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myoblasts incompetent encodes a zinc finger transcription factor required to specify fusion-competent myoblasts in *Drosophila*

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SUMMARY

We report a new gene, *myoblasts incompetent*, essential for normal myogenesis and myoblast fusion in *Drosophila*. *myoblasts incompetent* encodes a putative zinc finger transcription factor related to vertebrate Gli proteins and to *Drosophila* Cubitus interruptus. *myoblasts incompetent* is expressed in immature somatic and visceral myoblasts. Expression is predominantly in fusion-competent myoblasts and a loss-of-function mutation in *myoblasts incompetent* leads to a failure in the normal differentiation of these cells and a complete lack of myoblast fusion. In the mutant embryos, founder myoblasts differentiate normally and form mononucleate muscles, but genes that are specifically expressed in fusion-competent cells are not activated and the normal downregulation of *twist* expression in these cells fails to occur. In addition, fusion-competent myoblasts fail to express proteins characteristic of the general pathway of myogenesis such as myosin and Dmef2. Thus *myoblasts incompetent* appears to function specifically in the general pathway of myogenesis to control the differentiation of fusion-competent myoblasts.

Key words: *Drosophila*, Myoblasts, Cell fusion, Myogenesis, Transcription factor, *myoblasts incompetent*

INTRODUCTION

Myogenesis in *Drosophila* is initiated by the local segregation of founder myoblasts within muscle forming mesoderm. The founder myoblasts have the capacity to implement terminal differentiation through the expression of *Dmef2* (Bour et al., 1995; Lilly et al., 1995) and, by fusing with neighbouring fusion-competent myoblasts they seed the formation of multinucleate muscle fibres (Baylies et al., 1998). In the absence of fusion, fusion-competent cells cannot complete myogenesis (Rushton et al., 1995), so founder myoblasts act as local gates on the formation of muscles. The specific characteristics of individual myotubes are determined by the particular combination of transcriptional regulators such as *Krüppel* (*Kr*) and *S59* (also known as *slouch*) (Knirr et al., 1999) that each founder expresses (Baylies et al., 1998). Interestingly, however, fusion-competent myoblasts do express some aspects of terminal myogenic differentiation in the absence of fusion. In wild-type embryos, the contractile protein myosin is expressed in fusion-competent cells before they fuse with founders and in mutants where fusion is blocked, myosin is strongly expressed in clusters of unfused myoblasts. This, together with the expression of genes such as *sticks and stones* (*sns*), that are specific to fusion-competent cells (Bour et al., 2000) and essential for normal myogenesis, suggests that the fusion-competent myoblasts are a distinct population of muscle-forming cells and that there are regulatory factors that lead to their selective differentiation within the general pathway of myogenesis.

Here we report the first such factor, encoded by the gene *myoblasts incompetent* (*minc*). *minc* is expressed in fusion-competent cells and required for these cells to differentiate. In the absence of *minc* the fusion-competent cells remain as an immature population of mesodermal cells that cannot contribute to muscle formation. Thus in these mutants, fusion fails and no multinucleate muscles are formed. Interestingly our evidence suggests that *minc* not only controls the specific properties of fusion-competent myoblasts but that it is also required for myogenic differentiation itself, presumably acting in combination with genes such as *Dmef2*.

MATERIALS AND METHODS

Drosophila strains

Most of the deficiencies and lethals in the 94CD region used in this work were obtained from the *Drosophila* stock center at Bloomington and the Szeged stock center in Hungary (Flybase Consortium, 1999). *Df(3R)EB6* and *Df(3R)r90B* were provided by J. Mohler (Mohler and Vani, 1992) and *Df(3R)hhE23* was obtained from K. Basler (Basler and Struhl, 1994). The *minc[A388]* EMS-induced allele was generated on a multiply marked third chromosome following standard techniques. We used *Twi-GAL4* (Baylies and Bate, 1996), *En-GAL4* (Tabata et al., 1995) and *Sca-GAL4* (Budnik et al., 1996) to drive ectopic gene expression.

Molecular biology

Molecular biology experiments were performed following standard procedures (Sambrook et al., 1989). LD28538, LD47926 and LD0474 are expressed sequence tag clones from the Berkeley Drosophila Genome Project (BDGP). Cosmids in the 94CD region were obtained from the European Drosophila Genome Project (EDGP). Selection of *minc[A388]* mutant embryos used for genomic DNA sequencing was based on the use of a third chromosome balancer carrying a Kr-GFP construct, provided by I. Guerrero. For sequencing of *minc[A388]* genomic DNA, independent PCR amplification products covering the 3 exons of the gene *minc* were generated and subcloned using the TopoTA cloning kit (Invitrogen Corporation). Three independent PCR reactions were performed to confirm the transition responsible for the generation of a stop codon in the mutant DNA. DNA sequencing was performed with ABI chemistry in an automatic DNA sequencer and by the sequencing facility of Cambridge Bioscience. Custom synthesised oligonucleotides were obtained from ISOGEN (Bioscience BV, Maarsesen, The Netherlands). The conceptual protein sequence was analysed using MOTIF (http://www.motif.genome.ad.jp). Similarity searches for the Minc predicted protein were performed using the BLAST algorithm (Altschul and Lipman, 1990).

Germline transformation and ectopic expression of Minc protein using the UAS/GAL 4 system

Minc protein was ectopically expressed by means of the UAS/GAL4 system. The UAS-*minc* construct was made by subcloning a 2924n *Eco*RI-*Bgl*II fragment from LD47926 containing the entire *minc* ORF into the pUAST transformation vector (Brand and Perrimon, 1993). This construct was injected into *yw* embryos under standard conditions (Spradling, 1986). For ectopic expression of *minc*, males containing the *UAS-minc* transgene were crossed to females of different GAL4 lines at 29°C. For rescue experiments females containing *Twi-GAL4* combined with *minc[A388]* were crossed to males *UAS-minc; minc[A388]* or *UAS-Mef2; minc[A388]* at 29°C.

Other methods

Immunocytochemistry was as described previously (Ruiz-Gómez et al., 1997). The following primary antibodies were used: anti-muscle myosin, a gift from Dan Kiehart (Kiehart and Feghali, 1986), anti-Krüppel, a gift from Dave Kosman (Kosman et al., 1998), anti-β-galactosidase (Cappel), anti-Twist (Thisse et al., 1988), monoclonal anti-Hairy 24.1, kindly provided by D. Ish-Horowicz and anti-MEF2 (Bour et al., 1995). *minc* RNA localisation by means of in situ hybridisations using digoxigenin-labelled RNA probes was performed as described previously (Taylor, 2000). Double RNA in situ hybridisation and antibody staining was done sequentially with minor modifications of the previous procedures.

