

# Gene-Trapping to Identify and Analyze Genes Expressed in the Mouse Hippocampus

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**ABSTRACT:** Mice harboring random gene-trap insertions of a *lacZ* ( $\beta$ -galactosidase)-neomycin resistance fusion cassette ( $\beta$ -*geo*) were analyzed for expression in the hippocampus. In 4 of 15 lines reporter gene activity was observed in the hippocampal formation. In the *obn* line, enzyme activity was detected in the CA1-3 hippocampal subfields, in *hpk* expression was restricted to CA1, but in both lines reporter activity was also present in other brain regions. In the third line, *kin*, reporter activity was robustly expressed throughout the stratum pyramidale of CA1-3, with only low-level expression elsewhere. The final line (*glnC*) displayed ubiquitous expression of the reporter and was not analyzed further. Fusion transcripts for the first three lines were characterized; all encode polypeptides with features of membrane-associated signalling proteins. The *obn* fusion identified a human cDNA (*B2-1*) encoding a pleckstrin homology (PH) domain, while *hpk* sequences matched the Epstein-Barr Virus (EBV) inducible G-protein coupled receptor, EB1-1. *kin* identified an alternative form of the *abl*-related nonreceptor tyrosine kinase *c-arg*. Electrophysiological studies on mice homozygous for the insertions revealed normal synaptic transmission, paired pulse facilitation and paired-pulse depression at Schaffer collateral-commissural CA1 synapses, and normal long-term potentiation (LTP) in *obn* and *kin*. *hpk* mice displayed an increase in hippocampal CA1 long-term potentiation (LTP), suggesting a role for this receptor in synaptic plasticity. *Hippocampus* 1998;8:444-457.

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## INTRODUCTION

The purpose of this paper is to advocate the use of gene-trapping as a procedure to detect and identify genes whose expression is restricted to

particular regions of the adult mouse brain. The rationale for this is that such genes may be of utility, in future transgenic experimentation, to dissect diverse aspects of brain function.

Understanding how memory traces are recorded in the mammalian brain is a major goal of current neurobiological research. Much interest has focused on the hippocampal formation, a brain region held to play a central role in the formation of certain types of memory (Scoville and Milner, 1957; O'Keefe and Nadel, 1978; Squire, 1992). Hippocampal lesions impair spatial, relational, and other forms of explicit memory formation, without effect on procedural learning (Morris et al., 1982; Bunsey and Eichenbaum, 1996; Squire and Zola-Morgan, 1991).

Hippocampal synapses display a form of activity-dependent plasticity known as long-term potentiation (LTP; Bliss and Lomo, 1973), which has been widely discussed as a possible synaptic mechanism underlying learning. LTP is thought to be initiated by calcium influx through the NMDA (N-methyl D-aspartate) receptor (reviewed by Bliss and Collingridge, 1993). Calcium influx via NMDA receptors sets in train a cascade of signals: Particularly important players include the calcium-activated kinases PKC (protein kinase C) and the calcium- and calmodulin-dependent protein kinase type II (CaMKII) (Schwartz, 1993; Roberson et al., 1996).

In attempts to dissect the role of these and other kinases in learning and memory, transgenic mice have been engineered to harbor mutations in the genes encoding the alpha subunit of CaMKII (Silva et al., 1992a,b), *fyn* (Grant et al., 1992), and PKC gamma (Abeliovich et al., 1993a,b). Studies of these animals, and those of other mutant mice, have revealed abnormalities of LTP and/or learning (reviewed by Chen and Tonegawa, 1997). However, the anatomic and temporal nonspecificity of such genetic lesions complicates interpretation (Lathe and Morris, 1994). Potential problems include the genetic background of the animals and residual flanking genes from the strain used to generate

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For simplicity, no systematic distinction is made between the nomenclature for mouse, rat, and human genes and their protein products.

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the targeted mutations (Gerlai, 1996). Moreover, because the gene disruption is present in all tissues and at all stages of development, neither the observed memory deficits nor the impaired LTP can be uniquely ascribed to a loss of gene function in the hippocampal formation in the adult animal at the time of testing (Mayford et al., 1995; Lathe, 1996).

The recent application of the CRE-loxP system to restrict gene-targeted deletions to region CA1 of hippocampus represents one attempt to address some of these problems. This striking work exploited lines of mice in which expression of CRE from the  $\alpha$ CaMKII promoter is serendipitously restricted to specific brain areas (Tsien et al., 1996a,b). As  $\alpha$ CaMKII is not apparently expressed until late in development, the use of this promoter avoids certain developmental effects. Thus, the deletion of NMDAR1 achieved by Tsien et al. (1996a,b) and McHugh et al. (1996) was, to some degree, both site, cell-type, and temporally restricted. However, it is unclear to what extent this approach will be of general application because the site of integration is uncontrolled, the integration of multiple copies may lead to variegation of gene expression (Dobie et al., 1997), and large numbers of transgenic lines may need to be analyzed for each construct.

We have pursued the alternative route of attempting to identify genes expressed selectively in hippocampus. Not only is the identity of such genes of intrinsic interest, but ultimately it may be possible to use them to introduce (by homologous recombination in embryonal stem cells [ES cells]) expression cassettes designed to explore brain function. This approach was encouraged by the previous description of restricted gene expression in other brain regions, for instance of tyrosine hydroxylase in the locus coeruleus and substantia nigra (Berod et al., 1987). While region-specific genes do not in themselves avoid potential problems of expression during development, their ultimate combination with conditional expression cassettes would provide a very valuable research tool.

We have used several procedures to search for genes expressed selectively in mouse hippocampus. Differential screening previously revealed that transcripts corresponding to a new cytochrome P450, *Cyp7b*, are highly enriched in rat hippocampus though restricted expression of the corresponding gene in mouse was less clear (Stapleton et al., 1995). A similar discrepancy was noted for the 5-HT<sub>5B</sub> receptor that is expressed equi-abundantly in rat CA1 pyramidal cells and the medial habenula but which, in mouse, is strongly expressed in medial habenula but only faintly expressed in CA1 pyramidal cells (Wisden and Morris, 1994).

In a further approach, we have investigated the use of gene-trap insertions to detect genes selectively expressed in hippocampus. The procedure relies on random insertions of an expression-defective reporter gene such as the *lacZ* ( $\beta$ -galactosidase)-neomycin resistance fusion cassette  $\beta$ -*geo* (Friedrich and Soriano, 1991; Skarnes et al., 1995) into mouse ES cells. Integration into an endogenous gene can supply promoter and translation initiation signals lacking from the reporter (Skarnes et al., 1992; Skarnes, 1993) leading to expression of both *neo* (G418 resistance) and active  $\beta$ -galactosidase enzyme. These ES cells are then used to generate transgenic mice by blastocyst injection. A major advantage of this approach is that staining of transgenic mouse

tissues staining for  $\beta$ -galactosidase activity reveals the pattern of expression of the endogenous gene 'trapped' by the reporter gene insertion (Skarnes et al., 1992; Skarnes, 1993).

A feature of this technology is that no prior selection of the targeted gene is required, while more sophisticated gene-trap strategies can target insertions to a general class of protein-coding genes, such as those encoding secretory proteins (Skarnes et al., 1995).

The approach has three further advantages. The identity of the trapped gene can be determined by molecular cloning from either genomic DNA or expressed mRNA, taking advantage of linked reporter sequences. Further, insertions often disrupt the target gene: Inspection of the phenotypes of animals homozygous for the insertion can cast light on the biological role of the gene targeted. Finally, the presence of known gene sequences (the reporter) inserted into novel loci (the trapped gene) could be exploited to introduce, by homologous recombination with the reporter sequence, new expression cassettes into the same locus, so avoiding the need for detailed analysis of the target gene structure.

We describe here the study of different lines of adult gene-trap transgenic mice. We have set out to answer four central questions pertaining to the gene-trap approach. First, can random gene-trapping in ES cells detect genes expressed in the mouse hippocampus and, if so, at what frequency? Second, what are the expression patterns of the gene-trap fusions? Third, what are the identities of the genes detected by this method? Fourth, are any genes disrupted by hippocampal gene-trap insertions required for synaptic plasticity in this brain region? Answers to these questions will indicate whether gene-trapping can provide a viable strategy to the identification of genes for exploitation in directing region-specific transgene expression.

