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ORIGINAL ARTICLE

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Contribution of single somites to the skeleton and muscles of the occipital and cervical regions in avian embryos

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Abstract Controversy has surrounded the process of resegmentation of cervico-occipital somites. We have reinvestigated this topic by grafting single somites of quail embryos homotopically into chick embryos. Somites one to five contribute to the skull. Somites one and two contribute to the parasphenoid, which develops by direct ossification in a non-segmental fashion. All cartilaginous derivatives of the somites are segmental. Somite two forms a stripe of cells in the basioccipital, exoccipital and supraoccipital. Somites three to five give rise to the subsequent caudal parts of the basioccipital and exoccipital. Somite five forms the first motion segment including the occipital condyle, the cranial part of the atlas and the tip of the dens axis. Therefore, the border between head and neck is in the centre of somite five, and corresponds to the expression boundary of *Choxb-3*. Somite six forms the caudal part of the atlas and the cranial part of the axis. Somites two to eight all contribute to the cranio-cervical muscles with the exception of the Mm. rectus capitis dorsalis and ventralis and the M. biventer cervicis, which do not receive contributions from somite two. In contrast, the M. cucullaris capitis is exclusively formed by myogenic cells from somite two, which parallels its exclusive innervation by the accessory nerve. Our data confirm the segmental nature of the occiput, and show that resegmentation is a very regular process involving all except the four cranialmost somites. Except for somites one and two, all of the somites contribute to the muscles located at the appropriate levels.

Key words Somite · Occipital skeleton · Atlas · Axis · Cranio-cervical muscles

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Introduction

There has been interest in the development of the cervico-occipital transitional zone since abnormal development can lead to congenital malformations such as the assimilation of the atlas to the base of the skull, and the formation of an additional skeletal element, the Ossiculum terminale. Targeted mutations of *Hox* genes in mice have resulted in homeotic transformations in this region (Kessel et al. 1990) inducing the development of an additional skeletal element, the proatlas (resembling the Ossiculum terminale), or in the loss of the entire atlas (Condie and Capecchi 1994). The proatlas is a transient element of the vertebral column (Froriep 1883; Hayek 1924), and located between the basioccipital bone and the atlas. Its origin and fate have been a matter of debate. The development of the cervical and occipital regions have been studied experimentally in avian embryos, making use of the quail/chick chimera technique (Le Douarin 1969). The results have shown that the border between head and neck is located in the fifth somite but there are contradictory reports as to the number of somites contributing to atlas and axis (Couly et al. 1993; Christ and Ordahl 1995; Wilting et al. 1995), and the process of resegmentation has not been studied in detail in these cranial motion segments. The vertebral column and the basioccipital parts of the skull are derived from sclerotomes. The dermomyotomes of the somites give rise to the skeletal muscles of the trunk. The myogenic potency of the cervico-occipital somites has been studied previously (Noden 1983; Couly et al. 1993; Huang et al. 1997, 1999), but there is controversy as to the contribution of single somites to cranio-cervical muscles.

We have reinvestigated the development of the cervico-occipital region by grafting single somites of quail embryos homotopically into chick embryos. The hosts were reincubated until day 12 and serial sections were stained with an anti-quail antibody (QCPN; Selleck and Bronner-Fraser 1995; Wilting et al. 1995). The results show that the first five somites contribute to the basioccipital bone. Somite five also forms the cranial part of the atlas and

the tip of the dens axis. Somite six forms the caudal part of the atlas and the dens axis, as well as the cranial part of the body of the axis. Somite seven contributes to the caudal part of the axis and to the cranial half of the third cervical vertebra. We show that resegmentation takes place even in the cranialmost vertebral motion segments. In contrast to previous studies (Noden 1983; Couly et al. 1993) we have observed that somites two to eight form the cranio-cervical muscles and only the Mm. rectus capitis dorsalis and ventralis do not receive myogenic cells from somite two. However, somites one and two form the M. cucullaris, which is exclusively innervated by the accessory nerve.

Materials and methods

Embryos The experiments are based on interspecific grafting of single somites from embryos of the Japanese quail (*Coturnix coturnix japonica*) into the White Leghorn chick (*Gallus gallus*). The fertilized eggs were obtained from a local breeder and incubated at 37.8°C and 75% humidity.

Microsurgical procedures For the somite grafting the fertilized eggs were incubated for 30–40 h until stages 8–10 of Hamburger and Hamilton (1951). At these stages the embryos possess somites that have not yet been invaded by neural crest cells (Tosney 1982; Kuratani and Kirby 1991). Grafting was performed as described previously (Huang et al. 1996, 1997). Briefly, quail embryos served as donors and chicken embryos as hosts. Electrolytically sharpened tungsten needles (Dossel 1958) were used for the microsurgery. One of the somites 2–10 of the quail was isolated in ovo after application of two drops of a 0.4% trypsin solution (Sigma, Deisenhofen, Germany). The proteolysis was blocked with horse serum (Gibco, Eggenstein, Germany) after 5–10 min. The quail somite was transplanted homotopically after removal of the corresponding somite of the chicken host. During the operation, Locke's solution was regularly added to prevent the embryo from drying (Locke and Rosenheim 1907). The chimeras were reincubated for 4–10 days and fixed in Serra's solution (Serra 1946) overnight. At least four successful transplantations were evaluated for each somite level.

