Genetic ablation of a mouse gene expressed specifically in brain

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The *1B1075* gene was initially identified from a cDNA clone of a rat brain messenger RNA expressed in particular subsets of CNS neurons and pituitary cells. Although the protein encoded by this gene is of unknown function, its sequence suggests that it may be related to secretogranin proteins, which are found in association with secretory granules in a variety of peptidergic endocrine and neuronal cells. Here we show that the mouse *1B1075* gene is located between the *dilute* (*d*) and *short ear* (*se*) genes on chromosome 9. Many different deletion mutations have previously been isolated in the genetic region that includes these genes. By producing mice carrying two deletions that overlap at the *1B1075* locus, the gene for this brain-specific message can be completely eliminated from otherwise viable animals. The animals missing the *1B1075* gene provide an important new tool for determining the function of this gene in the brain. In addition, these results provide a new molecular entry point for detailed characterization of other genes in the *d-se* region.

Key words: chromosome mapping/gene ablation/mouse mutation/neurogenetics

Introduction

In many lower organisms, the functions of cloned genes can be determined by genetic inactivation of the corresponding sequences *in vivo*. This approach is common in bacterial and yeast experimental systems, where efficient strategies for homologous recombination can be used to replace endogenous genes with variants that have been generated in the laboratory (Shortle *et al.*, 1982; Rothstein, 1983). In higher organisms, systems for homologous recombination have only recently become available (Capecchi, 1989). However, in *Drosophila* some cloned genes have been inactivated using a classical genetic approach. For example, a *Drosophila* gene related to the Abelson oncogene has been isolated, mapped to a specific polytene chromosome band and ablated by combining existing chromosomal deficiency mutations that overlap at that region (Henkemeyer *et al.*, 1987). Here we report the use of a similar strategy to ablate a mouse gene encoding a brain-specific mRNA. This result

is based on the fortuitous location of the gene within the *dilute-short ear* (*d-se*) region, one of a small number of mammalian chromosome segments for which large numbers of deletion chromosomes are currently available.

The gene used for these studies was originally identified in a rat brain cDNA library and has been named *1B1075* (H.-P.Ottiger *et al.*, submitted). Transcripts from the *1B1075* gene represent ~0.05% of poly(A)⁺ rat brain and pituitary RNA, but are not detected at significant levels in other tissues of either the rat or the mouse (H.-P.Ottiger *et al.*, submitted). *In situ* hybridization studies have shown that the *1B1075* mRNA is expressed in some anterior and all intermediate pituitary cells and in specific subsets of neurons in the brain, especially in cortical structures. The sequence of the *1B1075* cDNA clone suggests that the corresponding mRNA encodes a novel protein of 533 amino acids. This protein is rich in acidic and paired-basic residues, bears a hydrophobic N-terminal signal sequence, and shows significant similarities to members of the secretogranin protein family. A 57 kd protein encoded by the single *1B1075* gene has been immunologically identified in brain extracts, and appears in immunohistochemistry studies to be concentrated in regions of the brain rich in nerve terminals and to be associated with secretory vesicles (H.-P.Ottiger *et al.*, submitted). The *1B1075* gene thus appears to encode a secreted or compartmentalized, secretogranin-like protein that is expressed in a highly tissue-specific manner in both rat and mouse. Hybridization surveys suggest that *1B1075*-related genes are found in most mammalian species.

Despite extensive knowledge about the structure and the spatial and temporal expression of the *1B1075* gene product, the function of this gene in the brain is not yet known. The mouse analog of this gene has previously been mapped to mouse chromosome 9 in the vicinity of a neurological mutation called *Snell's waltzer* (*sv*). These results raised the possibility that the deafness and locomotor disturbances seen in *sv* mutant mice were the phenotypic consequences of mutations at the *1B1075* locus (Blatt *et al.*, 1985). Here we report additional mapping studies which show that these two loci must be distinct. By combining two previously isolated deletion mutations that overlap at the *1B1075* locus, we also show that the *1B1075* gene can be deleted from the genome without major effects on viability, fertility or locomotor behavior. These results suggest that many of the cell types that normally express the *1B1075* gene product do not require this gene for their own survival and function.

