Tissue interactions in the organization and maintenance of the muscle pattern in the chick limb

By ANNICK MAUGER¹, MADELEINE KIENY¹*, IHSAN HEDAYAT¹ AND PAUL F. GOETINCK²

From the Equipe de recherche associée au CNRS, no 621 'Morphogenèse expérimentale', Laboratoire de Zoologie et Biologie animale, Université Scientifique et Médicale de Grenoble, France, and Department of Animal Genetics, University of Connecticut, U.S.A.

SUMMARY

Recent investigations on a hereditary muscular dysgenesis (cn/cn) in the chicken (Kieny, Mauger, Hedayat & Goetinck, 1983) have suggested that limb muscle pattern development and subsequent maintenance are two independent steps in the formation of the musculature. The respective activities of muscle cells and connective tissue cells in the ontogeny of the musculature have been investigated in avian embryos 1) by in ovo administration of drugs interfering with collagen biosynthesis, and 2) by heterogenetic somite-exchange experiments between normal and mutant embryos.

None of the drugs administered to the chick embryo caused any disturbance of muscle pattern formation or maintenance whether treatment occurred before (5 days) or after (7.5 days) the muscle splitting period.

Heterogenetic implantations were performed at 2 days of incubation either at the leg or at the wing level. Somitic mesoderm from non-mutant quail embryo was grafted to replace a piece of somitic mesoderm in putative mutant (cn/cn) chick embryos. The introduction of normal myogenic cells into a mutant leg or wing led to a normally patterned musculature, which demonstrates that the muscular dysgenesis cn/cn results from a defect of the somitic myogenic cell line.

INTRODUCTION

The dual origin of the limb musculature is now well established. Myocytes are of somitic origin, whereas muscular envelopes and tendons are of somatopleural origin. For example, the development of the zeugopod muscle pattern can be described schematically as follows: somitic myogenic cells enter the somatopleural mesoderm and are immediately involved in the developing programme of the limb. They conglomerate into two premuscular masses, which split up progressively until all the muscles which constitute the zeugopod musculature

¹ Authors' address: Laboratoire de Zoologie et Biologie animale, Université scientifique et médicale de Grenoble, BP n° 68, 38402 Saint-Martin-d'Hères Cedex, France.
² Author's address: Department of Animal Genetics, University of Connecticut, Storrs, Connecticut 06268, U.S.A.
* To whom requests for reprints should be addressed.
are separate entities. This occurs according to a rigid chronological sequence between day 5 and 7.5 of incubation (Shellswell & Wolpert, 1977; Pautou, Hedayat & Kieny, 1982). Once the muscle pattern is developed it has to become stabilized.

Recent investigations (Kieny et al. 1983) have suggested that pattern development and subsequent pattern maintenance are not to be considered as a whole, but represent two independent steps in the organization of the musculature. The crooked neck dwarf mutation (cn/cn) (Asmundson, 1945), which results in a drastic muscular hypoplasia of the lower leg, interferes with the course of the second step only. In the mutant, the splitting and the spatial arrangement of the muscles progress normally up to 7.5 days of incubation (Fig. 1). At that time, however, the muscles coalesce and fuse. They finally end up in an unorganized muscular tissue which surrounds the tibiotarsus and fibula (Fig. 1).

The above-mentioned duality of the skeletal musculature raises the question of the respective activities of muscle cells and premuscular connective tissue cells not only in the development but also in the maintenance of the muscle pattern. All experiments described up to now (Chevallier, Kieny & Mauger, 1976, 1977; Kieny & Chevallier, 1980; Mauger & Kieny, 1980; Chevallier & Kieny, 1982) point to the equivalence of muscle cells (at least up to the myoblast state) and, hence, the non-equivalence of connective tissue cells in the muscle patterning. In other words, they emphasize the organizing role of the premuscular connective tissue cells in the muscular architecture.

This paper investigates the interaction between muscle cells and connective tissue cells which leads to the spatial organization of the musculature of the avian limb. The study is restricted to the musculature of the zeugopod. Two experimental series were undertaken to approach this problem.