## MATERIALS AND METHODS

### Gene-Trap Lines

The gene-trap vector pGT1.8 $\beta$ geo was transfected into mouse strain 129 ES cells, G418 resistant colonies selected, and separately introduced into the blastocysts of recipient blastocysts by previously described procedures (Skarnes et al., 1992; 1995). Animals were backcrossed to C57BL/6 for two generations prior to analysis. Homozygotes were generated by intercrossing between animals backcrossed for two generations; control mice were the nontransgenic or heterozygous mice emanating from this cross. *kin* animals showed reduced transgene transmission in which the transgene transmitted at a reduced rate (only 20/80 typed progeny emerging from multiple crosses between wild-type animals and *kin*/+ heterozygotes  $\pm$  of either sex were found to be transgenic [25% instead of the expected 50%]), though fertility and litter sizes were indistinguishable from controls (not shown). The number of homozygotes emerging from heterozygote intercrosses also appeared to be proportionally reduced, but the low number of such animals precluded statistical analysis.

## Chromogenic Staining for Reporter Gene Expression

10- $\mu$ M sections were fixed in 0.2% glutaraldehyde (Sigma) and stained with X-gal (BRL) as described previously (Beddington et al., 1989); sections were counterstained with neutral red.

## In Situ Hybridization

Antisense lacZ RNA probes (1 kb) were uniformly labelled with  $^{35}$ S-rUTP using T3 polymerase. 10- $\mu$ M serial sections of adult mouse brain were collected on aminopropyltriethoxysilane (Sigma)-coated slides and stored at  $-70^{\circ}\text{C}$ . Slides were processed, hybridized, and washed as described (Cox et al., 1984). Slides were then dehydrated, exposed to X-ray film (3 days), dipped into NTB-2 emulsion (Kodak, Rochester, NY), and exposed for 1–6 weeks. Sections were stained with 0.02% neutral red.

## PCR Cloning of Fusion Transcripts

The RACE (rapid amplification of cDNA ends) protocol (Frohman et al., 1988) as modified (Skarnes et al., 1992) was employed to amplify sequences 5' to the *lacZ* insertion. The protocol involves priming reverse transcriptase with a *lacZ*-specific primer (5'-dTAAATGGGATAGGTTACG), tailing the product with poly-dA, priming second strand synthesis with 5'-dGGTTGTGAGTTCTAGATGG-(T<sub>17</sub>)-3' and PCR successively between nested *lacZ* primers (5'-dAGTATCGGCCTCAGGAAGATCG-3', then 5'-dATTTCAGGCTGCGCAACTGTTGG-3') and the primer 5'-dGGTTCTGAGCTCTTCTAGATGG-3'. This protocol was adapted to permit amplification of sequences from material refractory to standard procedures. First, an expression-selection strategy was devised to allow detection of cloned cDNA products derived from the gene-trap insertion. pBluescript was engineered, by localized mutagenesis (oligonucleotide 5'-dTCTGACAACGATAGGAGGACCGAAG-3'), to remove the *PvuI* site within the ampicillin resistance gene, generating a unique *PvuI* site downstream of the *alpha* segment of the *lacZ* gene within the  $\beta$ -galactosidase coding sequence. Removal of the segment between the polylinker (at the 5' end of the *lacZ* gene) and the *PvuI* site generated a plasmid devoid of  $\beta$ -galactosidase activity. Appropriate insertion of cDNA from the gene-trap line, containing an open reading frame fused in phase with the segment encoding LacZ( $\alpha$ ), was found to restore  $\beta$ -galactosidase activity (not shown). PCR products were cleaved with *PvuI* within the *lacZ* segment (and *XhoI*; within the 5' primers employed) and inserted into the expression-selection plasmid. Following transfection into *E. coli* and growth in the presence of X-gal, colonies staining strongly or weakly with the chromogenic substrate were selected.

Second, poly-dA tailing of first strand cDNA synthesis was replaced by poly-dG tailing. To determine whether long poly-T or poly-dC tracts in the plus-strand cDNA might be incompatible with the development of  $\beta$ -galactosidase activity, artificial poly-T or poly-dC tracts were inserted at the 5' end of *lacZ*; the presence of a poly-phenylalanine or poly-proline stretch was compatible with the development of enzymatic activity (data not shown).

Third, reverse transcription was performed in the presence of 10% dimethylsulfoxide (DMSO), a  $50^{\circ}\text{C}$  heat pulse was used, and priming of second strand cDNA employed random hexameric oligonucleotides according to standard procedures.

PCR products (or cloned products) were subsequently cloned into pBluescript KS and dideoxy sequencing was performed using the Sequenase 2.0 kit (Amersham) according to the manufacturers' specifications.

## RNase Protection Assay

$^{32}$ P-labelled antisense run-off fusion transcripts were synthesized with T3 or T7 RNA polymerase; the reaction treated with DNase I, extracted with phenol/chloroform and unincorporated nucleotides removed by centrifugation through a Sephadex G-50 column. Probes were purified on 6% polyacrylamide gels. Total RNA was extracted from adult mouse brain according to the method of Chomczynski and Sacchi (1987). 5  $\mu$ g of RNA was hybridized (16 h) to  $3.5 \times 10^5$  cpm of purified probe, digested with RNases A and T1 using the RPAII kit (Amersham). Protected RNAs were separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

## Immunohistochemistry

Staining for glial fibrillary acidic protein (GFAP) was performed as described (Storm-Mathisen et al., 1983). Briefly, animals were perfused (30 min) and 200- $\mu$ M sections dehydrated and rehydrated before incubating sequentially with control ovine serum, rabbit anti-GFAP, porcine anti-rabbit, and peroxidase-conjugated rabbit anti-porcine. Slides were developed with diaminobenzidine/ $\text{H}_2\text{O}_2$  and mounted in glycerol/gelatin.

## Electrophysiology

Standard techniques were used to record fEPSPs in stratum radiatum and population spikes from stratum pyramidale of the CA1 region of 400- $\mu$ m thick hippocampal slides obtained from wild type (*wt*) and homozygous *obn*, *hpk* and *kin* mice using procedures in accordance with UK Home Office guidelines. Slices were maintained at room temperature in a submerged recording chamber perfused at 2–5 ml/min with a mouse artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 3 mM KCl, 26 mM  $\text{NaHCO}_3$ , 1.25 mM  $\text{NaH}_2\text{PO}_4$ , 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgSO}_4$ , 10 mM D-glucose and bubbled with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$ . In all experiments synaptic responses were recorded using microelectrodes (1–4 MW) filled with 4 M NaCl. fEPSPs were evoked at 0.017–0.033 Hz in response to stimulation delivered via two bipolar nickel-chromium stimulating electrodes placed in stratum radiatum either side of the recording electrode. Data capture was performed using pClamp 6 software run on a PC and all analysis was performed offline using Clampfit software. Baseline stimulation strengths were sufficient to evoke fEPSPs that were approximately 50% of the maximal fEPSP amplitude. LTP was induced using a single 100 Hz for 1 s tetanus delivered to one or both inputs at test intensity. Experiments were interleaved such that *wt*, *obn*, *hpk*, and *kin* mice were tested in a randomized fashion. Data

are presented as means  $\pm$  standard error of the mean (SEM) and statistical significance assessed using ANOVA performed on raw data with  $P < 0.05$  being taken as indicating statistical significance.  $n$  values refer to the number of times a particular experiment was performed in different slices (in some cases, two slices were taken from a single mouse).

## RESULTS

A promoterless reporter cassette, comprising a splice-acceptor site derived from *en-2* preceding the  $\beta$ -*geo* fusion (Skarnes et al., 1992) [see Figure 2A], was introduced into mouse ES cells. The  $\beta$ -*geo* open-reading frame lacks a translation initiation site: application of G418 selects for insertion events where the reporter has integrated by chance into an endogenous gene locus, generating a fusion linking the N-terminus of an endogenous protein to the reporter polypeptide. Briefly, G418-resistant ES cells were introduced into the cavity of mouse blastocysts, reimplanted into foster mothers, and chimeric males so produced mated to produce offspring heterozygous for the gene-trap insertion. Initially, 15 independent lines were produced by this method, as described (Skarnes, 1993; Wilson et al., in preparation).

