Normal development of mice carrying a null mutation in the gene encoding the L14 S-type lectin

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SUMMARY

The L14 lectin is a 14×10^3 M_r carbohydrate binding protein belonging to the family of S-type lectins. The pattern of expression of this protein during mouse embryogenesis suggests that it may have multiple roles during pre- and post-implantation development. Using the technique of homologous recombination in embryonic stem cells, we have introduced a null mutation in the gene encoding the L14 lectin and generated a strain of mice carrying the mutant allele. We report here that homozygous mutant animals that lack the L14 lectin develop normally and are viable and fertile. The absence of any major phenotypic abnormalities in these mutant animals suggests that other protein(s) potentially compensate for the absence of the L14 lectin. Here we show that a related protein termed L30, a lectin that has carbohydrate binding specificity similar to that of L14, is present in the same embryonic cell populations as L14 at the time of implantation, suggesting that the two S-type lectins may be capable of functional substitution at this early stage of embryogenesis.

Key words: L14 lectin, L30 lectin, CBP35, homologous recombination, gene targeting

INTRODUCTION

Lectins are generally defined as non-enzymatic, non-immunoglobulin proteins, that bind selectively to specific carbohydrate structures (Goldstein et al., 1980). Sugar-lectin interactions are implicated in diverse biological phenomena, from intracellular routing of glycoproteins to cell-cell adhesion (Sharon and Lis, 1989; Hughes, 1992; Drickamer and Taylor, 1993). For example selectins, a class of lectins anchored in the membrane, mediate adhesion between leucocytes and endothelial cells (Lasky, 1992). During embryonic development, the rapidity and complexity of changes in the distribution of specific carbohydrates suggest that they play important roles (Muramatsu, 1988). Thus, recognition by a specific lectin of a specific carbohydrate moiety may provide one means by which information carried by glycoconjugates can be processed during embryogenesis.

Most animal lectins can be classified into two protein families on the basis of their carbohydrate recognition properties (Drickamer, 1988). The first group of calcium-dependent lectins (C-type lectins), constitutes a large class of integral membrane proteins which include for example the mammalian asialoglycoprotein receptors (Spiess, 1990) and the selectins (Lasky, 1992). Lectins of the second group are calcium independent (S-type lectins) and display a specificity for lactose derivatives (Barondes, 1984). In mice, the S-type lectin family is limited to two well characterized members, L14 and L30 (also called CBP35), of 14×10^3 and 30×10^3 M_r respectively. These proteins share very similar carbohydrate recognition domains (Drickamer, 1988) and display distinct but similar glycoconjugate binding specificities (Leffler and Barondes, 1986). Additional members of this latter gene family have now been identified in rat and human tissues (Leffler et al., 1989; Gitt et al., 1992).

The L14 S-type lectin is a homodimeric protein that is not subject to posttranslational modifications (Sparrow et al., 1987; Tracey et al., 1992); the bivalent nature of the molecule may allow it to link glycoconjugates together. Several roles have been proposed for the L14 protein. During myoblast differentiation, the localization of L14 protein has been shown to shift from the cytoplasm to the extracellular medium where it is secreted by a novel mechanism (Cooper and Barondes, 1990). L14 is known to bind to laminin (Cooper et al., 1991; Zhou and Cummings, 1990) and following secretion from myoblasts, it exerts a ‘de-adhesion’ effect on myoblasts by displacing their attachment to laminin thus allowing fusion (Cooper et al., 1991).

L14 has also been assigned a growth promotion activity on smooth muscle cells and pulmonary arterial cells in vitro (Sanford and Haring-Hooker, 1990), as well as a growth inhibition activity on rat fibroblasts in vitro (Wells and Mallucci, 1991), although these studies await confirmation using highly purified protein preparations. It has also been shown
that overexpression of Lect14 leads to transformation of 3T3 cells in vitro (Yamaoka et al., 1991). Finally, L14 and L30 are found on the surface of different murine and human tumor cell lines, including melanoma, fibrosarcoma and carcinoma (Raz et al., 1987, 1990).

We previously described cloning of the mouse Lect14 gene following a differential screen of cDNA libraries that was designed to isolate genes regulated during embryonic stem cell differentiation (Poirier et al., 1991). The results of in situ hybridization studies and immunohistochemical analysis suggested that Lect14 may participate in several aspects of mouse embryogenesis. A number of rapid changes in expression of this gene were found, the most striking of which was observed on the fourth day of gestation (Poirier et al., 1992), when the onset of Lect14 expression in the cells of the trophectoderm coincided with the hatching of the blastocyst from the zona pellucida prior to implantation, suggesting a potential involvement of L14 in this process.

