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Gene Is Not Required for Anterior Pattern Formation

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***Cer1* is the mouse homologue of the *Xenopus* Cerberus gene whose product is able to induce development of head structures during embryonic development. The Cer1 protein is a member of the cysteine knot superfamily and is expressed in anterior regions of the mouse gastrula. A segmental pattern of expression with nascent and newly formed somites is also seen. This suggests an additional role in development of the axial skeleton, musculature, or peripheral nervous system. *Xenopus* animal cap assays and mouse germ-layer explant recombination experiments indicate that the mouse protein can act as a patterning molecule for anterior development in *Xenopus*, including induction of *Otx2* expression, and suggest it may have a similar role in mouse development. However, we present here genetic data that demonstrate that *Cer1* is not necessary for anterior patterning, *Otx2* expression, somite formation, or even normal mouse morphogenesis.**

INTRODUCTION

The *Xenopus laevis Cerberus* gene was identified by differential screening of cDNA enriched in Spemann's organiser and encodes a putative secreted protein belonging to the cysteine knot superfamily which includes TGF-β and BMP proteins (Bouwmeester *et al.*, 1996). The same authors show that microinjection of Cerberus mRNA into *Xenopus* embryos induces ectopic heads and occasionally causes duplicated hearts and livers, suggesting a role for the molecule in induction of head structures in the vertebrate embryo. The identification of mouse cDNAs with a predicted amino acid sequence 48% identical to the *Xenopus* protein has been reported (Belo *et al.*, 1997; Biben *et al.*, 1998; Shawlot *et al.*, 1998). In addition, there is a family of proteins in both mouse and *Xenopus* that are related to *Cerberus/Cer1*, which include *Gremlin/drm* and DAN/*Dana* and which all have patterning activity in the *Xenopus* embryo. All three proteins appear to act as BMP antagonists by directly binding to members of the BMP family (Hsu *et al.*, 1998; Stanley *et al.*, 1998b). Furthermore, Cerberus is also able to bind to and antagonise Wnt and Nodal signalling (Piccolo *et al.*, 1999). Gremlin can induce secondary axis formation, whilst DAN has dorsalising activity and can induce some anterior structures such as cement glands, but less efficiently than *Cer1* (Hsu *et al.*, 1998; Stanley *et al.*, 1998b). A fourth family member, PRDC, has been identified but no activity has been ascribed to it (Minabe-Saegusa *et al.*, 1998).

Detailed expression studies have shown that the mouse *Cer1* gene is expressed during early gastrulation in a region of primitive endoderm on the side of the egg cylinder essential for anterior patterning (Belo *et al.*, 1997; Biben *et al.*, 1998; Shawlot *et al.*, 1998). During a second phase of expression, *Cer1* is expressed in the most recently formed somites and anterior presomitic mesoderm (Biben *et al.*, 1998; Shawlot *et al.*, 1998). These specific expression patterns suggest a role in anterior patterning and somite formation during mouse embryogenesis. Further evidence in support of this idea comes from *in vivo* experiments. Although ectopic expression of mouse *Cer1* in *Xenopus* animal cap assays is not capable of inducing head-like

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structures it can induce cement glands and markers of anterior neural tissue and endoderm characteristic of BMP induction (Biben *et al.*, 1998). Furthermore, mouse germ-layer explant recombination assays show that only *Cer1*-expressing somitic or presomitic mesoderm can mimic the anterior neuralising ability of anterior mesendoderm and maintain *Otx2* expression in competent ectoderm (Biben *et al.*, 1998). However, in contrast to these expression and functional studies we present genetic data showing that *Cer1* is not required for anterior patterning, somite formation, or even normal mouse morphogenesis.

