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Cerberus-like is a secreted factor with neuralizing activity expressed in the anterior primitive endoderm of the mouse gastrula

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Abstract

We report the isolation of mouse *cerberus-like* (*cer-l*), a gene encoding a novel secreted protein that is specifically expressed in the anterior visceral endoderm during early gastrulation. Expression in the primitive endoderm starts before the appearance of the primitive streak and lasts until the head-fold stage. In later stages, a second region of expression is found in newly formed somites. Mouse *cer-l* shares some sequence similarity with *Xenopus cerberus* (*Xcer*). In *Xenopus* assays *cer-l*, like *Xcer*, mRNA acts as a potent neuralizing factor that induces forebrain markers and endoderm, but is unable to induce ectopic head-like structures as *Xcer* does. In addition to *cer-l*, anterior visceral endoderm was found to express the transcription factors *Lim1*, *goosecoid* and *HNF-3β* that are also present in trunk organizer cells. A model of how head and trunk development might be regulated is discussed. Given its neuralizing activity, the secreted protein Cer-l is a candidate for mediating inductive activities of anterior visceral endoderm. © 1997 Elsevier Science Ireland Ltd.

Keywords: Cerberus-like; Neural induction; *Lim1*; *goosecoid*; Primitive endoderm

1. Introduction

In the mouse embryo, grafts of the anterior end of the full-length primitive streak have been shown to induce secondary axes (Beddington, 1994). The grafted tissue, or node, gives rise mostly to notochord and definitive endoderm, whereas neural plate and somites are recruited from the host. The duplicated structures extend rostrally to the level of the hindbrain and therefore the anterior primitive streak may be considered equivalent to the trunk-tail organizer of the amphibian embryo (reviewed in Spemann, 1938; De Robertis et al., 1994). Grafts of the early blastopore lip of amphibian embryos have been shown to induce complete twinned axes including anterior head structures such as eyes, whereas late dorsal lips induce only trunk-tail structures, leading to the concept that the head organizer might be distinct from the trunk-tail organizer (Spemann, 1931).

In the mouse, there is genetic evidence suggesting the existence of a separate head organizer. Mutation of the *Lim1* organizer-specific homeobox gene results in mouse embryos lacking all head structures anterior to the auditory vesicle (Shawlot and Behringer, 1995). Targeted inactivation of *Otx2*, a homeobox gene initially expressed in the entire embryo that subsequently becomes restricted to the anterior third of the endoderm and overlying neural folds, also results in severe anterior head truncations (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Mutations in the winged-helix gene *HNF-3β* result mainly in dorsoventral defects along the entire anteroposterior axis, but in some mutant embryos the anterior head region is also missing (Ang and Rossant, 1994; Weinstein et al., 1994). These headless phenotypes were interpreted as resulting from failure of the development of prechordal plate mesoderm. However, recent findings suggest that the anterior visceral endoderm, referred to in the literature also as anterior primitive endoderm (primitive endoderm includes both parietal and visceral endodermal cells), may play a fundamental role in head organizer activity (Thomas and Beddington,

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1996; Varlet et al., 1997; reviewed by Bouwmeester and Leyns, 1997).

The *Hesx1/Rpx* homeobox gene is first expressed in a small patch of cells in the anterior primitive endoderm in the 6.5-day-old embryo (Hermesz et al., 1996; Thomas and Beddington, 1996). Lineage tracing studies showed that although the primitive endoderm is gradually displaced by definitive endoderm cells originating from the node, the patch of *Hesx1* expression remained in the region underlying the future forebrain (Thomas and Beddington, 1996). At day 7.5 *Hesx1* transcripts were activated in the overlying neurectoderm and, importantly, extirpation experiments showed that a signal from anterior primitive endoderm is required for the expression of *Hesx1* in the prospective forebrain (Thomas and Beddington, 1996).

The importance of endodermal signals in head development is also highlighted by experiments in which chimeric embryos, composed predominantly of wildtype cells in the epiblast and entirely of *nodal*$^{-/-}$ cells in the primitive endoderm, failed to form head structures anterior to the hindbrain (Varlet et al., 1997). Thus, *nodal*, a TGFβ family member expressed uniformly in the entire visceral endoderm, appears to be required for head formation.

In *Xenopus* a large number of genes specifically expressed in the dorsal lip of the blastopore, or Spemann's organizer, have been isolated. These comprise transcription factors, mainly homeobox genes, which in turn activate secreted factors that are proposed to mediate the effects of organizer grafts on the differentiation of neighboring cells (Lemaire and Kodjabachian, 1996; De Robertis et al., 1997). Cerberus is a secreted factor that is a good candidate for playing an inductive role in head formation in *Xenopus* embryos. Microinjection of *cerberus* mRNA into embryos can induce the formation of ectopic head-like structures containing forebrain, cyclopic eyes, olfactory placodes and cement glands. In animal cap explants *cerberus* mRNA induces anterior central nervous system (CNS) markers such as *Otx2*, as well as endodermal (*endodermin*) and heart (*Nkx-2.5*) markers (Bouwmeester et al., 1996).

Cerberus is expressed in the yolky cells that form the leading edge of the *Xenopus* gastrulating endoderm. This cell population will eventually give rise to foregut and midgut, including the liver, and is separate from the mid gastrula dorsal lip, which forms the trunk organizer. However, at earlier gastrula stages the domains of expression of some dorsal lip homeobox genes such as *goosecoid* (*gsc*) and *Xlim1* extend into the yolky cells that will form the leading edge of the dorsal endomesoderm, indicating that both domains may share common regulatory pathways (Bouwmeester et al., 1996).

Although a precise topological comparison is difficult due to the lack of extraembryonic tissues in the *Xenopus* embryo, it is likely that the *cerberus*-expressing cells represent the equivalent of the mouse anterior visceral endoderm (Thomas and Beddington, 1996; Bouwmeester and Leyns, 1997). This view is supported by the expression pattern of the *Xenopus Hesx1* homologue, *XANF1*, in leading edge endodermal cells during gastrulation (Zaraisky et al., 1995). In addition, both the anterior endoderm in mouse and the leading edge *cerberus*-positive cells in *Xenopus* come into close contact with the ectoderm of the prospective anterior neural plate during the course of morphogenetic movements in the gastrula.

In the present study we present *cerberus-like* (*cer-l*), a mouse gene encoding a novel secreted protein that shares sequence similarities with *Xenopus cerberus* (*Xcer*). *Cer-l* is expressed in anterior primitive endoderm early in mouse development and in definitive anterior endoderm at later gastrula stages. Like *Xcer*, microinjection of *cer-l* mRNA induced anterior CNS markers in injected animal cap explants. However, unlike *Xcer*, *cer-l* mRNA was not able to induce ectopic head-like structures in *Xenopus* embryos, suggesting that the mRNAs have overlapping but distinct biological effects. The region of *cer-l* positive primitive endoderm expresses, in addition to *Otx2* and *Hesx1*, other nuclear factors such as *Lim1*, *gsc* and *HNF-3β*. The possible roles of homeobox genes and of the Cer-l secreted factor in the induction of anterior neurectoderm are discussed in light of the emerging ideas on the role of anterior visceral endoderm in the mouse embryo.

