INTRODUCTION

The formation of the head is a multistep process beginning early in embryogenesis. Transplantation, gene knock-out and gene-transfer experiments indicate the existence of a head organizer as a separate entity, which is distinct from the trunk organizer (for reviews see Bouwmeester and Leyns, 1997; Harland and Gerhart, 1997). A common vertebrate characteristic is the successive appearance and the structural continuity of the definitive endoderm (prospective foregut, liver), the mesendoderm (prechordal plate) and the notochord. In the development of the amniota (reptiles, birds, mammalia), these three tissue types are preceded by the primitive endoderm, the visceral endoderm in mice and the hypoblast in the chick, which gives rise to the extraembryonic yolk sac. Classically, the prechordal mesendoderm cells were considered to be the cell population inducing and patterning the head. They populate the early dorsal blastopore lip in amphibia, or the tip of the fully extended primitive streak in amniota, from where they ingress to underlie the prospective forebrain region (for review see Ruiz i Altaba, 1993).

The importance of the endoderm for head development has become evident in Xenopus laevis. It expresses a secreted factor, Cerberus, which can induce a secondary head upon injection into the D4-blastomere of 32-cell embryos (Bouwmeester et al., 1996). However, on its own, the endoderm is not capable of head induction in transplantation experiments, which is in contrast to the prechordal mesendoderm (Bouwmeester et al., 1996). More recently, a murine gene related to Cerberus, the Cerberus-like gene, has been isolated (Belo et al., 1997; Thomas et al., 1997; Biben et al., 1998; Shawlot et al., 1998). After mid-streak stages, it is expressed in the definitive endoderm as it emerges from the node. This endoderm has the same fate, foregut and liver, as the non-involuting endoderm of Xenopus, in which Cerberus is expressed. However, in contrast to the frog gene, there is another expression domain of Cerberus-like in the anterior visceral endoderm of mammals whereas, in birds and amphibia, they reside in the prechordal mesendoderm, indicating a heterochronic shift of the head inductive capacity during the evolution of mammalia.

SUMMARY

Different types of endoderm, including primitive, definitive and mesendoderm, play a role in the induction and patterning of the vertebrate head. We have studied the formation of the anterior neural plate in chick embryos using the homeobox gene GANF as a marker. GANF is first expressed after mesendoderm ingestion from Hensen’s node. We found that, after transplantation, neither the avian hypoblast nor the anterior definitive endoderm is capable of GANF induction, whereas the mesendoderm (young head process, prechordal plate) exhibits a strong inductive potential. GANF induction cannot be separated from the formation of a proper neural plate, which requires an intact lower layer and the presence of the prechordal mesendoderm. It is inhibited by BMP4 and promoted by the presence of the BMP antagonist Noggin. In order to investigate the inductive potential of the mammalian visceral endoderm, we used rabbit embryos which, in contrast to mouse embryos, allow the morphological recognition of the prospective anterior pole in the living, pre-primitive-streak embryo. The anterior visceral endoderm from such rabbit embryos induced neuralization and independent, ectopic GANF expression domains in the area pellucida or the area opaca of chick hosts. Thus, the signals for head induction reside in the anterior visceral endoderm of mammals whereas, in birds and amphibia, they reside in the prechordal mesendoderm, indicating a heterochronic shift of the head inductive capacity during the evolution of mammalia.

Key words: Chick, Rabbit, Head, Visceral, Endoderm, GANF, BMP4, Noggin

INTRODUCTION

The formation of the head is a multistep process beginning early in embryogenesis. Transplantation, gene knock-out and gene-transfer experiments indicate the existence of a head organizer as a separate entity, which is distinct from the trunk organizer (for reviews see Bouwmeester and Leyns, 1997; Harland and Gerhart, 1997). A common vertebrate characteristic is the successive appearance and the structural continuity of the definitive endoderm (prospective foregut, liver), the mesendoderm (prechordal plate) and the notochord. In the development of the amniota (reptiles, birds, mammalia), these three tissue types are preceded by the primitive endoderm, the visceral endoderm in mice and the hypoblast in the chick, which gives rise to the extraembryonic yolk sac. Classically, the prechordal mesendoderm cells were considered to be the cell population inducing and patterning the head. They populate the early dorsal blastopore lip in amphibia, or the tip of the fully extended primitive streak in amniota, from where they ingress to underlie the prospective forebrain region (for review see Ruiz i Altaba, 1993).

