

The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD

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SUMMARY

We show that *Myf-5* and *mNotch* mRNA are both present in the presomitic mesoderm before muscle cell commitment and before muscle structural gene activation. The failure of presomitic mesoderm to respond to *Myf-5* and express myogenic properties implies that there may be a mechanism in presomitic mesoderm to suppress muscle differentiation. Here we show that ectopic expression of the intracellular domain of mNotch (mNotchIC) functions as a constitutively activated repressor of myogenesis both in cultured cells and in frog embryos. Mutagenesis experiments indicate that the target for inactivation by mNotch

is the MyoD basic helix-loop-helix domain. mNotchIC contains a nuclear localization signal and localizes to the nucleus. Removal of the nuclear localization signal (NLS) reduces nuclear localization and diminishes the inhibition of myogenesis caused by *Myf-5* or MyoD. Additional experiments show that the CDC10/SWI6/ankyrin repeats are also necessary for myogenic inhibition.

Key words: mNotch, myogenesis, MyoD, mouse

INTRODUCTION

The presomitic mesoderm in developing mouse embryos is subdivided into somitomeres, which mature into somites from anterior to posterior (Tam et al., 1982; Tam, 1988). Somites contain three cell types with different developmental fates: myotome, which generates skeletal muscle; sclerotome, which contributes to cartilage (e.g., the ribs and vertebrae) and dermatome, which forms the dorsolateral dermis. Cell marking experiments suggest that presomitic mesodermal cells are not committed to any particular cell fate and can yield progeny that contribute to all three cell types (Stern et al., 1988). A family of transcription factors collectively known as myogenic regulatory factors (MRF or the MyoD family) are expressed in the myotome of maturing somites and are thought to specify the muscle fate (Tapscott et al., 1988; Weintraub et al., 1990; Ott et al., 1991; Miner and Wold, 1990; Pownall and Emerson, 1992; Rudnicki et al., 1993; Weintraub, 1993).

In *Drosophila* ectoderm, specification of the neural fate involves a family of related genes – the proneural genes of the *achaete-scute* complex (*AS-C*). The myogenic genes and *AS-C* genes are all members of the bHLH gene family (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; Murre et al., 1989; Jan and Jan, 1993). *AS-C* genes are required for ectodermal cells to give rise to neural lineages. Interestingly, they are expressed in clusters of equivalent cells of which only a subset will differentiate into neurons and the rest will form

epidermal cells (Cabrera, 1990, 1992; Campuzano and Modolell, 1992; Skeath and Carroll, 1991, 1992). A second group of largely unrelated genes, the neurogenic genes, determine, by cell-cell interactions, which of these equivalent cells will choose the neural fate (Lehmann et al., 1983; Sternberg, 1988; Yochem and Greenwald, 1989; Artavanis-Tsakonas and Simpson, 1991; Goriely et al., 1991; Greenwald and Rubin, 1992). Notch appears to function as a receptor in this signaling pathway for a specific ligand(s) such as the product of the *Delta* locus (Kidd et al., 1989; De Cellis et al., 1991, 1993; Fehon et al., 1990, 1991; Heitzler and Simpson, 1991, 1993; Lieber et al., 1992; Rebay et al., 1991; Kooh et al., 1993; Lyman and Young, 1993). Activation of Notch inhibits the neural fate, whereas in its absence all equivalent cells expressing *AS-C* become neural cells (Heitzler and Simpson, 1991, 1993; for recent see reviews, Campos-Ortega, 1993; Ghysen et al., 1993). In the fly, Notch is also involved in differentiation of many other tissues, including the somatic follicle cell (Ruohola et al., 1991; Ruohola-Baker et al., 1994), the eye (Cagan and Ready, 1989) and muscle (Corbin et al., 1991). Vertebrate genes homologous to *Notch* have been cloned from the frog (Coffman et al., 1990), the rat (Weinmaster et al., 1991, 1992), the human (Ellisen et al., 1991) and the mouse (Franco Del Amo et al., 1992; Reaume et al., 1992; Kopan and Weintraub, 1993). We decided to ask whether Notch acts as an inhibitor of myogenesis in vertebrate myogenesis.

MATERIALS AND METHODS

Staging of mouse embryos, in situ hybridization and RT-PCR

CD-1 mice were kept under 12/12 hours dark/light regiment. Matings were set up in the late afternoon and vaginal plug inspection was done the next morning. For timing purposes, day 1 of pregnancy, or day 1 post coitum (pc), was the day on which the plug was detected. Whole-mount in situ hybridization was a modification of a previously described protocol (Harland, 1991; the probe and further modifications in Kopan and Weintraub, 1993). For RT-PCR, embryos from 8.5 pc to 10.5 pc were separated under a dissecting microscope into fragments containing caudal neuropore and posterior PSM (CN), presomitic mesoderm (PSM), four most posterior somites (1-4) and the four somites anterior to them (5-9). Sections from 5 to 10 embryos of each age were pooled for RNA extraction while sections from littermates were digested in trypsin and cells were counted (for 9.5 day, average number of cells in one embryo: CN: 2.2×10^4 ; PSM: 7.5×10^3 ; 1-4: 1×10^4 ; 1-5: 2×10^4). Five embryos were pooled from 9.5 day pc). The sections contained tissue derived from all germ layers. Pooled sections were immediately placed into 4.5 M guanidinium thiocyanate solution for RNA extraction (Chomczynski and Sacchi, 1987). After RNA preparation, the RNA precipitate was resuspended in 20 μ l of DEP-treated H₂O. Each RT reaction included 1 μ l of RNA, 1 unit each RNase-free DNase and RNasin (Stratagene) in 10 μ l total. The solution was incubated at 37°C for 10 minutes, 65°C for 10 minutes and then cooled to 4°C. Each sample was split in two, RT-PCR cocktail (Rupp and Weintraub, 1991) was added (final volume 10 μ l) but the enzyme was added to one reaction only (AMV reverse transcriptase, BRL). The reaction mix was incubated at 55°C for 30 minutes, then cooled to 4°C. PCR reaction mix was prepared during the incubation time for all the transcripts examined: (mNotch primers: 5'GCTGCTGACCTGCGCATG3'; 5'AGCAGCTGCATCTTCT-TCTTCTCAC3'; Myf-5 primers: 5'TCCTCAGGAATGCCATCCGC3'; 5'GACAGTAGATGCTGTCAAAG3' RPL7 primers and primers used in RT-PCR in Table 3 see Hollenberg et al., 1993). 1 μ l of the RT reaction was added to each 50 μ l of PCR mix. Conditions were determined empirically to permit exponential amplification of 10 to 10⁵ copies for each primer set, by amplifying known copy numbers of template plasmid in non-specific background of *Caenorhabditis elegans* cDNA (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds for all primers. Cycles: for RPL7, aliquots were taken at 25, 27 and 31 cycles; 33 for mNotch and 37 for Myf-5). Ribosomal protein L7 (RPL7) mRNA expression was used as an internal control for mRNA reverse transcription and PCR amplification. By plotting ³²P incorporation as a function of the increase in cycle number, we showed that, within the exponential amplification range, all samples differed in their RPL7 mRNA level by less than tenfold. Where possible, primers spanned an intron and the amplification in the absence of reverse-transcriptase was used to establish that the signal originated from mRNA and not from DNA contamination. The products of the amplification were separated on 6% TBS polyacrylamide gels. Dried gels were exposed on a phosphorimager and ³²P incorporation in the PCR products was determined and plotted. On average, each PCR reaction amplified cDNA of 5 to 15 cell equivalents. Quantitation of Myf-5 transcripts in PSM was based on comparing ³²P incorporation into amplified product from known starting number of plasmid copies to ³²P

incorporation into amplified product of the cDNA from the RT reaction. This estimated number of mRNA molecules was divided to the number of cell equivalents in the PCR reaction.