1) Since the premuscular connective tissue is primarily involved in the muscle organization, this involvement could be mediated through the extracellular matrix. Collagen, a principal constituent of the extracellular matrix, was selected for investigation by applying, in ovo, substances which interfere with its biosynthesis with the aim of demonstrating changes in the muscle arrangement. Such attempts to demonstrate a role for collagen in muscle arrangement were unsuccessful whether the drugs were applied before or after the muscle splitting period.

2) In order to determine which of the component tissues is affected by the crooked neck dwarf mutation, heterogenetic somite-exchange experiments were

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Fig. 1. Comparative representation of the zeugopodial hindlimb musculature in crooked neck dwarf mutants and in normal siblings between 5 and 12 days of incubation. Open arrows indicate the normal evolution; filled arrows indicate the dysgenic evolution of the musculature. In the mutant, the muscle individuation occurs normally between 5 and 7 days of incubation; thereafter most muscles progressively fuse together into a single mass. dm, dorsal premuscular mass; vm, ventral premuscular mass.
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performed. Somitic mesoderm from non-mutant quail embryo was grafted to replace a piece of somitic mesoderm in putative mutant chick embryos. These results clearly demonstrated a defect in the muscle cells of the mutant.

MATERIALS AND METHODS

1. Drug administration

Rhode Island Red × Wyandotte chick embryos were used in this study. Drugs were obtained from Sigma. Methods for drug administration and conditions of incubation were fully described elsewhere (Hedayat, 1982). In brief, 5- and 7-5-day embryos received a single dose of 100 μl of the vehicle containing one of the following compounds: insulin, β-aminopropionitrile, D-penicillamine, L-azetidine-2-carboxylic acid, α-α' dipyridyl and 4-methyl-umbelliferyl-β-D-xyloside. The latter drug was suspended in sterile corn oil; α-α' dipyridyl was dissolved in alcoholic saline (1 vol. ethanol: 3 vol. saline); the other drugs were diluted in Tyrode's solution. The solution or suspension was made fresh for every injection series.

Treated embryos were removed daily between 3 to 5 days after drug administration. After recording any morphological changes induced by the drug, the embryos were fixed in Bouin's fluid. Some of them underwent Lundvall's selective staining for cartilage before the hind limbs were cut out and processed further for histology. The limbs were embedded in paraffin, serially sectioned at 7 μm and stained with Mallory's triple stain. Histological analysis comprised recording the spatial arrangement of the lower leg muscles in transverse sections of the treated hind limbs.

2. Heterogenous transplantation

Experiments were performed on crooked neck dwarf chick embryos and on Japanese quail embryos from flocks reared at the University of Connecticut, Storrs. Eggs from a cross between parents heterozygous for the cn gene were obtained from the chick mutant stock maintained by the Department of Animal Genetics, and the quail eggs came from the stock maintained by the Nutritional Science Department.

Somitic grafts

At 2–2.5 days of incubation (13 to 23 pairs of somites) putative mutant chick embryo somitic mesoderm adjacent to the wing (somite level 13 to 20) or leg territory (somite level 24 to 32) was replaced by non-mutant quail somitic mesoderm of equivalent stages (12 to 21 pairs of somites).

Since previous work using heterotopic transplantation had shown that the muscle pattern develops appropriately to the host limb segment regardless of which group of somites is the source of the myogenic cells (Chevallier et al. 1976,
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1977; Mauger & Kieny, 1980), the grafted mesoderm in the present study was taken either from the neck level (somites 6 through 12), the wing level (somites or presumptive somites 13 through 21), or the leg level (presumptive somites 22 through 32).

Histological analysis

The host embryos were sacrificed 8 to 10 days after the implantation (stages 36 to 39 of Hamburger & Hamilton (1951)) and classified as normal, mutant or questionable embryos according to the curvature of their neck and to the filiform aspect of their tibiotarsal segment on the non-operated side. Both features are distinctive of the crooked neck dwarf mutant. Questionable embryos were found only among those sacrificed at stages 36 and 37 of H.H., when the mutant phenotype was not fully discernible externally.