The importance of the endoderm for head development has become evident in Xenopus laevis. It expresses a secreted factor, Cerberus, which can induce a secondary head upon injection into the D4-blastomere of 32-cell embryos (Bouwmeester et al., 1996). However, on its own, the endoderm is not capable of head induction in transplantation experiments, which is in contrast to the prechordal mesendoderm (Bouwmeester et al., 1996). More recently, a murine gene related to Cerberus, the Cerberus-like gene, has been isolated (Belo et al., 1997; Thomas et al., 1997; Biben et al., 1998; Shawlot et al., 1998). After mid-streak stages, it is expressed in the definitive endoderm as it emerges from the node. This endoderm has the same fate, foregut and liver, as the non-involuting endoderm of Xenopus, in which Cerberus is expressed. However, in contrast to the frog gene, there is another expression domain of Cerberus-like in the anterior visceral endoderm (AVE) prior to the onset of gastrulation. A specific role of the AVE in the mammalian head organizer is suggested by a number of recent findings (for review see Beddington and Robertson, 1998). Several genes are expressed in an independent domain in the AVE of the prospective head region before or at the onset of primitive streak formation, prior to their expression in the axial mesendoderm or the node during gastrulation. These include the homeobox genes Hesx1 (Rpx), Gsc, Lim1, Hex and Otx2, the forkhead gene HNF3β, the
nuclear protein gene Mrg1, the growth factor gene Nodal, and the antigen VE-1 (Ang et al., 1994; Rosenquist and Martin, 1995; Hermesz et al., 1996; Thomas and Beddington, 1996; Belo et al., 1997; Varlet et al., 1997; Dunwoodie et al., 1998; Thomas et al., 1998). Morphological indications for anterior patterning before gastrulation exist in those mammalian embryos, which have a flat embryonic disc during gastrulation. As demonstrated, for example for the rabbit, both the ectoderm and the visceral endoderm are significantly thickened at the anterior margin, forming the ‘anterior marginal crescent’ and allowing thus the recognition of the anterior pole before primitive streak initiation in the living embryo (Viebahn et al., 1995).

Functional evidence for a murine head organizer came from the genetic inactivations of Hesx1, Lim1, Otx2, HNF3β and Nodal, which resulted in severe head dysmorphologies (Ang and Rossant, 1994; Weinstein et al., 1994; Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995; Ang et al., 1996; Varlet et al., 1997; Dattani et al., 1998). However, since these genes are expressed both in the AVE and in the node/mesendoderm, the definitive cause of these defects remained unclear. Several other experiments have addressed the role of the visceral endoderm more directly. Chimeric mouse embryos composed predominantly of wild-type cells, but entirely of Nodal−/− or Otx2−/− cells in the visceral endoderm, did not form anterior head structures (Varlet et al., 1997; Rhinn et al., 1998). The mechanical ablation of the AVE prevented the later expression of Hesx-1 in the anterior neural plate and led to a diminution in the size of anterior cranial folds at later stages (Thomas and Beddington, 1996). The evolving concept suggests that mammals have an independent anterior signalling mechanism residing in the AVE.

In order to follow head formation in the avian embryo, we studied the chick homeobox gene GANF. This gene is a member of the ‘Anf’ (anterior neural folds) family, from which a single member was found in vertebrates, such as fish (Danf), amphibia (Xanf), chick (GANF), mice (Hesxl/Rpdx) and human (HANF/HESX1), but not in the invertebrate Drosophila melanogaster (Thomas and Rathjen, 1992; Beebe, 1994; Mathers et al., 1995; Zaraisky et al., 1995; Hermesz et al., 1996; Kazanskaya et al., 1997). Extensive screening and sequence comparisons led Kazanskaya and colleagues to the conclusion that the known ANF genes are most likely orthologues, and not non-orthologous homologues. All ANF genes appear to be predominantly expressed in the ectoderm of the anterior neural plate, and more weakly in the underlying mesendoderm (Mathers et al., 1995; Zaraisky et al., 1995; Kazanskaya et al., 1997). In addition, the murine Hesxl/Rpdx gene is also transcribed in the AVE at the onset of gastrulation (Hermesz et al., 1996; Thomas and Beddington, 1996).

In the chick, GANF induction occurs when the neural plate formation has already begun (Schoenwolf, 1991; Kazanskaya et al., 1997). We found that it was inhibited by manipulations that interfered with neural development, such as the exposure to BMP4, or the removal of the endoderm or the prechordal mesendoderm. GANF expression could be elicited by inducers of a neural fate, such as prechordal mesendoderm grafts or Noggin releasing implants, but not by the hypoblast or definitive endoderm transplants. To study the role of the mammalian visceral endoderm in head formation, we performed crosses-transplantation experiments. We demonstrate that the AVE prepared from prestreak rabbit embryos could indeed induce GANF expression and a thickening of the chicken epiblast, in contrast to the anterior hypoblast of the chick embryo. The striking difference in the timing of the head induction between the avian and the mammalian embryos, respectively, is discussed.

MATERIALS AND METHODS

Embryos

Fertilized chick eggs (White Leghorn, obtained from Lohmann Tierzucht, Cuxhaven) were incubated at 38°C in a humidified incubator. Chick embryos were staged according to Hamburger and Hamilton (1951) or Eyal-Giladi and Kochav (1976). Rabbit blastocysts were flushed from uteri of New Zealand White rabbits at 6.1 and 6.5 days postconception.

Reverse-transcriptase PCR (RT-PCR)

Total RNA was prepared from whole embryos (Micro RNA Isolation Kit, Stratagene) and 5 μg of the RNA were reverse transcribed (First-strand cDNA Synthesis Kit, Pharmacia). For the amplification of GAPDH and GANF the following primers were used: ACGCCATCACTCTCTCCAG and CAGCCCTCTACTCCC-TCTTG for GAPDH (Schultheiss et al., 1995) and CATCTGCAAATCCCTGTTGTTTCC and AGGCCACCTAGAGATCAAACG for GANF. The PCR was carried out at an annealing temperature of 56°C or 60°C with 26 or 30 amplification cycles for GAPDH or GANF respectively.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed essentially as described by Wilkinson (1992), except that the hybridization and the first two washing steps were performed at 70°C in the presence of 0.1% CHAPS detergent (Sigma) (Stein and Kessel, 1995), and no RNase treatment was done. The GANF riboprobe covered 335 bases (from the 181th to the 516th base of the GenBank sequence GGU65436, including the homeobox). The c-Otx2, c-SOX3 and Lim-1 riboprobe are described elsewhere (Tsuchida et al., 1994; Bally-Cuif et al., 1995; Rex et al., 1997). For paraffin sections (8 μm), stained embryos were dehydrated and embedded in Paraplast plus (Sherwood Medicals).