DNA constructs

The cloning of the *mNotch* gene is described in Kopan and Weintraub (1993). Plasmid containing MCK promoter driving β -galactosidase (MCK/ β -gal) was based on the 3300 kb promoter sequence of MCK described previously (Johnson et al., 1989). The Myf-5 expression vector was made by cloning the mouse *Myf-5* (provided to us by Dr A. Buonanno, 1992) into the CS2+6MT vector (Fig. 6). For transient transfection and synthetic RNA preparation, the CS vectors were used. These vectors are based on a Bluescript plasmid (Stratagene, CA) to which the following elements have been added: a simian cytomegalovirus (CMV) promoter/enhancer, followed by the SP6 RNA polymerase promoter, polycloning sites and SV40 polyadenylation signal (CS2+; Rupp et al., 1994; Turner and Weintraub, 1994). Derivatives with SV40 NLS and six Myc tags (Roth et al., 1991; CS2+NL6MT) or six Myc tags alone (CS2+6MT) were also used. These elements were followed by polycloning sites. Into the CS vectors we cloned the intracellular portion of the mouse mNotch (mNotchIC, a derivative of pksMotch, Kopan and Weintraub, 1993 with a *StuI* site in frame at the 5' end and cloned into a CS2+6MT vector). Derivatives of mNotchIC used in this study: mNotchICA-PEST: mNotchIC digested with *XhoI* and self-ligated to a polycloning site at the 3' end. mNotchIC Δ sNLS/NLmNotchIC Δ sNLS: PCR amplified segment (5'GCTGGGATCCGCTGACGTCGGCATG3'; 5'GGCCTCGAGGCCGTGTGTGGCAGACTTGAG3') was digested with *XhoI* and cloned into *StuI* and *XhoI* digested mNotchIC with or without the SV40 NLS. Point mutations in the CDC10/SWI6 repeats were introduced by PCR: overlapping oligos that include the nucleic acid substitutions (overlap in capital letters, substituted base in bold. M1: 5'GCCACAGCTCCAGCGatcctggc3'; 5'CGCTGCAGCT-

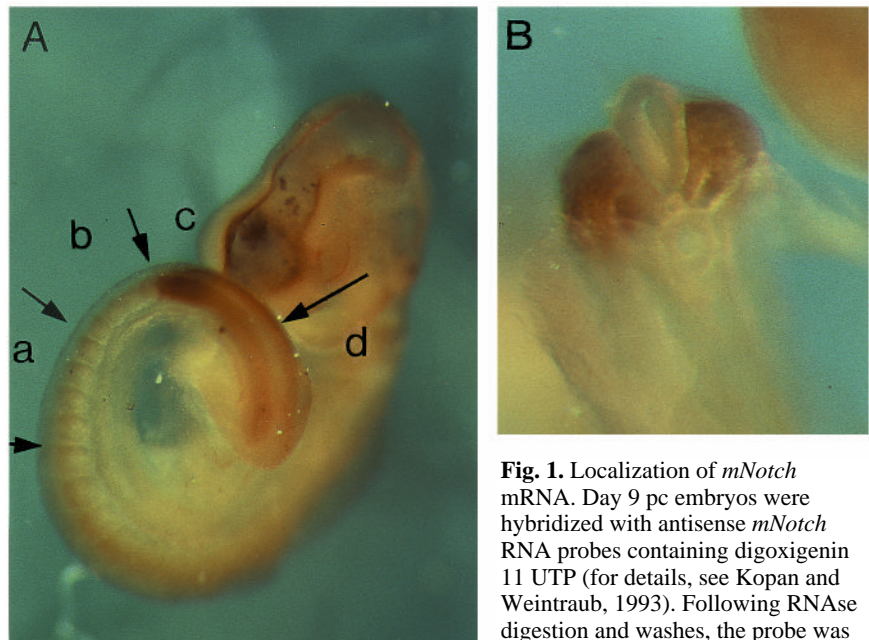


Fig. 1. Localization of *mNotch* mRNA. Day 9 pc embryos were hybridized with antisense *mNotch* RNA probes containing digoxigenin 11 UTP (for details, see Kopan and Weintraub, 1993). Following RNase digestion and washes, the probe was detected by alkaline-phosphatase-conjugated anti-digoxigenin antibodies and a colorimetric alkaline-phosphatase reaction. Lateral view (the head process on top, dorsal to the right) of an embryo from the antisense hybridization is shown in A. Probe-specific patterns are visible in a rostral-caudal gradient in presomitic mesoderm area. A section of the same embryo is presented in frame B. Dorsal is to the top. Staining reveals signal is located in the presomitic mesoderm and is missing from lateral plate mesoderm. Arrows in A demarcate the segments used for the RT-PCR analysis, anterior to posterior: a, 5-9; b, 1-4; c, PSM; d, CN.

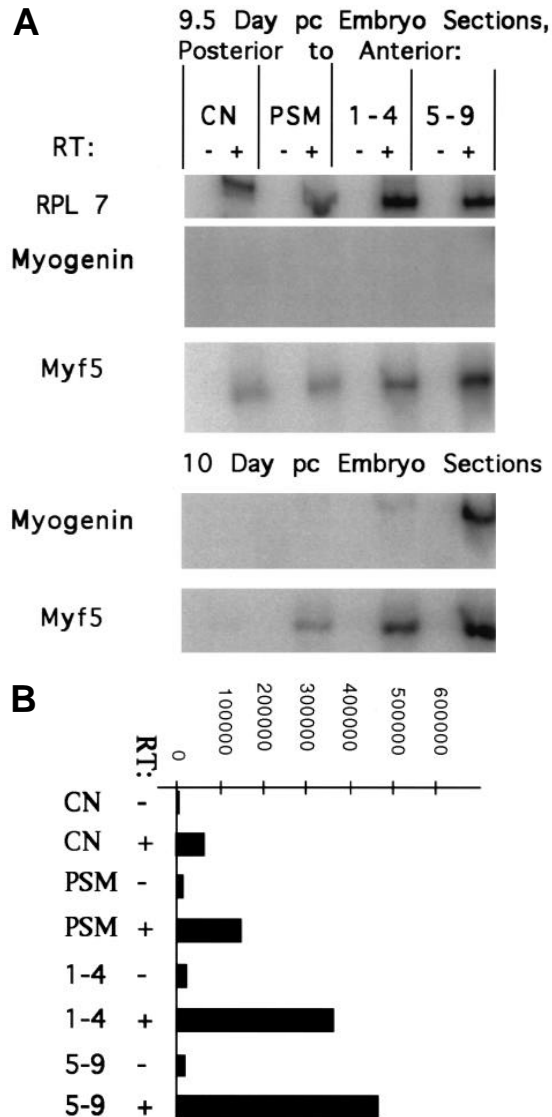


Fig. 2. RT-PCR analysis of Myf-5 expression in the PSM of mouse embryos. (A) RNA-dependent amplification of Myf-5 and myogenin in day 9.5 pc and day 10.5 pc. (B) A plot of RNA abundance in 9.5 day old embryo, normalized to cell number, shows that *Myf-5* transcripts in PSM was based on comparing 32 P incorporation into amplified product from known starting number of plasmid copies to 32 P incorporation into amplified product of the cDNA from the RT reaction. Estimated number of mRNA molecules was divided by the number of cell equivalents in the PCR reaction. RNA amounts in each reaction were estimated by RT-PCR amplification of ribosomal protein L7 (RPL7).

