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her1, a zebrafish pair-rule like gene, acts downstream of notch signalling to control somite development

Christina Takke and José A. Campos-Ortega*

Institut für Entwicklungsbiologie, Universität zu Köln, 50923 Köln, Germany
*Author for correspondence (e-mail: jcampos@novell.biolan.uni-koeln.de)

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SUMMARY

During vertebrate embryonic development, the paraxial mesoderm becomes subdivided into metameric units known as somites. In the zebrafish embryo, genes encoding homologues of the proteins of the *Drosophila* NOTCH signalling pathway are expressed in the presomitic mesoderm and expression is maintained in a segmental pattern during somitogenesis. This expression pattern suggests a role for these genes during somite development. We misexpressed various zebrafish genes of this group by injecting mRNA into early embryos. RNA encoding a constitutively active form of NOTCH1a (*notch1a-intra*) and a truncated variant of deltaD [*deltaD*$^{(Pst)}$], as well as transcripts of *deltaC* and *deltaD*, the *hairy-E(spl)* homologues *her1* and *her4*, and *groucho2* were tested for their effects on somite formation, myogenesis and on the pattern of transcription of putative downstream genes. In embryos injected with any of these RNAs, with the exception of *groucho2* RNA, the paraxial mesoderm differentiated normally into somitic tissue, but failed to segment correctly. Activation of NOTCH results in ectopic activation of *her1* and *her4*. This misregulation of the expression of *her* genes might be causally related to the observed mesodermal defects, as *her1* and *her4* mRNA injections led to effects similar to those seen with *notch1a-intra*. *deltaC* and *deltaD* seem to function after subdivision of the presomitic mesoderm, since the *her* gene transcription pattern in the presomitic mesoderm remains essentially normal after misexpression of *delta* genes. Whereas NOTCH signalling alone apparently does not affect myogenesis, zebrafish *groucho2* is involved in differentiation of mesodermal derivatives.

Key words: *her1*, Notch signalling, Somitogenesis, Zebrafish

INTRODUCTION

In *Drosophila*, the NOTCH signalling pathway has been studied chiefly from the point of view of neurogenesis, in particular with respect to its participation in the selection of neural progenitor cells (Campos-Ortega, 1993). However, in *Drosophila*, this regulatory pathway is also involved in several other developmental processes, including oogenesis (Ruohola et al., 1991; Xu et al., 1992), myogenesis (Corbin et al., 1991; Carmena et al., 1995), gut and heart development (Tepass and Hartenstein, 1994). Recent data indicate that homologues of the genes of the NOTCH pathway play crucial roles in vertebrate development as well. Besides their role in primary neurogenesis (Chitnis et al., 1995; Chitnis and Kintner, 1996; Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al, 1998; Takke et al., 1999), there is growing evidence that a similar regulatory network is also involved in segmentation of the paraxial mesoderm. However, the situation in vertebrates is more complex, in part due to the presence of multiple *Notch* and *Delta* genes (Del Amo et al., 1992; Lardelli and Lendhal, 1993; Bierkamp and Campos-Ortega, 1993; Westin and Lardelli, 1997; Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998). Mouse embryos deficient for *Notch1* or for the *Delta* homologue *Dll1* show severe somitic defects (Conlon et al., 1995; Hrabe de Angelis et al., 1997). In *Notch1*$^{\Delta 1}$ mutant embryos, condensed unsegmented mesoderm was often present near the presomitic mesoderm. Later on, epithelialization sometimes appeared incomplete, and most of the newly formed somites were not as tightly packed as in the wild type (Conlon et al., 1995). In *Dll1* mutant embryos, craniocaudal segment polarity appeared to be lost, no epithelialized somites were formed and myoblasts extended across the segment borders, suggesting that *Dll1* is involved in compartmentalization of the somites and that muscle cell differentiation itself is an independent process (Hrabe de Angelis et al., 1997). From studies of the expression pattern of *Dll1* and *Dll3*, Dunwoodie et al. (1997) concluded that the different members of this regulatory network act in a coordinate manner in establishing the intersomite boundaries. Similar somite defects to those described for the *Dll1* and *Notch1* mutants have been observed in mice that lack RBP-Jκ (Oka et al., 1995; de la Pompa et al., 1997). This vertebrate homologue of the *Drosophila Suppressor of Hairless* product (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) thus appears to be required for at least some aspects of NOTCH signalling (Wettstein et al., 1997; Jen et al., 1997). While these observations suggest that the NOTCH pathway is required for segmentation of the paraxial mesoderm, it is not

known how NOTCH performs this function, nor upon which step during segmentation it acts.

In addition to being involved in segmentation, studies in vertebrates and invertebrates suggest that the LIN12/NOTCH receptors inhibit cell differentiation when activated by members of the DSL (for DELTA, SERRATE, Lag2) ligand family. Thus, the NOTCH signalling pathway appears to play a role in myogenesis as well. Both HES-1, a mouse homologue of E(SPL), and the intracellular domain of murine NOTCH are able to block myogenesis in vitro (Sasai et al., 1992; Nye et al., 1994; Kopan et al., 1994). However, the data suggest that inhibition of myogenesis by NOTCH is mediated by an RBP-Jκ/CBF1-independent pathway (Shawber et al., 1996).

Here we discuss some aspects of the function of the NOTCH signalling pathway in zebrafish segmentation and muscle development. Our results suggest that one of the zebrafish *Notch* genes, *notch1* (Bierkamp and Campos-Ortega, 1993, corresponding to *notch1a* of Westin and Lardelli, 1997), acts during segmental prepatterning in the presomitic mesoderm. This function seems to be mediated by *her1* and *her4*, both of which encode bHLH proteins of the HAIRY-E(SPL) family (Müller et al., 1996; Takke et al., 1999) and are misregulated following ectopic activation of NOTCH. Misexpression of *deltaC* and *deltaD* variants also causes somitic defects. However, neither *delta* gene affects the transcription pattern of *her1* or *her4*; the *delta* genes thus appear to act on the establishment and/or maintenance of somite boundaries rather than on segmentation itself. Whereas NOTCH activation alone does not affect myogenesis, *groucho2* is essential for the differentiation of mesodermal cells.