Extritation of the lower layer tissue

Chick embryos were manipulated in a modified NEW culture (Stern, 1993). The hypoblast, the endoderm and the young head process were removed with a bent insect needle leaving the epiblast unaffected. The manipulation was repeated every 2 hours during an average incubation time of 10 hours in which the embryo reached the prechordal plate or the early headfold stage.

Transplantations of chick tissues

The grafts were excised with a bent insect needle from embryos submerged in Pannett-Compton saline. We carefully checked under the dissecting microscope that the ectodermal epithelium of the donor remained unaffected, ensuring that the graft was completely devoid of ectodermal cells. The grafts were transferred with a micropipette from the donor blastoderm onto the ventral surface of the host blastoderm and inserted between the endoderm and the ectoderm as indicated. The cultures were incubated for an additional 6-18 hours. Hypoblast grafts of the donor were labelled with fixative-stable Dil (1,1’-dioctadecyl 3,3,3’,3’-tetramethylindocarbocyanine perchlorate, Celltracker CM-Dil, Molecular Probes, Oregon). Dil was dissolved at 0.5% (w/v) in ethanol and was diluted 1:10 in 0.3 M sucrose. The dye was applied to the hypoblast by several microinjections using air pressure prior to the removal of the graft.
Transplantation of rabbit tissue in chick hosts

The chick host embryos were prepared as described above. The rabbit blastocysts were recovered by flushing the uterus with prewarmed (37°C) PBS (phosphate-buffered saline) of 15- to 18-week-old New Zealand White rabbits, which had been euthanised with 90 mg Nembutal (Bayer, Germany), at 6.1 and 6.5 days postconception. After being transferred into HAM’s F10 medium (GIBCO-BRL, Life Technologies) containing 20% fetal calf serum (Biochrom), the anterior end of the embryo was marked under the dissecting microscope by injecting a tungsten needle into the acellular blastocyst coverings juxtaposed to the anterior margin of the embryonic disc using the anterior marginal crescent as a landmark. The embryo was subsequently cut into an anterior and posterior half. The anterior or posterior visceral endoderm, or the anterior ectoderm was prepared with tungsten needles and marked by adhering particles of the water-insoluble dye Carmine (Sigma C-1022) prior to its transfer onto the blastoderm of the cultured chick embryo. Transplantation into the chick host embryos were either under the hypoblast at the anterior margin of the area pellucida or into the anterior, proximal area opaca. The chick host embryos were cultured for 6 to 10 hours until they reached the prechordal plate or early headfold stage.

Implantation of cell aggregates or beads

Both cell aggregates and beads were implanted into chick host embryos. Primary chick embryo fibroblasts (CEF) transfected with mouse BMP4 RCAS-A retroviral DNA were kindly provided by Paul M. Brickell (Duprez et al., 1996). As a control, untreated CEFs were used in the same way. Prior to the implantation, CEFs were grown on a tissue culture dish to 50-90% confluency and scraped into aggregates. The aggregates were cut into pieces with tungsten needles and washed in DMEM. These cell aggregates were implanted between the endoderm and the ectoderm of chick embryos in the modified NEW culture as described above. Heparin acrylic beads (Sigma H-5263) were washed in PBS and were soaked in recombinant human BMP4 (Genetics Institute; final concentration of 5 μg/ml, 1 mg/ml BSA in PBS) or recombinant human Noggin (Regeneron, 20-100 μg/ml, 1 mg/ml BSA in PBS) for 3-5 hours prior to implantation. Control experiments were undertaken without the recombinant BMP4 or Noggin, respectively. The beads were implanted in the same way as the cell aggregates.

RESULTS

Expression analysis of GANF

We extended the initial expression analysis of the GANF gene performed by Kazansksaya et al. (1997) using reverse-transcriptase PCR (RT-PCR) and whole-mount in situ hybridization combined with histology. No GANF transcripts were detectable at early streak stages (HH2) by means of RT-PCR, extremely low levels were found late during gastrulation, were detectable at early streak stages (HH2) by means of RT-PCR. After the appearance of the headfold (HH6), the expression of GANF persisted at a high level in the anterior neural plate. However, it started to decrease in the more posterior midline (Fig. 2C), from where it was lost when the neural tube started to close (Fig. 2D). Only the anterior neural folds and a small anterior medial region connecting the neural folds continued to express GANF (HH7; Fig. 2D). At HH9, GANF was expressed in the anterior dorsal part of the neural tube, the ventral surface ectoderm and more weakly in the most anterior ventral part of the neural tube (Fig. 2E,L). After the 10-somite stage (HH10), the expression persisted only in the oral ectoderm (Fig. 2F), which started to invaginate at the 22-somite stage (HH14; Fig. 2G,M,N) and developed into the primordium of the adenohypophysis, Rathke’s pouch (Couly and Le Douarin, 1987).

