Mouse-chick chimera: a developmental model of murine neurogenic cells

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SUMMARY

Chimeras were prepared by transplanting fragments of neural primordium from 8- to 8.5- and 9-day postcoital mouse embryos into 1.5- and 2-day-old chick embryos at different axial levels. Mouse neuroepithelial cells differentiated in ovo and organized to form the different cellular compartments normally constituting the central nervous system. The graft also entered into the development of the peripheral nervous system through migration of neural crest cells associated with mouse neuroepithelium. Depending on the graft level, mouse crest cells participated in the formation of various derivatives such as head components, sensory ganglia, orthosympathetic ganglionic chain, nerves and neuroendocrine glands. Tenascin knockout mice, which express lacZ instead of tenascin and show no tenascin production (Saga, Y., Yagi, J., Ikawa, Y., Sakakura, T. and Aizawa, S. (1992) Genes and Development 6, 1821-1838), were specifically used to label Schwann cells lining nerves derived from the implant. Although our experiments do not consider how mouse neural tube can participate in the mechanism required to maintain myogenesis in the host somites, they show that the grafted neural tube behaves in the same manner as the chick host neural tube. Together with our previous results on somite development (Fontaine-Pérus, J., Jarno, V., Fournier Le Ray, C., Li, Z. and Paulin, D. (1995) Development 121, 1705-1718), this study shows that chick embryo constitutes a privileged environment, facilitating access to the developmental potentials of normal or defective mammalian cells. It allows the study of the histogenesis and precise timing of a known structure, as well as the implication of a given gene at all equivalent mammalian embryonic stages.

Key words: mouse, chick, nervous system, chimera

INTRODUCTION

Shortly after it is formed, the neural plate folds up and its lateral ridges fuse on the mediadorsal line, giving rise to the neural tube, which later provides the central nervous system (CNS) and the neural crests from which the peripheral nervous system (PNS) is largely derived. The neural tube is initially flat epithelium (the precursor of the ependymal layer) and then becomes columnar. During CNS development, the cells generated by the neuroepithelium migrate, but remain confined within the epithelium itself. They form a germinal layer along the inner lining and then migrate, assembling at the outer margin to constitute the mantle layer in which the motor neurons develop. The tips of their axons penetrate through the external limiting membrane to constitute bundles (the motor nerves) and advance toward their target. The neural crest, in association with the neural tube, is a transient embryonic structure whose cells undergo migrations at precise periods of development, finally settling in specific locations where they differentiate into a variety of cell types, including neurons and glial cells of autonomic and sensory ganglia, endocrine cells of the carotid body and adrenal medulla, pigment cells, and skeletal and connective tissues of the craniofacial skeleton (Le Douarin, 1982). These results have been largely demonstrated in birds through the use of microsurgical procedures in chick and quail embryos.

For mammals, many questions about the origin and developmental potential of cellular components of the nervous system remain unanswered. Progress in this field requires an experimental system that will allow the isolation of identified progenitor cells and their study in vivo. There are difficulties in working on the mouse embryo that hinder analysis of cell lineage, so to aid identification several authors have labeled neural tubes and associated neural crest cells of mouse embryos with a solution of Dil, a fluorescent carbocyanine dye (Serbedzija et al., 1989; Trainor and Tam, 1995), or an iontophoretic injection of LRD, a lysinated rhodamine dextran (Serbedzija et al., 1994), prior to culturing them. Yet, as embryo cultures can be maintained for only 24 hours, the use of this technique is too restrictive for an efficient study of the developmental mechanisms in mammalian embryogenesis. Therefore, we recently devised a technique using chick embryo as a host for developing mouse cells. We first attempted to trace the development of mammalian somitic derivatives by replacing somites in chick embryo unilaterally with somites from mouse fetus (Fontaine-Pérus et al., 1995). Particular attention was given to the myogenic behavior of grafted mouse cells through the use of desmin nls lacZ transgenic mice (Li et
al., 1993), with transgene expression serving as a specific marker of mouse muscle cells. We demonstrated that the avian embryonic environment can influence the fate of the grafted somites. Mouse somitic cells gave rise in ovo to dermis, cartilage and muscle, and then migrated to participate in the formation of hypaxial muscles in the chick host. In the present work based on the same procedure, we examined the embryonic development of the mouse nervous system after mouse neural primordium was grafted into chick host embryo. We first evaluated the effect of signals emanating from grafted mouse neural tube on chick host myogenesis. Signals from the neural tube and the notochord have been shown to regulate myotome differentiation in ovo (Rong et al., 1992; Christ et al., 1992; Pownall et al., 1996) and in vitro (Packard and Jacobson, 1976; Vivarelli and Cossu, 1986; Kenny-Mobbs and Thorogood, 1987; Buffinger and Stockdale, 1994, 1995; Stern and Hauschka, 1995; Cossu et al., 1996). The initiation of the myogenic program is clearly controlled by a set of basic helix-loop-helix (BHLH) transcription factors including Myo-D, Myogenin, Myf-5 and MRF-4 (for review, see Buckingham, 1994). We analyzed Myo-D gene expression in chick myotomes forming at the level of mouse neural tube to determine whether mouse neural tissue in our chimeric system was capable of contributing to the signaling processes required for myogenic gene expression in somites. Accordingly, in the present study, mouse neural transplants were performed at the level of segmental plate mesoderm to determine whether chick somites adherent to mouse tube can form and differentiate. Our findings indicate that chick cells follow their normal differentiation in newly formed somites at the graft level. Finally, we attempted to trace the fate of mouse neural crest cells in the chick environment. Our findings indicate that these cells are able to migrate in ovo and reach the normal arrest sites of host neural crest. Numerous extracellular matrix substances that line the migratory pathway followed by neural crest cells are thought to be involved in their patterning, including tenasin, a glycoprotein closely associated with axons, which appears to play a prominent role in guiding their ingrowth. According to Tucker and McKay (1991), neural crest-derived cells associated with developing spinal ventral roots are the major source of tenasin (Wehrle and Chiquet, 1990), whereas Tan et al. (1991) have demonstrated that there is no causal link between the presence of neural crest-derived cells and the distribution of tenasin. A mouse line in which tenasin C gene expression was deleted and replaced by the reporter gene lacZ has been produced by Saga et al. (1992). These deficient mice make it possible to localize the protein, since transgene expression is indicative of its accumulation. In our mouse-chick neural chimera, chick host neural tube was replaced by that arising from mutant mice. Spinal roots manifesting lacZ activity appeared ventrally, and the enzyme was expressed precisely by the neural crest itself, clearly indicating that tenasin was synthesized by the glial cells accompanying the spinal roots.