2. Results

2.1. Cerberus-like encodes a secreted protein

During efforts to clone the murine homologue of *Xcer* an EST (AA120122), released by the WashU-HHMI mouse EST project, was identified from cDNA sequences derived from the Beddington day 7.5 embryonic region library. This partial clone had sequence similarities to *Xcer* and was named *cerberus-like*. The genomic region and full-length cDNA were cloned and sequenced (GeneBank AF012244). The mouse *cer-l* gene encodes a 272 amino acid protein and contains a single 2 kb intron (Fig. 1A). The deduced protein contains a typical hydrophobic signal sequence at its amino terminus and a cysteine-rich domain close to its carboxy terminus. Comparison to Xcer showed that the cysteine-rich domain is 58% identical, that the overall amino acid identity for the entire proteins is 31% and that the spacing of the nine cysteines is conserved (Fig. 1B).

To determine whether the full-length cDNA encoded a secreted protein, the 293T embryonic human kidney cell line was transiently transfected with a *cer-l* eukaryotic expression vector and metabolically labeled with [^{35}S]-methionine (see Section 4.6). As shown in Fig. 1C, lane 2, a broad band of 34–43 kDa was found in the culture medium. The Cer-l protein was presumably glycosylated, since Cer-l protein translated in the reticulocyte lysate system (in the absence of microsomal membranes) resulted in a pro-

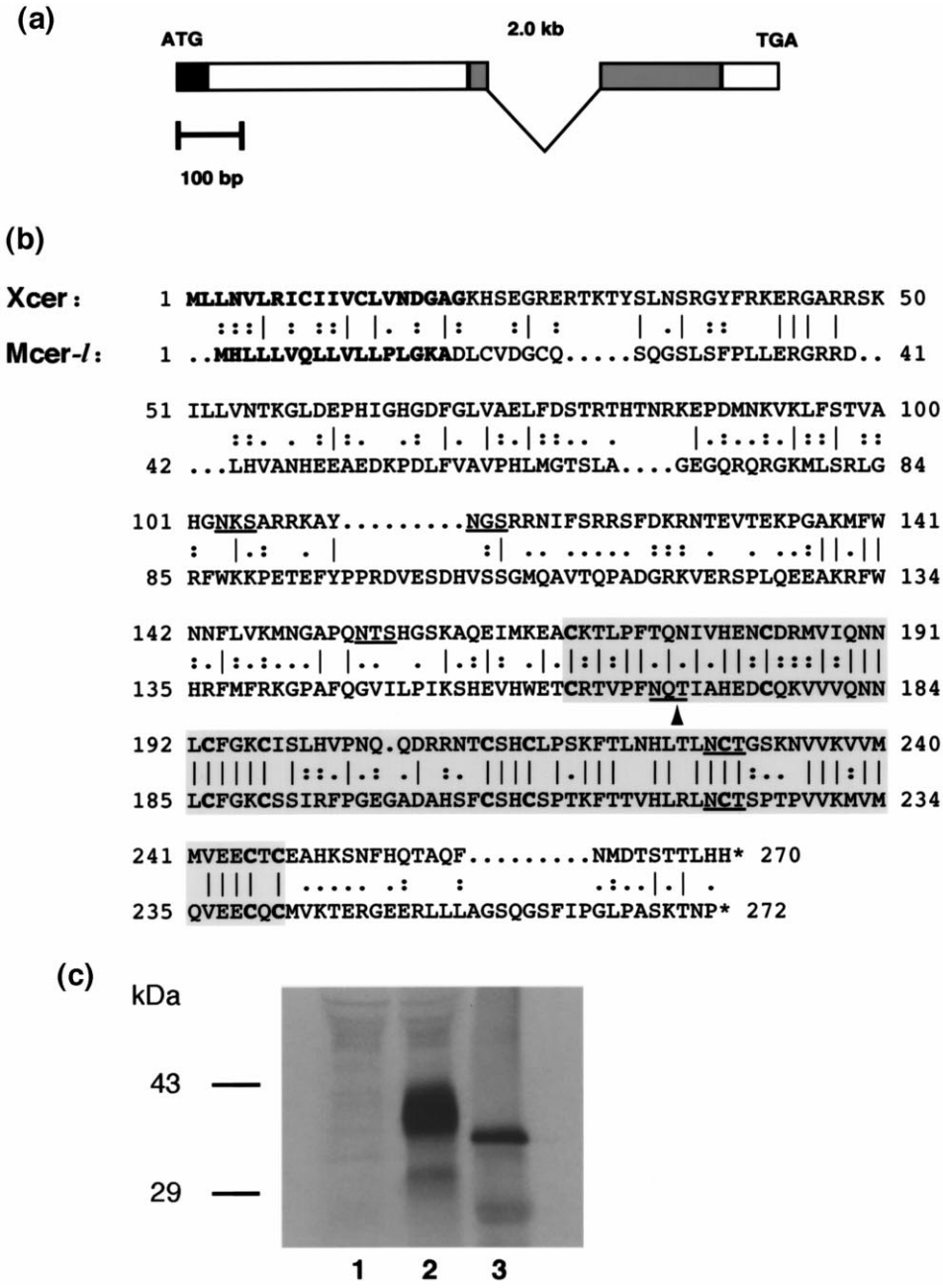


Fig. 1. The mouse *cerberus-like* gene encodes a secreted protein with a cysteine-rich domain. (A) Genomic organization of the *cer-l* gene. Translated regions are depicted as rectangles, with the signal peptide region filled in black and the cysteine-rich domain in gray. (B) Alignment of the amino acid sequences of Xcer and mouse Cer-l. Vertical lines indicate identical residues. The predicted signal peptides and the nine conserved cysteines are shown in bold. The cysteine-rich region is boxed in gray and the potential N-linked glycosylation sites are underlined. The arrowhead indicates the position of the *cer-l* intron and an asterisk indicates the stop codon. (C) ^{35}S proteins secreted by transiently transfected 293T cells into the culture medium and separated in reducing PAGE gels. Lane 1, supernatant from control cells transfected with pCS2 plasmid lacking an insert; lane 2, ^{35}S culture medium form cells transfected with *cer-l* in pCS2; lane 3, in vitro translation of *cer-l* mRNA in rabbit reticulocyte lysate. Smaller bands, presumably due to degradation products, are also seen in lanes 2 and 3.

duct of 33 kDa (Fig. 1C, lane 3). This size was in agreement with the molecular weight of 31 kDa predicted from the amino acid sequence. The Cer-l protein has two putative N-linked glycosylation sites (Fig. 1B). We conclude that the *cer-l* gene encodes a secreted protein with 58% amino acid identity with Xcer in the cysteine-rich domain but with low similarity outside of this region.

2.2. cer-l is expressed in anterior endoderm and somites

Fig. 2 shows the pattern of expression of *cer-l* in mouse embryos from pre-streak to head-fold stages (Kaufman, 1992; Downs and Davies, 1993). In pre-streak embryos (5.5 days post coitum), *cer-l* transcripts can be detected on one side of the visceral endoderm, including the distal tip of

the embryo (Fig. 2A). Histological sections confirmed that the patch was confined to the endodermal layer before primitive streak formation (Fig. 2A, inset). At the early primitive streak stage, expression is found in a patch of visceral endoderm cells on one side of the embryonic region and no longer extends to the tip of the embryo (Fig. 2B). This patch corresponds to the anterior side of the embryo, since in sections it is found in the endoderm opposite to the forming primitive streak which can be recognized by a thickening of the posterior epiblast (Fig. 2C, arrow). Importantly, cells expressing *cer-l*, which encodes a secreted factor, are in direct contact with the epiblast cells that will give rise to the fore- and midbrain region of the CNS (Quinlan et al., 1995). At the mid-streak stage, the *cer-l* positive patch remains in the anterior visceral endoderm (Fig. 2D). At the late-streak stage, when the primitive streak has reached the distal tip of the embryo, a second population of *cer-l* expressing cells can be seen in the region surrounding the tip of the embryo (Fig. 2E). These cells presumably correspond to definitive endodermal cells that are known to emerge from the node (Lawson and Pedersen, 1987; Lawson et al., 1991; Beddington, 1994). The endodermal nature of this cell population was confirmed by histological analysis (Fig. 2F–H).

