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Segregating expression domains of two *goosecoid* genes during the transition from gastrulation to neurulation in chick embryos

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SUMMARY

We report the isolation and characterization of a chicken gene, *GSX*, containing a homeobox similar to that of the *goosecoid* gene. The structure of the *GSX* gene and the deduced GSX protein are highly related to the previously described *goosecoid* gene. The two homeodomains are 74% identical. In the first few hours of chick embryogenesis, the expression pattern of *GSX* is similar to *GSC*, in the posterior margin of the embryo and the young primitive streak. Later during gastrulation, expression of the two genes segregate. *GSC* is expressed in the anterior part of the primitive streak, then in the node, and finally in the prechordal plate. *GSX* is expressed in the primitive streak excluding the node, and then demarcating the early neural plate around the anterior streak and overlying the prechordal plate. We demonstrate that the *GSX*-positive part of the primitive streak induces gastrulation, while the *GSC*-expressing part induces neurulation. After full extension of the streak, the fate of cells now characterized by *GSX* is to undergo neurulation, while those expressing *GSC* undergo gastrulation. We discuss the effect of a duplicated basic *goosecoid* identity for the generation of a chordate nervous system in ontogeny and phylogeny.

Key words: *GSX*, *GSC*, primitive streak, neural plate, segregation, transplantation, induction, chick

INTRODUCTION

Homeobox genes are involved in specifying position, fate, pattern formation and morphogenesis during embryogenesis; together these features define the 'identity' of cells or tissues. A prominent role during axis formation has been attributed to genes with a *goosecoid* homeobox. *Goosecoid* genes were found in vertebrates (Blum et al., 1992, 1994; Blumberg et al., 1991; Cho et al., 1991; Izpisúa-Belmonte et al., 1993; Stachel et al., 1993) and in *Drosophila melanogaster* (Goriely et al., 1996; Hahn and Jäckle, 1996). The earliest expression of the *Xenopus goosecoid* gene is observed in the blastula, specifically in the dorsal part of the marginal zone. The ventral part of the marginal zone, on the contrary, is characterized by overlapping domains of the two homeobox genes *Xvent1* and *Xvent2* (Gawantka et al., 1995; Onichtchouk et al., 1996). While *Xgsc* responds to TGFβ-like, dorsalizing factors (activin A, Vg1; Cho et al., 1991; Seleiro et al., 1996), the *Xvent* genes respond to the ventralizing factor BMP-4 (Gawantka et al., 1995; Onichtchouk et al., 1996). Thus, the *goosecoid* and the two *Xvent* homeobox genes seem to be involved in specifying dorsal or ventral identities, respectively, in the marginal zone.

In gastrulating vertebrate embryos, *goosecoid* is expressed in the dorsal/anterior blastopore margin, the embryonic shield in zebrafish, the dorsal blastopore lip in *Xenopus* or in Hensen's node in chick and mouse. *Goosecoid*-expressing cells migrate anteriorly and form the prechordal mesendoderm underlying the prospective forebrain ectoderm. The conspicuous expression patterns of the *goosecoid* gene has prompted studies addressing its role in the phenomenon of Spemann's organizer (Spemann and Mangold, 1924; Cho et al., 1991; Blum et al., 1992; Izpisúa-Belmonte et al., 1993; Niehrs et al., 1993). Expression of *goosecoid* RNA in ventral cells leads to the formation of a second blastopore and consequently to the generation of a secondary embryo.

Gene targeting experiments in mice did not reveal any function of *goosecoid* in gastrulation and neurulation (Rivera-Peréz et al., 1995; Yamada et al., 1995). The *goosecoid* null-mutants did not show the early phenotypes, instead the observed phenotypes corresponded rather to late expression domains, such as the first branchial arch. Surprisingly late phenotypes were also observed after inactivation of other homeobox genes expressed early in embryogenesis, and were generally interpreted as indications of redundancy. Thus, *Otx-2* gene inactivation resulted in head defects (Ang et al., 1996) and not early epiblast defects, the *Nkx2.5* gene inactivation resulted in heart morphogenesis abnormalities and not in defects of the precardiac mesoderm (Lyons et al., 1995), and also several *Hox* gene inactivations resulted in relatively late abnormalities (Stein et al., 1996a).

In vertebrates, many homeobox genes are present in paralogous pairs, with homeodomains of more than 75% identity. Possibly this is a result of massive genomic duplication, which occurred in the chordate lineage after the cephalochordates *(Amphioxus)* and thus are found in all vertebrates (Holland et al., 1994). Several examples exist where the expression

patterns of two paralogous genes overlap to a large extent, e.g. the *CNOT* genes (Ranson et al., 1995; Stein and Kessel, 1995; Stein et al., 1996b), the *Xvent* genes (Gawantka et al., 1995; Onichtchouk et al., 1996) or some *Hox* genes. A different combination of homeobox gene expression seems to be involved in the specification of cellular identity (Kessel and Gruss, 1991). This concept of a molecular code was derived from studies on the *Hox* genes, but may well be a more generally applicable principle. The duplication of information resulting from gene duplications allows an increased flexibility for the coding mechanism, leading to complexity, redundancy and safety typical for higher vertebrate development.

Here, we characterize a *goosecoid*-related gene in the chick. The expression of the *GSX* gene segregates strikingly from the expression of the previously described chicken *goosecoid* gene. *GSX* expression characterizes cells located in the middle and posterior part of the primitive streak, while *GSC* expression characterizes cells being located in the anterior part of the primitive streak, including Hensen's node. *GSX* activity correlates in the mid-streak phase (HH stage 3) with the induction of gastrulation, and from the extended streak phase (HH stage 4) onwards with a neural fate. On the contrary, *GSC* activity correlates with the induction of neurulation and a mesendodermal fate. We discuss the role of both homeobox genes, *GSX* and *GSC*, at the transition from gastrulation to neurulation.

MATERIALS AND METHODS

Isolation and characterization of genomic clones

A *Xenopus goosecoid* cDNA fragment, corresponding to the highly conserved homeobox was labelled with [^{32}P] CTP by random priming and used to screen a genomic chick library generated in Lambda Fix II (Stratagene). Hybridization under low stringent conditions allowed the isolation of three independent clones. After subcloning into pBluescript II KS and rescreening a 4853 bp *Eco*RI restriction fragment, containing the sequence of the entire *GSX* gene, and a 1618 bp *Apa*I restriction fragment, containing the 3′-part of the gene, were identified. The sequencing (Sanger et al., 1977) was performed with the Dye Terminator Kit (ABI) on a 377 DNA Sequencer (ABI).