In summary, GANF is initially transcribed in the anterior neural plate and the underlying mesendoderm late during gastrulation.

Extration of the hypoblast, the endoderm and the young head process

We analysed the role of the mid-streak hypoblast, the definitive endoderm and the young head process for the induction of GANF in the ectoderm by mechanical ablation. The anterior hypoblast at HH3 underlies the epiblast, which will express GANF from HH4 onwards. Its removal did not influence the expression of GANF in the anterior neural plate at later stages (HH5-8; n=11, 0/11; data not shown). In order to create a situation in which the epiblast develops in the absence of an underlying lower layer, we removed one anterior quadrant of the hypoblast/endoderm repetitively, and thus prevented also the anterior migration of any mesoderm (Fig. 3A). The analysis of such embryos at the prechordal plate stage (HH5) revealed the absence of GANF expression above the operated area and the inhibition of neural plate formation (n=9, 9/9; Fig. 3B,C).

![Fig. 1. The onset of GANF expression monitored by RT-PCR analysis. Total RNA of early streak (HH2), late mid-streak streak (HH3*), late streak (HH4) and prechordal plate-stage (HH5) embryos were taken for the analysis. The first lane on the left side shows the molecular weight marker (M), the length of the marker DNA fragments is indicated in base pairs. A control for the amount of RNA is shown in the following four lanes for the corresponding stages (glyceraldehyde-3-phosphate dehydrogenase GAPDH). The amplified product of the GANF RNA is shown on the right for the corresponding stages. At early streak stage (HH2), no GANF transcripts can be found. About 10 to 12 hours later, at HH3* and HH4, very low level of the transcripts are detected. At the prechordal plate stage (HH5), GANF expression is strongly increased.](image-url)
In a less radical operation, we repeatedly removed the head process, as well as the subsequently ingressing cells anterior of the node at the young head process stage (HH4+), without affecting the hypoblast and most of the definitive endoderm (Fig. 3D). Thus, we prevented the formation of the midline axial mesoderm while allowing paraxial development. In these embryos, the ectoderm overlaying the manipulated region did not undergo neural development as judged by the height of the epithelium and the absence of the anterior neural plate marker gene GANF (n=6, 6/6; Fig. 3E,F). The neural plate was, in effect, split so that two headfolds developed. This could be the result of axial mesendoderm cells redirected to both paraxial locations, or by the acquisition of a midline identity by paraxial cells relieved from the midline repression (Yuan et al., 1995).

In summary, the ablation of the endoderm/mesoderm layer or of the young head process inhibited neural development including the GANF expression domain.

Transplantation of the node, the hypoblast, the definitive endoderm, the young head process and the prechordal plate

We analysed the GANF-inducing potential of various tissues at different stages during chick development by transplantation to the outer margin of the area pellucida, where the epiblast cells are fated to become epidermis (Spratt, 1952; Rosenquist, 1966; Schoenwolf and Sheard, 1990; Bortier and Vakaet, 1992; Garcia-Martinez et al., 1993). When the anterior hypoblast from prestreak stages (EK XII/XIII; n=15, 0/15), or from mid-streak stages (HH3; n=4, 0/4), or the definitive endoderm from late streak stages (HH4; n=6, 0/6) was grafted, neither morphological alterations nor ectopic expression of GANF were elicited (Fig. 4A-C, and data not shown). Labelling with DiI demonstrated that the hypoblast cells remained together at the position of grafting during the incubation (Fig. 4B,C). Transplants of Hensen’s node (HH3+/HH4), the chick organizer, led to the induction of a neuroectodermal structure with a strong expression of GANF in its anterior margin, demonstrating again the capacity of the avian node to induce anterior identities (n=8, 8/8; Fig. 4D-F; Dias and Schoenwolf, 1990).

Grafting of the young head process (HH4+) to the lateral, anterior area pellucida caused a thickening of the epiblast and an induction of GANF expression in juxtaposed cells (n=12, 11/12; Fig. 4G-J). When these grafts came to lie more closely to the endogenous axis, the induced neural tissue fused with the neural folds of the host leading to two-headed embryos (Fig. 4K-M). We carefully checked the definitive endoderm directly anterior to the young head process for a GANF-
inducing potential, but we consistently obtained negative results \((n=10, 0/10, \text{data not shown})\). Since most cells of the young head process (HH4) are found in the prechordal plate at stage HH5, we also expected a GANF-inducing potential in this tissue, which turned out to be the case \((n=2, 2/2; \text{Fig. 4N-P})\). The induction of a neural epithelium and of GANF expression by prechordal mesendodermal transplants in the anterior margin of the area pellucida indicates a change of fate from epidermal to neural. They do not represent a mere maintenance of a previously induced neural specification, as demonstrated by the established fate maps. Previous investigations have shown that anterior neural induction by prechordal mesendoderm can also be elicited in the area opaca (Izpisúa-Belmonte et al., 1993; Foley et al., 1997; Pera and Kessel, 1997).

In summary, we could demonstrate neural and GANF gene induction by the node, the young head process and the prechordal plate, whereas the hypoblast and the definitive endoderm were inactive in this respect.