Results and Discussion

Interspecific backcross mapping

Previous mapping studies using recombinant inbred strains suggested that the mouse analog of the *1B1075* gene was linked to the *dilute* (*d*) coat-color locus on chromosome 9 (Blatt *et al.*, 1985). To confirm this finding, we have

Fig. 1. Position of *1B1075* locus by interspecific backcross mapping. The *1B1075* gene was positioned on mouse chromosome 9 using the interspecific backcross mapping data shown on the right. Each column of boxes represents a particular configuration of *dilute* and *1B1075* alleles transmitted to backcross progeny in a cross between (C57BL/6J $\times$ *M.spretus*)F₁ females and C57BL/6J males. C57BL/6J-specific and *M.spretus*-specific alleles are represented by black and white boxes respectively. Only alleles transmitted from the F₁ hybrid parent are shown. The total number of backcross animals observed with each allele configuration is shown beneath the columns. The single animal with a recombination between the *dilute* and *1B1075* loci has previously been analyzed with probes that recognize the *thymus cell antigen-1* (*Thy-1*) locus (a proximal chromosome 9 marker) and the *transferrin* (*Trf*) locus (a distal chromosome 9 marker; Kingsley et al., 1989). Because this animal inherited *M.spretus*-specific alleles of the *Thy-1* and *dilute* loci, and C57BL/6J-specific alleles of the *1B1075* and *Trf* loci, the most likely gene order is centromere-*Thy-1-dilute-1B1075-Trf*-telomere. The map of chromosome 9 shown here is aligned with conventional linkage maps (Davisson et al., 1988) at the *dilute* locus. Restriction fragment length polymorphisms for the various loci are described in Materials and methods.

compared the segregation pattern of the *1B1075* and *d* genes in progeny from an interspecific backcross between C57BL/6J and *Mus spretus* mice (Figure 1). In this system, meiotic crossovers in (C57BL/6J $\times$ *M.spretus*)F₁ females are scored by backcrossing to C57BL/6J males and then examining the resulting progeny for the inheritance of C57BL/6J- or *M.spretus*-specific alleles of particular genes. The wide evolutionary divergence between these two mouse species ensures that virtually any cloned DNA probe will recognize at least some restriction fragment length polymorphisms that can be followed in this cross (Avner et al., 1988). Probes for both the *1B1075* and *d* genes recognized DNA restriction fragment length polymorphisms that distinguished the C57BL/6J and *M.spretus* genotypes. Only one recombinant animal was detected in 173 progeny from the interspecific backcross, suggesting that the *d* and *1B1075* loci are closely linked (0.6 ± 0.6 centimorgans, cM). Analysis of flanking markers in the single recombinant animal indicated that the *1B1075* gene is distal to the *d* gene

(Figure 1). This map location is consistent with the deletion mapping results described below.

Deletion mapping in the *d-se* region

The approximate map location of the *1B1075* gene suggested that the *1B1075* locus fell within an extensively studied chromosomal segment that surrounds the *d* gene and the closely linked *se* gene (0.16 cM distal to *d* on mouse chromosome 9; Goodwins and Vincent, 1955; Russell, 1971). Both the *d* and the *se* mutations have been used as markers in an extensive series of specific-locus mutagenesis experiments designed to measure the effects of chemicals and radiation on the mammalian germ line (Russell, 1951). These experiments led to the isolation of hundreds of additional *d* and/or *se* mutations, many of which also affected surrounding genetic loci. Extensive complementation studies between the various mutations revealed that the *d-se* region contains at least six genes essential for embryonic development (prenatal lethal factors, *pl-1* through *pl-6*), two genes essential for neonatal survival (neonatal lethal factors, *nl-1* and *nl-2*), and one gene associated with deafness and locomotor disturbances (*sv*) (Russell, 1971; Rinchik et al., 1986). With few exceptions, these genes could be ordered in a linear complementation map of the *d-se* region (see Figure 2). Based on these genetic studies, many of the radiation-induced mutations were predicted to be deletion mutations (Russell, 1971). This has been confirmed at the molecular level using probes derived both from the *d* locus and from a proximal region between *nl-1* and *pl-3* (Rinchik et al., 1986).

To position the *1B1075* gene with greater precision in the *d-se* region, we determined whether *1B1075*-homologous sequences were present on chromosomes carrying the different mutations shown in Figure 2. All of these mutations were originally generated by irradiation of (C3H/Rl $\times$ 101/Rl)F₁ or reciprocal hybrids, and therefore must have occurred on either a C3H/Rl or 101/Rl chromosomal background. As shown (Figure 3), the *1B1075* probe detects a *Bam*HI polymorphism that distinguishes the C3H/Rl and 101/Rl genotypes.

