The mutant not enough muscles (nem) reveals reduction of the Drosophila embryonic muscle pattern

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SUMMARY

In a search for mutations affecting embryonic muscle development in Drosophila we identified a mutation caused by the insertion of a P-element, which we called not enough muscles (nem). The phenotype of the P-element mutation of the nem gene suggests that it may be required for the development of the somatic musculature and the chordotonal organs of the PNS, while it is not involved in the development of the visceral mesoderm and the dorsal vessel. Mutant embryos are characterized by partial absence of muscles, monitored by immunostainings with mesoderm-specific anti-β3 tubulin and anti-myosin heavy chain antibodies. Besides these muscle distortions, defects in the peripheral nervous system were found, indicating a dual function of the nem gene product. Ethyl methane sulfonate-induced alleles for the P-element mutation were created for a detailed analysis. One of these alleles is characterized by unfused myoblasts which express β3 tubulin and myosin heavy chain, indicating the state of cell differentiation.

Key words: muscle development, Drosophila, embryogenesis

INTRODUCTION

In the last decade much has been learned about pattern formation in the epidermis of Drosophila (for review see Ingham, 1988). In addition, the organization of the dorsoventral pattern and mesoderm formation have been studied extensively. A cascade of maternally active genes (for review see Anderson and Nüsslein-Volhard, 1986) gives rise to the dorsoventral axis of the egg. The maternal morphogen dorsal activates the zygotically expressed genes snail and twist, leading to ventral furrow invagination and thus to mesoderm formation (Simpson, 1983; Boulay et al., 1987; Thisse et al., 1988, 1991; Alberga et al., 1991; Leptin and Grunewald, 1990). Formation of the musculature, however, has been less well studied, as such mutants are not detectable by their cuticle phenotype. So far, the information on muscle development is largely descriptive (Crossley, 1978; Leiss et al., 1988; Bate, 1990). Mutants affecting solely the somatic derivatives (Drysdale et al., 1993; Lai et al., 1993) or the visceral mesoderm, such as tinman (Bodmer, 1993; Azpiazu and Frasch, 1993), have been described recently. Thus, the regulatory pathways of splanchnopleura and somatopleura separate at the extended germ-band stage. This is also reflected in the regulatory program of structural genes expressing mesodermal derivatives, for which the regulation of the mesoderm-specifically expressed β3 tubulin gene is a good example. In this case upstream regions confer tissue-specific expression in somatic muscles and the dorsal vessel, while expression in the visceral mesoderm is dependent on enhancers in the large intron inter-acting with homeotic gene products (Gasch et al., 1988, 1989; Hinz et al., 1992).

The development of the musculature follows a pathway of considerable complexity requiring master regulatory genes, cell-cell recognition, cell adhesion, fusion processes and transcription factors activating muscle-specifically expressed genes. Several presumptive regulatory genes have been described for Drosophila, such as S59, a homeobox-containing gene, expressed in a subset of somatic muscle precursors (Dohrmann et al., 1990) or a paired-box-containing gene, pox-meso, specifically expressed in the somatic mesoderm (Bopp et al., 1989). For vertebrate systems the MyoD family has been proposed as the master regulatory genes (for review: Olson, 1990). In Drosophila, a gene homologous to the vertebrate MyoD gene, named nautilus or Dmyd, has been isolated and characterized (Michelson et al., 1990; Paterson et al., 1991). It has been shown to be expressed in a subset of somatic muscle precursors (Abmayr et al., 1992). Two other genes, the homeobox-related gene S59 (Dohrmann et al., 1990) and apterous (Bourguin et al., 1992; Cohen et al., 1992), a member of the LIM domain family, are specifically expressed during early fusion processes in a subset of muscle precursors. Also for the visceral mesoderm, homeobox-containing genes are described, such as tinman (Bodmer et al., 1990) or H2.0 (Barad et al., 1988; 1991). tinman is functionally required for differentiation of the visceral musculature and gut morphogenesis (Bodmer, 1993; Azpiazu and Frasch, 1993). Later in embryogenesis, homeotic genes of the Bithorax and Antenna -pedia complex are expressed in the visceral mesoderm in a
region-specific manner (for review see Andrew and Scott, 1992). These homeotic genes are involved in gut morphogenesis (Immerglück et al., 1990; Reuter and Scott, 1990; Reuter et al., 1990). Gut constrictions are characterized by high levels of microtubules and, recently, it was shown that expression of the mesoderm-specific β3 tubulin isotype is controlled by the homeotic gene Ultrabithorax (Hinz et al., 1992). Thus, in the case of the visceral mesoderm, a functional interaction between homeotic selector genes and a structural gene has been demonstrated.