**BMP4 inhibits neural plate formation**

The ectoderm surrounding the neural plate in late streak embryos (HH4) expresses BMP4 prior to the onset of the GANF expression (Schultheiss et al., 1997). Therefore, we asked if the expression domain of BMP4 limits the extension of the neural plate judged by the transcription of GANF and another anterior neural plate marker, c-OTX2 (Bally-Cuif et al., 1995). We used both chicken embryonic fibroblasts producing the murine BMP4 under the control of a retroviral promoter (Duprez et al., 1996) and heparin-acrylic fibroblasts loaded with recombinant human BMP4 as an artificial source of BMP4. As a control, we used untreated fibroblasts and BSA-loaded beads.

Neither the control cells nor the BSA-loaded control beads affected the expression of GANF \((n=6; \text{Fig. 5A and Fig. 5C and E, respectively})\) or \(c\text{-OTX2 (}n=4; \text{Fig. 5G,M,N and Fig. 5H,O,P) at prechordal plate (HH5) or at late headfold stage (HH7)}\) even if the cells or beads were located close to, or directly within, the endogenous expression domain. Moreover, the morphology of the embryos containing implanted control beads was not significantly altered at late headfold or 4-somite stages (Fig. 5E,H), thus indicating that the presence of the beads did not physically interfere with the appearance of the neural folds.

However, an artificial source of BMP4 inhibited neural plate formation judged by the absence of GANF and \(c\text{-OTX2 expression and the morphology of the ectoderm. The suppression in the anterior neural plate occurred in a radial zone around the cell aggregates (}n=5; \text{Fig. 5B,J})\), or around the BMP4-loaded beads \((n=12; \text{Fig. 5D,K})\). They suppressed the expression of GANF in both the anterior neural plate and the underlying prechordal plate. Moreover, both the ectodermal and the endodermal expression of \(c\text{-OTX2 were suppressed by the BMP4 beads (}n=6; \text{Fig. 5G,N})\). Embryos that reached the late headfold stage or the 4-somite stage still exhibited strong attenuation of GANF or \(c\text{-OTX2 expression (Fig. 5F,L and Fig. 5H,O,P respectively). In addition to the suppression of the anterior neural markers, the epiblast exposed to the artificial source of BMP4 exhibited an epidermal ectoderm character in many cases (Fig. 5N,O,P). It remained thin and did not develop into properly formed neural folds at later stages.}

In summary, BMP4 was able to suppress expression of the anterior neural plate marker GANF and \(c\text{-OTX2 and inhibited the morphological appearance of a neural plate and its development into neural folds.}

**Noggin enlarges the neural plate**

Since the presence of BMP4 was sufficient to inhibit neural identity in the anterior ectoderm, the endogenous ring of BMP4 expression surrounding the prospective neural plate in late streak embryos might serve to limit the extension of the neural plate. We then asked if a BMP4 inhibitor such as Noggin (Zimmermann et al., 1996) is able to interfere with this effect and enlarges the neural plate. Noggin is normally expressed in the anterior part of the primitive streak in mid-streak embryos (HH3) and is upregulated in the anterior node at late streak stages (HH4). The young head process and the prechordal

![Fig. 3. Extirpation of the lower layer affects neural plate formation. Two different manipulations are shown schematically in A and D. The removed tissue of the lower layer is indicated in red. The scale bar in B represents 270 \(\mu\text{m in B,E and 135 \(\mu\text{m in C,F. (A-C) The endoderm and the hypoblast of the left anterior lateral quadrant in front of the primitive streak was removed five times during 10 hours of incubation. The manipulation prevents neural plate formation judged by the absence of GANF and the thin character of the ectoderm juxtaposed to the extirpated region indicated by black arrowheads. (D-F) Only the young head process with adhering endoderm was removed without affecting the hypoblast and most of the definitive endoderm, which already lies anterior to the manipulated region. This is sufficient to prevent neural plate formation in the midline over the extirpated region. The lower layer has already been closed by a thin endodermal layer since the last operation. Note that the ectodermal GANF expression was closely associated with the prechordal plate, which was separated into two parts and seems to induce two independent headfolds.}

---

Head induction in the chick 819
plate, which appear slightly later as mesendodermal products of the node also express Noggin (HH4+ and HH5) indicating that Noggin is present in the anterior region of the chick embryo during neural plate formation (Connolly et al., 1997). For this purpose, we implanted heparin-acrylic beads loaded with human Noggin protein into the area pellucida of chick embryos.

The Noggin beads were able to enlarge the expression domain of GANF in the anterior neural plate (Fig. 6A,B). When the implanted beads were located close to the endogenous expression domain of GANF, the ectodermal cells above and close to the Noggin beads also expressed GANF (n=12, 9/12; Fig. 6). At no time did we observe any effect on the GANF expression domain when control beads were used (n=8; data not shown). However, when the Noggin beads were not located close to the neural plate, neither GANF was induced nor the morphology affected (n=6; data not shown) even if the highest concentration of Noggin was used. Clearly, Noggin was not sufficient to induce an independent, ectopic expression of GANF.

When we implanted an aggregate of untreated chicken embryonic fibroblasts between the Noggin beads, the extensions of the GANF domain pointed precisely to the beads and did not enclose the region around the cells (n=10, 7/10; Fig. 6B). These findings suggest that the endogenous signal that is responsible for the induction of GANF in the anterior neural plate is able to spread more widely if the endogenous ring of BMP4 is disrupted by the ectopic source of the BMP4-antagonising agent Noggin.

In summary, we demonstrate the capacity of Noggin to induce the enlargement of the endogenous GANF expression domain in the anterior neural plate.