PCR strategies

Total RNA from HH stage 3/3$^+$ embryos was prepared by the LiCl procedure (Auffray and Rougeon, 1980) and 5 μg were reverse transcribed (Pharmacia). The conserved exon-intron boundaries in the homeobox were confirmed by the isolation of a 163 bp and a 120 bp fragment of the spliced homeobox by RT-PCR using gene-specific primers for the amplification of cDNA derived from embryos of the intermediate primitive streak stages.

Two specific primer pairs were synthesized to amplify *GSX* sequences from chick RNA. Primer pair LL67/66 amplifies a 163 bp fragment of the spliced *GSX* homeobox: 5′-primer, CAT CGC ACC ATA TTC ACC GAG, 3′-primer, GGC TCG ACG GTT CTT AAA CCA and primer pair LL68/66 amplifies a 120 bp fragment of the spliced *GSX* homeobox: 5′-primer, CTG GAA ACA CTT TTC CAC CAG, 3′-primer, GGC TCG ACG GTT CTT AAA CCA. The PCR cycling parameters were 25 cycles of 94°C for 1 minute, 60°C for 2 minutes, 72°C for 1 minute and a final extension at 72°C for 10 minutes. PCR products were ligated into the pCRII vector (Invitrogen) and identified by dideoxy sequencing.

Exon trapping and cloning

The cDNA sequence of the internal exon and the corresponding splice donor and acceptor sites were deduced from exon trapping experiments (Buckler et al., 1991). The exon trap procedure was applied to the genomic *GSX* sequence. By subcloning a fragment of the genomic clone into the pSPL1 vector, transfecting COS-7 cells and applying PCR amplification to first strand cDNA prepared from the resultant COS-7 cell mRNA, we obtained the internal exon, which was then subcloned into the pAMP10 vector (Gibco BRL).

Staging of embryos

Fertilized chick eggs (White Leghorn, obtained from Lohmann Tierzucht, Cuxhaven) were incubated at 37.8°C in a humidified incubator until they had reached the desired stage of development. Embryos were staged according to Eyal-Giladi and Kochav (roman numbers for preprimitive streak stages; Eyal-Giladi and Kochav, 1976) and Hamburger and Hamilton (arabic numerals after appearance of the primitive streak; Hamburger and Hamilton, 1951). Fertilized quail eggs were obtained from Heinrich Linnenschmidt, Wiedenbrück and quail embryos were staged using the chick tables.

Whole-mount in situ hybridization

Antisense *GSX* riboprobes were synthesized from a 1.6 kb genomic *Apa*I-fragment and a 163 bp fragment of the spliced *GSX* homeobox isolated by RT-PCR using T3 or SP6 RNA polymerase and incorporating digoxigenin-UTP (Boehringer Mannheim) according to manufacturer's instructions. Whole-mount in situ hybridization was performed essentially as described by Wilkinson (Wilkinson, 1992), except that the hybridization and the first two washing steps were performed at 55°C in presence of 0.1% CHAPS detergent (Sigma; Stein and Kessel, 1995), and no RNase treatment was done. For paraffin sections (8 μm), stained embryos were dehydrated and embedded in Paraplast plus (Sherwood Medicals). Sense control probes were negative in all experiments.

Embryo manipulations and primitive streak grafts

Preparation of host embryo cultures

White Leghorn chicken eggs were incubated in a humidified incubator for 12-15 hours. Embryos, together with large portions of the adjacent vitelline membranes, were removed from the eggs and prepared for a modified New culture (Stern, 1993). The blastoderm, still attached to its vitelline membrane, was oriented ventral side up in a 35 mm Petri dish on a fresh albumen substrate. A glass ring was positioned round the blastoderm and the vitelline membrane was draped over the ring. Such cultured chick blastoderms at HH stage 2-4 were used as hosts.

Preparation of donor embryos

Chick or quail embryos were used as donors. These were removed from their vitelline membranes and placed dorsal side up in a Petri dish, using Pannett-Compton saline. Primitive streak grafts were obtained from these embryos at HH stage 2-4. Pieces of primitive streak were excised with a tungsten needle. Each excised graft was transferred with a micropipet from the donor blastoderm and placed on top of the host blastoderm. A small hole was made in the hosts hypoblast in the germinal crescent, and the graft was inserted between hypoblast and ectoderm at the level of the anterior margin of the area pellucida. The cultures were incubated for 6-18 hours.

For the generation of chick/quail chimeras, chick embryos grafted with quail tissue were fixed in Zenker's fixative and embedded in Paraplast plus (Sherwood Medicals). Paraffin sections (8 μm) were processed for quail nucleolar histochemistry according to the Harris's haematoxylin staining described by Stern (1993).

Microscopy and photography

Embryos processed with the whole-mount procedure were viewed with a Zeiss Stemi SV 11 using a combination of reflected light from two fiber optic sources and transmitted illumination. Sections through the whole mounts were viewed and photographed using Nomarski differential interference contrast optics. Photographs were taken on Fuji 64T tungsten-balanced color film.

RESULTS

The *GSX* gene

By screening a chick genomic library under low stringent conditions with a *Xenopus goosecoid* cDNA fragment comprising the entire homeobox, we isolated a *goosecoid*-related gene, *GSX*, and characterized the locus by RT-PCR, exon trapping and sequence comparison. The *GSX* gene has a three exon organization, with an intron at a conserved position in the homeobox (Fig. 1, indicated by an arrowhead in B), similar to the known vertebrate and the *Drosophila goosecoid* genes (Fig. 1A). The initiator methionine for *GSX* was assigned based on its close vicinity to a sequence motif (FSIENIL) near the N terminus, that aligns with the highly conserved domain in the known *goosecoid* genes from frog, chick, mouse, human (FSIDNIL), zebrafish (FSIDSIL) and the *D-gsc* gene of the fruitfly (FTIDSIL).

