

# The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation

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## Summary

The aim of this work was to investigate the role played by the axial organs, neural tube and notochord, on the differentiation of muscle cells from the somites in the avian embryo. Two of us have previously shown that neuralexomy and notochordectomy is followed by necrosis of the somites and consecutive absence of vertebrae and of most muscle cells derived from the myotomes while the limbs develop normally with muscles. Here we have focused our attention on muscle cell differentiation by using the 13F4 mAb that recognizes a cytoplasmic antigen specific of all types of muscle cells. We show that differentiation of muscle cells of myotomes can occur in the absence of notochord and neural tube provided that the somites from which they are derived have been in contact with the axial organs for a defined period of time, about 10 hours for the first somites formed at the cervical level, a duration that progressively reduces caudalward (i. e. for thoracic and lumbar somites). Either one or the other of the two axial organs, the neural tube or the notochord can prevent somitic cell death and fulfill the requirements for myotomal muscle cell differentiation.

Separation of the neural tube/notochord complex from the somites by a surgical slit on one side of the embryo gave the same results as extirpation of these organs and provided a perfect control on the non-operated side. A striking finding was that limb and body wall muscles, although derived from the somites, differentiated in the absence of the axial organs. However, limb muscles that develop after excision of the neural tube started to degenerate from E10 onward due to lack of innervation. In vitro explantation of somites from different axial levels confirmed and defined precisely the chronology of muscle cell commitment in the myotomes as revealed by the in vivo experiments.

Key words: avian embryo, myogenesis, somites, neural tube, notochord, muscle marker.

## Introduction

Our knowledge of the early steps of skeletal muscle differentiation at the molecular level has progressed considerably in recent years. This progress has been brought about essentially by studies carried out in vitro on myogenic cell lines and by the discovery of a family of transcription factors specific for the myogenic lineage. Such are MyoD1 in mammals (Davis et al., 1987; Tapscott et al., 1988), CMD1 (Lin et al., 1991) and qmf1 (Charles de la Brousse and Emerson, 1990) in chick and quail, respectively, as well as myogenin (Wright et al., 1989) and other myogenic regulatory factors like myf5 (Braun et al., 1989). These factors are good candidates for playing a role in the commitment of somitic cells to the myogenic differentiation pathway.

However, in the embryo, the complex microenvironment in which the future myogenic cells of the paraxial mesoderm evolve is significantly different from that offered to cells dif-

ferentiating in culture. In this developmental system, as in many others, environmental influences have been shown to play a role in the final expression of the differentiated phenotype. This was demonstrated by a series of studies including those performed by two of us (Teillet and Le Douarin, 1983), in which chick embryos were totally deprived of their neural primordium and notochord at 2 days of incubation (E2) with the aim of studying the influence of these axial organs on the differentiation of neural-crest-derived peripheral ganglia. A side observation was the consecutive disappearance of the sclerotomes and myotomes and of the tissues normally differentiating from them, namely cartilage and dorsal muscles. The death of sclerotomal and myotomal cells was prevented by immediately reimplanting either the neural tube or the notochord. In this case, sclerotomes and myotomes could reach a more or less advanced differentiated state but exhibited a disturbed morphology. Many years ago, Strudel (1955) made similar observations on embryos where the

neural tube and notochord were excised along a more restricted area of the neuraxis. This led him to conclude that the axial organs had an inductive role on cartilage and muscle differentiation from somitic mesenchyme. Another observation of Teillet and Le Douarin's experiment was that, apparently, the limbs developed normally in the neuralec-tomized/notochordectomized chick embryos. Although smaller than normal, limb muscles were present at least up to E10.

The aim of the present work was to investigate further the role of neural tube and notochord during the period of time covering the metamerisation of the segmental plate, up to the expression of muscle structural proteins in the myotomes and the vertebral muscles. Moreover, since limb muscles also originate from the somites (Chevallier et al., 1977; Christ et al., 1974; 1977), it was interesting to compare the influence of the axial organs on the vertebral and appendicular striated muscles. This interest was reinforced by the observation, in the mouse embryo, of differences in the pattern of expression of MyoD1 and myogenin in these two groups of muscles (Sassoon et al., 1989).

In the experiments reported here, the paraxial mesoderm, segmented or not, was isolated from the neural tube and/or the notochord at E2 by different means either *in vivo* or *in vitro*. Differentiation of muscle cells was recognized through the use of a monoclonal antibody (mAb) called 13F4 prepared in our laboratory (Rong et al., 1987; 1990); 13F4 mAb identifies a still unknown muscle-specific protein that appears in all types of muscles (skeletal, cardiac and smooth). We demonstrate here that a signal arising from the neural tube/notochord complex is strictly required for survival and differentiation of myotomal cells whereas the somitic derived myoblasts of the limb buds survive, migrate and differentiate in the complete absence of these axial organs.

## Material and methods

The experiments were performed with chick (*Gallus gallus*) and quail (*Coturnix coturnix japonica*) embryos. Eggs were from a commercial source. We defined the stages of the embryos up to E4 by the number of somite pairs, and thereafter according to the morphology of the limbs, referring to the stages of Hamburger and Hamilton (HH; 1951).

### (1) Microsurgery

Six types of operations listed (a) to (f) were performed on chick embryos incubated for 30 to 60 hours, having 8 to 30 pairs of somites (Table 1 and Fig. 1).

#### (a) Excision of neural tube plus notochord in a one-step operation

Axial organs were excised *in ovo*, at 10- to 17-somite stages, using microscalpels according to the technique described by Teillet and Le Douarin (1983). The neural tube was separated from the somitic mesenchyme in the trunk and from the cephalic mesenchyme at the mesencephalic and rhombencephalic levels. The prosencephalon was excised with optic vesicles and neighbouring tissues. Then the whole neural tube and notochord present at this stage were removed by aspiration using a micropipette, and discarded.

#### (b) Complete removal of neural tube and notochord in a two-step operation

In order to remove the entire cephalocaudal neural tube and notochord, the excision was carried out in two steps. First, at the 15- to 18-somite stage, the neural tube/notochord complex was extirpated down to the non-segmented region and the embryo was put back in the incubator until it had reached the 29- to 30-somite stage. Then the caudal part of the neural tube/notochord complex, which had developed after the first operation, was excised in a second operation.

#### (c) Separation of somites from the axial organs by a slit

In a third series of experiments, the somites were separated, from neural tube and notochord, *in ovo*, by cutting a longitudinal slit down through all three embryonic layers (ectoderm, mesoderm and endoderm), at the 8- to 19-somite stage.

#### (d) Selective excision of neural tube or (e) notochord

Neural tube and notochord were excised as in (a) at the 14- to 20-somite stage. Then we back-grafted either the neural tube or the notochord separately, after dissociating them from one another using 20% pancreatin (Gibco) in Tyrode saline solution. In some cases, two or three notochords were implanted in the place of the neural tube/notochord complex.

#### (f) Replacement of the neural tube/notochord complex in chick by various quail embryonic tissues

Neural tube and notochord were excised as in (a) at the 14- to 23-somite stage, but only at the trunk level. A piece of one of the following tissues or organs of quail embryos of different ages was then grafted into the space created by the excision: the fibula, or a segment of ureter at E7, the Remak ganglion at E6, a piece of digestive tract or mesonephros at E4.

After the operations, the embryos were reincubated at 38°C in a humidified and ventilated incubator, for various periods, according to the experimental series.

## (2) Histology

The embryos were fixed at E3 to E20 in Bouin's fluid, embedded in paraffin and sectioned. The sections, 7.5 µm thick, were treated for immunocytochemistry: they were deparaffinized, washed in phosphate-buffered saline (PBS), incubated overnight with 13F4 mAb (undiluted hybridoma supernatant) at room temperature, then treated for 45 minutes with secondary fluorescent goat anti-mouse immunoglobulins coupled either to fluorescein isothiocyanate (GAM-Ig-FITC) or to tetramethyl rhodamine isothiocyanate (GAM-Ig-TRITC), from Nordic Tebu, and mounted in glycerol (70% in PBS). In certain instances, 13F4 immunoreactivity was revealed by a secondary antibody coupled to peroxidase or to alkaline phosphatase.

In some sections, the presence or absence of nerves was determined using an antibody to neurofilaments, or the BEN mAb (Pourquié et al., 1990) that binds to a surface determinant carried by certain neurons.