Immunohistochemistry After dehydration, the embryos were embedded in paraffin and sectioned serially at 8 µm in coronal and sagittal planes. The sections were stained with a monoclonal antibody (QCPN) recognizing a nuclear epitope of quail cells. The antibody was received from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, Iowa, USA) as partially purified IgG and diluted 1:500. An alkaline phosphatase conjugated goat anti-mouse antibody (Dako, Hamburg, Germany) was used as secondary antibody (1:1000), and nitroblue tetrazolium and X-phosphate (Boehringer, Mannheim, Germany) as chromogens. Parallel sections were double-labelled with the anti-quail antibody (QCPN) and with a polyclonal anti-desmin antibody (dilution 1:400). The desmin antibody was detected by a peroxidase-conjugated goat anti-rabbit IgG (dilution 1:300; Sigma), and diaminobenzidine (DAB) was used as chromogen.

Whole-mount in situ hybridization. Chick embryos of stages 10–15 were washed in PBS and then fixed overnight in 4% paraformaldehyde at 4°C. Sense and anti-sense RNA probes of *Choxb-3* (a 400 bp probe was kindly provided by Dr. Linda Ariza-McNaughton and Dr. Robb Krumlauf, London) were labelled with digoxigenin and whole-mount in situ hybridization was performed as described recently (Wilting et al. 1995; Nieto et al. 1996).

Results

The skeletal elements

Somites 1–5 form the basioccipital skeletal elements of the skull. The fate of the first somite has been described recently (Huang et al. 1997). It contributes to the parasphenoid and a segment of the basioccipital, exoccipital and supraoccipital. The parasphenoid develops by direct (mesenchymal) ossification and is of dual origin. It is derived from the preotic mesoderm (Couly et al. 1993) and from somites one and two (Fig. 1B). The cartilaginous parts of the occiput do not show such mixing of cells but are segmental. Somite two forms a stripe of chondrogenic cells in the basioccipital, exoccipital and supraoccipital (Figs. 1A, 3). In the exoccipital, the cochlear duct may serve as a landmark (Fig. 1A–E). The cartilage immediately caudal to the cochlear duct is derived from somite one (Huang et al. 1997). The next, more caudal, segment is from somite two (Figs. 1A, 3). Accordingly, the following two segments of the basioccipital and the exoccipital are derivatives of somites three and four (Figs. 1C–F, 3). This can be seen by the increasing distance of the quail graft from the cochlear duct. Somite four gives rise to that part of the exoccipital that is traversed by the hypoglossal nerve (Fig. 1E, F). Somite five is highly specialized because it is the first somite forming a motion segment, and is starting the process of resegmentation (Fig. 2A–C, 3). It is the anlage of the most caudal part of the exoccipital and the occipital condyle, articulating with the body of the atlas and the dental process of the axis (Fig. 2A). The latter two are also derivatives of somite five (Fig. 2A). Resegmentation becomes obvious because somite five forms the cranial half of the dens axis, the cranial part of the dorsal atlantic arch (Fig. 2C), and the cranial half of the atlantic spinous process (Fig. 2B). The caudal half of the dens axis and the cranial half of the body of the axis are derivatives of somite six (Fig. 2D). These two structures are transiently separated by dense connective tissue. The caudal half of the body of the axis and the cranial half of the third cervical vertebra are formed by somite seven (Fig. 2E). The results are schematically illustrated in Figure 3. They show that the border between head and neck is located in the fifth somite. This border can be visualized by whole mount in situ hybridization with the chick homeobox gene *Choxb-3*. The occipital four somites of day 2 embryos do not express this gene. *Choxb-3* is gradually expressed in somite five and is found in the more caudal somites (Fig. 4).

The cranio-cervical muscles

The occipital bones and the cervical vertebrae are connected by cranio-cervical muscles that consist of six paired muscles. These muscles are summarized in Table 1 and schematically illustrated in Fig. 5A. The muscles can be demonstrated in transverse sections stained with an anti-