GTGGCcatcatgcatcg3'. M2: 5'ATCCTGGAATTCCgcctggccc3'; 5'GGAATTCCAGGGATcagtggagt3') and outside primers were used in the first round in separate reactions to amplify short segments of mNotchIC. The PCR products were gel purified, combined and amplified again with the outside primer pair to generate a single longer segment. This segment was digested with *Bgl*II and *Eco*RV and cloned into a similarly digested CS2+6MT containing mNotchIC. Orientation, frame and mutations were verified by sequencing. Other

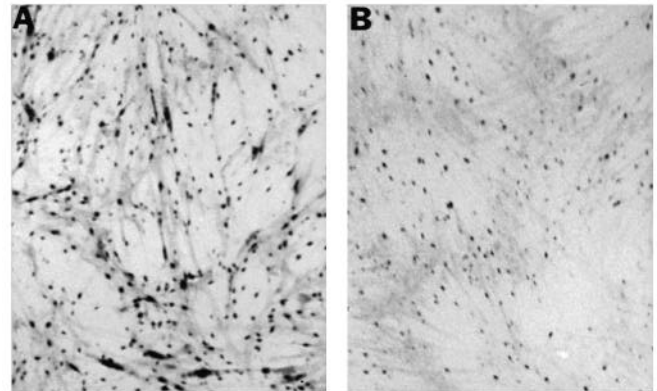


Fig. 3. Assay for activated mNotch in C2 cells. (A) C2 myoblasts were transiently transfected with a plasmid containing sCMV promoter driving β -gal modified to localize to the nucleus ($n\beta$ -gal, the reporter plasmid). Following transfection the cells were washed, kept for 24 hours in 10% bovine calf serum and then transferred to differentiation media. After an additional two days the cells were fixed and cells reacted with anti-myosin antibodies. Following washes the antibodies were detected by alkaline-phosphatase-conjugated secondary antibodies and lightly stained (light gray in A and B). The media was replaced for x-gal staining and identification of transfected, β -gal-containing cells. (A) Stained nuclei (black) were detected both in myotubes and in mononucleated cells. (B) An example of a representative field transfected with mNotchIC. Stained nuclei are predominantly in mononucleated cells. Notice the presence of myotubes (light gray) as background in both A and B. Transfections were done by the calcium phosphate precipitate method as described previously (Weintraub et al., 1991). C2 myoblasts were transiently transfected in 10 cm² plates with 10 μ g CS2+n β -gal (β -gal modified to localize to the nucleus, the reporter plasmid) and 10 μ g CS2+, or a mix of 10 μ g reporter and 10 μ g inhibitor.

plasmids used in this study, such as the MyoD/VP16 fusion and the Gal-MyoD fusions, were described in Weintraub et al. (1991).

Transfections and CAT assay

Transfections were done by the calcium phosphate precipitate method as described previously (Weintraub et al., 1991). C2 myoblasts were transiently transfected in 10 cm² plates with 10 μ g CS2+n β -gal (β -gal modified to localize to the nucleus, the reporter plasmid) and 10 μ g CS2+, or a mix of 10 μ g reporter and 10 μ g inhibitor (see text). Following transfection, the cells were washed, kept for 24 hours in 10% bovine calf serum and then transferred to differentiation media containing 5 μ g/ml transferin and 10 μ g/ml insulin. After an additional 2 days, the cells were fixed and cells incubated with mouse monoclonal anti-myosin antibodies (MF20, Bader et al., 1982). Following washes, the antibodies were detected by alkaline-phosphatase-conjugated secondary antibodies and lightly stained in NBT/BCIP (Harland, 1991). The media was replaced for x-gal staining and identification of β -gal-containing cells (Kopan and Weintraub, 1993). 3T3 cells were transfected following the same protocol, with the addition of a MyoD inducer plasmid at 1.5 μ g. For CAT assays, each experiment was done in four or five independent precipitates. Plasmid ratios were kept constant at 1.5 μ g MyoD (or other inducers, like MyoD/VP16 etc.), 8 μ g MCKCAT and 8 μ g CS2+ or mNotchIC derivatives. Some experiments varied the ratio of MyoD to mNotchIC derivatives while keeping total DNA amounts constant. The content of half a 10 cm² plate was scraped and the cells spun in a tabletop centrifuge to remove growth media. Cells were lysed in SDS-PAGE buffer and used for western blot to verify that the various constructs are expressed at comparable levels. The other half was used

for CAT assay (compensating for the reduction in cell number) as were another three of the five plates. The results of such experiments were averaged and considered as one data point. Experiments were repeated 4 to 20 times. The remaining plate was fixed in 4% paraformaldehyde (5 minutes), washed in PBS, and cells were permeabilized with 0.25% Triton X-100 in PBS for 5 minutes. Cells were stained with rabbit polyclonal anti-CAT antibody (5' 3') and a monoclonal mouse antibody against Myc tag (9E10, Evan et al., 1985) or MyoD (5.8, Shakhovich et al., 1992). Primary antibodies were detected with fluorescein-conjugated donkey anti-mouse (Jackson ImmunoResearch) and Texas red-conjugated goat anti-rabbit antibody (Molecular Probes). Cell counting and photography were done with the help of a two-channel confocal microscope (Bio-Rad). The CAT assay protocol was published previously (Weintraub et al., 1991).

Frog embryo injections

Capped synthetic RNA (SP6 RNA polymerase, SP6 promoter in the CS2+6MT vectors) or a DNA expression plasmid (same plasmid used for transfection and RNA synthesis) were injected (Rupp and Weintraub, 1991; Rupp et al., 1994 and Turner and Weintraub, 1994) with basically the same results. 2-cell-stage albino *Xenopus* embryos were injected twice in opposite poles of one cell (or in two of four cells) to produce ectopic expression on the dorsal side of the developing embryo. After embryos reached stage 25, they were fixed in MEMFA (2 hours) and transferred to methanol at room temperature. For staining, embryos were rehydrated and stained as described (Hemmati-Brivanlou and Harland, 1989) with anti-Myc monoclonal antibody 9E10 (1:5) or anti-myosin monoclonal antibody MF20 (1:5). The primary antibodies were detected with a goat anti-mouse, alkaline-phosphatase-conjugated antibody (Boehringer Mannheim) followed by NBT/BCIP staining.

RESULTS

Both *mNotch* and *Myf-5* are expressed in presomitic mesoderm

Previously, expression of *Myf-5* was found by in situ hybridization (ISH) in newly formed somites but not in the PSM (Ott et al., 1991). To examine whether *Myf-5* might be present at lower levels in PSM, we detected its expression by a more sensitive detection protocol based upon reverse transcription polymerase chain reaction (RT-PCR) (Rupp and Weintraub, 1991; Fig. 2). Both posterior PSM (including the caudal neuropore, CN in Figs 1A,2A) and, to a greater degree, anterior PSM express *Myf-5* RNA in day 8.5 day post coitum (pc) embryos (not shown), day 9.5 pc and day 10.5 pc (Fig. 2). The mean number of *Myf-5* RNA molecules per cell equivalent

in the PSM is at least 10 (Fig. 2B, see materials and methods). In agreement with prior work, *Myf-5* is also expressed in the more anterior, mature somites (Ott et al., 1991). In contrast, myogenin RNA is first expressed later in somite maturation (Fig. 2A; see also Sassoon et al., 1989).

mNotch mRNA is most highly expressed in the PSM, posterior to the most posterior somite (Fig. 1A, see also Franco Del Amo et al., 1992; Reaume et al., 1992; Kopan and Weintraub, 1993, Swiatek et al., 1994). The *mNotch* expression zone moves posteriorly in the embryo as new somites are added (Franco Del Amo et al., 1992; Reaume et al., 1992; Kopan and Weintraub, 1993). Collectively, these data show that both *Myf-5* and *mNotch* are expressed in the PSM. Whether the two genes are expressed in the same cells in the PSM will require a much more sensitive analysis. Recent experiments using a β -gal reporter fused into the endogenous *Myf-5* locus indicate that *Myf-5* is activated in a few individual cells present in the PSM (M. Buckingham and S. Tajbakhsh, personal communication).

