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Ectopic Expression of MyoD1 in Mice Causes Prenatal Lethalities

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ABSTRACT **A variety of differentiated cell types can be converted to skeletal muscle following transfection with the myogenic regulatory gene MyoD1. To determine whether MyoD1 is a dominant muscle regulator in vivo, mouse fertilized eggs were microinjected with a β-actin/MyoD1 gene. Ectopic expression of MyoD1 during mouse embryogenesis led to embryonic lethalities, the cause of which is not known. Transgenic embryos died before midgestation. The majority of tested embryos between 7.5 and 9.5 days, although retarded compared to control littermates, differentiated normally into tissues representative of all three germ layers. In most transgenic embryos there was no indication of myogenic conversion. The expression of the introduced gene was detected in all ectodermal and mesodermal tissues but was absent in all endodermal cells. Forced expression of MyoD1 was associated with the activation of myogenin and MLC2 (but not myf5 or MRF4) genes in non-muscle cell types, demonstrating the dominant regulatory function of MyoD1 during development. These results demonstrate that ectopic MyoD1 expression and activation of myogenin and MLC2 have no significant effects in the determination of cell lineages or the developmental fate of differentiated mesodermal and ectodermal cell lineages.** © 1993 Wiley-Liss, Inc.

Key words: Myogenin, MLC2, Transgenic embryos, Embryonic lethality

INTRODUCTION

MyoD1 is a member of the myogenic regulatory gene family, which also includes myf5, myogenin, and MRF4 genes (Davis et al., 1987; Wright et al., 1989; Edmonson and Olson, 1989; Braun et al., 1989; Braun et al., 1990; Rhodes and Konieczny, 1989; Miner and Wold, 1990). The products of these genes have common structural motifs, the basic and helix-loop-helix domains, required for binding to the consensus DNA sequence CANNTG found in the enhancer or promoter of many muscle specific genes, and for hetero- or homodimer formation (Murre et al., 1989; Lassar et al., 1989, 1991; Sartorelli et al., 1990; Weintraub et al., 1990; Wentworth et al., 1991; Piette et al., 1990). Members of this family function as transcriptional activators and are able to regulate their own transcription (Weintraub et al., 1991).

During mouse embryonic development, the MyoD1 gene is activated first at 10.5 days of gestation in the myotomal part of rostral somites and in the visceral arches, whereas at day 11.5 it is also expressed in the muscle cell masses of the limb buds (Sassoon et al., 1989; Faerman and Shani, submitted). The distinct temporal and spatial pattern of expression of this myogenic regulator as well as other members of this family in the myotome, the craniocervical, and limb bud regions suggests that multiple myogenic programs exist for myoblasts differentiating in these regions. This could be attributed to different muscle cell lineages. The recent quail-chicken chimera studies of Ordhal and Le Douarin (1992), demonstrated that two myogenic cell lineages could clearly be distinguished in the somites, one confined to the myotomal muscles and the other migrating out of the somite and giving rise to the skeletal cell masses in the limb buds.

To elucidate the function of the myogenic regulatory genes, a variety of somatic cell types have been transfected. These in vitro studies have shown that forced expression of MyoD1 gene in several somatic non-muscle cells (derived from the three germ layers) leads to the stable conversion of these cells to myoblasts, capable of differentiation into multinucleated myotubes, or at least to the activation of a number of muscle specific genes (for review see Olson, 1990; Emerson, 1990; Weintraub et al., 1991). Recently we have shown that forced expression of MyoD1 in totipotent embryonic stem cells results in the conversion of 30–50% of the cells to skeletal muscle cells, when induced to differentiate under specific conditions (Dekel et al., 1992). Thus, even uncommitted embryonic cells can respond to the myogenic conversion ability of this gene. However, not all embryonic cells responded to MyoD1, and a large variety of non-myogenic cell types continued to appear in these cultures despite MyoD1 expression.

The fact that the 4 myogenic regulators appear to function similarly in vitro, and that no known muta-

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tion is associated with the MyoD1 locus or with any of the other myogenic regulatory gene loci, makes it difficult to assess the developmental role of each of these genes during embryogenesis. One approach devised to test for their function is gene targeting in ES cells. Surprisingly, mice lacking a functional MyoD1 gene were found to be viable and fertile and exhibited no morphological or physiological abnormalities in skeletal muscle (Rudnicki et al., 1992). Alternatively, the cloned genes (or specifically modified genes) can be reintroduced into the mouse genome and expressed constitutively. In this case, the normal function of these genes and the interrelationships between them can be inferred from abnormal phenotypes resulting from a change in the copy number, or from inappropriate expression in ectopic positions. Using a slightly different approach, to study the function of the myogenic regulators in *Xenopus laevis*, excess copies of the corresponding mRNAs were microinjected into ectodermal cells of fertilized eggs (Hopwood and Gurdon, 1990; Hopwood et al., 1991). These results demonstrate that an excess of MyoD1 or myf5 mRNAs in these embryos is insufficient to irreversibly commit embryonic *Xenopus* cells to myogenesis. However, the ectopic expression of MyoD1 in *Xenopus* embryos results in the transient activation of the muscle-specific actin gene in ectodermal cells, which do not normally form muscle.

