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Original Article

Desmin transgene expression in mouse somites requires the presence of the neural tube

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ABSTRACT Transgenic mice were used to study the effect of the neural tube on somite myogenesis. These mice express a transgene in which the 1 kb DNA 5' regulatory sequence of the desmin gene is linked to a reporter gene which codes for *E. coli* ß-galactosidase. In order to determine whether the developmental fate of cells, specifically the prospective myogenic population, in newly developed somites was pre-determined, newly formed somites were isolated from the caudal region of day 9.5 transgenic embryos and transplanted into 8.5 day non-transgenic host embryos. Even though the implanted somites were not oriented in the host embryos, all the specimens examined developed normally at the graft site forming a dermatome, myotome and sclerotome in the correct anatomical positions. The myotome even expressed the desmin transgene. In addition, we isolated the 3 most caudal somites, that is, the most recently developed somites, from day 9.5 transgenic embryos and maintained them on gelatin-coated coverslips in culture for up to 4 days. While these somite explants did not develop myoblasts, it was possible to induce myogenesis by introducing pieces of neural tube into the explant cultures. These results suggest that the developmental fate of cells within the newly formed somite is not pre-determined, but is dependent on the microenvironment surrounding the developing somite.

KEY WORDS: *myogenic cells, somites, neural tube, transgenic embryos*

Introduction

During embryogenesis, the paraxial mesoderm becomes segmented in a cranio-caudal sequence, to give rise to the somites. These somites are established in pairs, each flanking the neural tube and notochord. A newly-formed somite is a spherical structure surrounded by a sheet of pseudostratified epithelial-like cells and filled with cells that will develop into the vertebral cartilage and the vasculature of the limb and body axis (Huang *et al.*, 1994). As somitogenesis proceeds down the length of the body axis, each somite differentiates and becomes subdivided into 3 basic tissues, the dermatome, myotome and sclerotome. The dermomyotome develops into dermal connective tissues and the skeletal musculature of the limbs (Chevallier *et al.*, 1977; Christ *et al.*, 1977; Hayashi and Ozawa, 1991; Lee and Sze, 1993; Goulding *et al.*, 1994; Sze *et al.*, 1995). The myotome forms the musculature of the body wall (Christ *et al.*, 1983; Kaehn *et al.*, 1988; Ordahl and Le Douarin 1992) while the sclerotome becomes the vertebral cartilage (Bagnall *et al.*, 1989).

The onset of myogenesis is observed initially in the myotome of the developing embryo. Kaehn *et al.* (1988) have demonstrated that cells proliferating at the cranial end of the dermatome gradually shift in a caudal direction to become the early myotome. The myotome can easily be identified with myogenic markers such as desmin antibodies (Christ *et al.*, 1992; Borman *et al.*, 1994). In the chick, contact between the axial structures, neural tube and notochord, and the newly-formed somites is apparently vital for the development of the myotome; if the neural tube and notochord are extirpated, the myotome does not form in the adjacent somites (Teillet and Le Douarin, 1983; Christ *et al.*, 1992; Rong *et al.*, 1992). This inhibitory effect can also be reproduced by inserting an impermeable barrier between the somites and the axial complex (Borman and Yorde, 1994). In explant culture experiments, it has been revealed that newly developed somites, obtained from the caudal region of chick embryos cannot produce myoblasts unless they are co-cultured with the neural tube (Kenny-Mobbs and Thorogood, 1987; Buffinger and Stockdale, 1994). Consequently, these authors proposed that myogenic