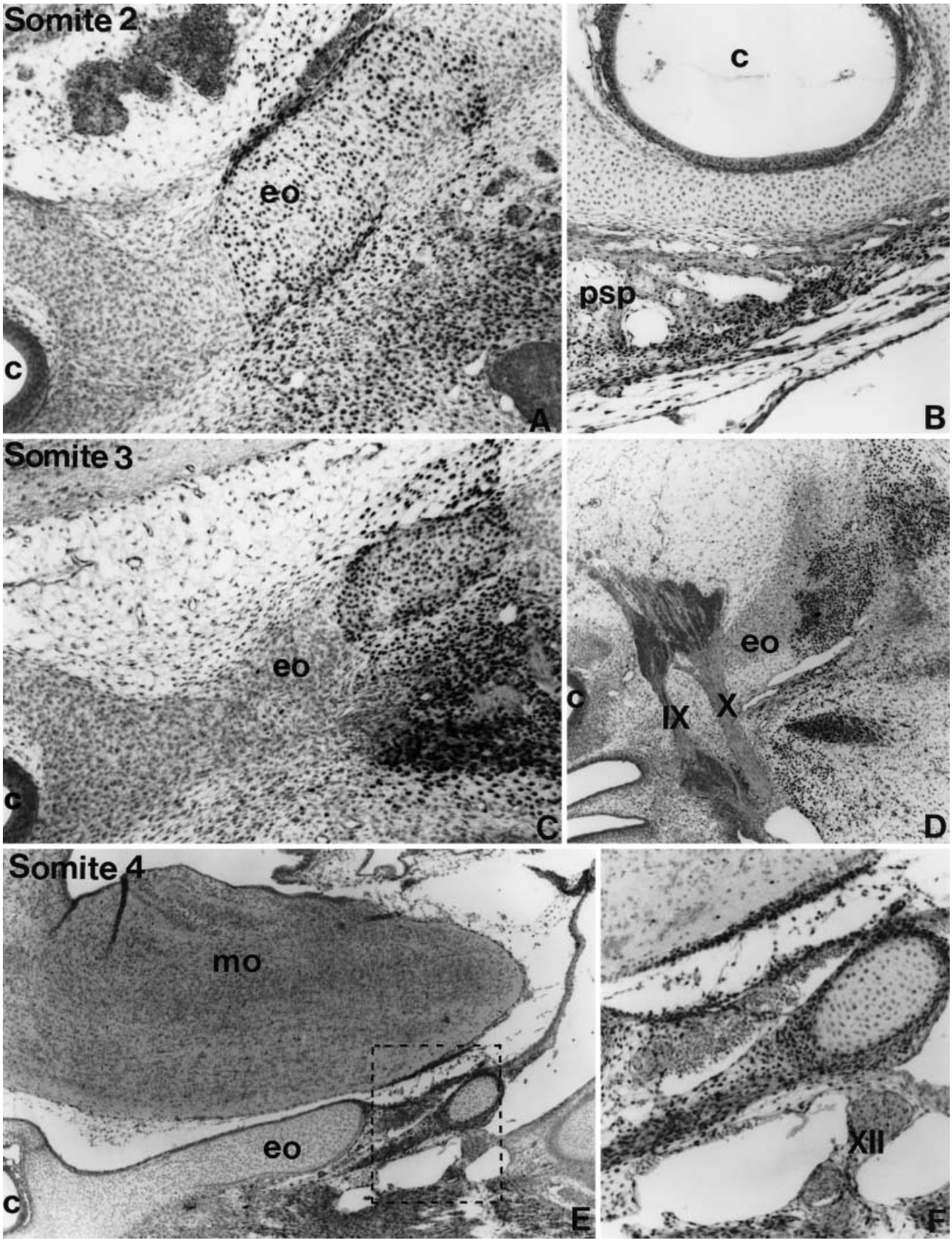

Fig. 1 Sagittal sections through the head of chimeras after grafting of somite 2 (**A, B**), somite 3 (**C, D**) and somite 4 (**E, F**). **A** Reincubation 6 days, **B** reincubation 9.5 days, **C, D** reincubation 5 days, **E** and **F** reincubation 8 days. **F** Enlargement of the frame in **E** showing the Canalis hypoglossus. Nuclei of grafted quail cells are stained with the QCPN antibody (*c* cochlear duct, *eo* exoccipital bone, *mo* medulla oblongata, *psp* parasphenoidal bone, *IX* glossopharyngeal nerve, *X* vagus nerve, *XII* hypoglossal nerve)