MATERIALS AND METHODS

Zebrafish embryos were obtained from spontaneous spawnings. Adult fish were kept at 28.5°C on a 14 hour light/10 hour dark cycle. The embryos were staged according to Warga and Kimmel (1990) and Kimmel et al. (1995).

mRNA injections

Capped RNA was prepared from cDNAs cloned into the pCS2+ expression vector (Turner and Weintraub, 1994), as described in Takke et al. (1999), using a Message Kit from Ambion. 1-2 ng of the RNA were injected in a volume of 5 nl into one of the first two blastomeres.

A 2976 bp cDNA clone (2/1 d) of *deltaC* (Haddon et al., 1998) was kindly provided by Julian Lewis (ICRF, London). PCR was used to obtain a full-length cDNA of *deltaC* from a randomly primed cDNA library made with RNA from 3-15 h (hour) zebrafish embryos. To facilitate cloning, a *Cla*I site was introduced into the 5′ primer CTACTCTCACAGTCTGCT**ATCGAT**CAGTAGC, designed from a *deltaC* genomic clone kindly provided by Stefan Hans (Köln). The 3′ primer GCAGAAATCCGAGCATTCCTCGCCGTGG was based on the 2/1 d cDNA clone. A PCR fragment of 562 bp was cloned into PCRII-Topo vector (Invitrogen). A 518 bp fragment was isolated from this insert by cleavage with *Xba*I and *Bsm*I and ligated in frame to the 3′ end of the 2/1 d *deltaC* cDNA, thus completing the full-length *deltaC* coding sequence. Cloning into pCS2+ made use of the *Cla*I and *Xho*I sites.

In situ hybridization and antibody staining

Hybridization of digoxigenin-labelled RNA probes to embryo whole mounts was performed as described (Bierkamp and Campos-Ortega, 1993). Digoxigenin-labelled probes were prepared from cDNA clones of *her1*, *her4*, *MyoD*, *deltaC*, *deltaD* and *pax9*, using RNA labelling kits (Boehringer Mannheim). Embryos injected with RNA were subjected to in situ hybridization using the probes above, and to antibody staining, as described in Dornseifer et al. (1997). Anti-myosin staining was performed using a 1:10 dilution of the monoclonal anti-myosin heavy chain antibody (Developmental Studies Hybridoma Bank).

A note on terminology

Throughout this paper, we use italics to designate genes and mRNA molecules, and capitals to designate proteins. *Notch* and *Delta* refer to the *Drosophila* genes, and NOTCH and DELTA to their products, whereas the corresponding zebrafish homologues are designated by lower case italics or small capital letters. The NOTCH signalling pathway, when referred to in general terms, is designated with a capital N, as in *Drosophila*, where it was initially characterized; lower case and small capital letters are used to refer to the zebrafish pathway.

RESULTS

Embryos injected at the 2-cell stage with synthetic mRNAs were collected either at the 4- to 10-somite or at the 24 h stage, and analysed by in situ hybridization and/or antibody staining. Since no modifications of the somitic pattern were observed following control injections of *lacZ* mRNA alone, even at very high concentrations (Table 1), in all experiments *lacZ* mRNA was injected at low concentration together with the RNA to be tested. β-galactosidase was detected by antibody staining. Embryos that were negative for β-galactosidase showed no detectable effects of injection; consequently, we assumed that β-galactosidase-expressing cells also express the products of the co-injected RNAs. Therefore, throughout the text the term "injected embryos" refers to those that expressed β-galactosidase.

In the experiments reported below, a variant of zebrafish *notch1a*, *notch1a-intra*, was used, which encodes a NOTCH receptor that is constitutively active in neurogenesis (Takke et al., 1999). However, it is not clear whether the effects of misexpression of this variant reflect the normal function of *notch1a*, or mirror the results of activating other members of the zebrafish NOTCH family as well (Bierkamp and Campos-Ortega, 1993; Westin and Lardelli, 1997). Therefore, throughout the text, the consequences of injection of *notch1a-intra* mRNA are attributed to activation of NOTCH in general rather than to activation of NOTCH1a specifically.

Misexpression of DELTAC and DELTAD variants and activation of NOTCH perturb somitogenesis

As shown previously, misexpression of *deltaD* by mRNA injection leads to fusions and other malformations of the somites and myotomes (Dornseifer et al., 1997; Figs 1B, 2C,H; Table 1). The same effect was observed following injection of a mRNA encoding a DELTAD variant that lacks the intracellular domain (*deltaD*^{Pst}). This variant acts in a dominant-negative fashion in primary neurogenesis, where it promotes the formation of supernumerary *islet*-1-positive cells within *deltaD* expression domains in the neural plate (Takke et al., 1999). In embryos injected with *deltaD*^{Pst} mRNA, somite development, as judged by in situ hybridisations with *MyoD*, a marker gene expressed in all somites and myotomes, was perturbed (Figs 1C, 2E,K; Table 1). Misexpression of *deltaC*, another *Delta* homologue, causes somitic defects that are similar to those described for DELTAD variants. In situ

Table 1. The effects of mRNA injections on expression of *MyoD*

	mRNA injected								
	1.6 μg/μl *lacZ*	200 ng/μl *deltaC* 40 ng/μl *lacZ*	240 ng/μl *deltaD* 100 ng/μl *lacZ*	200 ng/μl *deltaD(Pst)* 40 ng/μl *lacZ*	300 ng/μl *nic* 40 ng/μl *lacZ*	400 ng/μl *her1* 40 ng/μl *lacZ*	200 ng/μl *her4* 40 ng/μl *lacZ*	200 ng/μl *her1* 100 ng/μl *groucho2* 40 ng/μl *lacZ*	200 ng/μl *groucho2* 40 ng/μl *lacZ*
Number of embryos injected	80	109	71	45	76	58	114	95	41
Number of embryos with β-gal activity	65	65	29	41	52	48	92	66	39
Number of embryos with abnormal *MyoD* expression	2 (3%)*	61 (93%)*	25 (86%)*	30 (73%)*	38 (73%)*	30 (62%)*	79 (86%)*	49 (74%)*	38 (97%)*
Number of embryos with wild-type pattern	63 (97%)*	4 (7%)*	4 (14%)*	11 (27%)*	14 (27%)*	18 (38%)*	13 (14%)*	17 (26%)*	1 (3%)*

*Percentages refer to embryos with β-gal expression.

hybridisation with *MyoD* shows that somites are formed, but their pattern is very irregular. Somite boundaries are poorly defined, i.e. they are frequently interrupted or do not develop at all, such that neighbouring somites are partially fused with each other (Figs 1D, 2D,I; Table 1).