In the vertebrate Msx1, a homeobox-containing gene, is associated with epithelio-mesenchymal interactions at many sites (for a review, see Davidson, 1995). This gene has also been described in ecto-mesodermal structures of the head formed by superficial ectoderm and neural crest-derived mesenchyme (Couly et al., 1993), thereby accounting for the abnormalities in craniofacial development found in Msx1-deficient mice (Satokata and Maas, 1994; Houzelstein et al., 1997). In our experiments, Msx1 gene expression was used to mark the early steps in mouse neural implant development.

**MATERIALS AND METHODS**

Experiments were performed using JA657 chicks and Swiss mice from commercial sources. Homozygous tenasin-deficient mice were obtained from Dr S. Aizawa (Saga et al., 1992). Embryos were staged according to the somite number before day 3 of incubation (E), and then by the incubation day number for chick embryos and the gestation period for mouse embryos (Rugh, 1968).

Pregnant mice from the Swiss strain and transgenic lines were killed by cervical dislocation before removal of embryos at 8 (6 somites), 8.5 (10-15 somites) or 9 (15-20 somites) dpc (day of vaginal plug=day 0 of gestation). Host embryos formed between 6 and 20 somites at the time of surgery.

**Microsurgical production of chimeras**

Reciprocal exchanges of precisely defined regions of the neural tube were performed between chick and mouse embryos (Fig. 1). After withdrawal of 0.5 ml of albumin, the upper part of the shell was opened to gain access to the chick embryo. A mixture of Tyrode’s solution and drawing ink was injected beneath the embryonic area to allow its visualization. The vitelline membrane was removed, and Tyrode’s solution was laid on the embryo to prevent dehydration. The neural tube region to be grafted was first delimited in the mouse donor and the chick host and then removed from the host and replaced by the donor graft. The chimeras were killed at various stages of development between E1.5 and E14, depending on the series (Table 1).

**Series I:** In a first series of experiments (involving 200 embryos), a fragment of mouse neural tube was implanted into 14- to 19-somite chick embryo over a length corresponding to the last six somites.

**Series II:** In a second series (involving 50 embryos), a fragment of mouse neural tube was implanted into 16- to 22-somite chick embryo at the adrenomedullary level, including the level of somites 18-24.

**Series III:** In a third series (involving 81 embryos), mouse neural tube was implanted into 15- to 21-somite chick embryo at the adrenomedullary level, including the level of somites 18-24.

**Series IV:** In a fourth series (involving 30 embryos), the prosencephalon (including a part of the mesencephalon) of 8 dpc mouse embryo was implanted into 6-somite chick host embryo (Series IV), or the anterior rhombencephalon of

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<th>Series</th>
<th>Surgery levels</th>
<th>Number of operated embryos</th>
<th>Number of observed embryos</th>
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<tr>
<td>Series I</td>
<td>Truncal level (Somites 14-19)</td>
<td>200</td>
<td>150</td>
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<tr>
<td>Series II</td>
<td>Adrenomedullary level (Somites 18-24)</td>
<td>50</td>
<td>37</td>
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<td>Series III</td>
<td>Unsegmental plate</td>
<td>81</td>
<td>57</td>
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<tr>
<td>Series IV</td>
<td>Prosencephalon (Mesencephalon)</td>
<td>20</td>
<td>16</td>
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<td>Series V</td>
<td>Rhombencephalon</td>
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8.5 dpc mouse embryo (10-somite stage) was implanted into 10- to 11-somite chick host embryo (Series V).

**Histological analysis**

Host embryos were fixed at 4°C in Carnoy’s solution before being embedded in paraffin, cut in transverse serial sections and stained according to the Feulgen-Rossenbeck (1924) procedure, which allowed us to distinguish chick from mouse nuclei (see Fontaine-Pérus et al., 1995) (Fig. 2). The chimerism of cryostat-cut sections was analyzed after acridine orange (Fontaine Pérus et al., 1985) or bis-benzimide staining (Hoechst No. 33258).