## (3) Culture of somites

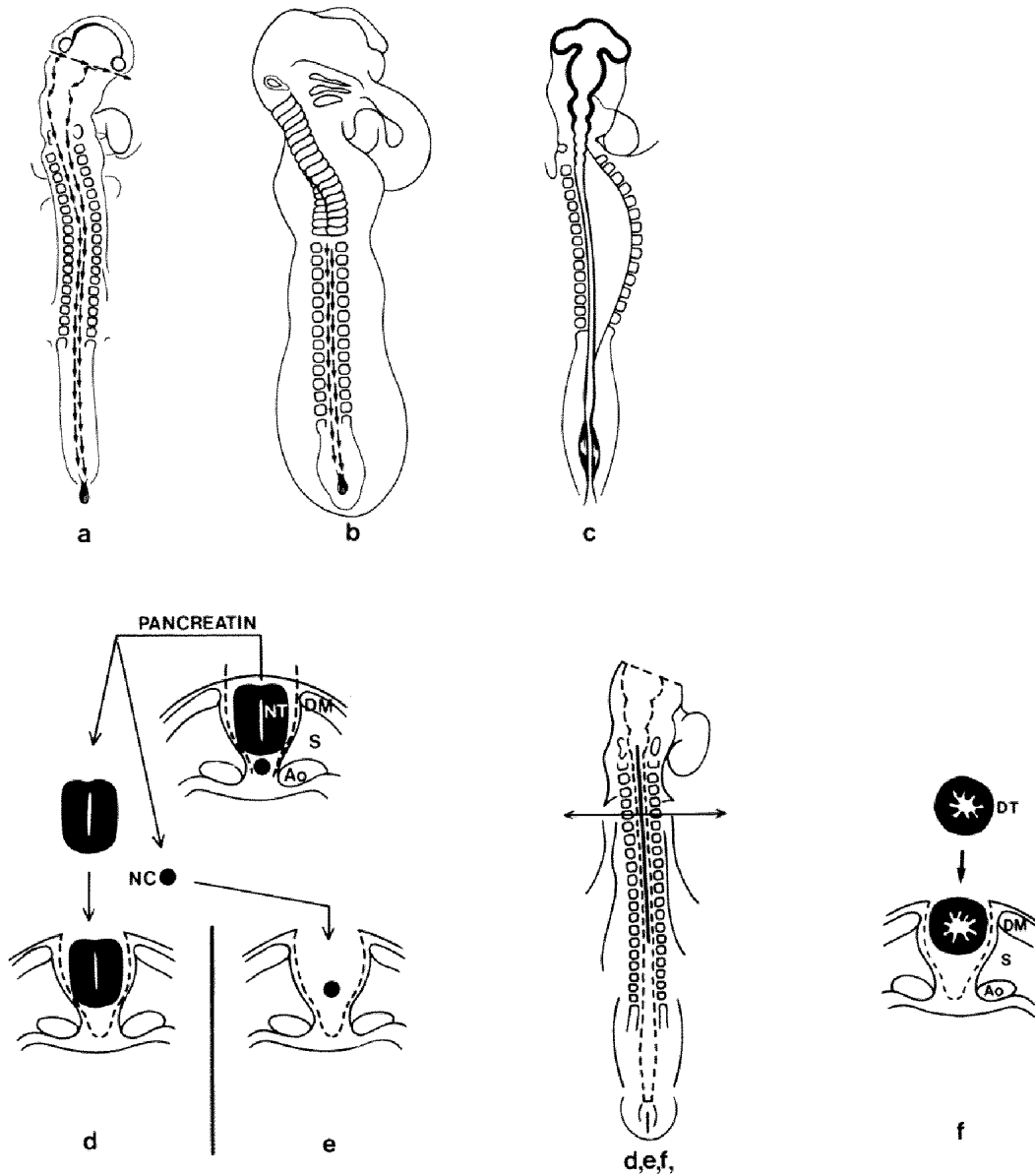
#### (a) *In vitro* cultures of isolated somites (Table 2)

Quail and chick embryos with 8 - 31 somite-pairs were explanted and pinned onto paraffin-coated Petri dishes, their dorsal side upwards, in a drop of Tyrode solution. An incision was made with a microscalpel in the ectoderm over the whole length of the segmented somitic mesoderm, and the embryos were treated for 5 - 10 minutes with 50% pancreatin in Tyrode. The somites could then be dislodged one by one, and transported with a micropipette to Terasaki culture wells containing 25 µl of serum-free culture

**Table 1.** *In ovo* experiments

Type of operation	Stage of operation	Stage of fixation	Number of embryos analyzed	
a) Excision of NT and NC* in one step	10-17 somites	E3-E4	9	
	11-16 somites	E5-E6	3	
	15-17 somites	E7-E9	3	
	14-16 somites	E13-E20	8	
b) Excision of NT and NC* in two steps	1st step 15-18 s	E5	3	
	2nd step 29-30 s			
c) slit between NT and paraxial mesoderm	8-19 somites	E3-E5	27	
d) back-graft of NT	14-16 somites	E4-E7	3	
e) back-graft of NC	14-20 somites	E4-E14	8	
f) substitution of NT and NC by:	mesonephros (E4)	E3-E4	7	
	digestive tract (E4)	E4	2	
	Remak ganglion (E6)	E4	2	
	ureter (E7)	E4	1	
	fibula (E7)	15-23 somites	E4-E6	2

\*NT, neural tube; NC, notochord.



**Fig. 1.** Schematic representation of the microsurgical operations performed on E2 chick embryos. (a) One-step operation: ablation of neural primordium and notochord at 18-somite stage. (b) Two-step operation: the drawing represents the second step, i.e. the ablation of neural tube and notochord in the region caudal to the 18th somite-pair. (c) Separation of the paraxial mesoderm (somites and a portion of the segmental plate) from the neural tube/notochord complex by a slit. (d-f) Back-transplantation of neural tube (d), notochord (e) or an embryonic organ: here the digestive tube (f) from an E4 quail embryo, after ablation of neural primordium and notochord. Ao: aorta; DM: dermomyotome; DT: digestive tube; NC: notochord; NT: neural tube; S: sclerotome.

medium (BBM, Brazeau et al., 1981, or SFRI4 from SRFI, France). Each well was seeded with one defined somite-pair or with one single somite.

The cultures were incubated for 2 days at 38°C, then fixed for 1 hour with 4% paraformaldehyde in PBS, washed, treated for 20 minutes with 0.25% Triton-X 100, incubated for several hours at room temperature with the 13F4 mAb and for 45 minutes with GAM-Ig-FITC.

The effect of the growth factors bFGF and TGF-1 was tested on somite cultures; recombinant human bFGF was a gift from G. Neufeld (Haifa, Israel) and porcine TGF-1 was provided by Dr. H. Moses (Nashville, USA). The factors were added separately to BBM or to SFRI4. The concentrations were 1 or 2.5 or 5 ng/ml for bFGF and 10 ng/ml for TGF-1. It should be noted that the BBM contains 0.2 ng/ml FGF.

#### (b) Co-cultures of somites with neural tube and/or notochord

*In vitro* cultures. Somites and segmental plates were dissociated from 31 quail and chick embryos with 10–20 somite-pairs and cultured as described in (a). In a first series, the 3 caudalmost somites and fragments of the segmental plate of one side of the embryos were seeded individually in Terasaki wells. In each well, a segment of the neural tube and/or the notochord of the same embryo was added to the explanted somite, the size of the neural tube or notochord explant being approximately the same as the size of the somite. The contralateral somites were cultured without neural tube and notochord, as controls. In a second series, the somites and segmental plates were put into wells that had been previously conditioned by a pre-culture of fragments of neural tube and/or notochord. A third experiment was performed with cell suspensions of somites to which dissociated neural tube and/or notochord cells were added. Somitic cell suspensions without neural tube/notochord cells served as controls. Cell suspensions were obtained from the 6 caudalmost somites of 13- to 19-somite stage embryos and from the corresponding segments of neural tube and notochord: the explants were incubated in a trypsin-EDTA solution (0.025–0.12%) in Ca<sup>2+</sup>Mg<sup>2+</sup>-free PBS for 5 minutes at 37°C. The dissociated cells were plated in 35mm Nunclon plastic dishes containing 2 ml BBM and cultured for 2 days. Each culture consisted of cells from 10 to 14 somites, with, or without, an equivalent number of cells from neural tube/notochord.

*Organotypic cultures on the chorioallantoic membrane (CAM) (Table 4).* Somites and segmental plates of quail embryos with 3 to 6, 12, and 18–22 somite-pairs were isolated as described in (a). Groups of 10–20 somites and segmental plates were assembled on pieces of Millipore filter (0.45 µm pore size), and fragments of neural tube and notochord of the same embryos were added or not to the explanted somitic mesoderm. The associations were cultured for 24 hours on the surface of a culture medium consisting of Dulbecco's minimum essential medium supplemented with 10% foetal calf serum and 2% chick embryo extract and then transferred together with the Millipore filter support onto the exposed CAM of E7 chick embryos. Grafts were grown for 2 to 3 days; they were then excised and fixed in Bouin's fluid and processed for immunocytochemistry with 13F4 mAb.

## Results

This study is based on observations made on 78 operated embryos, listed in Table 1, on *in vitro* cultures of isolated somites (Table 2 and 3) and on organotypic cultures on the CAM (Table 4).

#### *Experiment a: combined excision of neural tube and notochord (one-step operation)*

Ablation of neural tube and notochord was performed at E2, at stages ranging from 10 to 17 somite-pairs. The excised segment extended from the cephalic region to the posterior end of the formed segmental plate. In all surviving embryos, the neural tube and notochord were absent at the level of the operation. In contrast, the lumbar and sacral regions of the spinal cord developed normally. Therefore, in this section, we will focus on the region rostral to the hindlimbs, i.e. the cephalic, cervical and truncal regions. Twenty three embryos were examined at different stages (see Table 1).

As was noted by Teillet and Le Douarin (1983), the total absence of neural tube and notochord can be compatible with survival of a number of embryos even up to hatching time. The operated birds were always smaller than controls of the same age. Although the vertebral column was completely lacking in the operated region, the girdles and the forelimbs were well developed as well as the hind limbs.