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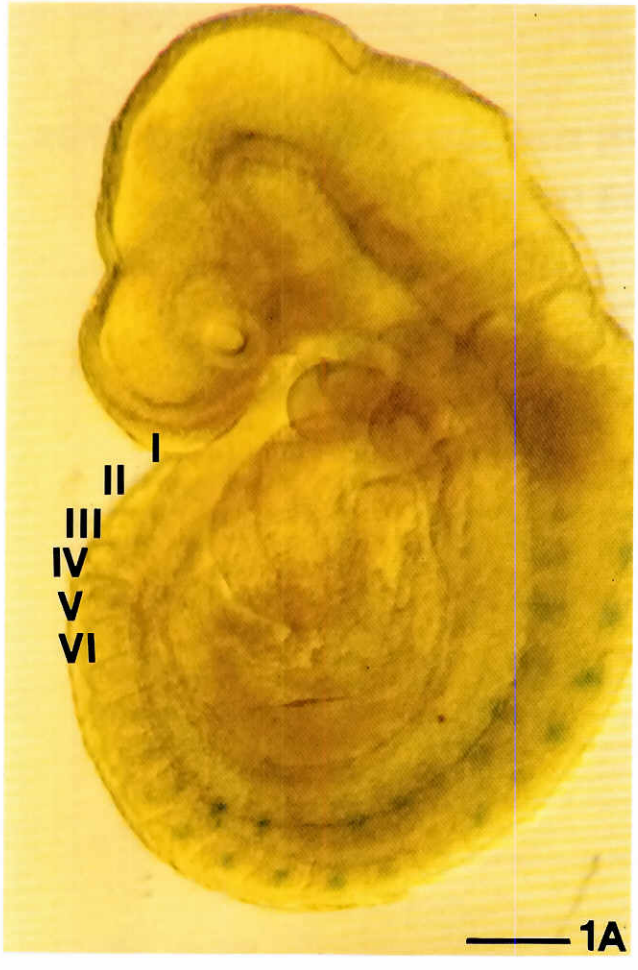

Fig. 1. A day 9.5 desmin transgenic embryo stained for ß-galactosidase. *LacZ expression is not visible in the 6 caudal most somites (I-VI) but is evident in the developing myotome located cranially. Bar, 500 µm.*

specification of the somites requires the inductive influence of the neural tube. Moreover, it has recently been demonstrated that the implantation of a fragment of neural tube or notochord near the vicinity of developing somites can dramatically influence the morphology of the myotome (Brand-Saberi *et al.*, 1993; Pourquie *et al.*, 1993).

In comparison with the chick, far less is known about how somites and the neural tube interact to determine myotome formation in mammalian embryos, although far is known about the molecular biology of muscles in the latter. However, recent improvements in mammalian embryo culture and transplantation techniques (Beddington and Martin, 1989; Lee, 1992; Lee and Sze, 1993) have now made it possible to examine somite development directly in mammals instead of relying on second-hand information extrapolated from Aves. One of the earliest studies to be performed on the mouse demonstrated that somitic cells, isolated from day 9.5 mouse embryos are capable of forming myoblasts when cultured in the absence of the neural tube, (Vivarelli and Cossu, 1986); however the presence of the neural tube dramatically increased the number of myogenic cells. Unfortunately the somites used in this study were obtained from cranial as well as caudal regions of the embryo and so a heterogeneous population of cells derived from both somites that already possessed a myotome and somites that did not have a myotome but were still specified myogenically, were used in the cultures. It is therefore unclear whether the neural tube was specifying the somitic cells to become myoblasts or merely enhancing the proliferation of existing myogenic cells.

In this study, we have made use of transgenic mice that bear a 1 kb 5' DNA regulatory sequence of the desmin gene attached to a reporter gene encoding for *Escherichia coli ß-galactosidase (Des1-nlacZ)* for explant and transplantation studies. Our particular desmin transgene is expressed exclusively in skeletal muscles and is one of the earliest known myogenic markers for the myotome (Li *et al.*, 1993). Using these transgenic mice we have studied (1) the regulatory potential of mouse somites, (2) the expression of the desmin transgene in somites *in situ*, (3) the ability of individual somites to produce myoblasts in the absence of the neural tube during culture and (4) the ability of the neural tube to induce somites, which have not been myogenically specified, to develop skeletal muscle cells.

Results

Myogenic potential of somites in vitro

Day 9.5 transgenic embryos have on average 20-22 pairs of somites. In order to identify the different somites, we assigned each pair a number, the most recently formed somite was designated somite I and the most mature somites at the cranial region were somites XX-XXII. *In situ*, X-gal staining revealed that all of the somites located at the cranial region of the embryo expressed the desmin transgene whereas somites I-VI were lacZ negative (Fig. 1 and Table 1).

In order to determine the myogenic potential of the somites, somites were isolated from both cranial and caudal regions of 9.5 day transgenic embryos and maintained individually on gelatin-coated coverslips for 4 days. All somites that expressed the desmin transgene *in situ* (somites VII-XXII) produced lacZ-positive cells in culture and these cells exhibited a typical myoblastic morphology, being highly elongated and bipolar in shape (Fig. 2A). Of the 21 somite explants analyzed from somites IV-VI, all produced lacZ-positive myoblasts (Fig. 2B); however, none of our 89 somite I-III explants developed myoblasts on examination between days 1 to 4 (Fig. 2C and D).