### Frequency Of Expression Of Gene-Trap Insertions In The Adult Mouse Hippocampus

To explore expression of the reporter gene in the adult central nervous system, sections of brain from these 15 gene-trap lines were incubated with the chromogenic  $\beta$ -galactosidase substrate X-gal. In 4 of these lines, *obn*, *kin*, *hpk*, and *glnC*, distinct staining was present in the hippocampus (Fig. 1). *glnC*, however, expressed ubiquitously and was not analyzed further. The remaining 11 lines, with the exception of *glnA* (that expressed at barely detectable levels in cerebellum), failed to exhibit reporter gene expression in adult brain (Table 1), of which 6 were negative in all tissues. A frequency of  $\frac{4}{15}$  lines expressing in hippocampus indicates that the gene-trap procedure is a feasible method to identify genes expressed in this brain structure.

### Distinct Expression Patterns in Hippocampal Subfields

In *obn* and *hpk* staining coincided with the cell bodies of the pyramidal neurons (Fig. 1E,F), while staining in *kin* appeared to be displaced from the cell body layer (Fig. 1I,J). Reporter activity was detected in several other brain regions of the *obn* and *hpk* lines, principally throughout the cortex, but staining in brain of *kin* mice appeared to be substantially, but not exclusively, restricted to the CA1–CA3 subfields of the hippocampus (Fig. 1A–D). Reporter activity was detected weakly in the molecular layer of the dentate gyrus and the lateral septum (Fig. 1G,H). Somewhat stronger expression was observed in an interstitial nucleus of the amygdala. Very weak reporter activity was also detected in amygdala, striatum, and olfactory bulb.

Chromogenic staining of brain slices from *kin* animals using the  $\beta$ -galactosidase substrate X-gal revealed that coloration was not localized to the cell bodies of the pyramidal cell neurons; instead, punctate staining was observed within the axo-dendritic fields of the CA1–3 regions of the hippocampal formation (Fig. 1I,J). This region is sparsely populated by cell bodies in normal brain; however, a plausible though unlikely interpretation was that glial expression, coupled with the induction of gliosis by the *kin* insertion, could have generated the observed expression pattern. To address this, slices from *kin* animals were stained with an antibody to an astrocytic marker (GFAP, glial fibrillary acidic protein) and X-gal. As shown (Fig. 1K), X-gal and GFAP staining were not colocalized, indicating that *kin* expression is not astrocytic in origin; there was also neither evidence of gliosis nor of unusual cell proliferation in the axodendritic field.

Staining patterns in non-neuronal tissues of the three primary lines were also analyzed. A number of organs outwith the central nervous system in *obn* and *hpk* mice showed expression (V. Wilson et al., in preparation); *kin* mice also exhibited significant expression of reporter gene mRNA in a number of other tissues in addition to brain (see below).

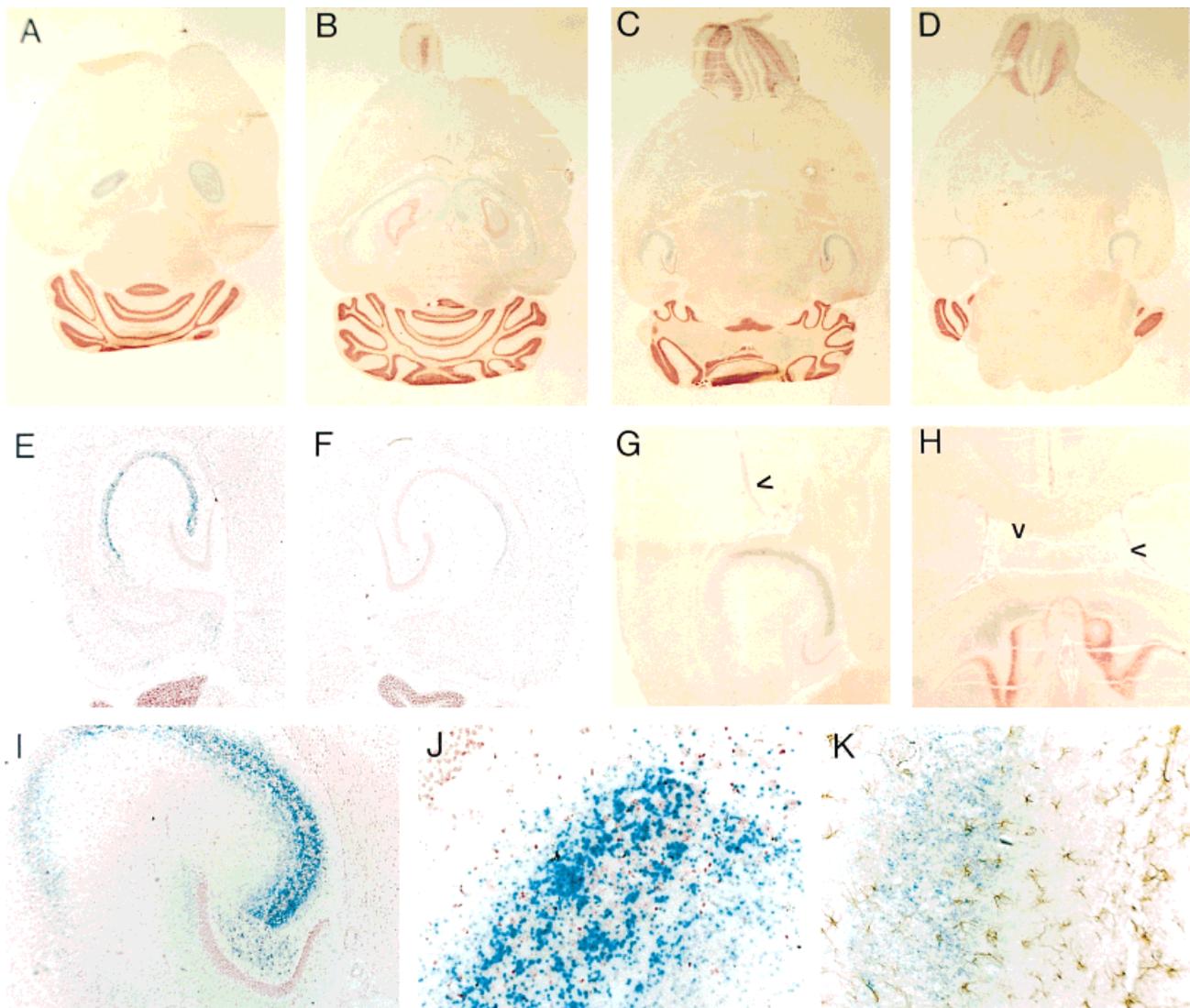
### Identification of Trapped Genes

cDNA cloning of the three gene-trap fusion transcripts was attempted by priming first strand cDNA synthesis with a specific primer (corresponding to the  $\beta$ -*geo* reporter), followed by homopolymeric tailing with A residues. PCR amplification between nested  $\beta$ -*geo* primers and an oligo(dT) primer was performed. For the *obn* and *kin* lines, no reliable PCR product was obtained (data not shown); the same result was obtained irrespective of whether mRNA from ES cell lines, or heterozygous or homozygous adult brain was employed (not shown).

To circumvent this problem, we explored three different modifications to select or enrich for longer PCR products. These involved an expression-selection strategy, tailing with a different deoxynucleotide, and a heat-pulse in the presence of DMSO (see Experimental procedures). All three approaches were successful, though the longest individual 5' cDNA PCR products derived from the DMSO/heat pulse approach. Inserts were sequenced and compared to DNA sequence databases by BLAST searching to identify the endogenous loci harboring the gene-trap insertions.