**Electron microscopic analysis**

The paraxial muscles from chimeras were dissected out and immersed in 2.5% glutaraldehyde in 0.1 M phosphate Milloning’s buffer, pH 7.2, for 1 hour at 4°C. Tissues were washed in 0.1 M Palade’s buffer (pH 7.4) and postfixed in 2% osmium tetroxide diluted in the same buffer. They were then rinsed in Millipore-filtered water, stained en bloc with aqueous 2% uranyl acetate at 4°C for 1 hour, dehydrated and embedded in Epon. Ultrathin sections were cut, stained with 2% uranyl acetate for 30 minutes at room temperature and stored in Reynold’s lead citrate for 7 minutes. Sections were observed at 80 kV and photographed on a Philips 410 transmission electron microscope.

**Skeletal preparations**

Skeletal preparations were done using an alcian blue-alizarin staining procedure, according to the method of Kessel et al. (1990).

**Immunohistochemical and histochemical procedure**

**Embryo preparation**

Chick host embryos were fixed in 4% paraformaldehyde solution in phosphate-buffered saline solution (PBS)+7% sucrose overnight at 4°C. Fixed embryos were then washed in PBS+7% sucrose for 24 hours at 4°C and immersed in a 15% saccharose/PBS solution for 24 hours before being frozen in liquid nitrogen-cooled isopentane. Sections (7 μm) were obtained along the whole length of the grafted region and collected on gelatin-coated slides. Every section corresponding to the implanted zone was treated.

**Immunocytochemical treatment**

According to the tissue types analyzed, different immunocytochemical treatments were used (anti-68 kDa neurofilament, anti-substanceP, anti-somatostatin and anti-Islet-1 protein monoclonal antibody; anti-
GFAP and anti-tyrosine hydroxylase polyclonal antibody). Sections were incubated overnight at 4°C with primary monoclonal or polyclonal antibody. They were then rinsed in PBS for 1 hour and incubated, according to the primary antibody type used, with fluorescein- or peroxidase-labeled goat anti-mouse, anti-rabbit or anti-rat secondary antibody for 1 hour at room temperature. In peroxidase-stained samples, primary antibody was visualized with 3,3'-diaminobenzidine as the peroxidase substrate and nickel-ammonium-sulfate (0.5%) for signal amplification.

**Histochemical procedure for β-galactosidase**
Sections were immersed in a solution containing 2 mM 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal, Sigma), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl₂ in PBS. Staining was performed for 4 hours at 30°C.

**In situ hybridization procedure**

 Probe preparation
Briefly, CMD1 (Chick Myo-D) and Msx 1 cDNA were linearized respectively with HindIII and PstI. T7 and T3 RNA polymerases were used, respectively, to transcribe cRNA probes. The digoxigenin UTP (Dig) analogue was incorporated into cRNA transcripts.

Hybridization analysis
Embryos were prepared as described above and then embedded in 7.5% gelatin/15% sucrose in buffer and serially sectioned with a cryostat (7-15 μm). The highly sensitive in situ hybridization procedure of Henrique et al. (1995) was used in our study. The probe was diluted in hybridization buffer and laid on sections for 24 hours at 65°C, which were then washed three times, treated with 2% Boehringer Mannheim blocking solution for 2 hours and incubated in conjugated anti-digoxigenin antibody. The staining reaction proceeded overnight at room temperature and was stopped by addition of PBS+EDTA. Sections were then dehydrated and mounted in Entellan.

Some samples of operated embryos were fixed for 24 hours in fresh 4% paraformaldehyde in PBS, washed once in PBT (PBS/0.25% Tween 20), dehydrated through a series of 5-minute washes in 25%, 50%, 75% and 100% methanol, and then stored at -20°C. After rehydration, hybridization was performed, and embryos were then photographed as whole mounts in PBT.

**RESULTS**

**In ovo development of mouse neural tube**
The development of in ovo implanted mouse neural tube was specifically analyzed after truncal neural tube exchanges (Fig. 1, Series I). In this series, surgery was performed on chick embryos in which neural tube at the 12- to 20-somite level (14- to 20 somite stage) was replaced by the caudalmost part of 15- to 20-somite mouse embryos (Fig. 1A,D). A total of 150 cases was examined between 5 hours (Fig. 1E) and 12 days postsurgery. Labeling with Feulgen-Rossenbeck and bisbenzimidizide nuclear staining allowed the behavior of implanted mouse cells to be studied up to the time of their total differentiation.

At 1 day postsurgery, histological examination indicated that mouse neural tube was well inserted into the host. The grafted tissue was covered with healing host ectoderm and underwent normal histogenesis (Fig. 3A). Immunoreactivity for neurofilament protein first appeared in restricted areas of the neural primordium, with 68 kDa antiserum staining a small number of cells and their processes located exclusively at the ventrolateral aspect of the neural tube near the outermost border of the neuroepithelium. Subsequently, implanted neural tube grew dorsoventrally. Its height became greater than its width, and the central canal was transformed into a slit. The dorsal and ventral walls became thinner.
than the lateral walls and formed the early roof and floor plates (Fig. 3B). At this time, neurofilament-positive cells formed a ring, with their processes running around the neural tube (Fig. 3C). The greatest dorsoventral growth in the graft occurred before the end of the second day. Feulgen-Rossenbeck and bisbenzimide mitotic staining showed that cells of implanted mouse epithelium proliferated, preferentially in the inner germinal zone of the neural epithelium. A few fusiform cells were also observed at this time, which became larger and multipolar in subsequent days, occupying the lateral motor columns (apparently as motoneurons). They induced axonal processes laterally and ventrally, which accumulated large amounts of neurofilament protein and later formed ventral roots.