The intracellular domain of mNotch (mNotchIC) prevents myogenesis in C2 myoblasts, MyoD-induced differentiation of 3T3 cells and in frog embryos

C2 myoblasts express MyoD and are capable of differentiating into mature myocytes and myotubes upon serum starvation. We constructed mutations of *mNotch* designed to act in a

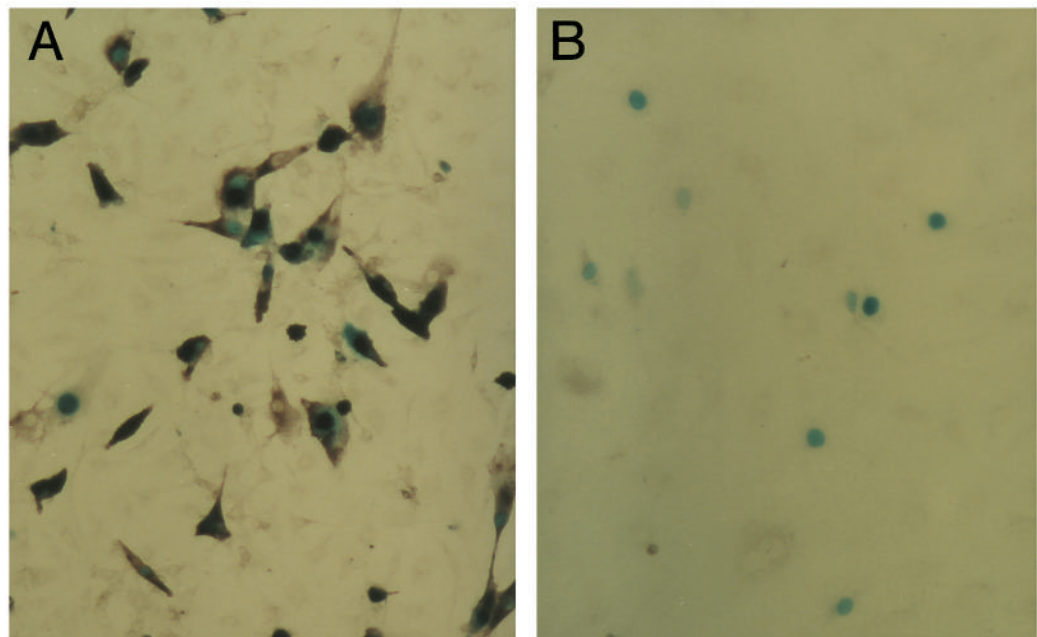


Fig. 4. Assay for activated mNotch in 3T3 cells. 3T3 cells were transiently co-transfected with a plasmid containing sCMV promoter driving n β -gal, the reporter plasmid, a second plasmid containing *Myf-5* and vector (A) or mNotchIC (B). Following transfection the cells were washed, kept for 48 hours in 10% bovine calf serum and then transferred to differentiation media. After an additional 2 days, the cells were fixed and reacted with anti-myosin antibodies. Following washes the antibodies were detected by alkaline-phosphatase-conjugated secondary antibodies and stained (brown; see Kopan and Weintraub, 1993). The media was replaced to permit for x-gal staining and identification of β -gal-containing cells. (A) Stained nuclei (blue) were detected both in myosin-positive and myosin-negative cells. (B) An example of a representative field co-transfected with mNotchIC. Notice the reduced presence of myotubes (brown). Stained nuclei (blue) were detected in cells, indicating the transfection efficiency was not altered. 3T3 cells were transfected as in Fig. 3, with the addition of a MyoD inducer plasmid at 1.5 μ g.

ligand-independent manner and asked whether these molecules affected C2 myogenesis.

When C2 myoblasts are transiently transfected with a plasmid directing constitutive expression of nuclear-targeted β -galactosidase (CS2+n β -gal) and triggered to differentiate, many n β -gal-positive nuclei (17%, $n=423$) are found in fused myotubes (Fig. 3A). These cells also express myosin. C2 cells were then cotransfected with CS2+n β -gal and a plasmid directing the expression of full-length or fragments of mNotch (Fig. 3A). Cotransfection with mNotchIC, the intracellular fragment of mNotch, significantly reduced the percentage of β -gal-positive nuclei present in fused myotubes (0.91%, $n=765$, Fig. 3B). However, little effects on distribution of n β -gal-positive nuclei in myotubes were seen following cotransfection with full-length mNotch (12.2%, $n=277$) or a construct bearing a deletion of EGF repeats 8 to LNR 2 (11.25%, $n=480$, not shown). Similarly designed intracellular constructs – Notch(intra) in *Drosophila* and lin-12(intra) in *C. elegans* – act as constitutively activated molecules in their respective organisms (Struhl et al., 1993; Lieber et al., 1993; Fortini et al., 1993). The present results suggest that the intracellular domain of mammalian Notch may also be sufficient to deliver a signal constitutively and that this signal prevents the differentiation of a committed myoblast.

We next tested whether conversion of the 3T3 fibroblast to muscle by myogenic regulatory factors is affected by an activated mNotch. 3T3 cells were cotransfected with an expression vector driving MyoD or Myf-5, a plasmid containing CS2+n β -Gal (Rupp et al., 1994; Turner and Weintraub, 1994), and an expression vector with or without mNotchIC. Following transfection the cells were transferred to differentiation medium and then stained for myosin and β -galactosidase. The frequency of cells doubly labeled for myosin and n β -gal was determined. 22.7% ($n=356$) and 41.7% ($n=304$) of the n β -gal-positive cells were also myosin positive in 3T3 cells cotransfected with expression vector (CS2+) and Myf-5 or MyoD, respectively (Fig. 4A). In contrast, in cells cotransfected with mNotchIC (Fig. 4B) only 1.3% ($n=302$; Myf-5) and 13% ($n=285$; MyoD) of the n β -gal-positive cells were also myosin positive. A 3T3 cell line stably expressing mNotchIC forms myosin-positive cells tenfold less frequently than control lines in response to MyoD transfection (not shown). Thus, the mNotchIC cotransfected cells acquired resistance to conversion to myosin-positive myocytes by MyoD or Myf-5. Similar observations were made in the pluripotent embryonal carcinoma cell line P19, where myogenic differentiation is

also affected by mNotchIC (reported in an accompanying paper; Nye et al., 1994).

To test whether mNotchIC can behave as an activated version of mNotch in an embryo, we injected one cell of 2-cell *Xenopus* embryos with capped mRNA coding for the Myc-tagged mNotchIC (Rupp and Weintraub, 1991). As shown in Fig. 5B,C, injected embryos develop myosin-positive, normal looking somites on the uninjected side but show reduced or absent myosin staining on the injected side. In addition, these embryos were curved towards the injected side, indicating abnormal development of somites. Neuronal defects on the injected side (split hind brain, lack of neural crest migration into pharyngeal arches, cement gland and eye disruption) are also observed and will be described elsewhere. Staining for the