At neural plate stages, *cer-l* transcripts are found in the endoderm underlying the anterior neural plate in a pattern comparable to the domain of *Otx2* expression (Ang et al., 1994 and data not shown); at this stage the *cer-l*-positive cell population presumably consists of both primitive and definitive endoderm (Lawson et al., 1986; Thomas and Beddington, 1996) and does not include the node itself (Fig. 2I). At the early head-fold stage, the *cer-l* signal in the endoderm starts to decrease (Fig. 2J) and by late head-fold *cer-l* expression is confined to the midline and adjoining endoderm (Fig. 2K). In histological sections expression is found in all cells of the midline from the rostral end of the embryo to the proximity of the node and includes anterior gut endoderm and mesoderm from the prechordal and notochordal plates. This is an important difference from *Xenopus*, in which *Xcer* is not expressed in the prechordal plate and notochord (Bouwmeester et al., 1996).

The expression of *cer-l* mRNA in this anterior domain continues until the start of somitogenesis and is then downregulated. Concomitantly, a late phase of expression begins in the somites. The last two somites to be formed and the most rostral portion of the presomitic mesoderm are prominently labeled (Fig. 3A,C). Expression in unsegmented presomitic mesoderm is indicated by arrowheads in the sagittal section shown in Fig. 3B. Expression in forming somites continues during tail formation until somitogenesis is completed (Fig. 3D). In *Xenopus*, *Xcer* expression was not detected in forming somites (Bouwmeester et al., 1996), pointing to another difference in the expression patterns of mouse *cer-l* and *Xcer*. It is worth mentioning, however, that somitogenesis in amphibians (which rapidly forms muscle) is known to have important mechanistic differences to that of amniotes (Keynes and Stern, 1988).

We conclude that mouse *cer-l* defines an anterior domain of the endoderm in early mouse embryos. Expression is found in the anterior endoderm that is in direct contact with the future neural plate. This anterior domain may be considered analogous to the region of *Xcer* expression in the leading edge of the *Xenopus* gastrula endoderm (Bouwmeester et al., 1996; Thomas and Beddington, 1996). Despite this similarity, important differences in expression exist since mouse *cer-l* is expressed in midline mesodermal structures (prechordal and notochordal plate) and forming somites, regions in which *Xcer* expression has not been detected (Bouwmeester et al., 1996).

2.3. cer-l mRNA has neuralizing activity in Xenopus assays

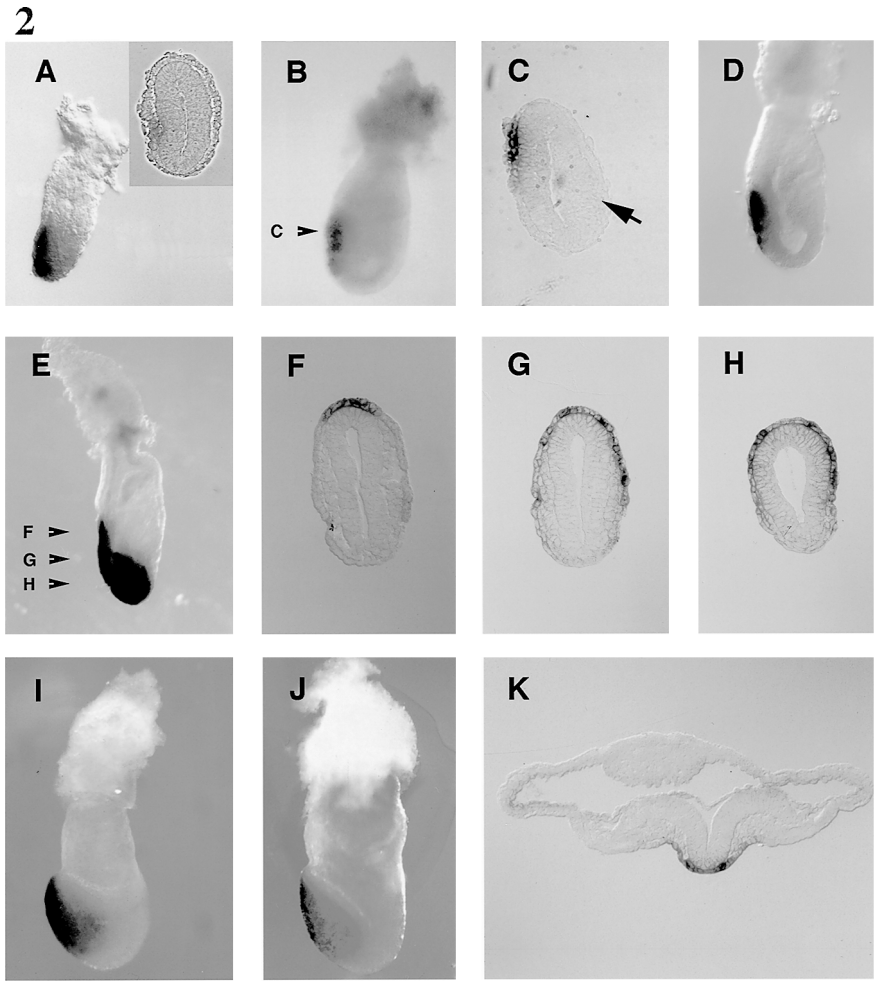
To assess the biological activities of mouse *cer-l* we used microinjection into *Xenopus* which, although an heterolo-

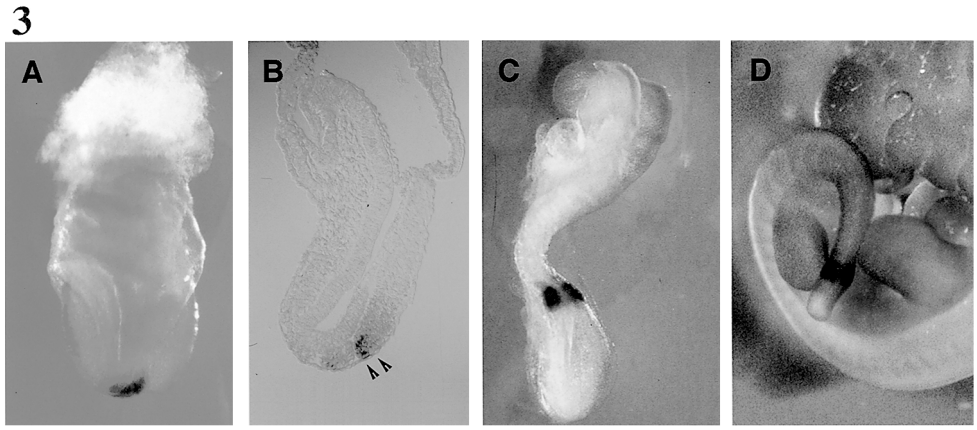
Fig. 2. Expression of *cer-l* during gastrulation and neurulation, as detected by whole-mount in situ hybridization. (A) Pre-streak embryo showing *cer-l* expression in the primitive endoderm on one side, including the distal tip. Inset, histological section at the same stage. (B) Early-streak embryo showing that the staining no longer extends to the distal tip. (C) Histological section taken at the level indicated by an arrowhead in the previous panel, showing that the expression of *cer-l* is confined to the anterior primitive endoderm cells, on the opposite side of the early primitive streak that can be recognized by a thickening of the epiblast (arrow). (D) Mid-streak stage embryo, *cer-l* is expressed in the anterior primitive endoderm. (E) Late-streak embryo, an additional population of endodermal cells expresses *cer-l* close to the node at the tip of the embryo. Arrowheads indicate the level at which the transversal histological sections shown in (F–H) were taken. (F) Proximal section showing *cer-l* expression on one small patch of the anterior primitive endoderm, extending laterally around approximately one-quarter of the circumference of the embryo. (G) *Cer-l* expression in the endoderm of the anterior half of the embryo. (H) *Cer-l* expression, still restricted to the endodermal layer, is seen extending laterally around three-quarters of the circumference in a section close to the tip of the late-streak embryo; this tissue presumably contains definitive endoderm. (I) At neural plate stage, *cer-l* expression becomes restricted to the endodermal cells of the anterior third of the embryo, underlying the prospective anterior neurectoderm. Note that the node is negative. (J) Early head-fold stage, *cer-l* expression in anterior endoderm weakens in lateral regions and perdures in the midline. (K) Transversal histological section from a late head-fold stage embryo showing *cer-l* expression in the single outermost layer of the anterior midline that forms the notochordal plate and flanking definitive endoderm. The background blue signal seen in sectioned material represents diffusion of staining product into nearby tissues.