Five independent *d-opisthotonus* (C class), one *se waltzing* (Dd1) and eight *se viable* (E class) mutations were available for study in the homozygous condition. *1B1075* sequences were present in DNA samples from animals carrying each of these mutations, and all hybridizing fragments were indistinguishable from those seen in the C3H/Rl or 101/Rl strains (Figure 3 and data not shown). Approximately half of the mutations (7 of 14) resembled the 101/Rl strain at the *1B1075* locus and the remainder resembled the C3H/Rl strain, confirming random induction of mutations on the two chromosomal backgrounds.

Deletion mapping with the other radiation-induced mutations was complicated by the fact that each covers one or more recessive-lethal loci and consequently must be maintained in the heterozygous state with a balancer chromosome. The balancer chromosomes used in these studies were marked with either the *d* or *d* and *se* mutations. Chromosomes of this type resembled the C3H/Rl chromosome at the *1B1075* locus (Figure 3 and data not shown).

Deletion mapping was unambiguous for those mutations that occurred on the 101/Rl background and retained the *1B1075* gene. For example, DNA samples from animals

Fig. 2. Localization of the *1B1075* gene in the *d-se* region. Radiation-induced mutations are grouped in five classes: *dilute-prenatal lethal*, *dilute-short ear-prenatal lethal*, *dilute-opisthotonic*, *short ear-lethal* and *short ear-viable* (A, B, C, D and E mutations respectively). Members of the same complementation group are indicated by the same second letter designation, and individual members of a complementation group are identified by numeral. Mutations that exhibit the 101/R1 *Bam*HI polymorphism at the *1B1075* locus are underlined. Horizontal bars represent the extents of the presumed deficiencies, with thick and thin bars denoting the presence or absence of *1B1075* sequences respectively (see text). The complementation map is drawn linearly, with the centromere at the left (indicated by ●); no correlation with physical distance is implied. Functional units include *pl-1* through *pl-6*, *prenatal lethals*; *nl-1* and *nl-2*, *neonatal lethals*; *d*, *dilute*; *se*, *short ear*; and *sv*, *Snell's waltzer*. 'Skipping' mutations are indicated by dotted lines, and mutations that behave aberrantly in complementation tests are indicated by an asterisk (Russell, 1971; Rinchik *et al.*, 1986).

carrying the Aa3, Ab1, Ac1, Ae1, Af1, Af2, Aj1 and Dc1 mutations each exhibited *Bam*HI fragments characteristic of both the 101/R1 and the balancer chromosome (Figure 3 and data not shown). These results indicate that *1B1075* sequences are present on chromosomes with deficiency mutations that cover the proximal half and the extreme distal end of the *d-se* complementation map (*pl-1* to *nl-2*, *pl-6*; Figure 2).

While DNA samples from approximately half of the *d^{pp}* (C class), *se viable* (E class) and *d-prenatal lethal* (A class) mutants exhibited the *Bam*HI polymorphism characteristic of the 101/R1 strain, all 18 samples from animals carrying the *d-se* (B class) deficiency mutations showed only the fragments characteristic of the balancer chromosome. The absence of 101/R1 fragments in DNA from this large group of mutants suggested that *1B1075* sequences were missing in *d* mutations that extend to or past the *se* gene. This conclusion was supported by the observation that the restriction fragments recognized by the *1B1075* probe appeared to be present at reduced copy number in all of the B class mutations (Figure 4 and data not shown). Similar results were observed for the Ah2, Da1 and Db1 mutations, but not for any of the other A, C, D or E class mutations. These results suggested that the *1B1075* locus was located in the region of common overlap between the B class and the Ah2, Da1 and Db1 mutations. This overlap region extends from *pl-4* to the *se* gene and does not include the *sv* neurological mutation (Figure 2).

Genetic ablation of the *1B1075* gene

The predicted location of the *1B1075* gene suggested that it should be possible to combine the Da1 and Db1 mutations to generate viable mice that lack the *1B1075* locus. Previous

Fig. 3. *1B1075* sequences are present in DNA samples from most A, C and E class mutants. DNA was prepared from frozen tissues of animals of the indicated types. Six microgram samples were digested with *Bam*HI, separated by agarose gel electrophoresis, transferred to nylon membrane and hybridized to the full-length *1B1075* cDNA clone (see Materials and methods). All mutations were originally generated by irradiation of (C3H/R1 × 101/R1)_{F1} or reciprocal hybrids. The Ce4, Ce6, Ea3, Ea8 and Dd1 mutations were analyzed in the homozygous condition. The other mutations, which cause embryonic death if homozygous, were analyzed in the heterozygous state with a balancer chromosome marked with either the *d* or the *d* and *se* mutations (Rinchik *et al.*, 1986). These balancer chromosomes resembled the C3H/R1 strain at the *1B1075* locus. The *d-se* lane is a control from a C57BL/6J-*dse* strain.

complementation studies indicated that the Da1 and Db1 deficiency mutations overlap at the *se* locus, but otherwise extend in opposite chromosomal directions, and do not include any of the same recessive lethal factors (Russell, 1971).

