Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 6198–6203, May 1998
Developmental Biology

Expression of the mouse *cerberus*-related gene, *Cerr1*, suggests a role in anterior neural induction and somitogenesis

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Communicated by R. L. Brinster, University of Pennsylvania, Philadelphia, PA, March 27, 1998 (received for review January 26, 1998)

ABSTRACT **The *Xenopus cerberus* gene encodes a secreted factor that is expressed in the anterior endomesoderm of gastrula stage embryos and can induce the formation of ectopic heads when its mRNA is injected into *Xenopus* embryos [Bouwmeester, T., Kim, S., Lu, B. & De Robertis, E. M. (1996) *Nature (London)* 382, 595–601]. Here we describe the existence of a *cerberus*-related gene, *Cerr1*, in the mouse. *Cerr1* encodes a putative secreted protein that is 48% identical to *cerberus* over a 110-amino acid region. Analysis of a mouse interspecific backcross panel demonstrated that *Cerr1* mapped to the central portion of mouse chromosome 4. In early gastrula stage mouse embryos, *Cerr1* is expressed in the anterior visceral endoderm and in the anterior definitive endoderm. In somite stage embryos, *Cerr1* expression is restricted to the most recently formed somites and in the anterior presomitic mesoderm. Germ layer explant recombination assays demonstrated that *Cerr1*-expressing somitic-presomitic mesoderm, but not older *Cerr1*-nonexpressing somitic mesoderm, was able to mimic the anterior neuralizing ability of anterior mesendoderm and maintain *Otx2* expression in competent ectoderm. In most *Lim1*−/− headless embryos, *Cerr1* expression in the anterior endoderm was weak or absent. These results suggest that *Cerr1* may play a role in anterior neural induction and somite formation during mouse development.**

Gastrulation in vertebrate embryos is accompanied by an anterior migration of mesendoderm tissue from the organizer. The organizer is a region of the gastrula embryo that can induce the formation of a second neural axis when transplanted to an undifferentiated region of a host gastrula stage embryo (1). During its migration, the anterior mesendoderm is thought to induce the overlying ectoderm to develop as anterior neural tissue. Subsequently, the posterior mesoderm that forms later in gastrulation is thought to transform a portion of the induced neural tissue into posterior neural tissue resulting in the anterior-posterior patterning of the central nervous system (2).

Classical experiments by Mangold (3) and Eyal-Giladi (4) support the hypothesis that the anterior mesendoderm induces the overlying ectoderm to form anterior neural tissue. Mangold transplanted different regions of dorsal mesendoderm by using the Einsteck method to the blastocoel cavity of host newt embryos. He found that the anterior dorsal mesendoderm induced head-specific structures whereas more posterior dorsal mesendoderm induced posterior brain structures. Eyal-Giladi (4) determined the specification states of the overlying ectoderm at different times during gastrulation in urodele embryos. She found that the first type of tissue to be induced after the initial involution of the dorsal mesendoderm was forebrain tissue in the posterior ectoderm. As the dorsal mesendoderm migrated further anteriorly, the anterior ectoderm was induced to become forebrain tissue and the posterior ectoderm now developed as posterior neurectoderm.

More recent experiments in *Xenopus* and mouse provide molecular support for the role of anterior mesendoderm in anterior neural development. In *Xenopus*, if anterior notochord tissue is recombined with ectoderm, it can strongly induce the expression of the midbrain marker *engrailed* (*en-2*) whereas posterior notochord tissue induces the expression of *en-2* to a lesser extent (5). In the mouse, germ layer recombination explant studies demonstrate that anterior mesendoderm, but not posterior mesendoderm, can induce the expression of the anterior neural marker genes *Engrailed-1* (*En-1*) and *Engrailed-2* (*En-2*) (6) and maintain and induce the expression of the forebrain-midbrain marker gene *Otx2* (7). These explant studies also demonstrate that the ectoderm becomes committed to form anterior neural tissue by the mid-streak stage that coincides with the anterior migration of the mesendoderm. Additional support for the involvement of anterior mesendoderm in anterior neural induction comes from mouse knockout studies. *Lim1* and *Otx2* are homeobox genes that are expressed in the anterior mesendoderm of gastrula stage mouse embryos (7–9). Both *Lim1*- and *Otx2*-deficient embryos were found to lack anterior head structures rostral to rhombomere 3 in the hindbrain (9–12). Analysis of early gastrulation stage mutant embryos using molecular markers suggests that the development of the anterior mesendoderm tissues is altered in both *Lim1* and *Otx2* mutants.

A number of genes encoding secreted factors have been identified in *Xenopus* that are expressed in the organizer and the anterior mesendoderm. These include *noggin*, *follistatin*, and *chordin*. (13–15). When mRNAs for these genes are injected into *Xenopus* embryos they are able to induce neural tissue development and axis formation. Both the noggin and chordin proteins have been shown to bind BMP4 and it is believed that their ability to induce neural development results from their ability to suppress BMP signaling (16, 17). Another gene encoding a secreted factor expressed in the organizer is *cerberus* (18). *cerberus* is expressed in the anterior endomesoderm of gastrula stage embryos and its expression is reported to overlap extensively with *Xlim-1* and *goosecoid* early in gastrulation. *cerberus* differs from *noggin*, *follistatin*, and *chordin* in that injection of *cerberus* mRNA into *Xenopus* embryos suppresses the formation of posterior mesoderm and specifically induces the formation of ectopic head structures. These results suggest that *cerberus* may be a component of the head induction pathway in vertebrates.

