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Gene Trap Expression and Mutational Analysis for Genes Involved in the Development of the Mammalian Nervous System

A. STOYKOVA, K. CHOWDHURY, P. BONALDO, M. TORRES, AND P. GRUSS*
Department of Molecular Cell Biology, Max Planck Institute of Biophysical Chemistry, Göttingen, Germany

ABSTRACT **We have used a large-scale gene-trap approach for the isolation and mutation of genes that might play roles in the developing nervous system. After in vitro integration of two different gene trap vectors (pGT1.8geo: Skarnes et al. [1995] Proc. Natl. Acad. Sci. USA 92:6592–6596; IRESβgeo: Chowdhury et al. [1997] Nucleic Acids Res. 25:1531–1536) in mouse embryonic stem (ES) cell lines, we created 64 transgenic mouse lines. The expression analysis of the reporter gene during embryogenesis of heterozygous embryos revealed 47 lines with a variety of patterns. Around one-third (36%) of these gene trap lines showed spatiotemporal expression that was either restricted predominantly in the developing nervous system (11 lines; 17%) or widespread but with very high levels of expression in the nervous tissue (12 lines; 19%). In most cases, a correlation was found between the in vitro and the in vivo patterns of the reporter gene expression. Thus far, preliminary mutant analysis of 16 gene trap lines with potentially interesting expression patterns in the developing nervous system showed that mice homozygous for eight (50%) insertions were lethal, whereas the homozygous mice from five gene trap lines (31%) showed a lower than expected Mendelian ratio of live homozygous animals. Analysis of β-galactosidase reporter gene expression during embryogenesis has shown that four transgenic lines are useful *lac*Z in situ markers for specific regions of the developing nervous system. Here, we discuss some in vivo and in vitro selection criteria that may increase the number of the trapped genes potentially involved in the control of neural development and some future strategies to improve further the efficiency of the gene trap approach. *Dev. Dyn. 1998;212:198–213.***

Key words: gene trap; mouse; development; nervous system; *lac*Z in situ markers

INTRODUCTION

During development, the vertebrate nervous system is faced not only with the task of generating the main cellular types, neurons and glia, but also with the production of enormous numbers of phenotypically diverse neuronal cells that are located in distinct brain structures and organized into functional units through specific axonal pathways. In a search for new genes that might have roles in establishing the complex organization of the developing nervous system, we recently applied a large-scale gene-trap approach. In this method, a promoterless reporter and selection marker gene (βgeo) containing a splice-acceptor site at the 5′ end is introduced into mouse embryonic stem (ES) cells. Subsequently, individual ES cell clones are isolated and used to generate viable mouse chimeras in which the expression patterns of the tagged gene can be visualized through β-galactosidase (β-gal) reporter gene activity. The gene trap approach has a number of already proven advantages compared with the classical methods for functional analysis of the genome: 1) The vector insertion into the genome is random (Chowdhury et al., 1997). 2) The trapped genes are tagged molecularly (Gossler et al., 1989). 3) Cloning of the tagged genes is achieved easily by using the 5′-rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) method (Frohman et al., 1988; Chowdhury et al., 1997). 4) Expression of the tagged gene can be traced by simple histochemical staining of the reporter β-gal gene (Friedrich and Soriano, 1991; Skarnes, 1992). 5) It is possible to enrich for specific classes of genes either in vitro, by a preselection of the tagged ES cells (Forrester et al., 1996), or in vivo, by the expression pattern of the reporter gene. 6) Finally, and most important of all, depending on the site of integration of the vector, a complete or partial knock out can be created (Friedrich and Soriano, 1991; Skarnes et al., 1992; Chowdhury et al., 1997).

Previously, we reported on the feasibility of large-scale cloning of trapped genes from cell lines generated by using a βgeo vector (pGT1.8geo; Skarnes et al., 1995) and a new IRESβgeo vector containing internal ribo-

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A. Stoykova, K. Chowdhury, P. Bonaldo, and M. Torres all contributed equally to this paper.

P. Bonaldo's current address is Institute of Histology and Embryology, University of Padova, Via Trieste 75, I-35121, Italy.

M. Torres' current address is Departmeno de Immunologia y Oncologia, Centro Nacional de Biotecnologia, Universidad Autonoma, Madrid 28049, Spain.

