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Positional signals: evidence for a possible role in muscle fibre-type patterning of the embryonic avian limb

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Summary

Current evidence favours the hypothesis that avian muscle fibre-type differentiation is intrinsically programmed during early embryogenesis and proceeds independent of neuronal influences (for review, Miller & Stockdale, 1987; Sanes, 1987). Previous interspecific (chick/quail) and intraspecific (chick/chick) chimaeric studies, involving the heterotopic transplantation of unsegmented somitic mesoderm, indicate that the ability of premyogenic cells to form specific limb muscles is determined by positional cues operative in the host embryo. To determine if similar influences are associated with embryonic fibre-type differentiation, the limb musculature of intraspecific (chick/chick) chimaeras was assessed using differential myosin–ATPase activity. The limb musculature analysed histochemically was derived from donor unsegmented thoracic somitic mesoderm transplanted to replace extirpated brachial somites of a host embryo at 48–52 h *in ovo*. Our results demonstrated that (1) the donor tissue formed, on schedule, an appropriate brachial musculature and (2) the experimental muscles duplicated the fibre-type patterning characteristic of control muscles. Thus, our data suggest that an extramyogenic, non-neural factor(s) operative in the host limb-forming region is associated with both muscle pattern formation and muscle fibre-type pattern formation. Whether or not the same putative factor(s) influences both processes remains to be determined.

Key words: fibre types, somitic mesoderm, somite transplants, chick embryo, myosin–ATPase, chimaera, limb muscle.

Introduction

The ability of peripheral motoneurones to determine muscle fibre-type characteristics, as first demonstrated by the elegant cross-reinnervation experiments of Buller *et al.* (1960), is well documented (for review, Jolesz & Sreter, 1981). Although these experiments were performed during the postnatal period, the influence of motoneurones on fibre-type determination had been extended to include early myogenesis (see Sanes, 1987). The latter assumption, however, has been challenged recently by the aneurogenic chick embryo model designed specifically to investigate the influence of peripheral nerves on the initial expression of muscle fibre types (Butler *et al.* 1982). Aneurogenic brachial muscles, prepared by deletion of the brachial neural tube at day 2 *in ovo*, exhibit fibre-type profiles equivalent to those characteristic of innervated brachial muscles. This observation favours the position that primary generation myogenic cells are intrinsically committed to distinct fibre-type cell lineages. Current evidence in support of this hypothesis is derived from further analyses of aneurogenic avian muscles *in vivo* (Phillips & Bennett, 1984; Bloom *et al.* 1985; Phillips *et al.* 1986; Sohal & Sickles, 1986) as well as embryonic *in vitro* clonal analyses (Miller & Stockdale, 1986, 1987), functional denervation studies (Crow & Stockdale, 1986) and embryonic cross-innervation experiments (Laing & Lamb, 1983; Butler *et al.* 1986; Vogel & Landmesser, 1987), using either myosin–ATPase profiles or monoclonal antibodies as markers to distinguish fibre types. Although the experiments cited

eliminate neuronal influences and imply an intrinsic commitment of myogenic cells to distinct cell lineages, the possibility exists that a non-neurogenic, extramyogenic factor(s) may be associated with early embryonic fibre-type diversification. The present study, designed to explore this possibility, assessed the fibre-type composition of brachial muscles derived from myogenic precursor cells of the thoracic region of a donor embryo.

Our rationale for selecting this experimental model was based on previous analyses of intraspecific (chick/chick) and interspecific (chick/quail) chimaeras formed by transplanting unsegmented thoracic somitic mesoderm to replace extirpated brachial somites at 48–52 h *in ovo*. Recently reported motility analyses of intraspecific chimaeras demonstrate that the donor tissue is viable and capable of forming a functional wing musculature (Cauwenbergs *et al.* 1986*a*). These findings correlate with previous histological analyses of interspecific chimaeras. Historically, the latter studies were the first to determine that 'the site of implantation ... controls the destiny of somitic presumptive muscle cells' (Chevallier *et al.* 1977; Chevallier, 1979) and emphasize that the 'myogenic potentials of the somitic mesoderm are not regionalized' (Chevallier *et al.* 1978). Thus, both functional and structural data attest to the highly plastic nature of somitic premyogenic cells and suggest that their ultimate ability to form specific individual muscles is determined by positional signals of the host embryo. Consequently, we now query whether or not positional information also influences the differentiation of specific muscle fibre types.

To answer this question, the brachial musculature of intraspecific chick/chick chimaeras previously analysed for functional activity (Cauwenbergs *et al.* 1986*a*) has now been examined histochemically to determine the fibre-type composition of muscles derived from heterotopically transplanted unsegmented thoracic somitic mesoderm. This model was highly suitable for achieving our objective since (1) unsegmented somitic mesoderm represents the earliest source of cells destined to form muscle fibres *in vivo* (Wachtler *et al.* 1982); and (2) the fibre-type profiles expressed by muscles derived from *in situ* thoracic somites differ from those exhibited by *in situ* brachial muscles (Butler & Cosmos, 1981*b*; Butler *et al.* 1986).