In an effort to identify genes that may be downstream of *Lim1* in the head induction pathway in the mouse, we have searched for a mouse homolog of *cerberus*. Here we report the existence of a mouse *cerberus*-related gene that we have designated *Cerr1*. *Cerr1* is expressed in the anterior visceral and definitive endoderm of early gastrulation stage mouse embryos and later in development it is expressed in the two most newly formed somites and in the

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Abbreviations: E, embryonic day; EST, expressed sequence tag.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF031896).

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anterior presomitic mesoderm. Because anterior mesendoderm, which expresses *Cerr1*, has previously been shown to have anterior neuralizing ability (6, 7), we tested whether *Cerr1*-expressing somitic-presomitic mesoderm might also have anterior neuralizing ability. We show that *Cerr1*-expressing somitic-presomitic mesoderm, but not *Cerr1*-nonexpressing somitic mesoderm, can maintain the expression of the anterior neural marker gene *Otx2* in ectoderm that is competent to respond to anterior neuralizing signals. In addition, we find that *Cerr1* expression in early gastrula stage *Lim1*−/− headless embryos is weak or absent in most mutant embryos. These results suggest that *Cerr1* may be involved in the induction or maintenance of anterior neural fates and somite formation during mouse embryogenesis.

MATERIALS AND METHODS

Isolation of Mouse Cerr1 cDNA Clones. A mouse *cerberus*-related cDNA sequence was identified from the dbEST database at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/dbEST/index.html) by using the TBLASTN program (19). The expressed sequence tag (EST) clone 538769, was obtained from Genome Systems (St. Louis). To obtain additional 5′ cDNA sequence we performed 5′ rapid amplification of cDNA ends PCR using embryonic day (E) 10.5 total RNA. The reverse transcription and amplification reactions were performed by using a commercial kit according to the manufacturer's instructions (GIBCO/BRL). The reverse transcription primer used was 5′-CCGATGCCAGAACCTCTTGG-3′. The PCR amplification primer used was 5′-CTTCCTCCTGTAGAGGTGAT-3′. The PCR amplification conditions used were 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 55°C for 40 sec, 72°C for 1 min, followed by 72°C for 10 minutes. A PCR product of ≈500 bp was amplified and subcloned into Bluescript II KS$^-$ (Stratagene) and sequenced by the DNA Sequencing Core at the M. D. Anderson Cancer Center. Additional cDNA clones were identified by screening a mouse E7.5 cDNA library (provided by Akihiko Shimono, M. D. Anderson Cancer Center) with a 0.45-kb *Sal*I-*Pst*I fragment from the EST clone.

Chromosomal Mapping. The chromosomal location of *Cerr1* was determined by Southern hybridization to a mouse interspecific mapping panel obtained from The Jackson Laboratory. The mapping panel was composed of genomic DNA from 94 backcross progeny from an interspecific cross between (C57BL/6J × SPRE/Ei) F1 hybrid female and SPRET/Ei male mice (20). Using a 0.45-kb *Sal*I-*Pst*I DNA fragment from the EST clone as a probe, a *Sst*I restriction fragment length polymorphism was identified between C57BL/6J and SPRET/Ei genomic DNA. A Southern blot membrane containing DNA samples from the backcross progeny digested with *Sst*I provided by Yuanhao Li (21) was then hybridized with the 0.45-kb *Sal*I-*Pst*I DNA probe. The restriction fragment length polymorphism distribution pattern of *Cerr1* in the backcross progeny was submitted to The Jackson Laboratory for analysis.

Whole Mount *in Situ* Hybridization. Whole mount *in situ* hybridization reactions were performed essentially as described by Wilkinson (22). Single-stranded RNA probes were labeled with digoxigenin-UTP according to the manufacturer's instructions (Boehringer Mannheim). Preparation of whole mount embryos for paraffin embedding and sectioning was performed as described by Sasaki and Hogan (23).

Germ Layer Explant Recombination Assays. Explant and recombination assays were performed essentially as described by Ang and Rossant (6). Ectoderm was isolated from the distal tip region of early primitive streak stage embryos using glass capillary needles. Tissue fragments containing somitic and presomitic mesoderm were isolated from E9.5 embryos with the aid of tungsten needles (0.5 mm, Goodfellow, Cambridge, U.K.). The tissues layers were enzymatically separated by incubation in 0.5% trypsin, 0.25% pancreatin in phosphate buffered saline for 5 min at 4°C. The enzymatic reaction was stopped by transferring the fragments into DMEM containing 15% fetal calf serum. The mesodermal and ectoderm components were cultured alone or together in a 25 μl drop of DMEM supplemented with 15% fetal calf serum, 2 mM glutamine and 0.1 mM 2-mercaptoethanol for 30 hr. The explants were then fixed in 4% paraformaldehyde for 1 hr at 4°C. Whole mount RNA *in situ* hybridization was performed as described above.