Embryos injected with mRNA encoding a variant of NOTCH1a that lacks the extracellular and the transmembrane domain (*notch1a-intra, nic*), which in neurogenesis behaves as a constitutively active form (Takke et al., 1999), were fixed at the 8- to 10-somite stage and stained for *MyoD*. Injected embryos exhibit severe somite defects, which are much stronger than those seen following misexpression of *delta* variants. Embryos injected with *notch1a-intra* show a diffuse pattern of *MyoD* expression in the paraxial mesoderm, and no somitic organization is apparent (Figs 1E, 2B,G; Tables 1, 2). To test whether muscle differentiation is affected in these embryos, we stained older embryos (22-24 h) with an antibody against myosin heavy chain. Myosin expression was normal in the injected embryos, although muscle fibres were seen to extend through the regions in which somite borders should have developed (Fig. 1F), i.e. the embryos showed pattern defects corresponding to the somite defects described above. Therefore, we conclude that NOTCH is required for the establishment and/or maintenance of somites and their boundaries in zebrafish, but that myogenesis itself is not affected by activation of NOTCH.

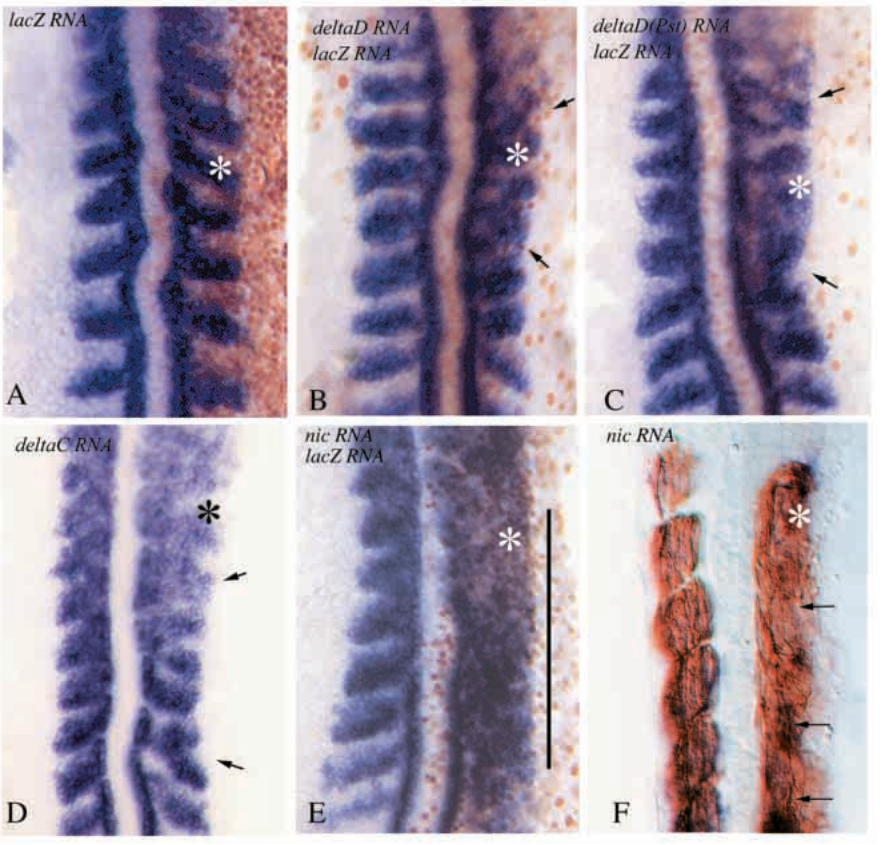

Fig. 1. (A-G) Flat preparations of embryos injected with various RNAs. Asterisks label the affected side. (A) 10-somite stage control embryo injected with *lacZ* RNA alone. (B,C) 10-somite-stage embryos injected with full-length *deltaD* (B) and with *deltaD(Pst)* (C) and *lacZ* RNA. Both embryos have been stained for *MyoD* (blue, in situ hybridization) and β-galactosidase (brown, antibody staining) expression. Somites are irregularly shaped on the affected side, arrows point to somite fusions. (D) 10-somite-stage embryo injected with full-length *deltaC* RNA and stained by in situ hybridization for *MyoD* expression. Somitic defects are similar to those in B and C. Arrows point to somite fusions. (E) 10-somite-stage embryo injected with *notch1a-intra* (*nic*) mRNA and *lacZ* RNA and stained for *MyoD* (blue, in situ hybridization) and β-galactosidase (brown, antibody staining) expression. Notice the poor somitic organization on the affected side, *MyoD* expression is diffuse and no somite boundaries can be distinguished in the territory labelled by the vertical line. (F) 22-h-stage embryo that had been injected with *notch1a-intra* (*nic*) mRNA and *lacZ* RNA, and stained for myosin heavy chain. Notice that the outlines of the somites appear blurred and muscle fibres are not packed into somitic groups. The arrows point to individual muscle fibres extending through regions in which somite borders should have developed

Table 2. The effects of *notch1a-intra* mRNA injections on transcription of putative target genes

	Probes for in situ hybridisations			
	her1/MyoD	*deltaC*	*deltaD*	*her4*
Number of embryos injected	151	105	163	65
Number of embryos with either β-gal staining or *MyoD* expression defects (#)	86#	52	135	40
Number of embryos with abnormal expression of marker genes	79 (92%)*	51 (98%)*	130 (96%)*	35 (88%)*
Number of embryos with essentially wild-type pattern	7 (8%)*	1 (2%)*	5 (4%)*	5 (12%)*

All embryos were injected with 200 μg/μl *notch1a-intra* mRNA or 40 ng/μl *lacZ* mRNA.
#Number of embryos with defects in *MyoD* expression.
*Percentages refer to embryos either with defective *MyoD* or β-gal expression.