### Hpk

Database searching revealed that the *hpk* fusion transcript is homologous to a previously described mouse G protein-coupled receptor, EBI-1 (Schweickart et al., 1994). Homology was 98% over the 40 nucleotides sequenced (Fig. 2C). Taking PCR errors and/or strain variation into consideration, we surmise that the *hpk* insertion is within the *EBI-1* transcription unit. However, the *hpk* insertion appears to have taken place into the 5' untranslated region of *EBI-1*, outwith the recorded open reading frame, translation instead taking advantage of an upstream ATG in a favorable context. It is unknown whether this site, possibly in concert with alternative mRNA splicing, is used in vivo to



**FIGURE 1.** Regional expression of  $\beta$ -galactosidase activity in brain sections of gene-trap mice. A–D: Expression in *kin* mice showing expression pattern from most dorsal (A) to most ventral (D) levels of the brain cut in horizontal sections. Note prominent expression of  $\beta$ -gal activity (blue) at all septo-temporal levels of areas CA3 and CA1 of the hippocampus, but absence of strong expression elsewhere in the brain. Original magnification (om)  $\times$  10. E: Expression in *obn* mice in layers II and IV of entorhinal cortex, para-subiculum, within the pyramidal cell layers of CA3 and CA1, and in subiculum. Note absence of staining in dentate and some thinning at border of CA1/CA3.  $\beta$ -gal activity was also apparent in several other areas of the brain, notably the cortex (not shown). om,  $\times$  20. F: Low-level expression of *hpk* in CA1 of the hippocampus and

overlying neocortex. om,  $\times$  20. G,H: Expression of  $\beta$ -gal in *kin* was almost but not quite exclusive to the hippocampal formation and nearby structures. At the temporal pole of hippocampus, prominent expression was also apparent in an interstitial nucleus of the amygdala (arrows). om,  $\times$  20. Expression in *kin* is also apparent in the septum (arrows). I,K: Higher magnification of  $\beta$ -gal expression in *kin* reveals displacement of protein expression from cell-body layer in both CA3 and CA1. Cell bodies stained with haematoxylin and eosin. om  $\times$  40 and 160 respectively. K: Immunocytochemical staining for GFAP showing absence of glial hypertrophy in *kin* mice and no colocalization of *kin*  $\beta$ -gal expression with astrocytic staining (brown). om  $\times$  160.

produce an isoform of EBI-1 protein that is not membrane-associated, as it would appear to lack the N-terminal signal sequence.

### **Obn**

The *obn* insertion demonstrated striking homology to an unknown human cDNA, denoted B2-1 (Liu and Pohajdak, 1992). The extent of homology (89% at the amino acid sequence level) (Fig. 2B) indicates that *obn* is the mouse equivalent of B2-1.

The function of the *obn*/B2-1 gene is unknown. While several suggestive homologies were detected on database searching, most homology was detected (42% at the amino acid level over a 150 residue central domain; Lui and Pohajdak, 1992) between B2-1 and a yeast protein, SEC7, required for intracellular protein traffic (Julius et al., 1984; Tschopp et al., 1984).

Previous database searching identified a number of domains within the B2-1 polypeptide shared by SEC7, a *C. elegans* protein,

TABLE 1.

**Brain Expression Patterns of Lines of Gene-Trap Mice\***

Line	Regions expressing the reporter
<i>kin</i>	CA1–3 (amygdala, striatum, olfactory bulb)
<i>obn</i>	CA1–3, cortex
<i>hpk</i>	CA1, cortex
<i>glnA</i>	(cerebellum)
<i>glnC</i>	Ubiquitous, including hippocampus, dentate gyrus, cortex and cerebellum
	Plus 4 lines negative for brain expression
	Plus 6 lines not expressing detectably in any tissue

\*Cryostat sections of brains from transgenic lines carrying random insertions of the pGT1.8 *geo* gene trap vector were stained with X-gal. Principal brain regions in which detectable  $\beta$ -galactosidase activity are listed; regions in parentheses gave barely detectable staining.

and EMB30, an essential protein in *Arabidopsis* (Shevell et al., 1994). We note a further domain conserved between *obn/B2-1*, SEC7, guanine nucleotide-releasing factor, and a mouse *src*-homology domain (SH3)-binding protein—as well as other proteins (Fig. 2B). It appears likely that the subregion conserved between *obn/B2-1* and other proteins identifies part of the pleckstrin homology (PH) domain (Haslam et al., 1993) that binds phosphatidylinositol 4,5 bisphosphosphate (Harlan et al., 1994), so serving to anchor polypeptides containing PH domains to cellular membranes (Hyvönen et al., 1995; see Discussion). This argues that the *obn/B2-1* protein associates with cellular membranes.

**Kin**

Sequence comparison (Fig. 2D) revealed striking homology between the *kin* cDNA clones and one form of the alternatively spliced gene encoding a nonreceptor tyrosine kinase of the *abl* family, *arg* (Kruh et al., 1986, 1990). Homology was 85% at the nucleotide sequence level. Allowing for interspecific variation in the *arg* sequence, the extent of the homology argues that *kin* identifies the mouse ortholog of *arg*. Expression of the *arg* kinase in brain has been described previously, but no regional localization was reported (Perego et al., 1991).

Both *arg* and *abl* are expressed from alternatively-spliced genes: transcripts originating from distinct upstream exons are spliced to the main body of the transcript comprising the conserved kinase domains (Shtivelman et al., 1986; Ben-Neriah et al., 1986; Kruh et al., 1990). The upstream region of the *kin* fusion transcript is homologous to the 5'-terminus of the MN2 cDNA described by Kruh et al. (1986), indicating that the *kin* insertion is located between an alternative upstream exon and the main coding region of the gene; it is thus possible that the *kin* insertion has not produced a true null mutation within the *arg* gene.

The MN2 alternative splice form of *arg* trapped by the *kin* insertion is most similar to the type B *abl* cDNA of Shtivelman et

al. (1986). In view of the N-terminal homology to consensus myristoylation signals (Fig. 2Dii), both *arg* (*kin* variant/MN2) and *abl* (type 4) are likely to encode proteins that harbor N-terminal additions of myristic acid. Indeed, modification of the type B/MN2 isoform of *arg* by myristic acid addition has been confirmed (Wang and Kruh, 1996). We conjecture that in *kin* animals this modification directs transport of the hybrid protein from CA1–CA3 hippocampal neurons into the axo-dendritic fields surrounding the pyramidal cell bodies.

**Translational Control of Expression of the *kin* Fusion**

We wished to determine whether the enzymatic activity mirrors the expression pattern of the endogenous gene. However, all three lines appear to identify short 5' exon sequences belonging to the trapped genes: The use of such probes would compromise the sensitivity of in situ hybridization. Instead, hybridization using a  $\beta$ -*geo* specific probe was performed. For *hpk* (Fig. 3A,B) and *obn* (Fig. 3C,D), the hybridization pattern mimicked the staining with X-gal, confirming a neuronal site of transcription. In *kin* animals, the hybridization signal also localized to the cell bodies of pyramidal CA1–3 neurones (Fig. 3E,F,H)—confirming that the observed punctate  $\beta$ -galactosidase activity in the axo-dendritic field is neuronal in origin. However,  $\beta$ -*geo* mRNA was much more widely distributed than enzyme activity (compare Fig. 1I with Fig. 3F), suggesting that in many brain regions, including dentate gyrus, the fusion gene is transcribed but not translated (see Discussion).

To exclude the possibility that, for instance, differential splicing might be responsible, RNase protection was used to identify hybrid transcripts in different body tissues. The identical *arg/B-geo* fusion transcript was found to be widely expressed in brain and a variety of body tissues (Fig. 4) that do not stain for beta-galactosidase activity (V. Wilson, personal communication), confirming region-specific translational control of the hybrid transcript.

**One of Three Gene-Trap Insertions Analyzed Affects Synaptic Plasticity**

Gene-trap insertions are often associated with disruption of the targeted locus; for instance, the GT4–2 insertion results in homozygote lethality (Skarnes et al., 1992). To our knowledge, however, there have been no previous studies on gene-trap lines expressing in the mammalian central nervous system. Accordingly, animals of all three lines were crossed to homozygosity and analyzed for a number of physiological parameters. All homozygous animals were normal with respect to gross morphology (body weight unchanged, no indication of runting), overt behavior (normal gait, exploration and grooming in the home cage), and reproduction/fertility and litter sizes [but see comments on the *kin* transgene transmission deficit, below]. Further, there was no evidence of structural abnormalities in hippocampus (Fig. 1) or other brain regions, as revealed by inspection of stained brain sections at the light-microscope level.