Between 3 and 4 days postsurgery, the development of neuroblasts and the nerve fiber system resulted in cytoarchitectural changes (Fig. 3D,E). The lateral motor columns became well developed (Fig. 3E) and stained by their neurofilament content. Older developmental stages were characterized by a progressive thickening of the neurofilament marginal layer, separated into ventrolateral and dorsal white columns, and an enlargement of areas containing neurofilament-reactive cell bodies (Fig. 3F). Between 5 and 6 days postsurgery, the lateral white column receiving the axons of commissural and associational neuroblasts (which had increased in number) underwent marked development. From day 6, the dorsal white columns became readily distinct and the central canal smaller, whereas the ventral white columns had enlarged somewhat (Fig. 3F). At this time, the first signs of differentiation of astrocyte glial cells occurred, as indicated by their fibrillary acidic protein content. Abney et al. (1981), who studied the differentiation of cell cultures of embryonic rat brain, found a correlation between the first expression of galactocerebrosides by oligodendrocytes and of GFAP by astrocytes. Although no oligodendrocyte marker was used in our experiments, the identification of astrocytes by their GFAP content was relevant to oligodendrocyte differentiation. Assessment of motoneuron differentiation was ascertained by the use of a marker of the postmitotic motoneuron, Islet 1 (Karlsson et al., 1990; Ericson et al., 1992). The expression of this marker, as analyzed in the spinal cord of E9 chick host embryos (Fig. 3G,H), showed that motoneuron nuclei had developed in the ventral horns of the mouse spinal cord. Throughout the study period, chick host vertebra organized around the mouse spinal cord by differentiation of sclerotomal somitic cells at the graft site (Fig. 4A). This was confirmed by examination at E10 of the skeletons of two chimeras obtained after neural tube exchange at the 12- to 20-somite level (Fig. 4B).

Attention was given to ventral horn development which was analyzed between days 2 and 10 postsurgery. At days 3-4, motoneurons could be readily identified because of the large size of their nuclei and their position as a densely packed population, easily distinguished from the rest of the gray matter. The total number of lateral motoneurons in every fifth section throughout the entire implanted spinal cord region was counted on one side of 12 chick host embryos ranging in age from E4 to E12 of incubation. During this period, mouse motoneuron nuclei increased in size from 7-9 μm at E6 to 10-12 μm at E10, but decreased in number. Calculation of the mean number of...
motoneurons was based on counts made for two cords per stage (E4, 180; E5, 240; E6, 200; E8, 130; E9, 100; E10, 75; E12, 80), the peak number being reached at day 3 postsurgery. 68% of the motoneurons initially present in the motor column died between days 3 and 8 postsurgery.

**Mouse neural tube and chick myogenesis**

During the anteroposterior progression of somite differentiation, myogenic bHLH genes are activated in myotomal cells (Sassoon et al., 1989; Charles de la Brousse and Emerson, 1990; Bober et al., 1991; Hinterberger et al., 1991; Ott et al., 1991; Pownall and Emerson, 1992). In birds, *CMD1 (Myo-D)* expression is considered to be the earliest marker of cells engaged in the myogenic lineage. In our study, the Dig hybridization method was used to assay Myo- D gene regulation during somite formation. In chick embryo, the first manifestation of Myo-D gene occurred from the 12-somite stage in the first nine somites and then progressively invaded other somites with advancing age. Between the 14- and 18-somite stages, the gene was expressed in all somites formed along the anterior-to-posterior axis but not in the segmental plate. Expression was restricted at first to a small cluster of dorsal medial somite cells corresponding to myotomal cell progenitors and then increased, progressively affecting the entire myotome. In a first series, the expression pattern of Myo-D gene was studied in the absence of any neural influence. Microsurgical removal of the neural tube was performed at the level of the unsegmented paraxial mesoderm, caudal to the youngest newly formed somites (Fig. 1, Series III). 25 embryos were examined between 1 and 9 days following neural tube extirpation. Myo-D transcripts were detectable in all embryonic stages examined 2 days after excision of the neural tube. From day 3 postsurgery, a decrease in Myo-D transcripts occurred in myotomes developing at the surgery level as compared to those in control embryos. This difference was maintained throughout the period considered (Fig. 5A,B).