Comparative histochemical analyses of experimental and control embryos from stage 34 (day 8E) through stage 45 (day 19–20E) demonstrated that an appropriate brachial musculature formed, on schedule, when unsegmented thoracic somitic mesoderm was grafted to replace extirpated brachial somites at day 2 *in ovo*. Moreover, each experimental brachial muscle, derived from transplanted thoracic somitic mesoderm, expressed a fibre-type profile equivalent to that characteristic of its control counterpart. Since the site of transplantation influenced the fate of grafted myogenic cells from the perspectives of muscle formation and fibre-type expression, we propose that non-neuronal environmental cues are associated with embryonic fibre-type determination. A preliminary report of these experiments has been presented (Butler & Cosmos, 1987*a*).

Materials and methods

Surgical procedures

Experimental embryos

The unilateral replacement of brachial (Br) somites by unsegmented somitic mesoderm of the thoracic (Thor) region was accomplished using the following protocol. Fertile eggs homozygous for the dystrophic (am^{++}) gene, obtained from the University of Connecticut (Storrs), and normal White Leghorn fertile embryos, obtained from either Storrs or a local hatchery (Martindale Farms), were incubated at 37·4°C and 56% humidity until potential donor embryos reached stage 14 (50–53 h) and host embryos were at stage 13 (48–52 h). Embryonic age was determined by the criteria of Hamburger & Hamilton (1951).

Donor embryos at the appropriate stage were removed from their eggs, placed in a Petri dish containing sterile Hank's Balanced Salt Solution (HBSS) and the thoracic region extending from somite 22 to presumptive somite 26 inclusive was excised. The excised tissue was then placed in a sterile solution of 0·25% trypsin/Ca^{2+}- and Mg^{2+}-free HBSS for 10–15 min, while the host embryo was being prepared. Following trypsinization, the thoracic somitic mesoderm excised from the right side of the donor embryo was dissected free from surrounding tissues (ectoderm, endoderm, neural tube, mesonephric duct and lateral plate mesoderm), transferred by pipette to trypsin inhibitor (5% fetal bovine serum/HBSS) for approximately 10 s, and then transplanted to the host embryo. Host embryos at the appropriate stage were prepared by extirpating the brachial somites (somite 16 to presumptive somite 21 inclusive) from the right side of the embryo. Removal of host brachial somites was achieved by using an electrophoretically sharpened tungsten needle. After penetrating the vitelline membrane, a longitudinal incision extending the length of the removal site was made through the surface ectoderm dorsal to the right lateral edge of the brachial neural tube. Transverse incisions through the ectodermal layer were then made to join this initial cut, first at the cranial end of the removal site (between somites 15 and 16) and then at the caudal end (between presumptive somites 21 and 22). The flap of ectoderm overlying the brachial somites was then folded laterally to expose the somites below. Two deeper longitudinal incisions were then made medial and lateral to the brachial somites, being careful not to damage the posterior cardinal vein, underlying aorta or mesonephric duct. Transverse incisions were then made at the cranial and caudal limits of the removal site and the brachial somites were gently lifted away with fine forceps. After

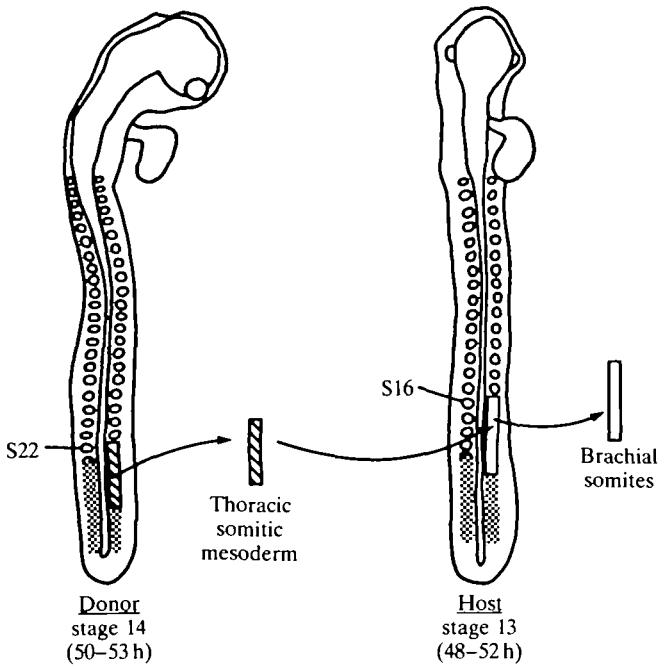

Fig. 1. Diagram to illustrate the heterotopic transplantation of thoracic somitic mesoderm from a donor chick embryo to replace extirpated brachial somites of a host chick embryo. S refers to somite number and stippled area represents unsegmented somitic mesoderm. (From Cauwenbergs *et al.* 1986*a*, by permission.)

removal of the brachial somites, the operative site was suctioned with a finely drawn glass pipette to remove any possible fragments of somitic mesoderm; the removal site was inspected for injuries to either the brachial neural tube or lateral plate mesoderm. Damaged embryos were discarded.