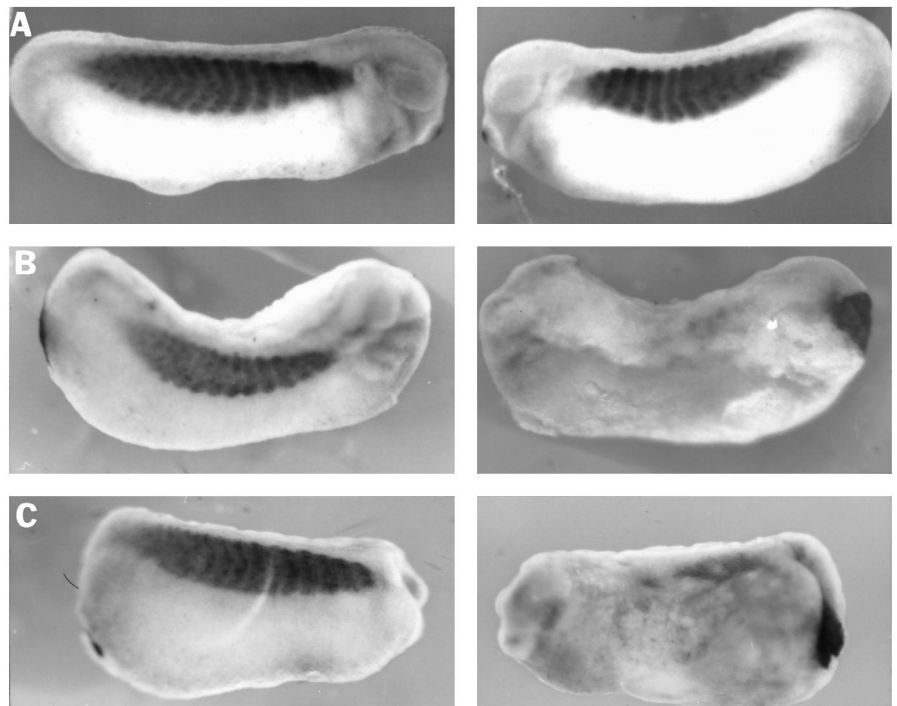


Fig. 5. Injection of mNotchIC into *Xenopus* embryos results in inhibition of myogenesis. (A) Uninjected control embryo at stage 25, shown with its left side on the left and its right side on the right. (B,C) Injected embryos. The uninjected side is shown to the left and the injected to the right. Notice the myosin staining of somites in the uninjected animal is bilateral, but only the uninjected side shows myosin staining in the injected embryo. In addition, the animals are curved towards the injected side (compare A with B,C) indicating unilateral defects in somitogenesis. Also visible are unilateral defects in the neural crest-derived pharyngeal arches and the neural tube. The water injected controls developed normally (uninjected: 40 normal (N), 1 mutant (M), 11 dead (D)); water injected: 22 (N), 3 (D)). The RNA-injected group had more severely affected individuals than the DNA-injected group. The phenotypes in the complete injected pool (RNA+DNA) were 39 (N), 88 (M), 60 (D). The specific breakdown of one RNA experiment: 5 (N), 9 (D), 27 (M): bilateral myosin, spina bifida, normal head, 4; unilateral myosin, head defects, 10; head and/or spinal cord unilateral defects with bilateral myosin, 5; other, 8. Capped synthetic RNA (SP6 RNA polymerase, SP6 promoter in the CS2+6MT vectors) or a DNA expression plasmid (same plasmid used for transfection and RNA synthesis) were injected (21) with basically the same results. 2-cell-stage albino *Xenopus* embryos were injected twice in opposite poles of one cell (or in two of four cells). After embryos reached stage 25, they were fixed in MEMFA (2 hours) and transferred to methanol at room temperature. For staining, embryos were rehydrated and stained as described with anti-Myc monoclonal antibody 9E10 (1:5) or anti-myosin monoclonal antibody MF20 (1:5). The primary antibodies were detected with a goat anti-mouse, alkaline phosphatase-conjugated antibody followed by NBT/BCIP staining.

Myc tag reveals a correlation between the degree of mosaic expression in the dorsal portion of the injected side and the severity of the phenotype (data not shown). These results clearly establish that expression of mNotch1C inhibits myogenesis in vivo.

Suppression of myogenesis by mNotch1C: mutagenesis

As described above, transient transfection of 3T3 cells with MyoD and mNotch1C suppresses the activation of endogenous muscle promoters. We devised a co-transfection assay based on MyoD (or Myf-5)-dependent activation of a MRF-binding site-promoter construct, either the muscle creatine kinase (MCK) gene enhancer (MCKCAT: Johnson et al., 1989) or a thymidine kinase promoter containing only four MyoD-binding sites derived from the MCK gene enhancer (4RCAT; Weintraub et al., 1991). To identify the protein product of mNotch1C and its derivatives on a single cell basis, we used our fusion proteins with the Myc tags at the amino terminus.

As with P19 cells (Nye et al., 1994) and frog embryos, immunofluorescence staining of Myc tagged mNotch1C transfected into 3T3 cells reveals prominent nuclear staining (Fig. 6A) with >90% of cells showing exclusively nuclear staining. The intracellular domain of Notch is similarly localized to the nucleus (Struhl et al., 1993; Lieber et al., 1993; Fortini et al., 1993). mNotch1C reduces MCKCAT expression to 6% of control (Table 1A). More importantly, cell-by-cell analysis reveals a strong correlation between nuclear protein localization of mNotch1C and suppression of myogenesis. No cells that are positive for nuclear staining ($n=0/90$) are also positive for CAT, whereas cells with cytoplasmic mNotch1C show activation of MCKCAT ($n=5/5$). This indicates that the residual uninhibited CAT activity (6%) resides solely in cells expressing mNotch1C in the cytoplasm or both nucleus and cytoplasm (see below). We do not understand why cells expressing both nuclear and cytoplasmic Notch still express some CAT protein; however, the levels of CAT staining are qualitatively reduced compared with those seen with cells expressing only cytoplasmic mNotch. Nevertheless, we have chosen to score these cells as CAT positive and therefore the overall level of inhibition is probably greater than we present.

To test whether cytoplasmic mNotch variants can interfere with nuclear mNotch1C, we have co-transfected mNotch1C with various mutants that are exclusively cytoplasmic. We found no interference with mNotch1C inhibition of MyoD by the co-transfected cytoplasmic mNotch even in 2:1 molar excess (data not shown). We also do not understand why, in any given experiment, a variable number of individual cells can express mNotch1C in the nucleus, or in the cytoplasm or in both. Variations in the subcellular distribution of mNotch1C and its derivatives may be indicative of some, as yet undefined physiological state of a cell.

To identify regions of mNotch that are required for its function in inhibiting myogenesis, we generated several mutant mNotch derivatives. Several domains have been tested, including the putative NLS (Stifani et al., 1992); CDC10/SWI6 repeat 4 (a repeat that seems critical for function of the related gene *glp-1* in *C. elegans*; Kodoyianni et al., 1992); and a deletion of mNotch1C that lacks sequence elements downstream of the putative NLS (mNotch1C Δ PEST) including the PEST (Rechsteiner, 1988) and OPA sequences.

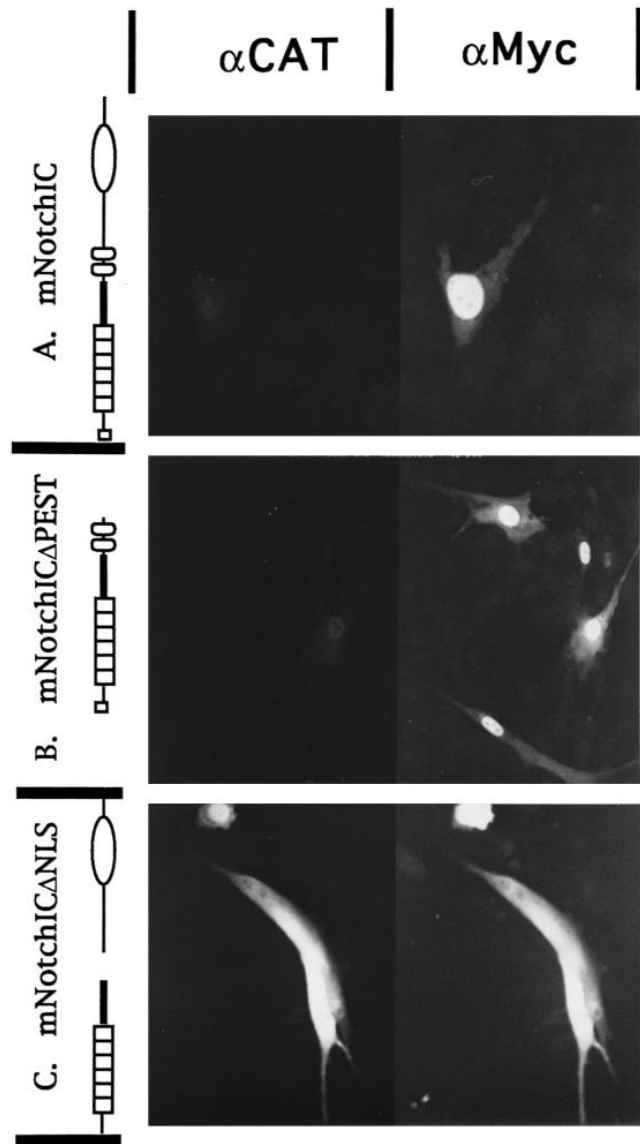
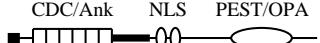
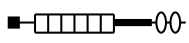
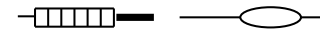




