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Delta 1-activated Notch inhibits muscle differentiation without affecting *Myf5* and *Pax3* expression in chick limb myogenesis

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SUMMARY

The myogenic basic helix-loop-helix (bHLH) transcription factors, Myf5, MyoD, myogenin and MRF4, are unique in their ability to direct a program of specific gene transcription leading to skeletal muscle phenotype. The observation that Myf5 and MyoD can force myogenic conversion in non-muscle cells in vitro does not imply that they are equivalent. In this paper, we show that *Myf5* transcripts are detected before those of *MyoD* during chick limb development. The *Myf5* expression domain resembles that of *Pax3* and is larger than that of *MyoD*. Moreover, *Myf5* and *Pax3* expression is correlated with myoblast proliferation, while *MyoD* is detected in post-mitotic myoblasts. These data indicate that *Myf5* and *MyoD* are involved in different steps during chick limb bud myogenesis, *Myf5* acting upstream of *MyoD*. The progression of myoblasts through the differentiation steps must be carefully controlled to ensure myogenesis at the right place and time during wing development. Because Notch signalling is known to prevent differentiation in different systems and species, we sought to determine whether these molecules regulate the steps occurring during chick limb myogenesis. *Notch1* transcripts are associated with immature myoblasts, while cells expressing the ligands *Delta1* and *Serrate2* are more advanced in myogenesis. Misexpression of *Delta1* using a replication-competent retrovirus activates the Notch pathway. After activation of this pathway, myoblasts still express *Myf5* and *Pax3* but have downregulated *MyoD*, resulting in inhibition of terminal muscle differentiation. We conclude that activation of Notch signalling during chick limb myogenesis prevents *Myf5*-expressing myoblasts from progressing to the *MyoD*-expressing stage.

Key words: Myf5, MyoD, Notch, Delta, Chick, Limb bud, Myogenesis

INTRODUCTION

In vertebrates, all the myogenic cells that form the striated skeletal muscles of the limb and trunk originate from the somites. The medial halves of the somites give rise to back and intercostal muscles or the epaxial musculature (Ordahl and Le Douarin, 1992). Cells derived from lateral dermomyotomes migrate lateroventrally to produce the muscles of the body wall and the limbs, forming the hypaxial musculature (Ordahl and Le Douarin, 1992; Christ and Ordahl, 1995). Formation of limb skeletal muscle during vertebrate embryogenesis involves cellular commitment, migration, proliferation, growth arrest and differentiation. Each step involves the expression and activity of a specific panel of factors (Olson, 1992). The myogenic bHLH transcription factors, Myf5, MyoD (also known as Myod1), Mrf4 (also known as Myf6) and myogenin, which are also called myogenic regulatory factors (MRFs), have been shown to initiate the skeletal muscle differentiation program. Ectopic expression of these MRFs is able to convert several non-muscle cell types into skeletal muscle in tissue culture (Weintraub et al., 1991), in transgenic mice (Miner et al., 1992; Santerre et al., 1993) and in *Xenopus* (Ludolph et al., 1994). Conversely, knockout of these genes leads to various muscle defects (Rudnicki et al., 1992, 1993; Braun et al., 1992a,b; Hasty et al., 1993; Nabeshima et al., 1993). Moreover, cells deprived of *Myf5* or *MyoD* assume a non-muscle fate (Tajbakhsh et al., 1996; Kablar et al., 1999). These properties have led to the notion that MRFs trigger the successive events leading to skeletal muscle formation. Gene targeting has clearly defined a hierarchy among the MRF family members. Mice lacking *Myf5* and *MyoD* (Rudnicki et al., 1993) do not form myoblasts or skeletal muscle. In contrast, in myogenin-null mice, myoblasts do form, as assayed by *Myf5* and *MyoD* expression, but do not differentiate into muscle fibres (Hasty et al., 1993; Nabeshima et al., 1993). *Myf5* and *MyoD* therefore appear to lie in a genetic pathway upstream of myogenin, the latter

having a role in activating muscle cell terminal differentiation (Hasty et al., 1993; Nabeshima et al., 1993). In mice, there are numerous and consistent studies concerning the sequential expression of myogenic factors during somite and limb bud development, *Myf5* being detected before *MyoD* in somites and limbs (Ontell et al., 1995; Tajbakhsh and Buckingham, 1999). In birds, studies of the timing of the expression of these factors in axial regions have led to the conclusion that, in contrast to the situation in mice, *MyoD* expression occurs before that of *Myf5* by a few hours (Pownwall and Emerson, 1992; Boricky et al., 1997; Denetclaw and Ordahl, 2000). However, Hacker and Guthrie (1998) found that *Myf5* transcripts were expressed first, followed by those of *MyoD*. In the limb, studies using in situ hybridisation of tissue sections (Williams and Ordahl, 1994), RT-PCR (Lin-Jones and Hauschka, 1996) and whole-mount in situ hybridisation (Hacker and Guthrie, 1998) have led to conflicting results concerning the timing of appearance of *MyoD* and *Myf5* transcripts.

The progression through discrete developmental steps has been studied in muscle cell lines. The presence of Myf5 and MyoD is not itself sufficient to trigger differentiation in cell culture, since myoblasts exposed to growth factors continue to proliferate and to express *Myf5* and/or *MyoD* (Yutzey et al., 1990). One well-described general mechanism influencing differentiation events during development is the Notch signalling pathway (reviewed in Artavanis-Tsakonas et al., 1999). The Notch pathway has been shown to operate at different steps during *Drosophila* myogenesis (Baylies et al., 1998). However, although mice bearing null mutations in the different Notch signalling components exhibit defects of somite formation, they do not display any muscle defects (Swiatek et al., 1994; Conlon et al., 1995; de Angelis et al., 1997). The lack of effect probably reflects the functional overlap among the Notch family members. In mammals, the only direct evidence of Notch involvement in myogenesis comes from in vitro studies where activated Notch or ligand-induced Notch signalling suppresses muscle differentiation in various mouse cell lines (Kopan et al., 1994; Nye et al., 1994; Lindsell et al., 1995; Shawber et al., 1996; Jarriault et al., 1998; Nofziger et al., 1999; Kuroda et al., 1999). No such evidence has been obtained in vivo.

Knockouts and studies on cell lines have proved very powerful in determining the genetic hierarchy of MRFs and giving clues about their functions. However, the exact functions of the proteins coded by *Myf5* and *MyoD* during development are still not fully understood. Based on in vitro studies, *Myf5* and *MyoD* are widely considered to have overlapping function associated with myoblast proliferation (Lassar et al., 1994; Molkentin and Olson, 1996). In order to gain insight into the respective roles of Myf5 and MyoD in avian limb myogenesis, we have characterised the cellular expression patterns of these genes. We found that in the chick limb, *Myf5* can be detected at stage 20 with an expression domain similar to that of *Pax3*, *MyoD* being detected a few hours later (stage 22) in a more restricted domain. Activation of the Notch pathway in vivo led to a downregulation of *MyoD* expression, without affecting *Pax3* and *Myf5* expression, followed by an inhibition of terminal differentiation. Together, these results suggest that *Myf5* acts upstream of *MyoD*, and that the Notch pathway is involved in the progression from the *Myf5*-expressing stage to the *MyoD*-expressing stage, during chick limb bud myogenesis.

MATERIALS AND METHODS

Chick embryos

Fertilised White Leghorn eggs (HAAS, Strasbourg, France) were incubated at 37°C. All grafting experiments were performed in ovo. Young embryos were staged according to Hamburger and Hamilton (HH) (1951), while old embryos were staged according to embryonic days in ovo. To facilitate comparisons, we report both staging for young embryos.