The genes described as being important in the mesodermal differentiation pathway so far are mainly genes coding for DNA binding proteins. This is due to the experimental strategies applied to isolate these genes. Here, we chose an experimental approach which should allow us to isolate genes essential for mesodermal differentiation without a preselection of the kind of molecule encoded. We reasoned that mutations in the musculature should confer late embryonic lethality while early processes like segmentation should not be affected. We made use of β3 tubulin, which is specifically synthesized in mesodermal tissues during mesoderm development from the extended germ-band stage until hatching of the larvae (Leiss et al., 1988). An antibody against β3 tubulin was used to stain late embryonal lethals in order to select for mesodermal defects. Here, we describe the phenotype of a P-element-derived mutation showing a severe reduction of the body wall musculature while the visceral mesoderm and the dorsal vessel develop normally.

MATERIALS AND METHODS

Drosophila stocks

P-element-induced embryonic lethals were obtained from T. Orr-Weaver and A. Spradling (Cooley et al., 1988). To screen for mutants with defects in muscle development, we stained 66 P-element mutant lines, prescreened for dying late in embryonic development, with the mesoderm-specific β3 tubulin antibody. Three of the P-element insertions seemed to have a muscle-specific defect. Furthermore, we stained mutants with chromosomal deficiencies covering 40% of the genome. However, most deletions revealed such severe distortions that the specificity of muscle phenotypes remained to be determined. The P-element-induced mutant, nemP8, revealed deficiencies of different somatic muscles and distortions of the chordotonal organs of the PNS, while the visceral mesoderm and the dorsal vessel are not disturbed.

EMS mutagenesis

The P-element insertions described in this paper were selected from a P-element (pUChsneo) mutagenesis of the second chromosome, which was done in parallel with a mutagenesis of the third chromosome (Cooley et al., 1988). The nem (l(2)neo113) P-element insertion and a second P-element insertion causing the mutation rolling stone (abbr.: rost = l(2)neo114) (Burchard et al., unpublished data were localized by in situ hybridization to polytene chromosomal regions 57B (nemP8) and 30A (rostP20), respectively. Prior to the isolation of mutant lines allelic to nem and rost we wanted to label the mutagenized second chromosomes with genetic markers, which would then allow the combination of the two P-element mutations on the same chromosome. At first, the rostP20 chromosome was marked with bw and sp and the nemP8 chromosome with b pr and cn. The following crossing of the marked chromosomes resulted in a chromosome with the two P-element mutations but without genetically detectable markers. This chromosome served as a test chromosome for the mutagenesis experiment. All marker mutations and balancer chromosomes used in these experiments have been described by Lindsley and Zimm (1992).

The following strategy was used to isolate new alleles for the two P-element mutations in one mutagenesis experiment: 1,000 males, homozygous for an isogenized second chromosome, were treated with 30 mM EMS (ethyl methane sulfonate) according to the method of Lewis and Bacher (1968). The isogenized chromosome was marked with cn bw sp. These males were crossed with 3,000 females with the genotype b pr cn vgΔ bw sp/CyO; 10,000 of the resulting cn bw sp/CyO males were tested for lethality by crossing with females of the genotype nem rost/CyO. These lines were again tested for lethality with nemP8 and rostP20 successively.

Complementation tests were performed between all new nem alleles in order to define complementation groups. For the determination of the lethal phase of the nem embryos we counted the percentage of embryos not being able to hatch, which represented 25% of a nem/CyO progeny.

Reversion of the P-element insertion

The excision of the P-element of the mutants not enough muscles should reverse their homozigous lethality to vitality. This reversion experiment was performed by microinjection of the transposase-producing helper plasmid p25.7wc (Karess and Rubin, 1984) in the germline of embryos with the genotype nemP8/CyO. The resulting flies were backcrossed to males or females of the line nemP8/CyO, which contains the P-element insertion. Crossing of flies in which the P-element was excised by transposase results in homozygous viability, recognizable by wild-type wings.