*Correspondence to: Peter Gruss, Department of Molecular Cell Biology, Max Planck Institute of Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany. E-mail: pgruss@gwdg.de

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somal entry sites (IRES) between the splice acceptor and the βgeo sequences (Chowdhury et al., 1997). We found that the IRESβgeo vector is a more efficient tool for trapping large numbers of potentially interesting *lac*Z-positive ES clones. In this paper, we report the observed in vivo *lac*Z expression patterns during the gene trap screen and the results obtained so far from the phenotypic analysis of the corresponding homozygous mutant mice. We also present a more detailed expression analysis of two known [microtubule-associated protein 1B (MAP-1B) and receptor protein tyrosine phosphatase kappa (R-PTPκ)] and two novel trapped genes that reveal these lines as useful *lac*Z in situ markers for neurobiological studies.

RESULTS AND DISCUSSION

In Vivo Expression of the Trapped Genes and Criteria for Selection of "Potentially Interesting" Genes

The ES cell clones were obtained by application of either the newly designed IRESβgeo vector (Chowdhury et al., 1997; electroporations XVI and XVIII) or the conventional βgeo vector (PGT1.8geo; Skarnes et al., 1992). The ES cell clones were preselected according to their in vitro expression patterns and, in several cases, also according to their responsiveness to various growth/ differentiation factors, as described in Experimental Procedures.

Different criteria have been applied for in vivo selection of "potentially interesting" lines in enhancer-trap (Soininen et al., 1992) or in gene trap (Skarnes et al., 1992; Wurst et al., 1995) expression screens. In most of these studies, screening for expression was performed at only one or a few developmental stages by using chimeric embryos, which, depending on the task of the study, may give satisfactory results. However, the production of chimeric embryos is a time-consuming process that severely limits the speed of characterizing the expression patterns. In addition, the β-gal staining may show large variability in chimeric embryos, depending on the contribution of the ES cells, and it may hinder the interpretation of the expression pattern. To circumvent these problems, we performed a large-scale analysis on the dynamics of *lac*Z expression during embryogenesis (and, in several cases, after birth) by using heterozygous mice for the gene trap insertion. This allowed us to

Abbreviations

1-VI	layers 1-VI of cerebral cortex
3V	third ventricle
AA	amygdala
ACC	anterior commissure
Bra	branchial arches
CA1–CA3	areas of hippocampal Ammon's horn
Cbll	cerebellum
CC	corpus callosum
ChPl	choroid plexus
CNS	central nervous system
CP	cortical plate
Cx	cortex
DG	dentate gyrus of hippocampus
DRG	dorsal root ganglion
DT	dorsal thalamus
Ent	entorhinal cortex
Ey	eye
FP	floor plate
Fr	frontal cortex
GE	ganglionic eminence
Gl	glomerular layer of olfactory bulb
GrL	granular layer of cerebellum
Hb,HB	hind brain
Hi	hippocampus
HM	homozygous offsprings
Hph	hypophitis
Ht	heart
HT	hypothalamus
IC	internal capsule
I–VI	layers I–VI of cerebral cortex
Kd	kidney
L	liver
Lm	limb, limbs
Ln	lungs
Ls	lens
LV	lateral ventricle
MB	midbrain
Mi	mitral cell layer of the olfactory bulb
Mn	mandibula
Ms,MS	mesencephalon
Mt	metanephros
Mx	maxilla
Nch	notochord
NCX	neocortex
Ne	neuroepithelium
NE	nasal epithelium
NP	nasal pit
NR	neural retina
NT	neural tube
Ob,OB	olfactory bulb
Occ	occipital cortex
OpV	optic vesicle
OT	olfactory tract
OV	otic vesicle
Par	parietal cortex
PC	posterior commissure
Pir	piriform cortex
PL	Purkinje cell layer of cerebellum
Pn	pons, pontine nuclei
PNS	peripheral nervous system
Ps	pancreas
PT	pretectum
R	red nucleus
RA	retinoic acid
Rb	ribs
RP	roof plate
Sc,SC	Spinal cord
SCl	superior colliculus
Scm	sclerotome
SE	septum
Sm	somites
Sn	snout
T	telencephalon
Tc	tectum
Tg	tegmentum
Tng	tongue
Vb,VB	vertebrae
VG	trigeminal ganglion
VIIG	fascial ganglion
VZ	ventricular zone
WM	white matter of the brain
ZL	Zona limitance intrathalamica

TABLE 1. *lac*Z Expression Patterns in Heterozygous Embryos/Pups Obtained With Gene Trap Cell Lines