32 chimeras with neural tube graft implanted at the unsegmental plate level were analyzed (Fig. 1, Series III), and myogenesis was studied in chick somites differentiating at the implantation site. In all operated embryos examined between 1 and 9 days following the graft, levels of Myo-D transcripts were equivalent all along the paraxial zone (Fig. 5C). At the surgery level, Myo-D transcripts were equivalent in myotomes of chimeras and control embryos. The extent of the Myo-D-positive zone was greater

![Fig. 6. Macroscopic views of mouse-chick chimeras implanted at the 6-somite stage, at the prosencephalon-mesencephalon level, with the equivalent zone from 8 dpc mouse embryo and examined (A) 1 day postsurgery and (A1) 3 days postsurgery. (A2) In toto hybridization with Msx1 probe of the embryo examined in (A1). (B-E) Transverse sections of the embryo observed in A after Feulgen-Rossenbeck staining. (B) Mouse cells (arrows) were identified in the maxillary process. (C) The telencephalon was derived from the graft. (D) Adenohypophyseal tissue was of mouse type. (E) Presence of mouse cells was noted in the facial skull. (F) Transverse section of a mouse chick-chimera implanted at the 10-somite stage, at the rhombencephalon level, with the equivalent zone from 8.5 dpc mouse embryo: examination 4 days postsurgery, after Feulgen-Rossenbeck staining, revealed the IX root cranial sensory ganglion near mouse rhombencephalon. Chick host notochord (N) and vertebra (V) developed at this level. (G) The IX cranial ganglion appeared to be totally composed of mouse crest-derived cells.](image-url)
in mouse-chick chimeras than in denervated chick embryos. The presence of mouse neural tube allowed restoration of a normal level of Myo-D transcription in the grafted zone (Fig. 5C,D).

**In ovo development of mouse neural crest cells**

The neural crest contains a population of cells primarily defined in terms of their migratory ability and the derivatives they form. The vast repertoire of the neural crest, and the fact that environmental cues influence cell fate, makes the differentiation of the neural crest an excellent developmental model.

**Prosencephalic-mesencephalic neural crest**

In 16 cases examined between 1 and 4 days after isotopic and isochronic implantation of mouse prosencephalon and a part of the mesencephalon (Fig. 1B, Series IV,F), the chick host head was observed macroscopically. During this period, the general morphology of the host head showed features strikingly similar to those of mouse head during embryogenesis, indicating that the in ovo graft conditions allowed mouse head development to occur (Fig. 6A,A1). 1 day postsurgery, Msx1 gene expression, specifically visualized after in toto hybridization at the mouse implant level, was restricted to the dorsal area of the graft (Fig. 6A2). Subsequent histological examination showed that cells from the implanted mouse neural crest reached the facial aspect of the head and participated in the maxillary process (Fig. 6B). The anterolateral neural ridges gave rise to the superficial ectoderm lining the nasal cavity. In the brain, the telencephalon was derived from the graft (Fig. 6C). The adenohypophyseal glandular cords were also composed of mouse cells (Fig. 6D), and mouse cells from the graft were found in the facial part of the skull (Fig. 6E).

**Anterior rhombencephalic neural crest**

In this experimental series, we replaced the mid-rhombencephalon (Fig. 1, Series V) of 10-somite stage chick hosts with equivalent levels isolated from 8.5 dpc mouse embryos. Seven chimeras were examined between days 4 and 7 postsurgery. The mid-rhombencephalon is responsible for the formation of the cranial sensory ganglia (Ayer-Le Lièvre and Le Douarin, 1982; D’Amico Martel and Noden, 1983), notably the proximal jugular superior ganglionic complex and the distal petrosal and nodose ganglia. In our experiments, the root sensory ganglia of cranial nerves IX and X were entirely derived from the graft (Fig. 6F,G), since both neurons and glia belonged to the mouse species. Mouse cells of glial type were identified in the petrosal and nodose ganglia of cranial nerves IX and X, respectively (Ayer-Le Lièvre and Le Douarin, 1982). In one chimera examined at day 7 postsurgery, a few mouse-derived neural crest cells were found in the chick host carotid body.

**Truncal neural crest**

A total of 150 cases was examined between 18 hours and 12 days postsurgery after truncal neural tube exchanges (Fig. 1, Series I). At 18 hours postsurgery, neural crest cells had left the mouse neural tube and invaded the cell-free spaces between the graft and the inner side of host somites (Fig. 7A). As in quail-chick chimeric embryos, it is likely that most of the cells that migrated between the neural tube and the somites aggregated to form the dorsal root ganglia, whereas those that passed between two adjacent somites migrated further ventrally and were quite probably involved in building up the sympathetic nervous system.
trunk structures (sympathetic chain and plexus and the adrenal medulla) (Le Douarin, 1982).