Development of transgenic somites in non-transgenic hosts

To determine the regulatory potential of newly-formed somites, somites I-III were isolated from day 9.5 transgenic embryos and

TABLE 1

DESMIN TRANSGENE EXPRESSION IN WHOLE DAY 9.5 EMBRYOS AND IN SOMITE EXPLANT CULTURES

	Caudal somites		Cranial somites
Desmin transgene expression	I-III	IV-VI	VII-XX
In situ	-	-	+
In vitro	-	+	+
Co-cultured with neural tube	+	ND	ND

-, no lacZ positive cells; +, cells with lacZ expression; ND, data not available.

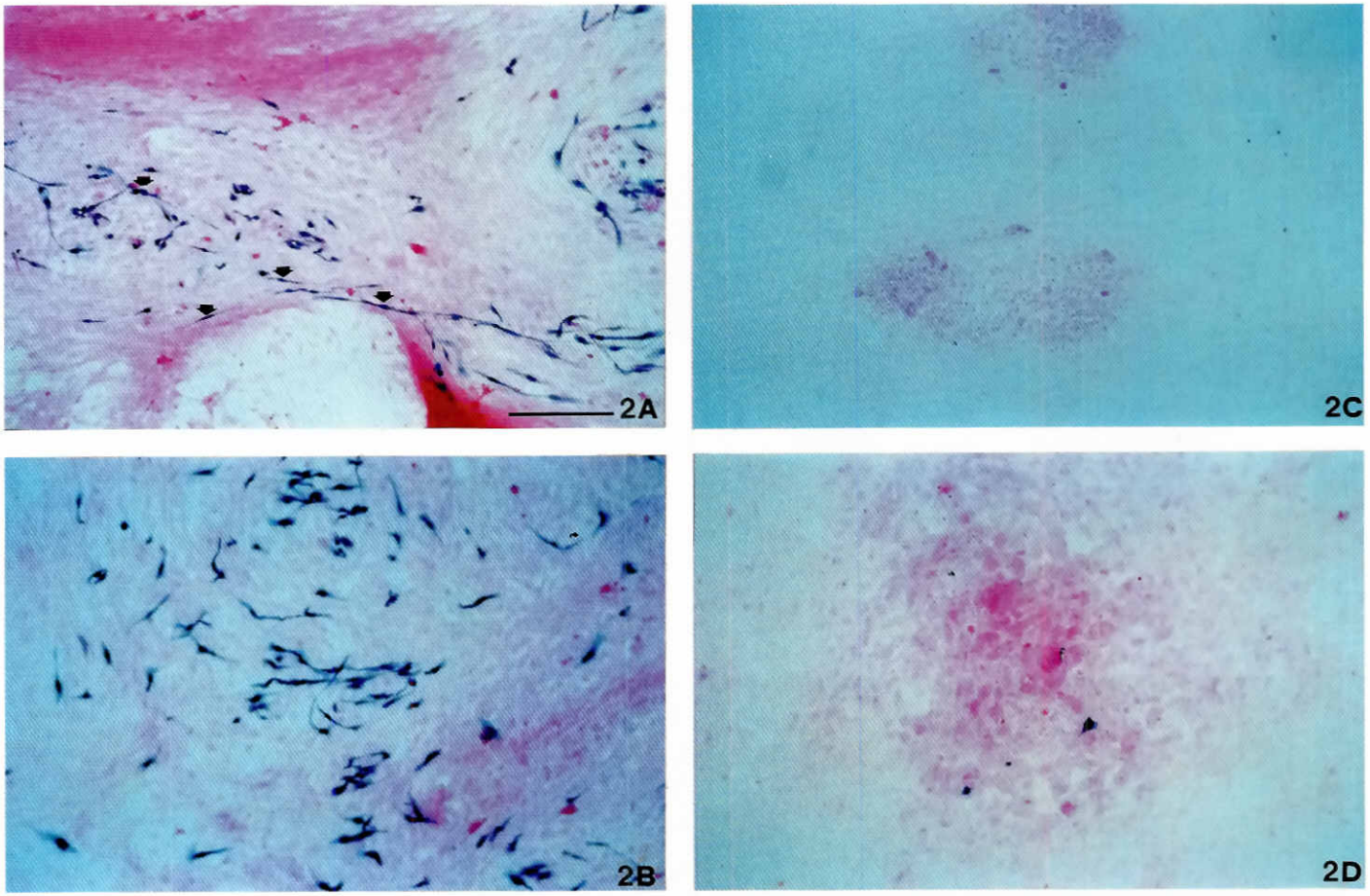

Fig. 2. Myogenic potential of somites isolated from different levels of 9.5 day transgenic embryos and cultured for 4 days. (A) *Somites VII-XX/XXII are capable of producing lacZ-positive myoblasts, (arrows).* **(B)** *Somites IV-VI which do not express the Des1-nlacZ transgene in situ can autonomously develop myoblasts in vitro. Somites I-III are incapable of developing myoblasts on day 2* **(C)** *and day 4* **(D)** *of culture. Bar, 50* μm.