Production of control/RCAS- or Delta-expressing cells

Infectious Delta/RCAS (Henrique et al., 1997) and control/RCAS viruses were produced in Chick Embryo Fibroblasts (CEF) as described by Duprez et al. (1998). Briefly, CEF were isolated from E10 O-line embryos (BBSRC, Institute for Animal Health, Compton, Berkshire, UK) and grown in DMEM (Gibco, BRL) containing 8% (v/v) fetal calf serum and 2% (v/v) chick serum supplemented with antibiotics. CEF were transfected transiently with retroviral recombinant DNA using Transfectam (Gibco, BRL) according to the manufacturer's instructions.

Grafting of retrovirus-infected cells

Retrovirus-expressing cells were prepared for grafting as described by Duprez et al. (1998). Pellets of approximately 50 to 100 μm in diameter were grafted into the limb field of White Leghorn embryos around stage HH 16 of development (E2.5). Embryos were harvested at different times after grafting and processed for in situ hybridisation of whole mounts or tissue sections. Embryos grafted with control/RCAS-expressing cells did not exhibit any change in morphology (see also Duprez et al., 1996) or gene expression (data not shown). The numbers of embryos processed for in situ hybridisation of whole mounts are given in the text or in Table 1. In each experiment, two to six specimens were used for in situ hybridisation of tissue sections.

Bromodeoxyuridine (BrdU) labelling in ovo

E3 embryos were injected in the amnios (near the heart and wing) with 200μl of 10mM BrdU (Amersham, Life Science), and were re-incubated for another 15 minutes. 1 μl of 10 mM BrdU was directly injected in the circulation of E7 embryos and fixed 1 hour after. The embryos were then fixed and processed for in situ hybridisation of sections.

In situ hybridisation of whole mounts and tissue sections

Embryos were fixed in 4% (v/v) formaldehyde and processed as previously described for in situ hybridisation of whole mounts and paraffin sections (Duprez et al., 1998, 1999). Antisense digoxigenin- and fluorescein-labelled RNA probes were prepared as follows: *Myf5* (Saitoh et al., 1993); *Pax3* and *MyoD* (Duprez et al., 1998), *Delta1* (Henrique et al., 1997), *Serrate2* and *Notch1* (kind gift from Dominguos Henrique). For double in situ hybridisation, the fluorescein probe was revealed with NBT/BCIP reagents (Roche) first, then the digoxigenin probe with INT/BCIP (Roche).

Immunohistochemistry

Differentiated muscle cells were detected on sections and in cultures using a monoclonal antibody against sarcomeric myosin heavy chain, MF20 (Developmental Hybridoma Bank, University of Iowa, Iowa City). Proliferating cells were detected using a monoclonal antibody against BrdU (Amersham). In situ followed by immunohistochemistry was performed using successively the probes (*Notch1*, *Serrate2*, *Delta1*, *Pax3*, *MyoD and Myf5*) and the monoclonal antibody against BrdU or MF20 antibody.

RESULTS

Myf5 transcripts can be detected before those of *MyoD* during limb development

In order to clear up the controversy concerning the timing of appearance of *Myf5* and *MyoD*, we performed in situ hybridisation on serial transverse limb sections (Fig. 1). *Myf5* and *MyoD* transcripts were not detected during the migration of the muscle progenitors from the somites to the limb bud (data not shown). This is in agreement with chick (Williams and Ordahl, 1994; Lin-Jones and Hauschka, 1996) and mouse data (Tajbakhsh and Buckingham, 1994; Ontell et al., 1995). In contrast, as soon as the *Pax3*-expressing myoblasts had reached their destination (Fig. 1A), defined as stage 20 (Chevalier et al., 1977; Christ et al., 1977), we were able to detect *Myf5* transcripts (Fig. 1B), but no *MyoD* mRNA was observed (Fig. 1C). At that stage the ventral and dorsal muscle masses had not yet separated, as visualised by *Pax3* expression (Fig. 1A). Using this in situ hybridisation technique, *MyoD* expression was first detected unambiguously at stage 22/23 (see Fig. 2F and Duprez et al., 1998).

Myf5 and *MyoD* show different expression domains during limb development

Whole-mount in situ hybridisation at stage 22/23 (E4) showed the *Myf5* expression domain was larger than that of *MyoD* and closely resembled that of *Pax3* (Fig. 2A-C). The *Myf5* expression domain matched that of *Pax3* throughout development (Fig. 1, Fig. 2 and data not shown). *MyoD* transcripts (Fig. 2F,G) were located in a subregion of the ventral and dorsal muscle masses expressing *Myf5* mRNA (Fig. 2D,E). The *Myf5* expression domain extended to near the ectoderm, while *MyoD* mRNA was located more centrally within the limb. In order to understand whether *Myf5* and *MyoD* transcripts were located in the same cells where their expression domains overlapped, we performed double in situ hybridisation at stage 23. These experiments on transverse wing sections confirmed that the expression domain of *MyoD* mRNA (orange) was more restricted than and contained within that of *Myf5* (purple) (Fig. 2H,I). All three possible expression combinations were observed (Fig. 2I): (1) $Myf5^{+}/MyoD^{-}$ cells (black arrows) were preferentially located near the ectoderm, (2) $Myf5^{-}/MyoD^{+}$ cells were preferentially found near the centre of the limb (black arrowheads) and (3) cells expressing both genes were found at the interface between these regions (white arrowhead). After stage 23, the *MyoD* expression domain spread to include all muscle masses, while *Myf5* and *Pax3* transcripts were progressively downregulated (see Figs 6, 8 and Duprez et al., 1998).

Myf5 expression is associated with myoblast proliferation whereas *MyoD* transcripts are detected in postmitotic myoblasts

The *Myf5* mRNA expression domain appeared identical to that of *Pax3* (Fig. 1A,B; Fig. 2A,B). The similarity in the expression patterns of *Pax3* and *Myf5* transcripts suggested that their expression might be linked to the proliferative state of the cell. Since *Pax3* expression has already been linked with proliferation (Epstein et al., 1995; Amthor et al., 1998), we studied the proliferative/differentiation state of *Myf5*-positive cells by performing BrdU incorporation experiments at stage 23 (see Materials and Methods). These experiments showed that some *Myf5*-expressing cells had indeed incorporated BrdU, similar to *Pax3*-expressing cells (Fig. 3A-D, arrows). In contrast, most of the cells expressing *MyoD* transcripts did not incorporate BrdU (Fig. 3E,F, arrowheads).

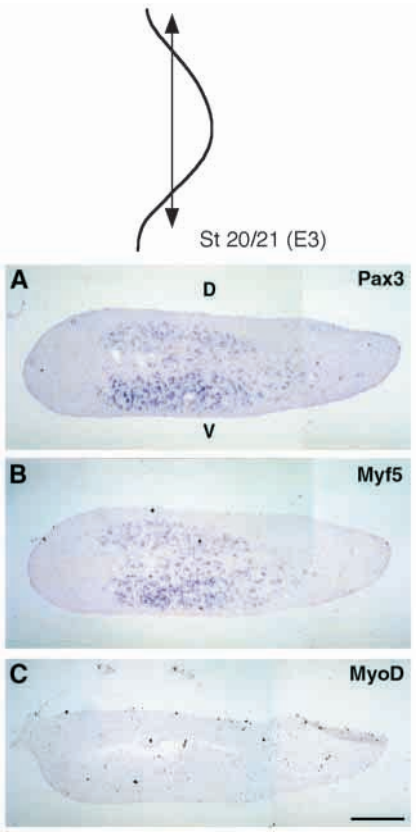

Fig. 1. *Myf5* transcripts are detected before those of *MyoD* in chick limb bud. Adjacent transverse wing sections from stage 20/21 (E3) embryos were hybridised with digoxigenin-labelled antisense probes for *Pax3* (A), *Myf5* (B) and *MyoD* (C). Scale bar, 160 μm. D, dorsal; V, ventral.