Carbohydrates in general (Kimber, 1990) and LNF1 (lacto-ß-N-fucopentaose 1) in particular (Lindenberg et al., 1988) are thought to play a role in implantation. The distribution of LNF1 is regulated during pregnancy: it appears in small ‘patches’ along the uterus on the fourth day of gestation (Kimber et al., 1988), presumably forming potential sites of attachment for the blastocyst. In addition, LNF1 has been shown to mediate, at least partly, the attachment of blastocysts to cultured endometrial cells (Lindenberg et al., 1988). As the L14 protein exhibits specificity for LNF1 in vitro (Leffler and Barondes, 1986; Sparrow et al., 1987), this raises the possibility that L14 might act as a high affinity ‘receptor’ for LNF1 in vivo. This hypothesis was further supported by the fact that L14 and the LNF1 ‘receptor’ activity appear at the same time in the blastocyst and could therefore be the same molecule (Poirier et al., 1992; Lindenberg et al., 1990). We had thus postulated that implantation might in part be mediated by an interaction between L14 expressed on the trophectodermal cells of the blastocyst and LNF1 present on the endometrial cells of the uterine wall.

To study further the role of this S-type lectin, we decided to introduce a null mutation in the Lect14 gene by homologous recombination in ES (embryonic stem) cells (Capecchi, 1989). Following transfer of this mutation into the germ line, suggesting a potential involvement of L14 in this process.

THE MATERIAILS AND METHODS

Construction of the targeting vector

Plasmids containing Lect14 locus genomic fragments were obtained from J. Chan (NIMR, London, UK). The construction of the replacement vector was achieved in four steps. First, Bam linkers were added to the 1.1 kb XhoI-BamHI neo fragment from pMC1 poly(A) (Thomas and Capecchi, 1987) which was then inserted into the Bam site of Bluescript KS. This plasmid was designated BS-neo. Next, the recessed ends of a 1.7 kb EcoRI-XbaI genomic fragment comprising exon 1 of the Lect14 gene were filled in and the fragment ligated into the Sma site of BS-neo, thus generating BS′-neo. The following step consisted of ligating the TK gene cassette (XhoI-HindIII fragment from MC1 TK) to BS′-neo digested with Xho and HindIII, to give BS-TK′-neo. Finally a 5.3 kb genomic Sal/Xba fragment containing exons 3 and 4, with the Sal end filled in, was ligated to BS-TK′-neo that had been cut with Xba and Spe, the Spe end having been filled in. The final replacement vector, BS-TK′-neo-3′, which is shown on Fig. 1, was linearized with XbaI before electroporation.

Cell culture and electroporation

CCE ES (Robertson et al., 1986) cells were maintained on mitomycin-treated STO neo fibroblast feeder cells as previously described (Robertson, 1987). Electroporation was performed under the following conditions. Cells were collected by trypsinization, washed once in PBS and resuspended in PBS at a concentration of 4×10^7/ml. Aliquots of this cell suspension (0.5 ml) were mixed with 15 µg of linearized DNA. Electroporation was carried out using a Bio-Rad Gene Pulser apparatus at 220 V and 960 Ω. Under these conditions, cell survival was approximately 50%. Cells were plated onto 10-cm feeder plates (approximately 3×10^6 surviving ES cells per plate), 24 hours later, selection was applied using medium containing G418 (GIBCO) at 200 µg/ml (active ingredient) and 3 days after that the electroporation medium containing both G418 at 200 µg/ml and gancyclovir (Syntex Research Co.) at 2×10^{-6} M was applied to cultures. The medium was replaced daily. After 9-12 days under selection, individual drug resistant colonies were picked into 24-well trays. 2-3 days later, individual cultures were expanded into 3-cm well trays for freezing and isolation of DNA.