Mesodermal expression of *her1* and *her4* depends on NOTCH signalling

her1 is a zebrafish *hairy-E(spl)* homologue that is expressed in a pair-rule like pattern within the presomitic mesoderm (Müller et al., 1996). This expression pattern suggests a role for *her1* in formation and/or differentiation of somites. To test whether *her1* is a target of NOTCH, we analysed the spatial pattern of transcription of *her1* following injection of *notch1a-intra* mRNA. Under normal conditions, *her1* is transcribed in stripe-like domains corresponding to the primordia of odd-numbered somites, starting with the 5th, while the areas corresponding to the even-numbered somites are devoid of transcripts (Müller et al., 1996). In embryos injected with *notch1a-intra* mRNA, ectopic activation of *her1* transcription was observed such that the pattern of presomitic stripes was lost and transcripts were diffusely distributed. However, ectopic expression was restricted to the presomitic mesoderm (Fig. 2B; Table 2). This result suggests that *her1* expression is dependent on NOTCH signalling. To assess patterning in presomitic and somitic territories of the mesoderm after *notch1a-intra* mRNA

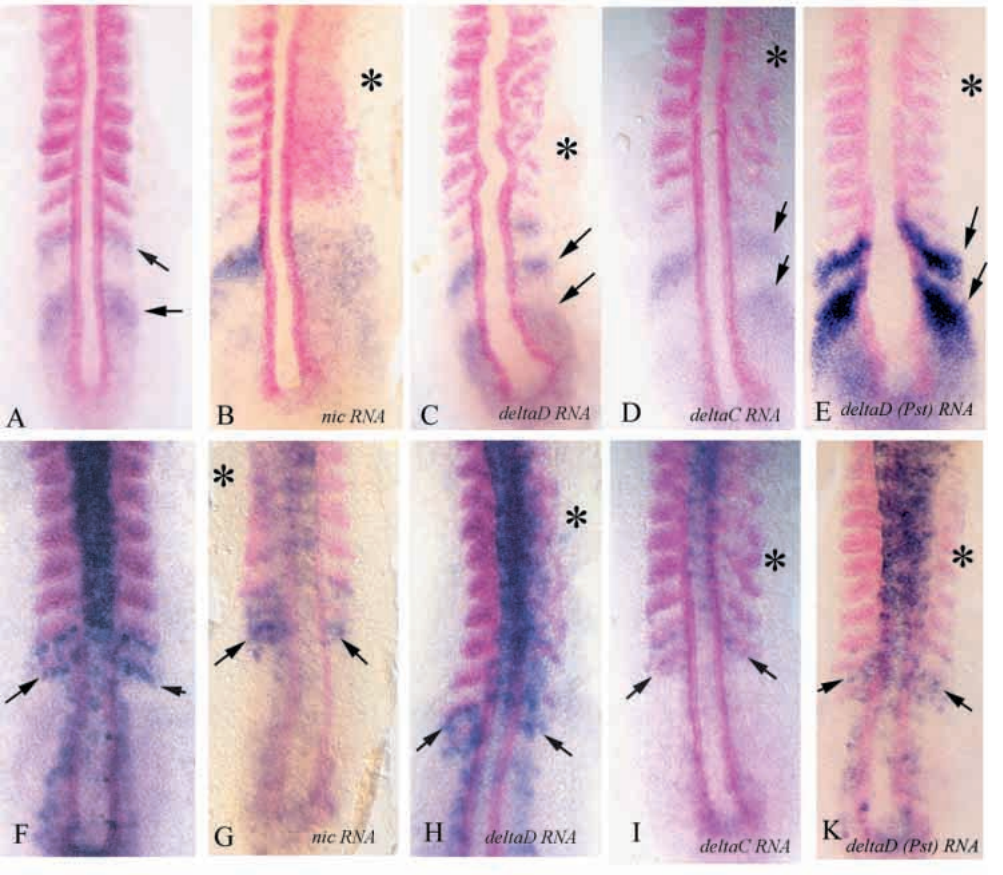

Fig. 2. Flat preparations of 8- to 10-somite-stage embryos injected with *notch1a* (*nic*) (B,G), *deltaD* (C,H) and *deltaC* (D,I) and *deltaD(Pst)* RNA (E,K). (A,F) Normal embryos. All embryos have been processed for in situ hybridization with two probes, either *MyoD* (red) and *her1* (blue, A-E) or *MyoD* (red) and *her4* (blue, F-K). Asterisks in B-E and G-K label the affected side; arrows point to *her1* and *her4* domains (blue staining). (B) On the affected side of *notch1a-intra* (*nic*) RNA-injected embryos, *her1* is upregulated within the presomitic mesoderm, but not in the immediate neighbourhood of the somitic territory, and the characteristic *her1* stripes cannot be distinguished. Notice that *MyoD* expression is also unpatterned in the somitic mesoderm. Following *nic* RNA injection, *her4* expression is also upregulated in the presomitic mesoderm on the injected side (notice that there are more blue stained cells on the injected side), *MyoD* expression in the somitic territory is diffuse (G). Following injection of *deltaD* (C,H), *deltaC* (D,I) or *deltaD(Pst)* RNA (E,K), somitic defects are visible as manifested by the *MyoD* expression (asterisks). However, *her1* and *her4* expression are essentially normal. The *her1* stripes are clearly separated from each other and the presomitic expression of *her4* is as weak as in normal embryos (arrows point to blue cells, compare with F). The diffuse blue colour on the injected side of the embryos in H, I and K is due to activation of *her4* transcription in the neural plate (Takke et al., 1999).

injection, double in situ hybridizations with *MyoD* and *her1* probes were performed. They showed that a diffuse distribution of *her1* transcript in the presomitic mesoderm invariably correlates with patterning defects in the somitic territory, as manifested by *MyoD* expression, suggesting a causal relationship between the two effects (Fig. 2B).

her4, another zebrafish gene of the *hairy-E(spl)* family, is mainly expressed within the neural plate. However, *her4* is also expressed, albeit very weakly, in one or two groups, depending on the time point, of presomitic mesodermal cells located in the vicinity of the last somite formed (Takke et al., 1999). In embryos injected with *notch1a-intra* mRNA, presomitic expression of *her4*, which is hardly detectable in the normal embryo at the 10-somite stage, becomes more prominent on the injected side (Fig. 2G; Table 2). Activation of *her4* following *notch1a-intra* injection remains restricted to the region of the normal expression domain, i.e. in the presomitic mesoderm immediately adjacent to the last complete somite. Double in situ hybridisations with *MyoD* and *her4* probes showed the same correlation between presomitic and somitic effects of the *notch1a-intra* mRNA injections as in the *her1* case (Fig. 2G; Table 2).

Misexpression of *her1* or *her4* perturbs mesodermal segmentation but not myogenesis

To test whether ectopic expression of *her1* and/or *her4* is causally related to the effects of NOTCH1a activation on somite development, we analysed *MyoD* and myosin expression following either *her1* or *her4* mRNA injections. In embryos injected with *her1* mRNA, somitic pattern defects and somitic fusions, similar to those observed following *deltaC/deltaD* injections, were found (Fig. 3A; Table 1). The same results were observed following injections of *her4* mRNA (Fig. 3C; Table 1). However, although the organisation of muscle fibres was perturbed, myogenesis was not affected, as judged by anti-myosin antibody staining (Fig. 3B), even following injections with higher concentrations of *her1* mRNA.