Cell line	Gene	Stage	Expression pattern[a]	Phenotype, remarks
VII-28 (Fig. 3A)	Spnr	E9.5 E10.5 E12.5	Entire CNS++; body mesenchyme+ As above; T, Hb, SC+++; Di, Ms++; Ey, OV+++; Bra, Lm+++; Ht, genital ridge, L+ As above	Live HM* (71 pups: 13 −/−, 40 −/+, 18 +/+)
VII-45 (Fig. 1B)	αE-catenin	E9.5 E11.5 E13.5	VZ of entire CNS+; VZ-T++; Ey, OV++ VZ-Cx, Ls, NR+++; Ms, Hb, SC, RP++; Sm, Lm, Mt+ As above; epithelium of Ln, Ps, Kd, gut, urogenital system++	Preimplantation lethal (Torres et al., 1997)
VIII-6	nd	E8.5 E10.5 E12.5	Ne of NT+++; paraxial mesoderm++ VZ of alar NT, RP, FP, motoneurons, DRG, VG+++; NR, Ls+++; Sm (myotome), caudal sclerotomal condensations, Ht+++ As above, body mesenchyme++	Sublethal (108 pups: 19 −/−, 57 +/−, 32 +/+)
VIII-15	nd	E9.5 E11.5 E13.5	Entire Ne+++ T, Ms, Hb, SC, Ey, Lm, Ht+++; body mesenchyme+ As above; epidermis, mesenchyme++	nd
X-7	Multiple RACE products	E9.5 E10.5	Liver++ Liver++	nd
X-91	New (ORF)	E9.5 E11.5 E13.5	Entire CNS, PNS+; body mesenchyme++ As above; SC; motoneurons, Lm, Sm+++ As above, stronger; digits++; L−	nd
X-218 (Fig. 1A)	EST (T31439, D20245)	E9.5 E11.5 E13.5	VZ of entire CNS+ VZ of CNS++; RP, Ey, caudal Sm+ As above; NR, Ls, VG, DRG++; Ht+	Live HM*
XIII-43	EST (R40887)	E10.5 E11.5	Brain, SC, Scm, Ht+ As above; body mesenchyme+	Live fertile HM (19 pups: 3 −/−, 11 +/−, 5 +/+)
XIII-45	New (ORF)	E10.5 E12.5 E14.5	T, Ms, Hb, SC+ Basal T, Ms roof, nasal Ne++; Hb, SC, DRG++; caudal Sm, Lm+ As above; Rb, Vb+; mesenchyme++	nd
XIV-109 (Fig. 2A)	New (ORF)	E10.5 E12.5 E14.5	Bra, Sn, OV, Lm+++; body mesenchyme++ Epithelia+++ (ear, oral cavity, gut, bronchi, pericardium, genitourinary tract); Lm (perichondrion), connective tissue++ As above, stronger; vibrissae, Ln, esophagus, intervertebral disks+++.	Live HM*; (29 pups: 5 −/−, 14 +/−, 10 +/+)
XIV-138 (Fig. 3B)	New (ORF)	E9.5 E11.5 E13.5	Entire CNS+, Sm+ SC, Hb, Tg-Ms+++; Bra, Sn, Lm, Ht+++; body mesenchyme++; DRG, Sm+ As above, stronger; Tc of Ms++	Exencephalus; lethal (in preparation)
V-1 (Fig. 2C,D)	EST (T66211, F12483)	E10.5 E12.5 E14.5	L+++; Ht++ L+++ L+++	nd
XV-53	New (ORF)	E12.5 E14.5 E16.5	T (basal), Ms, Hb, SC, Bra, Ht+ As above; Lm, Sn, body mesenchyme++ As above; RP, nasal Ne, Tng+; Kd, Vb+++	Lethal; (48 pups: 0 −/−, 32 +/−, 16 +/+)
XVI-16	New (ORF)	E9.5 E11.5 E13.5	T, Ms, Hb, SC, body mesenchyme+ Entire CNS, PNS+++; Bra, Scm, Lm+++ As above; body mesenchyme++	Repressed by RA Lethal
XVI-21	New (ORF)	E10.5 E12.5 E14.5	T (medial wall)+; tip of tail+++ As above; Bra+ As above; caudal Sm++	Activated by RA nd
XVI-23 (Fig. 3D)	NF-κB	E11.5 E12.5 E13.5	Ubiquitous mesh-like+ As above; Mx, Mn, Bra++ As above++	nd

TABLE 1. (Continued.)