transplanted orthotopically into the caudal region of day 8.5 non-transgenic hosts. A total of 20 host embryos were implanted and cultured whole on a roller culture system for 36 h. To test the ability of the culture system to support normal embryonic development, 20 unoperated day 8.5 embryos were also cultured and in all 20 cases the development of the embryos was normal (Table 2). All the embryos had turned, developed a pair of forelimb buds and 2 pairs of branchial arches and possessed a strong heart beat and an active yolk sac circulation. Similarly, the majority of operated embryos also developed normally during culture (Fig. 3A) with just one embryo being abnormal with a twisted body axis, probably as a result of the transplantation procedure. This abnormal embryo was discarded.

The transgenic somites were not oriented when implanted into the hosts. Despite this, all 19 of them developed normally at the graft site, forming a dermatome, myotome and sclerotome (Fig. 3B). Moreover, these tissues were laid down and arranged in their correct anatomical positions, i.e. with the dermatome situated laterally and myotome situated medially to the neural tube. In all 19 of these specimens, the desmin transgene was actively expressed in the myotome but not in the dermatome or sclerotome (Fig. 3B).

Effect of neural tube on somite myogenesis

Tissue fragments containing a single somite along with its associated ectoderm, endoderm and axial complex were dissected from somite level I-III of day 9.5 transgenic embryos and maintained in culture for 4 days (Fig. 4A). A total of 28 tissue fragments were cultured and lacZ-positive myoblasts and myotubules were found within all the explants (Fig. 4B). In order to determine whether the neural tube was responsible for inducing

TABLE 2

THE DEVELOPMENT OF CONTROL AND EXPERIMENTAL EMBRYOS *IN VITRO*

	No. of specimens	No. & % normal[1] embryos	Initial somite No.	Final somite No.	Percentage of embryos with HB/YS[2]	FLB[3]	O Ves[4]	3rd BA[5]
Control	10	10 (100%)	9.7±0.2	22.8±1.3	100	100	100	100
Transplanted	20	19 (95%)	9.7±0.2	21.2±1.5	100	100	100	100

[1]Grossly normal, embryos without distorted body axis, heart enlargement and open neural folds. [2]HB/YS, active heartbeat and yolk sac circulation. [3]FLB, fore-limb bud. [4]O Ves, otic vesicle. [5]3rd BA, third branchial arch.