The mouse-specific locus test has been used for several decades as a method of assessing the mutagenic properties of environmental insults such as exposure to radiation or chemicals (Russell, 1951; Russell, 1989; Davis and Justice, 1998). By crossing mice that have been treated with a potential mutagen with animals that are homozygous for a number of recessive visible mutations, rare newly induced alleles of these visible mutations are readily identified in the offspring. The most commonly used tester line of mice is homozygous for seven recessive mutations, six of which are coat colour mutations and include the brown mutation of *Tyrp1*. Use of the test has resulted in the identification of over 30 overlapping deletions on mouse chromosome 4 that encompass the *Tyrp1* locus, along with varying amounts of the genome flanking the locus. Almost all of the deletions when homozygous, or compound heterozygous with other deletions, confer a phenotype due to the removal of additional genes. We have previously mapped some of these phenotypes (Rinchik, 1994, Rinchik *et al.*, 1994) and have generated a detailed map of the deletion complex (Bell *et al.*, 1995). The mouse *Cer1* gene was recently mapped by interspecific backcross analysis to the central region of mouse chromosome 4, between *Tyrp1* and *Ifa* (Stanley *et al.*, 1998). We now show that certain deletions of *Tyrp1* also delete *Cer1* and that animals lacking *Cer1* survive to birth with apparently normal development.

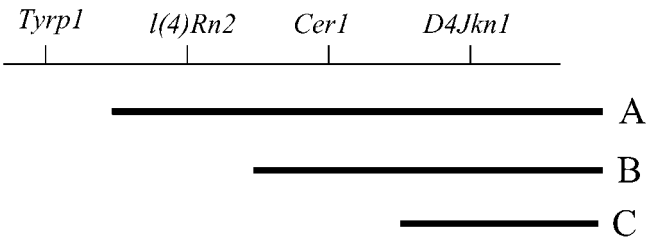

FIG. 1. Deletion mapping of *Cer1*. Simplified marker content of 25 deletions (not to scale). Four markers are indicated on the map across the top. Deletion chromosomes are indicated as three groups, A–C. Group A deletions remove only *Tyrp1* of these markers and comprise 173G, 1THO-IV, 37DTD, 47DthWb, 33G, 331K, 9Pu, 51DthWb, 11R30M, 49HATh, 13DT, 3YPSc, 1DF1OD, 55Cos, and 26R60L. Group B deletions remove *l(4)Rn2* but not *Cer1* and comprise 9R75VH, 37Pub, and 1OZ. Group C deletions remove both *l(4)Rn2* and *Cer1* and comprise 5CHLe, 8Pub, 46UTHc, 11Pu, 3YPSc, 13R75M, and 12Pu. Further details of markers and deletions can be found in Bell *et al.* (1995).

MATERIALS AND METHODS

Husbandry and Embryo Analysis

The origin and initial molecular characterisation of radiation- and chemical-induced lethal *Tyrp1* (brown) deletions is described by Rinchik *et al.* (1994). All mice were housed in a conventional facility at the Biology Division of Oak Ridge National Laboratory. Mice heterozygous for the $Tyrp1^{b\text{-}46UTHc}$ deletion were intercrossed and embryos dissected from pregnant females at E9 and E16.5. DNA was prepared from extraembryonic membranes (E9) or from tails (E16.5) for analysis by PCR amplification. Presence of the *Cer1* gene was detected by PCR amplification with primers 5′-TATGTGATGCCCCGACTGTA-3′ and 5′-GGGCACAG-TCCTGCAGGTCT-3′, which amplify a 591-bp fragment of exon 1. A control amplification was performed in the same reaction with primers 5′-AATGTGCTGGTTGTGATAGCC-3′ and 5′-CT-GACCATCCAGATGCCC-3′, which amplify a 349-bp fragment of the unlinked gene, *Mc1r*. Reaction conditions were as described below, except that the primer concentrations were approximately 0.3 μM. The PCR was carried out for 30 cycles with an annealing temperature of 55°C.

Mapping of Cer1

The *Cer1* sequence contains a run of repeated TC dinucleotides, beginning 135 bp downstream of the termination codon. Primers were designed to amplify a fragment containing this microsatellite by PCR from genomic DNA (5′-CTCAACAGAAA-GCAAAACCTCA-3′ and 5′-TGAGGCCAGCTGAGAATACA-3′). The product of amplification from laboratory strains of mice is 249 bp whilst from *Mus spretus* DNA the amplification product is approximately 210 bp. DNA from deletion-carrying mice that were the product of a cross between carriers of the deletion chromosome and *M. spretus* were used to locate *Cer1* within the deletion complex. The PCR was performed on 100 ng genomic DNA in 50 mM KCl; 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$ containing 200 μM each dATP, TTP, dCTP, and dGTP; both primers at approximately 0.5 μM; and AmpliTaq DNA polymerase (Perkin–Elmer) at 20 units/ml. The PCR was carried out for 30 cycles with an annealing temperature of 58°C, and products were visualised after electrophoresis through 4% NuSieve agarose (FMC Bioproducts).