All embryos were fixed in Helly’s solution. During the dehydration phase, the experimental limb (wing or leg) and the contralateral limb, which together make up a two-limb set, were severed and processed further for histology. In addition, in the case of somite exchange performed at the wing level, three-limb sets were made up by adding to the above-mentioned two-limb set one tibiotarsal segment from the same embryo, in order to fully ascertain the genotype of the embryo.

All two-limb and three-limb sets coming from mutant or questionable chick embryos were analysed histologically. Only a few sets derived from externally normal embryos were processed for histology. The 7 μm sections were cut either perpendicularly (legs) or parallel (wings) to the proximodistal axis of the zeugopod. They were stained according to Feulgen & Rossenbeck (1924) in order to reveal the distinctive nucleolar marker of the quail cells (Le Douarin & Barq, 1969).

Moreover, as the cn/cn mutation is less obviously expressed in wings than in legs, we examined 8- to 12-day mutant and normal embryos at the wing level, so as to become better acquainted with the muscular disorganization in the forearm. Both wings were fixed in Bouin’s fluid, embedded in paraffin and sectioned at 7 μm perpendicularly (one of the wings) or parallel (the contralateral wing) to the proximodistal axis. The sections were stained with Mallory’s triple stain. A histological study of the corresponding lower legs has been published in a previous paper (Kieny et al. 1982).

RESULTS

A. Failure to alter the muscular organization by interfering with collagen biosynthesis

The drugs used are listed in Table 1. With the exception of insulin which amplifies collagen production by stimulating proline and lysine incorporation into the molecule and by promoting hydroxylation of these residues (Bashey,
Table 1. *Effects of substances which interfere in collagen biosynthesis and distrubution, when administrated in ovo before the muscle splitting period in the lower leg*

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose(s)*</th>
<th>Number of embryos observed</th>
<th>General effects</th>
<th>Musculature</th>
<th>Effects on the hind limb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>growth (dwarfism)</td>
<td>development</td>
<td>Oedema</td>
<td>Flaccid-ness</td>
</tr>
<tr>
<td>β-aminopropionitrile</td>
<td>0·5–5 mg</td>
<td>29</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-penicillamine</td>
<td>10–30 mg</td>
<td>17</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>α-α'-dipyridyl</td>
<td>15 μg–1·5 mg</td>
<td>65</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-azetidine-2-carboxylic acid</td>
<td>0·8 mg</td>
<td>7</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>insulin (25·5 IU/mg)</td>
<td>10–400 μg</td>
<td>12</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4-methylumbelliferyl-β D-xylloside</td>
<td>0·5–1 mg</td>
<td>10</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Only those doses which led to teratological syndromes are indicated.
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Perlish & Fleischmajer, (1972), all the other drugs are known for their specific inhibitory effect on collagen synthesis. The latter compounds interfere with various steps of collagen biosynthesis. \textit{L-azetidine-2-carboxylic-acid} is incorporated into the peptide chains in place of proline, blocks triple helix formation and causes the synthesis of non-secretable protocollagen (Takeuchi & Prockop, 1969); the iron chelator \textit{\alpha-\alpha'-dipyridyl} inhibits hydroxylation of proline (Hurych & Chvapil, 1965); \textit{\beta-aminopropionitrile} and \textit{D-penicillamine} affect the maturation of collagen fibres by inhibiting cross-link formation (Pinnel & Martin, 1968; Nimni, Deshumkh & Gerth, 1972); \textit{4-methyl-umbelliferyl-\beta-D-xyloside} has a modifying action on the spatial distribution of the collagen fibres.

1) \textit{Treatment before muscle splitting period}

\textit{In ovo} administration of these drugs to 5-day chick embryos led to teratological syndromes characterized by reproducible morphological abnormalities (Table 1). These changes included slight dwarfism, retarded development, flaccidity, massive oedema of trunk and hind limbs, distortions of maxillary and skeletal elements of the limbs, particularly tibiotarsus and fibula.

The hindlimbs of 39 among the 140 affected embryos were examined histologically. In all cases, muscle patterning took place and the 15 muscles which constitute the proximodistally ordered lower leg musculature (Pautou \textit{et al.} 1982) developed as separate entities.