The coding sequence predicts a 213-residue protein, very similar in length to the vertebrate GSC proteins (chick 246 amino acids, mouse 254 aa, *Xenopus* 244 aa, zebrafish 240 aa and human 252 aa), but smaller than *Drosophila gsc* (419 aa). The open reading frame reveals that the gene product has a paired-type homeodomain near the C terminus, which is highly homologous to the other known *goosecoid* homeodomains: 73.7% identity between chick *GSX* and *Drosophila gsc*; zebrafish, chick, mouse and human *GSC*; and 75.4% identity between chick *GSX* and *Xenopus GSC* (Blumberg et al., 1991; Cho et al., 1991; Blum et al., 1992; Izpisúa-Belmonte et al., 1993; Stachel et al., 1993; Goriely et al., 1996; Hahn and Jäckle, 1996).

The GSX homeodomain contains, like all other members of the *goosecoid* family, a lysine residue at the ninth position of the DNA recognition helix (Fig. 1B, indicated by an asterisk), which is a critical residue for target-specific DNA-binding (Treisman et al., 1989; Blumberg et al., 1991). A lysine in the corresponding position is only found in five other types of homeodomain proteins in different species, as there are: Bicoid (Berleth et al., 1988), Otx/Orthodenticle (Finkelstein et al., 1990; Simeone et al., 1993), Six/Sine Oculis (Cheyette et al., 1994; Oliver et al., 1995) and UNC-30/Ptx (Jin et al., 1994; Lamonerie et al., 1996).

Expression analysis of *GSX*

GSX transcripts are already detectable before primitive streak formation and gastrulation, when the hypoblast sheet expands at stage EK XIII (Eyal-Giladi and Kochav, 1976). Expression is seen as a crescent associated with Koller's sickle at the posterior margin of the blastoderm (Fig. 2A). Sagittal sections reveal that the majority of *GSX*-positive cells are located in the epiblast (Fig. 3A). The deep layer of the posterior epiblast, the location of presumptive primitive streak cells, shows a weak staining for *GSX*.

The appearance of the primitive streak is accompanied by an increase in the level of *GSX* expression. At early to mid-streak stages (HH stage 2-3$^-$; Hamburger and Hamilton, 1951), expression is seen in the entire primitive streak including its tip (Fig. 2B). By HH stage 3-3$^+$, as the groove appears in the streak, *GSX* expression extends through the primitive streak, but excludes Hensen's node (Figs 2C, 3B, 4A).

When the streak has progressed to its maximal length by HH stage 4, *GSX* transcripts become less abundant in the primitive streak itself, with expression remaining in the primitive ridges (Fig. 2D). The clearly discernible primitive groove and the primitive pit, major sites of avian gastrulation, are negative for *GSX* expression (Fig. 2D). At this stage, a new domain arises around the anterior third of the primitive streak, with expressing cells in the epiblast surrounding Hensen's node in a circular and later pear-shaped expansion (Figs 2D and 3C). The limits of *GSX* expression reveal a sharp boundary between the strongly stained central neuroectodermal region and the unstained more peripheral epiblast (presumptive epidermis). Fate mapping studies have identified this domain as the early neural plate (Rudnick, 1935; Spratt, 1952; Garcia-Martinez et al., 1993).

During regression of the primitive streak, when it shortens by the movements of Hensen's node towards a more posterior

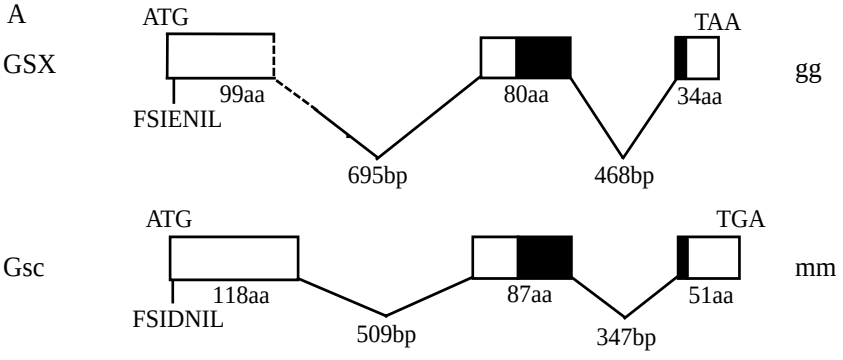

Fig. 1. The chick *GSX* gene. (A) Genomic organization of the chick *GSX* and mouse *Gsc* gene. Translated regions are depicted as rectangles, with the homeobox filled in black. Conserved motifs near the N termini of both proteins are indicated (FSIENIL, FSIDNIL). The 3′ end of the first exon was assigned based on the splice donor consensus (dotted lines). (B) Comparison of the chick GSX homeodomain with vertebrate Gsc and *Drosophila* gsc homeodomains. Dots indicate amino acid identity with GSX and the arrowhead points to the conserved position of an intron. An asterisk labels the lysine at the ninth position of the DNA recognition helix. Abbreviations: aa, amino acids; bp, base pairs; dm, *Drosophila melanogaster;* dr, *Danio rerio*; gg, *Gallus gallus*; hs, *Homo sapiens*; mm, *Mus musculus*; xl, *Xenopus laevis*.