Strikingly, coinjection of *her1* and *her4* mRNA causes a stronger perturbation of the *MyoD* expression, which is similar to that seen following misexpression of *notch1a-intra*: *MyoD* expression becomes diffuse and somitic boundaries fail to form (Fig. 3D).

her1 activity is specific for somite development

In early neurogenesis in *Drosophila*, the genes of the E(SPL)-C have partially redundant functions, such that they can substitute each other under specific conditions (Knust et al., 1992; Schrons et al., 1992). We tested whether *her1*, which is exclusively expressed in the presomitic mesoderm, has any effect on the development of primary neurons. This experiment was prompted by the fact that *her4* acts as a target of NOTCH1a during primary neurogenesis (Takke et al., 1999); thus we wanted to test whether *her1* can substitute for *her4* in neurogenesis. Whereas misexpression of full-length *her4* mRNA reduces the number of primary neurons in the neural plate, none of the embryos injected either with *her1* mRNA alone or with *her1* and *groucho2* mRNA together showed any alteration in the numbers of these cells (not shown). Since no defects other than those related to somitogenesis were observed, we conclude that *her1* is specific for somite development.

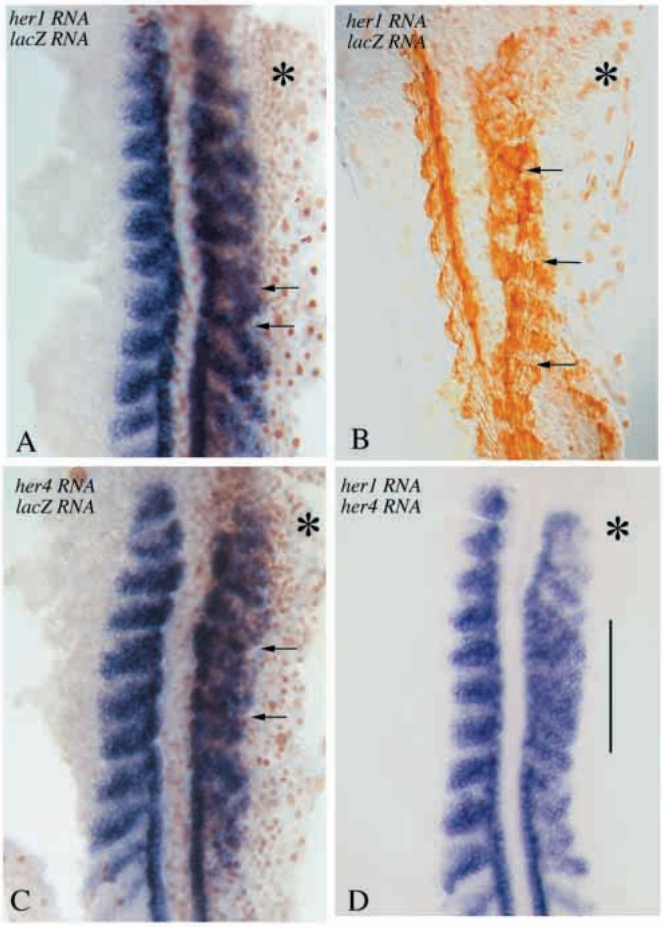

Fig. 3. Somitic defects following injection of *her1* (A,B), *her4* (C) or *her1* and *her4* (D) RNA. (A,C,D) In situ hybridizations with *MyoD*; (B) an anti-myosin staining. A-C have also been processed with an anti-β-galactosidase antibody. Asterisks label the affected side, arrows in A and C point to partially fused somites. The severity of defects in A-C is comparable to that in embryos injected with *delta* variants (see Fig. 1). Note that somite borders have formed and muscle fibres insert at the borders of neighbouring myotomes (arrows). However, somitic organization is less clearcut, in fact practically absent, in embryos injected simultaneously with *her1* and *her4* RNA (vertical bar in D).

A regulatory feedback loop in somitogenesis?

In *Drosophila*, lateral inhibition within a proneural cluster is regulated by a sensitive feedback mechanism (Haenlin et al., 1994; Kunisch et al., 1994; Oellers et al., 1994; Heitzler et al., 1996). There is evidence to support the existence of a similar regulatory feedback loop involved in primary neurogenesis in both *Xenopus* (Wettstein et al., 1997) and the zebrafish (Takke et al., 1999). To test whether such a feedback loop acts in somitogenesis as well, we injected *notch1a-intra* mRNA and probed for *deltaC* and *deltaD* transcription. There are three different *deltaC* transcription domains in the mesoderm (Haddon et al., 1998; Fig. 4A). First, there is a high density of transcripts within the tailbud; second, depending on the time point, one observes one, two or three pairs of stripe domains separated by areas that are devoid of transcripts, in the presomitic mesoderm; and, third, *deltaC* transcripts are found in the posterior half of each formed somite. All three types of