DNA analysis

Individual cell pellets (approximately 10^6 cells) were resuspended in 0.5 ml of lysis buffer (50 mM Tris pH 7.5, 50 mM EDTA, 100 mM NaCl, 5 mM DTT, 1% SDS, 100 µg/ml proteinase K) and high relative molecular mass DNA was prepared (Gross-Bellard et al., 1973). Clones were screened by polymerase chain reaction using a neo primer (5′ CCTGCGCCGTACACCGGA-ACACGG 3′) and a Lect14 primer corresponding to a 5′ genomic sequence located immediately upstream of the region of homology included in the replacement vector (5′ GGTTAGGTGTTGCGCTGCTGATGCATGCAG 3′) (see Fig. 1A). The PCR reactions were performed under standard conditions (35 cycles; denaturation at 94°C for 0.2 minute, annealing at 65°C for 1 minute, and polymerisation at 72°C for 1.5 minute) and amplification products visualized following staining of agarose gels with ethidium bromide. For Southern blot analysis, 10 µg of genomic DNA were digested to completion, electrophoresed on a 0.8% agarose gel, blotted on GeneScreen Plus (Dupont) filters and hybridized with 32P-labeled random primed probes.

Blastocyst injections and mating of chimeric mice

MF1 (Harlan Sprague-Dawley) blastocysts were injected with Lect14 targeted ES cells as described previously (Bradley, 1987). Male chimeras were mated with MF1 females to identify ES-cell derived agouti offspring. The heterozygous offspring were identified by Southern analysis and paired to obtain homozygous mice.

Antisera

The rabbit anti-rat L14 used for western blots and for immunohistochemistry had been affinity purified using recombinant rat L14
protein and cross-adsorbed against related rat S-type lectins (kind gift of D.N.W. Cooper, University of California, San Francisco). Immunochemistry experiments on L30 were performed using an affinity purified rabbit anti-L30 antiserum (kind gift of J. Wang, Michigan State University, East Lansing).

**Western blot analysis**

Individual tissue samples were mechanically homogenized (Dounce) in extraction buffer (50 mM Tris, pH 6.8, 2 mM EDTA, 1% SDS, 8% glycerol, 10% β-mercaptoethanol, 0.025% bromophenol blue), boiled for 10 minutes and centrifuged. Supernatants were electrophoresed on 15% acrylamide gel containing 0.1% SDS and blotted onto nitrocellulose. The blot was sequentially immersed in anti-L14 serum (1:125) for 1 hour, goat anti-rabbit IgG conjugated with alkaline phosphatase (1:7500), and developed in chromogen solution (350 µg/ml of nitroblue tetrazolium salt, 175 µg/ml of 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2 pH 9.5).

**Histological analysis**

Blastocysts were fixed in 4% (w/v) paraformaldehyde for 15 minutes, introduced in the swollen ampulla recovered from a female mouse 12 hours post coitum (procedure of Copp, 1978) and the tissue fixed for a further 15 minutes. 15 day old embryos were fixed for 3 hours. Samples were transferred in a solution of 30% (w/v) sucrose in 0.1 M sodium phosphate buffer, pH 7.5, and incubated overnight prior to embedding in Tissue Tek, and preparation of 13 µm frozen sections. Samples were rinsed twice in PBS and first treated for 10 minutes with 10% HIGS (heat inactivated goat serum), 0.1% Triton X-100 in PBS. The primary antibodies anti-L14 and anti-L30 respectively diluted at 1:250 and 1:60 in PBS containing 1% HIGS and 0.5% Triton X-100 were applied overnight at 4°C. After two washes in PBS, samples were covered slipped in glycerol:carbonate buffer (1:1) containing para-phenylenediamine (Placzek et al., 1993).

**RESULTS**

**Mutation of the Lect14 gene**

A ‘positive-negative’ gene targeting contract (Mansour et al., 1988) was used to generate a null mutation at the Lect14 locus. This strategy allows enrichment for the double crossover event that is necessary to correctly disrupt the target gene. The physical map of the mouse Lect14 locus is shown in Fig. 1A. The Lect14 transcription unit spans approximatively 5 kb and is composed of four exons encoding respectively 3, 27, 57 and 48 amino acid residues (Gitt and Barondes, 1991). A ‘replacement’ vector was constructed in which the neo gene was flanked by two fragments of Lect14 genomic sequences: a 1.7 kb fragment containing exon 1 on the 5’ side and a 5.3 kb fragment containing exons 3-4 on the 3’ side. The HSV TK gene was placed at the 5′ end of the homology (Fig. 1A).