Following removal of the host brachial somites, the donor thoracic somitic mesoderm was manipulated into the removal site of the host embryo (Fig. 1) using a fine wire probe. By cutting the caudal end of the donor tissue at a slight angle at the time of removal from the donor embryo, it was possible to maintain proper craniocaudal and dorsoventral orientation of the graft. After the donor somitic mesoderm was positioned correctly into the host removal site, some of the fluid overlying the embryo was suctioned away using a finely drawn glass pipette. This procedure prevented subsequent shifting of the grafted tissue within the operative site. The ectodermal flap produced at the start of surgery was then folded back over the transplanted somitic mesoderm using a wire probe. Following surgery, the eggs were closed with a glass coverslip sealed with soft dental wax and returned to the incubator to develop to the desired postoperative stage. A total of 177 embryos was subjected to the experimental (Thor–Br) manipulation, using either genotypically normal ($n = 34$) or genotypically dystrophic ($n = 143$) embryos as donors; in all cases, the hosts were of normal genotype.

Control embryos

A series of unoperated embryos of normal ($n = 123$) and dystrophic ($n = 90$) genotype as well as embryos surviving the homotopic somite transplantation procedure ($n = 5$) served as controls. Homotopic (Br–Br) somite transplants were performed by replacing extirpated brachial somites of a normal host embryo with brachial somites from either a normal or dystrophic donor embryo at stage 13 (48–52 h). In addition, the right (operated) brachial musculature of all surgically manipulated embryos was compared to that of the left (unoperated) side.

Histochemical analyses

Experimental (Thor–Br) and control (Br–Br, unoperated) embryos were removed from the egg, decapitated, eviscerated and frozen for cryostat sectioning. The exact developmental stage of each embryo was assessed according to the morphological criteria of Hamburger & Hamilton (1951). Serial longitudinal sections of frozen embryos were analysed by the myosin–ATPase reaction following alkali (pH 10·0) or acid (pH 4·35) preincubation to monitor differentiation of muscle fibre types, as described (Butler & Cosmos, 1981*a*,*b*). In addition, innervation patterns and end-plate formation were monitored by using our modification of the silver–cholinesterase reaction of Toop (Butler & Cosmos, 1981*a*) and the acetylcholinesterase reactions of Karnovsky & Roots (1964) or Koelle, as modified by Silver (1963).

Results

Viability of the transplant

Verification that thoracic somitic mesoderm is viable following heterotopic transplantation to a host embryo was derived from (1) functional and (2) anatomical criteria. (1) Using the distinctive wing motility patterns characteristic of dystrophic and normal embryos as our marker, we recently reported that transplants performed between donor and host chick embryos form a functionally viable wing musculature (Cauwenbergs *et al.* 1986*a*). To illustrate, comparative wing motility analyses performed after transplanting either normal or dystrophic thoracic somitic mesoderm to a normal host are presented in Fig. 2. Similar to the trends exhibited by unoperated normal and dystrophic wings (Cauwenbergs *et al.* 1986*a*,*b*), experimental muscles of dystrophic origin exhibit a significantly lower frequency of wing movements from day 10E to day 15E when compared to similarly aged experimental muscles of normal genotype. The observation that experimental wing muscles function according to the genotype of the donor embryo attests to the viability of the transplanted tissue. The present study represents histochemical analyses of nineteen experimental embryos from the series previously assessed for functional activity (see Fig. 2); without exception, experimental embryos that

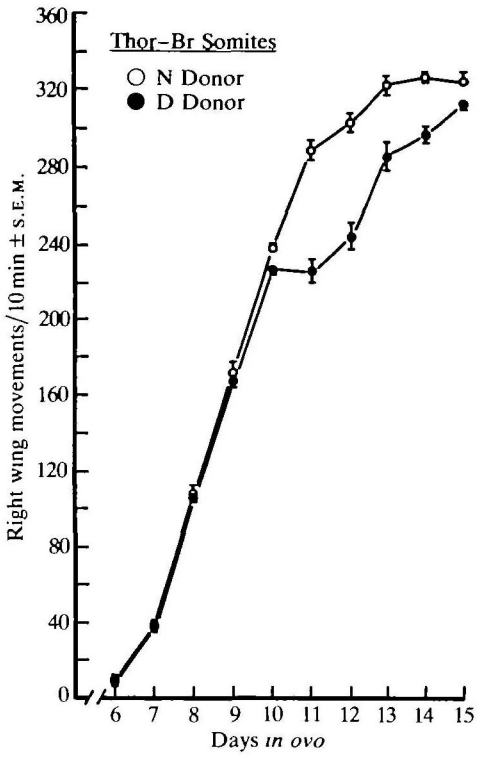

Fig. 2. Comparative functional (motility) analyses of experimental (Thor–Br) wing muscles derived from transplanted thoracic somitic mesoderm from normal (N) and dystrophic (D) donors, from day 6E through day 15E *in ovo*. Similar to the pattern characteristic of normal and dystrophic unoperated wings (Cauwenbergs *et al.* 1986*a*,*b*), genotypically dystrophic wing muscles exhibited impaired motility from day 10E through day 15E. (From Cauwenbergs *et al.* 1986*a*, by permission.)

