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Gene expression pattern

A hierarchy of gene expression accompanying induction of the primitive streak by Vg1 in the chick embryo

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Abstract

In the chick embryo, two secreted factors have recently been shown to cooperate in inducing the first axial structure, the primitive streak: cWnt8C (normally expressed around the circumference of the embryo, in the marginal zone) and the TGFβ superfamily member cVg1 (expressed in the posterior part of the marginal zone) (Development 128 (2001) 2915). Misexpression of Vg1 in the anterior marginal zone induces an ectopic primitive streak and recapitulates the morphological changes associated with normal primitive streak formation. Here, we analyse the time-course of appearance and disappearance of expression of 12 genes (*cVg1*, *Lef1*, *Nodal*, *FGF8*, *cWnt8C*, *cBra*, *cNot1*, *goosecoid*, *HNF3β*, *Chordin*, *Otx2* and *Sox3*, whose normal expression is also polarized at early stages of development) in response to cVg1 misexpression in the anterior marginal zone. We show that a hierarchy of gene expression accompanies induction of the ectopic axis, reminiscent of the order in which the same genes begin to be expressed in the normal embryo.

Keywords: Chicken embryo; Primitive streak; Spemann organizer; Hensen's node; Induction; Gastrulation; *cVg1*; *Wnt8C*; *cLef1*; *cFGF8*; *cNodal*; *Goosecoid*; *HNF3β*; *cNot1*; *Chordin*; *cBrachyury*; *Sox3*; *Otx-2*

1. Results

The posterior marginal zone of the chick embryo has the unique ability, when transplanted to an appropriate ectopic position, to induce an embryonic axis (which includes Hensen's node, the amniote equivalent of Spemann's organizer), without making a cellular contribution to the node (Bachvarova et al., 1998). This property defines the posterior marginal zone as the avian homologue of the Nieuwkoop centre. The competence of the blastoderm to respond to such an induction is restricted to the early stages after egg-laying (stage X; Eyal-Giladi and Kochav, 1976) and is lost some 10 h before the primitive streak forms (Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi and Khaner, 1989; Bachvarova et al., 1998). During this period of competence and up to the time when the primitive streak starts to form, many organizer and mesodermal genes are already expressed in the posterior marginal zone or in neighbouring regions of the embryo (e.g. Izpisúa-Belmonte et al., 1993; Ruiz i Altaba et al., 1995; Knezevic et al., 1997; Streit et al., 1998; Skromne and Stern, 2001; see also Lawson et al., 2001). Many of these genes encode either secreted proteins or transcriptional regulators, which raises the question: which of them are involved in the induction of the primitive streak?

Answering this is complicated by the fact that it is impossible to determine the precise stage when the first inducing events normally occur. We have therefore taken advantage of the finding that misexpression of Vg1 in embryonic regions expressing Wnt8C, such as the anterior marginal zone, leads to the formation of an ectopic axis by inducing a series of morphological changes that is remarkably similar to those observed during normal primitive streak formation at the posterior end of the embryo (Shah et al., 1997; Skromne and Stern, 2001). This provides an initial time point from which to measure the order in which other genes are upregulated during the process of primitive streak formation. We report the patterns and time-course of expression of 12 genes during the induction of an ectopic axis by Vg1, and correlate them with morphological changes accompanying the formation of the primitive streak.

1.1. Morphological changes following misexpression of Vg1

The earliest morphological consequence of Vg1 misex-

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pression visible in whole embryos is the appearance, 6 h after misexpression, of a translucent sector surrounding the Vg1-secreting cells; this was observed in 17/30 (57%) embryos examined. At 9 h, a short thickening appears in the area pellucida adjacent to the secreting cells, indicating the beginning of an ectopic primitive streak (13/25; 52%) (Fig. 1). By 12 h, the ectopic streak has elongated noticeably and resembles that of a normal stage 3 embryo (Fig. 1). By 15 h, the induced streak resembles that of a normal stage 4 embryo, with a discernible primitive groove and Hensen's node.

1.2. Time-course of expression of 12 markers following Vg1 misexpression

Twelve genes were chosen whose normal expression patterns indicate the polarity of the embryo at early stages of primitive streak formation (Fig. 1, see also Lawson et al., 2001): the signalling factors *cVg1*, *cNodal*, *FGF8*, *Wnt8C* and *Chordin*, the Wnt-pathway component *cLef1* (Skromne and Stern, 2001) and five transcription factors that mark specific regions of the embryo including *cBra* (for primitive streak/notochord; Kispert et al., 1995; Knezevic et al., 1997), *goosecoid* and *HNF3β* (markers for both hypoblast and the organizer; Hume and Dodd, 1993; Izpisúa-Belmonte et al., 1993; Ruiz i Altaba et al., 1995; Bachvarova et al., 1998; Foley et al., 2000), *cNot1* and *Otx2* (markers for pre-streak epiblast covered by hypoblast and later for the organizer; Bally-Cuif et al., 1995; Knezevic et al., 1995; Ranson et al., 1995; Stein and Kessel, 1995; Foley et al., 2000; Knezevic and Mackem, 2001) and *Sox3* (early neural marker; Uwanogho et al., 1995; Rex et al., 1997; Streit et al., 1998, 2000).