The designed mutation results in a deletion of a 1.0 kb region of the gene that encompasses exon 2, and replacement of this sequence by the 1.1 kb neo cassette containing a stop codon and a poly(A) addition signal (Fig. 1A). Moreover, as exon 2 encodes 27 amino acids of the protein and importantly includes a region required for carbohydrate binding activity (Abbott and Feizi, 1991), elimination of this exon alone would result in the loss of function. And finally, in the event of aberrant splicing around the neo cassette, translation of any resultant mRNA would result in the production of an irrelevant peptide since exons 1 and 3 of the human, chicken and mouse Lect14 gene are in different reading frames (Gitt and Barondes, 1991; Ohyama and Kasai, 1988; Chan and Rigby, unpublished results). Therefore, the mutation introduced by the gene targeting event will generate a null allele of the Lect14 locus.

CCE ES cells were transfected with linearized plasmid DNA by electroporation, and transformants selected in the presence of G418 and gancyclovir. 13% of G418-resistant clones proved to be resistant to gancyclovir selection, giving an enrichment of approximatively 7 fold against cells carrying randomly integrated copies of the construct. To identify clones that had undergone homologous recombination, 120 double-drug resistant clones were individually screened using a polymerase chain reaction (PCR) analysis. Two oligonucleotide primers were used that corresponded to sequences present in the neo gene and sequences 5′ to the extent of homology included in the replacement vector respectively (see Materials and Methods). Fig. 1B shows representative results from this analysis. Four of the 120 clones examined proved positive by this assay, giving the predicted 1.7 kb amplification product specific for the targeted junction fragment. Therefore, accounting for the 7-fold enrichment, the frequency of targeting at this locus is in the region of 0.5% of the integration events. The four clones determined to be positive by PCR were expanded and genomic DNA was analyzed further by Southern blotting. The structure of the targeted allele after homologous recombination between the vector and the endogenous Lect14 locus was verified by an extensive analysis using multiple digests and probes (data not shown). These data, which included a survey of the 5′ and 3′ flanking regions, showed that the predicted replacement event had occurred in 3 of the 4 PCR-positive clones (L14-6, L14-11, L14-31).

**Germline transmission of the Lect14 mutation**

A recombinant clone, designated L14-11, was injected into 3.5 dpc blastocysts to generate chimeric mice. From 12 embryos injected, 9 pups were obtained of which 6 were overtly chimeric. These chimeras were all males and two animals in particular showed very extensive agouti coat hairs, indicative of high levels of somatic chimerism. These were test bred to determine germline transmission, and both animals were found to transmit the dominant ES-cell derived agouti marker to all offspring, indicating that the germline had been completely colonized by ES cell derivatives. The resultant progeny were genotyped with respect to the Lect14 mutation by Southern blot analysis of tail DNA. Of 41 offspring screened, 15 proved to be heterozygous for the disrupted Lect14 gene.

The heterozygous animals were intercrossed to generate mice homozygous for the mutation. The genotypes of the resulting offspring were determined by Southern blot analysis. An example of an analysis of F2 animals is shown in Fig. 2. The 1.8 kb band derives from the wild-type Lect14 allele, and the 3.0 kb band is diagnostic for the disrupted Lect14 allele (see Fig. 1A). From a total of 87 pups screened, 25 (29%) proved to be genetically wild type, 36 (42%) were heterozygous, and 25 (29%) were homozygous for the disrupted Lect14 allele.
**Lect14 deficient mice are apparently normal**

The structure of the disrupted *Lect14* allele in the homozygous mice was confirmed by a series of Southern blots. Animals identified as homozygous for the mutation in this analysis were further examined using an affinity-purified polyclonal rabbit antiserum directed against L14. These experiments included embryos at different stages of development and various adult tissues. As described earlier, the targeted *Lect14* locus was designed to destroy the coding potential of the *Lect14* gene. Fig. 3 shows the results obtained from a western blot analysis of protein extracted from mutant and wild-type adult muscle tissue. As expected, there was no evidence of the L14 protein in homozygous mutant mice. Similarly, immunostaining of adult muscle tissue sections prepared from homozygous mutant animals failed to reveal immunoreactive L14 protein, which is normally localized on the surface of muscle fibers (Fig. 4E-H). Comparison of the histological appearance of the muscle tissue did not reveal distinct differences between wild-type and mutant samples.