Here we report the consequences of the ectopic expression of MyoD1 during mouse embryogenesis. These experiments address whether the transgenic expression of MyoD acts dominantly to convert non-myogenic cell lineages to skeletal muscle. Our results show that forced expression of MyoD1 leads to embryonic lethalities at mid gestation. Analysis of transgenic embryos at different stages after microinjection revealed no evidence for dominant activity of MyoD1 in myogenic conversion. The major effect of ectopic MyoD1 expression is developmental retardation, implying that MyoD1 expression can disrupt the entire developmental process.

RESULTS

Attempts to Produce Transgenic Mice Expressing the β-actin/MyoD1 Gene Construct

An expression vector including MyoD1 cDNA and the chicken β-actin promoter and its first untranslated intron and the SV40 polyadenylation signal (Dekel et al., 1992) was microinjected into mouse fertilized eggs. Surviving zygotes were implanted into the oviducts of pseudopregnant CD1 females and allowed to develop to term. Tail DNA of weaned offspring was analyzed by southern blot hybridization to identify transgenic mice. Of more than 80 offspring screened, none carried the injected DNA sequences. Since we routinely produce transgenic mice with an efficiency of 10 to 40% of the offspring, a likely interpretation of these results is that forced expression of MyoD1 during mouse development is deleterious to early development of embryos.

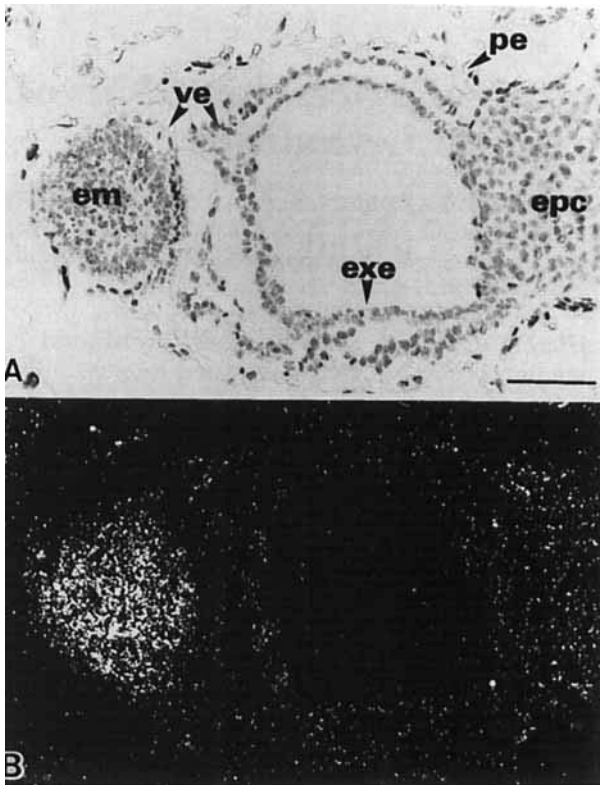


Fig. 1. Bright field (**A**) and dark field (**B**) microphotographs of a section of 7.5 day p.c. embryo expressing the β-actin/MyoD1 gene construct. The MyoD1 transcripts are detected in the embryonic ectoderm/mesoderm cells (em). No signal is detected in extraembryonic ectoderm (exe), ectoplacental cone (epc), and in the endoderm—both visceral (ve) and parietal (pe). Bar = 100 μm.

In Situ Hybridization Analyses of Transgenic Embryos Expressing the β-Actin/MyoD1 Gene Construct

To determine the stage of development at which transgenic conceptuses were dying and the possible cause of their death, we examined 12 such embryos from 7.0 to 9.0 days after microinjection, both histologically and by in situ hybridization. At these stages transcripts of the endogenous MyoD1 gene are not yet detectable (Sassoon et al., 1989).

Day 7.5 transgenic embryos. Figure 1 shows a transgenic embryo isolated 7.0 days after microinjection (equivalent to day 7.5 of gestation). Compared to control littermates, this embryo resembles day 6.5–7.0 mouse embryos, since the proamniotic cavity is not yet separated into the amniotic and ectoplacental cavities (Rugh, 1990; Theiler, 1972). This embryo is also bent along the border between the embryonic and extraembryonic regions. The dark field picture shows the hybridization to the MyoD1 riboprobe. Strong signal is found in the ectodermal/mesodermal cell layers of the embryonic region (Fig. 1B). Interestingly, no signal is detected in the endodermal layer of this embryo (Fig.