Cell line	Gene	Stage	Expression pattern[a]	Phenotype, remarks
XVI-43 (Fig. 1C)	− Strand of methyl malonyl CoA mutase promoter	E8.5 E9.5 E11.5 E12.5 E14.5 Adult brain	Extra- and embryonic tissues+ T, Hb, OpV, Bra, Ht++; Sm, Lm+ T++; Ms, Hb, SC, Lm+; Bra, Ht, Ey++ T+++; Ms, Hb, SC (RP, alarVZ)++; Ht+++; Bra, Sn, body mesenchyme++ As above; Ls, DRG+++; ChPl++ OB, Hi, CX− Occ+; ChPl in all ventricles++	Lethal (106E/12L; E10.5–E18.5; 0 −/−, 63 +/−, 38 +/+)
XVI-52 (Fig. 3E)	EST (D25304)	E10.5 E12.5	Widespread mesh-like++ As above; T, Nch, Ht, Sm++; Lm, epidermis++	Activated by NGF Live fertile HM (56 pups: 11 −/−, 35 +/−, 11 +/+)
XVI-56 (Fig. 2 E–H)	LINE (L1)	E11.5 E12.5 E14.5	Head and body mesenchyme, Lm, paraxial mesoderm++ As above, stronger As above; dermomyotomes, back and limb muscles+++; SC (intermediate columns)++	Live fertile HM
XVI-60	EST (R31173)	E10.5 E12.5 E14.5	Body mesenchyme, Sm++; T+ As above; Ht, VB, Rb++ As above; Kd, Ht++; L−	nd
XVI-75	New (ORF)	E8.5 E11.5 E12.5 E14.5 Adult brain	Extra- and embryonic tissue+; head++; presomitic mesoderm++ T, Ms, HB, SC+++; widespread++ As above; Sn, Lm, caudal Sm, Ht++ T, OB, Hb, SC+; Vb, Kd++; mesenchyme+ Hi (CA1+++; CA3, DG+); OB, Cx++; WM (ACC, IC, CC)++; GrL, WM of Cbl +	Activated by follistatin; lethal (in preparation)
XVI-78 (Fig. 1F–I)	MAP-1B	E9.5 E10.5 E13.5	Early axonal tracts, cephalic ganglia (VG, VIIG), T (basal plate)+ As above; stronger++; Ey++; Lm+ Whole CNS, PNS+++	Live HM* *lac*Z in situ marker for early axonal tract in CNS and PNS
XVI-80	2-Arylpropionyl-CoA mutase	E10.5 E12.5 E14.5	Nch+++; Bra, Sm++; body mesenchyme++ GE+; Tg-Ms, SC (FP, RP)+; Lm, Ht, ventral body mesenchyme, Mx, Mn++; As above	Live fertile HM (65 pups: 14 −/−, 35 +/−, 16 +/+)
XVI-87	Line (L1; X59224)	E10.5 E14.5	Ht++; nasal pit, Bra+ Tip of nose++; vibrissae+; DRG+	Activated by follistatin nd
XVI-91 (Fig. 1E)	New (ORF)	E8.5 E10.5 E12.5 E14.5	Neural fold++; Sm+ T (Cx anlage)+++; Ms, PT++; PC+++; DT+; ZL+++; NR (dorsal)++; Sm, Lm, Ht++ T+++; Ms (post.)++; Bra+++; SC (motoneuron column), Lm++ Faintly ubiquitous+; Lm (stripes), Rb+++; inner ear++; L, Ln−	Lethal (110E/9L; E10.5–E18.5; 0 −/−, 76 −/+, 37 +/+)
XVI-97	New (ORF)	E10.5 E12.5 E13.5 E15.5	T++; MS, Hb, border HB/MB, Sm, Lm+ As above; T+++; Ey, Ls, DRG, Sm++; body mesenchyme++ As above, stronger	Repressed by RA, activated by NGF; Lethal (11 pups: 0 −/−, 7 −/+, 4 +/+) E14.5: (15E: 0 −/−, 12 −/+, 3 +/+)
XVI-126	nd	E12.5 E13.5 E15.5	ChPl in all ventricles+ As above; Sm (caudal)+ As above; Sn, Tng, Rb, Lm, Kd++	nd
XVI-129 (Fig. 5A–C)	New (ORF)	E11.5 E13.5 E14.5 E16.5 Adult brain	SC, Hb, T++; Ht+++ As above, NR, RL, DRG, Bra, Lm++ As above, OB, Cx, Ms-roof++; Ln, Kd++ As above, lower zone of CP Lower (V/VI) layers of Fr Cx+++; upper (IV/III) layers of Par, Occ Cx++; Pn, SCl, R, GrL, PL ++	Live fertile HM *lac*Z in situ marker for developing Cx
XVI-132 (Fig. 2B)	nd	E11.5 E12.5 E13.5	Bra, Ls, Lm, Scm+; body mesemchyme++ As above; diffuse in entire CNS+ As above; Bra, Sn++; L−	nd

TABLE 1. (Continued.)