Fig. 6. Staining with anti-Myc tag antibodies reveals nuclear localization of mNotch1C correlates with inhibitory activity. The pictures were taken with a two-channel confocal microscope, one field with green channel (FITC, anti-Myc) another with red channel (Texas Red, anti-CAT). See Table 1, for additional information.

mNotch1C Δ PEST is a potent inhibitor of myogenesis and localizes to the nucleus of 3T3 cells (Fig. 6B, Table 1B, see also Mango et al., 1991). In contrast, two different clusters of point mutations in CDC10/SWI6 repeat 4 that alter the GxTpLxxAA consensus (AxApAxxAA; Kodoyianni et al., 1992; or GxTpLxxEF; Sidorova and L. Breeden, 1993) result in molecules that localize to the nucleus but fail to inhibit myogenesis or suppress MyoD-dependent MCKCAT activation (Fig. 7). Thus, the myogenic inhibition requires the CDC10/SWI6 domain (Lieber et al., 1993; Roehl and Kimbel 1993).

To determine if nuclear localization is required for mNotch to function, we deleted the two putative NLS sequences. An internal deletion was constructed removing 48 amino acids surrounding the putative NLS located between the CDC10/SWI6

Table 1. Deletion analysis of mNotchIC

Construct	Nuclear localization			%MCKCAT expression	%MCKCAT expression in single mNotch-positive cells	
	Nuc.	Cyto	Both.		mNotch Nuc. (exclusively)	mNotch Cyto or BOTH
A. 	+++	+/-		6 (5)*	0 (90)**	100 (5)**
B. 	+++	+/-		2 (1)*	0 (187)**	100 (4)**
C. 	+/-	+++	+++	75 (1)*	0 (14)**	50 (148)**
D. 	+++	+/-		ND	2.6 (191)**	30 (26)**
E. 				100		

■— Sequence starts at: **TKKFRFEPPVLPDLSQTDHRQWTQQHLDAADLRM**
 — Sequence starts at: **AADLRM**

*(Standard deviation); **(Cell number)

Table 1 shows that in the context of the whole intracellular domain, the NLS is required. Its function can be replaced by the SV40 large T antigen NLS. % MCKCAT expression is presented as percentage of the activity with MyoD and MCKCAT when cotransfected with vector alone (E). Each experiment was done in five independent transfection precipitates and repeated 4 to 20 times. Results from four plates of each experiment were averaged. Data points represent the average of different repeats. Single cell analysis was done after staining one of the transfected plates with an antibody mixture (the monoclonal 9E10 against Myc tag and a rabbit polyclonal against CAT) and fluorescent secondary antibodies. The protein distribution was determined simultaneously with a two-channel confocal microscope. Results from several transfections were compiled.

■— Sequence starts at: **TKKFRFEPPVLPDLSQTDHRQWTQQHLDAADLRM**
 — Sequence starts at: **AADLRM**

*(Standard deviation); **(Cell number)

Table 2. Selective inhibition of myogenic promoters by mNotchIC

Reporter or Inducer + Reporter	Activity with mNotchIC (% control)	Detection
(A) mNotchIC does not inhibit various promoters		
RSVβGAL	100	β-gal
RSVCAT	100	CAT
MSVCAT	100	CAT
SV2CAT (SV40 enhancer)	50-100	CAT
CMVGAL	100	β-gal
Actin-GAL (β actin promoter)	100-300	β-gal
HS-GAL (heat shock promoter)	100-200	β-gal
(B) mNotchIC inhibits myogenic promoters as well as Xash3a		
MD+MCKCAT	1-10	CAT
Myf5+MCKCAT	1-10	CAT
MD+4RCAT	1-10	CAT
Myf5+4RCAT	1-10	CAT
MD (Myosin HC, endogenous)	10	Antibody stain
Myf5 (Myosin HC, endogenous)	10	Antibody stain
Xash 3a+4RCAT	1-10	CAT

Constitutive promoters driving expression of reporter genes were transfected into 3T3 cells. Inducible promoters driving expression of reporter genes are co-transfected with MyoD. In all experiments, fold change reflects the difference between transfection of reporter alone (or inducer plus reporter) and co-transfection with mNotchIC. Each experiment was done in two independent transfection precipitates and repeated 2 to 5 times. Reporters used: **MCKCAT**; **MCKGAL**--3.3 kb Muscle Creatine Kinase promoter/enhancer driving CAT or β-gal, respectively. **4RCAT**--HSVTK promoter and 4 MCK MEF1 Ebox as enhancer.

repeats and the PEST region (Stifani et al., 1992). A basic stretch of amino acids (KKFR), conserved in vertebrate Notch molecules upstream of the CDC10/SWI6 repeats, was also

deleted, resulting in mNotchICΔNLS (Table 1C; Fig. 6C). mNotchICΔNLS localizes predominantly to the cytoplasm and does not repress MyoD-mediated activation of MCKCAT (Table 1C; Fig. 6C). Interestingly, in those few cells where mNotchICΔNLS is found in the nucleus, MCKCAT is inactive (0/14 cells show CAT).

We then replaced the missing NLS by positioning 14 amino acids encoding the SV40 large T antigen (NLS) at the amino terminus of mNotchICΔNLS (NLmNotchICΔNLS, Table 1D). This construct directs protein to the nucleus in the majority of cells (191/217). Replacing the NLS also partially restores the inhibitory activity, compared to mNotchIC (Table 1D); however, this molecule was not as potent an inhibitor as either mNotchIC or mNotchICΔPEST (compare Table 1D with 1A, B).


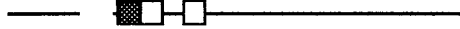

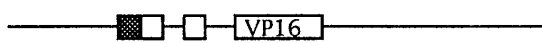

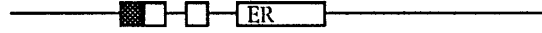
mNotchIC interferes with activation of target genes by MyoD but may not block MyoD binding to DNA

To address the specificity of the effects of mNotchIC on myogenic promoters, the SV40, MSV LTR, CMV, actin, heat shock promoters driving CAT or β-gal in 3T3 cells were tested. These promoters are not suppressed by mNotchIC (Table 2). To verify that MyoD and Myf-5 are indeed the site of action of mNotchIC inhibition, mNotchIC was evaluated in 3T3 cells with 4RCAT (see Tables 2, 3). Similar to MCKCAT, the activity of 4RCAT is reduced by mNotchIC to 1-10% activity, suggesting that the target for mNotchIC inhibition is the complex of MyoD and the E box-binding site. Achaete-scute proteins are negatively regulated by Notch in *Drosophila*. In similar experiments where a frog achaete-scute gene (Xash3; Turner and Weintraub, 1994) is cotransfected into 3T3 cells with a 4RCAT reporter, mNotchIC is also seen to be a potent inhibitor of trans acti-

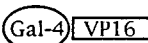
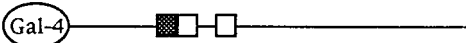
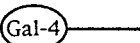
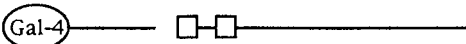
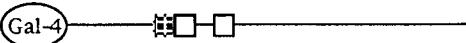
Table 3. Myogenic inhibition by mNotchIC is not targeting DNA binding, heterodimer formation or the activation domain of MyoD**(A) Inhibition is not rescued by addition of E12**