In situ hybridisations to polytene chromosomes using biotinylated labelled probes were performed as described previously (Ashburner, 1989).

RESULTS

A new gene required for myoblast fusion in *Drosophila*

In an EMS mutagenesis and screen for genes affecting myogenesis and muscle patterning in the *Drosophila* embryo, we isolated a recessive embryonic lethal mutation (*A388*) in a gene required for myoblast fusion. We mapped the allele meiotically to the interval between *ebony* (93D2-6) and *claret* (99B5-9) on the third chromosome. Subsequent analysis of deficiencies showed that *A388* fails to complement *Df(3R)hhGR2*, *Df(3R)EB6*, *Df(3R)M95A*, *Df(3R)GW2* and *Df(3R)23D1*, but complements *Df(3R)hhE23*, *Df(3R)5C1* and *Df(3R)r90B*. *A388* complements all known lethals in this region and identifies a new gene required for myoblast fusion, which is located proximal to *hedgehog* (*hh*), in the 94D3-13 region of the third chromosome.

A detailed analysis of the mutant phenotype in *A388* (see below) revealed that the failure in myoblast fusion reflects a specific defect in the differentiation of fusion-competent myoblasts, while there is no apparent requirement for the gene in the differentiation of founder myoblasts. For this reason we have called the new gene identified by the mutation, *myoblasts incompetent* (*minc*).

The GenBank accession number for the *minc* gene discussed in this paper is AJ311850.

Embryonic phenotype of mutations in *minc*

The mutagenesis and screen were designed to incorporate a marker for founder myoblasts (*rP298*) (Nose et al., 1998) in the genetic background. *rP298* is an enhancer trap insertion in the region of *dumbfounded* (*duf*) that drives the expression of β-galactosidase in all founder cells, their progenitors and the muscles they give rise to. Thus putative mutants could be assayed rapidly for failures of myogenesis or altered patterns of muscles, by scoring the expression of β-galactosidase. In *minc[A388]* we found that there was a complete lack of myoblast fusion and all muscles were mononucleated.

To show whether founder cells are specified normally in the mutant embryos, we used antibodies to a variety of transcription factors (e.g. *Kr*; Fig. 1A-C) that are expressed by subsets of founder myoblasts in normal embryos (Ruiz-Gómez et al., 1997). In all cases, these expression patterns in founder cells were normal. To characterise the phenotype in more detail, we used anti-myosin antibody to visualise the process of myogenesis in wild-type (Fig. 1D) and mutant embryos (Fig. 1E,F). Myosin staining showed that some mesodermal derivatives, including the heart, developed normally (Fig. 1F), but that multinucleate somatic muscles failed to form (Fig. 1E). Fusion also failed to occur in the muscles of the pharynx (Fig. 1F inset). Founder myoblasts appeared to differentiate normally, developing at appropriate positions, expressing myosin, elongating and attaching to the epidermis at their correct insertion sites (Fig. 1E). However, with myosin staining, fusion-competent myoblasts could not be detected at any stage (Fig. 1E,F) and this is in striking contrast to other non fusion mutants in which unfused myoblasts express myosin at high levels (Doberstein et al., 1997; Rushton et al., 1995).

The failure to detect fusion-competent myoblasts with myosin staining in the mutant embryos could reflect either the complete absence of these cells or, alternatively, it could be that the cells are present but have failed to differentiate. Because founder cells form normally in *minc* embryos, we assume that the segregation process that leads to the separation of a population of founders and fusion-competent cells has probably taken place in these embryos. Thus it seemed likely that the fusion-competent cells would be present but in some undifferentiated state. To explore this possibility further, we used an antibody to *Dmef2*, a gene that is expressed and required for normal differentiation in all myogenic cells (Bour et al., 1995; Lilly et al., 1994). Staining with this antibody revealed once again a normal pattern of founder cells, but an absence of expression in fusion-competent cells (compare Fig. 1G,H, and insets). This again is in marked contrast to other