Cell line	Gene	Stage	Expression pattern[a]	Phenotype, remarks
XVI-136	EST (Z19131, T7400)	E11.5 E13.5 E14.5	VG, VII–VIIIG++; Sm, Ht+++ As above; Bra, Sn++; L− T-roof, Ms, DRG+++; widespread++	nd
XVI-168 (Fig. 2K,L)	nd	(Chimera) E11.5 E12.5	 Widespread mesh-like+ As above; vessels, Sm, Lm++	 nd
XVI-169 (Fig. 4)	RPTPk	E9.5 E10.5 E11.5 E14.5 E18.5 Adult brain	Sm, FP, OpV, Neural folds+++ Brain (mesh-like staining)+; Bra++; FP of Ms; OV, NP++, Dorsal NR+++ Lm+++ As above; mesh-like expression in T-Cx+ Scm, FP, RP, Ht, Msph+++ As above; T-Ctx, SC, Vb, DRG, vibrissae Brain: Neo-Ctx+++; other domains, mesh-like staining++ Cx (layer IV)++	Live HM* E10.5–P1.5 (79 offspring/8L: 5 −/−, 54 −/+, 20 +/+) *lac*Z in situ marker for developing neocortex, dorsal NR, brain vascular system
XVIII-47 (Fig. 5D–F)	New (ORF)	E10.5 E12.5 E16.5 E18.5 P2.5	Ventral body mesenchyme, Ht++ As above; OB anlage++; SC, Hb, Basal T, DRG+ OB+++, nasal Ne++, OT++ As above OB (Mi, Gl, Gr layers)++; OT, Pir+++	Live fertile HM *lac*Z in situ marker for developing olfactory system
XVIII-72	TUPLE1 enhancer-like	E9.5 E11.5 E12.5	Ht+++; body mesenchyme+ As above; Bra, Lm, Sm, Rb++ As above; T, Ms, SC+	Live fertile HM
XVIII-73 (Fig. 3C)	New (ORF)	E9.5 E10.5 E12.5	SC, Hb, Ms, Sm++; Bra+ As above; T, Bra, Lm, Ey+++; body mesenchyme+; H, L− As above; Scm, Sn++	nd
XVIII-79	g-COP	E11.5 E13.5	T, MS, Hb, SC, Ey, Ht++; Lm, epidermis+ As above; Cx, Ms, RP, FP, DRG++; ear, Hph, Kd++; widespread++	nd

[a]+++, High; ++, moderate; +, low β-galactosidase staining; nd, not determined; live HM*: live homozygous offspring with lower than expected Mendelian ratio (further determination with probes specific for the isolated gene is needed to prove whether the phenotype is embryonic sublethal because of the insufficient statistics or because of the abnormal high number of the −/+ animals determined by quantitative Southern analysis); sublethal, based on convincing results for lower than normal Mendelian ratio for the −/− offspring; E, embryos; L, litter; HM, homozygous offsprings; NGF, nerve growth factor; MAP, microtubule-associated protein; RACE, rapid amplification of cDNA ends. For abbreviations, see list.

analyze the expression in as many stages as necessary to either discard or accept a line as "potentially interesting."

The complex organization of the nervous system is reflected in the far greater numbers of genes that are expressed either specifically or abundantly in nervous tissue compared with other tissue (Sutcliffe et al., 1984). Because these genes exert complex spatiotemporal expression profiles in the developing nervous system, we classified the whole-mount β-gal staining pattern as "potentially interesting" after analyzing at least two litters at three different embryonic stages: embryonic days 9.5/10.5, E11.5/12.5, or E13.5/14.5. Lines that scored negative at these time points were also analyzed at later stages up to E18.5 or even after birth. In several cases, the *lac*Z expression was also examined on vibratome or paraffin sections of isolated embryonic or postnatal brains. The potentially interesting gene trap lines were processed further for cloning of the integration site, whereas the transgenic animals were crossed for initial phenotypic analysis. A summary of the results of this screen is presented in Table 1.

Among 64 heterozygous lines that were analyzed for *lac*Z expression during embryogenesis, 17 (26%) were found to be negative (see Table 2). The spatiotemporal expression patterns detected in the remaining 47 gene-trapped lines were grouped into two main classes, which were determined by either restricted patterns (group 1) or widespread patterns (group 2). The lines with restricted patterns were divided mainly between two subgroups: those restricted predominantly to the developing nervous system, including few other tissues (subgroup 1.1; 11 lines; 17%), and those restricted predominantly outside the nervous system (subgroup 1.2; 11 lines; 17%). Because numerous genes that are important functionally for neural development are expressed abundantly, but not exclusively, in the nervous system (MAPs, enolases, adhesion molecules), we subdi-

TABLE 2. Classification of the *lac*Z Expression Pattern (Total 64 lines)