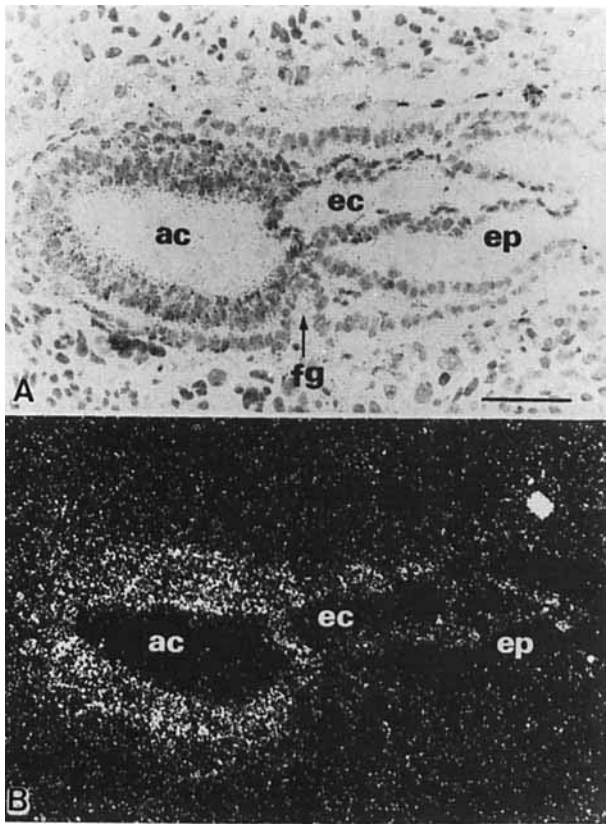


Fig. 2. Bright field (**A**) and dark field (**B**) microphotographs of a section of 8.5 day p.c. embryo expressing the β-actin/MyoD1 gene construct. The MyoD1 transcripts are detected in the embryonic ectoderm/mesoderm cells as well as in the extraembryonic mesoderm. ac, amniotic cavity; ec, exocoelom; ep, ectoplacental cavity; fg, foregut indentation. Bar = 100 μm.

1A,B), or any of the transgenic embryos that were analyzed in this study (see below).

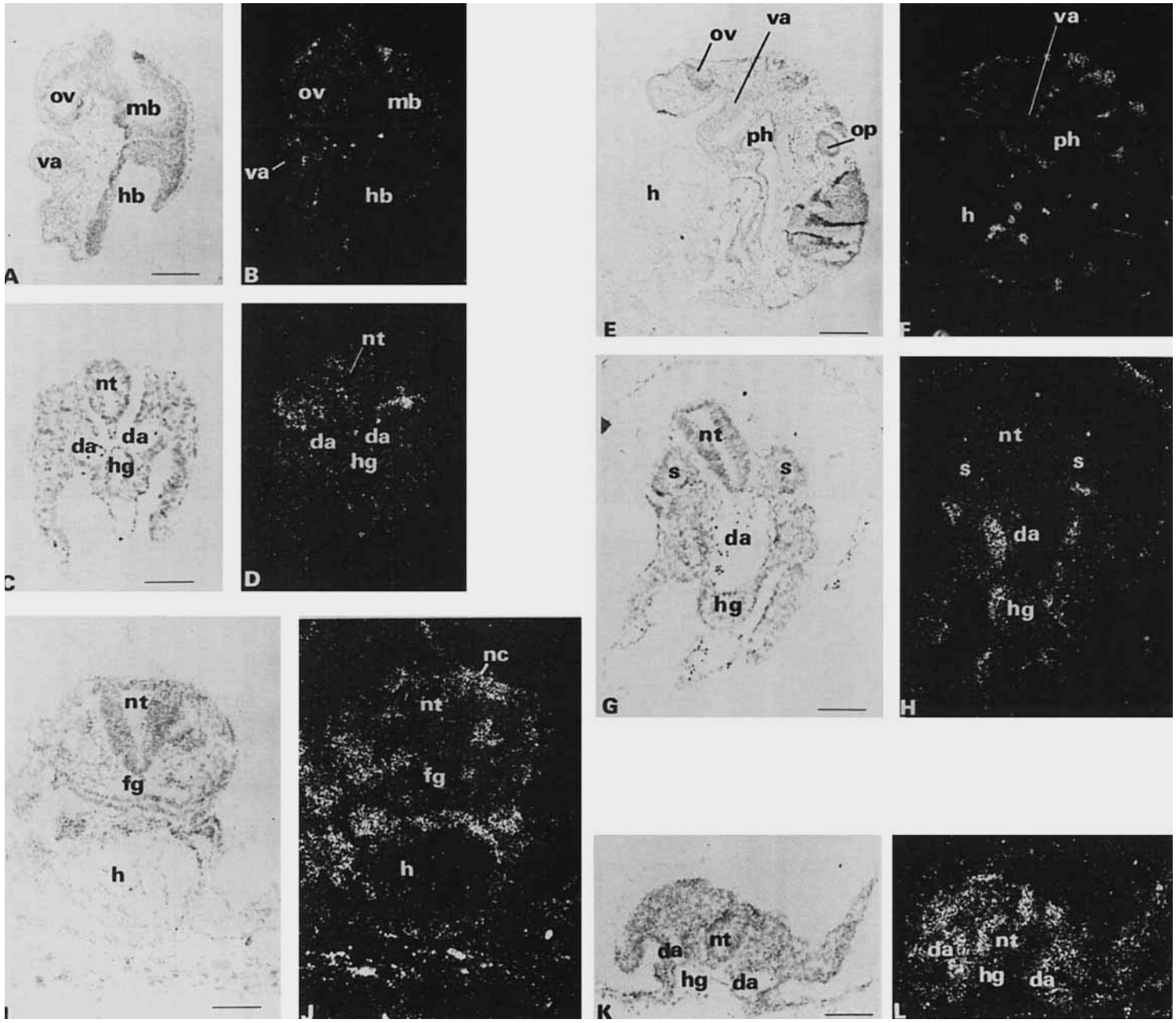
Day 8.5 transgenic embryo. Figure 2 shows a sagittal section of a transgenic embryo isolated 8 days after microinjection (equivalent to day 8.5 of gestation). Morphologically, this embryo resembles day 7.0–7.5 mouse embryos, since there is no sign of heart or somite formation. Expression of the MyoD1 transgene is confined to the ectodermal and mesodermal cell layers. No hybridization signal could be detected in the endodermal cell layer.