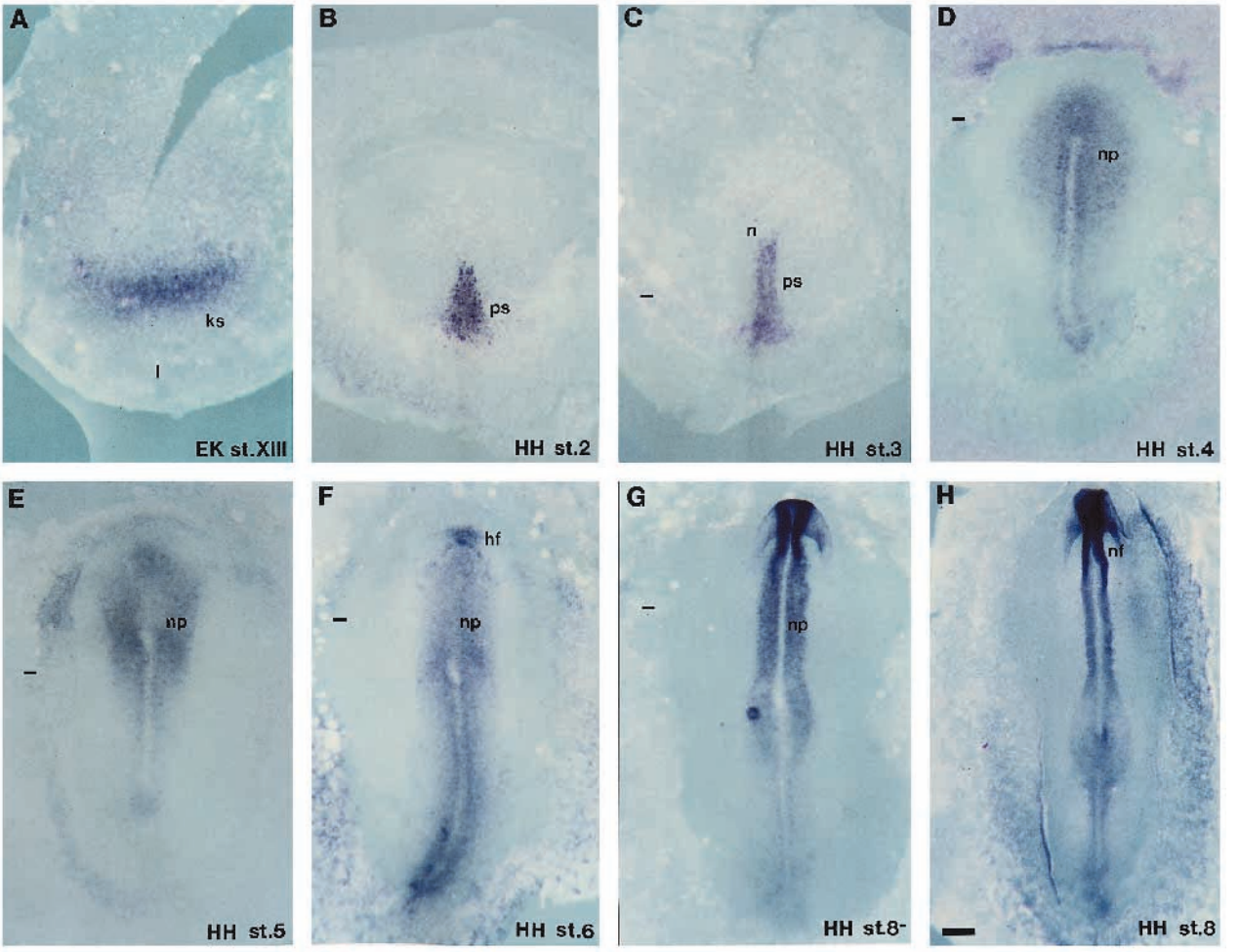

Fig. 2. Expression of the *GSX* gene during early chick development. Embryos were subjected to whole-mount in situ hybridization using a DIG-UTP-labelled riboprobe derived from a 1.6 kb genomic subclone, comprising the second and third exon. Embryos were photographed from the dorsal side, using both transmitted and incident light. Stages according to Hamburger & Hamilton (Hamburger and Hamilton, 1951) or Eyal-Giladi & Kochav (Eyal-Giladi and Kochav, 1976) are given in the right corner. The expression patterns (A-H) are explained in detail in the text. Indicated are head fold (hf), Koller's sickle (ks), node (n), neural folds (nf), neural plate (np), and primitive streak (ps). The scale bar equals 240 μm (A,B), 310μm (C,F) or 410μm (G,H), respectively. The level of sagittal or transverse sections of embryos in A,C,D,E and F are indicated by black dashes. Sections are presented in Fig. 3.

position, *GSX* expression is maintained in the neural plate, illustrating its convergence and extension (Figs 2E-H, 3D). As the head fold forms (HH stage 6), the elevating neural plate shows a strong expression of *GSX* (Figs 2F, 3E), which persists during neural tube closure (Figs 2G,H, 3F).

The comparison of *GSX* with *GSC* expression reveals segregating domains along the primitive streak, with *GSC* becoming confined to the anterior streak and the prechordal plate (Izpisúa-Belmonte et al., 1993), and *GSX* remaining in more posterior parts of the streak and then in the neural plate (Figs 4, 6). It is noteworthy, however, that after segregation *GSC* expression is induced in the developing forebrain above the prechordal plate, where it overlaps with *GSX* expression.

Transplantation experiments

We analyzed various parts of the primitive streak for their inductive potential at different times during chick development (Fig. 5). This type of experiments has been described before, mostly addressing the node as the chick organizer (for references, see Dias and Schoenwolf, 1990), but also more caudal levels (Gallera and Nicolet, 1969; Izpisúa-Belmonte et al., 1993; Waddington and Schmidt, 1933; summarized in Gallera, 1971; Waddington, 1952). We characterized the inductions with molecular markers and followed the fate in chick-quail chimeras. Grafts were taken from the tip or more caudal levels, in order to use cells expressing only *GSC*, only *GSX* or both genes simultaneously. They were transplanted to naive ectoderm in anterolateral positions of HH stage $3/3^{+}$ hosts. A total of 239 primitive streak grafts was made, of which 181 (76%) were used for analysis. Those that were not used include induced structures that merged with the host embryo or did not survive in culture. Two completely different outcomes were observed: either the induction of gastrulation or the induction of neurulation.

Induction of gastrulation

Induction of gastrulation was evident by formation of an ectopic primitive streak with a clearly discernible groove,