Location of Delta/Notch pathway components during chick limb myogenesis

One known mechanism involved in differentiation processes in many systems and species is the Notch pathway (Artavanis-Tsakonas et al., 1999). We set out to investigate whether this signalling pathway is involved in myogenesis. We first analysed the endogenous cellular expression pattern of the Notch pathway components. From the literature it appeared that the Notch receptor might be ubiquitously expressed, the specificity of its action being determined by its ligands (Artavanis-Tsakonas et al., 1999). Indeed, whole-mount in situ hybridisation showed that the ligands *Delta1* (Fig. 4A) and *Serrate2* (Fig. 4B) were located in the muscle areas of the chick limbs at E5, while *Notch1* transcripts were more uniformly distributed (Fig. 4C; Vargesson et al., 1998; Beckers et al., 1999). BrdU incorporation experiments indicated that high levels of the ligands *Delta1* (Fig. 4G, arrows) and *Serrate2* (Fig. 4D,E, arrows) were expressed in scattered cells that did not incorporate BrdU within the muscle masses. At E7, the ligand *Serrate2* was only detected in MF20-positive cells (Fig.

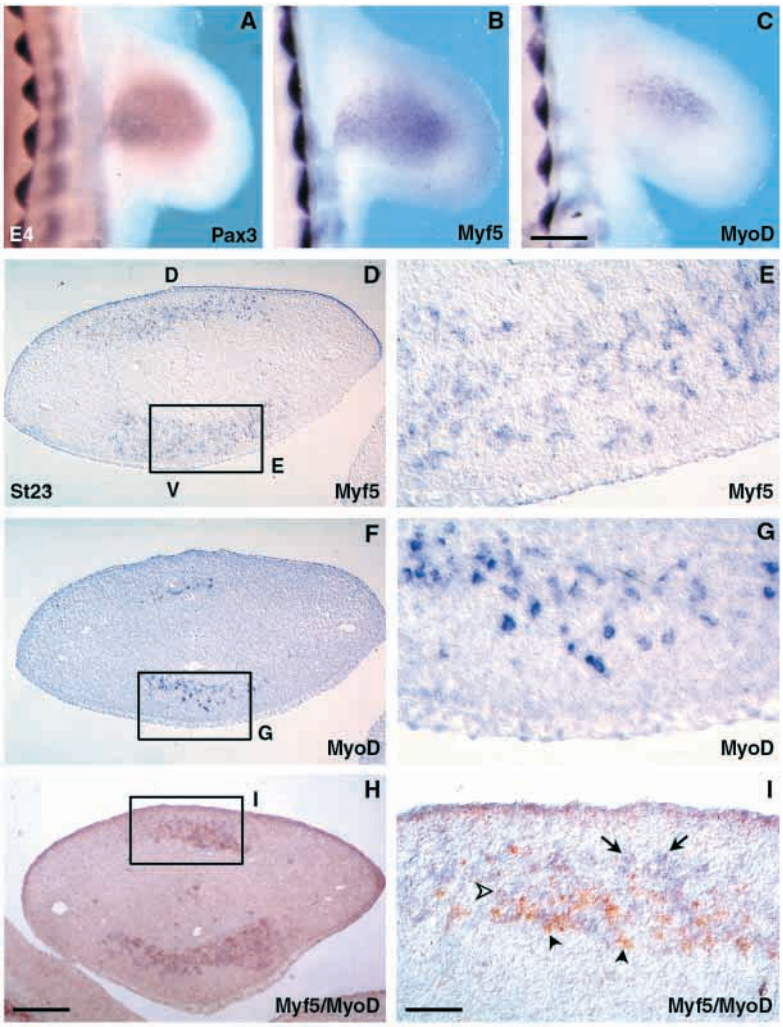

Fig. 2. *Myf5* expression domain differs from that of *MyoD*. Distribution of *Pax3* (A), *Myf5* (B) and *MyoD* (C) transcripts in whole-mount preparations from stage 22/23 embryos (E4). A-C are dorsal views focused on the wings. Consecutive transverse sections through the forelimb from a stage 23 embryo were hybridised with digoxigenin-labelled antisense probes for *Myf5* (D,E) and *MyoD* (F,G). On transverse sections through the forelimb from a stage 23 embryo, double in situ hybridisation was performed using the *Myf5* digoxigenin-labelled-probe (purple) and the *MyoD* fluo-labelled probe (orange) (H,I). Black arrows indicate the *Myf5*-positive cells. Arrowheads point to *MyoD*-positive cells. White arrowheads mark the double *Myf5*- and *MyoD*-positive cells. E,G,I show higher magnification of D,F,H, respectively. Scale bars: 1.2mm in A-C; 180 μm in D,F,H; 45μm in E,G,I. D, dorsal; V, ventral.

4H). *Delta1* was weakly detected at that stage (data not shown). The expression domain of the receptor *Notch1* was larger than that of the ligand *Serrate2* (compare the adjacent sections of Fig. 4E and 4F), suggesting that *Notch1* was also expressed in non-myogenic cells. We could detect *Notch1* mRNA in BrdU-positive cells (Fig. 4F, arrowheads), although most of the *Notch1*-positive cells were BrdU negative (Fig. 4F). In addition, *Notch1* transcripts were clearly detected in mononucleated cells around the muscle fibres (Fig. 4I). These results indicate that the receptor *Notch1* is expressed in immature myoblasts, while the cells expressing the ligands *Delta1*/*Serrate2* are more advanced in myogenesis (postmitotic myoblasts and muscle fibres).

Overexpression of *Delta1* affects *MyoD* expression without affecting *Myf5* expression

In order to understand the role of Notch signalling during the different steps of myogenesis, we activated the Notch pathway by over-expressing *Delta1* using the RCAS retrovirus. The Delta/RCAS construct has been shown to be effective in retinal (Henrique et al., 1997), cartilage (Crowe et al., 1999), feather bud (Crowe et al., 1998; Viallet et al., 1998) and scale (Crowe and Niswander, 1998) formation. Based on described functions of Notch signalling in different systems and species, we hypothesised that constitutive activation of Notch signalling in muscle cells would lead to an inhibition of terminal muscle differentiation. Aggregates of Delta/RCAS-transfected cells (see methods) were grafted to stage 16 (E2.5) wing buds. In situ hybridisation of *Delta1* transcripts in whole mounts showed the degree of virus spread 48 hours (Fig. 5A,B; n=5 out of 6) and 72 hours (Fig. 5C,D; n=2 out of 2) after grafting. In order to visualise the activation of Notch signalling, we looked for *Notch1* expression after grafting, since it has been shown that activation of Notch signalling enhances *Notch* expression (Lewis, 1996). Overexpression of *Delta1* in the limb bud led to an extension of the *Notch1* expression domain (Fig. 5I,J; n=4 out of 5), reflecting an activation of Notch signalling (Micchelli et al., 1997; Franklin et al., 1999). In such grafted embryos, the *MyoD* expression domain appeared reduced in the region where ectopic *Delta1* was detected (Fig.

Table 1. Muscle gene expression in whole-mount embryos following grafts of Delta1/RCAS transfected cells to the wing region at E2.5

	Gene transcripts		
Time after grafting	Pax3	Myf5	MyoD
24 hours	No change (2/2)	No change (5/6)*	No change (4/6)‡
48 hours	No change (7/8)§	No change (3/3)	Decrease (4/5)¶
72 hours	n.d.	No change (3/3)	Decrease (5/7)¶

Numbers in brackets indicate the number of cases giving the results out of the number of embryos examined.
*In one case *Myf5* expression appears downregulated.
‡*MyoD* expression was not detected in the control limb in four cases.
§In one case *Pax3* expression was upregulated.
¶The other cases were unchanged. n.d., not determined.