Day 9.5 transgenic embryos. Figure 3 shows three examples of day 9.5 transgenic embryos that differ in their intensities of hybridization to the MyoD1 probe and also in their morphologies. In the first transgenic embryo MyoD1 is expressed at a low level and the hybridization signal is concentrated in small patches of cells (Fig. 3A–D). The morphology of this embryo appears to be normal, with no apparent signs of developmental retardation. In the second transgenic embryo, the intensity of the hybridization signal is stronger. Patchy hybridization is more pronounced. The morphology of this embryo also appears normal (Fig. 3E–H). The third and more prevalent type of transgenic embryo shows a very strong hybridization to the MyoD1 probe, the pattern of the signal is very patchy, and its development is retarded (Fig. 3I–L). Retardation is displayed by the incomplete turning of the embryo and disruptions in the neural tube, which is closed only at the midbody region. According to these criteria, this transgenic embryo resembles day 8.5–9.0 normal mouse embryos.

An interesting abnormal transgenic embryo was identified at this stage (Fig. 4). The embryo consisted of 2 embryonic layers in the dorsal region and 3 embryonic layers in the ventral part. Some cells in the mesodermal layer resemble blood cell precursors while a large number of cells had a spindle shaped morphology. Based on the hybridization of serial sections with MyoD1 and MLC2 probes, MLC2 hybridization was confined to only a part of MyoD1 positive cells in the mesodermal layers, and many of those cells had spindle shaped morphology (Fig. 4). Interestingly, no signal could be detected with the myogenin probe (data not shown). In view of the expression of MyoD1 and MLC2 genes as well as the morphology of these cells it could be that in this abnormal transgenic embryo a large number of mesodermal cells were converted to myoblasts. However, more extensive analysis with similar embryos of this kind will have to be performed before definite conclusions can be drawn.