In Situ Hybridisation

The *Otx2* probe was made by amplifying by PCR the insert of the IMAGE cDNA clone 633802 (thanks to Tyler Cutforth), using vector primers for the T7 and T3 RNA polymerase promoters, and the amplified product was purified using the QIAquick PCR Purification Kit (Qiagen). T7 RNA polymerase was used to generate DIG-labelled antisense probe (Boehringer DIG labelling kit) and T3 polymerase to generate DIG-labelled sense (control) probe. The *Krox20* cDNA clone (Wilkinson *et al.*, 1989) was obtained from David Wilkinson and a DIG-labelled probe made in essentially the same way. Whole-mount *in situ* hybridisation was performed as described (Hammond *et al.*, 1998; Hecksher-Sorensen *et al.*, 1998) on E9 embryos from crosses segregating the $Tyrp1^{b\text{-}46UTHc}$ deletion.

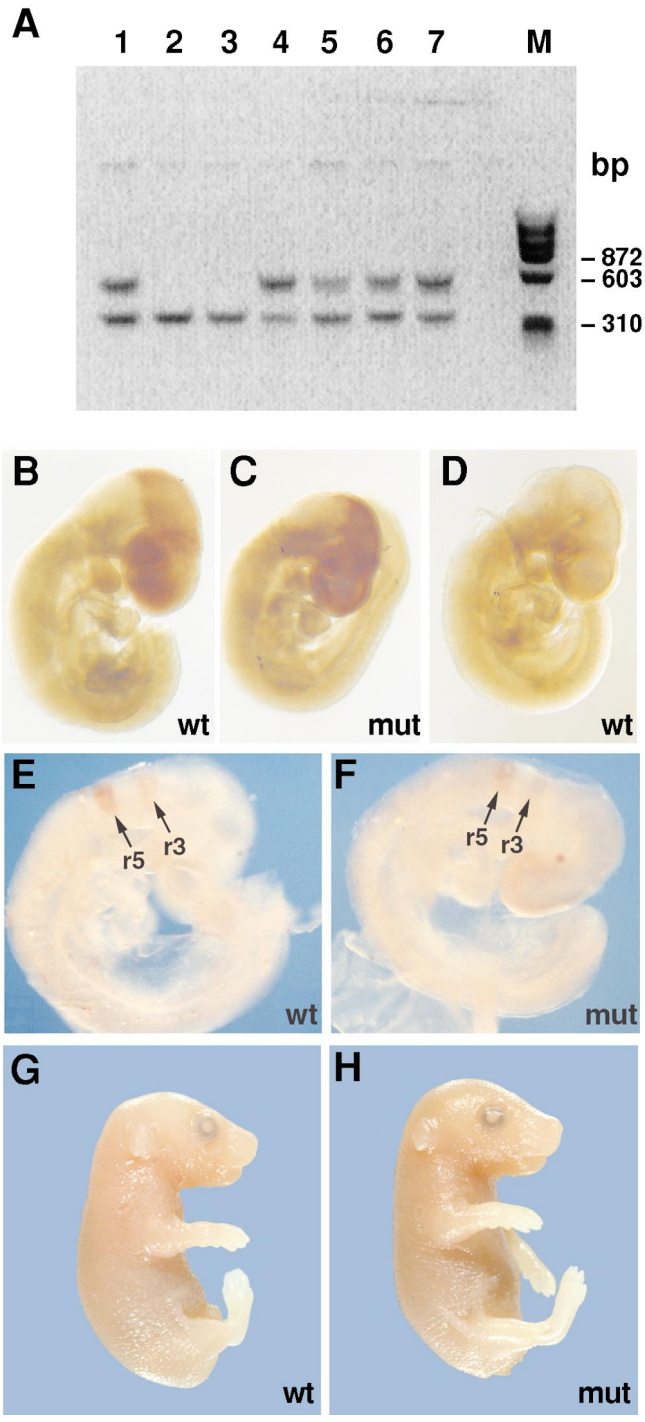

FIG. 2. Phenotype of *Cer1*-deleted embryos. (A) PCR analysis of an E16.5 litter segregating *Tyrp1*$^{b\text{-}46UTHc}$. Amplification of the *Mc1r* gene (lower band) indicates that all reactions were successful. Absence of the upper band in embryos 2 and 3 indicates that these are deleted for *Cer1*. (B) Hybridisation of *Otx2* to an E9 embryo, from a litter segregating *Tyrp1*$^{b\text{-}46UTHc}$, which is not deleted for *Cer1*. Note the hybridisation to fore- and midbrain and the posterior boundary of hybridisation that demarcates the hindbrain/midbrain boundary. (C) Hybridisation of *Otx2* to a littermate of B which is deleted for *Cer1* showing the same hybridisation pattern as B. (D) Control hybridisation of sense (control) *Otx2* probe to a nondeleted littermate of B and C. (E) Hybridisation of *Krox20* to an E8.5 embryo from a litter segregating *Tyrp1*$^{b\text{-}46UTHc}$, which is not deleted for *Cer1*. Note hybridisation to arrowed rhombomere 3 (r3) and r5. (F) Hybridisation of *Krox20* to a littermate of E which is deleted for *Cer1*. (G) Gross morphology of an E16.5 embryo, not deleted for *Cer1*, from the litter segregating *Tyrp1*$^{b\text{-}46UTHc}$ analysed in A. (H) Gross morphology of a littermate of G which is deleted for *Cer1*. Both embryos have had their tails removed for DNA analysis.

RESULTS AND DISCUSSION

Mapping Cer1