Although patterning had followed the normal splitting sequence, the subsequent growth and spatial localization of the muscles was often set back by about 24 to 36 h, when observed at 9 days of incubation (Table 1). However the delayed muscle development was always in accordance with the delay in the general development of the embryo.

Hindlimbs of non-affected embryos (10 cases) were also examined histologically. Muscular architecture was always normally developed.

2) \textit{Treatment after the termination of muscle splitting}

Twenty-one chick embryos constitute this series. They were treated with \textit{\alpha-\alpha'-dipyridyl} or with the lathyrogen \textit{\beta-aminopropionitrile}. Three to 5 days after drug administration, external morphological changes were slight and restricted to a more- or less-pronounced oedema. Histologically, a normal muscular arrangement was obvious although it was accompanied by some exaggeration of the intermuscular spaces in the oedematous embryos.

These experiments indicate that collagen does not have a primary role either in the establishment or in the maintenance of the muscle pattern.

B. \textit{Heterogenetic transplantation}

212 heterogenetic (homotopic and heterotopic) somite-exchange experiments were performed in which non-mutant quail somitic mesoderm was grafted in
Table 2. Homotopic and heterotopic somite-exchange between non-mutant quail embryos and putative crooked neck dwarf chick embryos

<table>
<thead>
<tr>
<th>Cephalocaudal level of origin of the quail graft</th>
<th>Host phenotype:</th>
<th>Host phenotype:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalocaudal level of origin of the quail graft</td>
<td>normal</td>
<td>mutant</td>
</tr>
<tr>
<td>neck</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>wing</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>leg</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>6</td>
</tr>
</tbody>
</table>

* or † respectively 1 or 2 cases in which the grafted quail cells did not enter the experimental limb.

place of a piece of limb-level somitic mesoderm in putative mutant chick embryos. Of 84 hosts (40%) which survived up to a minimum of 10 days of incubation, 58 had been operated at the leg level and 26 at the wing level (Table 2).

1) Heterogenetic somite-exchange experiments performed at the leg level

Among the 58 embryos which constitute this series and which are classified according to their external morphology and/or to the histological aspect of the lower leg, 12 were crooked neck dwarf mutants (21%). The histological observations were made in these 12 mutants and on 27 of the 46 normal siblings.

External morphology. The heterogenetic somitic mesoderm exchange led to a dramatic change in the morphology of the hindlimb on the operated side of those 12-day-old embryos which could be classified unambiguously as mutant. The lower leg and shank on the operated side were bulky and did not differ from those of the normal siblings in contrast to the legs of the unoperated side which had the characteristic filiform morphology (Fig. 2).

Histological observations. In 3 out of the 27 normal siblings, the participation of quail cells in the hindlimb tissues did not take place, just as if the grafts had been rejected. In the other 24 cases, the quail cells participated in a normal (Fig. 3) hindlimb musculature, where they constituted almost the totality (15 cases) or the vast majority (9 cases) of the myogenic population. In the latter cases, the quail myogenic cells were intermingled with chick myogenic cells. This mixture took two aspects: a) all muscles contained both types of myocytes with a predominance of quail; b) there was a segregation between the muscles. Some contained only a few quail cells and were made up almost exclusively of chick myocytes, like the gastrocnemius, pars interna muscle or the gastrocnemius, pars
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Figs 2, 3. Results of heterogenetic homotopic (Fig. 3) and heterotopic (Fig. 2) somite-exchange experiments performed at the leg level in 2-day putative mutant chick embryos.

Fig. 2. 12-day chick embryo, 10 days after the heterotopic implantation of quail wing-level somitic mesoderm (stage-19 pairs of somites) at the left leg level (stage-18 pairs of somites). The mutant phenotype of the embryo is externally discernible by a filiform lower leg and shank on the right unoperated side. On the left side, the bulky lower leg and shank of the experimental limb (el) are morphologically similar to those of normal siblings.