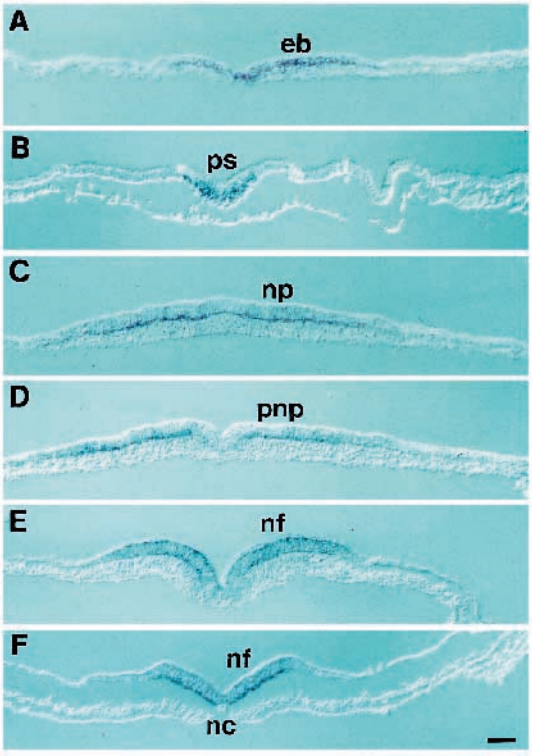

Fig. 3. Spatial and temporal expression of the *GSX* gene. (A) Mid-sagittal section (anterior to the left) through the posterior margin of a EK stage XIII embryo (Fig. 2A, indicated by a black dash). *GSX* expression is mainly seen in the epiblast, along with weaker expression in the deeper layer at the level of Koller's sickle. (B-E) Transverse sections of embryos shown in Fig. 2C-F at levels indicated in Fig. 2 by black dashes. (B) Transverse section through a HH stage 3 embryo (Fig. 2C), *GSX* expression is restricted to the primitive streak. (C) Transverse section through a HH stage 4 embryo (Fig. 2D), *GSX* expression labels the early neural plate. (D) Transverse section through a primitive streak of a HH stage 5 embryo (Fig. 2E), *GSX* expression labels the postnodal neural plate and excludes the primitive streak. (E) Transverse section through the folding neural plate of a HH stage 6 embryo (Fig. 2F), *GSX* expression labels the neural plate. (F) Transverse section through a HH stage 8 embryo (Fig. 2G), *GSX* expression labels the neural plate. Indicated are epiblast (eb), notochord (nc), neural folds (nf), neural plate (np), postnodal neural plate (pnp) and primitive streak (ps). The scale bar equals 50 μm.

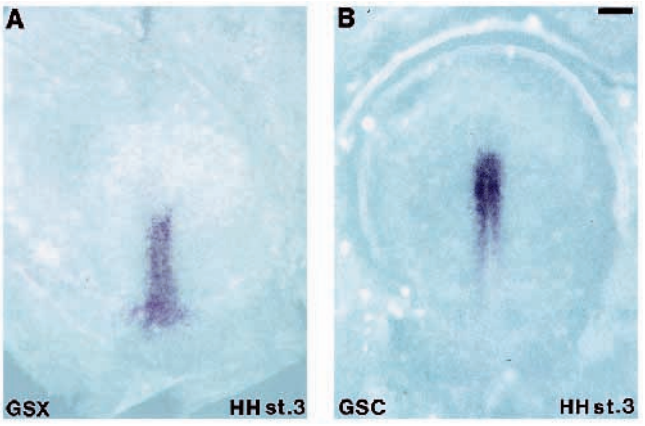

Fig. 4. Comparison of the *GSX* and *GSC* expression in mid-primitive streak stages. (A) During the elongation of the primitive streak *GSX* expression concentrates on the middle and posterior part of the primitive streak. (B) In a comparable mid-streak stage embryo, the *GSC* expression is confined to the anterior part of the primitive streak. The scale bar equals 310 μm.

which, in sections, revealed cells leaving the streak exactly as found in primary streaks (92 positive inductions of 128 operations). Gastrulation was induced by grafts from early streaks (HH stage 2/3$^-$) whether taken from the tip or more caudally. This result was also obtained with various grafts derived from the posterior half or end of the streak using HH stage 3/3$^+$ donors, but not using HH stage 4 or stage 5 donors. For the majority of the experiments described below, we decided to use grafts from the middle of the primitive streak (HH stage 3/3$^+$), which do not express *GSC*. Middle streak grafts induce nicely elongated streaks and represent the most posterior site being involved in bona fide axis formation, while the second half is mainly concerned with the production of extraembryonal mesoderm (Psychoyos and Stern, 1996). At HH stage 3/3$^+$, the capacity for the induction of gastrulation disappears from the tip of the streak (Gallera and Nicolet, 1969), where it is replaced by a novel capacity, inducing neural structures (see next section).

Induced streaks of various stages were analyzed with molecular markers. All were positive for the pan-mesodermal marker *Brachyury* (*Ch-T*; Kispert et al., 1995), with strong expression in the streak itself, in the ingressing mesoderm and in axial mesoderm, which is produced after prolonged incubation (Fig. 5A,B). *GSC* (Izpisúa-Belmonte et al., 1993), *HNF3β* (Ruiz i Altaba et al., 1995) and *CNOT1* (Stein and Kessel, 1995) are markers for the anterior portion of the primitive streak, a region deliberately avoided in the grafts. Expression of these three markers was not typically observed in the induced streaks (Fig. 5D-F,H-J). However, a few ectopic primitive streaks with either *GSC* (*n*=1) or *HNF3β* (*n*=3) expression were also obtained (data not shown), possibly indicating that anterior streak cells were unexpectedly present in the graft. *CNOT1* was only observed in epiblast cells around the tip of induced streaks, a signal corresponding to the described *CNOT1* expression in very young, prenodal, paranodal and postnodal neural plate (Fig. 5D,E,G), whereas the expression domain normally found in the anterior streak did not appear (Fig. 5D-F; Stein and Kessel, 1995). Induced gastrulation events showed *GSX* expression in the streak itself and in the surrounding neuroectoderm (Fig. 5K-M). In order to distinguish between self-differentiation and induction, grafts were taken from HH stage 3/3$^+$ quail embryos (Le Douarin, 1969). Quail cells derived from the graft were found in a horseshoe-like area anterior of the induced streak (Fig. 5N-P). Thus they behaved like the earliest mesoderm in chicken, the precardiac mesoderm, which normally reaches the future heart-forming region very anterior in the early embryo, either by passive displacement or by active migration. The fate of the grafted cells therefore was equivalent to the fate that they would have obtained if left in the primary embryo. Induced primitive streaks, including cells before, during and after gastrulation were completely derived from chick cells, indicating a true induction event (Fig. 5P).