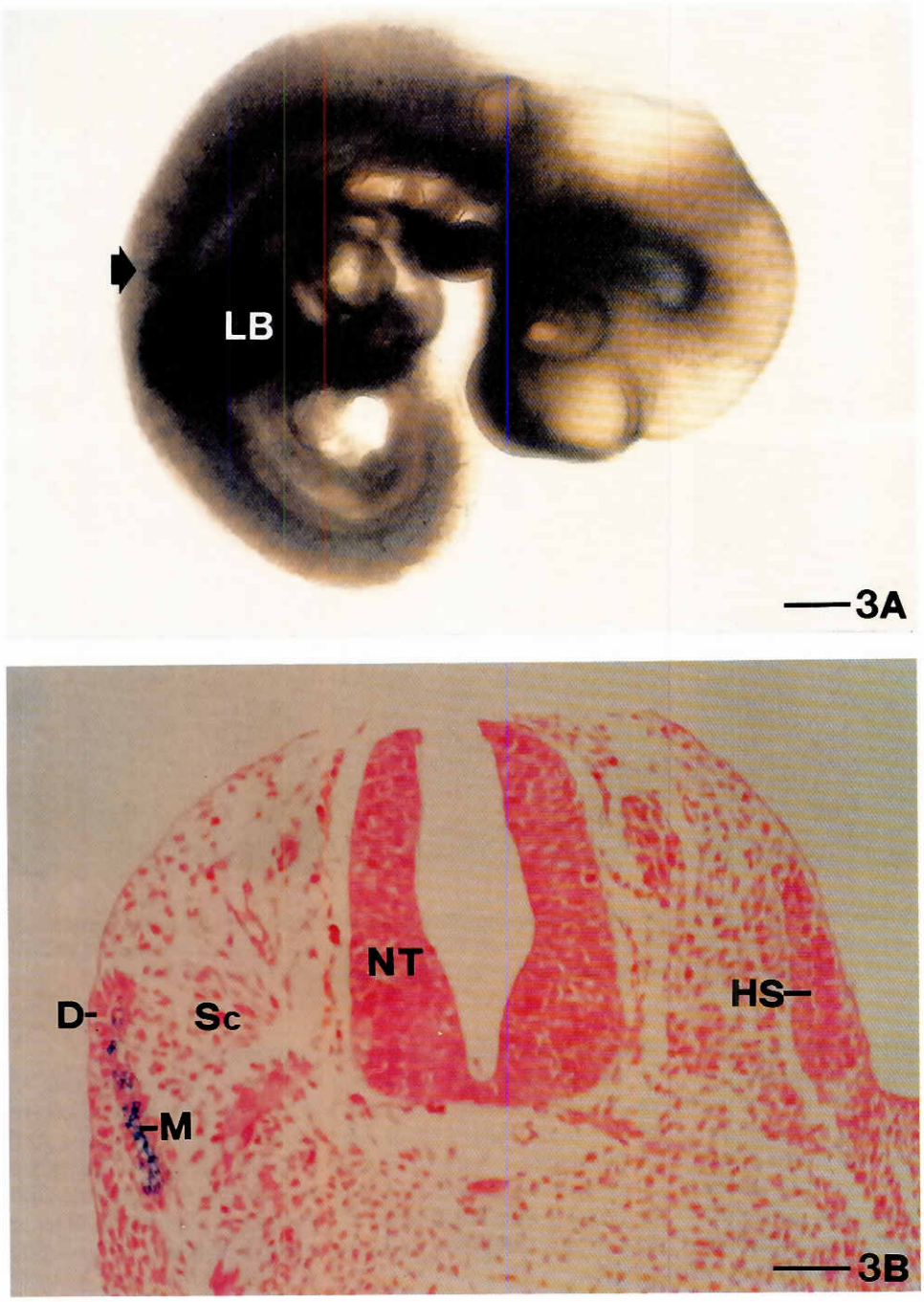

Fig. 3. Transplantation of transgenic somites into non-transgenic host embryos. (A) *Gross appearance of a 36 h cultured embryo with an implanted transgenic somite (arrow). Limb Bud (LB). Bar, 300 μm.* **(B)** *Transverse histological section of the same embryo demonstrating the normal development of the implant to produce dermatome, D, sclerotome, Sc, and a LacZ-positive myotome, M, even though the donor somite was not oriented when transplanted into host embryos. Host somite (HS), Neural tube (NT). Bar, 50 μm.*

the somites to produce myogenic cells, fragments of neural tube were isolated and co-cultured with somites I-III on gelatin-coated dishes for 4 days. Each piece of neural tube was combined with approximately 4 somites. A total of 12 specimens were examined and in all cases lacZ-positive myoblasts were found (Fig. 4C). As a negative control, 10 pieces of neural tube from the caudal region of transgenic embryos were cultured alone for 4 days. None of these cultures produced lacZ-positive cells. In addition, somites I-III were maintained for 4 days in culture medium that had been pre-conditioned by fragments of neural tube; however, none of the 21 somites examined was induced to develop myoblasts (Fig. 4D).

Discussion

The somite is a multipotent structure which differentiates to produce angioblasts, chondrocytes, myoblasts and soft connective tissue cells during development. It is generally accepted that the lateral region of the somite produces the dermatome whereas the medial region produces the sclerotome and myotome (Ordahl and Le Douarin, 1992). In our transplantation experiments, we grafted newly developed transgenic somites, in random orientation, into non-transgenic hosts. As the somites were not oriented, there was a possibility that the lateral aspect of the somite may confront