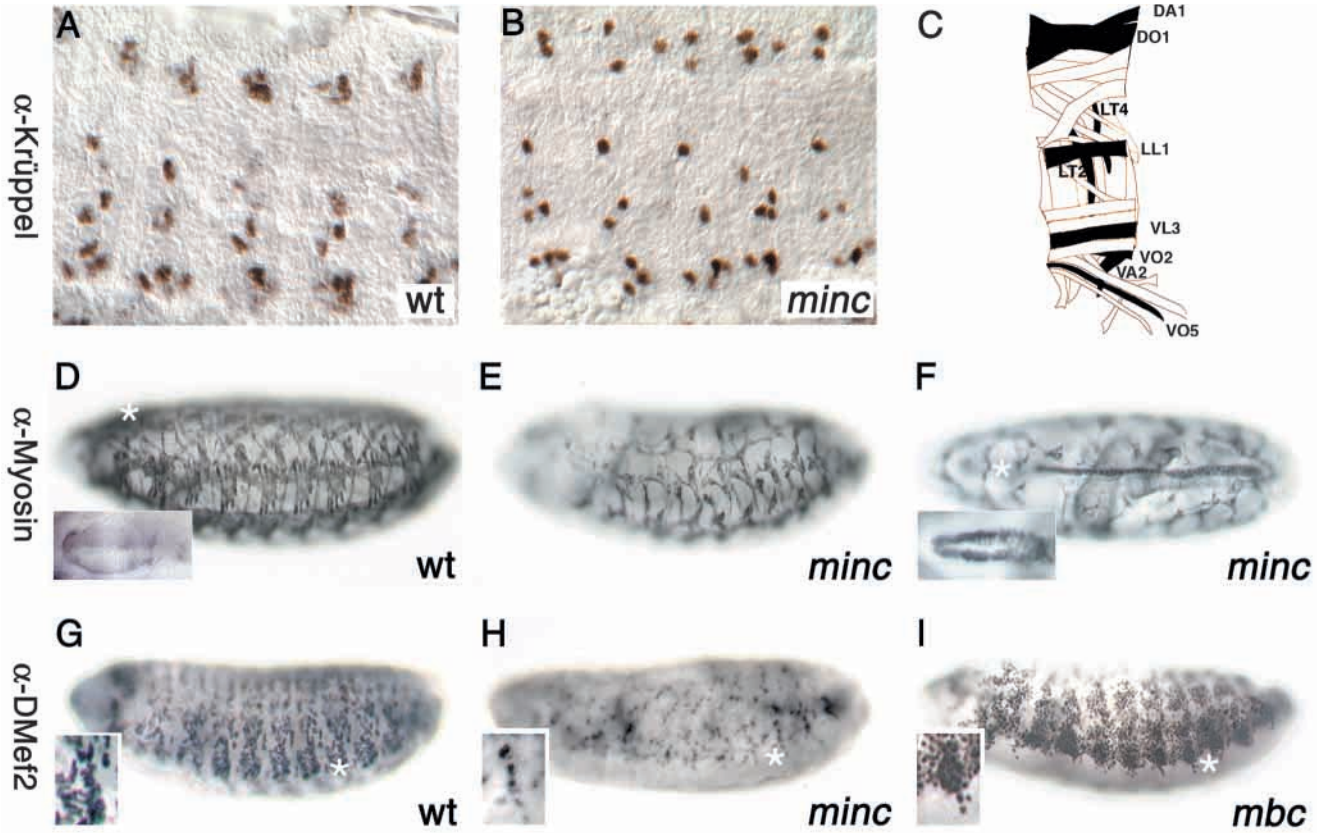

Fig. 1. Differentiation of mesodermal derivatives in wild-type and *minc* mutant embryos. (A,B) Anti-Krüppel staining confirms normal distribution of Kr-expressing founder cells in *minc* mutant embryos. Stage 14 embryos: wild type (A) and *minc[A388]* (B). (C) Diagram to show normal pattern of Kr-expressing muscles (black) in an embryonic hemisegment. (D-F) Anti-myosin staining in wild-type (D) and mutant (E,F) embryos to show normal muscle pattern (D), complete lack of myoblast fusion and differentiating mononucleated muscles (E,F) and normal heart (F). Inset in D shows enlarged view of pharynx and inset in F shows enlarged view of unfused pharyngeal muscles in this *minc[A388]* embryo. Note absence of myosin expression in unfused fusion-competent cells in E and F. (G-I) Expression of *Dmef2* in wild-type (G), *minc[A388]* (H) and *mbc1* (I) embryos. Note absence of *Dmef2* expression in fusion-competent myoblasts in H; compare with expression of *Dmef2* in these cells in I. Insets show enlarged views of regions indicated by asterisks in F-I.

non-fusion mutants such as *myoblast city* (*mbc*) (Fig. 1I and inset).

Because staining with an antibody to *Dmef2* did not resolve the issue of the fate of the fusion-competent cells in mutant embryos, we used instead an antibody to *twist*, which is a general marker for early undifferentiated mesodermal cells (Thisse et al., 1988). In normal embryos *twist* expression is lost from fusion-competent myoblasts during stages 11 and 12. In the mutant embryos, we find a striking persistence of *twist* expression in a population of cells whose arrangement is similar to the normal distribution of fusion-competent cells (compare Fig. 2A,B) (Bour et al., 2000). However there are fewer such *twist*-expressing cells in a *minc* mutant embryo than those detected by *Dmef2* expression in a non fusion mutant such as *mbc* (compare Fig. 2B and Fig. 1I). Interestingly therefore, it appears that in *minc* mutant embryos, *twist* expression persists in only a subset of the putative fusion-competent myoblasts. This expression is maintained throughout the normal period when fusion would be expected to occur. Thus, while in wild-type embryos founder cells are surrounded by fusion-competent myoblasts, with which they fuse, in *minc* embryos, normally differentiating founder cells are surrounded by an abnormal population of apparently undifferentiated mesodermal cells expressing *twist*.

To examine the extent to which these *twist*-expressing cells have been specified, we looked at the expression of two further genes, *hairy* (*h*) (Ingham et al., 1985) and *sticks and stones* (*sns*), whose products are characteristic of fusion-competent cells in the mesoderm of normal embryos. *sns*, in particular, is an early marker for this population and a critical determinant of the function of these cells in fusion (Bour et al., 2000). In both cases, we fail to detect any expression in the putative fusion-competent cells surrounding the founder myoblasts in mutant embryos (Fig. 2C,D, *h*; Fig. 2E,F, *sns*). *sns* is also expressed in a second population of fusion-competent cells (Bour et al., 2000) (Fig. 2G), that will contribute to the visceral muscles of the midgut (San Martin et al., 2001). However, we find that these cells do not lose *sns* expression in *minc* mutant embryos (Fig. 2H). Nonetheless, *minc* mutant embryos fail to form syncytial visceral muscles (data not shown).

It seems, therefore, that the process of segregation that generates two populations of somatic myoblasts, founders and fusion-competent cells, in normal embryos, has occurred in the mutant, but while founders develop normally, there is a complete failure of the fusion-competent cells to differentiate.