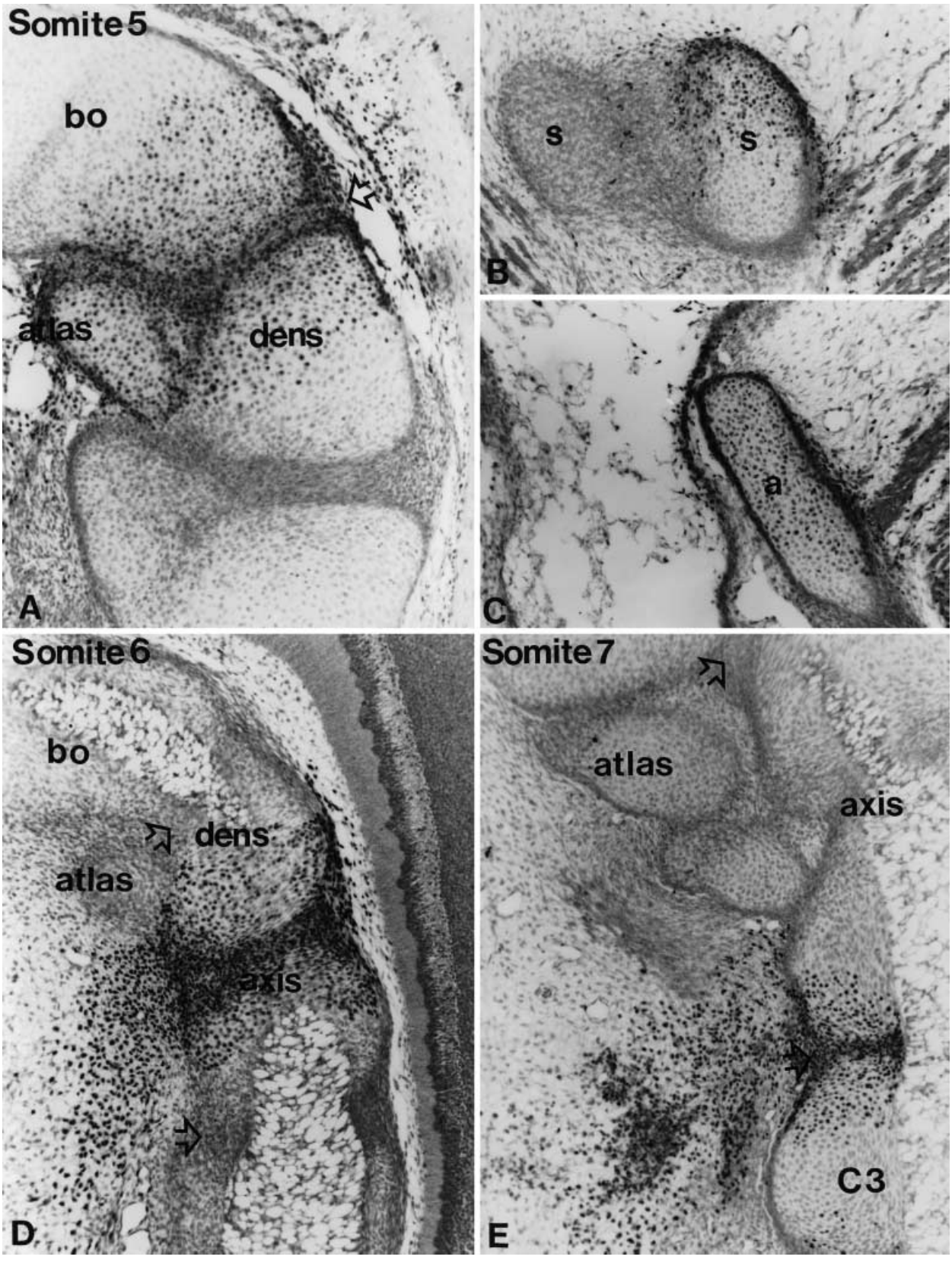

Fig. 2 Sagittal sections through basioccipital bone (*bo*), atlas and axis of chimeras after grafting of somite 5 (**A–C**), somite 6 (**D**) and somite 7 (**E**). Staining of the nuclei of quail cells with the QCPN antibody. **B** Coronal section through the spinous processes (*s*). **C** Coronal section through the posterior arch (*a*) of the atlas. **A–C** Reincubation 7 days, **D** reincubation 4 days, **E** reincubation 6 days (*arrows* boundaries of axis, *C3* the third cervical vertebra)