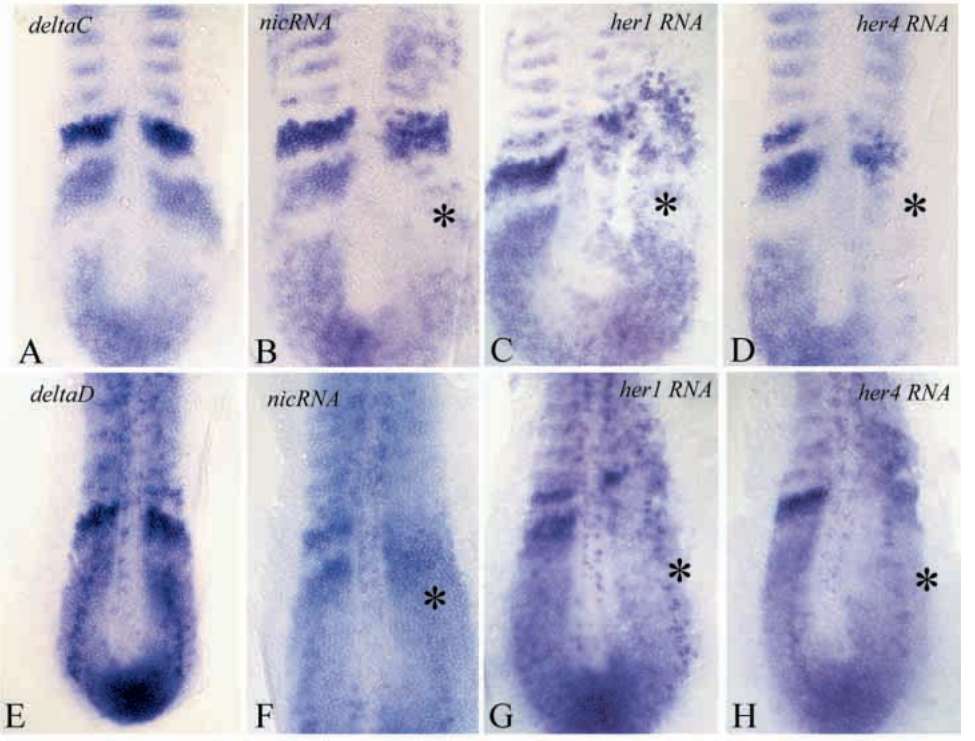

Fig. 4. Expression patterns of *deltaC* (A-D) and *deltaD* (E-H) following injection of *nic* (B,F), *her1* (C,G) or *her4* (D,H) RNA. (A,E) Normal embryos. Asterisks label the affected side. Notice considerable patterning defects and reduction of the amount of transcript on the injected side. Refer to text for further details.

expression domain are affected following misexpression of *notch1a-intra* (Fig. 4B; Table 2). We find that *deltaC* transcription is repressed within caudal regions of the presomitic mesoderm, whereas conspicuous patterning defects, rather than transcriptional repression, become evident towards cranial levels. The pattern of one to three presomitic stripes is perturbed: generally only one stripe is visible. Finally, *deltaC* transcripts are diffusely distributed in the somitic territory.

The pattern of *deltaD* transcription in the mesoderm of these embryos is similar to that of *deltaC* (Dornseifer et al., 1997; Haddon et al., 1998; Fig. 4E; Table 2). *deltaD* is normally transcribed in three different types of mesodermal domains: first, strongly within the tail bud; second, also very strongly in one or two pairs of stripes, depending on the time point considered, of presomitic mesodermal cells adjacent to the last formed somite, which probably correspond to a complete somitomere and part of the next one; and, third, at a lower level within the anterior half of each somite (Dornseifer et al., 1997). In embryos injected with *notch1a-intra* mRNA, transcription in the tailbud is not obviously affected, but the two presomitic pairs of stripes can no longer be distinguished; a more diffuse distribution of *deltaD* transcripts is observed instead. In addition, transcript density in the somitic territory is strongly reduced (Fig. 4F).

We tested whether this effect of *notch1a-intra* mRNA injection on *deltaC* and *deltaD* expression is mediated by *her1* and/or *her4* activation. Following injection of either *her1* or *her4* mRNA, a large proportion of injected embryos showed alterations in the *deltaC* and *deltaD* expression patterns that were similar to those observed after misexpression of *notch1a-intra* (Fig. 4C,D; Table 4). Obvious patterning defects were observed in the presomitic stripes, and transcript density within the somites was strongly reduced.

Mesodermal expression of *her1* and *her4* is independent of DELTAC and DELTAD signalling

Since both *deltaC* (Haddon et al., 1998) and *deltaD* (Dornseifer

Table 3. The effects of *deltaC*, *deltaD* and *deltaD*$^{(Pst)}$ mRNA injections on transcription of putative target genes

	mRNA injected						
	400 ng/μl *deltaC* 40 ng/μl *lacZ*		400 ng/μl *deltaD* 40 ng/μl *lacZ*		400 ng/μl *deltaD*$^{(Pst)}$ 40 ng/μl *lacZ*	800 ng/μl *deltaD*$^{(Pst)}$ 40 ng/μl *lacZ*	
	her1/MyoD	*her4/MyoD*	*her1/MyoD*	*her4/MyoD*	*her1/MyoD*	*her1/MyoD*	*her4/MyoD*
Number of embryos injected	49	53	29	44	74	84	51
Number of embryos with *MyoD* expression defects	27	25	19	25	10	33	23
Number of embryos with abnormal expression of target genes	0	0	3 (16%)*	0	2 (20%)*	5 (15%)*	2 (9%)*
Number of embryos with essentially wild-type pattern	27 (100%)*	25 (100%)*	16 (84%)*	25 (100%)*	8 (80%)*	28 (85%)*	21 (91%)*

*Percentages refer to embryos with defective *MyoD* expression.

Table 4. The effects of *her1* and *her4* mRNA injections on expression of *deltaC* and *deltaD*

	mRNA injected			
	600 ng/μl *her1* 40 ng/μl *lacZ*		600 ng/μl *her4* 40 ng/μl *lacZ*	
	deltaC	*deltaD*	*deltaC*	*deltaD*
Number of embryos injected	99	133	85	111
Number of embryos with β-gal expression	60	93	77	78
Number of embryos with reduced expression of marker genes	47 (78%)*	65 (70%)*	66 (86%)*	54 (69%)*
Number of embryos with wild-type pattern	13 (22%)*	28 (30%)*	11 (14%)*	24 (31%)*

*Percentages refer to embryos with β-gal expression.

et al., 1997) are transcribed in bilateral transverse stripes in the presomitic mesoderm (see above), either one or both of these genes might encode ligands for the NOTCH-dependent transcriptional activation of *her1* and *her4* (Fig. 2B,G). To test whether DELTAC and DELTAD act as ligands that allow NOTCH to activate the bHLH genes, we injected either *deltaC* or *deltaD* mRNA and scored for *her1* and *her4* expression. To assess patterning in presomitic and somitic territories, double staining using *MyoD* and *her1/her4* probes was carried out (Fig. 2C,D,H,I; Table 3). In all injection series, the mesodermal expression of *her1* and *her4* was essentially normal. In the case of *her1*, the pair-rule-like pattern was always distinguishable (Fig. 2C,D), contrary to the situation following *notch1a-intra* injections (Fig. 2B), although the alternating presomitic stripes were frequently irregularly shaped. This latter trait was apparently due to an additional effect on the size of the mesodermal and ectodermal germ layers, and is not directly related to the process of segmentation itself (Takke et al., 1999). Besides the effect on the size of the germ layers, the pattern of *her1* expression might be interpreted as manifestation of a developmental delay in the injected side, thus leading to differences between the injected and non-injected sides. The extent of the presomitic *her4* expression domain, i.e. the number of *her4*-expressing cells in the presomitic mesoderm, was as in the untreated embryos (Fig. 2H,I). These results suggest that neither DELTAC nor DELTAD functions as the ligand that causes NOTCH activation to stimulate *her1* and *her4* transcription.