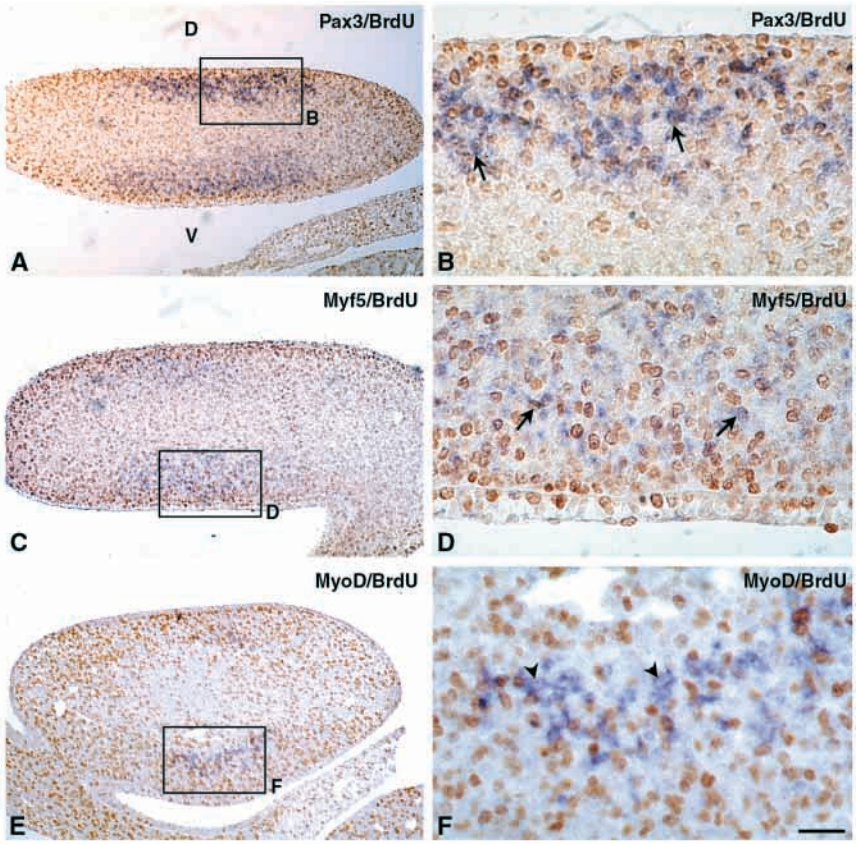

Fig. 3. *Myf5* and *Pax3* transcripts are associated with proliferative myoblasts while *MyoD* is detected in postmitotic cells. Transverse wing sections from stage 23 embryos incubated with BrdU 15 minutes before fixation were hybridised with *Pax3* (A,B), *Myf5* (C,D) and *MyoD* (E,F) probes and then incubated with the anti-BrdU antibody. B,D,F show higher magnification of the dorsal (B) and ventral (D,F) muscle masses of the sections shown in A,C,E, respectively. Arrows indicate the *Myf5*- (B) and the *Pax3*- (D) positive cells that are also BrdU positive. Arrowheads point to the *MyoD*-positive cells, which are BrdU negative. Scale bar: 160 μm in A,C,E; 40 μm in B,D,F. D, dorsal; V, ventral.

5B,D), compared with the control wing, after 48 hours (Fig. 5E,F; Table 1) and 72 hours (Fig. 5G,H; Table 1). In contrast, the *Myf5* (Fig. 5K,L; Table 1) and *Pax3* (Table 1) expression domains were unchanged compared to the control wing. This demonstrates that the Notch pathway operates between the *Myf5*/*Pax3*-expressing and the *MyoD*-expressing stages.

Delta-activated Notch inhibits terminal muscle differentiation, despite the presence of *Myf5* and *Pax3* transcripts

Seventy-two hours after the Delta/RCAS-expressing cells were grafted into the wing bud, the control and operated wings were cut transversely through the forelimb region and hybridised with the *Delta1* probe, revealing the extent of the spread of the virus (Fig. 6A,B,I,J). Adjacent sections hybridised with *MyoD* (Fig. 6C,D,K,L), *Myf5* (Fig. 6E,F,M,N) and *Pax3* (Fig. 6G,H,O,P) probes showed the normal and modified muscle pattern in the control and operated wings, respectively. The Delta/RCAS-infected right wing exhibited a downregulation of *MyoD* transcripts (compare Fig. 6C,D with 6K,L), while *Myf5* and *Pax3* expression appeared unaffected (compare Fig. 6E-H with 6M-P). Analysis of myosin expression using the MF20 antibody showed that there was a clear diminution in the number of differentiated muscle cells in the treated wing (Fig. 6O,P), compared with the control limb (Fig. 6G,H, in brown). This demonstrated that terminal differentiation is affected despite the presence of *Myf5* and *Pax3* transcripts.

Delta-activated Notch leads to disorganised muscles

We then examined the phenotype of the Delta/RCAS-infected limbs at E9.5 (7 days after grafting; *n*=4). Transverse sections were cut at the same level along the proximo-distal axis from the control (Fig. 7A,B) and manipulated (Fig. 7C,D) wings. In the limb shown in Fig. 7, only the posterior muscles were affected (Fig. 7D), although all muscles could be affected (data not shown). Examination of *MyoD* and myosin expression showed that the posterior muscles were reduced in size and disorganised (Fig. 7D), compared with the normal pattern (Fig. 7B). Moreover, the FDP (flexor digitorum profondus) muscle was absent in the *Delta1*-infected wing (Fig. 7D) but was clearly present in the control wing (Fig. 7B). Only certain muscles were affected (Fig. 7D) in this way by Delta1/RCAS infection despite the broad distribution of ectopic *Delta1* transcripts (Fig. 7C). We interpreted this result as Delta-activated Notch acting only within a specific time window between the *Myf5* and *MyoD* expression steps (see above). This time window is probably brief, so ectopic *Delta1* would have to infect myoblasts at a very specific time in order to affect their further differentiation. High magnifications of the *Delta1*-infected muscle FCU (flexor carpi unlaris) and control FCU (Fig. 8) confirmed the absence of myosin in the *MyoD*-negative region (Fig. 8C,D) of the infected FCU, while myosin expression could be detected in the *MyoD*-positive region (Fig. 8D; arrow). In the control muscles, we could detect the *MyoD* mRNAs (purple) and myosin (brown) in most of the cells (Fig. 8A,B).

DISCUSSION

Distinct expression patterns of *Myf5* and *MyoD* in the chick wing indicate different roles during myogenesis, *Myf5* acting upstream of *MyoD*

In situ studies of *Myf5* and *MyoD* expression during development in the chick have been apparently contradictory (see Introduction). We have shown that *Myf5* transcripts can be unambiguously detected before those of *MyoD* in the chick limb bud. A similar situation occurs in the chick somite (Hirsinger et al., 2001). These results are consistent with those obtained in mice. The expression domains of *Myf5* and *Pax3* essentially overlap during development. In contrast, *MyoD* mRNAs initially show