Three hours following Vg1 misexpression, the only change observed, as compared to control embryos, was a

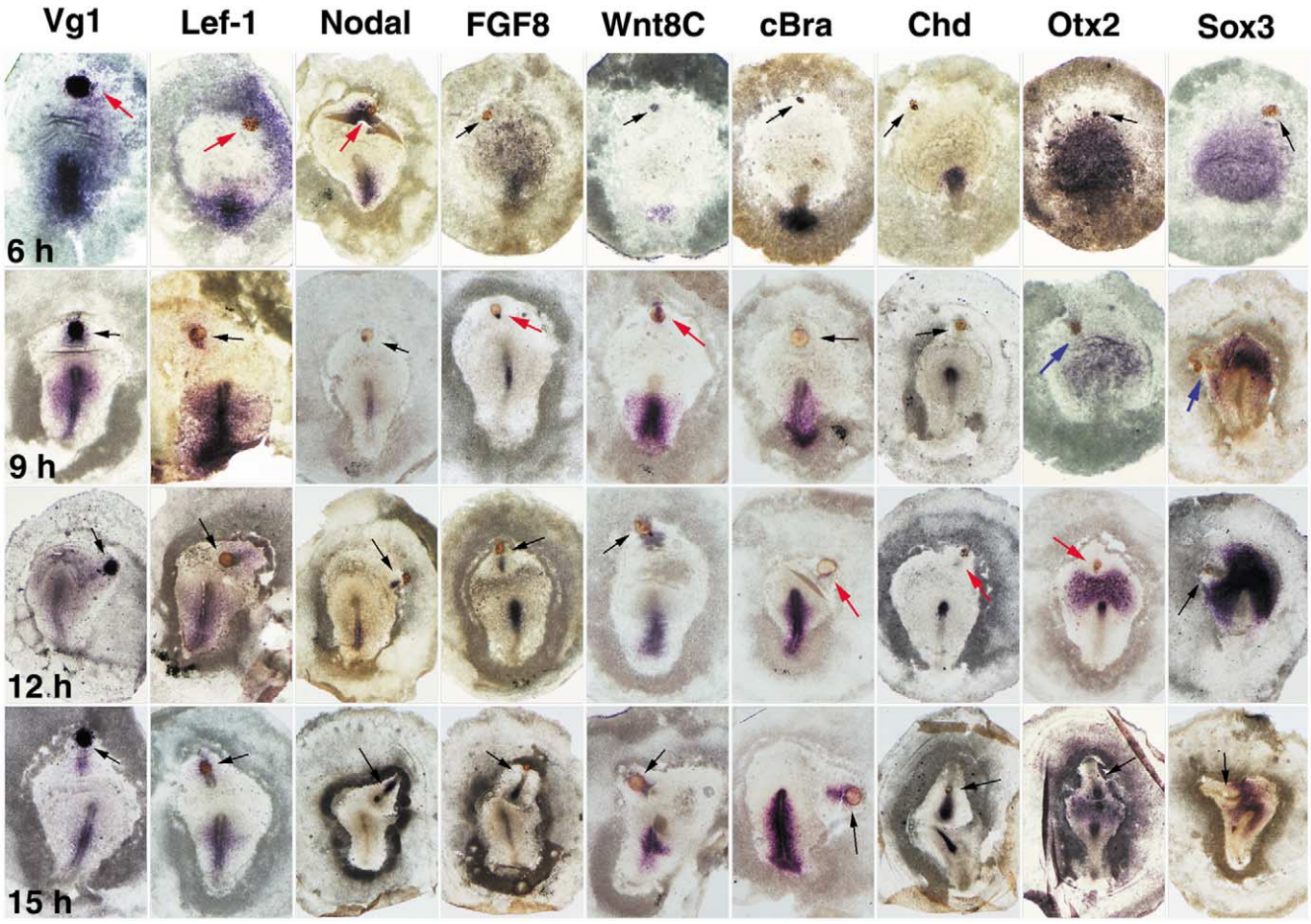

Fig. 1. Temporal sequence of gene expression during formation of an ectopic primitive streak in response to Vg1 misexpression in the anterior marginal zone. Hanging drop aggregates of cVg1-transfected COS cells were grafted to the anterior marginal zone of stage X–XIII hosts. Embryos were fixed at the times indicated on the left and processed for whole mount in situ hybridization (purple signal) for the genes indicated on top. Anti-myc immunohistochemistry (brown signal) was used to identify cell aggregates (arrows) and verify protein synthesis in transfected cells. Gene expression was confirmed in at least three independent experiments, each one containing a minimum of five embryos. Red arrows indicate the initial time point at which ectopic expression of a particular gene is detected in the host. Blue arrows indicate the time point at which *Otx-2* and *Sox3* expression starts to become downregulated in the epiblast. The expression profile of *goosecoid*, *HNF3β* and *cNot1* in the organizer (not shown) is similar to that of *chordin*. The probe used to detect expression of cVg1 in the embryo also hybridizes with the cVg1-transfected COS cells. The weak purple signal observed at 6 h in the *Wnt8C*, *cBra*, *chordin* and *Otx-2* panels is due to non-specific adsorption of the probe to the COS-cell aggregate.