**Prestreak visceral endoderm from rabbit embryos neuralizes and induces GANF in chick epiblasts**

In order to compare the inductive potential of the AVE of a mammalian prestreak embryo with avian tissues, we used rabbit embryos as donors. The principal feasibility of chick-to-rabbit, or rabbit-to-chick transplantations with resulting inductions of gastrulation or neurulation was pioneered by C. H. Waddington (1934). The prestreak-stage rabbit embryo is about 2.5 times larger than the prestreak-stage mouse embryo, when comparing sagittal ectoderm length between the anterior and posterior pole. Moreover, the thickened anterior end of the prestreak-stage rabbit embryo, the anterior marginal crescent, can be morphologically recognized prior to the onset of gastrulation (Viebahn et al., 1995). We grafted cells of the prestreak AVE from the rabbit embryos into the outer margin of the area pellucida of HH3 chick blastoderms (Fig. 7A,D), and cultured them until they developed to the prechordal plate stage (HH5). The AVE was capable of inducing an independent expression domain of GANF (n=9, 6/9; Fig. 7E,F) and of the general neural marker cSOX3 in the chick hosts (n=3, 3/3; Fig. 7B,C; Rex et al., 1997). The expression of GANF and cSOX3 was induced in the epiblast of the chick host embryos juxtaposed to the grafted rabbit AVE cells, which could be identified due to their different morphology compared to the host cells (Fig. 7C,F). The host epiblast was changed into a high columnar, neural-like epithelium above the grafted cells indicating the induction of GANF.

**Fig. 4.** GANF-inducing potential of the the hypoblast, the node, the young head process and the prechordal plate revealed by transplantation experiments. The transplantations are schematically shown in the diagrams A, D, G, K and N, the site of transplantation, the stages of the donor embryos and the hosts are indicated. The manipulated embryos are shown from a dorsal view with the anterior pole towards the top (B,E,H,I,O). The levels of the sections (C,F,J,M,P) are marked by black lines. The scale bar in B represents 230 μm in E,F,H,I,L,O,P, 115 μm in B and 60 μm in C. (A-C) The anterior hypoblast (EK XIII) is marked by a multiple injection of DiI before transplantation, indicated in A by a thin red arrowhead. The grafted cells of the hypoblast stay together during the incubation. Although they are in close contact to the epiblast they do not elicit neural thickening and expression of GANF in the epiblast. (D-F) Transplantation of the node to the anterior edge of the area pellucida leads to the induction of a neuroectodermal structure with strong expression of GANF in its anterior margin. (G,HJ) Transplantation of the anterior tip of the young head process induced an ectopic GANF expression domain in the host. (K-M) Transplantation of the anterior tip of the young head process more closely to the axis of the host induced an ectopic GANF expression domain which fused with the neural folds. (N-P) Grafting of the prechordal plate also leads to an ectopic GANF domain in the ectoderm of the host.
Head induction in the chick

821

a neural identity. No mesodermal cells of the chick embryo were found between the epiblast and the rabbit cells. The area opaca is never underlaid by the hypoblast, and is competent to respond to neural induction by node, prechordal plate and young head process grafts (Gallera, 1970, 1971; Storey et al., 1992; Izpisúa-Belmonte et al., 1993; Pera and Kessel, 1997; Streit et al., 1997). After transplantation of the prestreak rabbit AVE (Fig. 7G), we detected a significant, localized morphological response above the rabbit cells, where the squamous epithelium of the area opaca was changed into a high columnar, pseudostratified, neuroectoderm-like epithelium (n=10) with GANF expression (n=4; Fig. 7H,J).

As a control, we transplanted the anterior ectoderm overlying the visceral endoderm, and did not observe an ectopic expression of GANF and cSOX3 (n=8, 0/8 and n=2, 0/2 respectively, data not shown). We also attempted to transplant the posterior visceral endoderm of a prestreak-stage embryo. This tissue was difficult to isolate, because it was much thinner and more fragile than the AVE. Therefore, we had to separate the entire visceral endoderm from the epiblast starting with the anterior part, and then cutting the visceral endoderm in two pieces and grafting the fragile posterior visceral endoderm. We only succeeded in three of these manipulations and identified the expression of GANF in one case (n=3, 1/3), possibly due to a remnant of anterior visceral endoderm. Furthermore, we analyzed anterior visceral endoderm of mid-streak-stage rabbit embryos, but did not observe GANF induction by this tissue (n=5, 0/5). Judging from the mouse data, AVE at this stage would no longer be expressing Hesx1/Rpx (Hermesz et al., 1996; Thomas and Beddington, 1996) and no functional indication for a role in head induction was reported.

Previous studies have shown that the prechordal plate can anteriorize prospective hindbrain neuroectoderm towards a forebrain fate (Dale et al., 1997; Foley et al., 1997). We investigated if prestreak rabbit AVE possesses a comparable regionalising activity by transplanting it into the prospective hindbrain region at HH4. We performed transplantations of rabbit prestreak AVE (n=7), but did not observe significant GANF inductions, except for one case, where the primary GANF domain was weakly distorted towards the graft (not shown).

In summary, we found an activity inducing the chick GANF and cSOX3 gene in the rabbit visceral endoderm of prestreak embryos.

