.0 RNAs are located in approximately the same position and depth in the embryo (see Fig. 4J for a ventral view of the msh-2 expression pattern in a slightly younger embryo) and are most likely expressed by an overlapping set of cells. This overlap suggests that msh-2 could be involved in the regulation of H2.0.

As a first test of this hypothesis, we examined H2.0 expression in embryos lacking the region around 93E. H2.0 RNA was absent in the vicinity of the midgut in mutant embryos and only ectodermal patches of H2.0 expression remained (Fig. 7D). This finding indicates that mesodermal expression of H2.0 is dependent on a gene contained in the deficiency, possibly msh-2.

Discussion

Developmental profile and homeobox homology of msh-2 suggests a regulatory function in mesoderm specification
We hypothesize that the msh-2 gene specifies mesodermal cell fates for the following reasons: (1) msh-2 contains a homeodomain (Fig. 1). The gene product may act, therefore, as a sequence-specific transcription factor, as many other homeodomain-containing genes (see Levine and Hoey, 1988; Scott et al. 1989; Biggin and Tjian, 1989). Many homeodomain-containing genes were originally identified genetically as key regulators of development (Lewis, 1978; Kaufman and Abbott, 1984; Nüsslein-Volhard and Wieschaus, 1980). (2) msh-2 is transiently expressed (Fig. 4). msh-2 RNA is found in the majority of the mesodermal cells early during gastrulation. Expression persists in visceral and heart muscle primordia later in gastrulation. Except for the heart, expression ceases before muscles start to assemble. (3) Embryos that are deficient for the chromosomal region where msh-2 maps show reduced and abnormal somatic muscles and no differentiation of visceral or heart muscles (Fig. 6). These phenotypes are consistent with possible essential functions of the msh-2 gene in mesoderm development, but such a function has to be substantiated by the analysis of point mutations.

In deficiency embryos, the mesodermal movements appear normal during gastrulation and during the dorsal migration of a mesodermal cell layer until germband retraction (Fig. 7B). Since msh-2 is expressed first in most mesodermal cells and later only in a subset, it is speculated that msh-2 may serve significant functions in somatic as well as visceral muscle development, including possibly the determination of the fate of the mesodermal primordia that give rise to the viscera and the heart. It remains to be determined whether the muscle phenotypes found in these de-
iciency embryos are actually due to the absence of the
msh-2 gene, rather than other genes within 93C-F. This
can be tested by isolating and analyzing msh-2 point
mutations or by rescue of muscle defects of 93C-F
deficiency embryos by germline transformation of the
msh-2 gene.

Possible hierarchical position of msh-2 in mesoderm
determination

We propose a cascade of gene regulations that specifies
the mesoderm. The expression of the mesodermal
determinants twi and sna are independently initiated
but require each other to maintain expression (K.
Arora and C. Nüsslein-Volhard, personal communication).
twi directly regulates msh-2 (except for the head
region), whereas sna influences msh-2 expression
indirectly through the action of twi. msh-2 is upstream
of genes that are specific for visceral mesoderm, such as
H2.0. We speculate that the homeobox-containing gene
msh-2 may be a mesodermal selector gene that is
required for executing developmental programs during
pattern formation in the mesoderm. This proposed
regulatory hierarchy is only tentative, because only
relatively large deficiencies were available for the
mutant analysis. The evidence for the proposed
hierarchy is summarized below.

The onset of msh-2 and twi expression is closely
spaced: msh-2 turns on only 20–30 min after Twist
protein is detected in the mesodermal anlagen at
blastoderm. In addition, msh-2 expression is dependent
on twi function, because in twi mutant embryos msh-2 is
not expressed at any stage, except for a region in the
head which does not appreciably overlap with the twi
expression pattern (Fig. 5A,C). In addition, Twist is a
nuclear protein and has the HLH motif of DNA-
binding proteins (Thishe et al. 1988; see also Benezra
et al. 1990). These findings suggest that twi may be directly
upstream of msh-2. If this is true, there should be Twist-
binding sites in the regulatory region of the msh-2 gene.