Identification of mouse cDNAs highly homologous to the *Xenopus* Cerberus gene was reported by three independent laboratories. Cerberus-like, *Cer1* (AF012244; Belo *et al.*, 1997); murine Cerberus homologue, *mCer1* (AF035579; Biben *et al.*, 1998); and Cerberus-related gene, *Cerr1* (AF031896; Shawlot *et al.*, 1998) differ at only one amino acid position over the entire 272-amino-acid sequence predicted from the coding sequence present in the cDNAs. (At position 220 both *Cerr1* and *mCer1* have a methionine, whereas in *Cer1* an arginine is predicted.) This residue is not conserved in *Xenopus* Cerberus or in other related proteins and the difference is likely to be due to either a strain polymorphism or a sequencing error.

We mapped the *Cer1* gene to the series of radiation-induced deletions generated at the *Tyrp1* locus by the specific locus test (Bell *et al.*, 1995). We designed primers to amplify by PCR a fragment from the 3′ end of the *Cer1* gene that includes a microsatellite which is variant between laboratory strains of mouse and *M. spretus*. The fragment was amplified by PCR from DNA of hybrid mice carrying 1 of the 25 available deletions on one chromosome, opposite a chromosome derived from *M. spretus*. Amplification of all DNAs produced a fragment from the *M. spretus* chromosome, whilst presence or absence of the lab strain fragment indicated whether *Cer1* was deleted from the deletion-carrying chromosome. We found the *Cer1* gene to be absent from seven deletions whose end points lie distal to *Tyrp1*, between that locus and *Ifa*, but was present in another 18 chromosomes which have deletion end points closer to *Tyrp1* (Fig. 1). Many markers have been mapped to these deletions and most of the deletion end points can be distinguished by marker content mapping, but for simplicity we have here assigned them to three groups according to presence or absence of three loci critical for this study. We previously described a lethal phenotype, *l(4)Rn2*, that maps in the deletion complex distal to *Tyrp1*. Foetuses lacking *l(4)Rn2* reach term, but die shortly after birth (Rinchik *et al.*, 1994; Simpson *et al.*, submitted for publication). This phenotype, however, is not due to deletion of *Cer1* as there

are three chromosomes whose deletions include *l(4)Rn2* but which do not delete *Cer1*.