Fig. 4. Deletion mapping *IB1075* sequences in deficiency mutations. DNA samples from animals of the indicated types were analyzed as described in Figure 3 except that the hybridization mixture contained two radiolabeled probes: the *IB1075* cDNA clone and, as a loading control, a 2.7 kb mouse genomic probe (pPAD-1) that hybridizes to a locus on chromosome 11 (Buchberg *et al.*, 1989). The arrow indicates the *Bam*HI fragment recognized by the pPAD-1 probe. The A, B and D class mutations were analyzed in the presence of a balancer chromosome marked with the *d* or the *d* and *se* mutations (see above). The inbred strain DBA/2J is homozygous for the original *d* mutation.

Three matings were established between stocks carrying the *Da1* and *Db1* mutations [(+ *se*^{*Da1*}/*d* +) × (+ *se*^{*Db1*}/*d* +)]. The phenotypes and the presumed genotypes of 61 progeny included 13 dilute, long-eared animals (*d* +/*d* +); 30 dark, long-eared animals (+ *se*^{*Da1*}/*d* + or *se*^{*Db1*}/*d* +); and 18 dark, short-eared animals (+ *se*^{*Da1*}/+ *se*^{*Db1*}). As predicted, the viable, short-eared animals were missing the entire 45 kb set of genomic restriction fragments normally recognized by the full length *IB1075* cDNA probe (Figure 5). These results confirm the location of the *IB1075* gene near the *se* gene, and show that the *IB1075* gene is not essential for viability.

Although the mice missing the *IB1075* gene have short ears, analysis of other mutations has shown that the *IB1075* gene cannot correspond to the *se* gene. For example, the *IB1075* locus is also missing in the *Ah2* mutation, a deficiency chromosome that complements the defect in *se* animals. This result has been confirmed by generating *Ah2*/*Da1* double heterozygotes, which are viable, long-eared animals that lack the *IB1075* gene (data not shown).

Properties of mice missing the *IB1075* gene

To date, phenotypic analysis of the *Da1*/*Db1* heterozygotes has been limited to easily observable traits. The mice are indistinguishable from other short-eared mice, with no abnormalities of gait or balance and no tendency towards the circling behavior seen in many neurological mutations in the mouse, including the *sv* mutation (Green, 1981). Although the *IB1075* locus is widely expressed in extra-pyramidal motor pathways, and in Purkinje cells of the cerebellum, mice missing this gene do not have severe motor defects and do not display the ataxia typical of mice with Purkinje cell defects (Green, 1981). Both male and female *Da1*/*Db1* heterozygotes are fertile, confirming functional development of all neural and hormonal pathways controlling gametogenesis, mating, fertilization, gestation, birth and lactation. The fertility of the mice was of particular interest because the *IB1075* mRNA is normally expressed in cells of the anterior pituitary. Many important hormones are

Fig. 5. *IB1075* sequences are missing in *Da1*/*Db1* heterozygotes. Tail DNA samples were prepared from the progeny of a cross between mice carrying the *Da1* mutation and mice carrying the *Db1* mutation, and analyzed as described in Figure 3. Lanes 1 and 2 originate from dilute, long-eared animals (*d* +/*d* +) that inherited a balancer chromosome from both parents. Lanes 3 and 4 originate from dark, short-eared littermates that inherited both the *Da1* and *Db1* mutant chromosomes. Upper panel: hybridization with the full-length *IB1075* cDNA clone. Lower panel: subsequent hybridization of the same filter with the pPAD-1 control probe.

secreted from various cell types in this gland, including prolactin (required for mammary tissue development and lactation) and follicle-stimulating hormone and luteinizing hormone (required for maintenance of gonads and adult fertility). The absence of dwarfism or gigantism in the *Da1*/*Db1* animals suggests that the growth-hormone-secreting cells of the anterior pituitary can also function in the absence of the *IB1075* gene product.