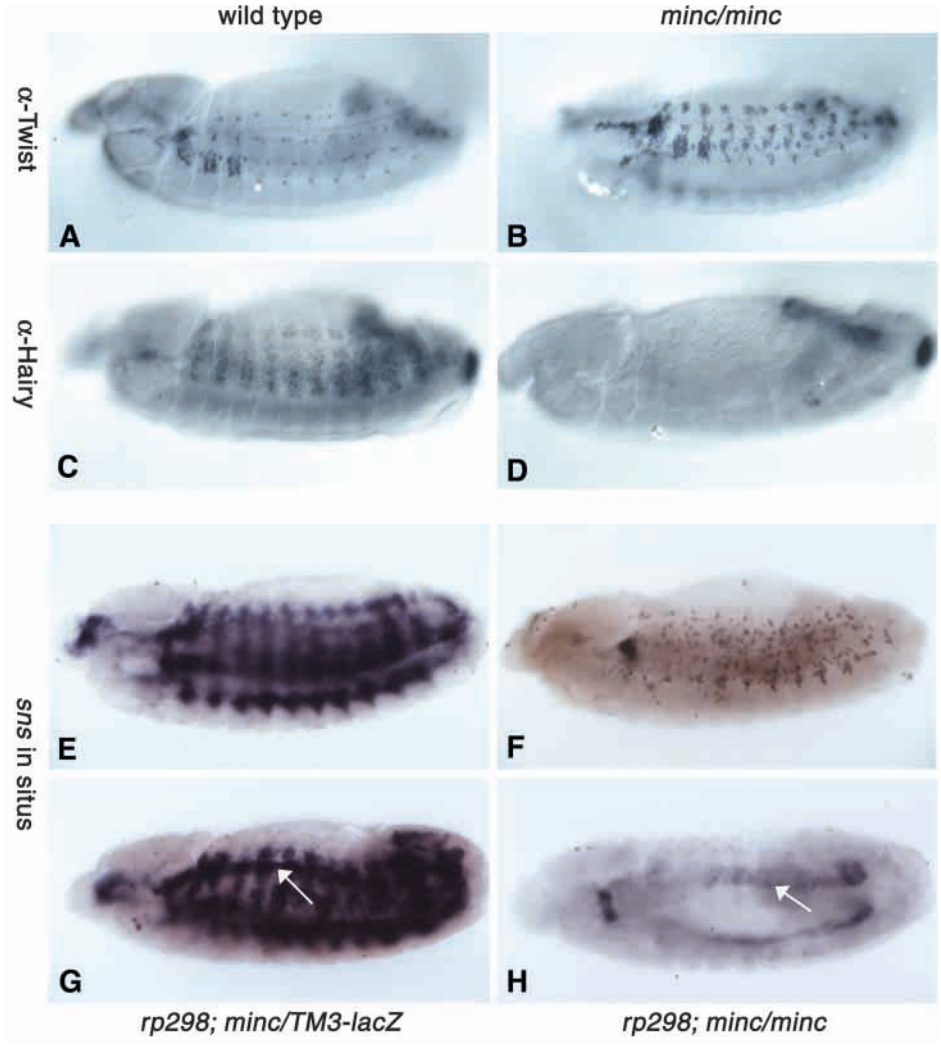

Fig. 2. Abnormal patterns of gene expression in putative fusion-competent cells in *minc* mutant embryos. (A,B) Anti-Twist staining in wild-type (A) and *minc[A388]* (B) embryos at stage 14. Note maintained expression of *twist* in fusion-competent population in B. (C,D) Anti-Hairy in wild-type (C) and *minc[A388]* (D) embryos. Hairy is present in fusion-competent cells in C but absent from these cells in the mutant (D). Note *hairy* expression is unaffected in other tissues in these embryos. (E-H) RNA in situ hybridisations for *sns* and anti-β-galactosidase staining in *rP298;minc[A388]/TM3ftzlac* (E,G) or *rP298;minc[A388]/minc[A388]* (F,H) embryos. Note loss of *sns* expression from somatic mesoderm in mutant embryos (F) but maintained presence in visceral mesoderm in the mutants (H). Arrows in G and H indicate midgut visceral mesoderm.

The putative fusion-competent cells remain as an undifferentiated population that continues to express *twist* and does not express any of the normal markers for the differentiation of this class of myoblasts. It appears that in *minc* embryos an essential step in the specification of fusion-competent cells has failed to occur, and that they therefore fail to differentiate.

Molecular characterisation of *minc*

The phenotype that we observe in *minc* mutant embryos indicates that the novel gene identified by this mutation has an important function in myogenesis. To investigate the nature of the defect in this mutation we analysed the mesodermal phenotype of those deficiencies (see above) that fail to complement *A388*. In all cases we find a comparable mesodermal defect namely a complete lack of fusion and maintained expression of *twist* in the somatic mesoderm. These results suggest that *minc[A388]* is a null allele for *minc* in agreement with the finding that the transheterozygote *minc[A388]* with *Df(3R)hhGW2* has a phenotype that is indistinguishable from the *A388* homozygote.

All the known lethals in the region defined by noncomplementation with deficiencies complement *minc*. To identify the region of DNA corresponding to *minc* we obtained cosmid clones spanning 94D3-13. Using in situ hybridisations to polytene chromosomes from deficiencies in the region, we identified cosmids partly or wholly removed by the deficiencies. We looked for transcription units with mesodermal patterns of expression within the genomic region encompassed by such cosmids and identified a fragment 3 kb long (present in cosmids 12A20, 47F9 and 43K19) with such expression. However, this fragment corresponds to DNA that is not removed by the smaller deficiencies that remove *minc*. The fragment was sequenced and used in BLAST searches of the *Drosophila* genome to place a proximal limit on the region containing *minc* (http://www.fruitfly.org). Using the location of *hh* (see above) as a distal limit, we concluded that *minc* was within a region of DNA where 34 predicted genes map.

Because the phenotype of *minc[A388]* suggests a role in regulating patterns of gene expression in fusion-competent myoblasts, we chose to concentrate on two of these predicted genes, CG4677 and CG4639, both of which correspond to predicted transcription factors, (both with putative zinc finger domains). We used the EST (expressed sequence tag) clones identified by The Berkeley Drosophila Genome Project (BDGP) for both genes (LD28538 and LD47926 for CG4677 and LD05474 for CG4639) in in situ hybridisations to embryos, and found that one of them, LD47926 (CG4677), has a mesodermal

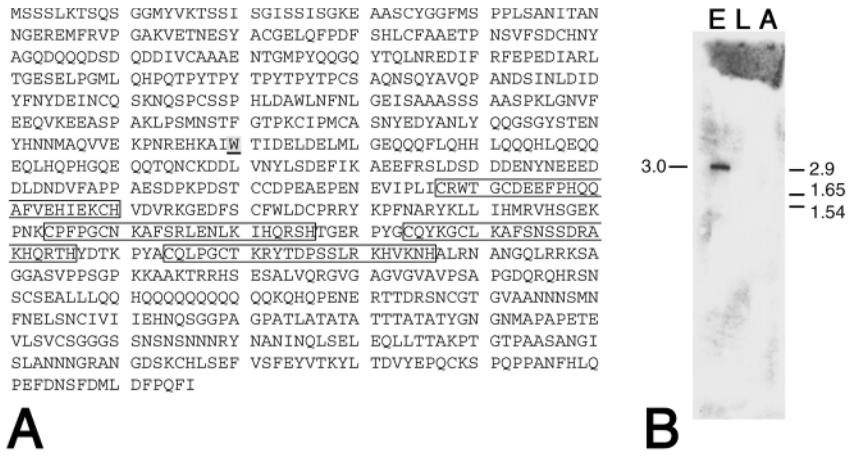

Fig. 3. (A) Predicted amino-acid sequence of Minc protein showing four C2H2 zinc finger motifs (boxes). The shaded, underlined box indicates the position of the amino acid change associated with the *minc[A388]* mutation. In addition a conservative change of F to I at amino acid 140 was also present in the mutant. (B) Northern blot prepared from samples of embryonic (E), larval (L) and adult (A) poly(A)$^+$ RNA hybridised to DNA probe LD 47926. A single transcript 3 kb long is revealed uniquely in RNA from embryos.