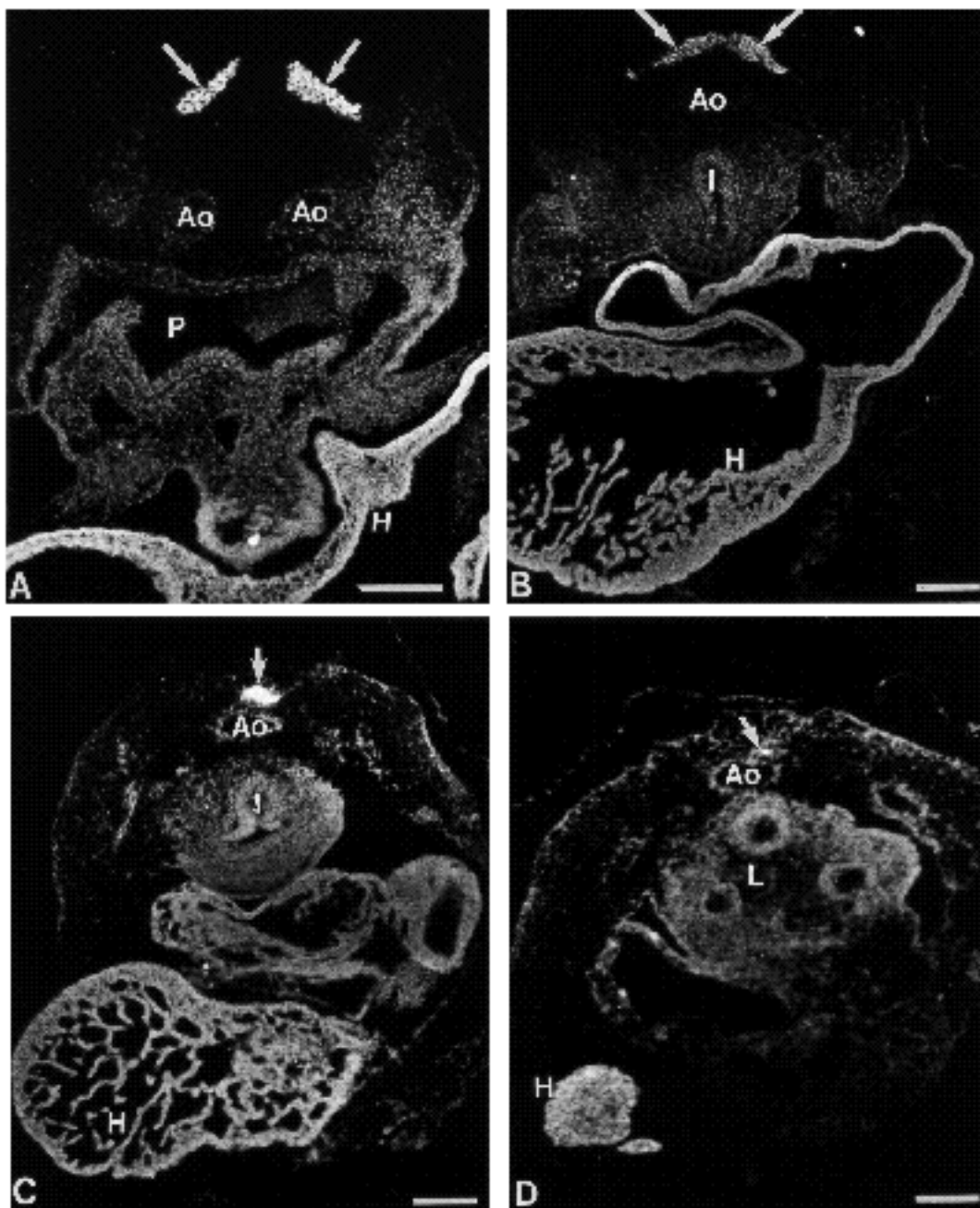
At E3, the operated embryos had no more than 30 somite-pairs. Often, the two somites of one pair were fused together across the midline and the limits between two consecutive somites became unclear. In the caudal region of the body, where a segment of the neural tube developed after the operation, the somites were normally segmented and well formed. In embryos fixed at E4.5, the somites could not be counted. In contrast, the anterior and posterior limb buds were normal in appearance although the neural tube was totally absent at the level of the forelimbs and downward at least to the midregion of the trunk. It is known that motor innervation of the forelimbs arises from the neural tube at the level of somites 15 to 19 (Wenger, 1951) and somites 15 to 19 give rise to the muscles of the wings (Chevallier et al., 1978). At E6–8, the forelimbs were normally developed and contained muscles. From E9 onwards, the dorsal skin became transparent and inflated. The pattern of feather development was different from that of controls, particularly in the dorsolateral region. In contrast, the caudal region, where the neural tube persisted, was usually well formed, with spinal cord and caudal vertebrae. At E15 to E20, the body of the operated embryos was globular and the inner organs could be seen through the transparent skin, dorsally as well as ventrally; however, well-formed feathers were present on most parts of the body including the limbs. The wings were smaller than normal and linked together through the scapular girdle.

13F4 expression was analyzed in the myoblasts and muscles of operated embryos.

At E3–E4, the cardiac muscle of the operated embryos was normally stained by 13F4 as was also the case at all the subsequent stages. In contrast, the myotomes exhibited an abnormal development, varying according to the stage of the operation: in the embryos operated at the 10-somite stage no somitic structure was recognizable at E3.5. However, cells labeled by 13F4 were present over a length of 150 µm in the rostral part of the trunk. The immunoreactive cells were found as small clusters of less than 10 cells above the aorta. Their number diminished caudally. In the embryos operated at the 12-somite stage, somites were still present at E3, but the myotomes were very reduced. Immunoreactive cells were found underneath the dermatomes which retained their

typical structure. Both dermatomes and myotomes of a same somitic pair were close to one another and sometimes fused forming a single mass of cells above the aorta. More caudally 13F4-positive myotomal cells became less numerous, and at the level of the oesophagus, no immunoreactive cells were found. In embryos operated at the 16- and 17-somite stages, the same anteroposterior decrease in the number of 13F4-

positive cells was observed (Fig. 2). However, the rostral myotomes were well developed presenting many 13F4-positive cells. They were not fused across the middorsal line. In contrast, more caudally, at the level of the oesophagus, the myotomal pairs were often fused on the midline. At the level of the lung anlagen downwards, no 13F4-labeled myotomes were seen.



**Fig. 2.** Immunolabelling with 13F4 mAb of four transverse sections of a chick embryo deprived of neural tube and notochord in a one-step operation. The ablation was performed at 17-somite stage, the fixation at E4. The sections are from different levels along the rostrocaudal axis of the embryo. (A) Level of the pharynx. Two almost normal immunoreactive myotomes are seen (arrows). (B) Level of the anterior gut. The myotomes are fused (arrows). (C) At a slightly more posterior level, immunoreactive myotomal cells are still present, but reduced in number and located dorsally close to the aorta (arrow). (D) Level of the lung anlagen. This is the last section in which very few 13F4-positive cells were seen (arrow). The immunolabelled heart is seen on all sections. Ao: aorta; H: heart; I: anterior gut; L: lung anlagen; P: pharynx. Bars=200  $\mu$ m.

At E5-E6 the distribution of 13F4-positive cells in the myotomes was significantly modified when compared with that described above. The important fact at that stage was the appearance of 13F4-positive cells in the limbs. In one embryo operated at the 11-somite stage and fixed at E6, scattered muscle cells were present in the axial region at the cervical level but not at the level of the wings. The wing buds, in contrast, contained well-developed premuscle masses stained by 13F4 mAb (Fig. 3).

In animals killed at E7 to E9, vertebrae and vertebral muscles were totally lacking at the levels where the notochord and spinal cord were absent, but the wing muscles exhibited a 13F4 labeling equivalent to that of controls. Smooth muscles appeared within the digestive tract and trachea, and were faintly labeled by 13F4, as in controls.

Interestingly, in all embryos killed at E5 to E9, masses of labeled cells were found dorsally to the mesonephros while sclerotome-derived structures were totally absent at this level (see Fig. 6A). To analyze the origin of these muscle masses, we performed a specific series of experiments described below in *experiment b*.

At the end of the incubation period (E13 to E20), defined parts of the body and the wings were examined for 13F4 expression. At E13, while the vertebral muscles were totally absent, the wings contained brightly labeled muscle structures, which, however, were smaller than those of normal embryos at the same age; moreover, some muscle fibers were disorganized and showed signs of degeneration with a significant level of cell death.

At E16, the muscles, in the non-innervated wings, became considerably degraded and reduced in size in comparison with the control (Fig. 4A,B). Accumulation of leucocytes in their vicinity was frequently noticed. At E17, E18 and E20, involution of the wing muscles continued without resulting, however, in a complete destruction at the end of incubation. In all the embryos fixed at later stages, the smooth muscles of the bowel and the heart muscle remained brightly labeled as in controls.

#### *Experiment b: complete excision of neural tube and notochord in a two-step operation*

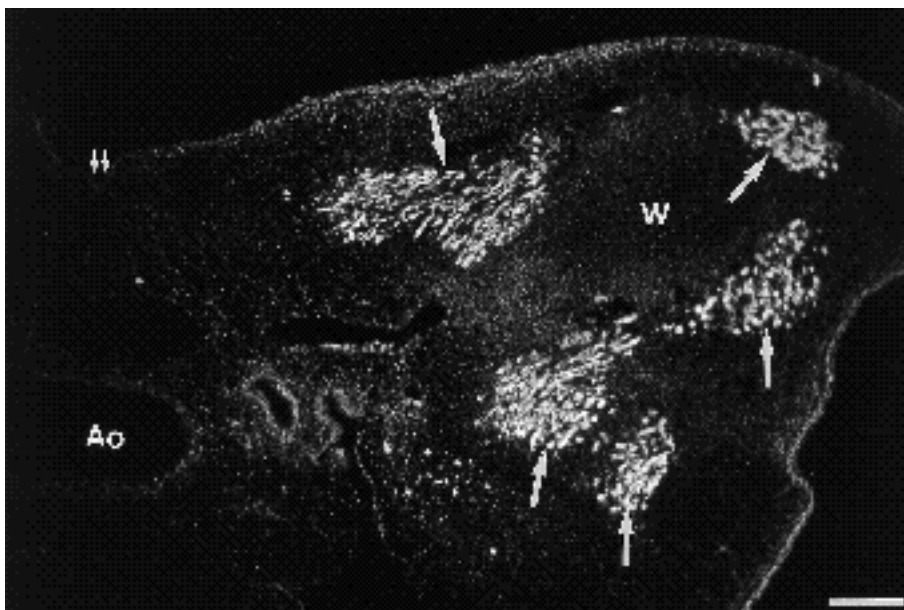
In the experimental embryos described up to now, the neural tube and notochord could be removed only down to the middle of the territory where the mesonephros develops. Therefore the masses of 13F4-positive cells that we observed in the previous experiment close to the mesonephros differentiated not far from the spinal cord, which developed caudally after the excision. At this level vertebrae and vertebral muscles form normally.