In contrast to twi, embryos that are mutant for sna
do initially express msh-2 in the hypothetical mesoderm
anlagen, but they soon turn off msh-2 transcription,
except for an expanded region in the head (Fig. 5B,C).
This observation suggests that only the maintenance of
msh-2 expression depends on sna. Because twi
expression is initiated transiently in sna mutant embryos,
it is likely that this twi expression is the immediate
cause for the transient expression of msh-2 in sna.
Even though strong alleles of sna and twi have been used
(Nüsslein-Volhard et al. 1984), it is not certain whether
or not they are functionally null. Therefore, a more
direct dependence of msh-2 expression on sna function
cannot be ruled out at this point.

Another indication that twi and sna are upstream of
msh-2 is the finding that in 93C-F deficient embryos
both the mesodermal movements during gastrulation
andTwist protein distribution appear normal (Fig. 7B).
On the other hand, in twi and sna mutants ventral
invagination is absent (Fig. 5).

The gene H2.0 is specifically expressed in the visceral
musculature and its anlagen (Fig. 7A,C; Barad et al.
1988). H2.0 expression in visceral derivatives through-
out development is dependent on functions included in
the chromosomal region that harbors msh-2 (Fig. 7D).
H2.0 is first detected in the visceral anlagen at the
dorsal margin at about the same time as msh-2 becomes
restricted to the exact same dorsal mesodermal region
(compare 4K and 7A). This temporal and spatial
coincidence of msh-2 and H2.0 expression in visceral
mesoderm, plus the dependence of H2.0 on the normal
function of a gene located in 93C-F, makes it tempting
to speculate that H2.0 is downstream of msh-2. We
would like to emphasize again that this chromosomal
region contains a number of unidentified genes.

In summary, based on the alterations of msh-2 and
H2.0 expression in different mutant embryos, we
speculate that msh-2 is part of an interacting network
of genes. The HLH gene twi and probably others may
regulate homeobox-containing genes such as msh-2,
which in turn regulate downstream genes such as the
homeobox-containing gene H2.0. It would be
interesting to see if in vertebrate myogenesis there exists
a similar cascade of gene regulation. For instance, among
the downstream genes of the HLH-containing myogenic
genes (MyoD, myogenin, etc. see Benezra et al.
1990), it would be of interest to find out if there are
homeobox-containing genes that specify different types
of muscles.

We would like to thank Drs W. McGinnis and M. Barad for
sending us a H2.0 cDNA clone, Dr S. Roth for the anti-Twist
antibody, Dr C. Nüsslein-Volhard for the twi and sna mutants,
S. Barbel, R. Carretto and S. Fried for technical help. We also
thank Drs K. Arora, M. Bate, E. Bier, E. Giniger and W.
McGinnis for helpful comments on the manuscript. L.Y.J.
and Y.N.J. are Howard Hughes Investigators.

References

analysis of transplanted individual cells in embryos of
BENEZRA, R., DAVIS, R. L., LOCKSHON, D., TURNER, D. L. AND
WEINRAUB, H. (1990). The protein Id: a negative regulator of
required for dorsal-ventral axis establishment and peripheral
nervous system development in Drosophila melanogaster. Genes
& Dev. 4, 190–203.
BIEBER, E., VAESSIN, H., SHEPHERD, S., LEE, K., MCCALL, K.,
BARBEL, S., ACKERMAN, L., CARRETO, R., UEMURA, T., GREGG,
E., JAN, L. Y. AND JAN, Y. N. (1989). Searching for pattern and
mutation in the Drosophila genome with a PlacZ vector. Genes
& Dev. 3, 1273–87.
BIGGS, M. D. AND TIANT, R. (1989). Transcription factors and the
BLOCHLINGER, K., BODMER, R., JACK, J., JAN, L. Y. AND JAN, Y.
N. (1988). Primary structure and expression of a product from
cut, a locus involved in specifying sensory organ identity. Nature
333, 629–635.
BODMER, R., BARBEL, S., SHEPHERD, S., JACK, J., JAN, L. Y. AND


(Accepted 3 August 1990)