received genotypically dystrophic donor tissue expressed the dystrophic phenotype of impaired wing motility whereas those that received genotypically normal donor tissue exhibited a normal motility pattern. (2) As shown previously (Kieny *et al.* 1972), the formation of supernumerary ribs serves as an anatomical marker for the viability of transplanted thoracic somitic mesoderm. Serial reconstruction of all chick/chick chimaeras analysed for the present study revealed that transplantation of thoracic somitic mesoderm to the brachial region resulted in the formation of supernumerary ribs, located anterior to the *in situ* rib cage. Fig. 3 compares the unoperated (left) and operated (right) sides of the brachial region of a stage-35 (day 8–9E) Thor–Br embryo. Note the absence of ribs opposite the left coracoid bone and the presence of supernumerary ribs (arrowheads) opposite the right coracoid bone. Serial reconstruction of control (Br–Br) embryos revealed the absence of supernumerary ribs on the operated side.

Once the viability of the donor tissue was confirmed using functional (Fig. 2) and anatomical (Fig. 3) criteria, the formation and fibre-type composition of experimental and control muscles were compared from stage-34 (day 8E) to stage-45 (day 19–20E). The latter analyses were restricted to proximal muscles derived from cleavage of the premuscle masses of the wing bud; distal (forewing) muscles or myotomally derived brachial muscles were not examined.

Formation of a brachial musculature from thoracic somitic mesoderm

Previous homotopic somite transplants, using the chick/quail chimaeric model (Christ *et al.* 1977),

Fig. 3. Photomicrograph of a longitudinal section from a stage-35 (day 8–9E) Thor–Br embryo to illustrate the formation of supernumerary ribs on the operated (right) side. Note the absence of ribs opposite the coracoid bone on the left (unoperated) side and the presence of supernumerary ribs (arrowheads) opposite the right coracoid bone. Arrows indicate bilateral formation of brachial plexus. Silver–cholinesterase reaction. *c*, coracoid bone. Bar, 0·5 mm.

Table 1. *Cleavage of the muscle masses of the wing bud**

Mass	Division	Muscle	Abbr.†
Dorsal	Deltoid	Tensor propatagii	
		Deltoideus	D
	Subscapular	Scapulohumeralis anterior‡	SHA
		Scapulohumeralis posterior	SHP
		Subscapularis et subcoracoideus	SS+SC
		Coracobrachialis posterior	
	Latissimus dorsi	Latissimus dorsi pars anterior‡	ALD
		Latissimus dorsi pars posterior	PLD
		Latissimus dorsi pars dorsocutaneous‡	DLD
		Latissimus dorsi pars metapatagial‡	MLD
	Triceps brachii	Triceps brachii pars scapularis	Ts
		Triceps brachii pars humeralis	
Ventral	Pectoral	Pectoralis	
	Supracoracoid	Supracoracoideus	
		Coracobrachialis anterior	
		Pars dorsalis‡	
		Pars ventralis	
	Ventral brachial	Coracobrachialis‡	
		Biceps brachii	
		Brachialis‡	

* Modified from Butler *et al.* 1982.
† Abbreviations for specific muscles discussed in text.
‡ Refers to muscles expressing dual acid- and alkali-stable myosin-ATPase activity in all fibres.

demonstrate that all brachial muscles are somitic in origin and that premyogenic cells migrate from the somites to form the primary dorsal and ventral muscle masses of the wing bud. Subsequently, the latter undergo a series of cleavages, according to a precise chronological schedule, to form the brachial musculature, i.e. intrinsic muscles of the wing as well as muscles associated with the pectoral girdle (Sullivan, 1962). To determine if transplanted thoracic somitic mesoderm was capable of forming, on schedule, an appropriate brachial musculature, experimental (Thor–Br) and control (Br–Br) embryos at stages 34 (day 8E), 35 (day 8–9E) and 36 (day 10E) were sectioned completely and compared to similarly staged unoperated embryos of both normal and dystrophic genotype. These specific stages were selected since the individualization of all muscles derived from the primary muscle masses of the wing bud is virtually complete during this period (Sullivan, 1962; Butler *et al.* 1982). Furthermore, since the process of cleavage is not impaired in the dystrophic mutant (Butler & Cosmos, 1987*b*), the transplantation of donor tissue from a dystrophic embryo was not a factor to be considered in this phase of our study. By stages 34 (day 8E)–36 (day 10E), the dorsal muscle mass of both unoperated normal and dystrophic wing buds has cleaved to form twelve individual wing muscles, whereas the ventral muscle mass has formed six distinct wing muscles (Table 1). A similar pattern was observed for all Thor–Br and Br–Br embryos examined during these stages. To illustrate, the stage-36 (day 10E) Thor–Br embryo presented in Fig. 4A shows the formation of distinct deltoideus (D), scapulohumeralis anterior (SHA) and posterior (SHP), subscapularis et subcoracoideus (SS+SC), anterior latissimus dorsi (ALD) and triceps brachii pars scapularis (Ts) muscles. This particular section of the right (operated) brachial region was selected because it includes muscles derived from each of the four divisions of the dorsal muscle mass, as shown in Table 1. More ventral sections from the Thor–Br embryo presented in Fig. 4A showed that all wing muscles originating from the ventral mass were also present and located in anatomically correct positions. Analyses of all Thor–Br embryos beyond stage 36 (day 10E) confirmed that transplanted thoracic somitic mesoderm was capable of forming an appropriate brachial musculature. The only anomaly observed was the absence of the right PLD muscle in one Thor–Br embryo examined at stage 42 (day 16E).