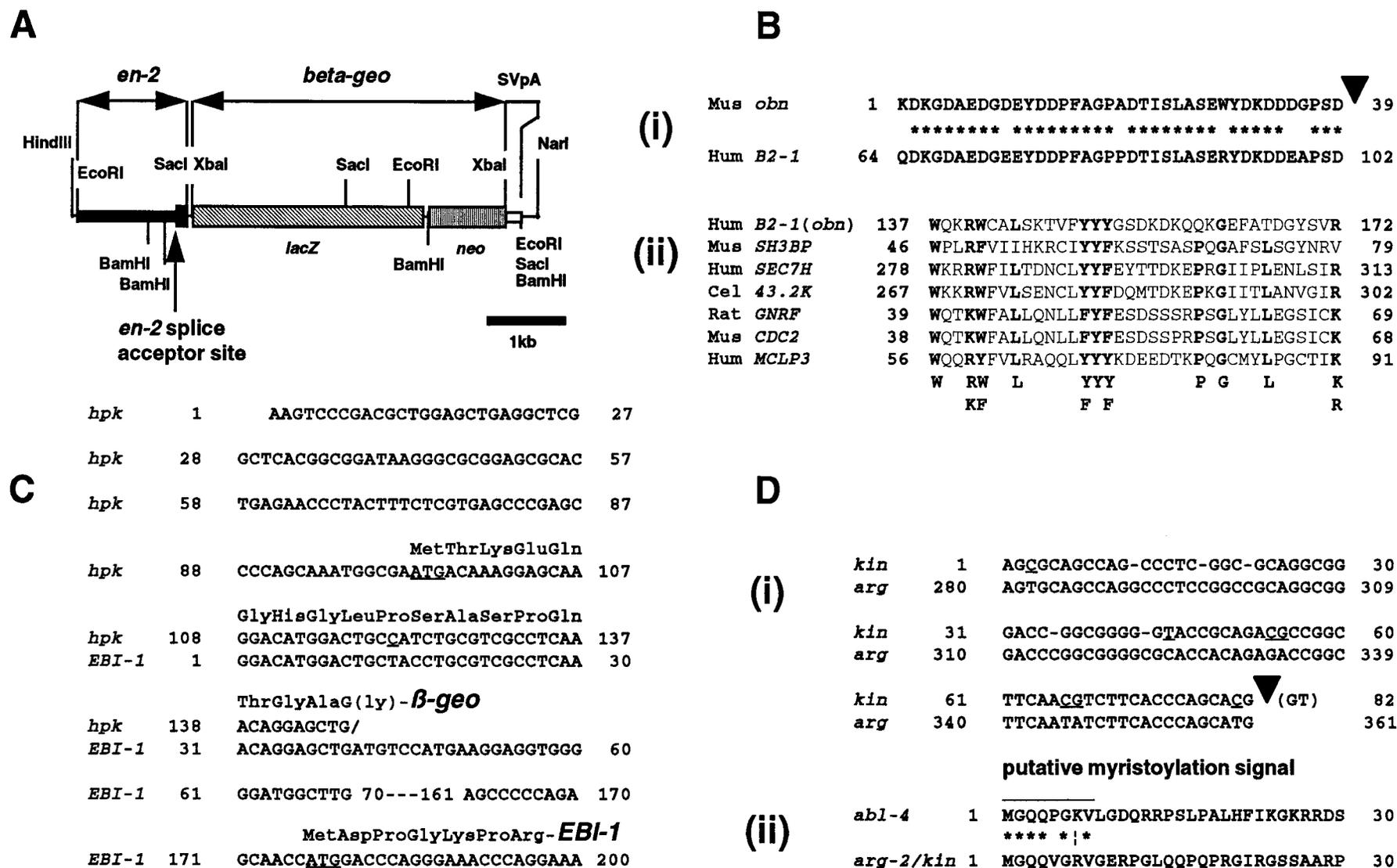
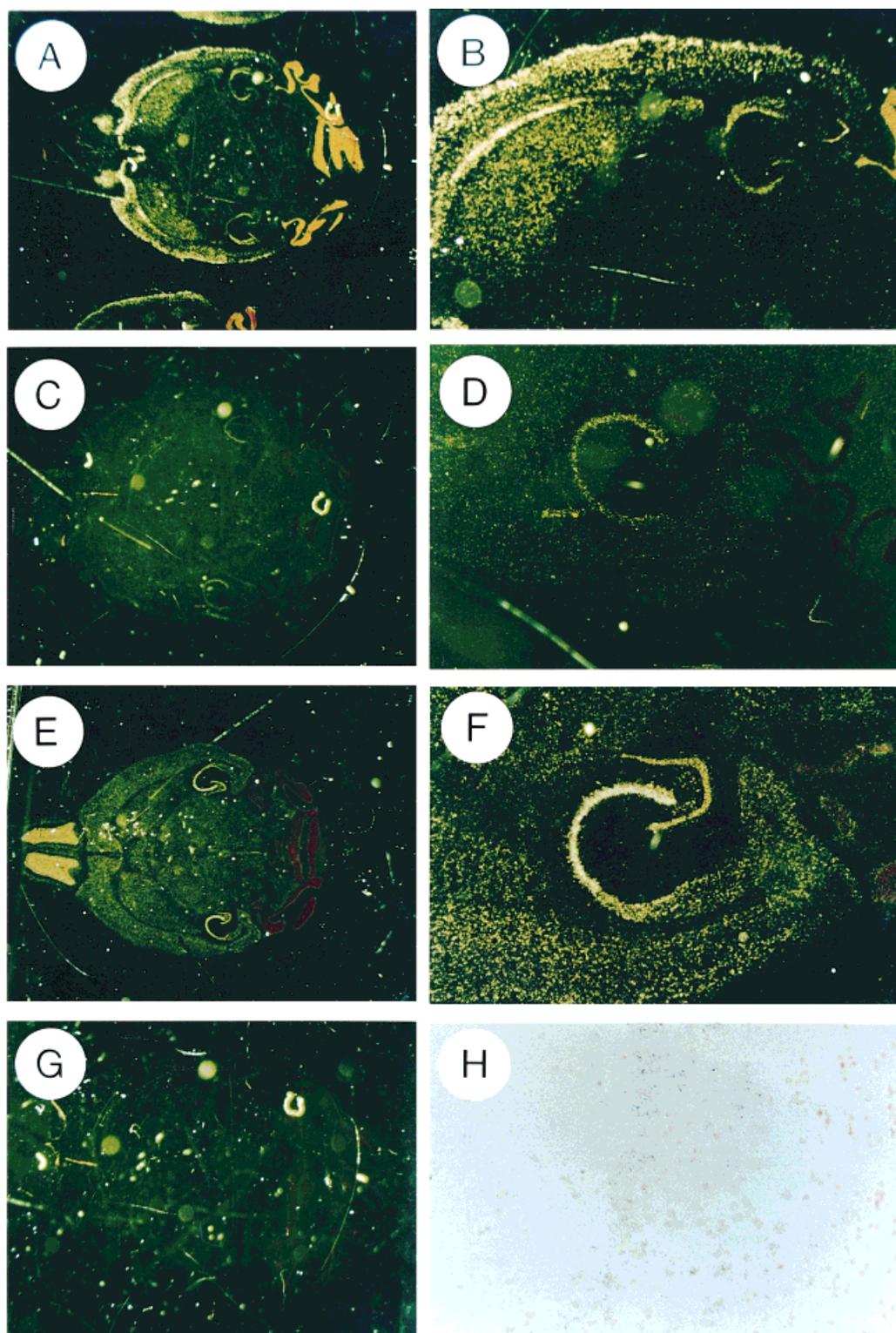
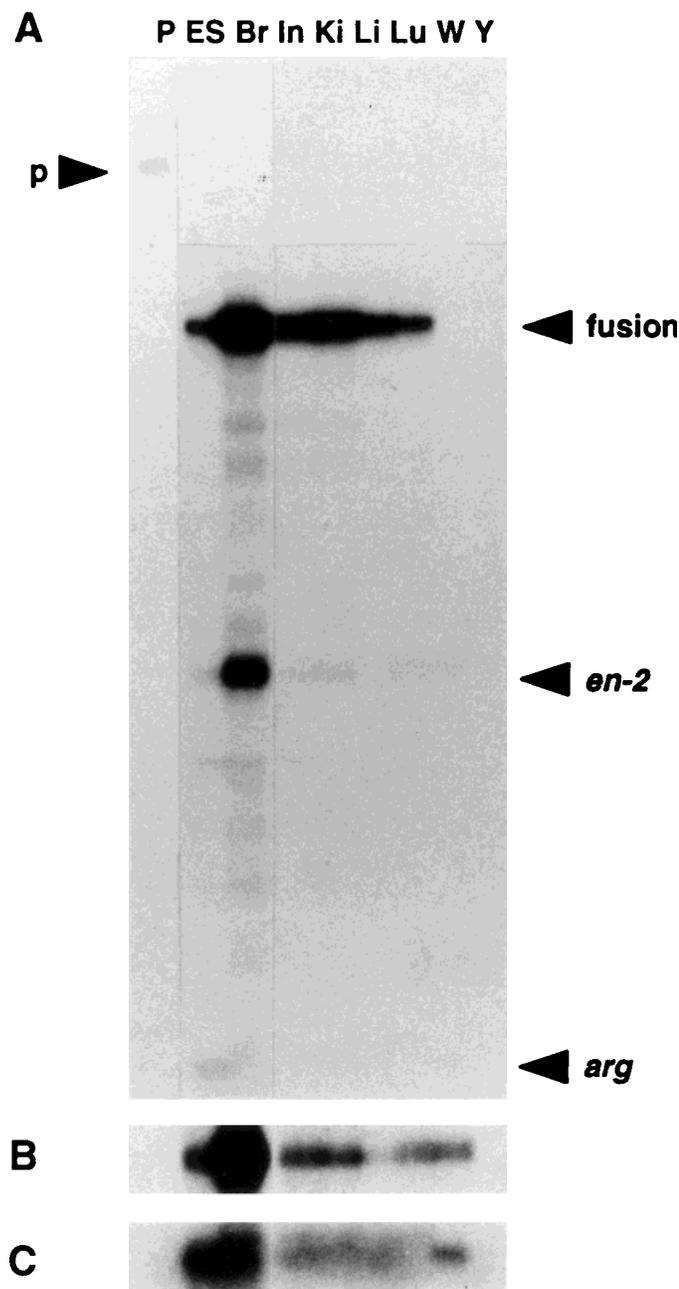