Trans-Activation of Myogenin and MLC2 Genes in Non-Muscle Regions

Since the morphology of most transgenic embryos (except the one described above) does not reveal any dramatic alteration or myogenic conversion, it was of interest to determine whether the ectopic expression of MyoD1 was associated with the activation of any skeletal muscle marker genes. To this end we have examined the hybridization to MyoD1, myogenin, myf5, MRF4, and MLC2 probes in serial or parallel sections of a 9.5 day mouse embryo. Figure 5 shows a 9.5 p.c. transgenic embryo in which regions that hybridized strongly to the MyoD1 probe also hybridized to MLC2 and myogenin probes. Signal with all three probes can be detected over mesenchyme and notochord, whereas in the brain wall there is hybridization only with the MyoD1 probe. Sections through the midbody region of this embryo had shown hybridization signal in cardiac muscle cells with all three probes (not shown). There was no detectable signal with the myf5 and MRF4 riboprobes (data not shown). Thus, MyoD1 expression leads to the activation of at least 2 skeletal muscle markers in non-muscle forming regions. In the myotomal region of this transgenic embryo no MyoD1 signal can be detected, while myogenin and MLC2 probes hybridize very strongly. Therefore, the hybridization signals with myogenin and MLC2 probes in this particular region at this developmental stage likely represent the normal expression of the endogenous genes.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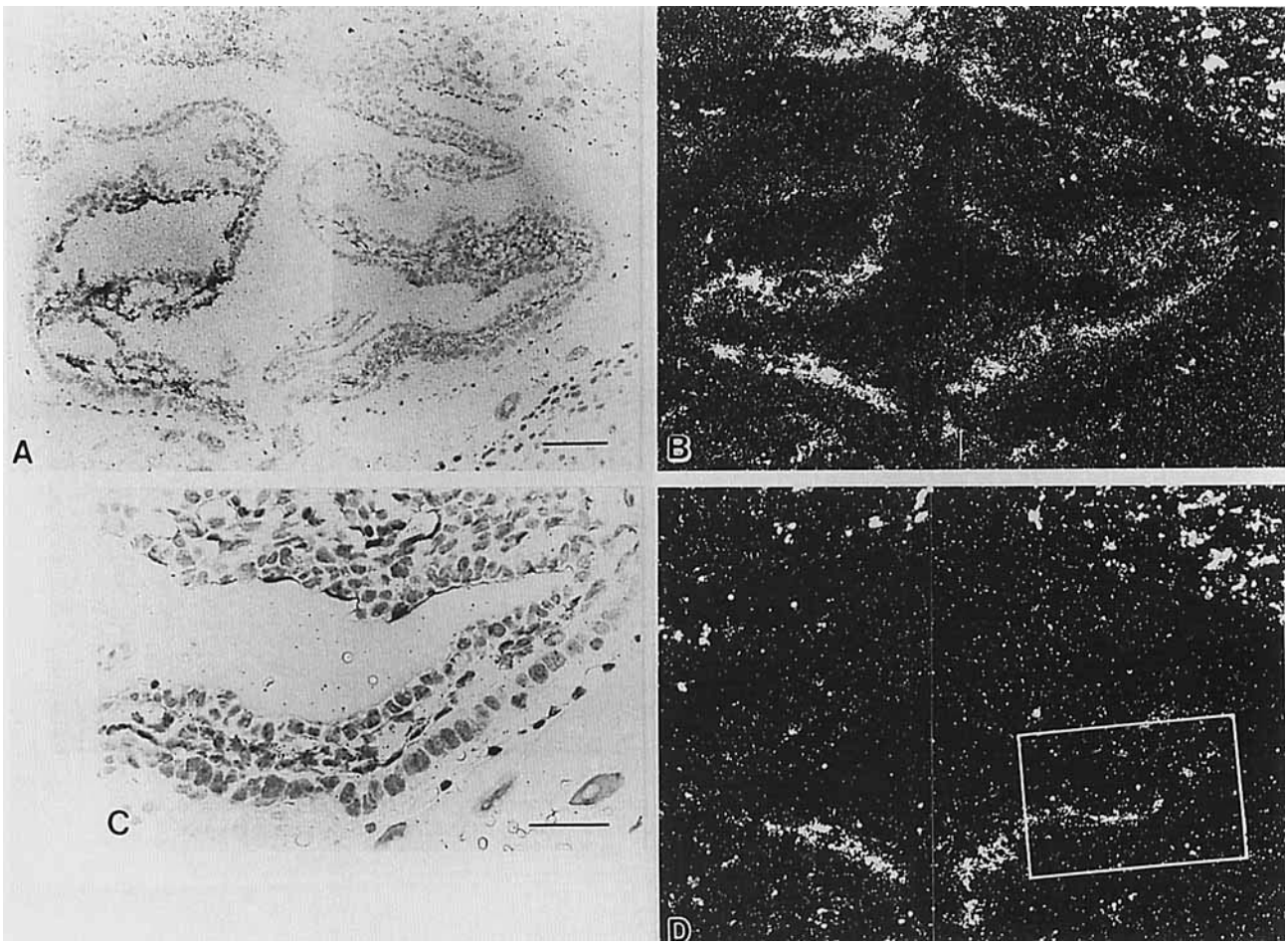

Fig. 4. Microphotographs of serial sections of an abnormal 9.5 day p.c. embryo expressing the β-actin/MyoD1 gene construct hybridized to MyoD1 (**A,B**) and MLC2 (**C**) probes. The dorsal part of the embryo is at the top. A and B, represent bright field and dark field images of the same section. C, represents magnified bright field image of the area boxed in **D.** Bar = 100 μm in A, B, and D and 50 μm in C.