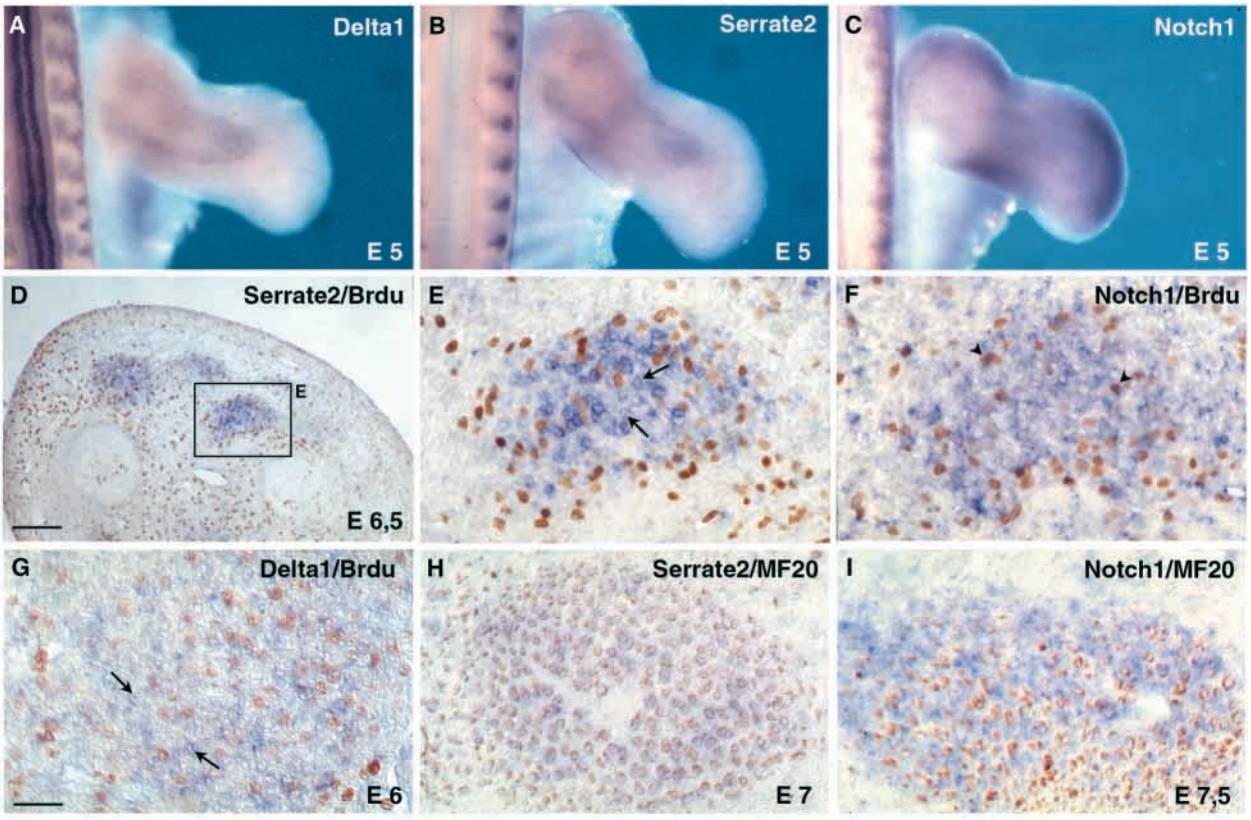

Fig. 4. Endogenous expression of Notch signalling components in the chick limb bud. Distribution of *Delta1* (A), *Serrate2* (B) and *Notch1* (C) transcripts in whole-mount preparations from stage 26/27 embryos (E5). (A,B,C) Dorsal views focused on the wings. Consecutive transverse wing sections from stage 29 embryos (E6.5) injected with BrdU 1 hour before fixation were hybridised with *Serrate2* (D,E) and *Notch1* (F) probes (blue), and then incubated with the anti-BrdU antibody (brown). (G) Posterior part of the ventral muscle mass indicates that the *Delta*-positive cells are BrdU-negative (arrows). Transverse wing sections from stage 31/32 (E7/E7.5) embryos were hybridised with *Serrate2* (H) and *Notch1* (I) probes (blue) and then incubated with the MF20 antibody (brown). Scale bars: 160 μm in D; 40 μm in E-I.

a more restricted pattern, in the centre of the limb. During subsequent limb development, the *MyoD* expression domain spreads to include all muscle masses, while *Pax3* and *Myf5* are downregulated, although a low level of *Pax3* transcripts can still be detected quite late (E10) (Duprez et al., 1998). Despite the absence of clear segregation between proliferative and postmitotic myoblasts in the limb, we observe a gradient of maturation from the ectoderm, where *Myf5*- and *Pax3*-expressing myoblasts proliferate (less differentiated), to the centre of the muscle masses, where myoblasts express *MyoD* and become postmitotic (more differentiated). There is probably an intermediate phase where the cells are $Myf5^{+}/MyoD^{+}$. This sequence of myogenic factor expression is shown in Fig. 9A.

In mice, *Myf5* and *MyoD* have been described as being activated in a mutually exclusive manner in the musculature, *Myf5* transcripts being first detected in the epaxial myotome and *MyoD* in the hypaxial myotome (Braun and Arnold, 1996; Cossu et al., 1996a, Tajbakhsh and Buckingham, 1999). Nevertheless, both genes are later co-expressed in the majority of cells with myogenic potential both in vivo and in vitro (Cossu et al., 1996b; Tajbakhsh and Buckingham, 1999). Moreover, *Myf5*-deficient embryos exhibit a 2-day delay in development of axial muscles, but normal formation of the limb musculature. Conversely, in *MyoD* mutant embryos, there is delayed limb muscle development and normal axial musculature formation (Kablar et al., 1997). This has been interpreted, in mice, as showing that *Myf5* has a primary function in the regulation of axial muscles whereas *MyoD* is involved in limb muscle formation. Our chick expression data (see Results) provide no evidence for this dichotomy of function (Myf5/axial muscles versus MyoD/limb muscles). Instead, our results suggest involvement at different steps during myogenesis, Myf5 acting before MyoD. This has been already suggested by gene targeting analysis: in the absence of *Myf5* and *Pax3*, mice do not express *MyoD* and fail to develop body skeletal muscles (Tajbakhsh et al., 1997). Moreover, it appears that *Myf5* is activated first in both epaxial and hypaxial domains of mouse somites (Tajbakhsh and Buckingham, 1999). In addition, mouse *Myf5* expression matches the main sources of myotomal precursors (Venters et al., 1999). These findings indicate that *Myf5* initiates the body skeletal muscle differentiation program in both chick and mouse. The absence of muscle phenotype in the *Myf5* knockout mice (Braun et al., 1992a,b) could be explained by *Pax3* replacing the absent *Myf5* and activating *MyoD*. Indeed, *Pax3* appears to be sufficient, in some cellular contexts, to activate *MyoD* expression and thus initiate the myogenic program in vitro (Maroto et al., 1997) and in vivo (Tajbakhsh et al., 1997; Bendall et al., 1999). Alternatively, paraxis could be another candidate to assume the role of *Myf5*, since the double mutation $paraxis^{-}/Myf5^{-}$ shows muscle losses not observed in the single mutations (Wilson-