RESULTS

Identification of a Mouse *cerberus*-Related Gene. We searched the dbEST database at the NCBI for genes related to *cerberus*. One mouse EST clone, no. 538769, from the Beddington E7.5 mouse cDNA library, was identified that shared statistically significant homology with *cerberus* at the amino acid level. We have named this cDNA clone *Cerr1*, for *cerberus-related gene 1*. The *Cerr1* EST clone is ≈1.8 kb in size. DNA sequencing of the EST clone indicated that it contained a Poly(A) tail but lacked the amino terminal coding region. 5′ rapid amplification of cDNA ends PCR was used to obtain ≈200 bp of additional 5′ sequence that contained a consensus translation initiation start site (24). Additional cDNA clones were identified by screening a mouse E7.5 cDNA library with a 0.45 *Pst*I-*Sal*I DNA fragment from the EST clone. Ten clones were plaque purified and subcloned. The cDNA clones ranged in size from ≈1.9 to 3.5 kb. Sequencing of portions of the cDNA clones revealed that the clones shared the same coding region but the length of the 3′ noncoding region was different between the clones. Translation of the ORF indicated that *Cerr1* can encode a protein of 272 amino acids. The composite DNA sequence of the EST clone and the 5′ rapid amplification of cDNA ends product has been deposited in GenBank database. Hybridization of a mouse genomic Southern blot with a *Xenopus cerberus* DNA probe encompassing the region conserved between *cerberus* and *Cerr1* (see below) at moderate stringency (final wash = 2× standard saline citrate at 55°; 1× standard saline citrate = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) failed to reveal evidence for additional *cerberus* family members.

Alignment of the predicted mouse Cerr1 protein sequence with *Xenopus* cerberus revealed a 110-amino acid region of overlap that is 48% similar (Fig. 1). Within this region the predicted proteins share a conserved spacing of nine cysteine residues. Outside this region, the two predicted proteins do not

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X-cer   MLLNVLRICI  IVCLVNDGAG  KHSEGRERTK  TYSLNSRGYF
                       ||    |  |             |   |
Cerr1   ~~~~~MHLLL  VQLLVLLPLG  KADLCVDGCQ  ..SQGSLSFP

X-cer   RKERGARRSK  ILLVNTKGLD  EPHIGHGDFG  LV.AELFDST
          ||| ||              |  |           |    |
Cerr1   LLERG.RRDL  HVANHEEAED  KPDLFVAVPH  LMGTSLAGEG

X-cer   RTHTNRKEPD  MNKVKLFSTV  AHGNKSARRK  AYNGSRRNIF
                                                  |
Cerr1   QRQRGKMLSR  LGRFWKKPET  EFYPPRDVES  DHVSSGMQAV

X-cer   SRRSFDKRNT  EVTEKPGAKM  FWNNFLVKMN  GAPQNTSHGS
                       ||       ||  |        | |
Cerr1   TQPADGRKVE  RSPLQEEAKR  FWHRFMFRKG  PAFQGVILPI

X-cer   KAQEIMKEAC  KTLPFTQNIV  HENCDRMVIQ  NNLCFGKCIS
        |  |    | |   | || | |  || |    | |  |||||||| |
Cerr1   KSHEVHWETC  RTVPFNQTIA  HEDCQKVVVQ  NNLCFGKCSS

X-cer   LHVPNQ.QDR  RNTCSHCLPS  KFTLNHLTLN  CTGSKNVVKV
            |    |     ||||  |  |||  ||  ||  ||    |||
Cerr1   IRFPGEGADA  HSFCSHCSPT  KFTTVHLMLN  CTSPTPVVKM

X-cer   VMMVEECTCE  AHKSNFHQTA  QFNMDTSTTL  HH*
        || ||||  |
Cerr1   VMQVEECQCM  VKTERGEERL  LLAGSQGSFI  PGLPASKTNP*
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FIG. 1. Comparison of *Xenopus* cerberus and mouse Cerr1 predicted amino acid sequences. The nine conserved cysteine residues in Cerr1 are underlined. Dots (·) indicate spaces introduced for optimal alignment. The stop codons are designated by asterisks (*). Sequences were aligned by the PILEUP program.

share extended regions of identity. A search of the PROSITE Dictionary of Protein Sites and Patterns, distributed by the European Molecular Biology Laboratory, failed to reveal any sequence motifs. Two potential glycosylation sites are present at amino acid positions 168 and 222 with the latter site being conserved with *cerberus*. The amino terminus of the predicted Cerr1 protein contains a hydrophobic region that may serve as a signal peptide suggesting that Cerr1 is a secreted protein.

Chromosomal Location of *Cerr1*. The chromosomal location of *Cerr1* was determined by Southern hybridization to a mouse interspecific mapping panel obtained from The Jackson Laboratory. The interspecific mapping panel is made up 94 DNA samples from the progeny of matings between female (C57BL/6J) and male SPRET/Ei mice (20). Southern blots of *Sst*I-digested DNA of the 94 backcross progeny were hybridized with the 0.45-kb *Sal*I-*Pst*I fragment from the EST clone. *Sst*I fragments of ≈6 and 3.2 kb were used to follow the inheritance of the *Cerr*1 allele from C57BL/6J and SPRET/Ei, respectively. The haplotypes of the backcross panel were compared with other previously typed markers (Fig. 2*A*). One mouse with a double crossover event in the region where *Cerr1* mapped was excluded from the haplotype count. The mapping results indicated that *Cerr1* cosegregated with a number of other loci including *interferon-alpha 1* (*Ifna1*) and *D4Mit26* on the central portion of mouse chromosome 4 (Fig. 2*B*). A classical mouse mutation called *Pintail* (*Pt*) also maps approximately to this region (Fig. 2*C*) (25). *Pt* mice have tails of variable length with a thin threadlike tip (26). The defect is thought to be caused by an abnormal rate of cell division in the notochord of E10.5 embryos (27). As *Cerr1* is not expressed in the notochord during development (see below) it is unlikely to be mutated in *Pt* mice. Based on the chromosomal location of the human *interferon alpha* genes (28), the predicted human syntenic region is chromosome 9p22.