Staining of embryos

Eggs laid by flies of the appropriate genetic constitution were collected on agar/apple juice plates. In order to obtain an age distribution allowing visualization of the different stages of muscle development, eggs were collected over a 24 hour period. Eggs were dechorionated, permeabilized and fixed essentially as described by Leiss et al. (1988). After washing and blocking in BBT (0,15% crystalline BSA, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 40 mM MgCl2, 20 mM glucose, 50 mM sucrose, 0.1% Tween-20), the eggs were incubated overnight with a dilution of the appropriate antibody. For analysis of the mesoderm formation, the anti-twist antibody was used (Thiss et al., 1988; Leptin and Grunewald, 1990). For staining of mesodermal derivatives, the anti-β3 tubulin antibody (Leiss et al., 1988) and muscle myosin antibody (Kiehart and Feghali, 1986) were used. For the staining of the muscle precursors, the anti-nautilus antibody was used (Abmayr et al., 1992). The central and peripheral nervous system were stained with monoclonal antibody Mab22C10 (Zipursky et al., 1984). The bound antibody was detected with a biotinylated secondary antibody and stained with the Vectastain ABC Elite-kit (VectorLabs) using diaminobenzidine as detection agent. Single and double stainings were performed as described by Lawrence et al. (1987). The stained embryos were embedded in Epon and photos taken under Nomarski optics with a Zeiss Axioskop microscope (Kodak, Ekta 25).

RESULTS

The nem mutation causes a reduction in the larval muscle pattern

We aimed to isolate and characterize mutations involved in larval muscle formation. For that we tested 66 late embryonal lethal rosy- or neomycin-marked P-element-induced mutants from T. Orr-Weaver and A. Spradling, and 59 deletions of the Bloomington Stockkeeping center for defects in muscle development. Muscle development was followed with an anti-β3
tubulin antibody (Leiss et al., 1988), so that individual mesodermal derivatives could be visualized during embryonic development in deletion or P-element-induced mutations. Most of the investigated mutants had either no defect in muscle staining or clearly secondary effects; for example, as a consequence of segmentation distortions. We concentrated on P-element-induced mutations, as in our experience deletions which comprise several loci show such severe aberrations that muscle-specific defects are difficult to define. The P-element mutant nemP8, however, revealed a striking abnormality in muscle development in that the number of muscles was strongly reduced and in addition there was a distortion of the PNS.

Before describing the muscle phenotype of nem mutants in detail, a short description of the wild-type situation as it was observed with β3 tubulin antibody staining will be given. β3 tubulin was first detected in large mesodermal cells in every segment (Fig. 1A). When germ-band retraction begins the β3 tubulin antigen allows visualization of the separation of somatic and visceral mesoderm (Fig. 1B). During germ-band retraction, fusion of myoblasts to myotubes starts, in that small groups of di- and trinucleate cells form (Bate, 1990), and at stage 14 the fusion process is completed (Fig. 1C). The final muscle pattern is shown in a lateral view of stage 17 embryo (Fig. 1D). In dorsal view, the dorsal vessel and the dorsal muscles are visualized (Fig. 1E).

In the abdominal segments A2 to A7 the muscle patterns are identical; every segment contains a number of muscles arranged in ventral, pleural and dorsal groups. In the description of the mutation nemP8 (Fig. 2), we will focus on the muscle pattern in these abdominal segments. The mutation is clearly visible at late stage 13 after the completion of the fusion process. In this stage, the muscles were concentrated in the middle of the segment (compare Fig. 2A with the wild-type embryo in Fig. 1C). Compared to stage 17 embryos, the muscle distortions are clearly evident between wild-type embryos (Fig. 2B) and nemP8 mutants (Fig. 2C and D). In the abdominal segments, the ventral muscles pl1, vio1, vio2 and vio3 are characteristic of the wild-type (for nomenclature see Campos-Ortega and Hartenstein, 1985). In the mutant nemP8 only three of these muscles were present, pl1 was most

**Fig. 1.** Muscle development in the wild-type embryo as followed by the distribution of the β3 tubulin. (A) Staining with the β3 tubulin-specific antibody (Leiss et al., 1988) is first observed when germ-band extension is completed (stage 10 according to Campos-Ortega and Hartenstein, 1985). (B) Shortly later, when germ-band retraction begins, splanchnopleura (visceral mesoderm) and somatopleura (somatic mesoderm) are visible as separate units. The visceral mesoderm forms a contiguous cell layer on each side of the embryo. The somatic mesoderm is visible as a small group of cells in every segment. (C) During germ-band retraction, the cells of the somatic mesoderm start to fuse. At stage 14, most fusions are completed and the ventral, pleural and dorsal groups of muscles are distinguishable. Also, precursor cells of the dorsal vessel are labelled with β3 tubulin-specific antibody. (D) In lateral view of a stage 16 embryo the complete muscle pattern is visualized by the β3 tubulin-specific antibody. (E) In a stage 16 embryo the dorsal vessel and dorsal musculature are visible. cms, cephalic mesoderm; ms, mesoderm; dm, dorsal musculature; dv, dorsal vessel; pm, pleural musculature; sm, somatic mesoderm (somatopleura); vem, ventral musculature; vm, visceral mesoderm (splanchnopleura).
probably missing (Fig. 2C). Also the ventral muscles veo1, veo2 and veo3 were often missing (Fig. 2C). Furthermore, the pleural group of muscles was disorganized in this mutant. In the wild-type pet1, pet2, pet3, pet4 and pet5 are members of this group. Often, however, there were only two or three muscles present. The muscle phenotype of this P-element-induced mutation was to a certain degree variable (an example is shown in Fig. 2D). Also, here the pleural muscle set was reduced, but the muscles were in much closer contact compared to those in the embryo shown in Fig. 2C.