It is possible that defects will be revealed by future studies of the physiology and behavior of animals missing the *IB1075* gene, by examining the morphology of neurons that normally express the *IB1075* gene, or by examining the detailed function of other cells in the pituitary, such as those that produce adrenocorticotrophic hormone. The overtly normal appearance, behavior and fertility of the animals, however, raises the possibility that the *IB1075* gene may be completely dispensable in mice, perhaps because its normal function can be replaced by the products of other genes. A recent survey reported that ~70% of yeast genes are dispensable for growth and viability (Goebl and Petes, 1986). Comparable numbers are not yet available for mammalian genomes, but the present results clearly indicate that the *IB1075* gene is not required for survival.

Analysis of other naturally occurring and induced mutations has shown that several other mouse genes are also dispensable for survival, including the *albino* locus, the *short-ear* locus, the *glycerol-3-phosphate-dehydrogenase* (*Gdc-1*) locus, the *hypoxanthine-guanine phosphoribosyl transferase* (*Hprt*) locus, several tissue-specific class I loci encoded by the *Q* region of the major histocompatibility complex (MHC) and probably the *agouti* locus and the *pink-eye* locus (O'Neill *et al.*, 1986; Hooper *et al.*, 1987; Kuehn *et al.*, 1987;

Ruppert *et al.*, 1988; Prochazka *et al.*, 1989; Russell, 1989). Mutations at the *Gdc1*, the *Hprt* and the MHC *Q* region loci, like mutations at the *IB1075* locus, also do not produce any obvious phenotypic abnormalities. We believe that these results suggest a need for caution in predicting the result of future genetic inactivation studies in mice. Many genes with interesting patterns of expression may not play an essential role in the development, survival or function of the cells that normally express them. As more and more genes are inactivated in mice using embryonic stem cell technology, it should be possible to determine whether the current results are typical of a large fraction of mammalian genes. The outcome of such studies will allow a critical assessment of the degree of disparity between estimates of gene number based on phenotypically detectable mutations and those based on discernible mRNA species.

Studies of other genes in the *d-se* region

The map location of the *IB1075* gene suggests that this gene will provide an important new entry point for molecular studies of other functional units in the *d-se* region. The *IB1075*, *nl-2* and *pl-4* genes are all predicted to lie within the small 0.16 cM interval between the *d* and *se* genes. Based on the average relationship between genetic and physical distance in the mouse, this interval may correspond to as little as 200–300 kb of DNA. By initiating a chromosome walk from the *IB1075* locus, it should be possible to isolate all of the genes in this interval, and to determine the role of these genes in the developmental and skeletal phenotypes associated with *nl-2*, *pl-4* and *se* mutations.

Materials and methods

Mice

Interspecific backcross progeny were generated from a cross between (C57BL/6J × *M.spretus*)F₁ females and C57BL/6J males (Buchberg *et al.*, 1988). The various mutations from the *dilute-short ear* complex were maintained as previously described (Russell, 1971).

Probes

The *IB1075* probe was a 2.3 kb full-length rat cDNA clone (H.-P.Ottiger *et al.*, submitted). Similar results have been obtained using a mouse brain cDNA clone from the *IB1075* locus (data not shown). The pPAD-1 control probe contains a 2.7 kb mouse genomic fragment mapped to mouse chromosome 11 (Buchberg *et al.*, 1989). The *dilute*, *thymus cell antigen-1* and *transferrin* probes used for the interspecific backcross mapping experiments have been described previously (Kingsley *et al.*, 1989).

DNA isolation and hybridization analysis

DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and probe hybridization were performed essentially as described (Jenkins *et al.*, 1982). Tail DNA samples were prepared as described (Siracusa *et al.*, 1987). All blots were prepared with Zetabind nylon membrane (AMF-Cuno). Probes were prepared by random-hexanucleotide priming (Feinberg and Vogelstein, 1984). Washing was done to a final stringency of 0.5–1 × SSPE, 0.1% SDS, 65°C.

Interspecific backcross mapping

DNA samples from the progeny of a cross between (C57BL/6J × *M.spretus*)F₁ females and C57BL/6J males were digested with various restriction endonucleases and typed for the inheritance of C57BL/6J-specific or *M.spretus*-specific alleles of various genes. A 1.0 kb internal *Hind*III fragment from the *IB1075* cDNA clone detected 10, 3.2, 1.8 and 1.25 kb *Msp*I fragments in *M.spretus* DNA and 3.6, 1.8 and 1.25 kb fragments in C57BL/6J DNA. Restriction fragment length polymorphisms for the *dilute*, *thymus cell antigen-1* and *transferrin* loci have been described previously (Kingsley *et al.*, 1989).

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