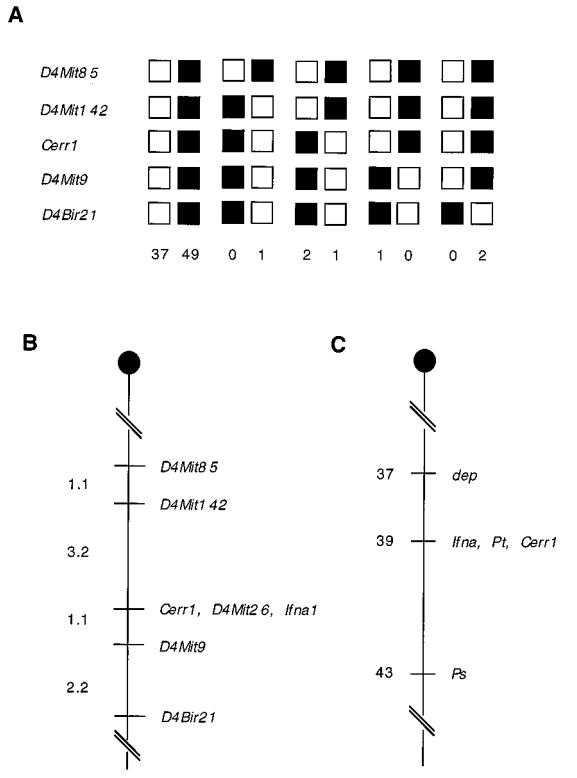

FIG. 2. Mouse *Cerr1* mapped to the central portion of chromosome 4 by interspecific backcross analysis. (*A*) Haplotype analysis of backcross data. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × SPRET/Ei) F1 mother. ■, The C57BL/6J allele; □, the SPRET/Ei allele. The number of offspring that inherited each type of chromosome are listed at the bottom. (*B*) Likely gene order, with recombination distances between loci shown to the left of the chromosome. (*C*) Location of *Cerr1* relative to the *interferon alpha* complex genes and several mouse mutations on chromosome 4.

Expression Pattern of Cerr1 during Early Development. To determine the pattern of expression of *Cerr1* during early mouse embryogenesis, whole mount *in situ* hybridization was performed on E6.5 through E9.5 embryos. At E6.5, in early and mid-streak stage embryos, *Cerr1* was expressed in the anterior visceral endoderm. The *Cerr1* expression domain extended from the extraembryonic–embryonic junction to approximately two thirds of the way down the epiblast (Fig. 3*A*). This pattern of expression is similar to that of a number of other anteriorly expressed genes including *Otx2* (7), *Lim1* (9), *Rpx/Hesx1* (29, 30), *nodal* (31), *goosecoid*, and *HNF3β* (32). By the mid-streak to late streak stage, *Cerr1* continued to be expressed in the anterior visceral endoderm but was also expressed in the definitive endoderm emanating from the anterior portion of the primitive streak (Fig. 3*B*). By the neural plate stage at E7.5, *Cerr1* expression was present throughout the anterior definitive endoderm layer including both the midline and anterior lateral endoderm but *Cerr1* was not expressed in the anterior-most lateral region that appears to correspond to the presumptive cardiac region (33, 34) (Fig. 3 *C–E*). At the early headfold stage, *Cerr1* expression was reduced in the anterior lateral region and expression was seen primarily in the foregut endoderm (Fig. 3*F*). In early E8.5 embryos, *Cerr1* expression was restricted to the two most recently formed somites (Fig. 3*G*). Expression elsewhere in the embryo was not detected. In E9 and E9.5 embryos, *Cerr1* continued to be expressed in the two newest formed somites and also in the anterior presomitic mesoderm (Fig. 3*H*). These results indicate that *Cerr1* is expressed in the anterior visceral and definitive endoderm and in the forming somitic mesoderm during early mouse embryogenesis.

Germ Layer Recombination Using *Cerr1*-Expressing Somitic-Presomitic Mesoderm. Germ layer recombination explant experiments demonstrate that anterior mesendoderm can induce and maintain the expression of anterior neural markers (6, 7). Because anterior mesendoderm expresses *Cerr1*, we asked whether *Cerr1* expressing somitic-presomitic mesoderm might also have anterior neuralizing properties. To determine whether *Cerr1*-expressing somitic-presomitic mesoderm has anterior neuralizing properties, germ layer recombination explants were performed by using *Cerr1*-expressing somitic-presomitic mesoderm from E9.5 embryos and the anterior ectoderm from E6.5 early streak stage embryos (Table 1). These tissues were cultured alone and together for 30 hr and then assayed by whole mount RNA *in situ* hybridization for the expression of the anterior neural marker gene *Otx2*. When *Cerr1*-expressing somitic-presomitic mesoderm was cultured alone, no *Otx2* expression was detected (Fig. 4*A*). Very weak *Otx2* expression was detected in ≈50% of the ectoderm pieces cultured alone (Fig. 4*B*). As *Otx2* is expressed in early streak stage ectoderm but is not maintained unless it receives a signal(s) from the anterior mesendoderm (7), the weak expression in the ectoderm explants cultured alone presumably represents residual *Otx2*

Table 1. *Otx2* expression in germ layer explants

Type of explant	No. expressing/ no. of explants
Somitic-presomitic mesoderm + early streak ectoderm	31/32
Mature somitic mesoderm + early streak ectoderm	0/12
Posterior presomitic mesoderm + early streak ectoderm	5/9
Somitic-presomitic mesoderm	0/11
Mature somitic mesoderm	0/11
Posterior presomitic mesoderm	0/11
Early streak ectoderm	6*/15

*6/15 early streak ectoderm explants had weak residual *Otx2* expression that was used as a baseline for determining expression in recombinants.