Fig. 3. Analysis of *cer-l* expression during somitogenesis. (A) At the onset of somitogenesis at day 7.5, *cer-l* expression in the anterior midline is downregulated, but a second domain appears in somitic mesoderm. (B) Sagittal section of the embryo shown in (A) in which the mesoderm of the forming somites and adjoining presomitic mesoderm (arrowheads) is stained by the *cer-l* probe. (C) Day 8.5 embryo, *cer-l* expression is restricted to the last two formed somites and presomitic mesoderm. (D) At day 11.5, *cer-l* expression is still restricted to the last two somites and the presomitic mesoderm in the tail region, forming three stripes.

gous system, permits a determination of inductive activities (for a recent example see Zeng et al., 1997). When 75 pg of *cer-l* synthetic mRNA were microinjected into the marginal region of each blastomere at the four-cell stage (radial injections) embryos with shortened axes and enlarged dorsoanterior structures were observed (Fig. 4A, see legend for details). The same amount of *Xcer* mRNA caused the formation of much enlarged, sometimes radial, cement glands (indicated by arrows in Fig. 4A) as described previously (Bouwmeester et al., 1996). The differential effects of mouse *cer-l* and *Xcer* were also seen in single injections into ventral-vegetal blastomeres, in which *cer-l* was unable to induce ectopic cement glands and head-like structures, as *Xcer* did, in parallel assays in the same experiment (Fig. 4B, see legend for details).

The enlarged head region caused by radial injection of mouse *cer-l* in *Xenopus* embryos had one cement gland, two eyes and greatly enlarged brain structures in histological sections (n = 11, data not shown). The shortened trunk contained a prominent notochord and somites, consistent with a