pattern of expression whose profile coincides with sites where *minc* function is required. Furthermore, LD47926 is not expressed in embryos homozygous for deficiencies that remove *minc* (data not shown). Probing a northern blot containing RNA samples from embryos, larvae and adults with the LD47926 clone we identified a unique transcript 3.0 kb long, which is expressed in embryos (Fig. 3B). The putative *minc* cDNA sequence was determined by sequencing the EST clone LD47926. The cDNA is 3192 nucleotides long, contains an open reading frame (ORF) of 866 amino acids and untranslated regions (UTR) on the 5′ and 3′ ends of 256 and 335 nucleotides respectively. The sequence of the cDNA differs from the predicted translation for the CG4677 gene. Thus the gene CG4677 has 3 exons instead of the 5 predicted, as predicted introns 1 and 4 are included in the cDNA sequence. The putative *minc* cDNA encodes a transcription factor that contains four C2H2-type zinc finger motifs (Fig. 3A) whose closest homologues are the zinc finger protein Gli1 (Kinzler et al., 1988) and the Cubitus interruptus protein (Orenic et al., 1990).

To verify that cDNA LD47926 corresponds to *minc*, we sequenced genomic DNA encoding the gene CG4677 obtained from *minc[A388]* embryos and found a change G to A in nucleotide 959 of the coding region that changes tryptophan 320 into a stop codon (Fig. 3A, shaded box, underlined). The truncated protein that is produced in *minc* mutant embryos lacks the four C2H2 finger domains and most probably its ability to bind DNA. This result is in agreement with *minc[A388]* being a null allele for *minc* as suggested (see above) from its mutant phenotype.

Pattern of *minc* expression

The earliest expression of *minc* that we detect is in cells of the visceral mesoderm – initially at stage 10 in a small patch of cells at the posterior tip of the embryo, that probably corresponds to the primordium of the caudal visceral mesoderm (Kusch and Reuter, 1999) and then in segmentally repeated clusters of cells that correspond to the *bagpipe* (*bap*)-expressing progenitors of the trunk visceral mesoderm (Azpiazu and Frasch, 1993) (Fig. 4A). These patches are joined ventrally by expression in the mesodermal cross bridges (Bate, 1993) (Fig. 4A, arrowheads). Expression in the visceral mesoderm includes both founders and fusion-competent myoblasts (Fig. 4B). By stage 11, expression starts in cells of the somatic mesoderm and begins to decline in cells of the visceral mesoderm (Fig. 4C). Expression is predominantly in cells that do not express the *rP298* marker for founder cells. There is probably residual expression of *minc* in *rP298*-expressing cells but it is clearly at a lower level than in the adjacent cells with which they will fuse (Fig. 4D). There is strong expression in the somatic mesoderm until stage 13 (Fig. 4E). Expression begins to decline in stage 14 and, apart from residual expression in a few cells, has disappeared by late stage 15. At this stage, however, *minc* is detectable in mesodermal cells of the gonad (Fig. 4F).

Mutations in *twist*, which eliminate all mesodermal derivatives also completely lack expression of *minc* (Fig. 5A,B), so confirming its exclusively mesodermal pattern of expression. Previous work has shown that founder cells segregate from the somatic mesoderm by a process of lateral inhibition that is mediated by the neurogenic genes and the activation of the *Notch* signalling pathway in neighbouring cells, that are thereby prevented from becoming founders themselves. In *Notch* mutant embryos, there is an overproduction of founder cells at the expense of other adjacent cells (Corbin et al., 1991). It seems likely that the cells that are inhibited from becoming founders are the cells that will enter the other myoblast class, namely the fusion-competent cells. This view is reinforced by the finding that *sns* expression, which is characteristic of these cells, is reduced in *Notch* mutant embryos (Bour et al., 2000). If this is so, and if *minc* is involved in the specification of fusion-competent cells, then we would expect that its expression too would be reduced in neurogenic mutant embryos. Accordingly, we assayed the expression of *minc* in mutants for *Notch* and *Delta*. In both cases there is a striking reduction in the expression of *minc* (Fig. 5C,D, *N*; E,F, *Dl*). There is some persistent *minc* expression in these embryos which is likely to be in cells of the visceral mesoderm.

minc mutant phenotypes are rescued by mesodermal expression of *minc*

To verify that the product encoded by the LD47926 cDNA corresponds to the missing function in *minc* mutant embryos, we used the GAL4/UAS system (Brand and Perrimon, 1993) to drive expression of this cDNA in the mesoderm of *minc[A388]* embryos. Using *Twi-GAL4* (Baylies and Bate, 1996) to drive expression throughout the mesoderm, we found substantial rescue of all aspects of the *minc* mutant phenotype, including fusion to form multinucleate muscle fibres, expression of myosin (compare Fig. 6A,B,C) and reduction of