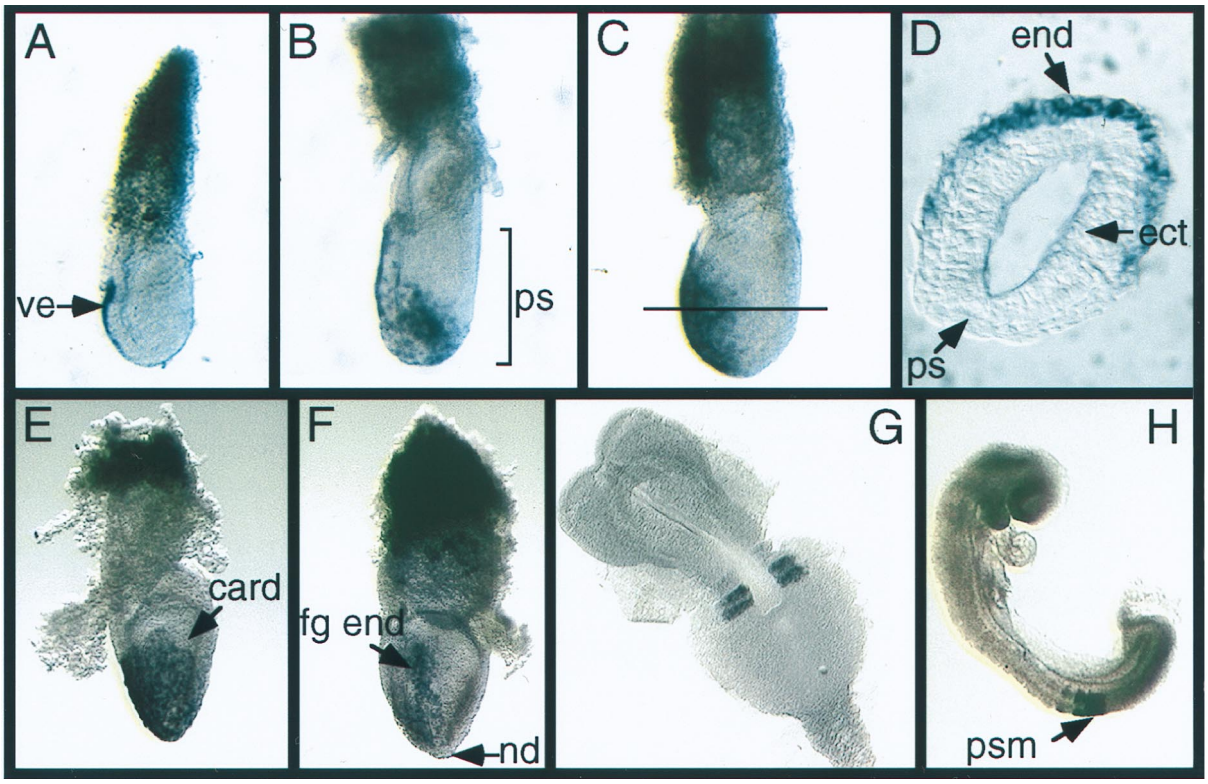

FIG. 3. Expression of *Cerr1* from E6.5–E9 revealed by whole mount RNA *in situ* hybridization. In E6.5 and E7.5 embryos, anterior is to the left and posterior to the right. (*A*) E6.5 early/mid-streak stage embryo. Expression is localized to the anterior visceral endoderm. (*B*) E7.0 mid- to late streak stage embryo. *Cerr1* is expressed in the anterior visceral endoderm and the definitive endoderm emanating from the anterior portion of the primitive streak. (*C*) E7.5 early neural plate stage embryo. *Cerr1* is expressed in both the anterior and the anterior lateral portion of the embryo. (*D*) Transverse section of an E7.5 early neural plate embryo showing *Cerr1* expression in the anterior endoderm layer. The approximate level of the section is shown by the line in *C*. (*E*) Frontal view of *Cerr1* expression in an E7.5 early neural plate stage embryo showing *Cerr1* expression in the midline and anterior lateral region but excluded from the presumptive cardiac region. (*F*) Frontal view of an early headfold stage embryo. Expression is localized to the foregut endoderm. (*G*) E8.5 early somite stage embryo. *Cerr1* is expressed in the two most newly formed somites. (*H*) E9 embryo. *Cerr1* is expressed in the last two newly formed somites and in the anterior portion of the presomitic mesoderm. card, presumptive cardiac region; ect, ectoderm; end, endoderm; fg end, foregut endoderm; nd, node; ps, primitive streak; psm, presomitic mesoderm; ve, visceral endoderm.

expression. When early streak stage ectoderm was combined with *Cerr1*-expressing somitic-presomitic mesoderm, strong *Otx2* expression was seen in 31 of 32 recombinants explants (Fig. 4*C*). To test whether this activity was specific to the *Cerr1*-expressing somitic mesoderm, non-*Cerr1*-expressing somitic mesoderm located 6–7 somites anterior to the somite-presomite boundary was combined with competent ectoderm. In these experiments, 0/12 recombinants expressed *Cerr1* above background levels (Fig. 4*D*). We also performed recombination assays using posterior presomitic mesoderm. In these recombinations, *Otx2* expression was detected in ≈50% of the recombinants (Table 1). As there are no morphological boundaries in the presomitic mesoderm, the mesodermal component may have contained some *Cerr1*-expressing cells or alternatively *Cerr1* may have become expressed in the posterior presomitic mesoderm after being explanted. These results indicate that the somitic-presomitic mesoderm that expresses *Cerr1* has anterior neuralizing ability.