1. Restricted expression[a]	
1.1 Predominantly in CNS	11 lines (17%)
1.2 Predominantly outside CNS	11 lines (17%)
2. Widespread	
2.1 Highly abundant in the CNS and widespread	12 lines (19%)
2.2 Faintly ubiquitious	7 lines (11%)
2.3 Strongly ubiquitous	6 lines (9%)
3. Negative	
During embryogenesis (E9.5–18.5)	17 lines (26%)

[a]CNS, central nervous system; E, embryonic day.

vided the widespread expression patterns into three subgroups: those with extremely abundant expression in the nervous system plus faint-to-moderate, widespread expression (subgroup 2.1; 12 lines; 19%); those with faintly ubiquitous patterns (subgroup 2.2; 7 lines; 11%); and those with strongly ubiquitous expression patterns (subgroup 2.3; 6 lines; 9%). Representative examples of the expression patterns of each group are shown in Figures 1–3. Obtaining wider information for the reporter-gene expression was of greatest importance for the discrimination of lines with ubiquitous/widespread but abundant expression in the developing nervous system (subgroup 2.1) from those that appeared uniformly ubiquitous. In some cases, the strikingly higher level of reporter gene expression in the nervous system compared with other tissues was visible only after examination of sections, especially when prominent staining was present in the epidermis, giving the impression of a completely ubiquitous pattern after whole-mount staining. The main difference between the faintly or strongly ubiquitous expression patterns was seen in the level of expression. For further molecular and phenotypic characterization, we selected the trapped lines with expression patterns restricted predominantly to the nervous system (subgroup 1.1) and those with particularly strong expression of the reporter gene in the developing nervous system, although it was widespread otherwise (subgroup 2.1). Together, these account for 23 germ lines that represent 36% of the transgenic lines tested in this expression screen.

By using this strategy, we learned that performing a more detailed in vivo expression analysis in heterozygous gene trap embryos during a wide developmental window contributed significantly to the enlargement of the numbers of potentially interesting genes. For instance, a large gene trap screen that tested only at E8.5 of embryogenesis showed that most of the expression patterns were either ubiquitous or negative (Wurst et al., 1995). Such a screen, for instance, would also consider the expression of the follistatin-inducible gene trapped in the line XVI-75 as constantly ubiquitous, although its expression pattern becomes highly restricted to specific brain domains at prenatal stages and after birth (see Table 1). An expression screen limited only to early embryonic stages would miss the intriguing expression patterns in the developing nervous system of the genes captured in lines XVI-129, XVI-169, and XVIII-47 that suggest important functions for these genes in cortical patterning and for development of the brain olfactory system, as described below.

In Vivo Versus In Vitro *lac*Z Expression Patterns of the Gene-Trap Lines

The comparison of the observed in vivo and in vitro β-gal staining in ES cell clones obtained by using the two vectors revealed a good correlation that is in accordance with previous findings (Baker et al., 1997). Ubiquitous/widespread expression patterns in vivo were found in about 70% of the lines with ubiquitous staining in vitro. In contrast, most lines with restricted in vitro staining showed highly restricted spatiotemporal expression patterns in developing embryos (Bonaldo et al., in preparation). For example, the ES clones XVI-78, XVI-91, and XVI-168 showed a "mosaic" of strongly and faintly stained cells in distinct colonies that correlated with a restricted in vivo expression, which was confined predominantly to the developing central nervous system for the lines XVI-78 (Fig. 1F–I) and XVI-91 (Fig. 1E) or to the developing vascular system for the line XVI-168 (Fig. 2K,L). Similarly, the observed highly restricted, in vivo *lac*Z expression in the developing olfactory system of the gene trap line XVIII-47 (Fig. 5D–F) correlated with a faint "dot-like" staining of only a portion of the cells in the colonies. We also tested the embryonic *lac*Z expression of 13 lines that were negative for β-gal staining in vitro to ascertain whether the tagged genes could be activated later during development. Twelve of these lines were still negative in E9.5–18.5 embryos.

We previously reported on the usefulness of the IRESβgeo vector for capturing a wide range of distinct type of genes effectively (Chowdhury et al., 1997). By using this vector and a simple protocol for the detection of genes that are regulated by specific growth (nerve growth factor; NGF) and differentiation factors (follistatin, retinoic acid; see Experimental Procedures), we detected several gene trap integrations that were either activated or repressed, imply-