Fibre-type analyses

To determine if the brachial musculature derived from thoracic somitic mesoderm duplicated the fibre-type patterning characteristic of similar muscles derived from brachial somitic mesoderm, the fibre-type composition of brachial muscles was compared among Thor–Br, Br–Br and unoperated embryos

from stage 34 (day 8E) onward. Although the majority of brachial muscles of unoperated embryos are composed predominantly of fibres that exhibit alkali-stable myosin–ATPase activity only, specific muscles are composed entirely of fibres that exhibit dual alkali and acid stability (marked ‡ in Table 1; Butler *et al.* 1982). Experimental Thor–Br muscles demonstrated a similar pattern. The profiles of representative stage-42 (day 16E) experimental brachial muscles derived from grafted thoracic somitic mesoderm are presented in Fig. 5A–D. Experimental ALD, posterior latissimus dorsi (PLD), dorsocutaneous latissimus dorsi (DLD), metapatagial latissimus dorsi (MLD) and SHP muscles all exhibit alkali stability. Only the ALD, DLD and MLD, however, are also acid stable; the PLD and SHP are essentially acid labile (Fig. 5A,B). Thus, similar to unoperated embryos (see Table 1), three of the four muscles derived from the latissimus dorsi division expressed dual stability in all fibres. Sections from a more ventral region of the same Thor–Br embryo shown in Fig. 5A,B demonstrate that, typical of the unoperated SHP (Butler *et al.* 1982), acid stability is noted in fibres located on the ventral surface of the SHP, adjacent to the scapula (Fig. 5C,D). A similar restriction of acid-stable fibres to specific regions within the predominantly alkali stable D muscle is also observed. At stage 42 (day 16E) all experimental muscles were well-innervated and exhibited endplates.

Examination of younger Thor–Br embryos between stage 34 (day 8E) and stage 39 (day 13E)