In Fig. 2E, the typical phenotype of the dorsal musculature is shown. Often small gaps were observed; the positions of the gaps varied from one embryo to the other and they may have been caused by malplacement of muscles or by lack of muscles. The question arises of whether all mesodermal derivatives are disturbed or whether the defects are specific for somatic derivatives. Fig. 3A shows a mutant embryo focused on somatic derivatives; in comparison with the same embryo focused on the visceral musculature in Fig. 3B. Clearly, the visceral musculature has developed rather normally and gut morphogenesis has occurred. Formation of the dorsal vessel (Fig. 2E, nem\textsuperscript{P8}; Fig. 4B, nem\textsuperscript{3}; Fig. 5C, nem\textsuperscript{8}; Fig. 7D, nem\textsuperscript{22}) is normal as is evident from comparison of a wild-type embryo (Fig. 4A) with a mutant embryo. The lethal phase of all nem mutants has been determined as being shortly before hatching.

It has often been observed that P-elements integrate into the control regions of genes, with the consequence of a certain variation in the severity of the phenotype. However, variation in the manifestation of the phenotype was also observed in the EMS-induced nem mutations (see below and Fig. 6).

The P-element insertion causing the muscle phenotype is localized at 57B on the second chromosome

The nem\textsuperscript{P8} mutation originates from a P-element mutagenesis of the second chromosome performed in parallel with a screen of the third chromosome (Cooley et al., 1988).

As a prerequisite for further genetic and molecular characterization of the nem gene, the chromosomal site of integration was determined by in situ hybridizations to polytene chromosomes using the P-element as a probe. A single chromosomal integration site on the right arm of the second chromosome at position 57B was found (data not shown). None of the muscle-specifically expressed genes analyzed so far are localized at this position. We wanted to exclude the possibility that a

Fig. 2. Muscle pattern of the P-element-induced nem\textsuperscript{P8} allele as followed by staining with the β3 tubulin antibody. (A) The homozygous nem\textsuperscript{P8} embryo at stage 14 shows separation between ventral, pleural and dorsal muscle groups. Muscles have fused; however, individual myotubes are lying too close to each other. (B) A lateral view of a stage 16 embryo showing the fully developed muscle pattern in the wild-type embryo. (C) A stage 16 nem\textsuperscript{P8}/nem\textsuperscript{P8} embryo shows a reduced muscle pattern, many muscles of the ventral group and the pleural group (compare arrowheads in 2B and C) are missing. (D) Another stage 16 homozygous nem\textsuperscript{P8} embryo shows a disorganized but also reduced muscle pattern. (E) In a dorsal view of a stage 17 nem\textsuperscript{P8} embryo the dorsal vessel (dv) is visible and shows no major defect.
**Fig. 3.** The development of the midgut visceral musculature is not disturbed in the nem³ mutant. The embryos were stained with the β3 tubulin antibody. (A) The disorganized somatic muscles of a stage 16 homozygous nem³ embryo are shown. (B) The same homozygous nem³ embryo shows normal development of the midgut visceral musculature.

**Fig. 4.** The dorsal vessel develops properly in the nem³ allele. The embryos were stained with β3 tubulin antibody. (A) A stage 16 wild-type embryo, which shows the dorsal vessel (arrowhead) and dorsal musculature. (B) The dorsal vessel of a homozygous nem³ mutant is not affected by the mutation.