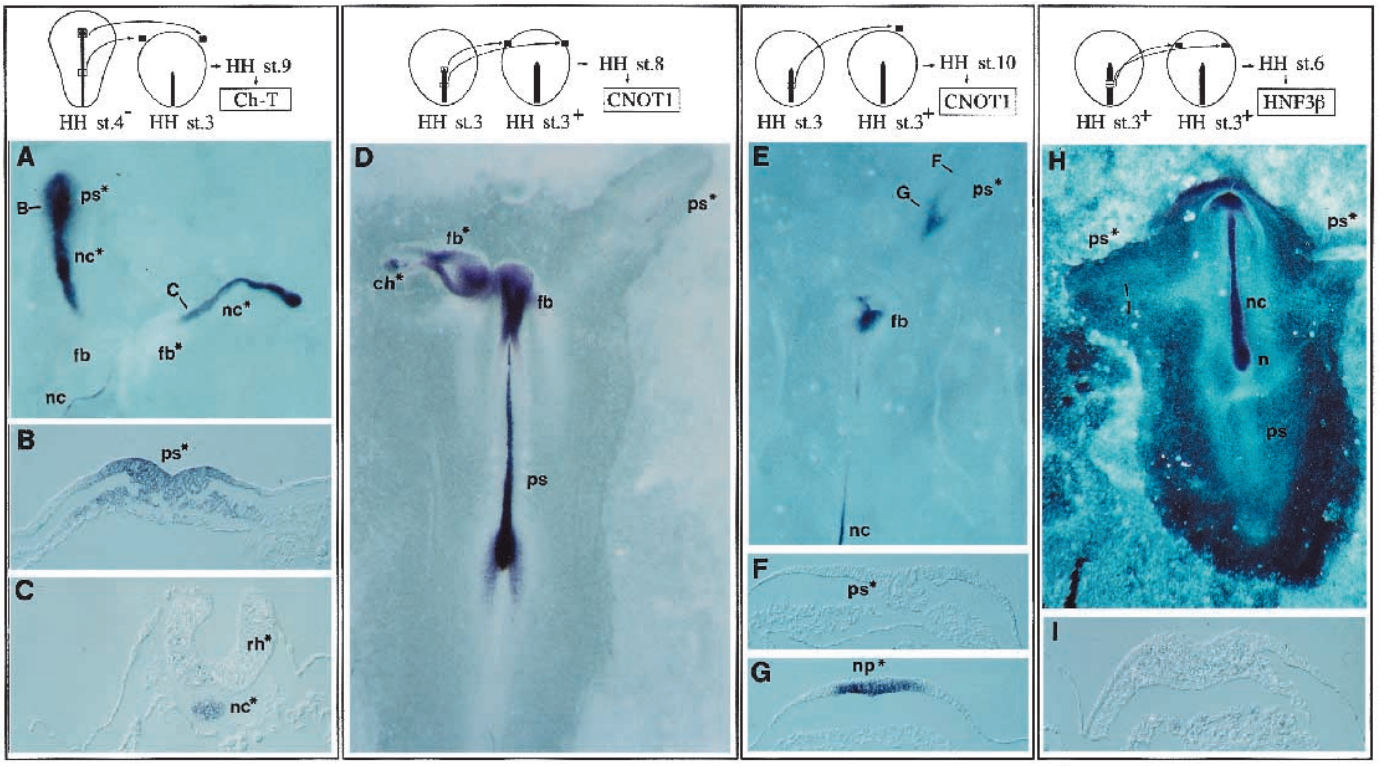

Fig. 5 (A-H) – See legend opposite.

Induction of neurulation

The induction of neurulation proceeded quite differently (89 positive inductions of 111 operations). Neural induction by node transplantations represents the chick equivalent of Spemann´s organizer experiments using the Einsteck procedure and have been characterized in depth elsewhere (Dias and Schoenwolf, 1990; Storey et al., 1992; Beddington, 1994). We corroborated that node grafts (HH stage 3/4) induced in the overlying host epiblast a nervous system, which was clearly rostrocaudally patterned by morphological and molecular criteria (Fig. 5A,C,D). During this process, the epiblast thickened and neural folds elevated, but it appeared that the basal membrane never opened to allow gastrulation of epiblast cells to contribute to notochord or paraxial mesoderm. The grafts themselves grew out, depending on their precise stage, to form either a globular, chordoid mass (HH stage 3 nodes, Fig. 5D), or an elongated notochord (HH stage 4⁻, Fig. 5A). By using quail donors, we demonstrated the absence of graft-derived cells from the neuroectoderm and their presence in the chordoid material (not shown). Secondary embryos were analyzed with a number of molecular markers: *Brachyury*, *CNOT1*, *GSC* and *EN-2*. In all cases, they were expressed in their expected patterns: prospective dorsal forebrain was indicated by *CNOT1* (Fig. 5D), prospective midbrain by *EN-2* (not shown), notochord-like cells by *Brachyury* (Fig. 5A,C) and *CNOT1* (Fig. 5D). *Brachyury* or *CNOT1* expression was never induced in cells of the epiblast overlying the graft. In conclusion, we demonstrated the segregation of inductive potential along the primitive streak, with neural induction becoming confined to the anterior streak and the capacity for induction of gastrulation localized towards more posterior parts (Fig. 5).

DISCUSSION

Parallel segregation of expression patterns and inductive potentials

Based on the primary structure of the homeodomain, the conservation of a second N-terminal domain and on the genomic organization, we consider *GSX* to be closely related to the previously described chicken *goosecoid* gene (GSC). It remains to be seen, however, if, like *GSC*, the *GSX* gene also confers an axis-inducing potential after injection of *GSX* mRNA into *Xenopus* embryos.

The two chicken genes, *GSX* and *GSC*, are expressed simultaneously in early primitive streak stages, when only gastrulation is occurring and neurulation is not yet induced. We assume that *GSC* expression within the prospective mesoderm, the *Brachyury* (*Ch-T*)-positive zone (Kispert et al., 1995), is related to the definition of dorsal values, as has been demonstrated in the amphibian *Xenopus laevis* (Cho et al., 1991).

With the further extension of the streak, a new inductive capacity segregates and appears in the tip of the streak. The forming node now acquires the potential to induce neurulation, as evident in transplantation experiments and in the appearance of neural commitment around the node (Dias and Schoenwolf, 1990; Gallera and Nicolet, 1969; Storey et al., 1992, Waddington and Schmidt, 1933). In parallel, the initially overlapping expression domains of the related genes segregate, so that *GSC* expression is correlated with the new inductive potential, while *GSX* characterizes the remaining part of the streak, i.e. the older, gastrulation-inducing area. Thus, when the primitive streak approaches its maximal extension, *GSC* and *GSX* do not form a nested pair of expression domains. The difference concerns an area of major importance for pattern formation