In order to know whether the newly formed neural tube was responsible for the differentiation of dorsal muscles located above the anterior part of the mesonephros, we removed the caudal neural tube/notochord complex in a second step of the operation (see Material and methods and Table 1). In these embryos, which completely lacked a neural tube and notochord (3 embryos at E5) (Fig. 5), a large crescent of labeled cells extending to the lateral walls of the body was still present above the mesonephros (Fig. 6A). This crescent of muscle cells seems to represent the fusion of both pectoral muscle masses normally situated, at that stage, laterally in the somatopleure (Fig. 6B). The neural tube/notochord complex therefore is not necessary for these muscles to develop and the absence of all axial structures including the myotomes and sclerotomes results in their dorsal fusion.

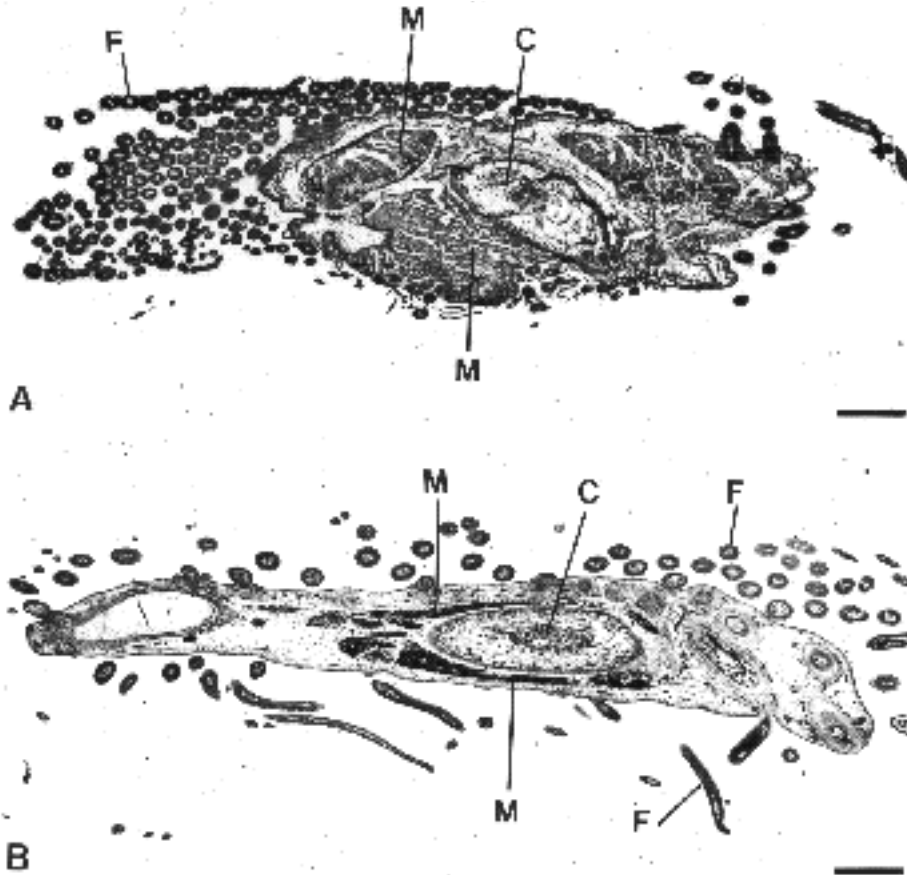
The embryos operated in two steps exhibited a normal amount of 13F4-positive cells in the limbs when examined at E5, although neither the forelimbs nor the hindlimbs were innervated, as demonstrated by the absence of neurofilament and BEN immunoreactivity (not shown).

#### *Experiment c: separation of the somitic mesoderm from the neural axis by a slit through the three germ layers*

In order to compare accurately, in the same embryo, the development of the myotomes with and without contact with the axial organs, a slit was opened between the paraxial mesoderm and the neural tube/notochord complex unilaterally in 8- to 19-somite-stage chick embryos. The slit was



**Fig. 3.** Transverse section at the brachial level of a chick embryo deprived of neural tube and notochord at the 11-somite stage and fixed at E6. The wing bud contains muscle masses labelled by 13F4 mAb (arrows). In the axial region of the embryo (double arrow) no fluorescent cell is seen. Ao: aorta; W: wing bud. Bar=200  $\mu$ m.



**Fig. 4.** Immunoperoxidase staining by 13F4 mAb of wing sections at E16. (A) Wing of a normal chick embryo with large muscle masses. (B) Wing of a chick embryo deprived of neural tube and notochord at 15-somite stage. The muscle masses are reduced in size. C: cartilage; F: feathers; M: muscles. Bar=1 mm.

made microsurgically at the cervicobrachial level over the length of 10 somites or more. No enzymatic digestion was used, so that some mesodermal cells were often left adherent to the neural tube on the operated side. We observed that the metameres of the segmental plate occurred after its separation from the neural tube/notochord complex.

As in the previous experiments development of the somites on the operated side varied according to the stage of the operation. When the slit was made at the 8- to 16-somite stage, no 13F4-positive cells appeared in the separated somites whatever the time of fixation (8-10 or 20 hours after the separation). In contrast, on the contralateral non-operated side, 1 to 18 somites exhibited 13F4-positive cells, according to the stage reached by the embryos at fixation. When somitic cells were left adherent to the neural tube at the level of the incision, most of them were labeled by 13F4 (Fig. 7A). Interestingly, the somites on the separated side were not segregated into sclerotome and dermomyotome, while the control somites on the non-operated side differentiated normally. When the slit was made at later stages, i.e. at the 17- to 19-somite stage, 13F4-positive cells were found in 1 to 6 rostral segments according to the time of fixation (8 to 20 hours after the operation). In these somites, a normal differentiation of dermatome, myotome and sclerotome was observed. In these embryos also, when somitic cells of the operated side were left in contact with the neural tube, many of them were 13F4-positive (Fig. 7B).

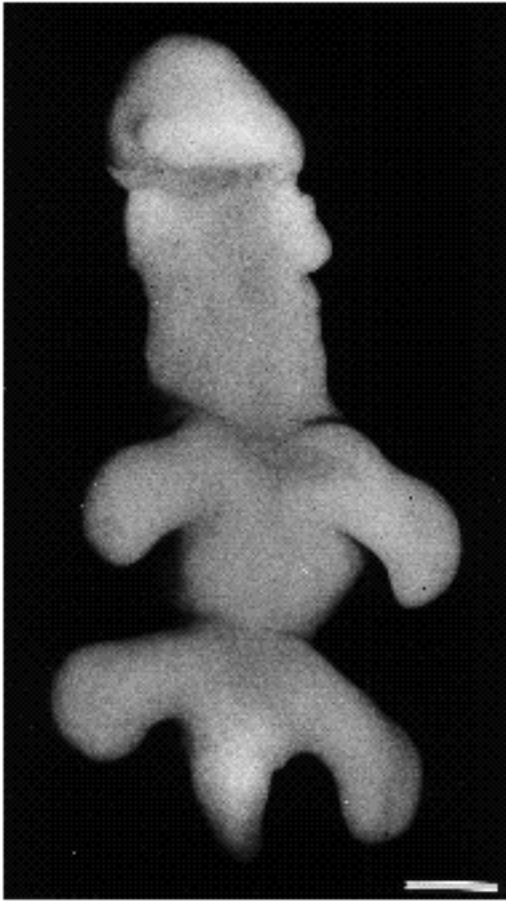
Whatever the stage of the operation, in the embryos fixed 3

days after the incision (E5), myotomes and sclerotomes were totally lacking in the separated region of the embryos. In contrast, the development of the wing, even separated from the axial organs at a very early stage, was unaffected by the operation. Thus, a consequence of the separation of the paraxial mesoderm and the neural tube/notochord complex was the total absence of axial muscles.

#### *Experiments d and e: selective excision of either neural tube or notochord*

In order to determine the respective roles of the neural tube and notochord in the survival and differentiation of 13F4-positive myotomal cells, we selectively back-grafted either the neural tube or the notochord after the excision of both (see Material and methods and Table 1). In some embryos, two or three notochords were grafted into the groove formed after excision of the axial organs. In contrast to the embryos in which both the neural tube and the notochord had been ablated, the embryos of this experimental series were almost normal in size and morphology.

At E4 to E6, in both cases, the labeling of muscle cells by 13F4 was normal in the heart and limb buds, and the myotomes also were well developed in size, although their morphology was altered in some respects. When the neural tube was present and the notochord absent, the myotomes were almost perfectly normal, but when the notochord only was present, the myotomes that developed dorsally to the notochord were fused in a semi-circular mass of 13F4-posi-



**Fig. 5.** Dorsal view of a chick embryo at E5 completely deprived of neural primordium and notochord. The two-step operation was performed at the 18- and 30-somite stages. The dorsal structures are totally lacking, while the limb buds are normal. Bar=1 mm.

tive cells. In Fig. 8 the myotomes of embryos deprived of either neural tube or notochord are shown; they should be compared with myotomes of a normal embryo (see Fig. 5B) and with those of an embryo deprived of both neural tube and notochord (see Fig. 2). In two embryos that received, respectively, two and three notochords, the amount of 13F4-positive myotomal cells was approximately the same as in the embryos in which one notochord only had been grafted. Thus, the quantity of notochord does not regulate the amount of muscle tissue produced in the myotomes.

When fixed from *E7 onward*, embryos in which either a notochord or a neural tube had been grafted displayed cartilaginous vertebral structures together with muscles, as was observed before (Teillet and Le Douarin, 1983).

At *E10*, the dorsal muscles presented signs of degeneration in embryos deprived of neural tube.