In the dorsal margin of the *Drosophila* wing, DELTA appears to require *fringe* in order to activate NOTCH (Panin et al., 1997; Klein and Martinez-Arias, 1998). To exclude a possible requirement of *fringe* homologues for DELTA-mediated signalling in somitogenesis of the zebrafish, we injected *deltaD*$^{(Pst)}$ RNA and probed for *her1/4* expression. Since the *deltaD*$^{(Pst)}$ RNA encodes a truncated DELTA peptide that acts as dominant negative mutation (Takke et al., 1999), its misexpression might affect the function of the endogenous DELTA proteins irrespective of the presence or absence of putative co-factors. Two different concentrations were used (Table 3). None affected the *her1* stripe pattern nor the number of *her4*-expressing presomitic mesodermal cells (Fig. 2E,K).

It is important to emphasize that embryos with a pair-rule like distribution of *her1* transcripts (Fig. 2C-E) and essentially wild-type expression of *her4* (Fig. 2H-K) exhibited fusions and other irregularities in somite pattern. Furthermore, since *her1* and *her4* expression domains adjacent to the last-formed somite are normal, but the somites themselves are abnormal, as shown by the *MyoD* patterning defects, *deltaC/D* misexpression is likely to exert its effect after segmentation of the presomitic mesoderm.

groucho2 affects mesodermal cell differentiation

In *Drosophila*, members of the HAIRY-E(SPL) family of proteins form complexes with GROUCHO, which are functionally active in various ontogenetic processes (Paroush et al., 1994; Fisher et al., 1996). Batches of embryos were injected simultaneously with mRNA derived from the zebrafish homologue *groucho2* (Wülbeck and Campos-Ortega, 1997) and with *her1* mRNA, to test whether *her1*-dependent activity requires *groucho*. The percentage of injected embryos with *MyoD* expression defects was not significantly increased. However, the phenotype of such embryos differed from that observed following *her1* misexpression alone. In the embryos in which *her1* was coinjected with *groucho2* mRNA, two distinct phenotypic traits were observed, a disorganisation of the somitic borders and a strong repression of *MyoD* expression. The same reduction in *MyoD* expression was observed in embryos injected with *groucho2* alone (Fig. 5A,B; Table 1). On the injected side,

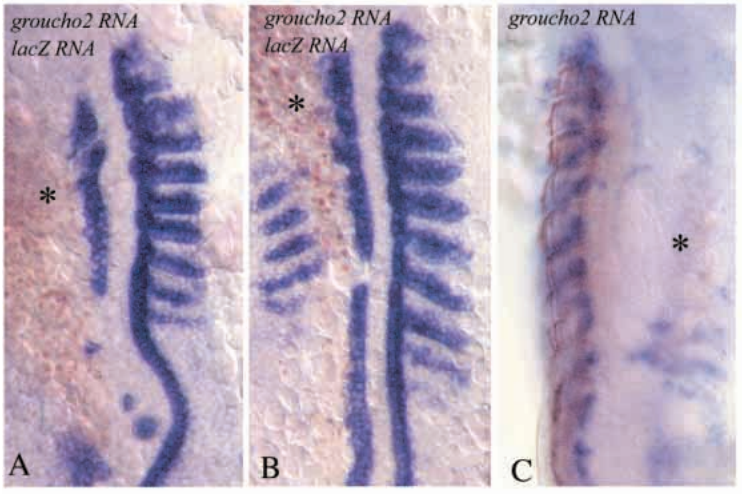

Fig. 5. Flat preparations of embryos injected with *groucho2* and *lacZ* mRNAs and stained for *MyoD* (blue, in situ hybridization) and β-galactosidase (brown, antibody staining) expression (A,B), and for *pax-9* (blue, in situ hybridization) and myosin heavy chain (brown, antibody staining) expression (C). Asterisks label the affected side. Mesodermal differentiation is strongly reduced.

mesodermal differentiation appeared to be completely blocked within the territory of β-galactosidase expression. Staining with anti-myosin antibody of embryos older than 24 h revealed that muscle development was inhibited (Fig. 5C); the same was observed after staining with antibody F59, which recognises the myosin heavy chain (Miller et al., 1989) in the adaxial mesodermal cells (Devoto et al., 1996). *pax-9* expression was essentially abolished (Fig. 5C; Nornes et al., 1996).

DISCUSSION

Genes of the NOTCH signalling pathway control somitogenesis at two different stages in the zebrafish

Preliminary results regarding the effect of misexpressing wild-type *deltaD* had suggested a function for this gene in somite development (Dornseifer et al., 1997). We have shown above that misexpression of a dominant negative variant of *deltaD*, wild-type *deltaC*, or an activated form of NOTCH1a, or misexpression of the *hairy-E(spl)* homologue *her1* or *her4*, leads in all cases to considerable disruption of somitogenesis. However, whereas the mesodermal effects of perturbing *deltaC* and *deltaD* activity are similar, those observed following either NOTCH activation or coinjection of *her1* and *her4* appear to have a different basis. In the former case, patterning defects are evident, but the presomitic mesoderm seems to be subdivided into somitomeres, as incomplete somite borders are visible; in the latter case, somites apparently do not form, as no somite borders at all can be seen. Since both *her1* and *her4* are ectopically activated by *notch1a-intra* within the presomitic mesoderm and misexpression of both *her* genes causes defects similar to those seen with *notch1a-intra* mRNA, we propose that both *her* genes are targets of NOTCH during somitogenesis. It follows then that, during normal development, NOTCH-mediated activation of *her* genes may be causally related to the initial subdivision of the paraxial mesoderm into somitomeres.