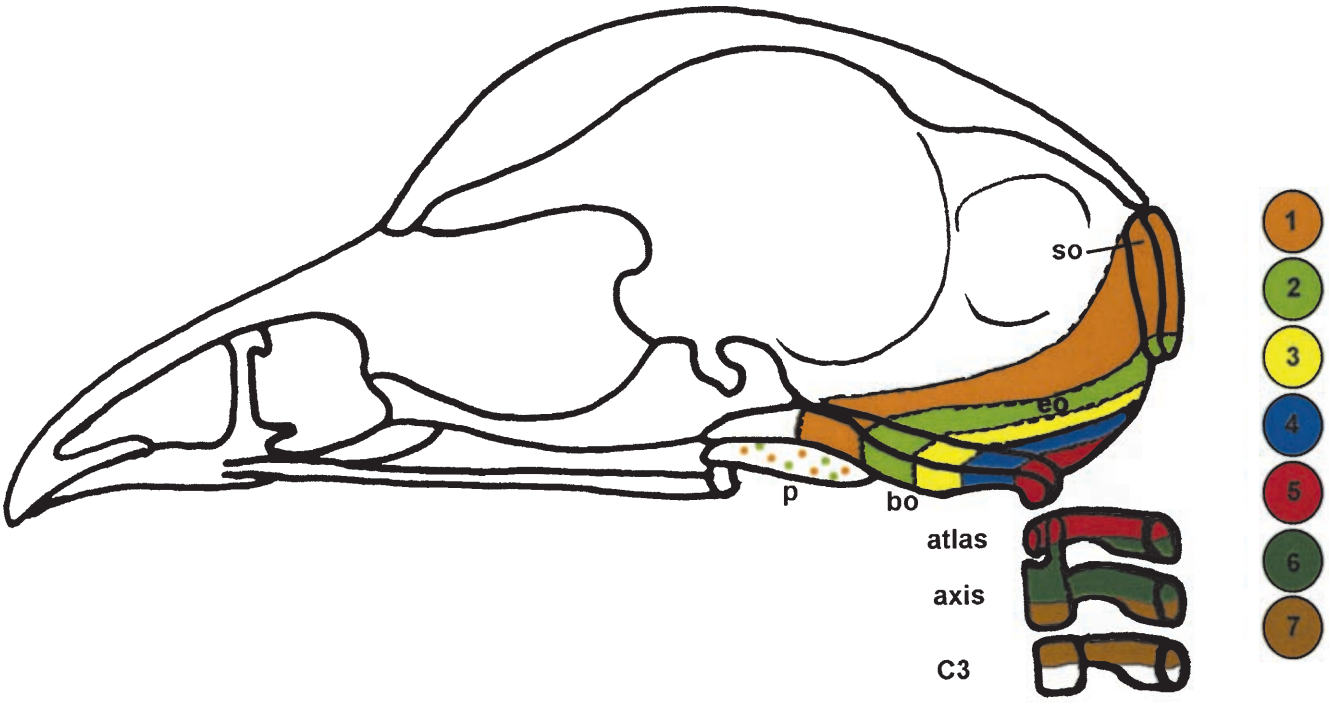

Fig. 3 Schematic illustration showing the distribution of somite-derived cells (labeled by different colours) to the neurocranium and the first three cervical vertebrae. Somites are numbered consecutively from cranial to caudal (*bo* basioccipital bone, *C3* third cervical vertebra, *eo* exoccipital bone, *p* parasphenoidal bone, *so* supraoccipital bone)

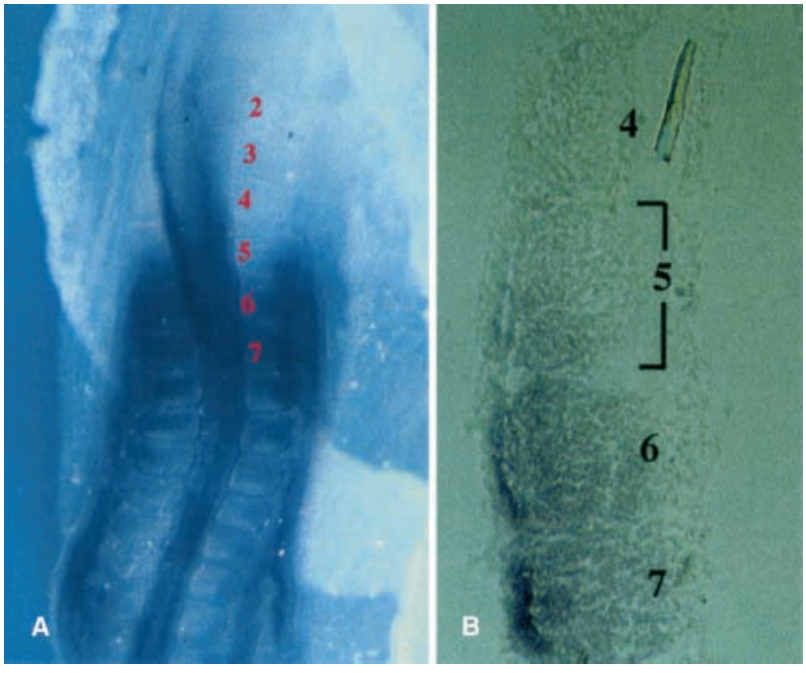

Fig. 4A,B Expression of *Choxb-3* in the head region of a chick embryo of HH-stage 13. **A** Whole mount. Somites 2–7 are numbered. **B** Sagittal section through somites 4–7 of the embryo in **A**. Note the absence of *Choxb-3* expression in the first four somites. Gradual expression starts in somite 5

body against desmin (Fig. 5B). The numbers of the muscles in Fig. 5A,B correspond to those in Table 1. The M. biventer cervicis (6) is the only muscle connecting the thorax with the cranium. It is separated from all other cervical muscles by a fascial sheath. The M. cucullaris capitis (No. 7 in Fig. 5B) is a superficial muscle and innervated by the accessory nerve. It extends from the temporal and occipital bones, dorsal to the Meatus acusticus externus, to the shoulder region.