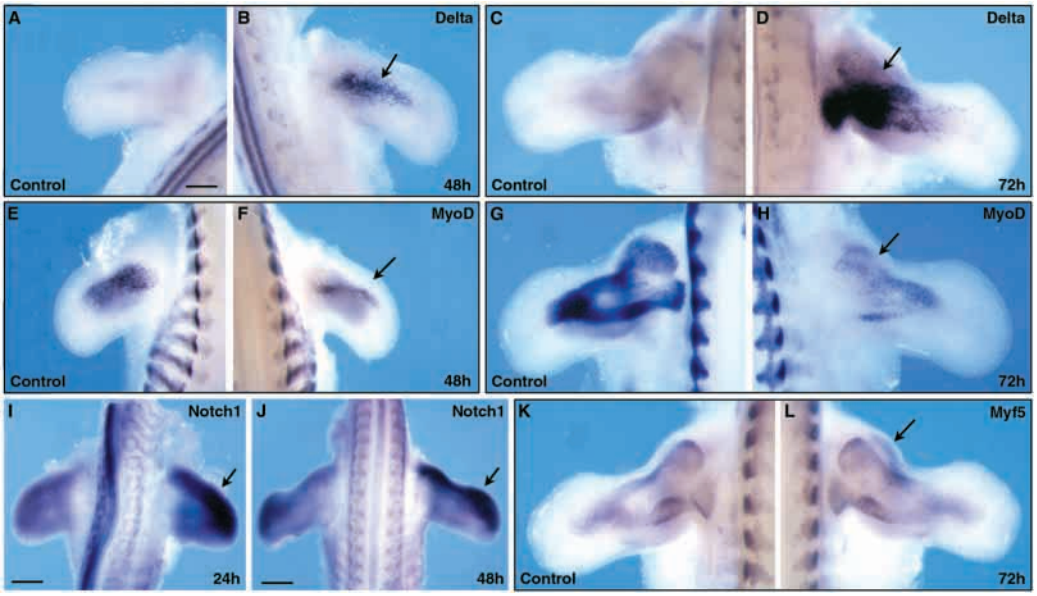

Fig. 5. Ectopic *Delta1* expression downregulates *MyoD* without affecting *Myf*5 expression. Viral transcripts were detected in whole-mount preparations by in situ hybridisation with a probe against *Delta1*, 48 hours (A,B) and 72 hours (C,D) after grafts to the wing at E2.5 of Delta/RCAS-expressing cells. *MyoD* transcripts are downregulated in the grafted wings 48 hours (F) and 72 hours (H) after similar grafts compared with the respective control limbs (E,G). *Notch1* transcripts are upregulated in the grafted wings (right) 24 hours (I) and 48 hours (J) after similar grafts compared with the control limbs (left). Distribution of *Myf*5 transcripts in whole-mount preparations is unchanged 72 hours (K,L) after similar grafts. Arrows indicate the ectopic *Delta1* expression (B,D), the downregulation of *MyoD* (F,H), the upregulation of *Notch1* (I,J) and the unchanged *Myf5* (L) domain in the manipulated wings. Scale bars: 500 μm in A-G,K,L; 350 μm in I; 1mm in J.

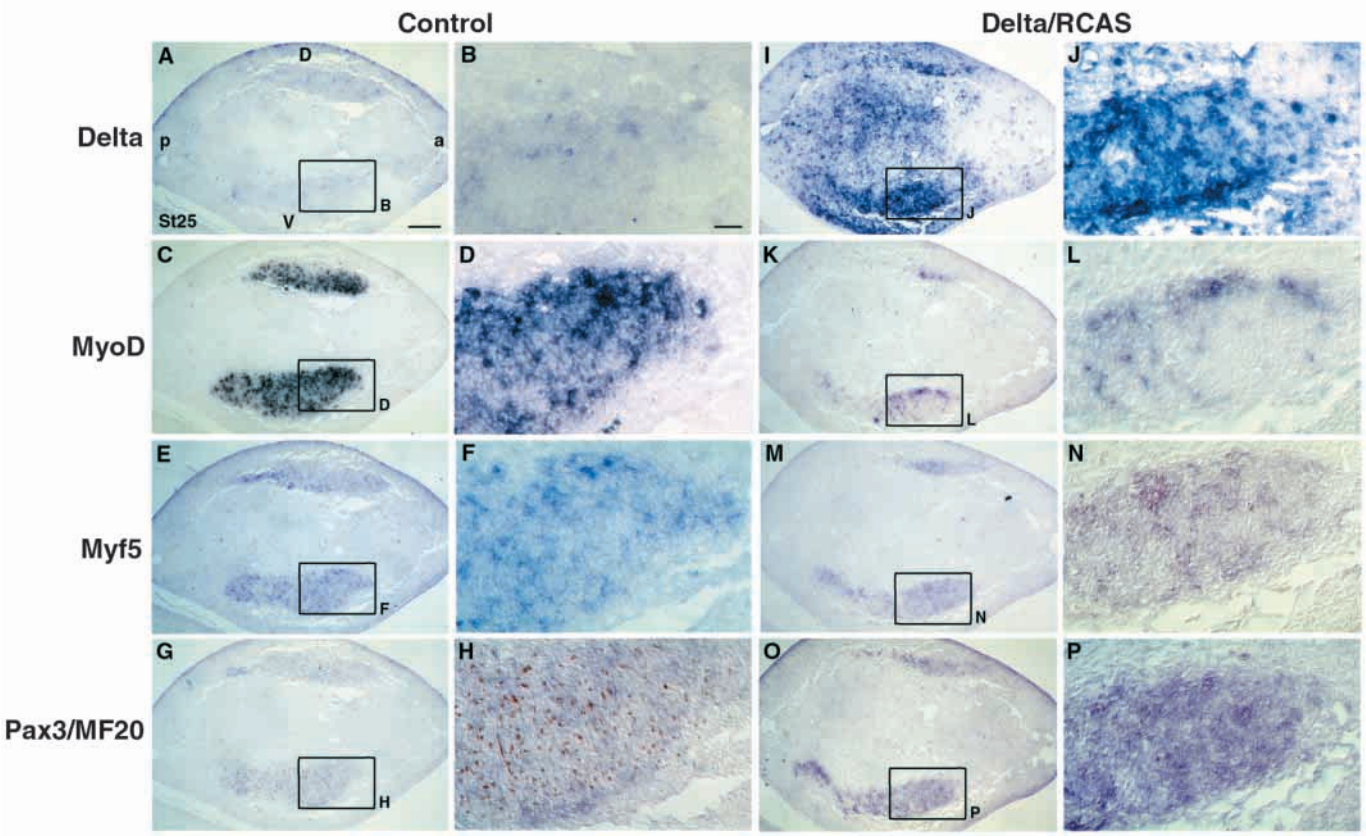

Fig. 6. Overexpression of *Delta1* inhibits myogenesis despite the presence of *Myf5* and *Pax3* transcripts. Adjacent transverse sections of the control (A-H) and infected wings (I-P) from the same embryo 72 hours after grafting Delta/RCAS-expressing cells in E2.5 limbs were hybridised with Delta1 (A,B,I,J), MyoD (C,D,K,L), Myf5 (E,F,M,N) and Pax3 (G,H,O,P) probes. The *Pax3* in situ hybridisation was followed by an incubation with the MF20 antibody (G,H,O,P). All the pictures are orientated similarly: dorsal towards the top, ventral towards the bottom, posterior towards the left and anterior towards the right. (B,D,F,H,J,L,N,P) High magnifications of the anterior parts of the ventral muscle masses from the control limb (A,C,E,G) and infected limb (I,K,M,O). Scale bars 240 μm in A,C,E,G,I,K,M,O; 60 μm in B,D,F,H,J,L,N,P.