Regulation of *Cerr1* Expression in *Lim1*−/− Embryos. *Lim1*-deficient embryos lack anterior neural structures including the forebrain, midbrain and part of the hindbrain (9). To determine whether *Cerr1* expression was altered in *Lim1*−/− embryos, whole mount RNA *in situ* hybridization was performed on E6.5 and E7.5 wild-type and *Lim1*−/− embryos. In 12/14 *Lim1*−/− embryos examined, *Cerr1* was either not expressed or was weakly expressed in a few scattered cells in the presumptive anterior visceral endoderm and the definitive endoderm regions (Fig. 5*A*). In two *Lim1*−/− embryos however, a patch of cells that strongly expressed *Cerr1* was seen just anterior of the primitive streak (Fig. 5*B*). These results demonstrate that *Cerr1* expression is altered in *Lim1*−/− embryos but indicate that *Lim1* is not absolutely required for *Cerr1* expression.

In *Xenopus*, *follistatin*, *chordin*, and *noggin* can up-regulate *cerberus* expression (18). To determine whether the altered *Cerr1* expression seen in *Lim1*−/− embryos might be a consequence of altered *follistatin*, *chordin*, or *noggin* expression, we analyzed the expression of these genes in mid- to late streak *Lim1*−/− embryos. Mid- to late streak embryos were chosen for study because germ layer explant experiments indicate that anterior ectoderm becomes committed to form anterior neural tissue by these stages (6). Although the primitive streak in *Lim1*−/− embryos is shifted more posteriorly, perhaps due to the constriction between the embryonic and extraembryonic regions, we found that both *follistatin* and *chordin* were expressed in the appropriate part of the primitive streak relative to other primitive streak specific markers like *Brachyury* and *goosecoid* that were analyzed previously (9). *Follistatin* was expressed in the posterior portion of the primitive streak of wild-type embryos (35) and in the equivalent region of the streak in *Lim1*−/− embryos (Fig. 5*C*). *Chordin* expression was localized to the anterior portion of the primitive streak in wild-type and in the corresponding region of *Lim1*−/− embryos (Fig. 5*D*). We were unable to detect *noggin* expression in mid- to late streak wild-type or *Lim1*−/− mutant embryos (data not shown). These results suggest that *Cerr1* is unlikely to be regulated by *follistatin*, *chordin*, or *noggin*.

DISCUSSION

We have identified a mouse *cerberus*-related gene that we have named *Cerr1*. As *Cerr1* and *cerberus* share modest sequence

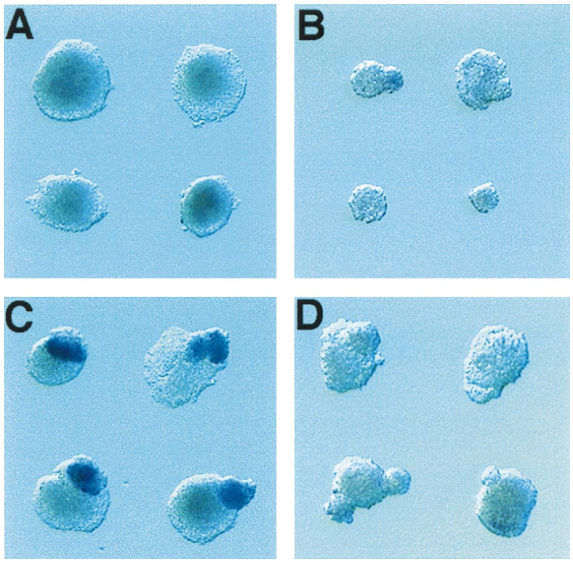

FIG. 4. Maintenance of *Otx2* expression in ectoderm explants recombined with *Cerr1*-expressing somitic-presomitic mesoderm. (*A*) Somitic-presomitic mesoderm region explant from an E9.5 embryo cultured alone and assayed for *Otx2* expression by whole mount RNA *in situ* hybridization. No *Otx2* expression was detected in the explants. (*B*) Early streak ectoderm explant cultured alone and then assayed for *Otx2* expression. Weak expression can be detected in some ectoderm pieces. As *Otx2* is expressed in early streak ectoderm before isolation, the weak expression seen in the top two explants presumably represents residual expression present after 30 hr in culture. (*C*) Somitic-presomitic mesoderm that expresses *Cerr1* is able to maintain *Otx2* expression in early streak ectoderm. (*D*) Non-*Cerr1* expressing somitic mesoderm is unable to maintain *Otx2* expression in early streak ectoderm. The weak signal present in the bottom two recombinants presumably represents residual *Otx2* expression in the ectoderm.