**Fig. 5.** The muscle pattern of the nem⁸ allele is reduced and disorganized. The embryos were stained with β3 tubulin antibody. (A) The wild-type muscle pattern is shown for comparison. (B) A mutant nem⁸ embryo with focus on the ventral and pleural muscles shows severe reduction in the muscle pattern. (C) The mutant phenotype of the pleural muscles shows variability (compare B). (D) A dorsal view of the nem³/nem³ embryo. The dorsal vessel (dv) is fairly normal. Most dorsal muscles are formed and inserted appropriately in the apodemes (iap).
mutation other than the P-element insertion at 57B might have caused this mutation. Therefore, we mobilized the P-element, which resulted in its excision from 57B. Flies bearing one second chromosome with an excised P-element and one chromosome with the original nemP8 allele were viable. Thus, excision of the P-element reverses lethality. This makes it very likely that the P-element insertion has indeed caused the nemP8 mutation.

The pleural musculature reveals unfused myoblasts in the muscles of the EMS-induced nem22 allele

To gain more insight into the function of the nem gene, new alleles were induced by EMS mutagenesis. We were interested in a further P-element-induced mutation (rolling stone (rost); Burchard et al., 1994, and unpublished data) on the left arm of the second chromosome. nemP8 and rostP20 were first combined on a single chromosome by recombination (see Materials and Methods). This double-mutant chromosome was used to select EMS-induced alleles; 16 alleles were obtained for rost and three alleles for nem. A complementation analysis between the three EMS-induced nem alleles (nem3, nem8 and nem22) and the P-element-induced allele revealed that all alleles fell into the same complementation group, indicating that it was very likely that a single gene was causing the mutant phenotype as well as embryonic lethality.

The phenotype of the allele nem3 was similar to the original P-element-induced mutation, while nem8 and nem22 revealed stronger phenotypes. The nem3 mutation represented a weak nem allele, which often shows muscle reduction in one or two segments only. An example of distortions in the pleural musculature is shown in Fig. 6B and the distortions of the ventrolateral musculature are shown in Fig. 7F.

The nem8 mutation exhibited strong defects in the ventral, pleural and dorsal musculature in comparison to the wild-type (Fig. 5 and Fig. 6A). The muscle pattern was often more incomplete than in the case of nemP8 (Fig. 5B and C). The dorsal vessel developed normally while the dorsal muscles exhibited irregularities, in that small gaps appeared in the muscle pattern (Fig. 5D).

A particularly striking feature of the nem22 allele was the presence of large gaps in the ventral, pleural and dorsal musculature (Fig. 7B-D). In the phenotype of this allele, unfused single myoblasts were found in late embryonic stages (Figs 6D, E and 7B), in which in the wild-type situation the muscle differentiation program is completed (Fig. 7A). These single myoblasts expressed β3 tubulin and muscle myosin, which

Fig. 6. The three nem alleles show high variability in phenotypic expression. The staining of the musculature was done with β3 tubulin antibody. (A) The nem8 allele show severe muscle distortions in the ventral lateral and dorsal musculature (compare with Fig. 5). (B) The nem3 allele is the weakest of the three nem alleles. It often shows defects only in the pleural musculature. (C) A dorsal view from the nem8 allele presents distortions in the dorsal musculature. The dorsal vessel is not disturbed. (D) In a stage 16 nem22 allele there are a lot of myoblasts, which give the impression that the fusion of myoblasts, to myotubes is not complete. In comparison to the wild-type (Fig. 1D) the β3 tubulin is not localized at the edges and tips of the fused myotubes but is organized in the individual cells as microtubules of the cytoskeleton. (E) Higher magnification of D showing the aberrant β3 tubulin distribution in each cell.
identify these cells as muscle-forming cells (see below). In the wild-type situation the β3 tubulin-containing microtubules are concentrated in the distal parts of the myotubes and at the sites where attachment to the epidermis occurs. This distribution is also characteristic of most nem alleles (Fig. 7E for wild-type and F for distribution in the mutant). However, in this nem22 allele the β3 tubulin distribution is disturbed (Fig. 6D). At a higher magnification the β3 tubulin is localized in the myotubes (Fig. 6E), so individual myoblasts are still recognizable.