#### *Experiment f: replacement of chick neural tube and notochord by various quail embryonic tissues*

The aim of these experiments was to establish whether tissues other than neural tube and notochord could replace these structures in inducing the survival of myotomal cells. In place of the removed neural tube and notochord of 14- to 23-somite-stage chick embryos, we implanted the following

quail tissues: nervous tissues, i.e. fragments of E6 Remak ganglion; bone tissue, i.e. E7 fibula or other mesodermal tissues, i.e. E4 mesonephros and E4 ileum or E7 ureter (Table 1). Operated embryos were fixed from E3 to E6. Grafted quail tissues survived and differentiated in the chick host where the specific structures they gave rise to, could be recognized.

In comparison with the mere ablation of neural tube/notochord complex, none of these tissues allowed more myotomal cells to survive and/or to become 13F4-positive, except for the E7 fibula, in the vicinity of which bilateral rows of well-differentiated 13F4-positive muscle masses were found at E4 and E6 in two operated embryos (Fig. 9).

In these experiments where foreign tissues were inserted into the groove between the somites, 13F4-positive cell masses belonging to the pectoral muscles, observed after ablation of the axial organs (see above, *experiments a and b*), were prevented from fusing and they appeared as two separate lateral groups distributed like pectoral muscles in normal embryos. When the grafted tissue was E4 mesonephros, the quantity of 13F4-positive cells was not augmented but distributed in two lateral masses of 13F4-positive cells, as described above. This shows that neither the caudal remnant of neural tube nor the host's mesonephros are responsible for the presence of these muscle masses and support our interpretation that they represent pectoral muscles, the development of which is therefore independent of neural tube/notochord influence.

#### *In vitro cultures of isolated somites*

In view of the dramatic influence of the neural tube/notochord complex on myotomal development, somites were isolated *in vitro* and muscle cell differentiation was assessed by 13F4 mAb after 2 or 3 days of culture. Single somites or two somites of one pair were isolated and cultured separately in Terasaki wells (Material and methods and Table 2). After 24 hours, somitic cells had formed a monolayer.

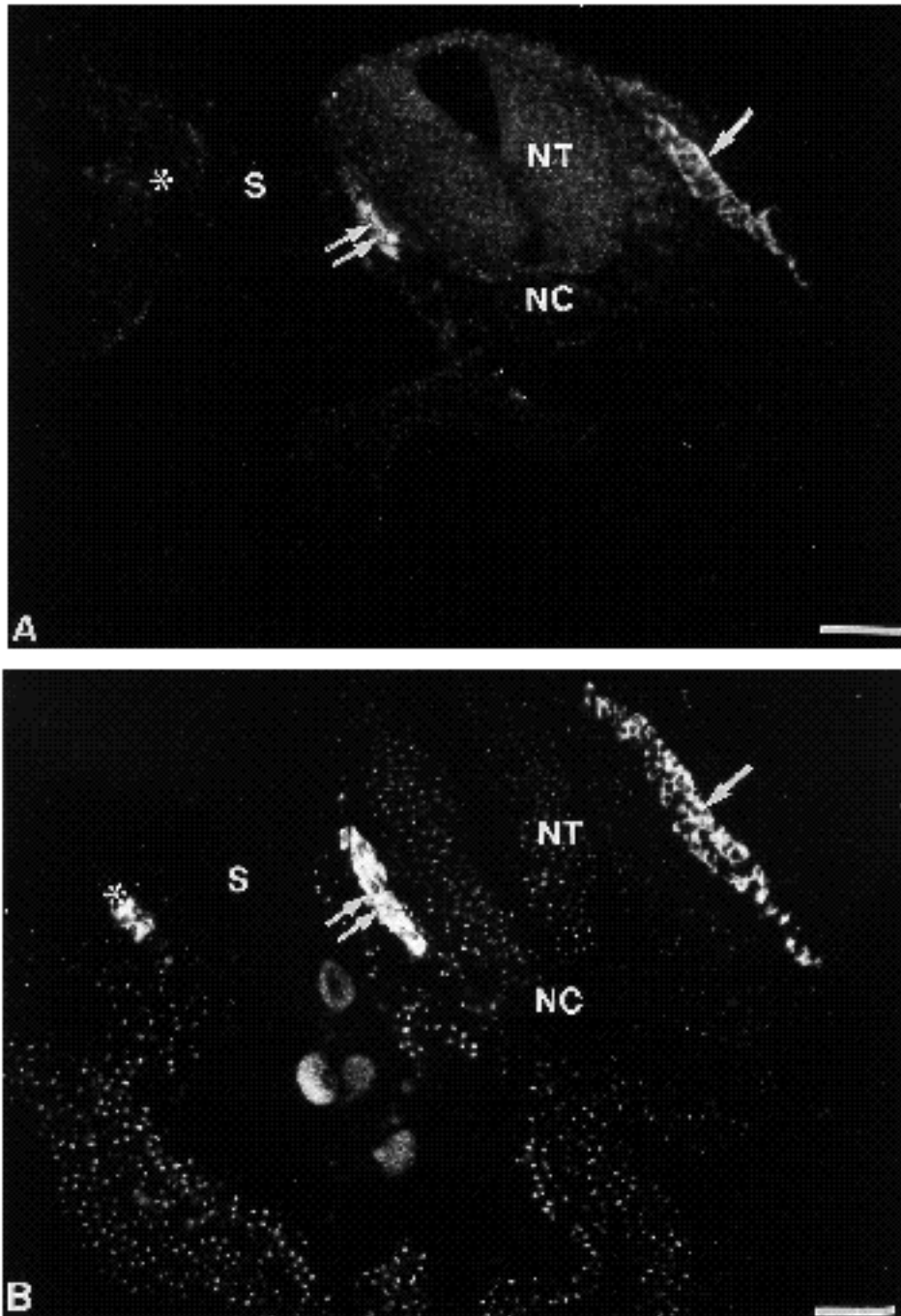
The two rostral somite-pairs that disappear precociously from 12-somite stage onward were not included in the experiments dealing with embryos older than 12-somite stage and the most rostral somite taken at these stages was counted as number 3.

No somite excised from 8- to 11-somite-stage embryos and cultured for 2 or 3 days showed 13F4-positive cells, whereas a few cells labeled by the muscle antibody were consistently found in the cultures of the third pair of somites taken from 12-somite-stage embryos; occasionally, one or two positive cells appeared in cultures of somites 4 to 6 of the same embryos.

When somites of embryos with 14-15 somite-pairs were cultured, 13F4-positive cells appeared in the somite-pairs 3 to 10 inclusively. It is generally accepted that at E2, approximately one pair of somites appears per hour (see Hamburger and Hamilton, 1951). Thus, 5-10 hours after their segmentation, the rostral somites contain muscle precursors able to differentiate into myoblasts when withdrawn from their normal environment.

Single somites were also isolated from embryos with 16 to 31 pairs of somites (see Table 2). 13F4-positive cells appeared in somites 3-15 taken from 16- to 19-somite-stage embryos and in somites 3-22 to 3-31 in cultures from 20- to



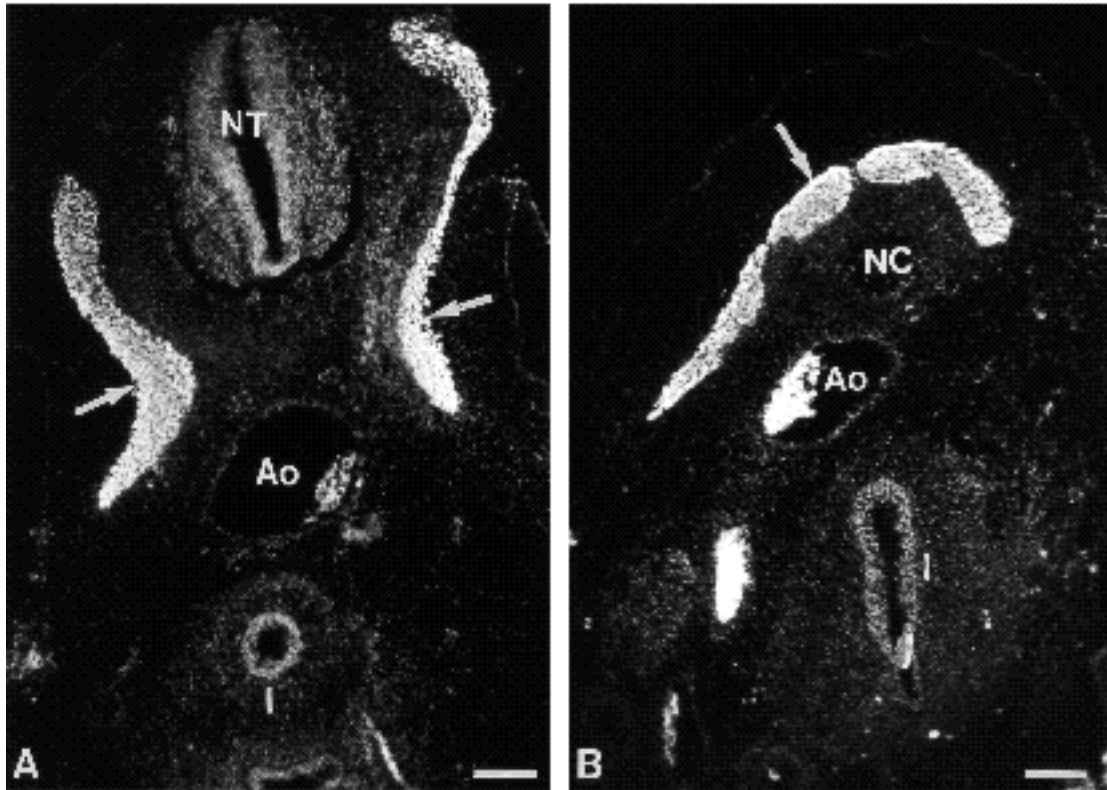


**Fig. 7.** Results of the separation of the paraxial mesoderm from the neural tube/notochord complex. Cross sections of chick embryos stained by 13F4 mAb. (A) The operation was performed at the 14-somite stage and the embryo fixed at the 29-somite stage (E3). A normal immunofluorescent myotome is seen on the non-operated side (arrow). 13F4-positive cells are also present close to the neural tube on the operated side (double arrow) but no 13F4-positive cell is formed in the separated mesoderm (asterisk). (B) The slit was made at the 18-somite stage and the embryo fixed at 30-somite stage (E3). 13F4-positive cells are present in the separated mesoderm (asterisk), as well as in contact with the neural tube on the operated side (double arrow). A normally developed myotome is seen on the control side (arrow). NC: notochord; NT: neural tube; S: slit. Bar=200  $\mu$ m.