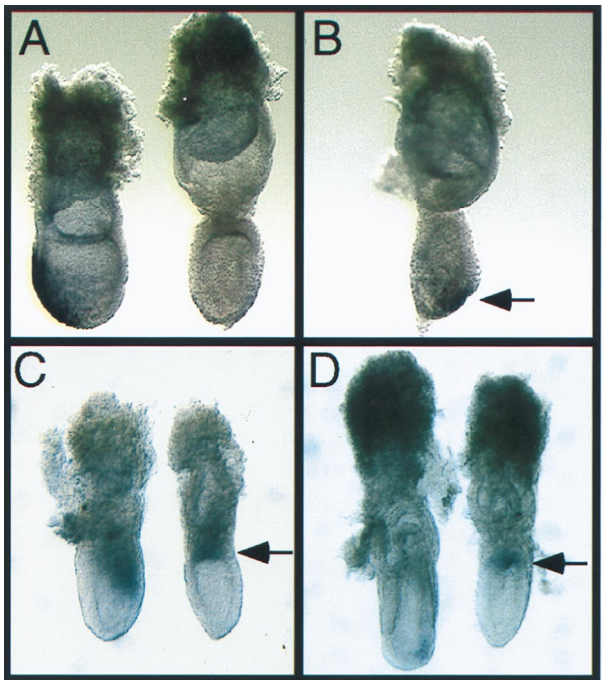

FIG. 5. Expression of *Cerr1* in *Lim1*−/− embryos. (*A*) E7.5 wild-type (*Left*) and *Lim1*−/− (*Right*) embryos assayed for *Cerr1* expression by whole mount RNA *in situ* hybridization. Weak or no expression was observed in 12/14 E6.5 and E7.5 *Lim1*−/− embryos. In some mutant embryos, a few *Cerr1*-positive cells were seen in the central portion of the epiblast and also near the distal tip region that may correspond to the anterior visceral endoderm region. (*B*) Late streak stage *Lim1*−/− embryo expressing *Cerr1* in a region just anterior to the primitive streak. (*C*) *follistatin* expression in mid-streak embryos. In the wild-type embryo (*Left*), *follistatin* is expressed in the posterior portion of the primitive streak. In the *Lim1*−/− embryo (*Right*) *follistatin* is expressed in a region of the *Lim1*−/− embryo that corresponds to the posterior region of the primitive streak (9). (*D*) *chordin* expression in mid-streak stage embryos. In the wild-type embryo (*Left*), *chordin* is expressed in a small region at the anterior portion of the primitive streak. In the *Lim1*−/− embryo (*Right*), *chordin* is expressed like *goosecoid* (9) in the region between the embryonic and extraembryonic region. Arrows indicate regions of gene expression. In all embryos, anterior is to the left and posterior to the right.

homology, it is unclear whether *Cerr1* is the mouse homolog of *cerberus* or only a *cerberus* gene family member. The predicted amino acid sequences of the two vertebrate genes are 48% identical over a 110-amino acid region and share nine conserved cysteine residues. Despite the modest homology, the expression of *Cerr1* in the anterior endoderm of early gastrula stage embryos, its colocalization in tissues with anterior neuralizing ability and its altered expression in *Lim1*−/− mice suggest that *Cerr1* may indeed be the mouse homolog of *cerberus*.

The initial phase of *Cerr1* expression in early gastrulation stage mouse embryos is similar to *cerberus* expression in *Xenopus* gastrula stage embryos. At the start of gastrulation in the mouse, *Cerr1* is expressed in the anterior visceral endoderm. A number of other genes with a role or a suspected role in anterior axis formation are also expressed in this region. These genes include *Otx2* (7), *Lim1* (9), *Rpx/Hesx1* (29, 30), *nodal* (31), *goosecoid*, and *HNF3β* (32). The visceral endoderm is a layer of primitive endoderm that surrounds the pregastrula epiblast (36). It does not ingress through the primitive streak and is replaced by the definitive endoderm from the anterior portion of the streak during gastrulation (37). In *Xenopus* gastrula stage embryos, *cerberus* is expressed in the anterior-most endoderm that does not involute during gastrulation. It is tempting to speculate that this noninvoluting anterior endoderm in *Xenopus* is homologous to the noningressing anterior visceral endoderm in the mouse. The fate of the cells in these two regions are different however. In *Xenopus*, the anterior endoderm that expresses *cerberus* is fated to form foregut, liver, and midgut (18) while in the mouse, the anterior visceral endoderm is fated to form yolk sac endoderm (30, 37).

During the second phase of expression, at the mid- to late streak stage, *Cerr1* is expressed in the anteriorly migrating definitive endoderm. The fate of the anterior definitive endoderm is gut endoderm (37), therefore this phase of *Cerr1* expression in the mouse may be most similar to *cerberus* expression in *Xenopus*. By the early neural plate stage, *Cerr1* is expressed in both the midline mesendoderm and the anterior lateral endoderm. In *Xenopus*, *cerberus* is expressed in both the anterior and lateral endoderm (18). The expression patterns of the two gene do differ somewhat at this stage though. In mouse, *Cerr1* is expressed in the midline but not in the presumptive cardiac region. In *Xenopus*, *cerberus* is excluded from the midline mesendoderm and is expressed in the presumptive cardiac region. The discrepancy in the midline expression may be due in part to staging differences because by the early headfold stage *Cerr1* expression is excluded from the presumptive notochord region of mouse embryos.