**Mesoderm formation is normal in nem mutants**

In the P-element-induced allele nemP8 and the three EMS-induced alleles a reduction in the muscle number was consistently found. This may have been a consequence of defects in mesodermal differentiation or it may be speculated that not enough mesodermal cells were formed during gastrulation. Mesoderm formation depends on a cascade of maternally active genes as well as on two zygotically active genes, *twist* and *snail* (Thirse et al., 1987; Boulay et al., 1987). The twist antigen allows mesodermal cells to be followed during gastru-

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**Fig. 7.** Muscle pattern in the EMS-induced nem22 and nem3 alleles. (A) A stage 17 wild-type embryo is shown for comparison. (B) A lateral view of a homozygous nem22/nem22 embryo reveals a reduced muscle pattern. Several single β3 positive myoblasts are visible (arrowheads). (C) In this mutant nem22 embryo the muscle pattern of the pleural group is heavily reduced (arrowhead). (D) In a dorsal view the heart (dv) of a homozygous nem22 embryo is visible. The dorsal group of muscles is less affected than the ventral and pleural group. (E) Higher magnification of a stage 17 wild-type embryo with the focus on the ventrolateral musculature is shown (arrows, veo 1-3; arrowhead, pl 1 and vio 1-3; nomenclature of the muscles according to Campos-Ortega and Hartenstein, 1984). (F) In the homozygous nem3 mutant, the ventral muscles veo 1-3 (arrows) and, to a variable extent, the pleural longitudinal muscle pl 1 and ventral internal muscles vio 1-3 (arrowhead) are missing.
lation (Leptin and Grunewald, 1990). Thus, an antibody recognizing the twist antigen was used to follow mesoderm formation in nem mutants. For the nem mutants, no major difference in the pattern of twist expression in comparison to that in the wild-type was found (data not shown). However, we cannot exclude the possibility that single cells were missing which may be essential for initiating the formation of specific muscles (see Discussion).

**The muscle phenotype of nem mutations is not caused by distortions in epidermal differentiation**

The development of the musculature does not necessarily depend solely on the differentiation of the mesoderm itself but may be dependent on the nervous system (see below) or the differentiation of the epidermis in which the muscles are inserted at their attachment sites, the apodemes. To check the differentiation of the epidermis, the pattern formation of the cuticle in the nem mutants was analyzed (data not shown). In comparison to wild-type embryos, no significant distortion of the cuticle was found, so the nem mutation had no influence on epidermal pattern formation in the cuticle itself. This suggested that the observed muscle phenotype was not a consequence of epidermal defects.

*In nem mutations the chordotonal organs of the peripheral nervous system are incomplete*

The question arises of whether nem mutations are selectively acting in the mesodermal cells or if mutations in the nem gene have direct or indirect consequences upon the differentiation of other tissues such as the nervous system. We used the Mab22C10 antibody (Zipursky et al., 1984) to stain the central and peripheral nervous systems. The musculature was stained with the β3 tubulin antibody. These double-staining experiments have been performed for all nem alleles and exhibit the same situation as depicted in Fig. 8. The peripheral nervous system reveals differences between wild-type and all nem mutants. As an example the nem3 allele is shown (compare Fig. 8A and B). Specifically, the pentascolopidial chordotonal organs (lch5) were not developed. All other cells of the peripheral nervous system, such as dorsal hair sensilla and lateral sense organs, were correctly formed. The central nervous system reveals no major distortions (compare Fig. 8C and D), but appears compressed and malformed which may be a consequence of the reduced set of muscles. The failure of development in the pentascolopidial cells, however, is very specific, raising the question of whether the nem gene has a dual function during muscle development and chordotonal organ development.

**Fig. 8.** Analysis of the central and peripheral nervous system in nem3/nem3 embryos. The embryos were stained with the β3-specific antibody to detect the muscle system (black) and the antibody Mab22C10 (Zipursky et al., 1984), which stains both the central and peripheral nervous system (red). (A) A lateral view of a stage 17 embryo focused on the peripheral nervous system. (B) In the mutant nem3/nem3 embryo (stage 17) the peripheral nervous system is reduced compared to the wild-type. Specifically, the chordotonal organs are affected (arrowheads), while the dorsal hair sensilla (dh1) are unchanged. (C) A ventral view of a wild-type embryo showing the ventral cord (nc). (D) The ventral cord of nem3/nem3 embryos exhibits no gross defects. dh1, dorsal hair sensilla; lch 5, lateral chordotonal organs; nc, nerve cord; vem, ventral musculature.
Muscle myosin is expressed in nem alleles

In addition to β3 tubulin distribution, the expression of another muscle-specific protein, muscle myosin, in the nem alleles was checked. Fig. 9A shows the myosin staining for the weak nem³ allele. The muscle myosin distribution in the somatic musculature was identical to that shown by the β3 stainings. Fig. 9B shows the stronger nem²² allele with unfused β3 positive myoblasts, which in the wild-type did not occur at this embryonal stage. Although in young wild-type embryos myoblasts did not express muscle myosin, single unfused myoblasts in the nem²² allele revealed muscle myosin (Fig. 9C), as was observed previously in neurogenic mutants (Corbin et al., 1991) and deletion mutants of the X-chromosome (Drysdale et al., 1993).