Fig. 3. 11-day normal sibling. Histological aspect of the lower leg musculature, whose pattern is normal despite the interspecies chick/quail combination in the muscle tissue. Operation performed with donor and host at stage-20 pairs of somites. f, fibula; fdl, flexor digitorum longus muscle; fpIII, flexor perforati digitii muscle; npp, peroneus profundus nerve; t, tibiotarsus. x40.

externa muscle, whereas others, like the ventral flexor and dorsal extensor muscles were made up predominantly of quail cells.

In 3 out of 12 mutant hosts, no quail cells had entered the developing experimental limb, which thus expressed the cn phenotype in an identical fashion to the contralateral limb. In the 9 remaining cases, a comparative (Figs 4 to 8) histological analysis of the tibiotarsal segment from the operated (Figs 6 to 8) and non-operated (Figs 4, 5) sides showed a drastic difference between both limbs, i.e., the experimental limb contained a patterned musculature similar to that of the normal siblings (compare Figs 3 and 6) whereas in the contralateral leg the tibiotarsus and fibula were surrounded by an unorganized muscular tissue. Tendons (Fig. 8) and nerves were normally located in normally arranged muscles, while in the contralateral limb they were displaced. For example, the peroneus
profundus nerve (npp), a convenient landmark for detecting the mutant phenotype as early as 7.5 days of incubation (Kieny et al. 1982) topped the flexor perforatus digitii (fpdi) muscle, whereas, contralaterally, it had glided internally between the flexor digitorum longus (fdl) muscle and the confluent flexor muscle mass.

The quail cells were confined to the musculature, where they constituted the totality (3 cases) or the vast majority (Fig. 7) (6 cases) of the myogenic population, as mentioned above for normal hosts.

These results clearly demonstrate that the implanted non-mutant quail cells led to a normal muscle patterning in crooked neck dwarf hosts.

2) Heterogenetic somite-exchange experiments performed at the wing level

Before going on with this experimental series, it was necessary to check that the wing musculature was also affected in the crooked neck dwarf mutant.

a) Ontogeny of the forearm muscle tissue in the cn/cn embryo. The muscular disorganization in the mutant forearms, observed from 8 to 12.5 days of incubation in transverse sections (Figs 9A, 10A) is less obvious than in the mutant lower legs, but it is easily discernible in longitudinal sections (Figs 9B, 10B). Muscles become gradually reduced in volume, lose their external connective tissue envelope and their scattered myotubes become wavy. Some muscles, although not fully fused together, are nevertheless coalescent. But this coalescence, up to 12.5 days of incubation, does not involve more than two neighbouring muscles at the same time. Tendons are blurred or absent.

One difference between the expression of the mutation at the zeugopodial level in wings and legs is that in the wing zeugopod the skeletal elements (radius–ulna) are widely separated from one another, while in the leg the skeletal elements (tibiotarsus–fibula) are close together. Thus, muscles surround each skeletal element in the wing, whereas they surround simultaneously both

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Figs 4–8. Lower leg musculature in an 11-day mutant embryo, 9 days after the heterotopic replacement of leg-level somitic mesoderm in a putative mutant chick embryo (stage–21 pairs of somites) by non-mutant quail somitic mesoderm from the wing level (stage–18 pairs of somites).

Figs 4, 5. Unoperated side showing the characteristic disorganized musculature of the crooked neck dwarf phenotype.

Fig. 4. Section through the distal half of the tibiotarsal segment. ×40.

Fig. 5. Higher magnification of the posterior muscle structures of Fig. 4. ×100.

Figs 6–8. Operated side. Illustration of the restoration of a normal muscle pattern (compare with Figs 3 and 4) by exogenous non-mutant myogenic cells.

Fig. 6. Section through the distal half of the tibiotarsal segment. ×40.

Fig. 7. Higher magnification of a portion of the extensor digitorum longus (edl) muscle showing the interspecies combination of quail myogenic cells (single arrows) and chick connective tissue cells (double arrows). ×400.

Fig. 8. Higher magnification of the posterior muscles (m) and tendons (t) of Fig. 6, which are normally distributed in space. ×100.

npp, peroneus profundus nerve.
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Moreover, and this could be another reason for the difference, in the forearm the muscles remain tiny and therefore spaced out for a longer period of time than in the lower leg.

b) Muscular architecture in the heterogenetically chimaeric wings. Among the

Figs 4–8
26 embryos which survived after the heterogenous somite-exchange experiments, 6 were *crooked neck dwarf* mutants (23%) (Fig. 11). Histological observations involved these 6 mutants and 3 out of the 20 normal siblings.