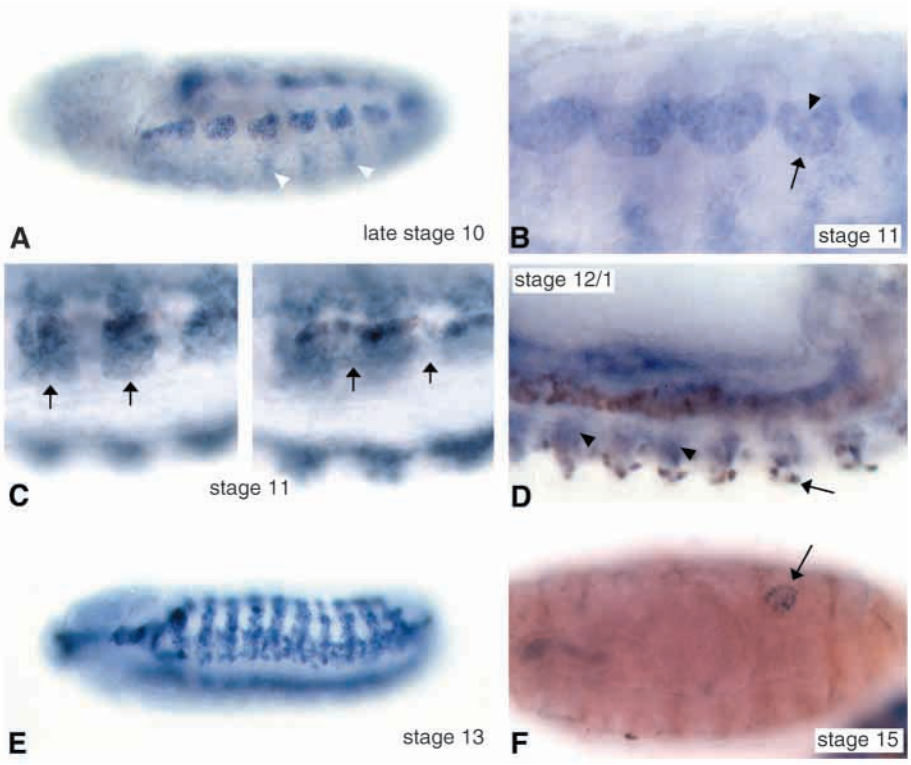

Fig. 4. Embryonic pattern of *minc* expression. (A,B,E,F) RNA in situ hybridisations with the LD47926 probe. (C,D) as other panels but with anti-β-galactosidase (brown) staining to reveal *rP298* expression. (A) Embryo at late stage 10 showing *minc* expression in repeated clusters of cells corresponding to primordia of trunk visceral mesoderm. There is faint expression in mesodermal cross bridges below the clusters (arrowheads). (B) Stage 11 embryo showing *minc* expression in both founders (arrow) and fusion-competent cells (arrowhead) of visceral mesoderm. (C) Superficial (left) and deeper (right) planes of focus in stage 11 embryo to show transition from expression in visceral to expression in somatic fusion-competent cells. Expression in visceral mesoderm (arrows in right panel) is reduced as strong expression begins in somatic mesoderm (arrows in left panel). The location of the visceral mesoderm is indicated by the ribbon of *rP298*-expressing visceral founder cells. (D) Late stage 12 embryo: founder myoblasts express *rP298* (arrows) but little if any *minc*; non *rP298*-expressing fusion-competent cells express *minc* at high levels (arrowheads). (E) Stage 13 embryo showing continued expression of *minc* in the somatic mesoderm. (F) By stage 15, *minc* expression is lost from the somatic mesoderm but now appears in the mesodermal cells of the gonad (arrow).

twist expression in fusion-competent cells (compare Fig. 6D,E,F). The rescue is incomplete and the reason for this is likely to be the use of the *Twi-GAL4* driver. The expression of *minc* itself downregulates *twist* in those cells that express it and therefore the effectiveness of the driver is reduced.

We used *En-GAL4* (Tabata et al., 1995) and *Sca-GAL4* (Budnik et al., 1996) to drive ectopic expression of *minc* in the ectoderm. Ectodermal Minc leads to the expression of *Dmef2* in the CNS (*En-GAL4* Fig. 6G) and *sns*, which is normally confined exclusively to fusion-competent cells, in the epidermis (*En-GAL4* Fig. 6H) and CNS (*Sca-GAL4* Fig. 6I).

From the rescuing effects of driving *minc* expression in the mesoderm and the ectopic activation of genes normally expressed in fusion-competent myoblasts we conclude that cDNA LD47926 is responsible for functions that are lost in *minc[A388]* mutant embryos.

Mesodermal expression of Dmef2 fails to rescue minc mutant phenotypes

Dmef2 is required for the normal differentiation of all myogenic cells (Bour et al., 1995; Lilly et al., 1995). However, we

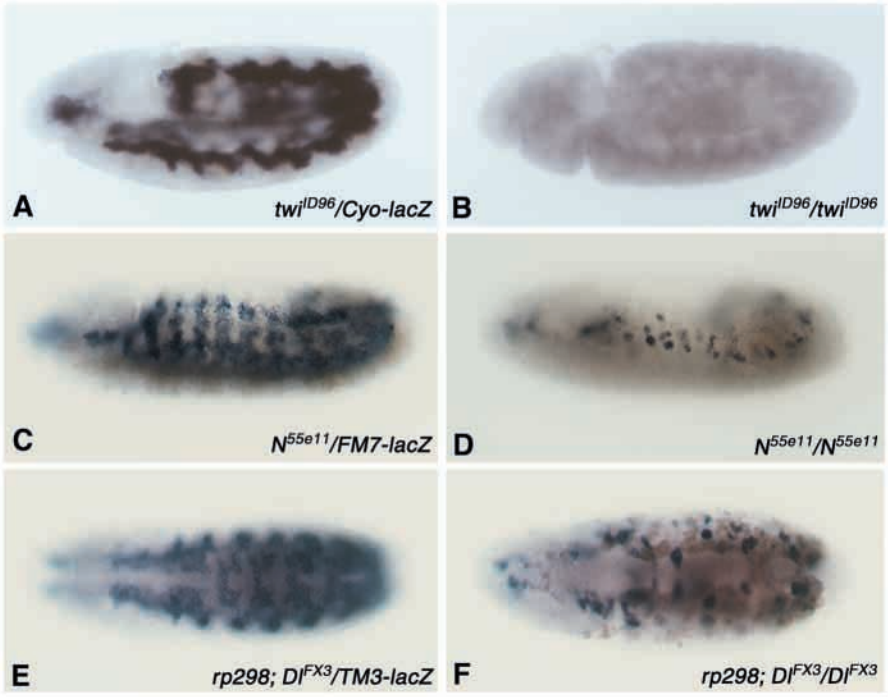

Fig. 5. Regulation of *minc* expression. RNA in situ hybridisation using LD 47926 and anti-β-galactosidase staining. (A) Control (*twistID96/TM3lac*) and (B) *twistID96* embryos, showing *minc* expression is absent from *twist* mutant embryos. (C) Control (*N55e11/FM7lac*) and (D) *N55e11* embryos to show marked reduction of *minc* expression in the *N* mutant. (E) Control (*rP298*; *DlFX3/TM3lac*) and *rP298;DlFX3* to show residual *minc* expression as in D and enlarged population *rP298*-expressing (brown) founders, which do not express *minc*.