FIGURE 2. Structure of the gene-trap vector and homologies detected between gene-trap cDNA products and database entries. A: Structure of the gene-trap vector employed (Skarnes et al., 1992). B: Sequence homologies between *obn* and other gene products. B1, homology with human protein B2-1; B2, homologies with SEC7H (human homolog of the yeast SEC7 protein), guanine nucleotide releasing factor (GNRF), a *src*-homology (SH3) domain binding protein (SH3BP), a myeloid cell line protein (MCLP3), and a hypothetical 43.2 kD protein from *C. elegans* reveal a highly conserved subregion within the larger pleckstrin homology (PH) domain (see text for details). C: Homology of *hpk* to the 5' region of the Epstein-Barr Virus (EBV) inducible G-protein coupled receptor (*EBI-1*). D: Homology of *kin* to the MN2 splice variant of the abelson-related tyrosine kinase *arg* (D1). N-terminal homologies between MN2 and the type 4 *abl* N-terminal polypeptide indicate conserved residues similar to a consensus signal for myristoylation; the  $\beta$ *geo* fusion polypeptide in *kin* (D2) may harbor an N-terminal myristic acid. In (B) and (D) the arrow (inverted triangle) indicates the point of reporter gene insertion where the hybrid cDNA sequence changes from endogenous gene to reporter gene sequence.



**FIGURE 3.** In situ hybridization analysis of hybrid *lacZ* mRNA expression in gene-trap lines. Following hybridization a *lacZ* probe, sections were emulsion coated and exposed for autoradiography. *hpk* (A,B), *obn* (C,D) and *kin* (E,F,H) animals; note expression in hippocampus (CA1, CA3) in all three lines; *hpk* shows low expression in area CA2 at the boundary between CA1 and CA3; *obn* shows no expression in dentate. In *hpk*, there is strong expression in frontal areas of cortex and striatum; *obn* shows scattered expression outside hippocampus; *kin* shows strong expression in olfactory bulb. Panel

(G) is the signal generated by a nontransgenic littermate. Panel (H) [bright-field] is a high-power magnification of the CA1/3 boundary in *kin*, hybridization grains are superimposed over pyramidal neurons (but not over adjacent neurons in the stratum oriens) rather than being displaced into the axo-dendritic fields as observed for *lacZ* reporter gene activity (Fig. 1J). This reveals different patterns of expression for *lacZ* reporter activity and mRNA. Original magnifications were  $\times 8$  (A,C,E,G),  $\times 16$  (B,D),  $\times 20$  (F) and  $\times 160$  (H).



**FIGURE 4.** Analysis of expression of fusion transcripts in *kin* gene-trap animals by RNase protection. **A:** Lanes were P, probe alone (no RNase), ES, *kin* heterozygote gene-trap ES cells; tissues from *kin* heterozygote animals: Br, brain; In, intestine; Ki, kidney; Li, liver; Lu; lung. Control lanes were W, wild type nontransgenic brain and Y, total yeast RNA. The positions of the probe (p), the fusion transcript and the endogenous *en-2* and *arg* protected fragments are indicated. Exposure time, 16 h. **B,C:** the *en-2* and *arg* regions of the autoradiogram exposed for 60 h.

The hippocampus plays a role in selected types of memory processing, and synaptic plasticity at hippocampal synapses, particularly long-term potentiation (LTP), has been suggested to implement aspects of information storage. Expression in hippocampus raises the possibility that the trapped gene might be involved

in these processes. Accordingly, we examined whether any of the gene manipulations affected hippocampal synaptic transmission using standard extracellular electrophysiological recording techniques.

Field-potentials evoked in area CA1 in response to stimulation of stratum radiatum were found to be comparable in all homozygous mice (i.e., wild-type [*wt*], *obn*, *hpk*, and *kin*). There were no apparent differences in paired-pulse facilitation of fEPSPs at intervals of 25–50 ms (Fig. 5A) or paired-pulse depression (PPD) of population spikes at intervals of 15 ms (Fig. 5B). The apparent slight decline in PPD magnitude in *kin* was not statistically significant.