**Ontogeny of the dorsal root ganglia (DRG)**

24 hours postsurgery, the early spinal ganglionic primordium appeared to be composed of densely packed small cells (Fig. 7B). Between 24 and 48 hours, mouse ganglionic cells had actively proliferated, as indicated by mitosis after nuclear staining. Differentiation of mouse neuroblasts, as visualized by their neurofilament content, occurred from day 2 postimplantation. By the third day, two distinct groups of neurons began to form: some that increased in size and others that remained small. Both types of neurons, as well as their corresponding glial cells, belonged to the mouse species (Fig. 7C). At this time, cell death occurred, as revealed by the presence of pycnotic nuclei. In avian embryos, small neurons are referred to as mediodorsal and large neurons as ventrolateral, depending on their spatial distribution. Substance P-containing neurons are restricted to the mediodorsal zone. In mammals, there is evidence that substance P accumulates in a population of (probably small) DRG neurons with no particular distribution pattern. In our mouse-chick chimera, substance P nerve cells that developed throughout the graft-derived DRG belonged to the small population of ganglion cells (Fig. 7D). It is noteworthy that the morphology of the ganglia derived from implanted crest cells resembled that of mouse embryo at an equivalent age, being spindle-shaped and generally smaller than host sensory ganglia (see Fig. 7D). 4 days after surgery, mouse spinal ganglia developing in vivo were comparable to 12-day mouse spinal ganglia, and became maximal in size at day 7 postsurgery. Mouse ganglia were dorsally connected to chick host spinal cord by the dorsal roots. At the ventral level, ganglionic cells, through their processes, combined ventral roots into ramus communicans, which penetrated the axial muscle primordia by dorsal and ventral rami. At the graft site, mouse neural crest-derived glial cells accompanied growing nerve rami (Fig. 8A) and later developed into myelinating Schwann cells (Fig. 8B). These cells are thought by some authors to be the main source of tenascin C (Wehrle and Chiquet, 1990; Tucker and McKay, 1991), whereas others (Yip et al., 1995) consider that most of the tenascin around nerves originates from cells of the somitic mesoderm. A recent report has demonstrated that mice lacking tenascin develop normally (Saga et al., 1992). In these mice, the lacZ gene inserted just before the translational codon in exon 2 of the tenascin gene deleted about two-thirds of exon 2 and a part of intron 2, so that the lacZ gene was expressed under the control of a regulatory unit of the tenascin gene, i.e. lacZ expression precisely mirrored tenascin expression. Eight chimeras in our study, which received a neural tube graft at the truncal level arising from tenascin-deficient mouse embryo, were examined between days 4 and 10 postsurgery. lacZ gene activity, visualized by blue staining of β-galactosidase, was detected from day 7 postsurgery in mouse spinal cord differentiated from implanted neural tube. The enzyme, scattered throughout the graft, was also expressed along the axons infiltrating paraxial muscles where mouse glial cells, clearly seen in association with these axons, expressed lacZ gene (Fig. 8C,D). Throughout the study, transgene expression was accumulated by Schwann cells along nerves.

**Ontogeny of sympathetic ganglia**

At the same time that mouse ganglia would normally condense to form the primordia of spinal ganglia, mouse-

![Fig. 8. Same experiment as in Fig. 7. (A) Examination 7 days postsurgery: mouse neural crest-derived satellite cells (arrows) were observed along neurofilament-positive nerves (arrowheads) emerging from the implanted tube. (B) These cells later differentiated into myelinating Schwann cells (M, electron microscopic observation). (C) The same experiment as in Fig. 7 was conducted using 9 dpc tenascin lacZ-deficient mouse neural tube: examination 10 days postsurgery showed blue staining of β-galactosidase in mouse neural tube (NT) and motor nerves infiltrating paraxial muscles at the graft level (arrows). (D, inset) Mouse neural crest-derived cells located along the nerves activated lacZ expression and stained blue (arrows).]
derived neural crest cells appeared ventrally in the chick (Fig. 9A), indicating the first condensation of the primary sympathetic ganglia that had accumulated neurofilament protein (Fig. 9B1, B2). The authenticity of their differentiation into adrenergic derivatives was demonstrated by their anti-tyrosine hydroxylase immunoreactivity (Fig. 9B3, B4). Other cells that also accumulated neurofilaments remained around the aorta, eventually forming the aortic plexus. From day 2 postsurgery, sympathetic ganglia consisted of immunoreactive cells whose neuritic processes participated in spinal nerves. 4 days after mouse neural tube implantation, graft-derived neural crest cells aggregated close to ramus ventralis axons and ventrally to host vertebra to help form secondary sympathetic ganglia, which subsequently developed, whereas primary sympathetic ganglia progressively disappeared.

Adrenomedullary cells and suprarenal plexus

37 embryos grafted at the 18- to 24-somite level (Fig. 1, Series II) were examined between days 1 and 12 postsurgery. At graft level, examinations were carried out essentially on the adrenal gland. From day 3 postsurgery, some mouse neural crest cells were observed in the vicinity of the gland. 1 day later they had penetrated it and were infiltrated between host cortical cells (Fig. 9C). They increased during subsequent development, generally compacting into several groups scattered throughout the gland (Fig. 9D). Some mouse-derived crest cells in the host gland expressed somatostatin and could be considered as adrenal cells (Fig. 9E). Mouse neural crest cells located laterally to the aorta formed two dense masses, which reacted strongly with antineurofilament antibody and participated in the formation of adrenal plexus (Thiéry et al., 1982).

Remak’s ganglion

In two embryos of this series examined at E12, the mouse neural tube graft was implanted into 25-somite chick embryos at a level including the two posteriormost somites and the segmental plate. Only a few mouse cells from the graft were found in the adrenal gland. Some were identified in Remak’s ganglion (Fig. 9F), which originates from the neural crest posterior to the level of somite 28 and develops in the dorsal mesentery (see Le Douarin, 1982). This provided indirect proof that mouse crest cells can participate in the formation of enteric ganglia, since at least part of the lumbosacral intramural ganglionic supply migrated through Remak’s ganglion.