Our findings on the contribution of single somites to the cranio-cervical muscles are summarized in Table 2. The results show that myogenic cells from somites 2–8

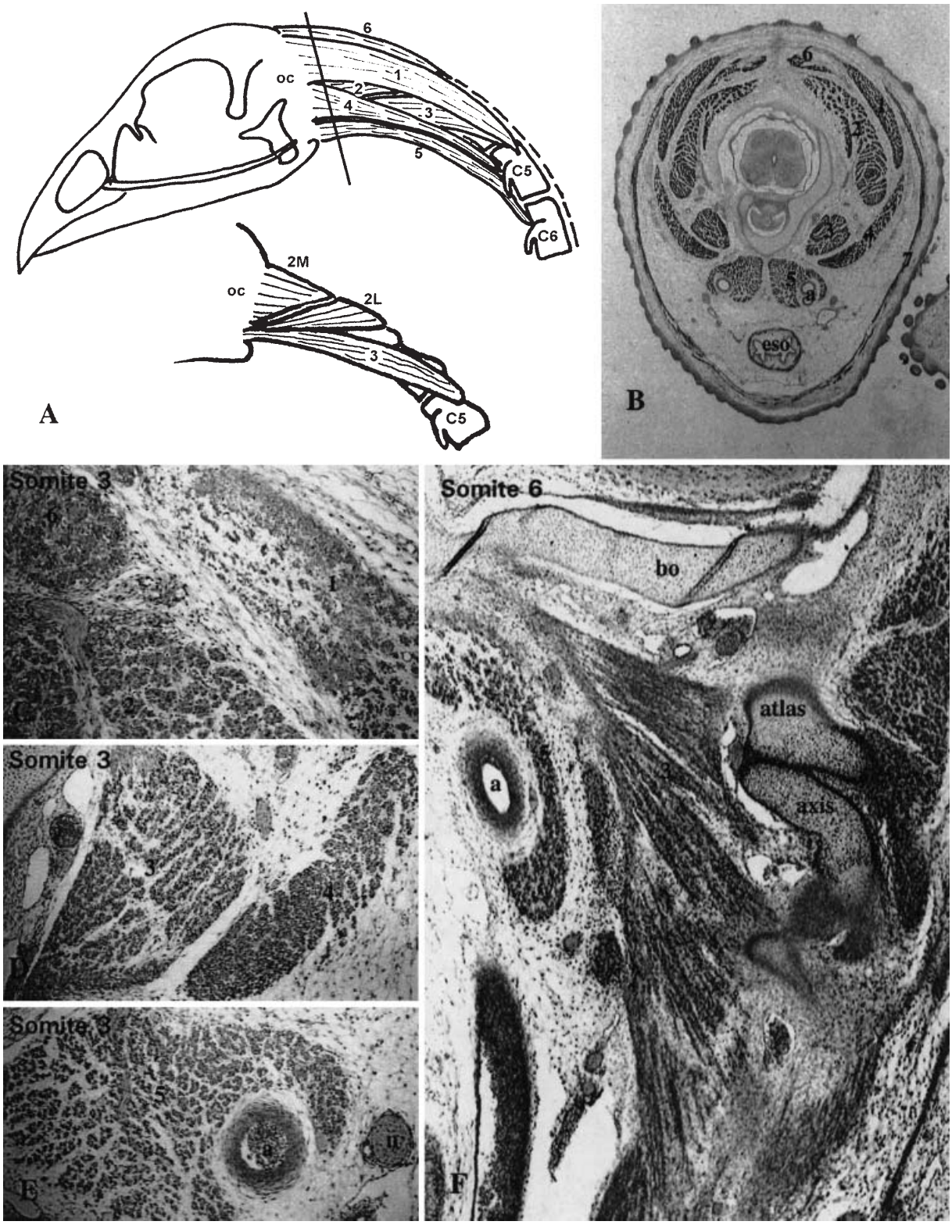

Fig. 5A Schematic drawing of the cranio-cervical muscles of a chick (modified from Vanden Berge and Zweers 1993). The numbers of the muscles correspond to those in Table 1 (*a* carotid artery, *eso* esophagus). **B** Transverse section through the cervical region (as indicated by the black line in **A**) of a 10-day-old chick embryo stained with an anti-desmin antibody. The numbers of the muscles correspond to those in Table 1. **C–E** Transverse sections of a chimera after transplantation of somite 3 and a reincubation period of 8 days (*n* vagus nerve). Quail cell nuclei are stained with the QCPN antibody. Quail cells are found in all of the cranio-cervical muscles (1–5) and the M. biventer cervicis (6). **F** Sagittal section of a chimera after grafting of somite 6 and a reincubation period of 7 days. Quail cells are found in the M. rectus capitis ventralis (*5*) and the M. rectus capitis dorsalis (*3*). Quail cells are also located in the caudal part of the atlas with its processus articularis caudalis, and in the cranial part of the axis with its processus articularis cranialis. (*bo* basioccipital bone)