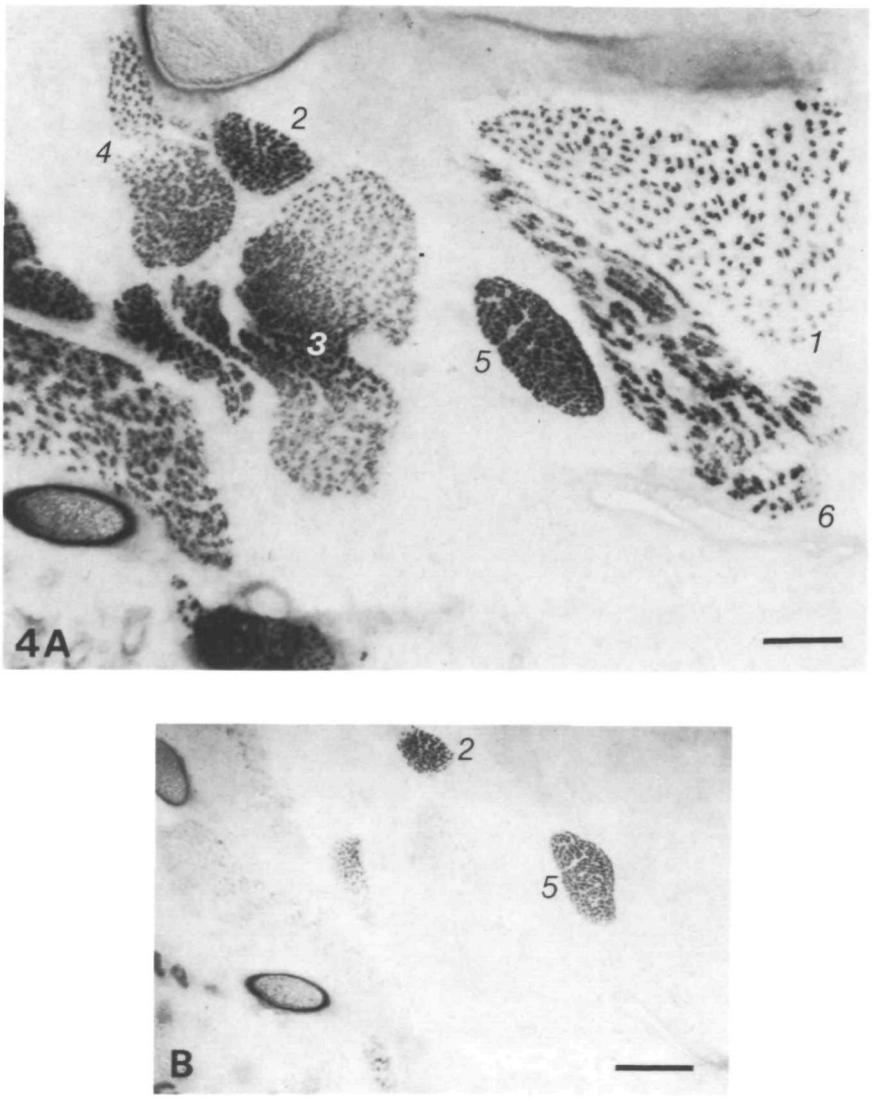

Fig. 4. Photomicrographs of longitudinal sections from the operated side of a stage-36 (day 10E) Thor–Br embryo to illustrate (A) the formation of individual muscles derived from cleavage of the four divisions of the dorsal muscle mass (see Table 1), namely, the D(*1*), SHA(*2*), SHP(*3*), SS+SC(*4*), ALD(*5*), and Ts(*6*). Note also that similar to unoperated embryos, the SHA(*2*) and ALD(*5*) exhibit dual alkali-(A) and acid-(B) stable myosin–ATPase activity; at this stage, the other numbered muscles express alkali stability only. Bar in A, 0·25 mm; bar in B, 0·5 mm.