Melanoblasts

Attention was also given to the role of mouse crests in the production of pigment cells. The quail-chick chimeric system previously showed that prospective melanocytes migrate essentially through the mesenchyme and that those which seed the skin are derived from neural crest cells, passing between the dermomyotome and the ectoderm (Teillet, 1971). The most active migration in skin occurred in E6 chick embryo, whereas certain cells remained in the dermal mesenchyme. As in avian

Fig. 9. Transverse sections of mouse-chick chimeras implanted at the 17-somite stage, at the adrenomedullary level (somites 18-24), with the equivalent zone from 9 dpc mouse embryo. (A) Examination at day 4 postsurgery of mouse neural tube (NT), mouse dorsal root ganglion (DRG), mouse sympathetic ganglia (S), chick host vertebra (V) and chick host notochord (N) after Feulgen-Rossenbeck staining. At the graft level, a sympathetic ganglion is seen after neurofilament immunodetection (B1) and mouse nucleus staining (B2). Adrenergic differentiation was apparent after tyrosine hydroxylase immunodetection (B3) of mouse crest-derived cells (B4). (C) Mouse crest cells were located near the host adrenal gland, and some (arrows) penetrated it; Feulgen-Rossenbeck staining. (D) At day 6 postsurgery, mouse cell groups (arrows) were examined within the host adrenal gland; Hoechst staining. (E) In this location, mouse crest cells accumulated somatostatin (arrow). (F) Examination of an E12 chick host embryo operated at the 25-somite stage at a level including the segmental plate: mouse-derived crest cells infiltrated the host Remak’s ganglion (Feulgen-Rossenbeck staining) and also migrated into the dermis; acridine orange staining (G).
embryo, the migratory pathways used by melanoblasts in mouse embryo lie within the dermal mesenchyme. Migration occurs between 8 and 12 dpc, and at 13 dpc melanoblasts are present in both components of the skin. One chimera at E12 was specifically searched for the presence of mouse crest-derived cells in the chick host. Grafted cells were in fact scattered throughout the subcutaneous mesenchyme (Fig. 9G). Some were found within the epidermis, and others were apparently inserted into feather germs. In view of the sites involved, all these cells were presumably included in the melanoblast lineage.

**DISCUSSION**

The present work applied the principle of mouse-chick chimera construction, previously used to study the behavior of grafted mouse somitic cells (Fontaine-Pérus et al., 1995), to an exploration of developmental processes in the mouse nervous system. Our intention was to address problems in mammalian development that had previously been inaccessible, due to the difficulty of performing transplantation experiments. Mouse neural tube fragments were isotypically and isochronically implanted into chick embryo to follow the fate of nervous system precursors.

**Mouse neural tube is able to develop in a chick environment**

The technique of grafting fragments of neural primordium was derived from that of Le Douarin (1982, for review), who transplanted quail neural tube segments into chick embryos. As in the quail-chick chimera, the identification of grafted cells among host cells was based on differences in DNA nuclear distribution in the mouse and chick species, which made it easy to follow the fate of mouse cells in histological serial sections obtained at all stages of incubation.

We demonstrated that the various components of the spinal cord derived from the mouse neural implant differentiated in the chick. Marginal, mantle and ependymal layers developed in the first 2 days following surgery, with the ependymal layer exhibiting considerable mitosis. Subsequently, the mantle layer became the thickest one. Among the neuroblasts composing it, motoneurons and their motor nerves developed ventrally, undergoing differentiation, as expressed by a neurofilament marker (NF 68 kDa), and recognizing Islet-1 antibody. The main changes in size, shape and number occurred in graft-derived motoneurons between days 2 and 6 after implantation of mouse neural tube. During this period, their number decreased considerably (68% died), whereas the size of the surviving motoneurons increased (7 μm versus 12 μm in nuclear diameter). This period corresponded to the normal phase of motoneuronal cell death observed at day 13-14 in *in situ* mouse development (Flanagan, 1969; Normes and Carry, 1978; Lance-Jones, 1982). The spatial distribution of motoneurons within the motor columns did not change radically beyond day 6 postsurgery. We thus demonstrated that neurogenesis occurred in mammalian cells transplanted into a chick embryonic environment. If we consider the timing of cell death in in ovo developing mouse spinal cord, the peak for motoneuron cells occurs in E7-8 chick host embryo, i.e. 5-6 days postgrafting, corresponding to an equivalent age of 13.5-14.5 dpc. Lance-Jones (1982) described the maximal rate of cell death in mouse spinal cord as 13.5-14.5 dpc. These data suggest that mouse neural tube in ovo maintains its native developmental pace.

During early development, the homeobox-containing gene *Msx1* is transcribed in the mesoderm and ectoderm of the primitive streak. Up to the neural-dermal derivatives, *Msx1* gene expression is restricted to the neuroepithelium that will form the dorsal part of the neural tube and the brain (see Davidson, 1995). The expression of *Msx1* early in the differentiation of organs suggests that it might play a fundamental role in development. In our experiments, in which the prosencephalon-mesencephalon of the chick host was replaced by its mouse counterpart, dorsalization of the graft was attested by *Msx1* gene expression. Interestingly, the chimeras assumed a rodent-like shape, with a snout and forehead bulges. Taken together, these results suggest several possible explanations. In neurogenesis, it may be admitted that the key information is provided early to ensure future programs. Thus, cells participating in the development of prosencephalon-derived structures would be committed to developmental fates by early prepatterning cues that are not overcome by environmental influences. This supports the idea of Nieuwkoop (1952) on the patterning of the *Xenopus laevis* nervous system, in which prosencephalon was considered as a baseline fate. Moreover, whether environmental signals outside the nervous system are required or not for its development, our experiments, like those of Itasaki et al. (1996), provide direct support for the notion that fundamental processes are conserved in the patterning of spinal cord (in both mouse and chick species).