Table 1 Origin and insertion of the muscles that connect the head with the neck, according to Vanden Berge and Zweers (1993; *C* cervical vertebrae, *T* thoracic vertebrae)

Muscle	Origin	Insertion
Mm. craniocervicales		
M.complexus (1)	Proc. articularis caudalis of C4 Proc. articularis cranialis of C5	Crista nuchalis transversa of Os supraoccipitale
M. splenius capitis (2)	Arcus atlantis Proc. spinosus of axis and C3	Os supraoccipitale (medial and lateral portion)
M. rectus capitis dorsalis (3)	Proc. articularis caudalis of axis Lateral surface of C3 and C4 Proc. articularis cranialis of C5	Lamina basiparasphenoidalis
M. rectus capitis lateralis (4)	Ventral surface of C2–5	Os exoccipitale
M. rectus capitis ventralis(5)		
Pars medialis	Ventral surface of C1–4	Lamina basiparasphenoidalis
Pars lateralis	Ventral surface of C5–6	Lamina basiparasphenoidalis (lateral to Pars medialis)
M. biventer cervicis (6)	Proc. spinosus of T2	Crista nuchalis transversa
Pars cranialis		
Pars caudalis	(The intermediate tendon is in the level between C3 and C7)	
M. cucullaris capitis (7)	Os temporalis, Os squamosum, dorsal to Meatus acusticus externus	Caudal extension to the shoulder region
Pars interscapularis		
Pars propatagialis		
Pars clavicularis		

Table 2 Contribution of single somites to the cranio-cervical muscles, the M. biventer cervicis and the M. cucullaris capitis (*So*/*n* somite number, *Em*/*n* embryo number, *numbers 1–5* correspond to the muscles in Table 1, + grafted quail cells present, – no grafted quail cells present)

So (n)	Em (n)	Mm. craniocervicales					M. biventer cervicis (6)	M. cucularis capitis (7)
		1	2	3	4	5		
2	495	+	+	–	+	–	–	+
	499	+	+	–	+	–	–	+
	505	+	+	–	+	+	–	+
3	479	+	+	+	+	+	–	–
	500	+	+	+	+	+	+	–
	502	+	+	+	+	+	+	+
4	488	+	+	+	+	+	+	–
	496	+	+	+	+	+	+	–
	498	+	+	+	+	+	+	–
5	471	+	+	+	+	+	+	–
	476	+	+	+	+	+	+	–
	477	+	+	+	+	+	+	–
	491	+	+	+	+	+	+	–
6	456	+	+	+	+	+	+	–
	468	+	+	+	+	+	+	–
	486	+	+	+	+	+	+	–
7	421	+	+	+	+	+	+	–
	428	+	+	+	+	+	+	–
	485	+	+	+	+	+	+	–
	454	+	+	+	+	+	+	–
8	422	+	+	+	+	+	+	–
	449	+	+	+	+	+	+	–
	450	+	+	+	+	+	+	–
9	431	–	–	–	–	–	+	–
	451	–	–	–	–	–	+	–
10	430	–	–	–	–	–	+	–

contribute to all cranio-cervical muscles. A typical example is shown in Fig. 5C–E, which demonstrates the contribution of myogenic cells of somite three to the cranio-cervical muscles. Although there was some migration of grafted cells in cranial and caudal directions, the main contribution to the individual muscles was found at the level of the grafting site. Figure 5 F shows the distribution of quail cells after grafting of somite six. This somite forms the caudal part of the atlas and the cranial half of the axis. The myogenic cells are mainly confined to the adjacent muscles (Fig. 5F). There are only two cranio-cervical muscles that do not receive myogenic cells from all of the cranial somites (Table 2). The Mm. rectus capitis dorsalis and ventralis are formed by somites 3–8, but do not receive myogenic cells from somite two. Similar observations were made for the M. biventer capitis (Table 2). In contrast, the M. cucullaris capitis is exclusively derived from myogenic cells from somites one (Huang et al. 1997) and two.

Discussion

Segmentation of the skeleton

The cervico-occipital region is a specialized part of the axial skeleton ensuring high motility of the head (Christ and Wilting 1992). The cranialmost vertebrae are the atlas and the axis. In the human, the atlas consists of an anterior and a posterior arch and the lateral masses. The anterior arch possesses an articular facet for the dens axis, and the lateral masses form the superior articular facets that articulate with the occipital condyles. The inferior facets articulate with the superior articular facets of the axis.

The principal structure of the cervico-occipital region is the same in birds, but modifications reside in the fact that there is only one rostro-medially located occipital condyle. This articulates with a facet on the anterior arch of the atlas (Baumel and Witmer 1993). This arch is therefore quite massive and has been called the atlantic body, although it is not homologous to the vertebral body. Like in mammals, the "body" of the atlas is the dens axis (Hamilton 1952). The dens is formed as an entity that fuses with the body of the axis during the cartilaginous stage of vertebral column development (Christ and Wilting 1992). Therefore, although there are some morphological modifications, the principal structure of the axial skeleton is comparable to mammals, and birds may serve as a model system.