**DISCUSSION**

**The chicken GANF gene and anterior anlage fields**

Ectodermal patterning in the chick blastoderm can be followed from prestreak stages onwards (Pera et al., 1998). One aspect of the ectodermal patterning is the induction of the neural plate, which probably begins before the primitive streak has reached its full length, and before axial mesendoderm or mesoderm inresses (Schoenwolf, 1991). Compared to these processes, the initiation of GANF gene transcription occurs later, coinciding with the appearance of the prechordal mesendoderm. The half-moon domain at HH5/6 demarcates...
Martinez et al., 1993). The diencephalon and telencephalon (Spratt, 1952; Garcia-

Fig. 6. Noggin enlarges the expression domain of GANF. Scale bar in A represents 220 μm in A,B and 110 μm in C-F. The levels of cross sections are marked by a black line, the Noggin beads by black arrowheads and the chicken fibroblasts by white arrowheads. (A,C,D) The expression domain of GANF enlarges over the implanted Noggin-loaded beads (black arrowheads). (B,E,F) The presence of untreated chicken fibroblasts disrupts this enlargement and the GANF domain points in stripes towards the implanted Noggin beads.

the anlage field of the forebrain, including prospective ventral diencephalon and telencephalon (Spratt, 1952; Garcia-Martinez et al., 1993). The GANF-expressing anterior neural folds give rise to the basal structures, the striatal subdivision, of the telencephalic vesicle (Fernandez et al., 1998). The medial part of the ectoderm expressing GANF includes a common hypophyseal field, which later splits into two separate anlagen (Couly and Le Douarin, 1987). The neurohypophyseal anlage in the ventral diencephalon reduces GANF expression relatively early (HH7), while coming under the patterning influence of the prechordal plate (Pera and Kessel, 1997). In later stages (HH14), the adenohypophysis anlage remains the only domain of GANF expression, when the oral ectoderm begins to invaginate as Rathke’s pouch. Apart from the different onset of Anf gene expressions, their patterns appear to be highly conserved in vertebrates (Kazanskaya et al., 1997).

Inductive influences on head development in the chick

The inductive role of the avian hypoblast has been investigated in several studies. An extensive inhibition of the nascent hypoblast at early stages (EK XI) prohibits formation of the primitive streak and thus further development (Spratt, 1946). Rotation experiments indicated that the hypoblast might dictate the direction of the primitive streak (Waddington, 1932, 1933; Azar and Eyal-Giladi, 1981). It has remained unclear and debated if the hypoblast could indeed induce an ectopic origin of the primitive streak (Khaner, 1995). For this function, the major source appears to lie in the posterior marginal zone and Koller’s sickle (Bachvarova et al., 1998). These inductive processes are, however, completed when the whole epiblast is covered by a hypoblast (EK XIII). If the hypoblast is removed at this stage, a primitive streak will form, a lower layer will regenerate and normal embryogenesis can recover. The observation of enhanced (but not de novo) forebrain tissue development after primitive streak induction by hypoblast replacement can possibly be explained as such a secondary response (Eyal-Giladi and Wolk, 1970). Up to now, no clear induction of a new fate by the avian hypoblast has been described and no molecular responses to hypoblast grafts are reported. Also our transplantation experiments failed to demonstrate that the anterior hypoblast and the definitive endoderm are capable of inducing GANF. However, our extirpation experiments suggest that an intact lower layer (hypoblast/definitive endoderm) is required for proper formation of the prechordal mesendoderm and thus also for GANF expression. The mesendoderm (preingression: node; post- ingression: young head process, prechordal plate) is both required and sufficient for GANF expression. Our further investigations demonstrate that expression of GANF depends on the correct formation of a neural plate. This is prevented by BMP4, as shown not only by suppression of the GANF gene, but also by the anterior neural plate marker c-OTX2 (Fig. 5), the general neural plate marker cSOX2, and by the activation of the two epidermal markers BMP-4 and DLX5 (Pera et al., 1998). The BMP-antagonist Noggin (Zimmermann et al., 1996), on the contrary, has a positive effect on the GANF expression domain and extends neural plate formation at its anterior margin, probably by allowing the GANF-inducing signal to act in the BMP4 expression domain (Fig. 6). However, in avian embryos neither Noggin, nor Chordin are on their own sufficient to induce a neural fate (Connolly et al., 1997; Streit et al., 1998), or to induce an independent GANF expression domain. Therefore, we believe that other or additional signals are required and they should stem from the axial mesendoderm and/or the definitive endoderm. A candidate molecule in this context would be the chick orthologue of Cerberus or Cerberus-like (Bouwmeester et al., 1996; Belo et al., 1997; Thomas et al., 1997; Biben et al., 1998; Shawlot et al., 1998). However, RNA of the chicken Cerberus-related gene C-cerb is nearly undetectable at HH4+, and not detectable at HH5 and HH6 by whole-mount in situ hybridization (A. Gardiner, M. Marvin and A. Lassar, personal communication). In preliminary experiments, we were not able to obtain GANF induction by either Xenopus Cerberus or murine Cerberus-like (data not shown). Therefore, we assume that a signal not related to Cerberus is responsible for the induction of GANF in the anterior neural plate.

In conclusion, we found that the prechordal mesendoderm and not the anterior hypoblast is the source for signals inducing GANF in the anterior neural plate of chick embryos.