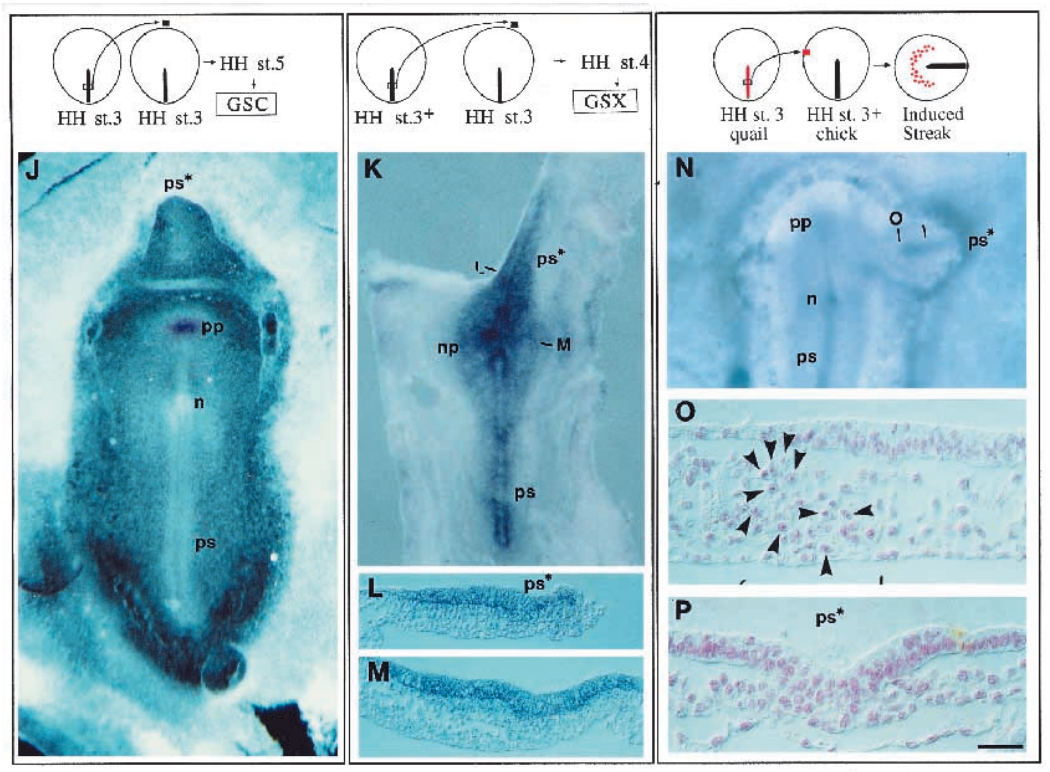

Fig. 5. Inductive potentials of primitive streak grafts. Diagrams indicate sites of transplantation, stages of grafts and stages of hosts at operation or fixing, and the respective probe for whole-mount in situ hybridization. Stereomicroscopic views are displayed in A,D,E,H,J,K and N (bar, 400 μm), interference contrast microscopic views in B,C,F,G,I,L and M (bar, 50 μm), and O and P (bar, 25 μm). An asterisk (*) labels the induced structures and abbreviations indicate chordoid mass (ch), forebrain (fb), node (n), notochord (nc), neural plate (np), prechordal plate (pp), primitive streak (ps) and rhombencephalon (rh). (A-C) An induced primitive streak producing axial mesoderm (left, cross section in B), an induced nervous system (right, cross section in C), and the anterior end of the host embryo are shown after analysis with a chicken *Brachyury* riboprobe (*Ch-T*). *Brachyury* transcripts are found in the induced primitive streak itself, in the corresponding ingressing mesoderm (cross section in B) and in the notochord of the induced nervous system (cross section in C). (D) An induced nervous system (left) and an induced primitive streak (right), together with the host embryo (middle), are shown after analysis with a chicken *CNOT1* riboprobe. In the host embryo, *CNOT1* expression is found in the node, notochord, parts of the prenodal and postnodal neural plate and the prospective forebrain and midbrain regions. The induced nervous system (left) is positive for *CNOT1* staining, indicating that the anterior streak graft induced prosencephalic vesicles in the overlying epiblast of the host embryo. Note the absence of *CNOT1* staining from the induced streak (right), but the presence of weak staining in parts of the neural plate. (E-G) An induced primitive streak in an anterolateral position to the host embryo is shown after analysis with a chicken *CNOT1* riboprobe. In the host embryo, the *CNOT1* signals focus to the closing anterior neuropore in the neuroectoderm of the prospective forebrain and to the notochord. In the induced embryo, the *CNOT1* signal is confined to the postnodal neural plate (cross section in G), but is not seen in the induced primitive streak (cross section in F). (H,I) Two induced primitive streaks on both sides of the host embryo are shown after analysis with a chicken *HNF3β* riboprobe. In both cases (left and right), the induced structures do not stain with this anterior streak marker. A cross section through an induced primitive streak (left) is shown in I. (J) An induced streak anterior to the host embryo is shown. A chicken *GSC* riboprobe does not stain the induced streak in the expected manner, but the prechordal plate of the host embryo, after prolonged time of incubation in the developing solution. (K-M) An induced streak together with the host embryo is shown after the analysis with a chicken *GSX* riboprobe. A cross section through the induced primitive streak is shown in L. Note that both embryos contribute to a common neural plate (cross section in M), that is positive for *GSX* expression. (N-P) A primitive streak induced by a quail graft with the anterior part of the chick host is shown. Note the absence of quail cells from the streak and the ingressing mesoderm (P). The displaced quail cells (O) are found mostly anterior of the induced streak (see red dots in diagram and arrowheads pointing to the quail nucleolar marker).

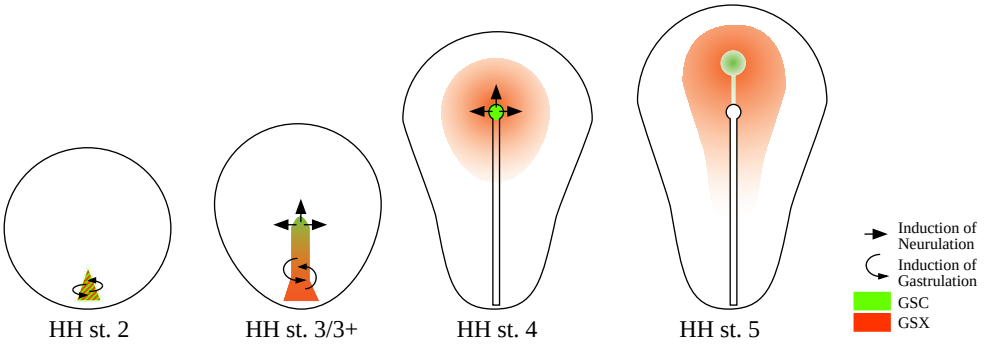

Fig. 6. Segregation of *GSX* and *GSC* expression parallels segregation of inductive potentials. The shared expression of *GSX* (red) and *GSC* (green) in early primitive streak stages (HH stage 2), segregates into posterior, *GSX* and anterior, *GSC*, domains during streak elongation (HH stage 3/3+). After full extension of the streak (HH stage 4), *GSC* cells populate Hensen's node and gastrulate, while *GSX* cells remain in the epiblast and neurulate. During regression of the primitive streak (HH stage 5), *GSC* expression is confined to the prechordal plate and the anterior head process, while *GSX* expression is maintained in the developing neural plate. In parallel, the common potential to induce gastrulation segregates into an anterior, neurulation-inducing (straight arrows), and a posterior, gastrulation-inducing (curved arrows) potential. The segregation phenomena allow the generation of a complex nervous system with *goosecoid* cells on the inducing (*GSC*) as well as on the responding side (*GSX*).