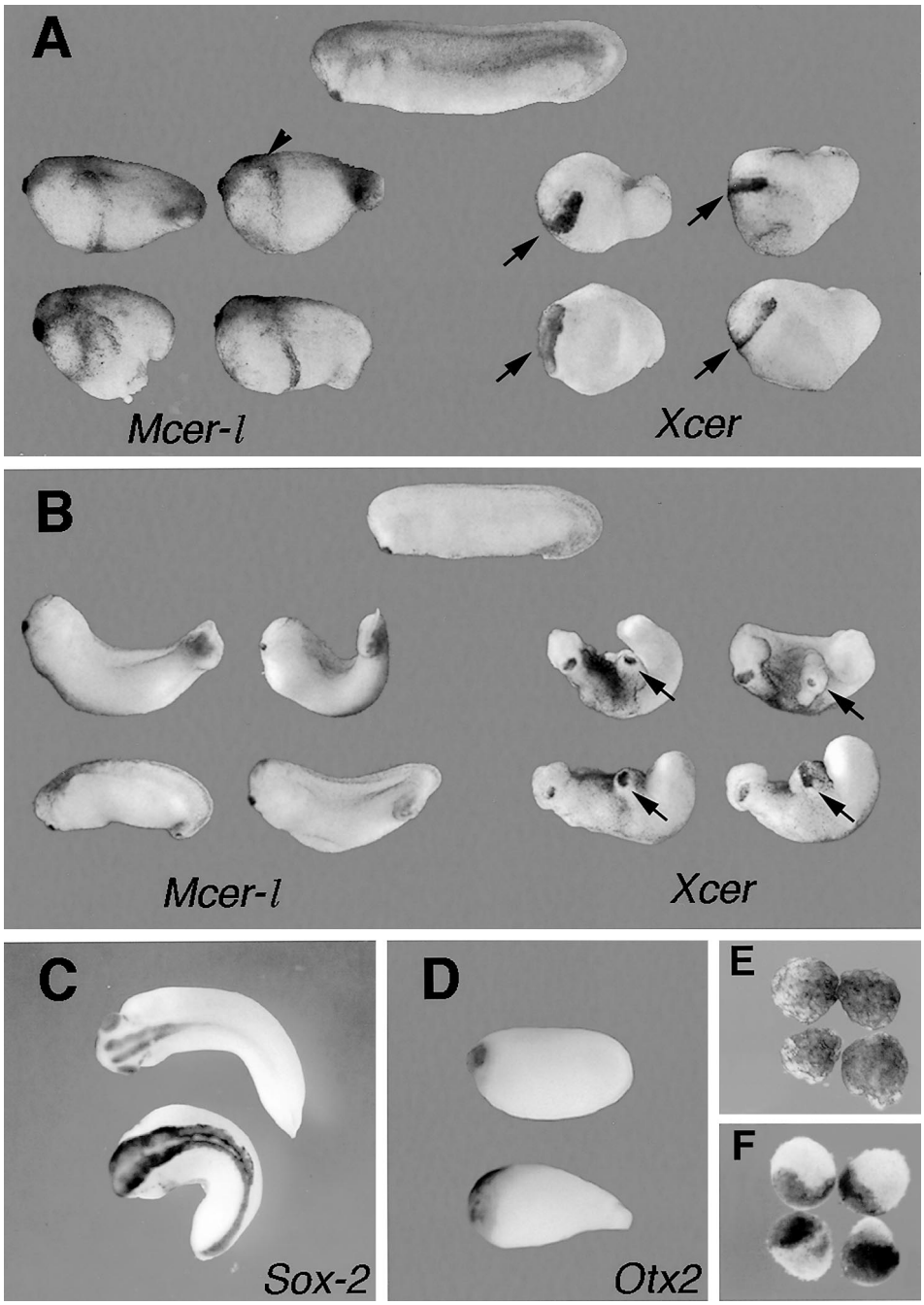


Fig. 4. Microinjection of *cer-l* mRNA into *Xenopus* embryos leads to enlarged head and neural structures. (A) Control stage 27 (top), four embryos microinjected with 75 pg of *cer-l* mRNA into the marginal region of each blastomere at the four-cell stage (left) and four embryos injected with the same amount of *Xcer* mRNA (right). *Cer-l* caused embryos with enlarged dorso-anterior structures and shortened axes. On the skin surface, accumulations of pigmented cells were observed in the head region (arrowhead) which histologically were not cement gland cells and, although their nature is unknown, could correspond to hatching gland cells. A range of concentrations of *cer-l* were tested (30–150 pg/blastomere, seven independent experiments, $n = 151$); slightly dorsalized phenotypes were observed in 95% of the embryos injected at 30 pg/blastomere. Representative embryos as shown were observed in 92% of injections at 75 pg/blastomere and in 73% of injections at 150 pg/blastomere. Above 150 pg/blastomere a high incidence of abnormal gastrulation was observed. Note that *Xcer* (embryos on the right) lead to much enlarged cement glands, some of them radial, as described previously (Bouwmeester et al., 1996). (B) Ventro-vegetal injection of 150 pg of murine *cer-l* (four embryos on the left) or of *Xcer* (right) in the same experimental conditions. Note that *cer-l* does not induce ectopic cement glands nor head-like structures, whereas *Xcer* does (arrows). Mouse *cer-l* mRNA was tested in this way in five independent experiments at various stages and concentrations ($n = 271$) and ectopic heads were never observed. The embryos of the top row were injected at eight-cell and those on the bottom row at the 16-cell stage. (C) Expression of the pan-neural marker *Sox-2* in control stage 27 (top, dorsal view) and in an embryo microinjected radially with *cer-l* mRNA; the CNS is expanded. (D) Expression of the anterior neural marker *Otx2* in lateral views of a control stage 19 (top) and a radially *cer-l* injected sibling (bottom). Note the expansion of the prospective forebrain region. (E) Control uninjected animal cap explants showing atypical epidermis differentiation. (F) Animal caps from embryos microinjected with *cer-l* mRNA become strongly polarized with a large pigmented cement gland on one side and pale cells on the other.

moderate dorsalization. In situ hybridization analyses with the *Xenopus* pan-neural marker *Sox-2* (De Robertis et al., 1997) detected a considerable expansion of neural tissue (Fig. 4B) and the anterior marker *Otx2* (Blitz and Cho, 1995; Pannese et al., 1995) detected an increased amount of anterior brain tissue (Fig. 4D).

The phenotypes observed in murine *cer-l* mRNA microinjections in *Xenopus* embryos differ in important aspects from those caused by *Xcer* injections. First, microinjection of *cer-l* into ventral-vegetal blastomeres never induced the small ectopic heads that are characteristic of *Xcer*. Second, ectopic or radial cement glands, a very frequent finding with *Xcer*, were not observed. Third, *cer-l* injected embryos have notochords and somites, whereas *Xcer* blocks mesoderm formation (Bouwmeester et al., 1996).

Xenopus animal cap explants that normally give rise to epidermis are widely used as an assay system for studying cell differentiation induced by growth factors. Animal caps from embryos microinjected with *cer-l* become polarized with the pigment clustered in a large cement gland (Fig. 4E,F). A comparison of the molecular markers induced by mouse *cer-l* or by *Xcer* in animal caps is shown in Fig. 5. Both mRNAs strongly induced the pan-neural markers *N-CAM* and *Sox-2*. The neural tissue was of the anterior type, as indicated by the activation of *Otx2*, the lack of expression of the posterior marker *Hoxb-9* and of *En-2*, a midbrain-hindbrain border marker. Consistent with the external appearance of the caps (Fig. 4F), the cement gland marker *CG-13* was activated. Neuralization took place in the absence of mesoderm induction, as indicated by the lack of expression of *α-actin* and *α-globin* markers. Neural induction by *noggin*, *chordin* and *Xcer* is accompanied by the formation of endoderm in animal caps (Bouwmeester et al., 1996; Sasai et al., 1996) and *cer-l* mRNA also activates the pan-endodermal marker *endodermin* (*Edd*). In initial experiments *cer-l* was found to be a weak inducer of the cardiac homeobox gene marker *Nkx-2.5*, but when the amount of mRNA injected per blastomere was decreased to 75 pg strong induction was observed (Fig. 5B). Thus, in animal cap assays *cer-l* is able to induce the entire set of molecular markers described previously for *Xenopus cerberus*. However, in whole microinjected embryos the two mRNAs generate distinct phenotypes. We conclude that

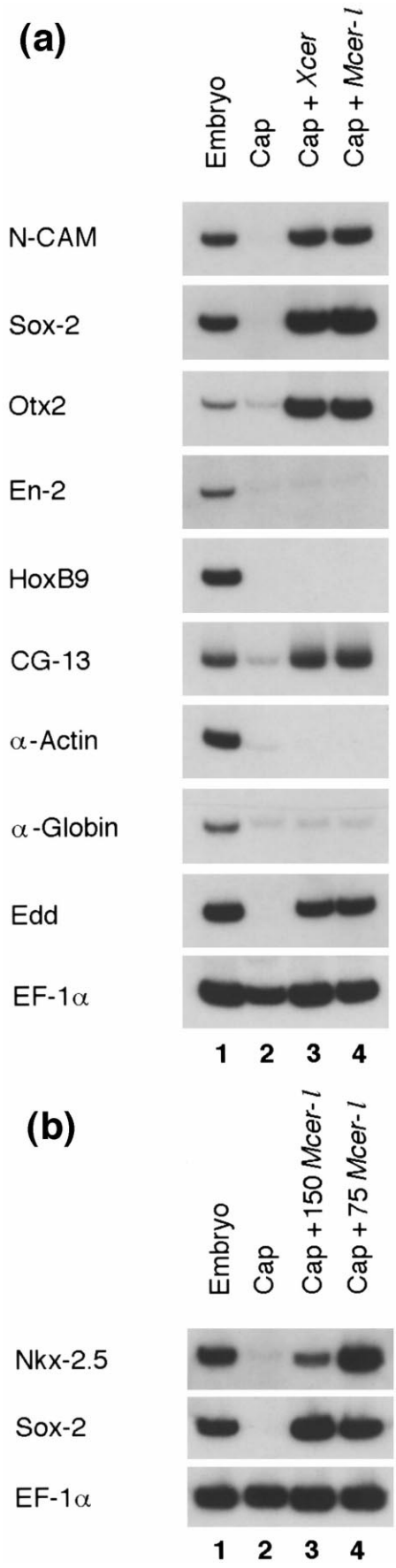


Fig. 5. Molecular markers induced by *Xcer* or *cer-l* in animal cap explants. (A) Molecular analysis by RT-PCR of the induction of neural markers and other gene markers in animal caps injected with 150 pg of *Xcer* or *cer-l*. Lane 1, RNA from whole embryos at stage 27; lane 2, uninjected sibling animal caps; lane 3, animal caps injected with *Xcer* mRNA; lane 4, animal caps injected with mouse *cer-l* mRNA. The various markers assayed (Bouwmeester et al., 1996) are indicated. Both *Xcer* and *cer-l* induce anterior CNS (*Otx2* positive, *En-2* and *Hoxb-9* negative), cement gland (*CG-13*) and endoderm (*Edd*) in the absence of mesoderm (*α-actin, α-globin*). *EF-1α* was used as an RNA loading control. (B) Independent experiment in which animal caps injected with 150 pg (lane 3) were found to be less effective in the induction of Nkx-2.5 than 75 pg/blastomere (lane 4). The neural marker *Sox-2* was strongly induced at both concentrations.

cer-l encodes a neuralizing factor that can lead to the formation of forebrain tissue in *Xenopus* assays.

2.4. Some organizer-specific genes are shared between trunk organizer and anterior visceral endoderm

In the mouse, *Hesx1* and *Otx2* are expressed in anterior endoderm (Ang et al., 1994; Thomas and Beddington, 1996) and therefore represent possible candidates for regulators of *cer-l* transcription. In *Xenopus*, their homologues *XANF1* and *Otx2* are expressed during gastrulation in regions overlapping with *Xcer* in deep endodermal cells (Blitz and Cho, 1995; Pannese et al., 1995; Zaraisky et al., 1995). The expression of organizer-specific genes in *Xenopus* is dynamic and it was noted that at early stages of gastrulation *Xlim1* and *Xgsc* partially overlap with *Xcer* transcripts (Bouwmeester et al., 1996). This prompted us to ask whether in mouse the *cer-l* positive cells in the anterior primitive endoderm domain also expressed homeobox genes associated with the anterior primitive streak.