31-somite-stage embryos (Fig. 10). Thus, at these later stage, somites of the truncal level contain cells that have the potentiality to become 13F4-positive in culture, in the absence of other embryonic tissues or organs, as early as 1 to 4 hours after their segmentation.

In two experimental series, the number of 13F4-positive cells per Terasaki well, i.e. per somite, was counted. Fig. 11 shows that, in cultures of somites taken from embryos at the 17- and 23-somite-stage respectively, the more rostral the somite, the more numerous the 13F4-positive cells produced. Besides this rostrocaudal gradient, it was also obvious that the number of muscle cells arising from each somite increased with embryonic age.

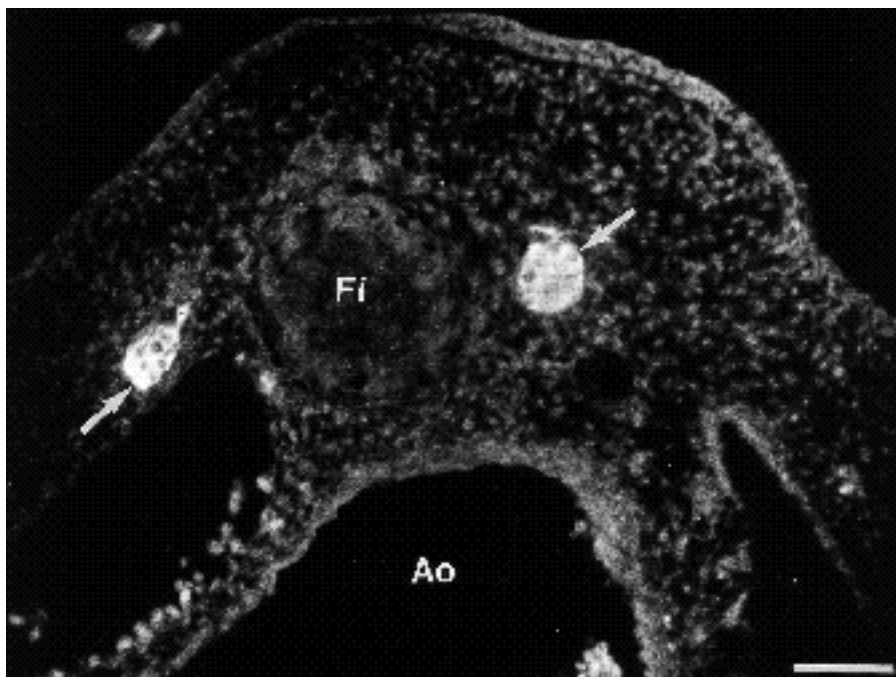
Molecules such as basic fibroblast growth factor (bFGF) and transforming growth factor (TGF- $\beta$ ) have been proposed as natural inducers of mesoderm, as evidenced by muscle differentiation in amphibians (Kimelman and Kirschner, 1987). We added bFGF and TGF- $\beta$  1 to the BBM or SFRI4 at the onset of culture. All the somites and segmental plates (i. e. unsegmented presomitic mesoderm) from one side of 6- to 11-somite-stage quail and chick embryos, the 10 caudalmost somites from 12- to 23-somite-stage embryos and the segmental plates from 15- and 18-somite-stage embryos were cultured individually in these conditions for 1 to 3 days, while the contralateral somites and segmental plates were cultured without growth factors, as controls. In



**Fig. 8.** Immunofluorescence labelling with 13F4 mAb of transverse sections of chick embryos at E4.5, at the level of the anterior gut. (A) Chick embryo deprived of notochord at 16-somite stage. The myotomes (arrows) are normally developed. (B) Embryo deprived of neural tube at 16-somite stage. 13F4-positive myotomes are seen (arrow); they are well developed but fused above the notochord. Erythrocytes in the aorta display a non-specific yellow fluorescence. Ao: aorta; I: anterior gut; NC: notochord; NT: neural tube. Bar=200  $\mu$ m.

experiments performed either with SFRI4 or with BBM (which contains 0.2 ng/ml bFGF) as culture medium (somites of 10 embryos), TGF- 1 had no visible effect either

on proliferation or on differentiation of somitic cells. In all cultures with bFGF, we observed a proliferative effect. For example, after 24 hours of culture in presence of bFGF, the



**Fig. 9.** Cross section of a chick embryo deprived of neural tube and notochord, with an implanted fibula. Ablation and graft were performed at the 15-somite stage and the embryo was fixed at E4. 13F4-positive cell masses are seen (arrows). Ao: aorta; Fi: fibula. Bar=100  $\mu$ m.

**Table 2.** *In vitro* culture of isolated somites: differentiation of 13F4+ cells in relation to embryonic stage and somitic level

(1) Number of embryos	8	4	6	18	13	20	8
(2) Stage of embryos (somite number)	8-11	12	13	14-15	16-19	20-23	26-31
(3) Level of the caudalmost somite in which 13F4+ cells were found	-	4th-6th	8th	10th	12th-15th	16th-22nd	25th-31st
(4) Number of somites without 13F4+ cells	8-11	8-6	5	4-5	4	4-1	1-0

(1) Number of embryos from which somites were isolated and explanted *in vitro*.

(2) The embryos were staged according to the number of somite-pairs that were present at explantation.

(3) 13F4+ cells were evidenced by immunofluorescence in the somite cultures.

(4) The number of last formed somites that are unable to produce 13F4+ cells decreases with increasing embryonic age.

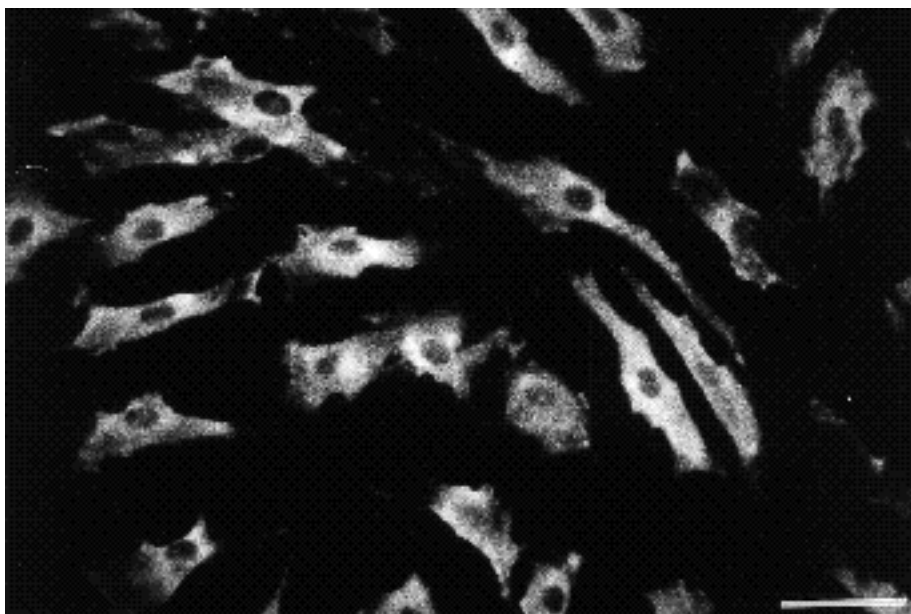
cells of one somite taken from a quail embryo of 12 somites had spread over a quarter of the Terasaki well surface, whereas in control cultures without bFGF, less than one sixth of the well was covered by cells. The presence of 13F4-positive cells was monitored after 1 to 3 days of culture. In no case, did we see immunoreactive cells in cultures of somites taken from embryos at 6- to 11-somite stage and in cultures of segmental plates from embryos at 15- and 18-somite stage, in presence and in absence of bFGF. This shows that bFGF has no inductive effect on muscle differentiation in early somites and segmental plates in these conditions. In cultures of somites taken from embryos at the 12- to 23-somite stages, 13F4-positive cells were seen and counted. As expected, the total number of 13F4-positive cells counted in the explanted somites of each embryo increased with increasing embryonic age, with the factor as well as without. In most cases, there were more positive cells in the absence of bFGF than in treated cultures (Table 3). This difference was significant in the group of 14-somite-stage embryos, thus reinforcing our conclusion that bFGF does not stimulate the differentiation of 13F4-positive cells. This result rather indicates that the

factor may even exert a negative effect on muscle differentiation in our conditions.

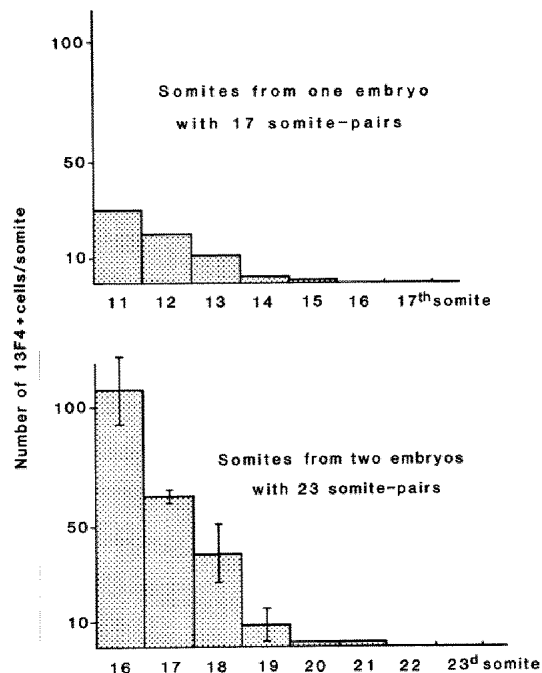
#### *Co-cultures of somites and presomitic mesoderm with neural tube and/or notochord*

In order to test whether the influence of the neural tube/notochord complex on muscle differentiation could be observed in early somites and presomitic mesoderm isolated from the embryonic environment, we performed co-cultures of somites and segmental plates with neural tube and notochord, either *in vitro*, or *in vivo* on the CAM.