DISCUSSION

The major aim of the present studies was to produce a "gain-of-function" mutation that through its phenotype the normal function of MyoD1 might be revealed. In contrast to the apparently normal phenotype of mice lacking a functional MyoD1 gene (Rudnicki et al., 1992), forced expression of MyoD1 during mouse embryogenesis is incompatible with normal development and leads to embryonic lethalities. It should, however, be noted that the phenotype of the "gain of function" is revealed prior to the activation of the endogenous MyoD1 gene, at day 10.5 of mouse development, and thus prior to the "loss of function" phenotype of Rudnicki et al. (1992). Except for one deformed transgenic embryo out of the 12 transgenic embryos that were analyzed in the present study, none showed any indication for myogenic conversion. Even in that deformed embryo, conversion was limited to some of the mesodermal cells into spindle shaped cells that expressed also the muscle specific gene MLC2 but not myogenin. Since the expression of myogenin was found in vitro in all cells undergoing terminal differentiation to skeletal muscle, it could be that in this transgenic embryo the activation of MLC2 and the acquisition of the typical morphology in a subpopulation of mesodermal cells

Fig. 3. Bright field (**A,C,E,G,I,K**) and dark field (**B,D,F,H,J,L**) microphotographs of the sections of 9.5 day p.c. embryos expressing the β-actin/MyoD1 gene construct. A–D: An embryo showing low level of transgene expression. E–H: An embryo showing the intermediate level of expression. I–L: An embryo with the high level of expression. da, dorsal aorta; fg, foregut; h, heart; hb, hindbrain; hg, hindgut; mb, midbrain; nc, neural crest; nt, neural tube; op, otic pit; ov, optic vesicle; ph, pharynx; s, somite; va, visceral arch. Bar = 200 μm in A and E, 100 μm in the rest of the photographs.

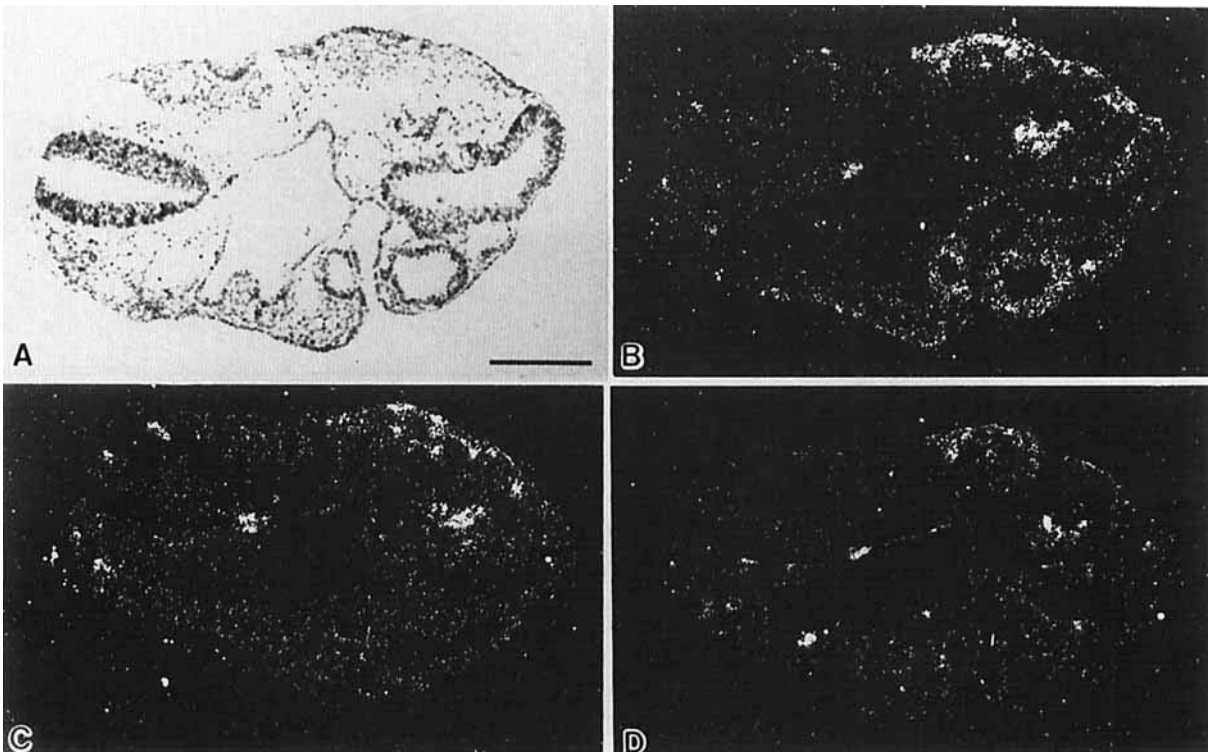

Fig. 5. Microphotographs of serial sections of 9.5 day p.c. embryo expressing the β-actin/MyoD1 gene construct hybridized to MyoD1 (**A,B**), and MLC2 (**C**), and myogenin (**D**) probes. A and B, represent the dark field and bright field images of the same section. Bar = 100 μm.

may occur without myogenin. That forced expression of MyoD1 is not sufficient to convert cells to muscle was also reported for *Xenopus* embryos (Hopwood and Gurdon, 1990; Hopwood et al., 1991) and for hepatocytes (Schafer et al., 1990). Whether ectopic expression of these regulators results in myogenic conversion may well be dependent on their level or the level of their partners (Rashbass et al., 1992).