We have previously shown that *Lect14* transcripts are expressed in a large number of tissues in the midgestation embryo (Poirier et al., 1992). As further confirmation of the absence of L14 protein, we examined mutant embryos at different developmental stages for any evidence for L14 expression. As shown in Fig. 4, the *Lect14m/m* embryos proved to be devoid of any immunoreactive L14. For example, Fig. 4B shows the immunofluorescent staining exhibited in the developing bone of a normal 15.5 dpc embryo as detected with anti-L14 serum, where the L14 protein is localized to the surrounding mesenchyme and the ossification center, but is not present in the cartilage. By contrast, no staining was detectable in a similar section taken from a homozygous *Lect14* mutant embryo (Fig. 4D).

Collectively, these experiments clearly verified that the mutation abolishes the *Lect14* gene function. The homozygous mutant animals appear anatomically indistinguishable from heterozygous and wild-type litter mates. Adult homozygotes of both sexes are fully viable and fertile and have been maintained in the laboratory as a homozygous mutant stock for several generations.
Fig. 2. DNA blot analysis of offspring from animals heterozygous for the disrupted Lect14 allele. DNA was digested with Spe and XbaI and hybridized with the S′ probe indicated in Fig. 1A. This probe detects a 1.2 kb fragment common to both wild-type and mutant allele as well as a 1.8 kb fragment unique to the wild-type allele and a 3 kb fragment unique to the disrupted allele. This blot shows the result obtained for 4 homozygous wild-type (+/+) , 6 XbaI and hybridized with the 5′ for the disrupted allele and a 3 kb fragment unique to the disrupted allele. This blot may partly account for our observation that Lect14 mutant embryos implant and develop normally.

DISCUSSION

The S-type lectin L14 is an abundant protein synthesized by the majority of adult tissues. It is also expressed in restricted subsets of cells in the early conceptus, suggesting several potential roles for this molecule at different stages of embryogenesis. These include the attachment of the blastocyst to the uterine wall, the differentiation of the muscle cell lineage and the development of the central nervous system (Poirier et al., 1992; Cooper and Barondes, 1990; Regan et al., 1986). To investigate further the role(s) of this lectin, we introduced a null mutation into the Lect14 gene of the mouse germline using the technique of gene targeting in ES cells.

Because our main focus was to establish the potential involvement of lectins in mediating the process of implantation, we initially examined whether L30 might also be expressed in the peri-implantation embryo. Fig. 5 shows a comparison of the staining patterns obtained using the anti-L14 (Fig. 5B) and anti-L30 (Fig. 5D) antibodies in similar tissue sections from a wild-type mouse blastocyst immediately prior to implantation. Expression patterns of both lectins are strikingly identical at this stage. Thus the cells of the trophectoderm display reactivity for both L14 and L30, whereas the cells of the ICM are negative. In this experiment, some tissues expressing only L14 or L30 were also identified (see for example, the maternal oviduct in Fig. 5) demonstrating the specificity of the antisera employed.

This initial characterization of L30 gene expression in the preimplantation mouse embryo has thus identified L30 as a second S-type lectin whose expression is confined to the trophectodermal lineage. Given the similarity in structure and carbohydrate binding specificity between L14 and L30, it is possible that both molecules have similar or overlapping functions during this developmental stage. This observation
We were particularly interested in a potential role for L14 during the implantation of the embryo. Implantation is a complex process that can be viewed as involving three distinct steps: coordinate maturation of the uterus and the embryo, (ii) attachment of the blastocyst, (iii) invasion of the maternal tissue by the trophectoderm. We postulated that L14 might play a role in the attachment step based on two lines of evidence. Firstly, the onset of Lect14 expression in the trophectoderm is coincident with hatching of the blastocyst just a few hours prior to implantation (Poirier et al., 1992). This observation suggests that L14 could be involved in the establishment of new cell-cell or cell-extracellular matrix interactions occurring at that time. Secondly, the onset of expression of L14 parallels that of LNF1-binding activity on the trophectoderm. LNF1 is a carbohydrate moiety present on the surface of endometrial cells which has been implicated in the attachment of the embryo (Kimber et al., 1988; Lindenberg et al., 1988; Lindenberg et al., 1990). Since LNF1 has been shown in vitro to bind L14 with high affinity (Leffler and Barondes, 1986; Sparrow et al., 1987), it is possible that L14 is the natural ‘receptor’ for LNF1 in vivo and thus mediates attachment to the uterine wall. Our observations that Lect14 m/m embryos implant normally rules out the simple hypothesis that L14 plays a unique role in the attachment of the blastocyst. It could mean that the function of L14 in the late blastocyst is irrelevant to the process of implantation; alternatively, L14 is important for implantation but it is only one of several functionally redundant proteins (perhaps S-type lectins) capable of mediating this step.