Using an improved whole-mount in situ hybridization protocol (see Section 4.6) it was found (Fig. 6A,A′) that *Lim1* was expressed in the anterior primitive endoderm, in addition to the previously reported expression in the primitive streak and its derivatives, the mesodermal wings and prechordal plate (Kaufman, 1992; Shawlot and Behringer, 1995). Mouse *gsc* is also expressed in anterior endoderm (Fig. 6B,B′). Although mouse *gsc* was initially reported as being expressed in anterior primitive streak (Blum et al., 1992), hybridization to sectioned embryos had also shown signal in anterior endoderm (Conlon et al., 1994) and this observation was confirmed here by whole-mount in situ analysis. Finally, the winged-helix gene *HNF-3β* was also found to be expressed, in addition to the primitive streak (Ang et al., 1993; Sasaki and Hogan, 1993), in anterior endoderm close to the junction between the embryonic and extraembryonic region where the anterior neural folds will form (Fig. 6C,C′).

We conclude from these studies that the mouse expression of transcription factor genes, some of which have been implicated in anterior neural specification, is shared by the primitive streak organizer and the anterior visceral endoderm. Mutations in *Lim1* and *HNF-3β* can lead to severe anterior truncations in the neural plate (Ang and Rossant, 1994; Weinstein et al., 1994; Shawlot and Behringer, 1995) and the present observations raise the possibility that these defects could be due to the loss of function of these genes in the anterior endoderm domain rather than in the node/prechordal plate domain.

3. Discussion

Cer-l is a secreted molecule expressed in anterior endoderm from pre-streak to head-fold stages during mouse development. In *Xenopus* animal cap assays, microinjection of *cer-l* mRNA leads to the formation of anterior neural tissue and to the activation of endodermal and heart markers. As this secreted factor is expressed at the right time and in tissue directly underlying the future anterior CNS, Cer-l is a good candidate to mediate possible neural inductive signals emanating from the anterior endoderm.

3.1. Is cer-l the homologue of Xcer?

Cer-l is 58% identical in amino acid sequence to Xcer in the cysteine-rich region and 31% over the entire protein. Although both proteins are clearly members of the same family and share common activities, significant differences exist. First, homologous growth factors from *Xenopus* and mouse tend to share higher conservation. For example, *Xenopus* and mouse BMP-4 are 96% identical in the mature growth factor region. Second, *cer-l* is expressed in mouse notochordal and prechordal plate, as well as in forming somites, but not in the corresponding *Xenopus* structures (Bouwmeester et al., 1996). Third, the most striking activity of *Xcer* is its ability to induce ectopic heads in ventral-vegetal microinjections, whereas *cer-l* does not have this activity. Instead, injection of mouse *cer-l* mRNA into *Xenopus* embryos results in dorso-anteriorized embryos with excessive CNS, enlarged heads expressing *Otx2* and shortened axes (Fig. 4).

Despite these differences, mouse *cer-l* and *Xcer* share similarities in function (such as neural and endodermal induction) and expression patterns. The leading edge of the involuting endoderm in *Xenopus* is considered to be the equivalent of the anterior primitive endoderm of the mouse (Thomas and Beddington, 1996; Bouwmeester and Leyns, 1997). In addition, in adult tissues of *Xenopus* and mouse the *Xcer* and *cer-l* genes were found to be expressed at high levels only in heart (data not shown). We have screened mouse libraries at low stringency with *Xcer* probes and have failed so far to isolate a murine homologue. Thus, there are arguments for and against *cer-l* and *Xcer* being homologues of the same gene. One could imagine that a mouse secreted product could work substandardly in *Xenopus* embryo assays. The expression of *cer-l* in notochord and prechordal plate would be more difficult to explain if the genes were true homologues. Although in our view the evidence to date suggests that *cer-l* and *Xcer* are not the murine and frog homologues of the same gene, definitive proof will require isolating the true mouse *cerberus* or the *Xenopus cerberus-like* genes.

3.2. Anterior visceral endoderm as a signaling center

During mouse gastrulation, the anteroposterior axis becomes morphologically evident when formation of the primitive streak starts (at about day 6.5) at the posterior end. Recently, an antigen defined by the visceral endoderm-1 (VE-1) antibody was found to mark the anterior

endoderm in pre-streak embryos (Rosenquist and Martin, 1995). This indicates that specification of the anterior region of the embryo starts before any anatomical asymmetries (i.e. a primitive streak in the posterior) are present. *Cer-l* is expressed in pre-streak embryos at day 5.5 in an asymmetric fashion reminiscent of AgVE-1. During early streak stages the expression patterns of *cer-l*, AgVE-1 and the homeobox marker *Hesx1/Rpx* (Hermesz et al., 1996; Thomas and Beddington, 1996) overlap in anterior visceral endoderm.

Several emerging lines of evidence suggest that anterior visceral endoderm plays an important role in patterning the anterior region of the embryo. As previously mentioned, the nodal growth factor is required in the primitive endoderm for proper head formation (Varlet et al., 1997). Furthermore, extirpation experiments have shown that removal of anterior visceral endoderm leads to the reduction of expression of *Hesx1* in the overlying forebrain (Thomas and Beddington, 1996), suggesting that a signal from the endoderm is required for forebrain patterning. Additional evidence for the existence of an endodermal signal comes from experiments in which *Otx2* was mutated. At the mid streak stage *Otx2* is normally transcribed both in anterior endoderm and in the overlying epiblast. In $Otx2^{-/-}$ embryos transcription of the locus, marked by a *lacZ* insertion, is present in the visceral endoderm but is lost from the overlying epiblast (Acampora et al., 1995). This suggests that the Otx2 homeodomain protein is required in visceral endoderm for the transmission of a secreted signal to the neurectoderm. The Cer-l factor is a candidate for such a signal, in view of its expression pattern in the mouse and its ability to induce *Otx2* in *Xenopus* ectodermal explants.

Mutation of *Lim1* leads to headless embryos (Shawlot and Behringer, 1995). It has been proposed that the lack of head organizer activity could be due to *Lim1* expression in prechordal plate and/or mesodermal wings. However, as shown in Fig. 6A, at early gastrula *Lim1* is clearly expressed in anterior primitive endoderm. It is possible that this early anterior expression domain plays a role in head specification, perhaps through the transcriptional activation of genes encoding secreted factors such as Cer-l. Expression in anterior endoderm was also noted for *gsc*, another homeobox gene expressed in the primitive streak organizer. Knockout of *gsc* does not cause gastrulation phenotypes (Rivera-Pérez et al., 1995; Yamada et al., 1995), although more recent analyses have identified deletions in the midline of the presphenoid cranial bone (J.A. Belo et al., in preparation). *HNF-3β* is expressed in the node and its derivatives (prechordal plate, notochord and definitive endoderm) and when mutated results in embryos lacking a node. Although a differentiated node is absent in $HNF\text{-}3\beta^{-/-}$ embryos, it is clear that a CNS can be formed, indicating that perhaps the source of neural induction might not be the trunk organizer (Ang and Rossant, 1994). Some of the $HNF\text{-}3\beta^{-/-}$ embryos have stronger phenotypes with anterior brain truncations (Ang and Rossant, 1994; Weinstein et al., 1994). These truncations could conceivably result from the lack of *HNF-3β* expression in the anterior primitive endoderm domain identified in Fig. 6C. The various lines of evidence from extirpation, chimeric and knockout experiments discussed above, as well as the expression pattern of the secreted factor Cer-l, are consistent with the view that the head organizer may reside in anterior visceral endoderm.