Surprisingly, most transgenic embryos up to day 9.5 of gestation appeared normal in terms of gross morphology. Every embryonic structure was formed. Therefore, the ectopic expression, presumably from the one-cell stage, did not interfere with the normal processes of determination and differentiation. This is in striking contrast to in vitro studies showing a dominant effect of MyoD1 expression in a large number of cell types (Choi et al., 1990; Davis et al., 1987; Edmonson and Olson, 1989; Lin et al., 1989; Miner and Wold, 1990; Rhodes and Konieczny, 1989; Tapscott et al., 1988; Thayer et al., 1989; Wright et al., 1989). The ability of early mouse embryos to tolerate the expression of the transgene may be due to the absence of positive regulators that are required for the formation of the functional heterodimers with E12/E47, or to the presence of negative regulators that block the formation of functional heterodimers (e.g., id; Benezra et al., 1990). In that respect, it is interesting to note that from 7.5 to 10.5 days p.c (when we see the onset of the phenotype) Id is abundantly expressed in all embryonic cell layers (Wang et al., 1992).

Forced expression of MyoD1 in these embryos was associated with the activation of myogenin and MLC2 (but not MRF4 or myf5) genes in a variety of non-muscle cell types such as mesenchyme, heart muscle cells, and the notochord. This demonstrates the dominant regulatory function of MyoD1 in the whole animal. Interestingly, ectopic expression of MyoD1 in ES cells resulted in the trans-activation of the same two genes (Dekel et al., 1992), implying that MyoD1 and myogenin are co-regulated in early embryos. These results support the notion that MyoD1 is a unique transcription factor with some role in muscle lineage determination as well as differentiation, and that MyoD1 and myogenin appear to be co-regulated in early embryos.

MyoD1 transcripts were found in most mesodermal and ectodermal cell types, but were not detected in endodermal cell lineages. The exclusion of endodermal cells is puzzling, since in vitro studies have demonstrated that MyoD1 can be expressed in endoderm derived liver cells (Weintraub et al., 1989, 1990; Choi et al., 1990; Schafer et al., 1990). However, the conversion of hepatocytes to muscle cells occurred only after fusion

with fibroblasts (Schafer et al., 1990). It could be that embryos expressing the transgene in endodermal cells are selected against and die earlier. Alternatively, it could be that the chicken β-actin promoter employed here is inactive in endodermal cells. Using the rat β-actin promoter to drive the bacterial β-galactosidase gene Beddington et al. (1989) have shown LacZ staining in cells of different germ layers including endodermal cells.

An interesting observation was also the patchy pattern of MyoD1 expression in many of the 9.5 days transgenic embryos, which express the exogenous MyoD1 at high levels. This may indicate that, while some cells can tolerate a high level of MyoD1 expression, others cannot. Alternatively, patchy expression may simply reflect different activity levels of the β-actin promoter in different cell lineages.

A common feature in many of these transgenic embryos was developmental retardation. Since morphologically these embryos appeared normal, retardation could be a general effect at the cell level. One possibility could be that ectopic expression of some transcription factor during mouse embryogenesis would result in a general toxicity and delayed development. Alternatively, retardation could be due to the role of MyoD1 in the control of the cell cycle (Sorrentino et al., 1990; Crescenzi et al., 1990). It was demonstrated that forced expression of MyoD1 in 10T1/2 cells resulted in a 10-fold reduction in the number of stable colonies compared to the selectable gene alone (Davis et al., 1987) and that the HLH domain is involved in the inhibition of cell proliferation (Sorrentino et al., 1990; Crescenzi et al., 1990). Interestingly, MyoD1 can suppress cell growth in cell types that are refractory to myogenic conversion (e.g., CV1 cells). Since no transgenic embryos could be identified later than 9.5 days of gestation, the precise cause of death remains to be determined.