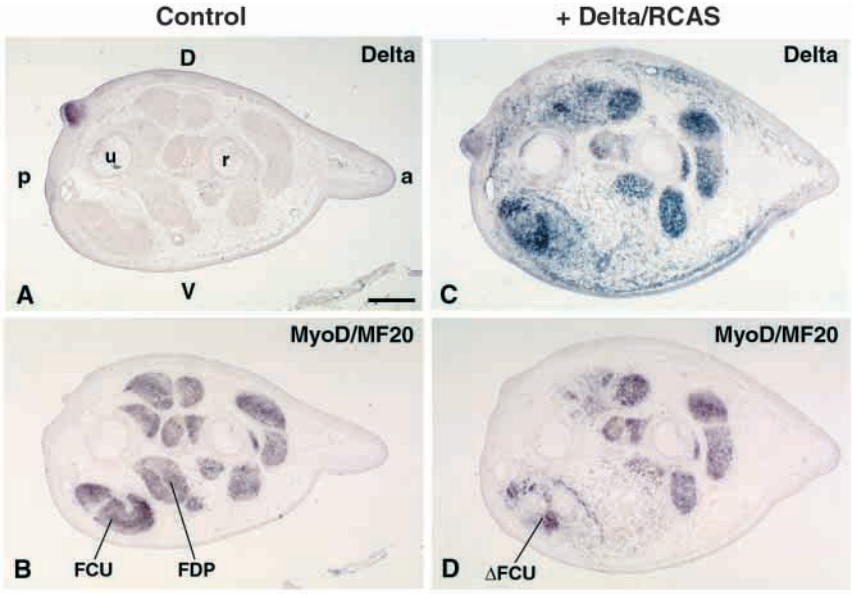

Fig. 7. Ectopic *Delta1* results in disorganised muscles. Adjacent transverse sections of the control (A,B) and infected (C,D) wings from the same embryo were hybridised with the RNA probes specific for *Delta1* (A,C) or *MyoD* (B,D) and then incubated with the MF20 antibody (B,D). The muscles in the posterior regions are disorganised or absent. a, anterior, D, dorsal; FCU, flexor carpi ulnaris; ΔFCU, the remains of the FCU; FDP, flexor digitorum profondus; p, posterior; r, radius; u, ulna; V, ventral;. Scale bar: 320 μm.

Rawls et al., 1999a). The presence of muscle in the absence of *MyoD* (Rudnicki et al., 1992) has been interpreted as showing that it can be replaced by *Myf5* (Rudnicki et al., 1993; Tajbakhsh and Cossu, 1997). However, *Myf5* alone is insufficient to activate the myogenic program in the absence of the other three myogenic factors (Valdez et al., 2000), suggesting rather an overlap in the functions of myogenin, MyoD and Mrf4. It has already been shown that *Mrf4* and *MyoD* can compensate for each other's absence in muscle differentiation in mice, since the *Mrf4*$^{-}$/*MyoD*$^{-}$ double mutant displays a severe muscle deficiency, whereas mice lacking either *Mrf4* or *MyoD* do not show defects in muscle development (Rawls et al., 1998). The absence of axial muscle defects in *MyoD*$^{-}$ mice could be explained if *Mrf4* were able to support muscle development. The transient expression of Mrf4 in myotome before its expression in late embryogenesis and postnatal muscles (Ontell et al., 1995) is consistent with this notion.

Delta-activated Notch signalling inhibits myogenesis in vivo

We have shown that Delta-activated Notch signalling in vivo downregulates *MyoD* expression and then inhibits terminal differentiation in the chick limb bud. This is the first demonstration in vivo of the involvement of Notch signalling in chick limb myogenesis. The cell-surface receptor Notch mediates communication between cells expressing Notch and cells expressing membrane-bound ligands such as Delta1 and Serrate2. Our examination of *Notch1* and *Delta1*/*Serrate2* expression shows that high levels of the ligands are detected in postmitotic cells and muscle fibres but that *Notch1* is associated with mononucleated cells surrounding the fibres. These results

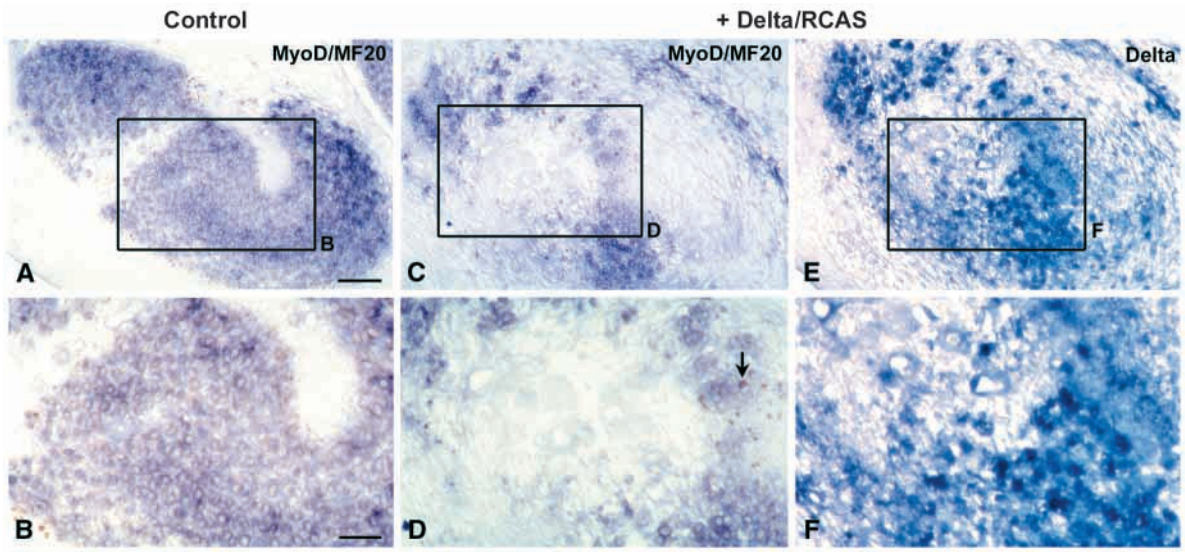

Fig. 8. Higher magnifications focused on FCU muscles from the control (A,B) and manipulated wing (C-F) from Fig. 7, hybridised with *MyoD* probe (purple) and then incubated with the MF20 antibody revealed in brown (A-D), or hybridised with the *Delta1* probe (E,F). The arrow in D indicates a myosin-positive cell in the *MyoD*-positive area. Scale bars: 80 μm in A,C,E; 40 μm in B,D,F.

are illustrated in Fig. 9B. Our overexpression data coupled with the in situ analysis can be interpreted as Notch signalling playing a role in maintaining the myoblasts in an undifferentiated state until myoblasts are correctly positioned to pursue their differentiation. This result is consistent with the known functions of Notch in other systems, e.g. retina, where progenitor retinal cells exposed to *Delta1* are prevented from undergoing neuronal differentiation (Henrique et al., 1997; Dorsky et al., 1995); cartilage, where misexpression of *Delta1* blocks chondrocyte maturation (Crowe et al., 1999); and feather, where overexpression of *Delta1* inhibits feather development (Crowe et al., 1998; Viallet et al., 1998). Moreover, during development of adult indirect flight muscles in *Drosophila*, Notch activation causes failure of differentiation (Anant et al., 1998).

The use of the dominant-negative form of *Delta1* that blocks Notch signalling (Henrique et al., 1997) failed to give any muscle phenotype when grafted into the limb (data not shown). This means that definitive proof of a physiological role for Notch in limb myogenesis is still lacking. However, successful block of Notch signalling using the dominant-negative form of *Delta1* has only been reported for one system, the retina (Henrique et al., 1997). An alternative explanation is that Serrate2, another Notch ligand that is expressed in differentiated myotubes (see Results), compensates for the lack of Delta1 activity. Serrate has been indeed shown to compensate for the loss of function of Delta in *Drosophila* (Gu et al., 1995). Moreover, overexpression of Serrate2 can inhibit the differentiation of C2C12 myoblast cells (Lindsell et al., 1995). *Serrate2* is expressed at the right time and place to play a role in limb myogenesis. However, it is not clear whether Delta1 and Serrate2 have identical functions or whether they use the same receptors during limb myogenesis.