The three caudalmost somites and the segmental plate from one side of 31 embryos with 11 - 24 somite-pairs were explanted individually *in vitro* with fragments of neural tube or notochord of the same embryos, while the contralateral somites and segmental plates were cultured alone as control. Cells expressing 13F4 immunoreactivity were counted in the cultures after 2 to 3 days. In co-cultures with neural tube (17 embryos), the numbers of 13F4-positive cells ranged from 0 to 42. In co-cultures with notochord (14 embryos), they ranged from 0 to 24. In control cultures they ranged from 0 to



**Fig. 10.** Immunofluorescence labelling with 13F4 mAb of a 3-day-old culture of a single somite taken from level 16 of a quail embryo at 23-somite stage. 13F4-positive cells can be easily counted. Bar=50  $\mu$ m.



**Fig. 11.** Histograms showing the distribution of 13F4-positive cells in culture of single somites, in relation to embryonic age and rostrocaudal level of explanted somites.

52. The mean values were 6, 6 and 7, respectively. Similar results were obtained when dissociated cells were cultured or when the medium had been conditioned by a culture of neural tube and notochord. No positive effect either of the neural tube or the notochord on muscle differentiation was ever observed in our *in vitro* culture conditions.

**Table 3.** Differentiation of 13F4-positive cells in cultures of isolated somites, in the presence and in the absence of bFGF

Stage of embryos	Number of 13F4-positive cells in somite cultures*	
	with bFGF	without bFGF
12 somite n=3	1±1	1.66±2.88
13 somites n=3	32±19	49±17.34
14 somites n=3	66.6±11**	168±46**
16 somites n=1	166	249
17 somites n=1	144	65
18 somites n=1	44	150
21 somites n=1	139	199
23 somites n=3	198±87.5	238±67.5

\*The numbers represent the sum of 13F4-positive cells counted in the cultured 10 caudalmost somites of one side of each embryo.

\*\* $P < 0.02$  (as estimated by the Student's test).

**Table 4.** Organotypic culture of somites and associations of somites and neural tubes and notochords (NT/NC) on the CAM. Differentiation of 13F4-positive cells

Embryonic stage (number of somites)	Somite levels	13F4 immunoreactivity	
		Somites without NT/NC	Somites associated with NT/NC
3-6	1-6	-(1)	nd
12	1-6	+(1)	+(1)
18-22	4 caudalmost	-(2)	+(1)
		-(2)	+(4)

The numbers of cultures examined are given in parentheses. Each culture consisted of 10-20 somites, to which 5-10 neural tubes and notochords were added, or not.

+ and -: reactivity to 13F4 mAb.

nd: not determined.

In co-cultures on the CAM, the results were totally different. As shown on Table 4, all the associations of somites and neural tube/notochord contained 13F4-positive cells after 3 to 4 days of organotypic culture, whatever the level and age of the somitic mesoderm. Somites cultured without neural tube and notochord produced 13F4-positive cells only when taken from the 6 anteriormost segments of 12-somite embryos. Caudal somites, or somites from very young embryos (3- to 6-somite-stage) were unable to do so.

The histological examination of the CAM grafts showed that the cultured tissues had retained organotypic structure. In particular, the organization of the neuroepithelium was recognizable (not shown).

In conclusion, the presence of the neural tube/notochord complex allows the differentiation of muscle cells in somites grown on the CAM, while the influence of the axial organs could not be evidenced in monolayers.

## Discussion

The aim of this work was to study the role played by the axial organs, neural tube and notochord, in the differentiation of muscle cells from somites. It has been previously demonstrated, through the use of the quail-chick chimera system, that the somites give rise not only to vertebral muscles but also to limb and body wall striated musculature (see Christ et al., 1986 and Gumpel-Pinot, 1984, for a review). It is also known that ablation of the neural tube/notochord complex in the avian embryo, at an early developmental stage, disturbs the development of sclerotomes and myotomes to such an extent that vertebral cartilage and muscle differentiation fails to occur while limb musculature is not affected (Strudel, 1955; Teillet and Le Douarin, 1983).

Here we investigated in more detail the behavior of myogenic somitic cells in the absence of notochord and neural tube, both in the axial and peripheral musculature. Three types of experiments were performed to deprive the somitic mesoderm of the influence of neural tube and notochord: microsurgical ablation of these organs, or unilateral microsurgical separation of the somitic mesoderm from the neural tube and the notochord, as well as culture of isolated somites

in various conditions. The differentiation of muscle cells was monitored in all the experiments through the use of 13F4, a mAb that reacts with a precocious and specific marker of muscle cells since it labels the myotomes of the most rostral somites before the embryo has reached the 20-somite-stage (Rong et al., 1987).

As shown before (Teillet and Le Douarin, 1983), neuralec-tomized/chordectomized chick embryos can survive up to hatching time. This obviously implies that the development of their heart muscle is not significantly affected by the operation; at all stages of development, 13F4 immunoreactivity is similar in the heart of operated and control embryos. This is also the case for the intestinal muscles. It should be remembered in this respect that this operation does not prevent development of the digestive tract intrinsic innervation. Moreover, striated muscles of non-somitic origin in the head differentiate normally in the operated embryos. Here we focus on the development of somite-derived muscles.

*Myogenesis in the myotomes requires a signal from the neural tube and/or the notochord*

All the *in vivo* experiments reported here show that the differentiation of 13F4-positive myotomal cells is impossible in the absence of neural tube and notochord, unless the somites have remained in contact with the axial organs during a certain period of time after segmentation. Thus, only the most rostral somites could produce 13F4-positive myotomal cells when separated from the axial organs at the 12-somite stage, i.e. about 10 hours after their individualization. When the embryos were operated on at later stages (16- and 17-somite stages), more somites produced 13F4-positive cells.

*In vitro* cultures of individual somites allowed muscle differentiation potentiality to be precisely determined for each somite-pair at every developmental time point. In 12-somite-stage embryos, only the third to the sixth pairs of somites were able to produce 13F4-positive cells in culture (the two first pairs disappear toward the 12-somite stage and were not explanted). In 18-somite-stage embryos, this capacity extends down to the 15th somite-pair and in 25- to 30-somite stage embryos, it involved all somites including the last one.

It has to be noted that absence of the axial organs does not prevent somite formation in the paraxial mesoderm unsegmented at the time of excision. This observation already made by Bellairs (1963) and Vardy et al. (1982) after extirpation of the neural tube and by Fraser (1960) after ablation of the notochord is in agreement with the opinion of Primmitt et al. (1989) who suggested that somitic segmentation depends on an intrinsic cell division program. However, in the absence of neural tube/notochord, the somites have only a very transient existence. In fact, the contact between the neural tube/notochord complex and the developing somites is necessary not only for the appearance of the muscle phenotype but also for the segregation of the somitic mesenchyme into sclerotome and dermomyotome. In the operated embryos, the most rostral somites only underwent a nearly normal maturation, resulting in differentiation of well-defined dermatome, myotome and sclerotome with normally distributed 13F4-positive myotomal cells, whereas in the more caudal ones, segregation of dermomyotome and sclero-

tome did not take place and the number of 13F4-positive cells decreased according to a craniocaudal gradient. Muscle cells that develop *in vivo* in the absence of neural tube and notochord have a transient existence. Two to four days after the operation (i.e. in E4 to E6 embryos) 13F4-positive cells progressively become less numerous and finally no muscle cells subsist in the paraxial mesoderm lining the area where excision was performed.

One of the striking observations made in experiments performed in 1983 by two of us (Teillet and Le Douarin), in which the axial organs were ablated in chick embryos, was that somitic cells were affected by a massive cell death starting during the first 24 hours and continuing until the virtually complete disappearance of somitic cells. Here, owing to the 13F4 muscle marker, we have been able to distinguish differentiated myogenic cells from degenerating somitic cells. Transient dermatomal structures were also recognized in the most rostral somites by their typical morphology, an observation already made by Teillet and Le Douarin (1983). Whether precartilaginous structures arise from sclerotomes was not determined.

Therefore, the primary role of the signal originating from the neural tube/notochord complex on myotomal cells is to ensure their survival. Both our *in vivo* and *in vitro* experiments show that the survival signal has to operate for a transient period after which presumptive myotomal cells can survive and further differentiate on their own. As already shown (Teillet and Le Douarin, 1983), the survival effect on somitic mesoderm can be fulfilled by one or other axial organ, the notochord or the neural tube. We demonstrate here that the notochord can induce muscle cell differentiation as efficiently as the neural tube. Survival of muscle cells later in development, however, can only take place if they become properly innervated, a function dependent on the neural tube (see below).

*Myogenesis in limbs and body wall does not depend upon a neural tube/notochord survival signal*

It has been established that the muscles of limbs and body wall, like the vertebral muscles, are derived from the somites (Gumpel-Pinot, 1974; Christ et al., 1974; Chevallier et al., 1977). Wing muscles arise from somites 15 to 19 and muscle precursors migrate to the wing territory by the 20- to 36-somite stage (Christ et al., 1977; Chevallier et al., 1978). However, even if the somites are separated from the neural tube/notochord complex before the 20-somite stage *in ovo*, scattered 13F4-positive cells are found in the wing buds at stage 24 HH, as in controls, and wing muscles develop normally.