As illustrated in Fig. 7, the mouse mid-gastrula appears to have two well separated signaling centers, the anterior visceral endoderm and the trunk organizer. The anterior primitive streak when grafted to an ectopic site is able to induce trunk structures and expresses genes such as *gsc*, *Lim1* and *HNF-3β*. Grafting experiments of the anterior visceral endoderm have not been carried out and therefore the organizing activity of this tissue remains to be assessed. However, this tissue is the site of the first anteroposterior asymmetry in the egg cylinder, marked by AgVE-1, and where homeobox genes such as *Hesx1* and *Otx2* and the secreted factor Cer-l are expressed.

One intriguing aspect of this division into separate anterior and posterior centers in the mouse gastrula is that some organizer-specific genes (*Lim1*, *gsc* and *HNF-3β*) are shared by both. In *Xenopus*, the leading edge endodermal cells that express *cerberus* are initially located in the vicinity of the trunk organizer located in the involuting dorsal lip and at early stages the expression of *Xgsc* and *Xlim1* spans both domains. In addition, the expression of *Xcer* is regulated by similar mechanisms to those that control trunk formation, as indicated by UV irradiation and LiCl treatment of early embryos (Bouwmeester et al., 1996). In the mouse, although the two proposed signaling regions are separate at the gastrula stage, as indicated in Fig. 7, it cannot be excluded that both centers might lie closer to each other at earlier stages, sharing common regulatory systems that lead to the expression of *Lim1*, *gsc* and *HNF-3β*.

In conclusion, the *cerberus-like* gene is expressed in anterior primitive endoderm before and during gastrulation. Once the primitive streak reaches the tip of the egg cylinder, *cer-l* transcripts are also found in definitive endoderm and axial mesoderm derived from the node. In microinjected *Xenopus* animal caps *cer-l* has a neuralizing function, inducing anterior CNS and in particular *Otx2* expression. Given its expression pattern in the early gastrula, in endoderm directly underlying the future head neurectoderm, this secreted factor could mediate in part the proposed inductive activities of anterior visceral endoderm. Determining whether *cer-l* has an essential role in the induction of rostral brain must await its inactivation by gene targeting.

4. Experimental procedures

4.1. Screening of libraries and databases

By searching the EST database for similarities with the amino acid sequence of *Xcer*, EST AA120122 was identified. This clone, released by the WashU-HHMI mouse EST

project, was derived from the Beddington 7.5 day mouse embryonic region library. This partial cDNA of 1.6 kb was sequenced on both strands and found to lack 5′ coding sequences. To obtain the missing 5′ region, the Beddington 7.5 day mouse embryonic region library (Harrison et al., 1995) was screened using the EST clone as a probe. Hybridizations under stringent conditions allowed the identification of 76 clones, but after restriction digests all of them were shorter than the original EST clone. This probe was used to screen a genomic mouse library generated in Lambda Fix II (Stratagene). Hybridizations under stringent conditions allowed the isolation of five independent clones. After Southern analysis and subcloning in pBluescript II SK, the genomic sequence of this gene was determined, revealing that it has two exons separated by a 2.0 kb intron. This sequence was confirmed by the cloning of a full-length cDNA clone obtained by screening a mouse ES cell library. Sequencing was performed with the Dye Terminator Kit

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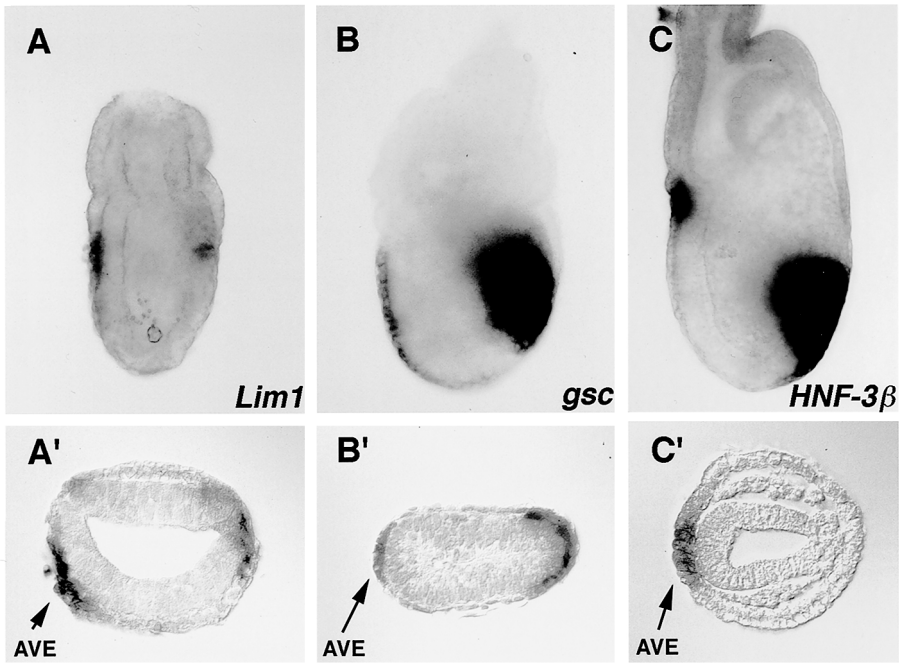

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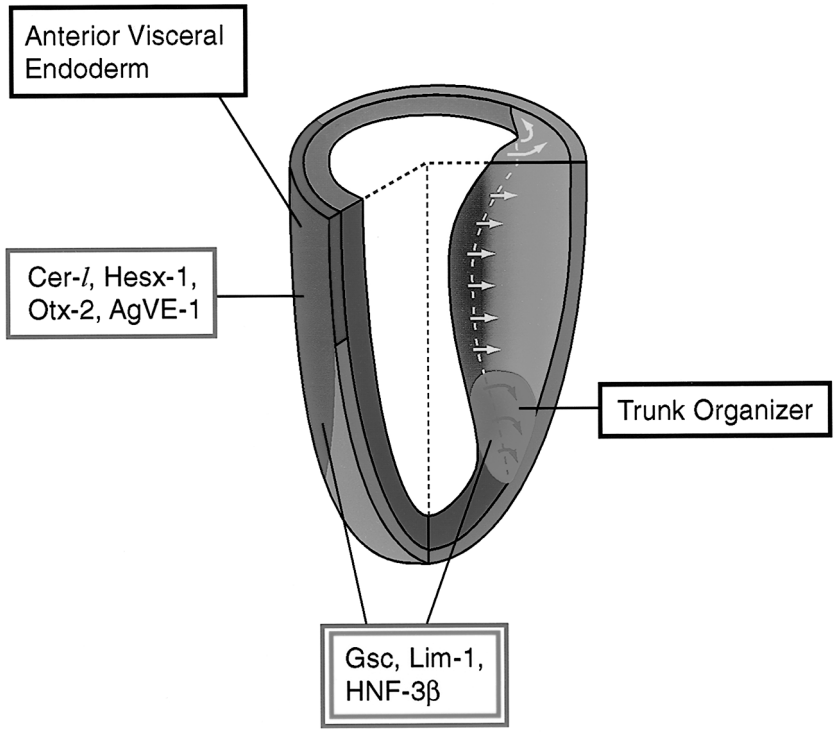

(ABI) on a 377 DNA Sequencer (ABI) and analyzed by the Wisconsin GCG program. The GeneBank accession number for mouse *cer-l* is AF012244.