Phenotype of Cer1 Deletion

As *Cer1* has been implicated, by overexpression studies in *Xenopus*, in anterior patterning and head development, we made crosses between mice that carried the *Tyrp1*$^{b\text{-}46UTHc}$ deletion and examined their litters. This deletion includes *Cer1* at its distal end (Fig. 1) and its proximal end is close to *Tyrp1*. It is the smallest deletion to include *Cer1* and in particular it does not delete the locus on the proximal side of *Tyrp1* (*l(4)Rn1*), which has been shown previously to result in early embryonic lethality (Rinchik, 1994; Rinchik *et al.*, 1994). We determined the genotype of each embryo by PCR analysis of DNA made from the extraembryonic membranes or from the tails of later foetuses. Figure 2A shows that embryos with a homozygous deletion of *Cer1* were readily detected in litters segregating *Tyrp1*$^{b\text{-}46UTHc}$ at embryonic day (E) 16.5. Figures 2G and 2H show embryos from this litter. Animals homozygous for *Tyrp1*$^{b\text{-}46UTHc}$ are viable to term but die shortly thereafter. Nevertheless *Tyrp1*$^{b\text{-}46UTHc}$ homozygotes (Fig. 2H) have grossly normal morphology compared to nondeletion littermates (Fig. 2G), and in particular they have developed normal head structures.

In order to see if early anterior patterning or somite formation is disrupted in *Cer1*-deleted embryos we took embryos from crosses segregating the *Tyrp1*$^{b\text{-}46UTHc}$ deletion at E9. Once again deletion homozygotes were morphologically indistinguishable from nondeletion embryos. In particular the somites, which can be clearly seen in the caudal region, and the head structures are normal in appearance (compare Figs. 2B and 2D with Fig. 2C).

Otx2 is one of two mouse homologues of the *Drosophila* gene orthodenticle, which is involved in patterning of anterior head structures in the fly. Expression of *Otx2* in mouse embryos is initially seen in the ectoderm before gastrulation, but later becomes restricted, through action of both positive and negative signals, to the anterior part of the developing brain (Ang *et al.*, 1994). In particular, expression of *Otx2* is found in the developing fore- and midbrain but not in the hindbrain, and there is a sharp boundary of expression between the mid- and hindbrain. *Otx2* expression is induced or maintained in these anterior regions by signals from the mesoderm (Ang *et al.*, 1994) and absence of *Otx2* results in embryos that lack anterior head structures (Acampora *et al.*, 1995; Ang *et al.*, 1996; Matsuo *et al.*, 1995). Injection of *Cer1* into *Xenopus* embryos induces expression of *Otx2*, and a simple model would be that *Cer1* acts to induce head structures by induction of *Otx2*. We therefore examined the expression of *Otx2* by *in situ* hybridisation to E9 mouse embryos wild type or deleted for *Cer1*. Figure 2B shows the normal pattern of *Otx2* in the fore- and midbrain, and the sharp demarcation between the mid- and the hindbrain. In Fig. 2C the hybridisation to an embryo deleted for *Cer1* shows the same pattern. Thus, although *Cer1* appears to induce *Otx2* expression in *Xenopus* embryos, *Otx2* is expressed normally in mouse embryos which lack *Cer1*.

In order to examine hindbrain development in *Cer1*-deleted embryos we used *in situ* hybridisation to assay *Krox20* expression. The *Krox20* gene encodes a zinc-finger protein that is specifically expressed in rhombomeres r3 and r5 (Wilkinson *et al.*, 1989; Irving *et al.*, 1996) and its expression is therefore a good indicator of anterior development of the neural tube and the hindbrain. Figures 2E and 2F show that the pattern of *Krox20* expression at E8.5 is indistinguishable between wild-type and *Cer1*-deleted embryos.

We have been able to utilise preexisting genetic resources to demonstrate that *Cer1* is not required for normal morphological development of the mouse embryo. *Cer1* was identified as the mouse homologue of a *Xenopus* protein that is capable of directing formation of ectopic anterior structures after injection into *Xenopus* embryos. The observation that absence of the *Cer1* gene product has little or no effect on development contrasts with the striking effect of addition of an excess and/or mislocalised product. The difference is most likely due to the nature of the assays; injection into *Xenopus* embryos indicates what the gene product is capable of doing, whereas genetic removal reveals for what the product is actually necessary. Several other molecules, such as goosecoid and activin, that were characterised by the *Xenopus* injection assay as having effects in early development in fact have little or no effect on early embryogenesis after deletion in mice, but do produce defects in later structures (Vassalli *et al.*, 1994; Matzuk *et al.*, 1995; Rivera-Perez *et al.*,1995; Yamada *et al.*, 1995; Ferguson *et al.*,1998). We are unable to determine whether *Cer1* deletion has a subtle effect on neonatal development or physiology as neonates die through the additional deletion of *l(4)Rn2*. In addition, there may be redundancy of *Cer1* through overlap of function with other molecules, either those already identified or others.

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