processes, the organizer. Therefore, the consequences are drastic. After full extension of the streak at HH stage 4, *GSC*-expressing cells gastrulate and ingress as axial mesendoderm, while *GSX*-expressing cells stay strictly ectodermal and demarcate the forming neural plate, for which *GSX* now represents a unique marker (see Fig. 6 for a schematic summary). In *Xenopus*, neural induction has been closely linked to the GSC target gene *chordin* (Sasai et al., 1994, 1995). Thus, the secreted factor chordin may represent a molecular link between *GSC* and *GSX* in the neural-inducing and the responding cells of the chick.

With the ingression of the axial mesendoderm, the definition of the anterior values begins. The most anterior aspect of the embryo is specified in the animal hemisphere, where a direct contact between endoderm and mesoderm occurs. The anterior endoderm is characterized by the homeobox gene *XANF1* in *Xenopus* (Zaraisky et al., 1995) and the related gene *Rpx* (*Hesx1*) in mouse (Hermesz et al., 1996; Thomas and Beddington, 1996). The overlying, also *XANF1*/*Rpx*-positive ectoderm, will form Rathke's pouch and later the adenohypophysis. Adjacent to the anterior endoderm lies the prechordal mesendoderm, consisting of *GSC*-expressing cells. The overlying, first only *GSX*-positive, but then also *GSC*-positive ectoderm will go on to form the neurohypophysis. An inductive interaction between the prechordal plate and the anterior neural plate, each expressing a different member of the *goosecoid* family, contributes to the patterning of the forebrain anlage. We have argued elsewhere that here again a neuralizing factor like chordin could be the molecular signal, emitted from the prechordal to the neural plate (Pera and Kessel, unpublished data).

During the patterning of the forebrain anlage, *GSC* expression appears also in the ventral neuroectoderm above the prechordal plate. By the 4-somite stage (HH stage 8), the neuroectodermal expression extends through the mesencephalon into the rhombencephalon. Since *GSX* labels the complete neuroectoderm at this stage, the two genes have finally generated a true nested expression pattern.

Embryogenesis without an organizer?

For the induction of gastrulation, we used medial, pure *GSX*-positive parts of primitive streaks and avoided the anterior, *GSC*-positive parts. The induced streaks were unusual with regard to genes expressed normally in anterior streaks or the node. Thus neither *GSC*, nor *CNOT1* or *HNF3β* expression was found in the tips of these streaks. Nevertheless, they seemed to proceed quite normally through gastrulation and to go into neurulation. We demonstrated an early neural plate forming in the ectoderm around the tip, expressing *GSX* and *CNOT1*. The tip of ectopically induced streaks produced axial mesoderm as evident by *Ch-T* expression (Fig. 5A). Taken together, the organizer with its typical genetic set up appears to be dispensable. This finding is further sustained by results from genetic inactivation of the murine *GSC* or *HNF3β* genes, suggesting the presence of redundant gene activities, replacing the inactivated gene in its function (Ang et al., 1994; Weinstein et al., 1994; Rivera-Peréz et al., 1995; Yamada et al., 1995). Both mutants passed quite undisturbed through gastrulation, neurulation and anteroposterior neural patterning, even though the *HNF3β* mutants did not generate a node or notochord. Mechanic ablation of the node or the complete anterior 40% of the primitive streak in chicken was also consistent with normal embryogenesis, although *GSC* expression was never regenerated in the healed, anterior streaks (Psychoyos and Stern, 1996). It remains to be seen how far, in these experiments, *GSX* is taking over the role of *GSC*.

Evolutionary implications of the related genes, *GSC* and *GSX*

The common beginning and later segregation of both gene activities during ontogeny could indicate a similar dynamic during phylogeny. This assumption is strengthened by findings concerning the *Drosophila goosecoid* gene (Goriely et al., 1996; Hahn and Jäckle, 1996). The primary structure of its homeodomain is equally distant (73.7%) from the chicken *GSC* and the *GSX* domains. The fly gene is expressed only in ectodermal structures, the brain anlage and the esophagus, and its derivative, the stomatogastric nervous system, and mutants have defects in neural development (Hahn and Jäckle, 1996). We suggest that the basic *goosecoid* information was split to two genes during the evolution of chordates, allowing the generation of novel fates and inductive potentials. Such a gene duplication most likely occurred together with many other duplications after the cephalochordates separated from the lineage leading to vertebrates (Holland et al., 1994). We speculate that gastrulation of *goosecoid* cells only became possible when, at the same time, a second *goosecoid* identity could be maintained in the ectoderm. As a consequence, a simple sequence, going from endoderm to notochord, was converted to a more elaborate system of endoderm-mesendoderm-notochord. The two lower groups of chordates, ascidians (Urochordata) and *Amphioxus* (Cephalochordata) indeed lack a prechordal mesendoderm, and their notochord is formed in continuity with the endoderm during gastrulation (Conklin, 1932; Satoh, 1994). A direct consequence of a prechordal plate in vertebrates is the more complex organization of the forebrain, as evident by the presence of a unique ventral forebrain or the presence of two eyes, instead of one (Pera and Kessel, unpublished data).

In the chick, the nervous system develops from *GSX* cells, which are induced and patterned under the influence of *GSC* cells. This interaction is exerted in a planar fashion, while still in the anterior streak, and vertically after gastrulation from the prechordal plate (Ruiz i Altaba, 1993). Thus, the two related genes are found on both sides of neural development in chicken: *GSC* on the inducer, *GSX* on the responder side. The segregation of both gene functions may represent a key feature at the beginning of a chordate nervous system not only during ontogeny, but also during phylogeny.

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