Head induction by prechordal mesendoderm in amphibia and birds, or by AVE in mammals

Several key genes for head induction appear to be conserved in vertebrates. An important role of signals from the cell layer below the prospective head ectoderm for induction and patterning has been demonstrated in classical experiments and was recently confirmed by molecular analysis (Bouwmeester and Leyns, 1997; Beddington and Robertson, 1998). These authors emphasize the common aspects of anterior patterning in frogs and mice, namely the signalling from endoderm to initiate anterior development. Based on the known topological differences, they suggest a unifying model delineating the putative AVE equivalents in chick, Xenopus and zebrafish (Beddington and Robertson, 1998).
out some remarkable differences between mice and chicken, and similarities between *Xenopus* and chicken. We confirm that similar signals and nuclear transcription factors are used, but that the timing of at least some reactions in head induction is significantly different. In order to compare the vertebrate model organisms, mouse, chick and frog, the endoderm can be conceptually divided into three types, best characterized by their fate. Firstly, the primitive endoderm as a prospective extraembryonic tissue is only present in the mouse and the chick, whereas amphibia generate no extraembryonic tissue at all. This endoderm is not a product of gastrulation, and its fate is to become the stalk of the yolk sac. Secondly, the definitive, anterior endoderm will develop into the foregut and the liver. In amphibia, it also comprises yolk cells outside the epithelial lining. Thirdly, the prechordal mesendoderm as an organizer-derived tissue migrates anteriorly to lie under the developing forebrain (Dale et al., 1997; Foley et al., 1997; Li et al., 1997; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997). In mice, it consists of a single cell layer, which is integrated, but morphologically distinct from the surrounding endoderm (Sulik et al., 1994). In chicks, the mesendodermal prechordal plate consists of a mesenchymal assembly of cells (the prechordal mesoderm), with the lowest layer integrated into the definitive endoderm (Fig. 2J; Adelmann, 1922). Avian prechordal mesoderm contributes later to extrinsic ocular muscles (Wachtler et al., 1984).

Several well-established molecular markers (*Lim-1, HNF3β, Cer-l, Otx2, Gsc, Hex, Hex1/Rpx*) are not only expressed in the murine endoderm or prechordal mesendoderm, but also have a spatially independent domain in the visceral endoderm (Ang et al., 1994; Shawlot and Behringer, 1995; Hermesz et al., 1996; Thomas and Beddington, 1996; Belo et al., 1997; Thomas et al., 1997; Varlet et al., 1997; Biben et al., 1998; Dunwoodie et al., 1998; Shawlot et al., 1998; Thomas et al., 1998). The same is true for some of the known orthologous genes in the chick (*Lim-1, HNF3β, c-OTX2, GSC*; Bally-Cuif et al., 1995; Ruiz i Altaba et al., 1995; H. K., unpublished data). However, there are significant differences, which point to alterations in amniote head induction. In the chick, *c-OTX2* is already expressed in the epiblast at the time when the primary hypoblast is forming by poly-delamination, making it impossible that a signal provided by the hypoblast is required for the ectodermal expression (Bally-Cuif et al., 1995). In contrast, *Otx2* expression in the murine visceral endoderm is essential for its induction in the ectoderm (Acampora et al., 1995). The early ectodermal expression in chicken is completely lost at late streak stages and reinitiated as the axial mesendoderm appears under the prospective neural plate (Bally-Cuif et al., 1995). In contrast, the murine *Otx2* remains active in the anterior ectoderm from pregastrulation stages onwards (Ang et al., 1994). Whereas *Hex1/Rpx* is strongly expressed in the murine AVE at the onset of gastrulation, its chicken orthologue *GANF* is only transcribed after the ingestion of the prechordal mesendoderm.

In our study, we demonstrated the remarkable potential of AVE from pregastrulation rabbit embryos to change the fate of epidermal or extraembryonal cells to an anterior neural identity, evident by the induction of *GANF* and *cSOX3*. These findings establish the inductive power of the mammalian visceral endoderm for the first time in a direct transplantation experiment. Taken together, our data indicate that a signal or a combination of signals sufficient for induction of anterior development reside in the mammalian, but not the avian primitive endoderm.

**Heterochrony in the evolution of head induction**

In *Xenopus laevis*, the onset of gastrulation coincides with the ingestion of the prechordal mesendoderm whereas, in mice and chicks, these events are temporally and spatially separated.
In terms of the timing and the location of the head organizer, the chick is more similar to the frog, where the signalling comes from the the organizer and its derivative, the mesendoderm. In contrast, head-inducing signals in mammals originate from the AVE. Hence, mouse embryos begin the patterning of the head long before the mesendoderm ingresses, whereas chick head development occurs only after mesendoderm formation. Only mammals have shifted the head-inducing signals into the primitive endoderm, and they begin the induction and patterning process of the head long before (about 24 hours in mice and rabbits) the mesendoderm ingresses.

Differential timing of developmental processes, or heterochrony, is not an uncommon evolutionary mechanism (Gould, 1977). The discussed example refers to the anterior neural plate, including the anlage field of the telencephalon, and to a gene type (Anf) which is tightly connected to telencephalic development (Dattani et al., 1998). The differences in inductive capacities demonstrated in the present study raise the question as to whether the major and principal differences between an avian and a mammalian telencephalon could originate from a heterochronic shift of inductive activities into the preстreak endoderm during the evolution of mammalia.


REFERENCES