Delta-activated Notch acts between the *Myf5* and *MyoD* steps

Our results show that neither *Myf5* nor *Pax3* is affected by ectopic activation of Notch signalling, indicating that Notch signalling acts after the *Pax3*/*Myf5* step (Fig. 9A). In addition, our results show that *Myf5* and *Pax3* are insufficient to allow further muscle differentiation in the absence of *MyoD*. This contrasts with the normal muscle phenotype in *MyoD* knockout mice (Rudnicki et al., 1992; Kablar et al., 1997). Our misexpression experiments do not allow us to exclude the hypothesis that Delta1-activated Notch acts on the MyoD-expressing lineage in the chick limb, leaving intact the Myf5-expressing pathway. But in that case we would have expected to observe normal terminal differentiation in our experimental limbs, as in the *MyoD* knockout mice (Rudnicki et al., 1992; Kablar et al., 1997). The Myf5 (and Pax3) pathway is insufficient, in our experimental context, to rescue terminal muscle differentiation in the absence of *MyoD*, since we observe fewer myosin-positive cells in Delta1-infected limbs. This reinforces the idea that myogenic factor(s) other than *Myf5* compensate for the absence of *MyoD* in *MyoD*$^{-/-}$ mice (Rawls et al., 1998; Wilson-Rawls et al., 1999b; Valdez et al., 2000). Alternatively, there might be a genuine difference between chick and mouse.

It is not clear whether activated-Notch acts on the transition (1) between *Myf5*-proliferative and *MyoD*-postmitotic cells or (2) between *Myf5*-postmitotic to *MyoD*-postmitotic cells. By analogy with the situation in the retina, where forced expression of *Delta1* maintains proliferating neuroepithelial precursors (Henrique et al., 1997), we would have expected an extension of the *Pax3* and *Myf5* domains concomitant with an increase of the BrdU incorporation. However, the fact that we do not observe any change in the *Pax3* and *Myf5* domains (Figs 5, 6) or of BrdU labelling (data not shown) after *Delta* misexpression favours an action of Notch on *Myf5* postmitotic cells. Whatever the situation, it is clear that Delta-activated Notch blocks further differentiation of Myf5-expressing cells.

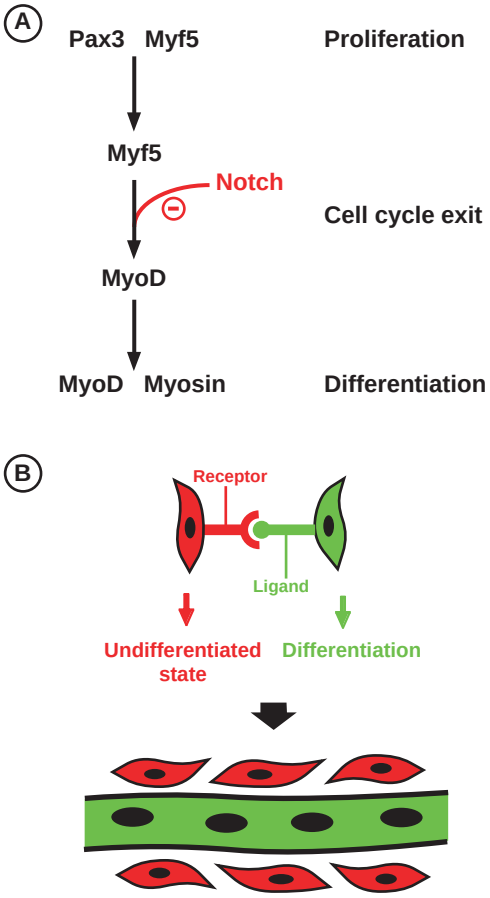

Fig. 9. Involvement of Notch signalling between the Myf5-step and the MyoD-step. **(**A) The sequence of expression of the myogenic factors during myogenesis is represented. *Pax3* and *Myf5* are associated with myoblast proliferation. *Myf5* remains expressed in post-mitotic myoblasts, which then express *MyoD* followed by the myosin proteins. Activated Notch inhibits the progression from the *Myf5* step to the *MyoD* step. (B) The myoblasts expressing the receptor *Notch1* remain in an undifferentiated state while the myoblasts expressing the ligands (*Delta1* or *Serrate2*) pursue their differentiation.

Relationship between MyoD and Notch signalling components

We found a decrease of *MyoD* transcripts after ectopic activation of Notch signalling. We cannot conclude from our experiments whether the downregulation of *MyoD* transcripts is the result of an inhibition of gene activation or a defect in the maintenance of *MyoD* expression. However, studies on transfected cell lines revealed that activated Notch is able to

inhibit *MyoD* transcription (Kuroda et al., 1999). Activated Notch also interferes with the muscle-inducing activity of the MyoD protein (Kopan et al., 1994). This interference has recently been shown to occur through a direct protein interaction between the ankyrin repeat region of Notch and MEF2C, an essential cofactor of MyoD, that blocks DNA binding (Wilson-Rawls et al., 1999b). Since MyoD and MEF2 participate in regulatory circuits involving positive transcriptional feedback loops (Thayer et al., 1989; Braun et al., 1989; Molkentin et al., 1995), the downregulation of *MyoD* expression we observed could also be the consequence of the inhibition of MEF2 activity.

It has been shown recently that MyoD is a direct, positive regulator of Xenopus Delta1 (Wittenberger et al., 1999). The detection of *MyoD* transcripts before those of *Delta1* in the Xenopus gastrula indicates that MyoD triggers Notch signalling in this species (Wittenberger et al., 1999). From our results it is not clear whether MyoD induces *Delta1*, which would then trigger Notch signalling in adjacent myoblasts, or if Notch signalling is activated before the onset of *MyoD* expression. Chick *MyoD* and *Delta1* expression seem to occur together in the limb (data not shown). However, *Serrate2* transcripts are detected before those of *MyoD* in the limb (data not shown). Thus, we favour the hypothesis that high levels of ligand (Delta1, Serrate2) expression in a few cells would activate Notch signalling in adjacent cells. The ligand-positive cells would then differentiate by activating *MyoD*. The existence of a positive feedback loop (*MyoD* towards *Delta1*) would enhance and lock the differentiation process.

Many arguments converge to suggest that MyoD expression is linked to cell-growth arrest

In vitro studies have generally concluded that *MyoD* is expressed in proliferative myoblasts (Lassar et al., 1994; Molkentin and Olson 1996), although this does not fit with the endogenous expression of *MyoD* in postmitotic cells of mouse and chick myotomes (Ontell et al., 1995; Brand-Saberi et al., 1996; Amthor et al., 1998; Hirsinger et al., 2001). In the chick limb, where the distinction between proliferative and postmitotic myoblasts is not obvious (in contrast to the situation in somites), we have shown that the majority of *MyoD*-expressing cells do not incorporate BrdU, indicating that they have withdrawn from the cell cycle and are postmitotic myoblasts. Moreover, experimental evidence from in vitro studies shows that MyoD induces withdrawal from the cell cycle independently of muscle differentiation (Davies et al., 1987; Crescenzi et al., 1990; Sorrentino et al., 1990; Trouch et al., 1993). No such effects have been reported for Myf5. In addition, Myf5 and MyoD show different expression profiles during the cell cycle in C2 cells. MyoD expression is maximal upon cell cycle exit (Kitzmann et al., 1998). A recent study of satellite cells reached the same conclusion that MyoD could be necessary for the transition from proliferation to differentiation (Yablonka-Reuveni et al., 1999). Interestingly, MyoD is able to activate myogenin (Hollenberg et al., 1993) and a cyclin inhibitor, p21, without any new protein synthesis (Otten et al., 1997; Cenciarelli et al., 1999). p21 activation is linked to cell-cycle exit (reviewed in Walsh and Perlman, 1997).

In conclusion, in vitro studies and genetic analysis, coupled with our in situ and in vivo experiments on chick limb, indicate that *Myf5* initiates the myogenic program and that *MyoD* expression is the manifestation of subsequent differentiation. The in vivo signal regulating the transition from the *Myf5* step to the *MyoD* step in the chick limb may involve Notch signalling.

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