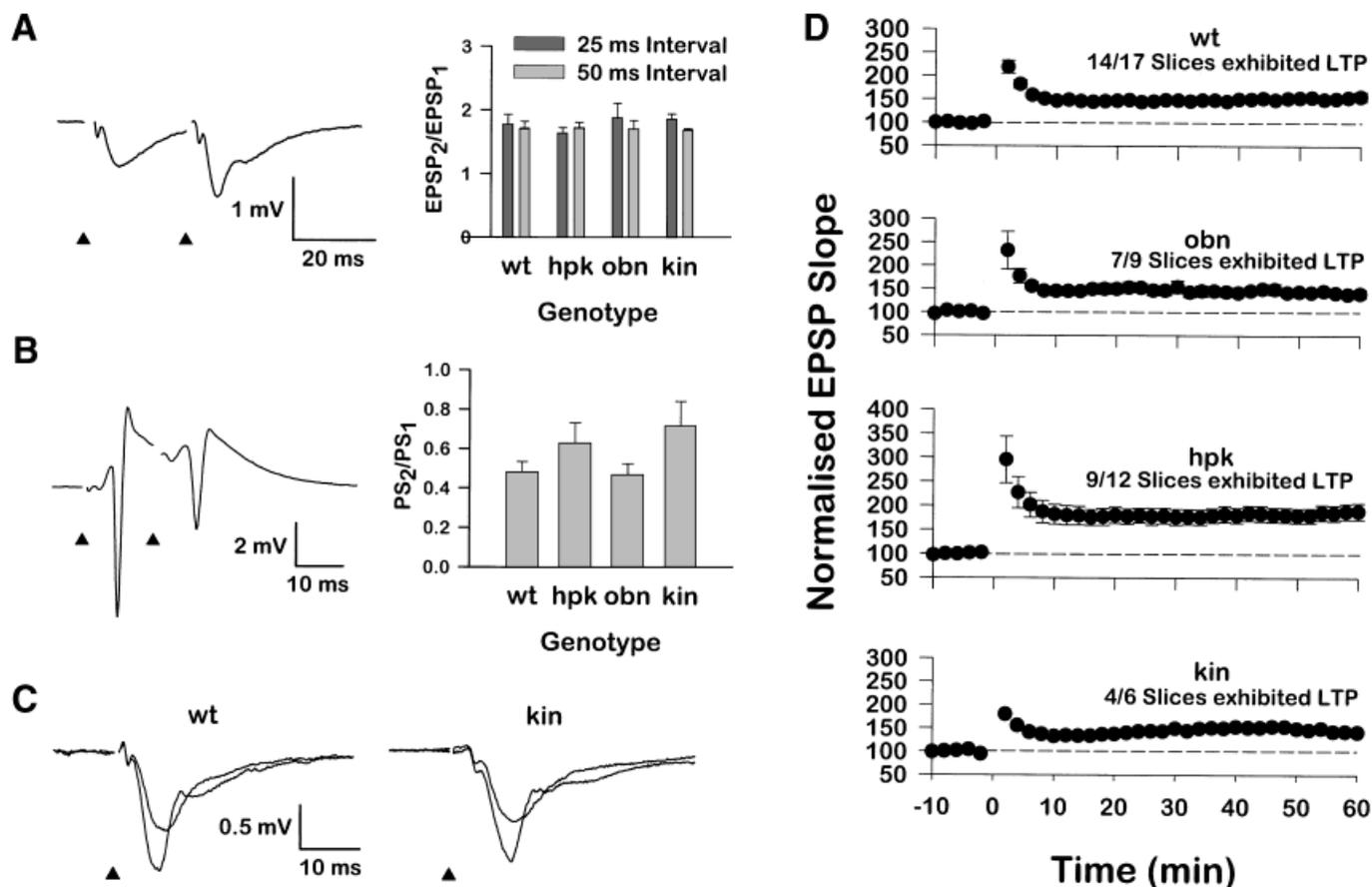
However, differences were observed in the magnitude of LTP. While there was no change in the percentage of brain slices in which LTP was observed (defined as a >10% increase in EPSP slope after 60 min), the magnitude of LTP elicited 60 min after a high-frequency tetanus of 100 Hz for 1 s (Fig. 5D) differed across groups. Statistical analysis showed an overall difference between groups (%LTP across groups: *wt* =  $153.5 \pm 4.5$ ; *obn* =  $140.4 \pm 9.2$ ; *hpk* =  $196.3 \pm 16.7$ ; and *kin* =  $141.8 \pm 12.3$ ;  $F = 4.29$ ,  $df = 3/37$ ,  $P = 0.01$ ). Further analysis using Newman-Keul's test revealed that *hpk* animals showed significantly greater LTP than *wt* ( $P < 0.05$ ), while neither *obn* nor *kin* differed from *wt* controls. The absence of any change in paired-pulse depression in *hpk* argues against a decrease in GABAergic neurotransmission being responsible for this enhancement of LTP. In addition, we initially evoked a maximal amplitude EPSP so that the stimulation intensity could be set to yield a baseline EPSP at 50% of maximum. In all slices (including those from *hpk* homozygous animals), we routinely saw the appearance of only a single population spike—indicating that GABA-ergic inhibition is intact.

## DISCUSSION

The primary findings of this series of experiments are: 1) the discovery of a gene-trap insertion (*kin*) directing reporter activity almost exclusively to the hippocampus, with minimal expression elsewhere; 2) the discovery of a gene (*hpk*) that is expressed in the hippocampus and neocortex whose disruption causes an enhancement of LTP; and 3) the finding that a significant fraction of genes identified by the gene-trap procedure are expressed, not only within the brain, but within the hippocampus. We consider these points in turn, together with additional findings concerning the identity of the genes targeted by the *kin*, *hpk*, and *obn* insertions, and then the implications for the exploitation of these genes in future work.

### Distinct Patterns of Staining in Hippocampus

The pattern of staining in the hippocampal formation was different in each of the three lines examined in detail. All showed reporter activity in CA1, two showed high levels of activity in CA3, and one showed low levels of activity in the molecular layer of the dentate gyrus (DG). These patterns indicate that the spectrum of genes expressed in DG neurones may differ substantially from that expressed in the CA fields, possibly reflecting differential presence of transcription factors in the different



**FIGURE 5.** Comparison of electrophysiological responses in homozygous *wt*, *obn*, *hpk*, and *kin* Mice. **A:** The synaptic trace represents extracellularly recorded field excitatory post-synaptic potentials (fEPSPs) evoked in a wild-type response to two stimuli delivered at an interval of 25 ms and demonstrates paired-pulse facilitation (PPF) of fEPSPs. The associated bar graph (right) plots the PPF ratio, defined as the ratio of the slope of the second fEPSP to that of the first fEPSP at paired-pulse stimulation intervals of 25 (speckled columns) and 30 ms (filled), for homozygous *wt*, *hpk*, *obn*, and *kin* mutant mice. The numbers in parentheses represent the number of slices tested. **B:** The synaptic trace represents extracellularly recorded population spikes evoked in response to two stimuli delivered at 70 Hz and demonstrates paired-pulse depression (PPD) of population spikes. The bar graph (right) illustrates pooled data for the extent of PPD, expressed as the ratio of the peak amplitudes for

the second and first spikes, observed in slices from the four mice genotypes examined. **C:** The superimposed traces represent typical examples of fEPSPs evoked just prior to and 60 min after a 100 Hz for 1 s tetanus in *wt* and *kin* mice. Note that the magnitude of LTP in each case was similar. **D:** Each graph plots the mean  $\pm$  SEM of the slope of the fEPSP, normalized with respect to a 10-min baseline immediately preceding the tetanus, versus time, for slices prepared from homozygous *wt*, *obn*, *hpk*, and *kin* mice. Each point is the average of four successive responses, obtained over a 2-min period, and has been pooled for the number of successful LTP experiments indicated above each graph; inputs in which LTP was not observed were excluded. Error bars are shown where larger than symbols. Tetani (100 Hz for 1 s) were delivered at  $t = 0$ . Note that the magnitude of the LTP in *hpk* mice was twice that of the other groups.

subfields. This specialization of the different subregions of the hippocampus argues that neurones in DG, CA1, and CA3 are functionally distinct at the cellular level, a conclusion borne out by other anatomical and electrophysiological studies (e.g., Hess et al., 1995).

**The *obn*, *hpk*, and *kin* Insertions Identify Membrane-Associated Proteins**

PCR cloning of hybrid transcripts was performed. Some difficulty was encountered in applying this procedure, but the problems experienced were surmounted by altering the experimental protocol (see Experimental procedures). Sequence information

was obtained from cloned products. In two cases, inspection of the sequence revealed regions unusually high in G+C residues, with tracts of greater than 85% G+C (see figures); we surmise that this alone may have hindered PCR cloning. Sequence searching revealed that all three trapped genes encode membrane-associated molecules likely to be involved in signalling processes.

***Kin***

This line was identified as an insertion at the locus encoding the Abelson (*abl*)-related tyrosine kinase, *arg*. This gene, like *abl*, is a non-receptor tyrosine kinase containing characteristic homologies to the *src* oncogene product (Kruh et al., 1990). In *Drosophila* it

appears that a single gene, termed *abl*, is the invertebrate equivalent of both *arg* and *abl* (Shtivelman et al., 1986). *abl* was originally identified in view of its ability to mediate oncogenic transformation; the oncogenic capacity of *arg* is so far unknown. *abl*-deficient animals primarily display immune system deficits and die shortly after birth (Schwartzberg et al., 1991; Tybulewicz et al., 1991) while *arg*-deficient mice have not been reported. Like *abl*, *arg* contains a src-homology (SH2) domain that is functional when exchanged into another protein, but which binds to the breakpoint cluster region (BCR) gene product 10-fold less strongly than when containing the *abl*/SH2 domain (Muller et al., 1992). This domain was also less effective in activating the oncogenic potential of *abl* (Muller et al., 1993).