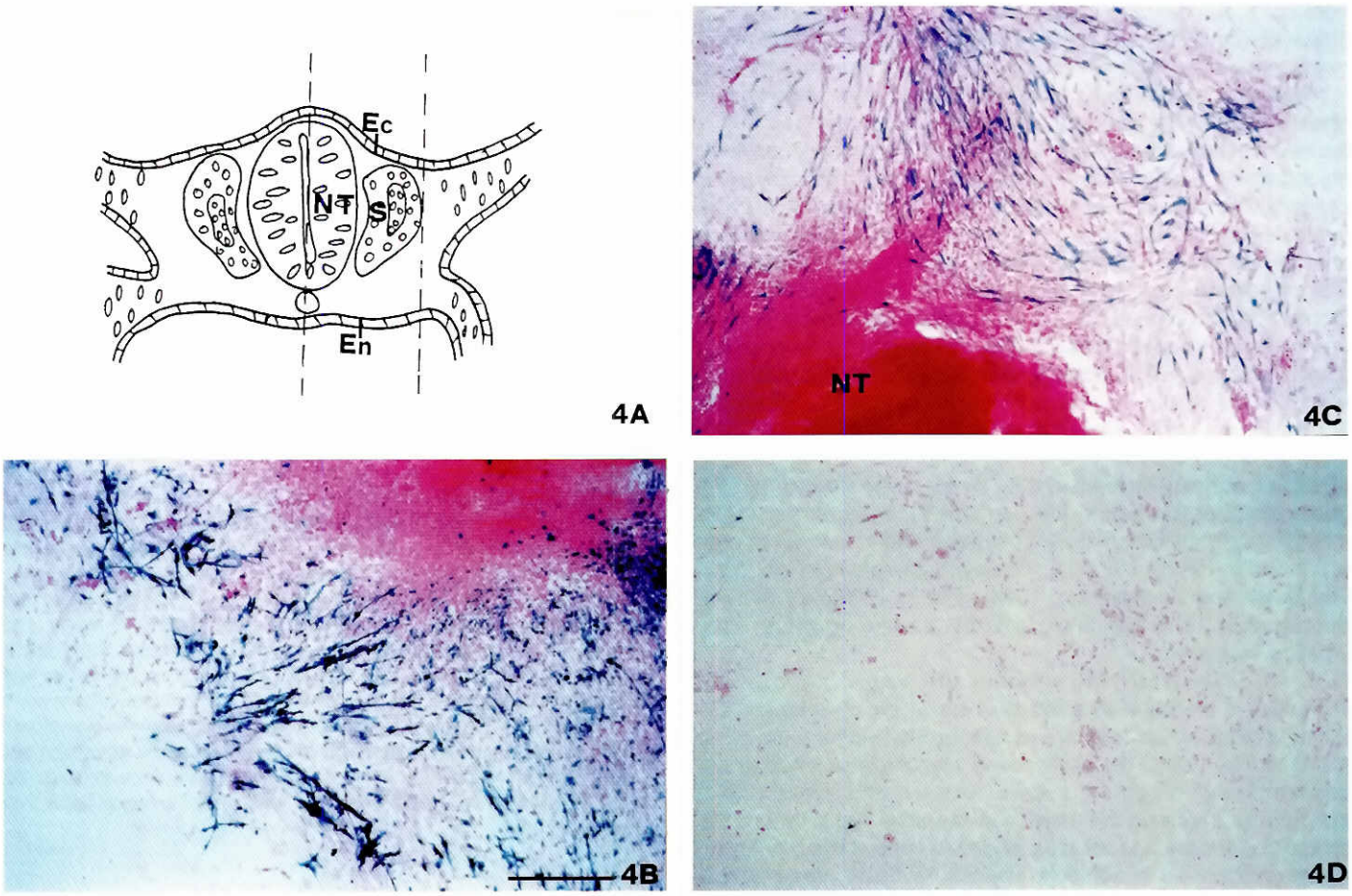

Fig. 4. Effect of the neural tube on somite myogenesis. (A) *Schematic diagram to illustrate the region of neural tube (NT) and somites excised for the culture studies. Ectoderm (Ec), Endoderm (En).* **(B-D)** *Explant cultures.* **(B)** *LacZ-positive myoblasts are found in mechanically dissected explants that contain somites I-III and their adjacent neural tube.* **(C)** *Neural tube, (NT), introduced into somites I-III explants can induce the development of lacZ-positive myoblasts.* **(D)** *Neural tube-conditioned medium does not elicit myogenesis in somites I -III cultures. Bar, 50 μm.*

tissues situated medially instead of laterally and vice versa; however, the implanted somite developed normally at the graft site. This suggests that rather than being pre-determined, the developmental fate of any region within a newly formed somite is interchangeable such that somitic cells, which under normal circumstances become chondrocytes, can instead differentiate into myoblasts. Thus, the developmental fate of somites is not intrinsically determined at the time of somite segregation but depends on information received from their microenvironment instead. This view is supported by recent findings which demonstrated that dorsal ectoderm, extracellular matrices and the neural tube (apart from the floor plate) all appear to stimulate myogenesis (Vivarelli and Cossu 1986; Kenny-Mobbs and Thorogood 1987; Buffinger and Stockdale, 1994). However, the insertion of a piece of notochord near the vicinity of a developing somite can suppress myogenesis but at the same time promote chondrogenesis (Brand-Saberi *et al.*, 1993; Pourquie *et al.*, 1993).