	Reporter or Inducer + Reporter	Activity with mNotchIC (% control)
	MD+MCKCAT	1-10
	Myf5+MCKCAT	1-10
	MD+MCKCAT+E12	1-10
	MD/E2-5(forced dimer)+MCKGAL	1-10

(B) mNotchIC does not inhibit DNA binding: MyoD/VP16 fusion protein activates MCKCAT in the presence of mNotchIC

Structure of MyoD derivatives	Reporter or Inducer + Reporter	Activity with mNotchIC (% control)
	MDΔC+4RCAT	1-10
	MDΔC/H+4RCAT	1-10
	MDΔN+MCKCAT	10
	MD/VP16+MCKCAT	50-100
	MD/VP16+4RCAT	50-100
	ERMD--MCK, endogenous	10 (by RT-PCR)
	ERMD--Myogenin, endogenous	10 (by RT-PCR)
	ERMD--Cardiac Actin, endogenous	10 (by RT-PCR)

(C) mNotchIC does not inhibit activation by the MyoD activation domain

Structure of MyoD-Gal derivatives	Reporter or Inducer + Reporter	Activity with mNotchIC (% control)
	GalVP16+GALCAT	100
	GalMD+GALCAT	100-200
	GalN+GALCAT	100-200
	GalMDΔB+GALCAT	100
	GalMDE12B+GALCAT	100

Constitutive promoters driving expression of reporter genes were transfected into 3T3 cells. Inducible promoters driving expression of reporter genes are co-transfected with MyoD. In all experiments, fold change reflects the difference between transfection of reporter alone (or inducer plus reporter) and co-transfection with mNotchIC. Each experiment was done in two independent transfection precipitates and repeated 2 to 5 times. GalMDΔB is a deletion of the basic region of MyoD fused to the Gal-4 DNA-binding domain. GalMDE12B is a substitution of the E12 basic region for that of MyoD. GalN is a fusion with the MyoD activation domain. ERMD is an estrogen-dependent version of MyoD. Reporters used: **MCKCAT**; **MCKGAL**--3.3 kb Muscle Creatine Kinase promoter/enhancer driving CAT or β-gal, respectively. **4RCAT**--HSVTK promoter and 4 MCK MEF1 Ebox as enhancer. **GALCAT**--E1b promoter and Gal4 UAS as enhancer. N, amino terminus of MyoD; bHLH, basic Helix-Loop-Helix domain; C, carboxy terminus of MyoD. C/H, Cystidine/Histidine rich region.

vation (Table 2). Thus, in our assay, negative regulation by mNotch appears to be specific to several tissue-specific bHLH transcriptional activators.

Supplying an excess of E12 or E2-5, ubiquitous bHLH proteins that heterodimerize with MyoD or Myf-5 to bind and activate 4RCAT, does not restore the activation by MyoD. Thus it appears that mNotchIC does not act by limiting E proteins. The inhibition by mNotchIC seems to be directed at a step after the dimerization of MyoD and E12 since mNotchIC can still inhibit a forced heterodimer of MyoD and E2-5, which is a fully functional fusion gene of MyoD and E2-5 (Neuhold and Wold, 1993; Table 3A).

The target for mNotchIC inhibition seems to be the bHLH region of MyoD, as MyoD derivatives missing the N terminus, C terminus and the C/H region (residues 63-98) are all inhibited (Table 3B). MyoD protein is localized to the nucleus in control cells and cells transfected with mNotchIC, indicating that mNotchIC does not interfere with nuclear localization of the transfected MyoD (data not shown).

MyoD mutants within the basic region were described that bind DNA but fail to activate target genes (Weintraub et al., 1991; Davis and Weintraub, 1992). Analysis of these 'positive control' mutants had led to the notion that in order for MyoD to activate muscle-specific promoters, it must interact with an

unidentified co-activator (see Weintraub et al., 1991; Davis and Weintraub, 1992; Tapscott et al., 1993) that fails to function with the positive control mutations. The viral activator VP16 fused to MyoD (MyoD/VP16) acts independently of the cellular co-activator and was therefore analyzed for its response to mNotchIC. In contrast to MyoD, MyoD/VP16 co-transfection with mNotchIC results only in a modest decrease of CAT activity (61%; s.d. 20%, $n=8$, Table 3). Sequences inserted into MyoD at the same *NarI* site where VP16 is inserted do not uniformly abolish the ability of mNotchIC to interact with MyoD; the hormone-binding domain of the estrogen receptor inserted into MyoD at the same site (Hollenberg et al., 1993) results in an estrogen-inducible MyoD that is inhibited by mNotchIC (Table 3B). Because the MyoD-VP16 fusion protein is relatively resistant to mNotchIC inhibition, it appears that mNotchIC does not directly interfere with the binding of MyoD to its target promoter.

To ask if the site of action of mNotchIC is the MyoD activation domain, we employed the DNA-binding domain of GAL4 fused to various derivatives containing the MyoD activation domain (residues 3-35). Rather than inhibiting, mNotchIC stimulates these fusion proteins (Table 3C). The mechanism of this stimulation is unclear. Maximal transcription efficacy of muscle-specific genes requires uncharacterized co-activators of MyoD (Davis and Weintraub, 1992; Tapscott et al., 1993). These present results imply that such co-activators could be the target of mNotchIC in the nucleus.

DISCUSSION

Holtzer and colleagues (e.g., Holtzer and Detwiler, 1953) clearly demonstrated that cell type specification in the early myotome is not absolute and can be redirected under the influence of the spinal cord. Several more recent studies (Rong et al., 1992; Ordahl and Le Douarin, 1992; Goulding et al., 1994; Williams and Ordahl, 1994) have confirmed and extended these conclusions. In contrast, it has been shown that dissociated chick blastoderm cells (see Holtzer et al., 1983) or dissociated PSM cells (George-Weinstein et al., 1994) can give rise to muscle cells in culture, suggesting that these early precursors are normally being inhibited from forming muscle by cell-cell interactions. Likewise, dissociated

frog dorsal ectoderm (animal caps) form nerve in the absence of induction when dissociated and plated into cell culture (Godsave and Slack, 1989). Previous observations made in allophenic mice (Gearhart and Mintz, 1972; Moore and Mintz, 1972) indicate that the number of founder cells for the