The cervico-occipital region has been studied descriptively in several species, including the human (Froriep 1883; Hayek 1923, 1924; Hadley 1948; Putz 1975; Müller and O'Rahilly 1994). Experimental studies were performed in the mouse (Kessel et al. 1990; Condie and Capecchi 1994), and in the chick (Bagnall et al. 1988, 1989; Couly et al. 1993; Wilting et al. 1995). Studies in the mouse have shown that the *Hox*-code determines the regional differences of vertebrae (Kessel et al. 1990). Overexpression of *Hoxa-7* induces a posterior homeotic transformation characterized by a manifestation of a proatlas and an atlantic "body". The combined deletion of *Hoxa-3* and *d-3* result in an entire loss of the atlas (Condie and Capecchi 1994). The importance of these paralog genes (Scott 1992) for the development of the head-neck transition is supported by our observation that the cranial expression border of *Choxb-3* is in the center of somite five. This somite forms the first motion segment and gives rise to those parts of the skull that surround the great foramen (including the occipital condyle), the cranial part of the atlas and the tip of the dens axis. In this respect, our studies are in line with previous observations (Couly et al. 1993; Wilting et al. 1995). However, our observations on the development of the atlas differ from those of Couly et al. (1993), who have reported that the anterior atlantic arch is a derivative of somite five and the posterior of somite six. We show that resegmentation is a very regular process, and somite five gives rise to the cranial parts of both the anterior and posterior atlantic arches. Somite six forms the caudal parts of the atlas and the cranial parts of the axis. This kind of resegmentation can be observed throughout the vertebral column, and even includes the distal parts of the ribs, which are of sclerotomal origin (Huang et al. 1996, 2000). The sclerotomes possess positional information, and all parts of the axial skeleton that develop by secondary (endochondral) ossification are segmental. This includes the basioccipital bones. The parasphenoid, which develops by primary (mesenchymal) ossification, is not segmental. It is a derivative of somites one (Huang et al. 1997) and two. The osteoblasts derived from these somites are lined up along capillaries. They do not form boundaries. Therefore our study confirms previous observations on the segmental nature of the skull (Couly et al. 1993), and the formation of the head-neck boundary by the fifth somite (Wilting et al. 1995). We additionally show that resegmentation is a very regular process, starting with somite five, which forms the first motion segment. We also provide evidence that osteoblasts do not possess segmental identity. Pattern formation seems to be imparted either by blood vessels or the connective tissue surrounding the dermal bones.

The cranio-cervical muscles

In the neck region of birds there are three groups of muscles: (1) The M. cucullaris capitis, which is located immediately beneath the skin, (2) the M. biventer cervicis, which is the only muscle connecting the head to the thorax, (3) the cranio-cervical muscles, which comprise a group of five muscles (see Tables 1, 2). The origin of these muscles from the paraxial mesoderm has been shown previously (Noden 1983; Couly et al. 1993), but there is controversy about the precise somitic levels from which each muscle arises. The M. cucullaris was reported to be derived from somites one to six (Couly et al. 1993), whereas we have observed that its progenitors are restricted to somites one (Huang et al. 1997) and two. This is paralleled by the fact that the M. cucullaris is innervated exclusively by the accessory nerve (Baumel and Witmer 1993). The accessory, glossopharyngeal and vagal nerves traverse the exoccipital in the region that is formed by somite one, underlining the close relationship between segmental origin and innervation of muscles. The M. cucullaris of birds is largely homologous to the Mm. sternocleidomastoideus and trapezius of the human. The M. biventer cervicis was reported to be derived from somites two to five (Couly et al. 1993) or somites three to four (Noden 1983). The muscle extends from the head to the thorax and we have observed that it is formed from myogenic cells of somites three to ten. Somite ten was the last somite included in this study and it is likely that further somites contribute to the M. biventer cervicis. Controversal results have also been obtained as to the segmental origin of the cranio-cervical muscles proper (Noden 1983; Couly et al. 1993). In contrast to previous studies we have observed that these five muscles (M. complexus, M. splenius capitis, Mm. rectus capitis dorsalis, lateralis and ventralis) receive myogenic cells from somite two to eight, with the exception that somite two does not contribute to the Mm. rectus capitis dorsalis and ventralis. Somite one does not contribute to any of the cranio-cervical muscles (Huang et al. 1997). This is consistent with the observation that the myotomes of somites one and two are considerably smaller than those of the more caudal somites, as can be demonstrated by the expression of myogenic determination genes (Huang et al. 1999). Therefore, the number of myogenic cells is obviously lower in somites one and two. In summary, our study shows that there is a contribution of myogenic cells from the somites to the muscles located at the corresponding levels. An exception is found at the level of so-

mites one and two, which contribute only to a small extent to the cranio-cervical muscles, but form the M. cucullaris, which is innervated by the accessory nerve.

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