Recently, Miner et al. (1992) reported on the ectopic expression of MyoD1 in transgenic mouse hearts. Their results demonstrated that forced MyoD1 expression in the heart resulted in abnormal heart morphology, leading to the death of transgenic embryos at 16–18.5 days of gestation. However, the abnormal morphology was not associated with the formation of multinucleated myotubes and the extent of conversion to skeletal muscle could have been restricted to a subset of cells (Miner et al., 1992). Nevertheless, the alterations in cellular organization were sufficiently substantial to cause morphological and perhaps functional abnormalities in the heart. It is also of interest that of the three additional myogenic regulators only myogenin was activated by the transgene very similar to our in situ hybridization results. In contrast to our approach, in which MyoD1 expression was driven by a strong constitutive promoter before determination of any myogenic program (skeletal or cardiac), the onset of MyoD1 expression in the heart of those transgenic embryos was rather late (day 13 of gestation), several days after the onset of cardiogenesis in the mouse. Therefore, it is not too surprising that only partial conversion to skeletal muscle was observed. As suggested by these authors, it could well be that in those cardiocytes both myogenic programs (skeletal and cardiac) co-exist.

In the experiments described here we expressed MyoD1 gene product ectopically to evaluate MyoD1 function in a broad range of cell types that do not normally express detectable MyoD1 in vivo. The results clearly demonstrate that MyoD1 expression and the activation of associated myogenic genes such as myogenin and MLC2 have no significant effects in the determination of cell lineages or the developmental fate of differentiated mesodermal and ectodermal cell lineages.

EXPERIMENTAL PROCEDURES

Construction of the β-Actin/MyoD1 DNA Fragment

Construction of the β-actin/MyoD1 hybrid gene was described previously (Dekel et al., 1992). Briefly, the chicken β-actin promoter, its first untranslated exon, and entire first intron were fused to the full length mouse MyoD1 cDNA (GeneBank M84918; Pinney et al., 1988) flanked by the SV40 polyadenylation signal.

Production of Transgenic Embryos

DNA was prepared for injection into fertilized mouse eggs by digestion with SmaI and BamHI, gel electrophoresed to separate the transgene from vector sequences, followed by purification through elutip.

Transgenic embryos were generated as described (Hogan et al., 1986; Shani, 1986), using FVB/N mice as embryo donors and CD1 mice as foster mothers. Transgenic embryos were identified based on the expression of the introduced MyoD1 gene determined by in situ hybridization.

In Situ Hybridization

Deciduas containing embryos were excised at different stages following microinjection and fixed in freshly prepared 4% paraformaldehyde in PBS. After dehydration in alcohol and clearing in chloroform, deciduas were embedded in paraplast. Five micron sections were cut and mounted onto polylysine-coated slides. Before hybridization, the sections were deparaffinized with xylene, rehydrated, and sequentially treated with 2 × SSC (70°, 30 min), pronase (0.125 mg/ml in 50 mM Tris-HCl, 5 mM EDTA, pH 7.5), 10% formalin in PBS (20 min), and 0.2% glycine in PBS. After washings in PBS (5 min) the slides were dehydrated through increasing concentrations of ethanol and air dried. Hybridization mixtures containing 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM $NaPo_4$ (pH 6.8), 5 mM EDTA, 1 × Denhardt, 10% dextran sulphate, 250 μg/ml yeast total RNA, 10 mM DTT, 50% (v/v) deionized formamide, and 10^4 cpm/μl of ^{35}[S]-UTP labeled antisense RNA were added to the sections, covered with plastic

coverslips, and sealed with rubber cement. The slides were incubated in a humidified chamber overnight at the appropriate hybridization temperature. Following hybridization the slides were incubated in 5 × SSC at 50°C for 40 min. During this incubation the coverslips were removed and the slides were washed in 50% formamide in 2 × SSC (65°C, 30 min), 2 × SSC (37°C, 15 min), RNase treated (20 μg/ml in 0.5 M NaCl, 10 mM Tris-HCl pH 8.0), washed again in 50% formamide, 2 × SSC for 30 min at 65°C, 2 × SSC for 15 min at 37°C, and dehydrated in ethanol containing 0.3 M ammonium acetate and air dried. For autoradiography, the slides were dipped in Kodak NTB-2 emulsion diluted 1:1 in water, dried, and exposed for 4–21 days (depending on the embryonic stage and the probe). The exposed slides were developed in Kodak D-19 developer and fixed with Kodak fixer. Sections were stained with hematoxylin, air dried, and coverslipped.

The MyoD1 and myogenin probes used were as described in Sassoon et al. (1989) and correspond to the 3′ untranslated regions of the mRNAs. The myf5 and MRF4 probes used were those described in Ott et al. (1991) and Bober et al. (1991), respectively. These probes were kindly provided by M. Buckingham. The MLC2 probe was prepared from the pGEM plasmid p103, containing 274 bp of the 3′ end of the rat skeletal muscle MLC2 mRNA (Katcoff et al., 1980).

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