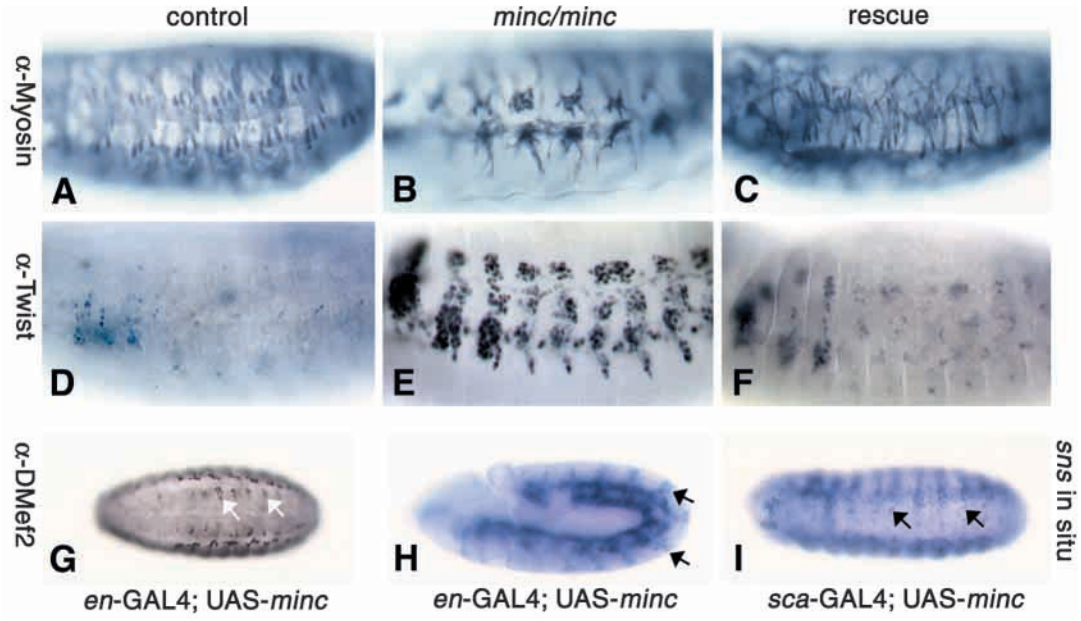

Fig. 6. Rescue of *minc* and ectopic *minc* expression. (A-F) *Twi-Gal4* used to drive *UAS-minc* in *minc[A388]* mutant background. A and D are controls for C and F, in which *UAS-minc* rescues *minc* mutant phenotype. B and E are *minc* mutant embryos for comparison. (A-C) Anti-myosin staining, (D-F) anti-Twist staining to illustrate (C,F) partial rescue of *minc* mutant phenotype shown by both recovery of fusion and myosin expression (C, compare B) and loss of *twist* expression from fusion-competent cells (F, compare E). (G,H) *En-Gal4/UAS-minc* drives ectopic *Dmef2* expression (arrows, G) in the CNS and *sns* expression in the ectoderm (arrows, H). *Sca-Gal4/UAS minc* drives CNS expression of *sns* (arrows, I).

know that *sns* expression is not lost in *Dmef2* mutants (own observations) (Bour et al., 2000) and therefore the early segregation and specification of fusion-competent cells appears to be independent of *Dmef2*. In addition, the downregulation of *twist* in fusion-competent cells occurs normally in *Dmef2* mutants (data not shown). Nonetheless, in the absence of *Dmef2*, fusion fails and myosin is not expressed (Bour et al., 1995; Lilly et al., 1995). Thus the failure of late steps in the differentiation of fusion-competent cells in *minc* mutant embryos might solely be caused by the absence of Dmef2 from these cells. To test this idea, we drove expression of *Dmef2* in the mesoderm of *minc* mutants using *Twi-GAL4*. In this experiment we find that there is no rescue of fusion or of myosin expression and that *twist* expression is maintained at a high level in the fusion-competent cells (data not shown). Thus in the absence of *minc*, *Dmef2* is incapable of eliciting myogenic differentiation from this population of mesodermal cells. We conclude that Minc is not only required for the initial specification of fusion-competent myoblasts but is also essential for the activation of terminal differentiation in these cells.

DISCUSSION

There is an essential subdivision of the population of muscle-forming cells during myogenesis in *Drosophila* (Fig. 7) (Baylies et al., 1998). Specific cells are selected locally to seed the assembly of particular muscles. These founder myoblasts endow muscles with characteristic properties such as size, innervation and orientation, by the operation of the particular constellation of transcription factors that they express. The other population of cells that contribute to the muscles are the fusion-competent myoblasts, which fuse with the founder myoblasts so that the local initiation of muscle formation by a founder leads to the development of a specific multinucleate muscle fibre. This subdivision of the muscle-forming cells into myoblasts of two kinds is essential for fusion. There is an intrinsic asymmetry to the fusion process that enables the founders to act as local gates on the myogenic process: founders will not fuse with each other, but only with fusion-competent myoblasts, fusion-competent cells will only fuse with founders. This asymmetry is driven by the differential expression of genes encoding two Ig domain proteins that are essential for fusion: founder myoblasts express *duf* (Ruiz-Gómez et al., 2000), whereas fusion-competent cells express *sns* (Bour et al., 2000). The diversification of myoblasts applies not only to the formation of somatic (body wall) muscles, but also to the myoblasts that contribute the visceral muscles lining the midgut (San Martin et al., 2001).

The specialisation of the fusion-competent cells revealed by their exclusive expression of *sns*, suggests that these myoblasts are not simply a naïve population of muscle-forming cells but that they have a specific programme of differentiation which must be implemented if myogenesis is to proceed. This programme includes the expression of *sns* and *hairy*, the down-regulation of *twist*, the maintenance of *Dmef2* expression and the synthesis of contractile proteins such as myosin. Some of these steps are specific to fusion-competent cells: the expression of *sns* and *hairy*; others such as the down regulation of *twist*, the maintained expression of *Dmef2* and the synthesis of contractile proteins, appear to be steps in a pathway of differentiation that is common to all myogenic cells.

In this paper we have identified a gene, *minc*, that is required for the proper specification of the population of fusion-competent myoblasts that contributes to the body wall muscles. *minc* is upregulated in fusion-competent cells and downregulated in founders and the key to this differential expression is the activation of the *N* signalling pathway in the fusion-competent cells as the progenitors of founder myoblasts segregate in the somatic mesoderm (Fig. 7). Our experiments show that *N* signalling leads directly or indirectly to the maintained expression of *minc* in fusion-competent cells. *minc*