In contrast, in the case of *deltaC* or *deltaD* misexpression the defects seem to be independent of the activity of the two *her* genes as it fails to perturb the transcription pattern of *her1* and *her4*. The same applies to misexpression of a truncated variant of DELTAD. This result is surprising and leads to two important corollaries. First, it suggests that neither DELTAC nor DELTAD acts as a ligand to trigger NOTCH-dependent activation of *her* genes. Second, it suggests that the DELTAC/D-dependent somitic defects do not depend directly on the activity of *her* genes. Accordingly, the *delta* function in somitogenesis appears to operate downstream of the component of NOTCH function that we have been able to assay, namely *her* gene activity. Double in situ hybridizations with *her1* or *her4* and *MyoD* probes following misexpression of *delta* variants suggest that the latter act within the somites once the presomitic mesoderm has been subdivided into somitomeres. By analogy to the situation in *Drosophila*, where the NOTCH regulatory network is required for maintenance of the epithelial state in several different instances (Hartenstein et al., 1992), as well as for the formation of borders in the wing disc (Couso et al., 1995; de Celis et al., 1996), we would like to propose that DELTAC and DELTAD act during the definition and/or maintenance of somitic borders in zebrafish embryos.

At least three important questions remain open in this scenario. First, whereas the proposed function of DELTA in controlling boundary development may rely on a mechanism similar to that operating in the wing margin of *Drosophila*, the mechanism by which NOTCH contributes to subdivide the presomitic mesoderm is unclear. Second, the ligand that activates the NOTCH1a receptor, and, consequently, the *her* genes, during the subdivision of the presomitic mesoderm is unknown. Although there are no less than four *delta* genes in the zebrafish, only *deltaC* and *deltaD* are expressed in the mesoderm (Haddon et al., 1998; Dornseifer et al., 1997; Appel and Eisen, 1998) and apparently neither one is capable of activating *her* genes under our experimental conditions. Therefore, there is no obvious candidate for this function. And, third, the receptor required for the DELTAC/D-mediated function during later stages of somite development is also not known. It is certainly not clear to what extent the defects associated with overexpression of *delta* genes are independent of NOTCH1a: indeed, our data do not preclude a late function for NOTCH1a in which DELTA proteins would act as ligands. Moreover, Westin and Lardelli (1997) have cloned three new zebrafish *notch* genes, i.e. *notch1b*, *notch5* and *notch6*. All four known zebrafish *notch* genes are expressed in the developing somites and all but *notch6* are expressed in the presomitic mesoderm (Bierkamp and Campos-Ortega, 1993; Westin and Lardelli, 1997). Therefore, any one of these, including *notch1a*, could encode the receptor for DELTAC/D-mediated functions in somitogenesis. However, we cannot assess such a putative late function of NOTCH since no candidate for an immediate target gene is known.

A linear regulatory pathway in somitogenesis?

In neurogenesis, both in *Drosophila* and the zebrafish, NOTCH-dependent signalling is organised as a closed network. In this network, a finely tuned feedback loop determines the intensity of NOTCH activation by regulating the amounts of proneural proteins and DELTA in the signalling cells (Kunisch et al., 1994; Heitzler et al., 1996; Takke et al., 1999). In zebrafish somitogenesis, the present evidence suggests that a simple linear relay, rather than a network, mediates NOTCH signalling. On the one hand, misexpression of *notch1a-intra* activates transcription of *her* genes; on the other hand, misexpression of *notch1a-intra*, or, equivalently, *her* genes, has an obvious effect on the transcription pattern of both *deltaC* and *deltaD*. However, there is no evidence that zebrafish *delta* genes are involved in modifying the activity of the *her* genes, i.e. the only targets of NOTCH activation known to us, the *her* genes, appear to be independent of DELTA. The present evidence is thus consistent with a linear epistatic chain, rather than a feedback loop: NOTCH activity is required for *her* gene transcription, which in turn represses *deltaC/D* transcription.

However, we would like to emphasize that our inability to demonstrate such a regulatory feedback loop does not necessarily mean that it does not exist. While the existence of a feedback loop cannot be postulated based on our present data, additional experiments may yet uncover evidence for such a mechanism.

Muscle cell differentiation is independent of NOTCH activity

Involvement of *Notch* genes in muscle differentiation has been postulated in a number of studies. Most of these studies,

however, have been carried out in vitro, using various mammalian cell lines (Nye et al., 1994; Kopan et al., 1994; Shawber et al., 1996). Thus, a constitutively active mouse NOTCH variant (mNotch1IC) blocks muscle cell differentiation in both 3T3 and P19 cells, as judged by the lack of *MyoD* expression. The only in vivo study in which a suppression of muscle development by NOTCH has been described was carried out under heterologous conditions (Kopan et al., 1994). Injection of *mNotchIC* mRNA into *Xenopus* was found to block *MyoD* expression. However, no such effect was obtained by injecting *Xenopus Notch* (*Xotch*) or *XDelta-2* mRNA into *Xenopus* (Coffman et al., 1990, 1993; Jen et al., 1997), nor were muscle differentiation defects observed in $Notch^{\Delta 1}$ mutants (Conlon et al., 1995). Misexpression of *XotchΔE* led in fact to the opposite effect. Suppression of myogenesis by mouse *Notch* seems to be independent of CBF1, insofar as overexpression of *hes1* does not have obvious effects on myogenesis (Shawber et al., 1996). The effect of *mNotchIC* can be explained by postulating the existence of an inhibitory cofactor that prevents activation of *MyoD* (Kopan et al., 1994). The lack of effect on myogenesis of the overexpression of *Notch* genes in both *Xenopus* and zebrafish (this study) points to differences in regulation of muscle differentiation between lower vertebrates and mammals.

However, misexpression of GROUCHO2 leads to strong downregulation of *MyoD* expression in the paraxial and adaxial mesoderm in zebrafish and to repression of muscle differentiation in later stages. In fact, these embryos show a virtually complete abolition of mesodermal differentiation, as revealed by all markers that we have tested. Since *groucho2* is expressed in the presomitic mesoderm, as well as in other regions of the body, but not in the differentiating somites (Wülbeck and Campos-Ortega, 1997), during normal development, it is likely to act in the process of segmentation itself, i.e. prior to the appearance of somites.

In *Drosophila*, there is evidence that GROUCHO requires the bHLH proteins of the E(SPL) complex for its function (Schrons et al., 1992), which form heterodimers (Paroush et al., 1994; Fisher et al., 1996) that act as transcriptional repressors (Oellers et al., 1994; Heitzler et al., 1996; Nakao and Campos-Ortega, 1996; Giebel and Campos-Ortega, 1997). Since the misexpression of *her* genes does not affect myogenesis, our present results do not provide any clues as to the mechanism by which *groucho2* acts in mesodermal differentiation. GROUCHO2 might of course act either on its own or in association with co-factors other than the products of the *her* genes involved in the regulation of mesodermal gene expression. However, the identity of these factors remains unknown.

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