nautilus expression is independent of the function of the not enough muscle gene

One key step in the process of muscle formation is the segregation of muscle precursors at the stage of germ-band retraction, as often certain muscles are not formed. nautilus, the Drosophila homologue of the vertebrate myogenic transcription factor MyoD (Michelson et al., 1990; Paterson et al., 1991; Abmayr et al., 1992) is expressed in a subset of muscle precursors. Transgenic flies containing a nau-promoter/lacZ fusion construct express the reporter gene during later stages in differentiated muscles, e.g. in the ventral external oblique muscles, the ventrolateral external oblique muscles, and the pleural external longitudinal muscles (Paterson et al., 1991). Some of these muscles are often missing in homozygous nem mutant embryos, for example pl1 and vio1 (see Table 1). To test whether the not enough muscle gene is essential for the formation of muscle precursors, we looked for this in cells by analyzing the expression pattern of nautilus in not enough muscle mutants (Fig. 10), assuming that the not enough muscle phenotype could be a result of the absence of muscle precursors. During germ-band retraction, when nautilus expression begins, the not enough muscle phenotype is not recognizable by β3 tubulin staining. Therefore, we changed the CyO balancer against the CyO⁷.¹ balancer in our strains. This balancer carries a β-gal fusion construct (Affolter et al., 1993), which allows detection of β-gal from the extended germ-band stage in the embryonic hindgut precursor, and later in the hindgut and the anal pad region. This expression pattern is overlapping with neither the nautilus nor the β3 tubulin expression pattern, so the muscle development can be observed in double stainings. Antibody stainings were done with an anti-lacZ and an anti-nautilus antibody. Only those embryos that lack expression of the β-gal fusion construct are homozygous for the nem mutation and allow us to observe nautilus expression already at the extended germ-band stage in the mutant embryos. The nautilus expression in nem²² homozygous mutants (Fig. 10A,C) is shown in comparison to that in wild-type embryos (Fig. 10B,D). We chose the nem²² allele for our experiments because this allele represents a strong phenotype. In lateral view no difference is visible at stage 10, when nautilus is first expressed in a single cell per hemisegment (Fig. 10A,B). At stage 13 or 14, a lot of cells arranged

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Table 1. Markers for muscle precursors and their correlation to defects in nem mutations

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<th>Muscles, which are often, but not always missing in nem mutants according to:</th>
<th>Expression in muscle precursors</th>
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<tr>
<td>Campos-Ortega* Bate†</td>
<td>Nautilus</td>
</tr>
<tr>
<td>pl1</td>
<td>12</td>
</tr>
<tr>
<td>pet1-4</td>
<td>21-24</td>
</tr>
<tr>
<td>pet5</td>
<td>18</td>
</tr>
<tr>
<td>vio1</td>
<td>13</td>
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<td>vio2,3</td>
<td>6,7</td>
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†Bate, (1990).
in ventral, lateral and dorsal groups express *nautilus* (Michelson et al., 1990; Paterson et al., 1991; Abmayr et al., 1992). Wild-type (Fig. 10B,D) and mutant embryos (Fig. 10A,C) reveal identical *nautilus* expression patterns, as far as is detectable by this method. The results show that the nem phenotype is not due to the absence of nautilus positive precursor cells. Therefore we have begun to search for certain muscle precursor cells in nem mutants.

**DISCUSSION**

The genetic hierarchy establishing the dorsoventral axis by maternally transcribed genes culminates in the expression of the ventral morphogen *dorsal* and the zygotically expressed genes *twist* and *snail* (Govind and Steward, 1991). The maternally encoded *dorsal* gene product regulates the expression of *twist* (Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991). At the extended germ-band stage, the twist protein disappears from most cells with the exception of precursors of the adult musculature (Bate et al., 1991). Soon afterwards, the splanchnopleura and the somatopleura separate and both reveal β3 tubulin expression as an early sign of differentiation (Leiss et al., 1988). We previously showed that β3 tubulin is an excellent marker for following mesoderm differentiation from the extended germ-band stage to hatching in all mesodermal cells (Leiss et al., 1988). Using the β3 tubulin antibody as a marker, we searched for mutations causing specific defects in the somatic musculature without preselection for the kind of molecule involved.