As some of the early studies, such as those reported by Vivarelli and Cossu (1986), make use of somites which have been pooled from various cranio-caudal levels, there is still limited information about the onset of myogenesis in newly formed somites. We have shown that somites I-III on their own cannot produce myoblasts even under optimal culture conditions. However, the situation can be reversed by co-culturing these somites with pieces of neural tube, and then, all the cultures develop myoblasts which express the desmin transgene. These results are in close correlation with those reported for the chick (Kenny-Mobbs and Thorogood, 1987; Rong *et al.*, 1992; Buffinger and Stockdale, 1994). During mammalian somitogenesis, approximately 1.5-2 h are required for a somite to be generated from the paraxial mesoderm (Sze, 1994). The fact that somites IV-VI are already competent to produce myoblasts autonomously, suggests that somites I-III probably require approximately 4.5-6 h of interaction with the neural tube *in situ* for these somites to be myogenically competent. In quail embryos, somites II and III apparently express the mRNA for a homolog of MyoD, qmf1 (Pownall and Emersin, 1992). This view is supported by recent findings in the chick *in ovo* which demonstrate that neuralectomy does not affect the ability of adjacent somites to express the muscle regulatory factor, CMDI and myogenin genes (Bober *et al.*, 1994). These somites can even synthesize desmin cytoskeletal proteins. However, the expression of these muscle specific genes is not maintained after 3 days unless the neural tube is present, and Buffinger and Stockdale (1994) could not obtain myoblasts

from chick somites on examination after 4 days culture, unless they were co-cultured with the neural tube. This suggests that a newly formed somite is already specified and that the neural tube is required to sustain transcription and allow the translation of the MyoD mRNAs. We have cultured somites I-III in the absence of the neural tube but have been unable to obtain desmin transgenic myoblasts on examination between days 1 to 4. As our results contradict the chick's neuralectomy data, such disparity may be explained by the fact that in the neuralectomized embryo the somites still reside within the embryonic microenvironment and factors present there may support limited myogenesis. Kenny-Mobbs and Thorogood (1987) have already indicated that in some instances the ectoderm and collagen can promote myogenesis in somite explants so it is possible that these factors are sustaining myogenesis. In this sense, our *in vitro* cultures reflect more accurately the myogenic potential of the somites because they were cultured free of the surrounding embryonic tissues. Moreover, our experimental system clearly demonstrates also the direct effect of the neural tube on somite myogenesis. In the mouse, the myf-5 regulatory sequence and transcript is also expressed in the caudal most somites (Buckingham *et al.,* 1992; Tajbakhsh and Buckingham, 1994), and so it is likely that the neural tube, as in the chick, acts by supporting rather than initiating, myogenesis.

In order to determine whether the somite/neural tube interaction is mediated by a diffusible signal, we cultured somites I-III in a medium that was pre-conditioned by neural tube. In no cases did we detect the presence of myoblasts expressing our desmin transgene during culture. Borman and Yorde (1994) inserted an impermeable barrier of tantalum foil between the caudal somites and the neural tube and found that this barrier can inhibit myogenesis in adjacent somites. However, the inhibitory effect could not be reversed by replacing the foil with a permeable barrier. Therefore, it appears that the neural tube does not exert its positive effect on somite myogenesis via a diffusible factor and most likely it involves cell to cell contact. With regard to the sclerotome, the situation may be different. It has long been known that the axial complex is required to obtain cartilage from early somites and the effect is mediated by a soluble factor (Grobstein and Parker, 1954; Lash *et al.,* 1957).

In conclusion, the developmental fate of cells within the newly formed somite is flexible and substantially influenced by the embryonic microenvironment. The neural tube, in particular, is an important inducer of myogenesis within somites during explant culture. Without doubt, the neural tube plays an important role in determining myotome formation during embryogenesis.

Materials and Methods

Embryos

Day 8.5 embryos were obtained from ICR pregnant female mice and day 9.5 hemizygous transgenic embryos were isolated from C57/BL females crossed with homozygous *Des1-nlacZ* males (Pasteur Institute, Paris, France). The presence of a vaginal plug was designated as embryonic day 0.5. The mice were killed by cervical dislocation and the embryos were released from their decidua in pre-warmed phosphate-buffered medium containing 0.4% BSA (PB1). As the ICR embryos were to be used for whole embryo culture, the Reichert's membrane was carefully removed with the yolk sac and ectoplacental cone left intact.