*External morphology.* Even in the oldest mutant embryos sacrificed at about

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Fig. 9. Histological aspect of the wing musculature in a 12-day *crooked neck dwarf* mutant. *A,* transverse section through the middle of the right forearm; *B,* longitudinal section through the left forearm and arm. Compare with Fig. 10 and note the relative lack of myotubes and the blurring or absence of epimysium and tendon (*t*). ×25. *h,* humerus; *u,* ulna.
12 days of incubation, there was no clearcut difference between the experimental and the contralateral wings.

_Histological observations_. At the histological level however, the difference was striking (Figs 12, 13). On the whole, the 6 experimental wings contained normally patterned muscles, which were normally connected to tendons. The muscle showed no coalescence nor any waviness of myotubes, as was observed in the contralateral wings.

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Fig. 10. Histological aspect of the wing musculature in a 12-day normal sibling. _A_, transverse section through the middle of the right forearm; _B_, longitudinal section through the left forearm and arm. ×25. _h_, humerus; _r_, radius; _t_, tendon; _u_, ulna.
Quail cells, in all cases and in all muscles, made up the totality of the muscle cells (Figs 14, 15). The same quail-cell distribution was also observed in the normal siblings.

Consequently, in wings as in legs, the introduction of normal quail cells leads to a normal muscle pattern in the mutant.

**CONCLUSION**

The work reported here leads to the following conclusions.

1) Collagen is not primarily implicated in either the development or the maintenance of the muscle pattern of the lower leg. Although substances interfering with collagen biosynthesis had general deleterious effects on the embryos and, in the case of β-aminopropionitrile, damaged specifically the limb skeleton, there was no specific effect on the muscular architecture. Following an indirect immunofluorescence study of the collagen distribution during and after the muscle splitting period of the chick forearm muscles, Shellswell, Bailey, Duance & Restall (1980) rejected the possibility of a primary role of collagen in the divisions and subdivisions of the muscle masses, *i.e.*, in the development of the muscle pattern.

2) In the *crooked neck dwarf* mutant, the inability of the muscular organization to become stabilized into definitive structures is linked to a defect of the striated myogenic cells. Indeed the replacement of the limb-level somitic mesoderm in mutant embryos by a piece of non-mutant somitic mesoderm leads to a normal and stable muscle architecture, and demonstrates that the connective tissue of the mutant is normal. The latter has the ability to organize, as well as to maintain, the muscle pattern.

As the myogenic cells which make up the striated musculature have been considered to constitute a distinct cell line (Kieny, 1980), the results reported here strongly suggest that the mutation alters the myogenic cell line.

The results also support the concept of a two-step formation of the musculature. The first step, which corresponds to the pattern development is controlled by the connective tissue cells. For the second step, which includes the

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*Figs 11–15. Result of heterotopic replacement of wing-level somitic mesoderm in a putative mutant chick host (stage–16 pairs of somites) by non-mutant quail somitic mesoderm (stage–16 pairs of somites) from the neck level.*

*Figs 11, 12. Histology of the muscular tissue in the tibiotarsal segment (Fig. 11, transverse section) and in the forearm on the unoperated side (Fig. 12, longitudinal section) vouching for the mutant phenotype of the host. ×30.*

*Figs 13–15. Restoration of a normal wing muscle pattern on the operated side. Fig. 13. Longitudinal section through the forearm. ×30.*

*Figs 14, 15. Higher magnification of the delineated portions of Fig. 13, showing the heterospecific constitution of the muscles. Tendons (t), perimysial (p) and epimysial (e) envelopes are made up by chick cells, whereas the myogenic cells (arrows) are quail cells. ×400. h, humerus; r, radius; u, ulna.*
pattern maintenance, a normal muscle cell population is required. The mechanism whereby one or the other step is accomplished is far from being understood. Nevertheless, tissue interactions undoubtedly occur in the organogenesis of the musculature.

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