**Somatic muscles are missing in the nem mutant while the visceral musculature and the dorsal vessel are formed**

The nem mutants revealed specific defects in somatic muscles while the development of the dorsal vessel and the visceral musculature was normal. Specifically, in the P-element-induced nemP8 mutant, as well as in the EMS-induced alleles, several muscles were missing from the pleural, ventral and dorsal groups. This reduction in the number of muscles was not caused by a significant amount of the mesodermal cells being missing as was shown by the unchanged distribution of the twist protein in these mutants. However, we cannot rule out the possibility that single cells of the mesodermal germ layer in the blastoderm stage are missing. It has been proposed that every individual muscle needs its own precursor cell to start fusion processes (Bate, 1990). Thus, one possible cause of the nem phenotype might be that some precursor cells are missing, so certain muscles fail to form. We began to check for muscle precursor cells with nautilus as a marker for precursors of muscles that are often missing in nem mutants (Table 1).

Using the anti-nautilus antibody we found no difference in the nautilus distribution between wild-type and nem homozygous embryos. Thus, the absence of individual nautilus positive cells is not detectable. The pattern of muscles which are missing in nem mutants is quite variable, only several indi-

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**Fig. 10.** nautilus is expressed in muscle precursors in homozygous nem22 embryos. nem22/CyO7.1 (see Materials and Methods) embryos were stained with an anti-nautilus and an anti-lacZ antibody to distinguish between homozygous nem22 embryos and embryos containing the balancer chromosome (wild-type). (A and C) Homozygous nem22; (B and D) wild-type embryos. The lacZ reporter gene expression in the hindgut/hindgut-precursor is demonstrated in D (arrowhead). (B and D) Lateral views of wild-type embryos (stage 10 and 13) selected by double staining. In nem22 homozygous embryos nautilus is expressed in the early precursors (A) and later in all precursors for the ventral, lateral and dorsal muscle group of the somatic muscles as described for the wild-type, as far as is detectable with this method (C). In comparison to wild-type embryos (B and D), at similar stages and orientation there is no obvious difference in the expression pattern.
vidual muscles are often, but not always, missing. The variable phenotype and the normal expression of nautilus might argue against a defect at the level of muscle precursors.

The analysis of the EMS-induced nem^{22} allele revealed an additional phenotype, in that there are free unfused β3 tubulin- and myosin-positive myoblasts. We propose that in this mutant muscle precursor cells have not been specified. As a consequence of the missing precursor cells, certain myoblasts would not find the precursor cell to which they have to fuse. This hypothesis is supported by the presence of unfused β3 tubulin- and muscle myosin-positive cells in the nem^{22} allele. We have started a molecular analysis of the nem locus, which will help to elucidate the cellular level of action on the nem gene.

The nem gene is essential for muscle development as well as for the formation of the pentascolopidial chordotonal organs of the peripheral nervous system, while the epidermal cell fate is normal

The defects observed using the β3 tubulin antibody showed displacement and reduction in the muscle pattern. Furthermore, we observed that the central nervous system appeared compressed and, specifically, the pentascolopidial chordotonal organs of the PNS were missing. This finding may be interpreted as showing a dual function for the nem gene, both in muscle formation and in determination of the pentascolopidial chordotonal organs. For neurogenic genes like Notch and Delta, which determine the fate of epidermal cells (for review see: Campos-Ortega and Knust, 1990), a dual function has also been shown. Notch protein is expressed both in the epidermis and in the mesoderm (Kidd et al.,1989) before and during the initial formation of bi- and trinucleate precursors of somatic muscles (Bate, 1990). For the formation of these precursors, the Drosophila MyoD homologue, nautilus, may be essential (Michelson et al., 1990). Corbin et al. (1991) looked at nautilus, β3 tubulin and muscle myosin distribution in neurogenic mutants. In these mutants they found an increased number of nautilus positive cells compared to the wild-type. Both nautilus and β3 tubulin staining revealed that cell shaping does not occur during muscle development. The authors proposed that neurogenic genes are needed for correct segregation of cell types in the mesoderm as well as in the ectoderm.

In conclusion we propose that the nem gene probably has a dual function in specifying certain muscles as well as the pentascolopidial chordotonal organs. Several other genes are known which have a dual function in the mesoderm and the nervous system: Lai et al. (1993) examined zh-I, the gene product of which has a zinc-finger motif and a homeodomain; a dual function in mesoderm and PNS was also shown for numb (Uemera et al., 1989).

We have started a molecular analysis of the wild-type nem gene and the EMS-induced mutant alleles to gain further insight into the level of nem function in the muscle determination program.

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