Somite isolation

Individual somites and their adjacent tissues were dissected from day 9.5 transgenic embryos using a pair of sharp tungsten needles. The somites were dissociated from their surrounding tissues by incubation with Modified Eagle's Medium (Sigma, St. Louis, USA) buffered with HEPES and containing 0.75% (w/v) trypsin (Gibco) and 0.09% (w/v) collagenase (Sigma) for 20 min at 4°C. The enzyme reaction was inhibited with MEM medium containing 10% fetal bovine serum. The somites were then carefully isolated by passing the partially digested embryonic tissue through a glass pipette in PB1 medium. We assigned each somite pair a number, somites XX-XXII being the most cranial somites and somite I being the most caudal and thus the most recently formed somite.

Somite transplantation

Only ICR embryos with an intact yolk sac were selected for use as hosts. The Reichert's membrane of these embryos was reflected but not removed. For transplantation, approximately 4 to 5 of these embryos were placed in a 1 ml drop of PB1 medium on a 60 mm culture dish lid (Falcon), together with 10 to 15 somites I-III which had been enzymatically isolated from the transgenic embryos. Using a pair of watchmaker's forceps, a host embryo was pinned securely by its Reichert's membrane against the bottom of the culture dish. The embryo was then oriented so that the somites were clearly visible under the dissection microscope. A glass micropipette (80 μm in diameter) attached via a metal holder to a controlling mouth-piece, was used to manipulate the somites. The micropipette was placed directly over a newly formed somite in the caudal region of the host and held in position by gentle suction. The somite was removed by passing the micropipette straight through the embryo to leave an empty space for the donor somite. A transgenic somite was then picked up with the same pipette, released directly over the somitic space and with a blunt tungsten needle the donor somite was gently implanted into the space in a random orientation. All the experimental embryos were then cultured for 36 h.

Embryo culture

The medium used for embryo culture was composed of equal volumes of Dulbecco's Modified Eagle's medium (DMEM, Gibco) and rat serum (extracted from the blood of adult Sprague-Dawley rats). The serum was heat-inactivated at 56°C for 30 min prior to use. The transplanted embryos were cultured in groups of 3 to 4 inside a 50 ml culture bottle containing 5 ml of culture medium. The cultures were maintained in a roller-incubator (BTC Engineering, Cambridge, UK) rotating at 30 rpm and at 37°C for 36 h. During the first 24 h of incubation, the cultures were gassed twice with a mixture of 20% O_2 and 5% CO_2 in N_2, the oxygen tension was then increased to 40% for the remaining 12 h.

Explant culture

Isolated somites obtained from all levels of the day 9.5 transgenic embryos were explanted onto glass coverslips (13 mm diameter) pre-coated with 0.1% gelatin and housed individually inside 4-well culture dishes (Nunc). The somites were cultured in DMEM supplemented with 10% fetal calf serum and the cultures were maintained at 37°C in 5% CO_2 for 4 days.

To determine the influence of the neural tube on somites I-III, segments of neural tube were isolated by the same enzyme-digestion method used for somite isolation, from the caudal region of the transgenic embryos. The segments were recombined with four of the caudal somites and the specimens were then cultured for 1 to 4 days under the same experimental conditions as the isolated somites.

In some experiments, somites I-III were maintained in neural tube-conditioned medium. The medium was prepared by culturing 9 pieces of neural tube isolated enzymatically from the caudal region of 9.5 day embryos in 500 μl DMEM containing 10% fetal calf serum. The conditioned medium was harvested for use after 4 days of culture.

Histology

The explant cultures and the whole embryos (both the ICR embryos, which had been transplanted with transgenic somites, and day 9.5 transgenic embryos, which had not been operated on), were fixed overnight in 4% buffered-paraformaldehyde at 4°C. All the specimens were washed in several changes of phosphate-buffered saline and incubated at 37°C for 24 h in X-gal solution (2 mM 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (Boehringer Mannheim), 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide and 1 mM magnesium chloride in PBS), after which the explant cultures were counterstained with eosin. These were then dehydrated in alcohol, cleared in xylene and then mounted under coverslips. The whole embryos were embedded in paraffin wax and sectioned at 6 μm prior to counterstaining with eosin and mounting.

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