**Mouse neural tube sends signals conducive to chick myogenesis**

The initiation of the myogenic program is controlled by a set of basic-helix-loop-helix (bHLH) transcription factors (Buckingham, 1994 for review). The inductive interactions necessary to activate their expression, as well as skeletal muscle differentiation, are partly known. Efforts to identify the source of the cues that dictate somitic cell fate have focused on the axial tissues, i.e. neural tube and the notochord.

In vivo myogenic specification in somites appears to be dependent on the presence of the neural tube (Teillet and Le Douarin, 1983; Rong et al., 1992; Christ et al., 1992; Bober et al., 1994). In vitro experiments have also provided evidence that neural tube is required for muscle cell differentiation (Kenny-Mobbs and Thorogood, 1987; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995; Münsterberg and Lassar, 1995; Stern et al., 1995). Indeed, neither immature somites (the caudalmost somites) nor the segmental plate, when cultured as explant, form muscle fibers unless cocultured with cells from the neural tube. In contrast, rostral somites can differentiate autonomously in explant cultures. Some studies have demonstrated a positive role for notochord in somitic myogenesis both in vivo and in vitro (Rong et al., 1992; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995), while others have suggested that notochord inhibits myogenesis and stimulates chondrogenesis (Brand-Saberi et al., 1993; Pourquié et al., 1993; Fan and Tessier-Lavigne, 1994). Like Bober et al. (1994), we removed the neural tube microsurgically at the level of unsegmented paraxial mesoderm, a technique that ensures that somites developing postsurgically
are deprived of any neural tube and neural crest influence. Despite the fact that Myo-D genes were activated in somites that developed without any contact with the neural tube (Pownall et al., 1996), further expression of the myogenic regulatory gene revealed that development of paraxial muscle did not proceed normally. In the 24 hours following neural tube ablation, a decrease in the level of Myo-D expression was noted in the mediadorsal somitic zone, resulting in impairment of paraxial muscle development at later stages. Recently, Pownall et al. (1996) used microsurgery, tissue grafting and in situ hybridization techniques to demonstrate that notochord provides the primary signals regulating the activation of myogenic regulatory genes when somites form from segmental plate mesoderm. Our results indicate that the neural tube would be required shortly after the notochord for stabilization of myogenic gene expression in newly formed somites. An interesting result of our experiments is that in ovo implanted mouse neural tube is able to rescue further epaxial myogenesis.

Although our experiments do not consider how mouse neural tube can participate in the mechanism required to maintain bHLH gene expression, they demonstrate that the grafted neural tube acts in the same manner as the chick host neural tube. Regardless of whether combinatorial signals from the neural tube, floor plate and notochord allow an induction of myogenic gene expression in somites (Münsterberg and Lasser, 1995), we have demonstrated here that mouse tissue is able to send the same signals as that of the chick.

**Neural crest associated with mouse neural tube participates in chick host neural crest derivatives**

In vertebrates, neural crest is the embryonic precursor of a variety of cell types. In avian embryos, interspecific exchanges between quail and chick species have allowed precise identification of the neural crest migration pathways and their fate in normal development, as well as insight into the mechanisms governing cell differentiation in the different lineages arising from the neural crest (for a review see Le Douarin, 1982). Intensive efforts have been made by several investigators to study the differentiation of mouse neural crest, but experimental handling of mouse embryo is rather complicated and the culture of mouse tissue limited. Our mouse-chick chimera overcomes all these difficulties since the implanted neural crest migrates into the chick host and reaches the normal crest arrest sites where differentiation normally occurs. At the truncal level, and after a migration phase, grafted mouse neural crest cells in our experiments formed spinal DRG ganglia and orthosympathetic chains and gave rise to Schwann cells lining the nerves. Additional mouse neural tube implanted at the precise level of 18-24 somites resulted in a participation of myogenic gene expression in somites (Le Lièvre, 1974; reviewed by Le Douarin, 1982; Couly and Le Douarin, 1985, 1987; Couly et al., 1996). Cells from mouse-implanted prosencephalic neural crest congregated in the face of the chick host, contributing to the frontal nasal process by forming the rostral end of the maxillary process, and participated in the formation of some skull pieces (Le Lièvre, 1978). Briefly, regardless of the graft level selected, mouse crest cells migrated into chick host and finally colonized the normal sites corresponding to crest graft level.

This study shows again that mouse-chick grafting experiments offer a wide range of potential solutions for developmental problems. Our system, combined with mutations affecting the differentiation of the neural crest, is well suited for the study of gene control over neural crest development. The use of genetically marked cells ensures the monitoring of the fate of a specific cell type expressing a particular gene. Owing to the ease of in ovo development, our chimeric model provides a powerful tool for examining the behavior of wild and mutant mouse cells in every location and development stage. For example, in the case of tenascin-C null mutation, we demonstrated that the protein is expressed by glial cells lining the nerves.

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**REFERENCES**


is induced by the neural tube and Myo-D by the dorsal ectoderm in mouse paraxial mesoderm. Development 122, 429-438.


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