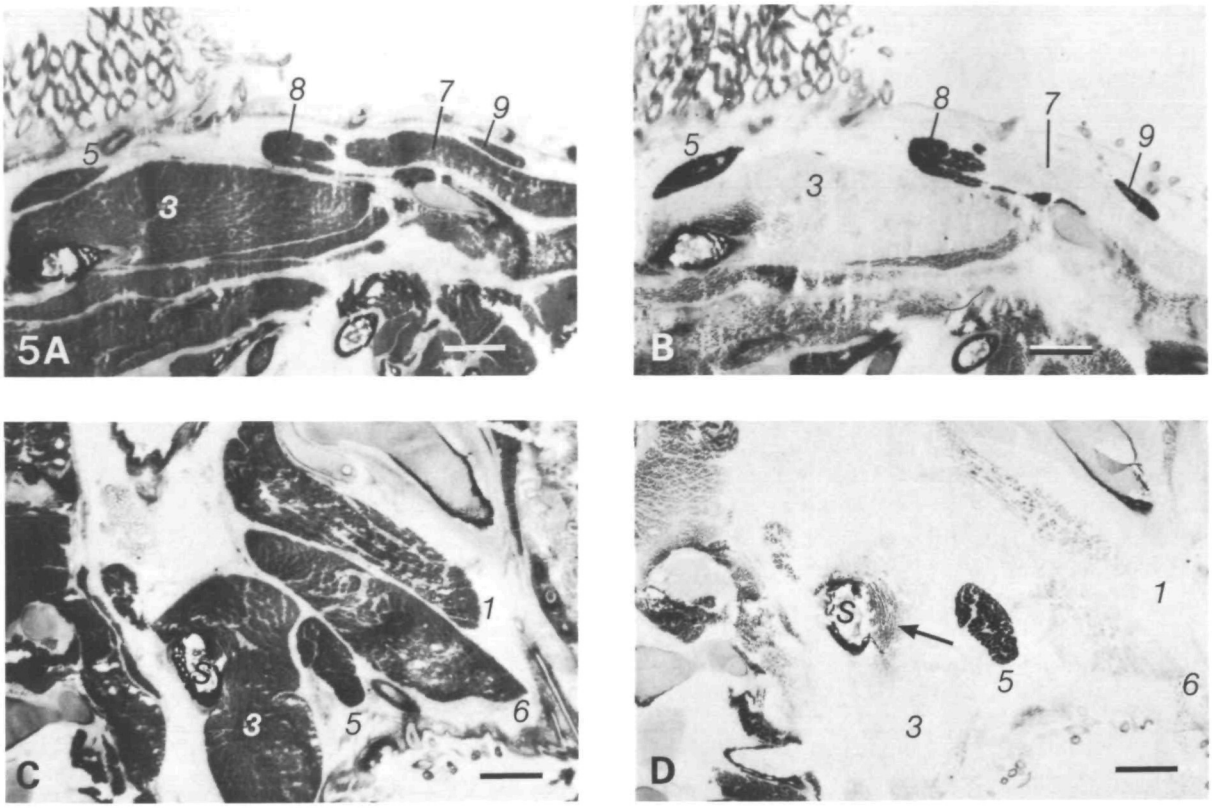

Fig. 5. Photomicrographs of longitudinal sections from the operated side of a stage-42 (day 16E) Thor–Br embryo to illustrate fibre-type profiles following alkali (A,C) and acid (B,D) preincubation. Serial sections from a more dorsal level demonstrate that the experimental ALD(*5*), DLD(*8*) and MLD(*9*) express dual alkali and acid stability whereas the PLD(*7*) and SHP(*3*) are alkali stable only (cf. A,B). Serial sections from a more ventral level show acid-stable fibres (D, arrow) localized on the ventral surface of the SHP(*3*) adjacent to the scapula. Likewise, at this level, the predominantly alkali stable only D(*1*) also exhibits a focal area of acid-stable fibres (cf. C,D). At both levels, the ALD(*5*) is alkali and acid stable (cf. A–D). Similar profiles were expressed by unoperated and Br–Br muscles at this stage. Unlabelled muscles are of myotomal origin. Myosin-ATPase. *s*, scapula. Bar in A,B, 1·1 mm; bar in C,D, 1·0 mm.

confirmed that brachial fibre-type profiles were expressed by muscles derived from thoracic somitic mesoderm. To illustrate, at stage 36 (day 10E), the experimental ALD and SHA exhibit dual stability whereas the SHP, Ts and D muscles are alkali stable only (Fig. 4A,B). At this stage, unoperated muscles express similar profiles. Analyses of Thor–Br embryos during earlier developmental stages was important since a previous study (Butler *et al.* 1982) determined that fibre-type differentiation proceeds independent of neuronal influences during this phase of development. Therefore, the possibility that innervation from brachial nerves influenced the fibre-type characteristics expressed by Thor–Br muscles was eliminated. Thus, throughout the developmental period analysed, individual brachial muscles derived from transplanted thoracic somitic mesoderm exhibited fibre-type profiles equivalent to those characteristic of their unoperated counterparts. Likewise, the profiles expressed by Br–Br muscles resembled those typical of unoperated muscles. Furthermore, these observations applied to donor tissue of either normal or dystrophic gentotype, confirming a previous observation that differentiation of appropriate myosin–ATPase profiles is not impaired in dystrophic embryos (Butler & Cosmos, 1987*b*).

Discussion

Investigations concerned with the fate of somitic mesoderm following either homotopic or heterotopic transplantation have contributed significantly to an understanding of myogenesis during early embryonic development. With the advent of the quail nuclear marker (Le Douarin & Barq, 1969), interspecific

chick/quail chimaeric studies resolved a long-standing controversy concerning the origin of limb musculature. Today, it is generally accepted that skeletal muscle fibres are somitic in origin whereas the tendon and connective tissue components of limb muscles are derived from the somatopleure (for review, Kenny-Mobbs, 1985). Furthermore, although homotopic transplantation of single somites indicates that individual somites contribute to the formation of specific muscles during normal embryogenesis (Beresford, 1983), heterotopic transplants demonstrate that individual somites are not predetermined to form specific muscles exclusively (Chevallier *et al.* 1977; Chevallier, 1979; Jacob *et al.* 1978; Cauwenbergs *et al.* 1986*a*; present study). Indeed, the heterotopic experiments cited emphasize the plasticity of myogenic somitic cells and suggest that positional cues associated with the site of transplantation control their destiny as far as muscle pattern formation is concerned.

The present study has now focused on the fate of heterotopically grafted somitic mesoderm from the perspective of muscle fibre-type patterning to foster an understanding of factors associated with embryonic fibre-type expression. More specifically, we questioned whether or not positional cues would also influence this developmental process. Therefore, the fibre-type composition of experimental muscles derived from heterotopically transplanted somitic mesoderm was determined.

Experimental design

Intraspecific chick/chick chimaeras were prepared by grafting unsegmented somitic mesoderm from the thoracic region of a donor chick embryo to replace extirpated brachial somites of a host chick embryo (Thor–Br model). Paramount to the interpretation of our experiments is evidence that (1) the donor tissue was viable and (2) the experimental muscles analysed were devoid of *host* myogenic cells. Evidence concerning the viability of the donor graft is inferred from the interspecific chick/quail study of Chevallier *et al.* (1977). Utilizing the distinctive quail nucleolus as a marker, they demonstrated that thoracic somitic mesoderm from a quail donor participates in the formation of the wing musculature of the chick host. Myofibres of the wing muscles were exclusively of donor (quail) origin whereas the tendons and connective tissue components were of host (chick) origin. For the present intraspecific chick/chick chimaeric study, viability of the donor tissue was verified directly by functional and structural criteria. The observation that thoracic somitic mesoderm transplanted from a dystrophic donor to a normal host forms muscles that express the dystrophic phenotype of impaired wing motility provides functional verification of graft viability (Cauwenbergs *et al.* 1986*a*). Anatomically, the operated brachial region of Thor–Br embryos exclusively was characterized by supernumerary ribs. As demonstrated previously (Kieny *et al.* 1972), the presence of these ectopic structures in the brachial region attests to the viability of transplanted thoracic somitic mesoderm. Thus, based on these parameters, the donor tissue was not only viable but capable of differentiating both myogenic and chondrogenic cell lineages.