Expression of *arg* tyrosine kinase in the central nervous system is significant because nonreceptor tyrosine kinases such as *fyn* have previously been implicated in hippocampal function and receptor phosphorylation (Grant et al., 1992; Moon et al., 1994; Rosenblum et al., 1996). Further, the *arg* binding protein ArgBP1 is highly expressed in brain and is the homolog of a brain-specific protein identified in *Xenopus* (Wang et al., 1996).

Although in situ hybridization confirmed that the *kin* insertion is transcribed in hippocampal neurones, reporter gene activity was localized to discrete subcellular structures within the axo-dendritic fields of the CA1–3 regions of the hippocampus. Because the alternative 5' exon trapped by the *kin* insertion encodes a myristoylated form of *arg* kinase, we surmise that axo-dendritic staining is due to transport of the myristoylated *arg*- $\beta$ *geo* fusion polypeptide into cell processes. Although the identity of the substructures targeted by the fusion polypeptide is unknown, myristoylated proteins are often localized in specialized internalization/transport vesicles known as caveolae (Anderson, 1993; Lisanti et al., 1995). However, the relatively sparse distribution of staining may argue that the fusion polypeptide is instead targeted to a more specialized structure of unknown identity.

It is also of note that, although  $\beta$ -galactosidase activity is most prominently expressed in the hippocampus,  $\beta$ *geo* mRNA was very much more widely distributed. This argues that the *kin* insertion is transcribed widely in brain, but is only translated in CA1–3 (though sophisticated alternative splicing patterns cannot be wholly excluded). We conjecture that the mechanism operating to transport the  $\beta$ -galactosidase fusion protein may only be present in hippocampal neurones, and transport failure in other cell types may lead to translational deficits. Such region-specific control of translation adds a further level of complexity to gene expression in the brain. It also has implications for the exploitation of *kin* in further studies (see below).

### Hpk

This gene-trap insertion identifies the mouse gene encoding the Epstein-Barr virus (EBV)-inducible Gprotein-coupled receptor EBI-1. This receptor is most homologous to a family of transmembrane proteins, including C—C chemokine, somatostatin, interleukin-8 (IL8), and neuropeptide Y receptors, and together these appear to constitute a closely-related subfamily within the superfamily of G protein-coupled receptors (Birkenbach et al., 1993).

Previously thought to be a lymphocyte-specific G protein, our data provide evidence for transcription within neurons of the central nervous system. While EBI-1 expression is upregulated by Epstein-Barr Virus infection (Birkenbach et al., 1993) and herpes virus infection (Hasegawa et al., 1994), both regulation of *EBI-1/hpk* transcription in the central nervous system and prospective ligand(s) for the encoded protein(s) are so far uncharacterized.

### Obn

A further gene-trap insertion was found to correspond to an uncharacterized human cDNA denoted B2–1 (Liu and Pohajdak, 1992). The polypeptide encoded by B2–1 is most similar to SEC7, a yeast protein required for intracellular protein traffic. SEC7 encodes a high molecular weight protein (230 kDa) involved in transport between functional compartments of the Golgi (Achstetter et al., 1988; Franzusoff and Schekman, 1989) and which may participate as a component of nonclathrin-coated vesicles that cycle to and from the cell surface (Franzusoff et al., 1992; Kean et al., 1993). We have identified a further homology box conserved between B2–1/obn, SEC7, and other proteins (Fig. 1B). This box comprises part of the pleckstrin homology (PH) domain (Haslam et al., 1993), equivalent to domains 2–4 of Musacchio et al. (1993) and Touhara et al. (1994). The C-terminal half of the PH domain interacts with beta-gamma subunits of G-proteins (Touhara et al., 1994), while the subregion conserved between B2–1 and other proteins is thought to bind phosphatidylinositol 4,5 bisphosphonate (Harlan et al., 1994) and tether the protein to cellular membranes, arguing that the gene identified by the *obn* insertion is also a membrane-associated protein.

### Phenotypes: Genetic Transmission Deficit in *kin* and Accentuated LTP in *hpk* Animals

To examine possible phenotypes generated by the *obn*, *hpk*, and *kin* insertions, the animals were crossed to homozygosity. No overt phenotype was recorded, and no neuroanatomical abnormalities were noted. In the *kin* line, however, there was a selective deficit of transmission of the (heterozygous) transgene from either male or female transgenic parents, with the frequency of transgenic offspring being approximately two-fold reduced over that expected. We have no explanation for this result, but because the same deficit is observed in the transmission of the transgene to heterozygotes, homozygote lethality cannot be invoked as an explanation.

None of the homozygous insertions appeared to affect paired-pulse facilitation or paired-pulse depression, nor the frequency at which LTP could be induced. However homozygous *hpk* mice displayed an intriguing increase in the magnitude of LTP, the degree of potentiation being approximately twice that of wild-type and the other mutant lines examined. This may be of some interest for, to our knowledge, of all the diverse gene disruptions analyzed to date (Chen and Tonegawa, 1997) only one, the disruption of the glutamate receptor GluR2 (Jia et al., 1996), has led to an increase rather than an impairment of LTP. Although we

cannot rigorously exclude contributing genetic background effects, an increase in the extent of LTP would appear unlikely to result from residual donor genome because mice of the inbred 129 and related strains display, if anything, deficits rather than improvements (Upchurch and Wehner, 1988; Gerlai, 1996).

This finding raises the possibility, therefore, that the G protein-coupled receptor, EBI-1, identified by the *hpk* insertion, may act normally to suppress some aspect of neuronal excitability and/or synaptic plasticity. This result is not unprecedented, for mice deficient in neuropeptide Y, a transmitter whose receptors are similar to EBI-1/*hpk*, are predisposed to spontaneous and pharmacologically induced seizures (Erickson et al., 1996); no LTP studies were reported with these mice. However, our results could suggest that drugs targeting the EBI-1/*hpk* receptor may have potential in conditions of excess neuronal excitation such as epilepsy and ischemia.

### Frequency of Gene Expression in Hippocampus

An unexpected outcome of this work is the high frequency of gene-trap expression in the hippocampus. In 4 of 15 lines analyzed, enzyme activity was present in the hippocampus though activity was detected in other brain regions in addition to the hippocampus. It is to be noted that gene-trapping appears to be largely unselective regarding the ES cell expression of the genes targeted, for insertions have been obtained into genes that express at extremely low levels (i.e., where  $\beta$ -galactosidase activity is not detectable); but this very low level of expression is still sufficient to confer resistance to G418.

To assess whether this elevated frequency is significant, we analyzed a further 13 independent lines (not presented here). Among these 13 new lines, 6 were confirmed nongenetrapped events (and were excluded), while 4 of the remaining 7 expressed in the hippocampus (not presented). Thus, in our studies to date, 8 out of 22 gene-trap lines have revealed expression in the hippocampal formation. Together our data argue that some 37% (21% to 59%, 95% confidence interval) of genes within the mouse genome, and transcribed by RNA polymerase II, are expressed in the hippocampus. This result is consistent with reports of unusually high RNA complexity in brain (e.g., Grouse et al., 1980; Snider and Morrison-Bogorad, 1992; Takahashi, 1992).

### Implications

The original objective of this study was to identify genes selectively expressed in the hippocampus. Once identified, our aim has been to exploit this regional specificity to investigate hippocampal function. For instance, we have attempted to exploit the *kin* insertion to engineer mice in which LTP would be disrupted, in selected subfields the hippocampus, through the specific expression of a transdominant inhibitory peptide of protein kinase C (PKCip). However, using two different constructs, we found that embryonal stem (ES) cells harboring the kin-PKCip fusion could not be propagated, suggesting that even low levels of expression of the inhibitor are incompatible with ES cell growth.

Another promising approach involves site-specific expression of CRE recombinase in order to engineer animals that could be cross-bred with other lines in which target genes have been flanked by LoxP sites (e.g., Tsien et al., 1996a,b). A further approach is likely to involve the use of inducible transcriptional activators, such as rtTA (Gossen et al., 1995) or the ecdysone receptor (No et al., 1996), in combination with region-specific gene expression.

This work underlines the power of the gene-trap approach to detect and define the function of genes expressed in specialized subsets of neurons in the brain. Further, they will ultimately permit the role of such specialized neurons in brain function to be addressed. It is hoped that the high frequency of gene-trap expression in the hippocampus and other neocortical structures reported here will encourage the study of further novel gene-trap patterns of brain expression.

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