Fig. 1. (Overleaf.) Examples of whole-mount β-galactosidase (β-gal) staining in heterozygous embryos showing restricted expression predominantly in the nervous system (Table 2, group 1.1). The embryos are from the following gene trap embryonic stem (ES) cell lines: X-218 (**A**), VII-45 (α-E-catenin; **B**), XVI-43 (**C**), XVI-21 (**D**), XVI-91 (**E**), and XVI-78 (MAP-1B; **F–I**). Embryos are shown at stages embryonic day 12.5 (E12.5; A), E11.5 (B–E,H), E9.5 (F), and E10.5 (G). In D, the arrows indicate the restricted *lac*Z expression in the medial wall of the telencephalon and at the midbrain/hind brain boundary, respectively. In F, the arrows mark the *lacZ*$^+$ axonal tracts extending from early differentiating neurons in the mesencephalon. Note the strong signal delineating the cephalic ganglia (trigeminal and fascial ganglia; VG and VIIG, respectively). In G, the large and small arrows point to extending axons from the cephalic ganglia and from the spinal cord, respectively. I is a view of a sagittal section of the whole-mount stained embryo shown in H illustrating the strong β-gal staining of axons (arrowheads) that extend from the trigeminal ganglion (VG) and from the neurons of nasal epithelium (NE). For other abbreviations, see list.

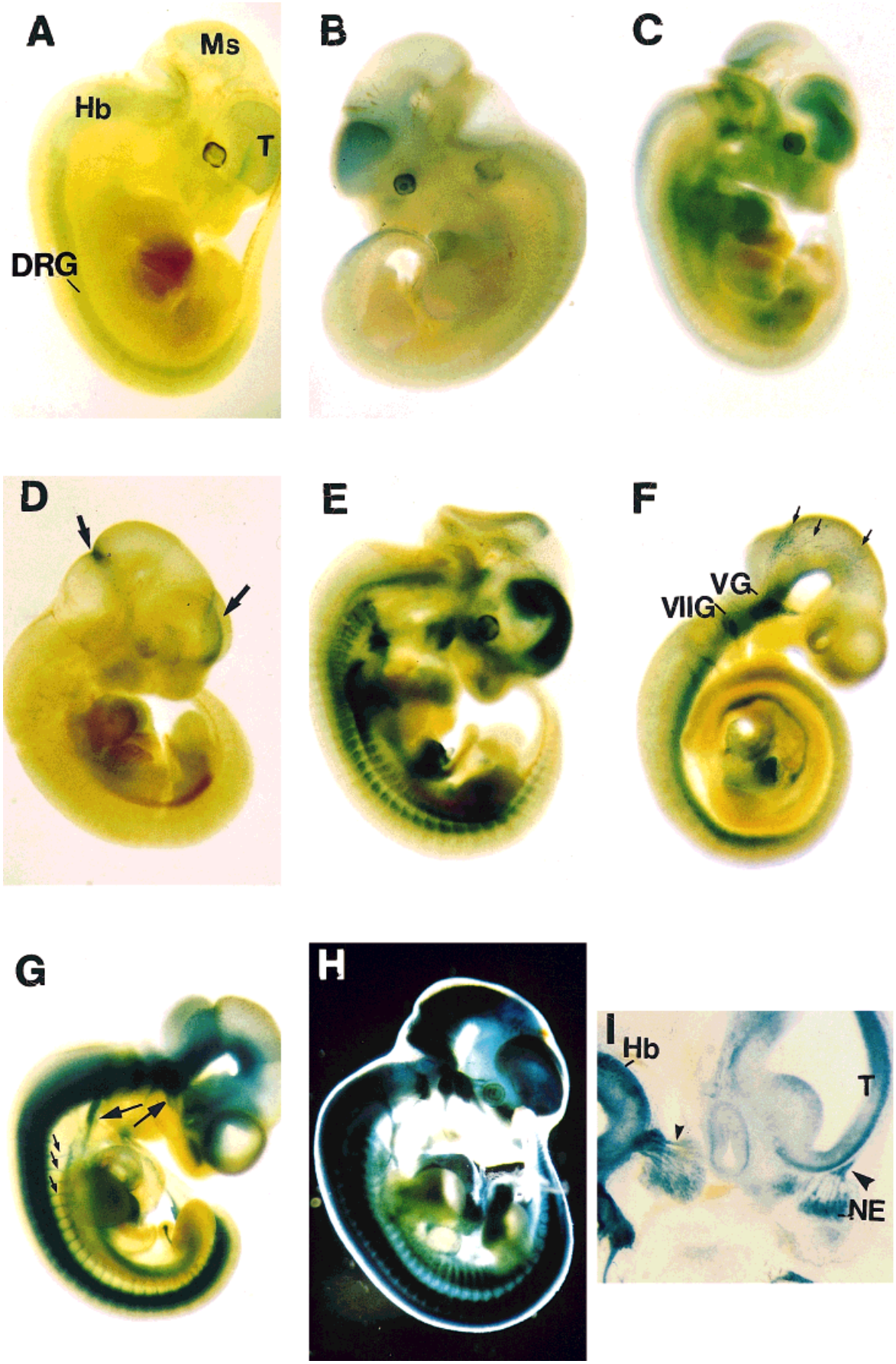

Figure 1.