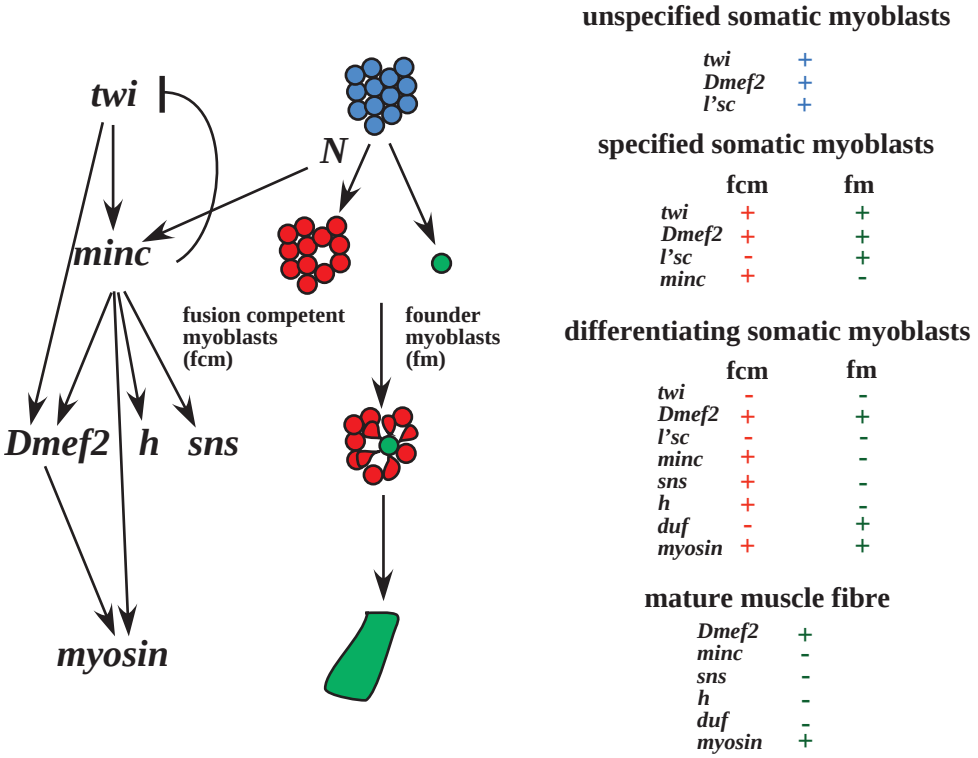

Fig. 7. Diagram illustrating the mode of action of *minc* in the myogenic pathway and patterns of gene expression underlying myoblast diversification within the somatic mesoderm. Fusion-competent myoblasts (red; fcm) and founder myoblasts (green; fm) segregate from unspecified somatic myoblasts (blue) through the activation of the *N* signalling pathway in the fusion-competent myoblasts. This leads to *minc* being expressed selectively in these cells, thus initiating patterns of gene expression that are characteristic of either fusion-competent cells (red) or founder myoblasts (green). *minc* expression in fusion-competent cells is required both for specific patterns of gene expression (*h*, *sns*) and for the implementation of myogenic differentiation in these cells (myosin).

in turn is required for the expression of genes that are specific to the fusion-competent cells, such as *sns* and *hairy*, and it is also required for the implementation of myogenic differentiation in these cells. In the absence of *minc*, *Dmef2* is not maintained in fusion-competent cells but the loss of *Dmef2* expression is not solely responsible for the failure of myogenic differentiation in fusion-competent cells that we observe in *minc* mutant embryos. If *Dmef2* alone were sufficient to activate myogenic differentiation in these cells then it would be possible to rescue this aspect of the *minc* phenotype simply by providing the potential fusion-competent cells with *Dmef2*. However, myosin expression in the fusion-competent cells is not rescued by *Dmef2* in the absence of *minc* and this shows that *minc* is an essential component in implementing the terminal differentiation of this population of muscle-forming cells, presumably in combination with *Dmef2*.

The fact that *minc* is required not only for specific patterns of gene expression but also for the implementation of what might be considered the common pathway of myogenesis in the somatic fusion-competent myoblasts is interesting and prompts us to speculate as to why there should be such a specific control mechanism for terminal myogenesis in these cells. We know that specific muscle properties within the somatic muscle lineage are regulated by the expression of particular transcription factors in subsets of muscle founder cells. However, it is likely that there are general characteristics of the somatic muscles that are not shared with other differentiated myogenic cell types such as the visceral and cardiac muscles. It might be that the implementation of these characteristics in the somatic lineage could depend, at least in part, on a particular programme of myogenic differentiation implemented only in the somatic fusion-competent myoblasts under the control of *minc*.

However, *minc* is also expressed in the visceral mesoderm. Here too it is expressed at relatively higher levels in fusion-competent cells than in founders, but curiously it is not required for *sns* expression in these cells, nor for the expression of *Dmef2* or markers of terminal differentiation such as myosin. In addition, the control of *minc* expression is probably exerted through a different mechanism. Instead of a response to *N* activation it seems likely that *minc* expression is controlled by general regulators of visceral mesoderm differentiation such as *bap*. Thus, *minc* expression appears in the *bap*-expressing clusters and in the region of *bap* expression in the caudal visceral mesoderm, and in mutations that reduce *bap* expression [such as loss of *hedgehog* function (Azpiazu et al., 1996)], there is a concomitant reduction in *minc* (data not shown). However, in the absence of *minc*, *hairy* expression is lost from the visceral fusion-competent myoblasts and visceral myoblasts fail to fuse to form either longitudinal or circular syncytial muscles (data not shown). Clearly *minc* has some function in the development of the visceral mesoderm but in these cells it does not operate as an essential regulator of terminal myogenic differentiation as it does in the somatic mesoderm. The fact that fusion fails in the visceral mesoderm, despite the expression of both *duf* and *sns* in founders and fusion-competent myoblasts respectively indicates that additional factors, regulated by *minc* are required, perhaps specifically in the fusion-competent myoblasts if fusion is to occur.

minc encodes a putative zinc finger transcription factor whose closest homologues are Gli proteins in vertebrates (Kinzler et al., 1988) and the Cubitus interruptus protein (Orenic et al., 1990) in *Drosophila*. Proteins of this class can act both as activators and repressors of transcription (Ruiz i Altaba et al., 1997). Our experiments indicate that Minc acts to down regulate the expression of *twist* and to activate the expression of *sns*, *hairy* and *Dmef2*. Since ectopic expression of *minc* can drive expression of both *sns* and *Dmef2* in ectodermal derivatives and

rescues the mutant phenotype in fusion-competent myoblasts we conclude that *minc* acts specifically within the myogenic lineage to endow fusion-competent myoblasts with their essential properties. Although *minc* appears to act at a high level in the hierarchy of regulators that control myogenesis it does not on its own convert cells of a different lineage – the ectoderm – to the myoblast cell fate. Some aspects of myoblast differentiation are initiated, such as *sns* and *Dmef2* expression but it is clear that additional factors, presumably present only in the mesoderm, are necessary for terminal muscle differentiation. This accords with the view that *minc* acts as a regulator that is intrinsic to the myogenic pathway, modifying the fate of cells in the muscle lineage to form a particular kind of myoblast.

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Note added in proof

Furlong et al. (Furlong et al., 2001) have reported a gene *gleeful*, which is identical to the gene *minc* described in this paper.

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