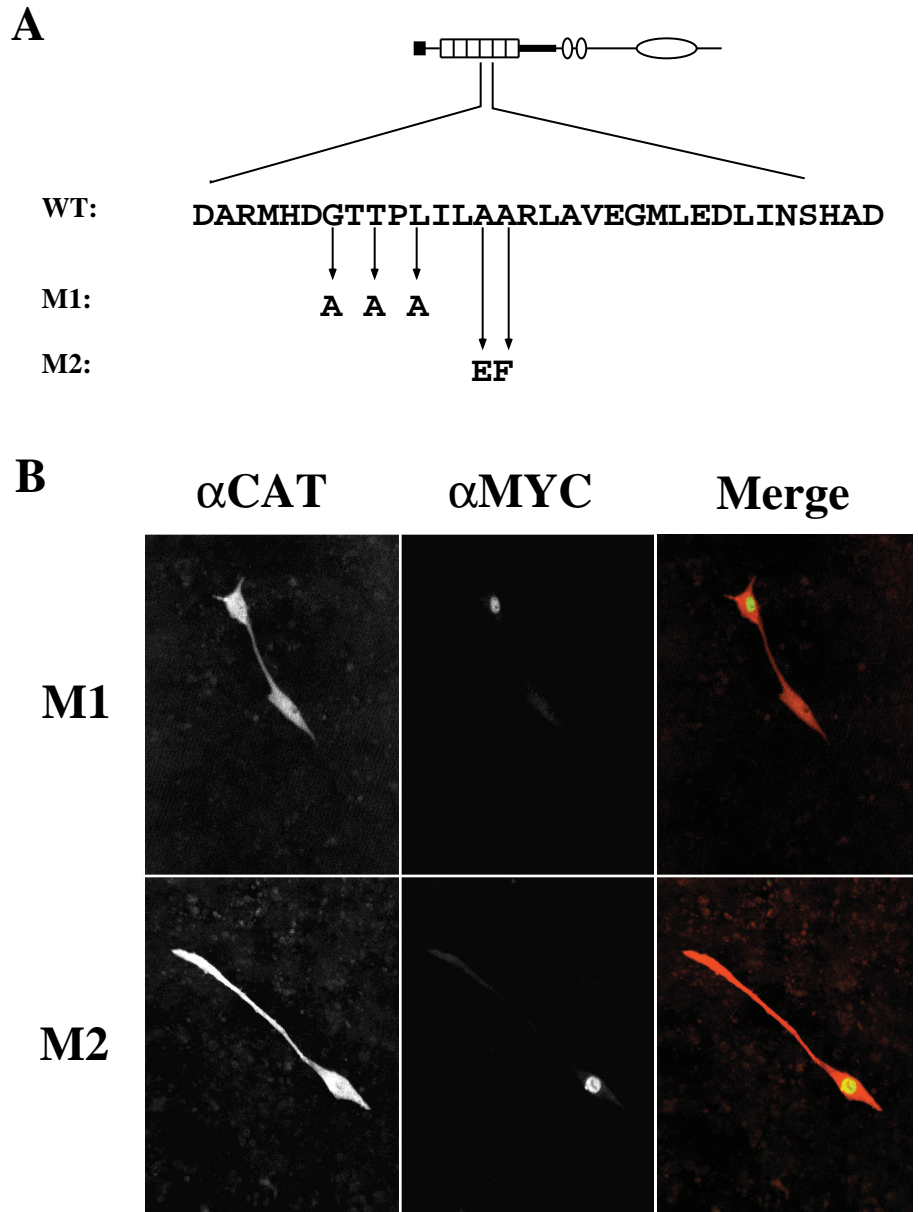


Fig. 7. Point mutation in CDC10/SWI6 repeats abolish myogenic inhibition but not nuclear localization: two independent mutations (A; M1 changes GxTpLxxAA into AxApAxxAA and M2 changes GxTpLxxAA into GxTpLxxEF) result in nuclear localizing mNotchIC but this time most cells (39/41; 95%) also express CAT in the cytoplasm and differentiate in response to MyoD (elongated, muscle-like morphology, fused cells in M1; B). Transfections and staining as described in materials and methods. Mutations were introduced by PCR: overlapping oligos that include the nucleic acid substitutions and outside primers were used in the first round in separate reactions to amplify short segments of mNotchIC. The PCR products were gel purified, combined and amplified again with the outside primer pair to generate a single longer segment. This segment was digested with *BglII* and *EcoRV* and cloned into CS2+6MT containing mNotchIC. Recently Diedrich et al. (1994) report a viable, similar new mutant in *Notch* that suppresses negative complementation of *Abruptex* alleles and may be deficient in interactions with *deltex*. This allele, however, is not reported to have a neurogenic phenotype, indicating it is functional in lateral inhibition.

myotome may be very small (two to five cells). This implies that the myotome could be specified by very local interactions involving only a few cells.

The availability of committed myoblasts (C2) as well as cells that can be induced (P19; Nye et al., 1994) or coerced with MyoD or Myf-5 (3T3) to become myoblasts facilitated testing the role of mNotch in negatively regulating myogenesis. Our functional assay used the intracellular domain of mNotch, mNotchIC, which acted as a constitutive inhibitor of myogenesis in all of these situations as well as during embryogenesis in frogs. Because the role of full-length mNotch is not known in mammals, we cannot be certain that our data with mNotchIC faithfully reflects the true function of full-length mNotch; however, in both flies and worms (Struhl et al., 1993; Lieber et al., 1993; Rebay et al., 1993; Fortini et al., 1993), this has been clearly demonstrated and given the conservation of this molecule it is likely that mouse NotchIC behaves similarly. Recent mouse knock-outs of mNotch1 (Swiatek et al., 1994) failed to show a specific phenotype interpretable as an altered cell fate; however, interpretation is made difficult since there are several Notch genes in mice. Coffman et al. (1993) have recently shown that when a Xotch derivative deleted of the extracellular domain (Xotch Δ E) is injected into frog embryos, extra muscle is produced – a phenotype different from the one that we describe. The activity of this Xotch derivative (which retains the transmembrane domain) has not been studied in detail in flies and worms and therefore it is not clear whether this is in fact a dominant active receptor. These authors suggested that their Xotch construct was in fact a constitutive signaling molecule which might initially inhibit myogenesis but after the Xotch Δ E mRNA and protein turn over, there are now more cells to enter the myogenic compartment. Their results and interpretation are consistent with ours if, under their conditions, Xotch Δ E mRNA and protein turn over more rapidly.

Deletion analysis of mNotchIC

To address the mechanism of signal transduction, we used our *in vitro* assay to dissect the functional elements in mNotchIC. Myogenic inhibition (but not nuclear localization) is lost if point mutations are introduced into the CDC10/SWI6 repeat, number 4, suggesting that the CDC10/SWI6 domain is required for myogenic inhibition, most likely by mediating protein-protein interactions (LaMarco et al., 1991).

Both inhibition of MyoD and nuclear localization are lost with deletions removing both the putative amino terminal NLS (KKFR) and the internal 48 amino acids containing the second putative NLS. Inhibitory and nuclear localization activity were both partially restored when the SV40 large T antigen NLS was inserted upstream of the epitope tag (Table 1C,D). *In vivo*, mNotchIC in frog embryos (not shown) and Notch(intra) in the fly also localize to the nucleus (Struhl et al., 1993; Lieber et al., 1993; Fortini et al., 1993). Taken together, the mutagenesis experiments suggest that both the nuclear localization signal and the CDC10/SWI6 domain 4 are required for myogenic inhibition. It should, however, be noted that attempts to show that *Drosophila* Notch localizes to the nucleus after activation have thus far failed to detect such a species (Lieber et al., 1993).

mNotch participates in a pathway that regulates transcriptional activation by MyoD and Myf-5

The MyoD/VP16 fusion protein acts independently of a

cellular co-activator (see below). The activation of both MCKCAT and 4RCAT by MyoD/VP16 fusion protein in our cell culture assay system suggests that DNA binding of MyoD can occur in the presence of mNotchIC. Together with the data from fusion of MyoD with Gal-4, which suggest that mNotchIC does not affect the MyoD activation domain, our data are best explained if mNotchIC inhibits a co-factor required for MyoD activation of myogenic genes. Supporting this conclusion is the observation that mNotchIC and MyoD do not interact in gel mobility shift assays using the MEF1 Ebox of the MCK promoter (data not shown). The co-factor is unlikely to be E12 as addition of excess E12 does not ameliorate the inhibition; forced heterodimers of MyoD and E12 (Neuhold and Wold, 1993) are inhibited as well (Table 3).

Mutagenesis of MyoD suggests such a co-factor works through the bHLH DNA-binding domain of MyoD and use of a reporter containing four MyoD-binding sites shows that the inhibition works through the MyoD-binding site. In *Drosophila*, it has recently been shown that the production of ectopic bristles by a minimal construct (the bHLH region of lethal of scute) is still subject to lateral inhibition (Hinz et al., 1994). Previous observations have identified MyoD basic region mutations that bind DNA but fail to activate muscle gene transcription. A co-factor that recognizes the basic region of MyoD was postulated to explain this result (Davis and Weintraub, 1992). Similarly, absence of a co-factor was postulated to explain why MyoD does not function in rhabdomyosarcoma cells (Tapscott et al., 1993). It is an intriguing possibility that such a factor might be the target for the repression of myogenesis (and maybe neurogenesis) by mNotch.

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