To ensure that experimental muscles were devoid of *host* myogenic cells, selection of an appropriately aged host embryo was critical since myogenic cells located in somites opposite limb-forming regions migrate to seed the developing limb musculature during a specific phase of early myogenesis. Based on previous reports that migration within the brachial region does not commence prior to stage 14 (Jacob *et al.* 1978, 1979; Kenny-Mobbs, 1985), brachial somites were extirpated from host embryos at stage 13, i.e. prior to migration. Indeed, recently Solursh *et al.* (1987) reported that migration within the brachial region does not occur until stage 15. Using a monoclonal antibody specific to vascular endothelial cells, they demonstrate that mesodermal cells at stage 14, previously described as migrating myogenic cells, are actually endothelial cells belonging to the vascular network. Morphologically identifiable migrating (myogenic) cells within the brachial region of the chick embryo do not appear until stage 15.

Fibre-type expression

Analyses of muscles derived from the donor tissue demonstrated that the somitic level of origin is associated with *neither* (1) the formation of specific muscles *nor* (2) the differentiation of specific fibre-type profiles. Unsegmented somitic mesoderm from the thoracic region of a donor embryo transplanted to the brachial region of a host embryo at 48–52 h *in ovo* formed, on schedule, an appropriate brachial musculature in all experimental embryos analysed. Furthermore, without exception, the experimental brachial musculature that formed duplicated the fibre-type patterning characteristic of unoperated and control (Br–Br) brachial muscles at all stages analysed. Therefore, we propose that commitment to distinct fibre-type cell lineages is not endogenously programmed, as previously suggested (for review, see Miller & Stockdale, 1987; Sanes, 1987), but is influenced by non-neuronal cues operative within a limb-forming region.

Although the mechanism whereby positional signals influence fibre-type expression is unknown at present, such cues appear to be operative during a temporally limited period if one correlates previous experiments performed by Laing & Lamb (1983) with

the present study. Laing & Lamb observed that a wing bud grafted at stage 18 (65–69 h) or 19 (68–72 h) to replace an extirpated leg bud develops a wing musculature with appropriate wing-muscle fibre-type profiles. Although designed to examine the influence of foreign innervation on fibre-type expression, the results of their study may be interpreted to indicate that myogenic cells within a transplanted *limb bud* are already committed to distinct fibre-type cell lineages. Myogenic precursor cells transplanted at the *unsegmented somitic mesoderm* stage lack such a commitment, as indicated by the present study performed at stage 13 (48–52 h). Therefore, we propose (1) that commitment to distinct fibre-type cell lineages may be irreversibly expressed during a temporally limited period of approximately 20 h. This specific period represents the interval between the stage when the present unsegmented somitic mesoderm transplants were performed and the stage when the limb bud grafts were done. (2) Alternatively, fibre-type determination may occur at an even later stage. A grafted limb bud is not composed exclusively of myogenic cells. Therefore, a non-neuronal, extramyogenic factor(s) associated with fibre-type determination, unique to each limb-forming region, may be present in the limb bud graft and, thus, may be operative during the post-transplantation period. The time frame for expression of this putative factor(s), however, would be limited to approximately 24 h post-transplantation since *in vivo* analyses (Miller & Stockdale, 1986) indicate that distinct classes of myogenic cells exist within the developing limb as early as day 4 *in ovo*.

In summary, heterotopic transplant studies indicate that somitic mesodermal cells destined for the chondrogenic cell lineage are already regionalized along the cephalocaudal axis at the unsegmented somitic mesoderm stage (Kieny *et al.* 1972; present study). In contrast, at this stage somitic mesodermal cells destined for the myogenic cell lineage are unspecified as to both muscle pattern formation and fibre-type pattern formation. Instead, these processes appear to be influenced by non-neural, extramyogenic cues associated with the site of transplantation.

Although limb skeletal patterning has been studied extensively and found to be influenced by positional cues derived from the apical ectodermal ridge (Summberbell, 1974) and the zone of polarizing activity (Summerbell, 1979), comparatively few studies have investigated limb muscle pattern formation. These limited studies, however, do indicate that neither peripheral nerves (Butler *et al.* 1982) nor limb skeletal elements (Lanser & Fallon, 1987) influence initial muscle patterning. Whether or not connective tissue derived from the somatopleure controls this process, as suggested by Kieny *et al.* (1986), remains to be proven. The elucidation of the mechanism(s) controlling limb muscle pattern formation may provide clues concerning our proposed relationship between environmental signals and incipient limb fibre-type pattern formation.

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