4.2. Expression constructs

The full-length cDNA was cloned in the *Eco*RI/*Not*I sites of the pBluescript II SK vector, generating pBS-*cer-l*. The coding region for *cer-l* was subcloned using *Eco*RI and *Xba*I into the CMV promoter driven eukaryotic expression vector pCS2 (a gift of R. Rupp), generating the pCS2-*cer-l* expression vector used for *Xenopus* injections and mammalian cell transfections. Capped synthetic mRNA was produced and microinjected as described (Bouwmeester et al., 1996). To obtain *cer-l* and *Xcer* sense mRNA, pCS2-*cer-l* and pCS2-X*cer* were linearized with *Not*I and transcribed with SP6 RNA polymerase using the Ambion Message Machine Kit. All RNAs produced were quantified by gel electrophoresis and spectrophotometry.

4.3. Embryo preparation

Xenopus embryos were obtained and microinjected as described (Bouwmeester et al., 1996). Mouse embryos were obtained from intercrosses of B6SJL/F1 hybrids (Jackson Labs) maintained on a 1900 h to 0500 h dark cycle and mated overnight. Noon on the day of finding the vaginal plug was designated day 0.5. Embryos were dissected from the uterus in PBS and further staged by morphological landmarks (Downs and Davies, 1993).

4.4. Cell culture

Embryonic human kidney cells (293T) were cultured in DMEM containing 10% fetal calf serum. Transfection with pCS2-*cer-l* was carried out overnight at 50–70% confluency by standard calcium phosphate technique. On the next morning, the medium was replaced by fresh one. For labeling, on the second day the cells were starved in medium free of methionine and serum for 2 h, incubated with [^{35}S]methionine (Amersham; >1.000 Ci/mmol) in methionine free medium at 100 μCi/ml for 8 h and conditioned medium was collected. A similar series of experiments was also carried out in COS cells (data not shown); Cer-l was also secreted into the culture medium and migrated as a broad band of 38–44 kDa in reducing gels.

4.5. RT-PCR analysis and in situ probes

Synthetic RNA transcribed from pCS2-*cer-l* or pCS2-*Xcer* was injected into each of the animal blastomeres of eight-cell embryos. Animal caps were explanted at stage 9–10 and grown in culture until siblings reached stage 27. Conditions and primers for RT-PCR were as described (Bouwmeester et al., 1996).

To generate the antisense *cer-l* probe the EST clone was linearized with *Eco*RI and used as a template for in vitro transcription with T7 RNA polymerase. The pBS-*Lim1* plasmid was digested with *Hind*III and transcribed with T7 RNA polymerase. The *gsc* probe was obtained by linearizing pBS-*gsc* full-length cDNA with *Sac*I and transcription with T3 RNA polymerase. After linearization with *Asp*700, pBS-*HNF-3β* was transcribed with T7 RNA polymerase, generating the *HNF-3β* antisense probe. The *Xenopus Otx2* riboprobe was synthesized digesting p*XOTX3.1* plasmid with *Not*I, followed by transcription with T7 RNA polymerase. The plasmid pBS-*Sox-2* was digested with *Eco*RI and transcribed with T7 RNA polymerase, generating the *Xenopus Sox-2* antisense probe.

4.6. In situ hybridization

A sensitive mouse whole-mount in situ protocol was modified from the *Xenopus* protocol of Harland (1991). The main modifications are listed below. The RNA probes were spun through Quick Spin Columns (Boehringer Mannheim) and stored at −70°C. All solutions were treated with DEPC and filtered. The hybridization vials and caps were treated with RNAZap (Ambion). All steps before pre-hybridization were performed on ice except the proteinase K treatment. Embryos were fixed in 4% paraformaldehyde/PBSw (PBS + 0.1% Tween-20) at 4°C overnight. The embryos were treated with proteinase K/PBSw (4.5 μg/ml) at room temperature (3 min for day 6 embryos, 5 min for day 7.5 embryos, 7 min for day 9.5 embryos, 9 min for day 10.5 embryos or 11 min for day 11.5 embryos). Diges-

Fig. 6. Transcription factors associated with the trunk organizer are also expressed in anterior visceral endoderm. (A) Early streak stage embryo showing *Lim1* expression in the anterior visceral endoderm in addition to the previously known expression domain in the primitive streak; (A′) transverse histological section of the embryo shown in (A). (B) Expression of the homeobox gene *gsc* in a mid-streak embryo; (B′) section showing *gsc* positive cells both in the anterior visceral endoderm and in the primitive streak. (C) *HNF-3β* can be seen in an anterior visceral endoderm domain close to the embryonic-extraembryonic junction, in addition to the previously described primitive streak expression domain; (C′) transversal section at the level of the anterior visceral endoderm domain of expression of *HNF-3β*. AVE, anterior visceral endoderm.

Fig. 7. Diagram indicating that the mouse mid-gastrula appears to have two distinct signaling centers, i.e. the anterior primitive streak, or trunk organizer, and the anterior visceral endoderm, which has been suggested to function in head induction. The homeobox genes *Hesx1* and *Otx2* are expressed in anterior primitive endoderm at gastrula. At this stage, the anterior primitive streak genes *gsc*, *Lim1* and *HNF-3β* have been found to be also expressed in the anterior visceral endoderm. It is thought that organizer-specific homeobox genes activate the expression of secreted signaling molecules. Mouse *cerberus-like* is the only secreted molecule known so far to be specifically expressed in the anterior primitive endoderm signaling center. *cer-l* transcripts are found directly underlying the prospective rostral brain and have neuralizing properties. Thus, mouse *cerberus-like* is a candidate to mediate the proposed role of the anterior primitive endoderm in the induction of the head.

tion was stopped by washing in freshly prepared 2 mg/ml glycine/PBSw. The embryos were re-fixed in 4% paraformaldehyde–0.2% glutaraldehyde/PBSw for 15 min, pre-hybridized (in 50% formamide, 5× SSC (pH 7.0), 1% Boehringer Mannheim blocking reagent, 100 μg/ml heparin, 0.1% Tween-20, 1 mg/ml Torula tRNA, 0.1% CHAPS and 5 mM EDTA) at 65°C for 3 h. The embryos were hybridized at 70°C overnight in probe solution (pre-hybridization solution containing 200 ng/ml of heat denatured probes). The embryos were washed in pre-hybridization solution, then in a 1:1 dilution of pre-hybridization buffer and 2× SSC (pH 4.5) at 70°C, then in 2× SSC (pH 7.0)/0.1% CHAPS at 70°C and finally in maleic acid buffer (MAB; 100 mM maleic acid, 150 mM NaCl (pH 7.5)) at 70°C (30 min each) and washed in PBSw at room temperature. The embryos were then incubated in blocking solution (10% heat inactivated goat serum, 1% Boehringer Mannheim blocking reagent/PBSw) for 2 h at 4°C. The antibody conjugate was pre-adsorbed in blocking solution at a dilution of 1:10 000 for 2 h at 4°C. Embryos were washed five times in 0.1% BSA/PBSw (45 min each) and in PBSw (2 × 30 min), changed to alkaline phosphatase buffer twice for 10 min at room temperature and stained in BM purple substrate (Boehringer Mannheim) at 4°C in the dark (a few hours to several days). A more detailed protocol can be found on the World Wide Web (http://stratus.lifesci.ucla.edu/hhmi/derobertis/).

After staining, some embryos were re-fixed in 4% paraformaldehyde and embedded in Paraplast (Fisher). After sectioning at 7 or 10 μm, some slides were counterstained with Contrast Red (Kirkegaard and Perry Labs) and mounted in Permount (Fisher).

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