To date, only the gene encoding the cytokine LIF (Leukemia Inhibiting Factor) has been directly demonstrated to have a role in implantation. LIF is normally expressed in the developing endometrial glands and its expression is regulated during pregnancy (Bhatt et al., 1991). Stewart et al. (1992) have shown that the uterus of a LIF m/m female is unable to support pregnancy by either wild-type or LIF m/m blastocysts. In contrast both types of embryos can develop in a wild-type uterus. This result clearly implicates LIF in the early step of maturation of the uterus.

While several S-type lectins have been described in various species, they are still very poorly defined at the molecular level. In the mouse, L30 is the only other S-type lectin to be cloned (Jia and Wang, 1988). L30 consists of a C-terminal carbohydrate recognition domain homologous to that of L14 coupled to a domain rich in proline and glycine residues. L30 has been independently identified as the macrophage surface differentiation antigen, Mac2 (Cherayil et al., 1990), a major IgE-binding protein in basophilic
flushed from the uterus and placed in an oviduct to aid sectioning the phase contrast image) and in the oviduct tissue (B). L30 is 4.5 dpc embryos at the time of implantation. Blastocysts were immunohistological detection of L14 and L30 in wild-type Fig. 5. (see Materials and Methods). (A,C) Phase contrast. (B,D) Immunofluorescence with anti-L14 and anti-L30 antisera respectively. L14 is present in the trophectoderm (see arrows in the phase contrast image) and in the oviduct tissue (B). L30 is found exclusively in the trophectoderm cells (D). Bar, 50 µm.

leukemia cells (Laing et al., 1989), and as a tumor-cell-surface lectin (Raz et al., 1990). L30 has been postulated to have a number of roles both inside (Wang et al., 1991) and outside (Cherayil et al., 1990; Woo et al., 1990) the cell. Using two independent anti-L30 antisera, we were able to show that this S-type lectin is indeed present at the time of implantation where it appears to be specifically localized to the trophectodermal cells in a fashion similar to L14. Recently, another group reported finding a similar distribution of L30 in the trophectoderm of the preimplantation embryo (Weitlauf and Knisley, 1992). This finding provides an indication that L30 may play a role at a very early stage of development and, taken together, these results reinforce the idea that lectin-carbohydrate interactions might be important at the peri-implantation stage.

The overlapping distribution of L30 and L14 in the implanting blastocyst suggests that both molecules may have similar biological roles at this stage and raises the possibility that expression of either one of the two S-type lectins may be sufficient to allow blastocyst attachment, thus accounting for the ability of Lect14 m/m embryos to implant normally. The observation that both L14 and L30 have high affinity for LNF1 in vitro (Leffler and Barondes, 1986; Sparrow et al., 1987) is consistent with this interpretation. To test this hypothesis, it will be necessary to investigate the ability of embryos harboring mutations in both genes to undergo normal implantation.

The observations reported in this paper have been made from a study of outbred mice (129 × MF1) that carry the Lect14 mutation. We have recently introduced the Lect14 mutation into the 129/Sv genetically inbred background in an attempt to reveal any subtle aspect of the phenotype. For example, it will be important to study the behaviour of the mutant mice as Lect14 is normally expressed in the nervous system. Analysis of these animals may identify subtle abnormalities. Nevertheless, it is clear from the present results that the absence of the L14 lectin in mice does not have any profound effect on normal development.

There are now several examples of null mutations in a wide variety of genes producing little or no mutant phenotype in the animal. These include PrP (Büeler et al., 1992), tenascin (Saga et al., 1992), MyoD (Rudnicki et al., 1992) or ß1 retinoic acid receptor (Li et al., 1993). The case of MyoD is particularly instructive since its role as an important regulator gene in muscle cell differentiation had been well established in vitro. Moreover, even among the mutations that have given an overt phenotype, in many instances those abnormalities are less pronounced than predicted by the nature or the distribution of the protein e.g. src (Soriano et al., 1991). The general idea that is emerging more and more clearly from these studies is that the mammalian developmental systems are very redundant. The accumulation of all these newly engineered mutations is going to be helpful to the unravelling of these complex circuits of regulation.

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