weak ectopic domain of expression of *cVg1* mRNA surrounding the implanted cells (8/8 embryos; not shown). Upon sectioning, ectopic *cVg1* expression was observed in both the hypoblast and the epiblast layers (data not shown). By 6 h, ectopic expression of *cVg1*, *cLef1* and *cNodal* was detected in host tissue around the grafted cells in about 50% of the cases (*cVg1*, $n = 7$; *cLef1*, $n = 5$; *cNodal*, $n = 4$). This ectopic *cVg1* is expressed in a broad domain including the anterior marginal zone and area pellucida (Fig. 1). By contrast, *cLef1* is expressed in the anterior marginal zone and area opaca and *cNodal* is restricted to the area pellucida adjacent to the grafted cell pellet (Fig. 1). No ectopic expression of *FGF8*, *gsc*, *chordin*, *cBra*, *cNot1*, *HNF3β* or *Wnt8C* is detected at this stage, nor are any alterations in the patterns of expression of *Otx2* or *Sox3* ($n = 123$).

By 9 h, embryos with a visible ectopic primitive streak ($n = 151$) express *cVg1*, *Wnt8C* and *cLef1* throughout this structure, and *FGF8* and *cNodal* at its anterior tip (Fig. 1). In addition, the area pellucida epiblast surrounding the induced streak shows downregulation of expression of *Otx2* and *Sox3* (Fig. 1, blue arrows). However, no ectopic expression of any organizer marker (*gsc*, *chordin*, *HNF3β*, *cNot1* or *cBra*) is observed (Fig. 1 and data not shown).

Expression of organizer markers begins at about 12 h ($n = 142$ embryos): *chordin*, *gsc*, *HNF3β*, *cNot1* and *Otx2* are first detected at the tip of the ectopic streak, accompanied by *FGF8* in a slightly larger domain, while *cVg1*, *cLef1*, *Wnt8C* and *cNodal* are transcribed in the posterior part of this streak, and *cBra*, expressed throughout its length (Fig. 1). Outside the induced streak, the domains of *Otx2* and *Sox3* show a pronounced clearing (Fig. 1). The hypoblast, which expresses *Otx2* and *gsc*, is now confined to a narrow region between the two streaks, consistent with it having become displaced by endoblast and endoderm arising from both axes (Fig. 1 and not shown; see Foley et al., 2000). By 15 h ($n = 216$ embryos), expression of the organizer markers intensifies, and the ectopic expression of all markers resembles that of the normal axis at an equivalent stage of development.

During normal development, all of these 12 markers are expressed before primitive streak formation begins, but for many of them it is difficult to ascertain the precise time of onset of expression. The characteristic sequence of induction of these genes following Vg1 misexpression allows us to rank them into a hierarchy. The most surprising finding is that the primitive streak marker *cBra* and the organizer markers *gsc*, *HNF3β*, *Otx2*, *chordin* and *cNot1* appear relatively late in the sequence, as the primitive streak and node become morphologically distinguishable.

2. Materials and methods

Stage X–XIII embryos (Eyal-Giladi and Kochav, 1976) obtained from briefly incubated fertile White Leghorn hens' eggs (SPAFAS, CT, USA) were set up in modified New culture (New, 1955; Stern and Ireland, 1981). Five hundred cell aggregates expressing myc-cVg1 construct were grafted to the anterior marginal zone of host embryos as previously described (Shah et al., 1997). Following transplantation, embryos were cultured at 38°C, fixed at the appropriate time in 4% formaldehyde in phosphate buffered saline (PBS) (pH 7.0) and processed for in situ hybridization (Streit et al., 1997) using the following probes: *cBra* (Kispert et al., 1995; Knezevic et al., 1997; gift of J.C. Smith), *chordin* (Streit et al., 1998), *cFGF8* (Kengaku et al., 1998; Streit and Stern, 1999; gift of J.C. Izpisúa-Belmonte), *goosecoid* (Izpisúa-Belmonte et al., 1993), *HNF3β* (Ruiz i Altaba et al., 1995; gift of A. Ruiz i Altaba), *cLef1* (Kengaku et al., 1998; gift of J.C. Izpisúa-Belmonte), *cNodal* (Jones et al., 1995; Levin et al., 1995; gift of M. Kuehn), *cNot1* (Stein and Kessel, 1995; gift of M. Kessel), *Otx2* (Bally-Cuif et al., 1995; gift of L. Bally-Cuif and E. Boncinelli), *Sox3* (Uwanogho et al., 1995; Rex et al., 1997; gift of R. Lovell-Badge and P. Scotting), *cVg1* (Seleiro et al., 1996; Shah et al., 1997) and *cWnt8C* (Hume and Dodd, 1993; gift of J. Dodd). After in situ hybridization, a monoclonal anti-myc antibody (9E10, Evans et al., 1985) was used to identify the cell aggregates as previously described (Shah et al., 1997).

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