A third phase of *Cerr1* expression occurs during somitogenesis. In early somite stage embryos and continuing through E9.5, *Cerr1* is expressed in the two most newly formed somites and also in the anterior presomitic mesoderm. In *Xenopus*, *cerberus* expression was undetectable by stage 14 (18) when somitic mesoderm begins to form (38). The *Notch1* and *Notch2* genes are expressed in a manner similar to *Cerr1* during somitogenesis. *Notch1* is expressed highly in the presomitic mesoderm (39, 40) while *Notch2* is expressed most highly in the most recently formed somites (41). The *Notch* family of genes encode cell surface receptors involved in cell-cell interactions in *Drosophila* and *C. elegans* (42). Targeted mutations of *Notch1* in the mouse affects somite formation and organization (41, 43). The association of *Cerr1* expression with anterior presomitic mesoderm and the most recently formed

somites suggests that *Cerr1* may also play a role in coordinating somite formation.

The expression of *Cerr1* in the anterior visceral endoderm and anterior definitive endoderm are suggestive of role in anterior neural induction in the mouse. Thomas and Beddington (30) have proposed that the anterior visceral endoderm is involved in the initial induction of anterior neural fates in the mouse. They demonstrated that if part of the anterior visceral endoderm is removed from mid- to late streak stage embryos cultured *in vitro*, the size of the anterior neural folds was reduced. Varlet *et al*. (31) also demonstrated a role for the visceral endoderm in anterior neural development. They injected wild-type cells into *nodal*-deficient blastocysts. As embryonic stem cells do not contribute extensively to the visceral endoderm when injected into blastocysts (44), the visceral endoderm in the chimeras was predominantly made up of *nodal*-deficient cells. Varlet *et al*. found that chimeras with a substantial contribution of wild-type embryonic stem cells to the embryo proper and containing *nodal*-deficient cells in the visceral endoderm had head defects affecting the rostral-most neural structures. *Cerr1* expression in the anterior definitive endoderm may also be important in neural induction as suggested by germ layer recombination experiments. Recombination experiments demonstrate that anterior mesendoderm from mid- to late streak embryos can induce *En* and *Otx2* expression in early streak stage ectoderm (6, 7). Thomas and Beddington (30) have postulated that in the mouse the anterior visceral endoderm is responsible for the initial induction of anterior neural identity and that subsequently the anterior mesendoderm maintains and reinforces this anterior neural identity.

A novel finding from our experiments was the observation that in germ layer recombination explants, *Cerr1*-expressing somitic tissue was able to maintain expression of the anterior neural marker *Otx2* in early streak stage ectoderm. This anterior neuralizing ability is specific to the *Cerr1* expressing somitic-presomitic mesoderm as more mature somites that no longer express *Cerr1* are unable to maintain *Otx2* expression. Although these recombination experiments do not demonstrate that *Cerr1* is the neuralizing factor, it does demonstrate that the developing somitic mesoderm expresses a factor(s) or a combination of factors that can direct early streak stage ectoderm to develop as anterior neural tissue. The neural tube adjacent to the *Cerr1*-expressing somitic-presomitic mesoderm does not express *Otx2* (7). At this time the neural tube may not be competent to respond to the anterior neuralizing signal or alternatively a posteriorizing neuralizing signal(s) may be dominant over the anterior neuralizing signal. Another intriguing possibility is that the newest formed somites are signaling to the overlying neural tissue and for that matter the other adjacent tissues, in a progressive anterior to posterior manner. Thus, this dynamic mesoderm-derived embryonic structure may provide an integrative mechanism for generating anterior-posterior patterning in the adjacent tissues of vertebrates.

Our rationale for searching for a mouse homolog of *cerberus* was that *cerberus* may be a downstream target gene of *Lim1* in the head induction pathway. In the mouse, *Lim1* is initially expressed in the anterior visceral endoderm like *Cerr1*. It is not known if they are expressed in the same cells. As gastrulation proceeds, *Lim1* is expressed in the node, the primitive streak, the mesodermal wing and the midline anterior mesendoderm (9) whereas *Cerr1* is expressed in throughout the anterior endoderm. Because *Cerr1* is expressed more widely in the endoderm as gastrulation proceeds, *Lim1* cannot be directly regulating *Cerr1* expression outside the visceral endoderm. When we examined E6.5 and E7.5 *Lim1*$-/-$ embryos, we found that in most embryos *Cerr1* expression was either absent or restricted to a few cells in the presumptive anterior visceral endoderm and definitive endoderm regions. These results suggest that the anterior endoderm cells may be incorrectly specified in *Lim1*$-/-$ embryos or alternatively that interactions between germ layers are required for the maintenance of *Cerr1* expression. Future studies will be directed at understanding the regulation of *Cerr1* during early gastrulation and the creation of knockout mice to determine whether *Cerr1* has an essential function in anterior neural development and somite formation.

We thank Randy Johnson for the *Cerr1* EST clone, Akihiko Shimono for the E7.5 mouse cDNA library, Yuanhao Li for RNA samples and the mouse interspecific mapping panel Southern membrane, and Yuji Mishina for computer graphics help. In addition, we thank Siew-Lan Ang, Hai Wang, Zhoufeng Chen, Marty Matzuk, Rosa Beddington, and Richard Harland for probes. We also thank Lucy Rowe of the Jackson Laboratory for assistance with gene mapping. The DNA Sequencing Core facility at the University of Texas M. D. Anderson Cancer Center is supported by the National Cancer Institute Grant CA16672. This work was supported by a research grant from the Human Frontier Science Program.

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