A recent study, performed in our laboratory with the quail-chick nuclear marker (Ordahl and Le Douarin, 1992), showed that the wing muscle progenitors reside in the lateral half of the brachial somites, at the time of their segmentation, while the medial half contains the precursors of the myotomes. 13F4-positive cells are detected in the myotomes of these somites only a few hours after the presumptive wing muscle cells have left them. The wing muscle progenitors, therefore, migrate as 13F4-negative cells and they become positive later when already in the wing buds (Rong et al., 1987). Thus, as already noted by other authors (Jacob and Christ, 1980), neuralectomy and notochordectomy do not

prevent wing muscle precursors migration from the somites to the somatopleura, even if the operation is made early, before the segmentation of the somites has occurred at the wing level.

In the wings of the operated embryos, the 13F4-positive cells differentiated normally into myotubes from E5-6 onward and they finally formed well-organized muscles. It is noteworthy that, at the level where the wings developed with normal muscles, the axial muscles were absent (see Fig. 3). Ordahl and Le Douarin (1992) showed that, when the medial halves of the somites were labeled by the quail marker, removal of the axial organs at this level resulted in the complete disappearance of the quail cells in the medial area as early as two days after the operation. Moreover, no quail cell was found in the developing wing. However, in these conditions, wing muscles developed normally. Therefore, in the somites, only the cells of the medial somitic half are affected by the cell death process caused by the absence of neural tube and notochord; it seems that none of them escapes death by migrating to the wing territory.

Early neural tube ablation at the trunk level results in the complete absence of wing innervation. Many authors have reported that the wing muscle pattern is unaffected by the lack of nerves (Jacob and Christ, 1980; Butler and Cosmos, 1981; Butler et al., 1982; Phillips and Bennett, 1984; Noakes et al., 1988). Our *in vivo* ablations are more radical than those mentioned above, since they encompassed the complete or nearly complete neural primordium together with the notochord and were done earlier in development, before the segmental plate at the level of the wing and pectoral muscle territory was segmented. Even in these circumstances, the wing muscles and the body wall (i.e. the pectoral and abdominal) muscles developed normally. Therefore, pectoral and abdominal muscles, also derived from the somites (Chevalier, 1979), behave like those of the limbs. In embryos deprived of neural tube and notochord they developed as a unique dorsal muscle at the truncal level in the vicinity of the mesonephros (see Fig. 6A). The body wall muscles, however, were abnormally fused on the dorsal midline, unless they were kept separate by the implantation of foreign tissues (*experiment d*).

From E10 onward, we observed the disorganization of 13F4-positive myotubes in the aneural muscles of the wings of operated embryos. This observation is in complete agreement with previous studies (Phillips and Bennett, 1984; McLennan, 1983). Indeed, E10 is the stage when the first secondary myotubes normally appear (McLennan, 1983). The classical view was that the absence of nerves has no influence on the formation of primary myotubes but that motor innervation is necessary for their maintenance and for the production of secondary myotubes. However, in a recent report, Fredette and Landmesser (1991) have shown that both populations of primary and secondary myotubes are affected by the lack of innervation, the latter being somewhat more sensitive than the former.

*Can other tissues be substituted for the neural tube/notochord complex in the process of myotomal myogenesis?*

From the above surgical experiments it was clear that the presence of one of the axial organs (either the neural tube or the notochord) is necessary for the development of

myotomal muscle cells (Strudel, 1955; Teillet and Le Douarin, 1983). The question was then raised as to whether they might be replaced in this function by other tissues. A variety of developing organs of either neural (ganglion of Remak), or non-neural origin, cartilaginous fibula, digestive tube, mesonephros or ureter, were used to answer this question.

None of these tissues except the cartilaginous fibula, permitted the survival and differentiation of a significant number of 13F4-positive cells, organized as muscle masses. Apparently the cartilaginous rudiment of the bones can to a certain extent fulfill the requirements of the myogenic cells, at least for the earliest steps of muscle differentiation and organization.

*How does the neural tube/notochord complex act on myotomal differentiation?*

We examined this question in cell and organotypic cultures. Very early somites, i.e. the last formed ones in 10- to 18-somite-stage embryos, or unsegmented segmental plate produce very few 13F4-positive cells when isolated in culture in defined medium while in cultures of older somites, many muscle cells differentiate. With the aim of stimulating myogenesis in cultured somites and segmental plates, pieces of neural tube or notochord were added to the explants.

In cultures where early somitic cells spread on the substratum and formed a monolayer, addition of neural tube or notochord did not promote 13F4-positive cells differentiation. In contrast, Peirone et al. (1977) and Vivarelli and Cossu (1986) obtained myogenesis *in vitro* from early somites by adding neural tube explants. The difference between their results and ours could be due to the fact that they explanted a much greater number of somites or somitic cells than we did. Our individual cultures consisted of one somite per well, whereas Peirone et al. (1977) explanted 30 chick somites together and Vivarelli and Cossu (1986) seeded  $2 \times 10^4$  mouse somitic cells per culture. We also performed organotypic cultures on the CAM, where we assembled 10 to 20 early somites taken from several embryos. In this case, muscle cells were found in the associations with neural tube and notochord, but not in early somites cultured alone.

Obviously, myogenesis in culture is controlled by neural tube and notochord. However, this effect takes place only when a sufficient mass of somitic tissue is present, and probably also if, as suggested by Kenny-Mobbs and Thorogood (1987), a three-dimensional organization of the tissues is established in the cultures.

Growth factors like bFGF and TGF- $\beta$  have been shown to be inducers of mesoderm from ectoblast of the amphibian blastula (Kimelman and Kirschner, 1987; Slack et al., 1987; Weeks and Melton, 1987; Smith et al., 1988; Kimelman and Kirschner, 1990). This induction is generally evidenced by muscle differentiation. On the other hand, the same factors are known to have an inhibitory effect on muscle differentiation of myoblasts while promoting their proliferation *in vitro* (Florini and Magri, 1989; Vaidya et al., 1989; Johnson and Allen, 1990; Moore et al., 1991). bFGF has been detected in the avian embryo neural primordium as early as E2 (Kalcheim and Neufeld, 1990). It was tempting to see whether bFGF could be one of the factors mediating the

effect of neural tube on myogenesis. We found that, as already shown on muscle cell lines by others (Johnson and Allen, 1990), bFGF was active on somitic cell proliferation in vitro. In contrast, myogenesis was not enhanced by this factor and was even reduced in some cases as compared to cultures without bFGF.

TGF- $\beta$  1 modified neither proliferation rate nor myogenic capabilities of the cultures, even when added to a medium containing bFGF. Therefore our in vitro culture experiments do not support the contention that the signal required for the myotomal differentiation may be mediated by one of these growth factors.

#### *Myogenic regulatory genes and muscle determination.*

The *MyoD* gene family has been shown recently to have a crucial role in the determination and the differentiation of the skeletal myogenic lineage (see Weintraub et al., 1991). Although all the members of this family have the same capacity to activate muscle-specific genes, there are differences between them concerning their time of expression during ontogenesis (Sassoon et al., 1989; Ott et al., 1991) and in different species (Hopwood et al., 1989; Harvey, 1990; Scales et al., 1990). *myf5* is the earliest myogenic regulatory gene to be expressed in the somites and in the limbs, long before the appearance of structural muscle proteins in either structure, and would be the first indication of muscle determination. Indeed, in the mouse embryo, Ott et al. (1991) showed that *myf5* is detected at a very early stage, when the somites still have a ball shape and are not yet subdivided into dermatome, myotome and sclerotome. *myf5* transcripts are localized in the dorso-medial region of the somites, in close contact with the dorsal neural tube. The medial part of the somite is precisely the territory which gives rise to the myotome, as shown by the experimental study of Ordahl and Le Douarin (1992). The effect of the neural tube/notochord complex on myotome differentiation might be chronologically correlated with the onset of *myf5* expression as described in the somite of the mouse embryo. Our in vivo and in vitro experiments show that a minimum time of contact between axial organs and somites is required for self-differentiation of myotomal muscle cells. The determination process might take place during this time.

The state of determination of the limb muscle precursors, when they leave the somites and migrate to the limb territory, is not known. During their migration, they are negative for all the known markers of the myogenic lineage (Sassoon et al., 1989; Charles de la Brousse and Emerson, 1990; Hinterberger et al., 1991), including the earliest one, *myf5*, in the mouse (Ott et al., 1991). In contrast to the myotomal cells, limb and body wall muscle precursors can survive and differentiate independently of the presence of the neural tube. It remains to be established if they receive a first signal from the neural tube before leaving the somites and if they need another environmental inductive influence during their migration and/or in their site of differentiation. Such an influence might be provided in the limb bud by the apical ectodermal ridge, a structure that has been shown to be critical for patterning and differentiation of the limb mesenchymal anlage. However, such a structure does not exist in the body wall where striated muscles of somitic origin also develop independently of the axial organs.

In any case, even if the axial organs, neural tube and notochord, play a role in the early determination of myotomal cells, they also have an additional role in ensuring the survival of those somitic cells which will give rise to vertebral muscle or cartilage. This function can be fulfilled equally well by the notochord or the neural tube. Both organs must be present however for proper vertebral morphogenesis to be established. Later in development, the neural tube, i.e. the spinal cord, has another role in muscle cells, providing them with innervation in the absence of which both vertebral and peripheral musculatures degenerate.

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**Fig. 6.** Cross sections of chick embryos at E5 at the trunk level. (A) Immunofluorescence labelling with 13F4 mAb of an embryo without neural tube and notochord (two-step operation). A large muscle mass (arrows) is seen above the kidneys. (B) Immunophosphatase staining with 13F4 mAb (in blue) of a normal embryo. The positive muscle masses (arrows) seen on both sides at the level of the kidneys are the anlagen of the body wall muscles. This section is double stained with BEN mAb (immunoperoxidase, in brown) which shows elements of the nervous system. Ao: aorta; DRG: dorsal root ganglia; K: kidneys; M: myotomes; NC: notochord; SC: spinal cord. Bars=100  $\mu$ m.