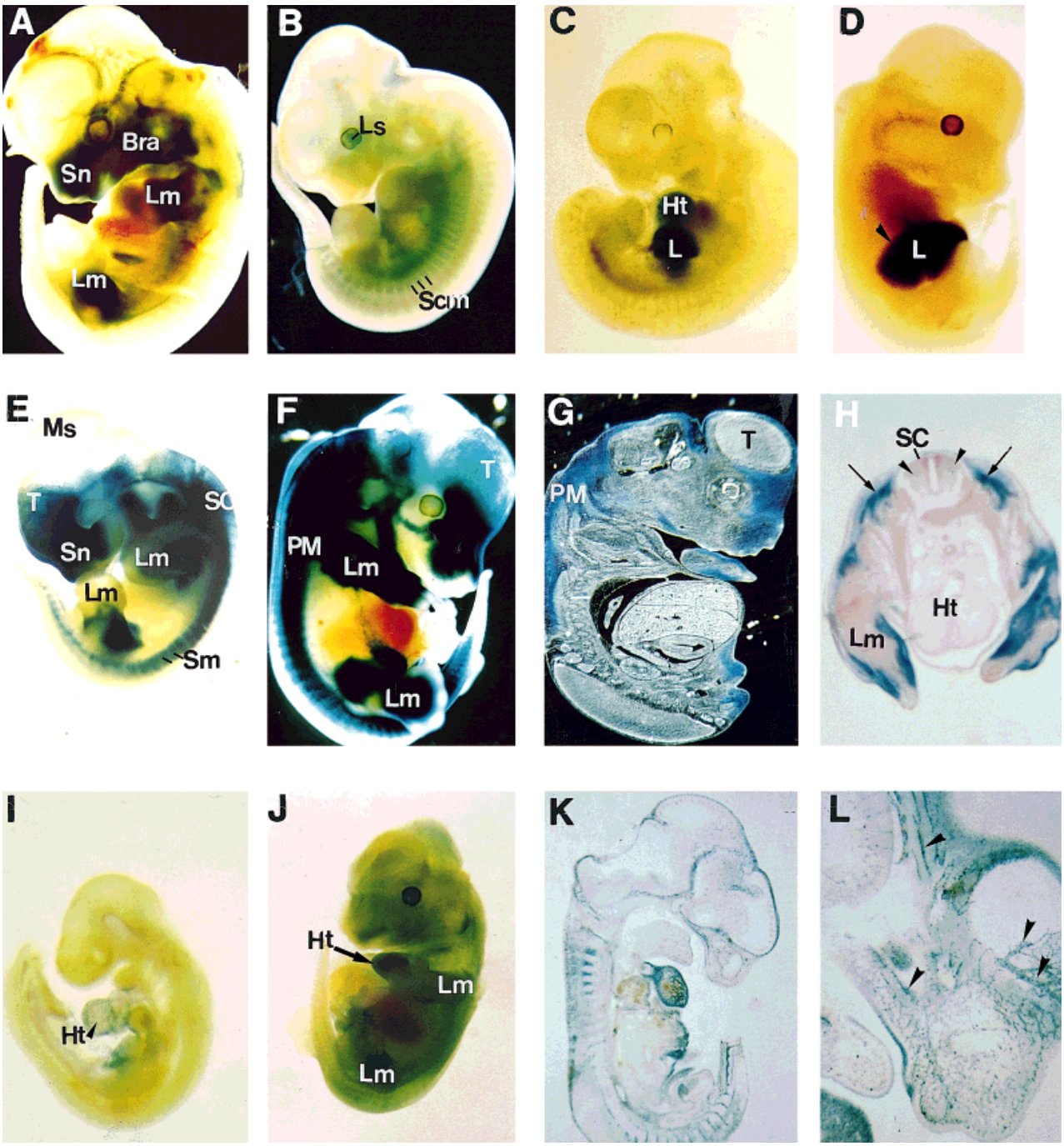

Fig. 2. Examples of β-gal staining in heterozygous embryos showing restricted expression outside the nervous system (Table 2, group 1.2.). The embryos were obtained with the following gene trap ES cell lines: XIV-109 (**A**), XVI-132 (**B**), XV-1 (**C,D**), XVI-56 (**E–H**), XVIII-72 (**I,J**), and XVI-168 (**K,L**). Embryos are shown at stages E9.5 (I), E10.5 (C,E), E11.5 (A,B,J,K,L), E12.5 (F–H), and E13.5 (D). A–F,I,J are photomicrographs of whole-mount β-gal staining of the embryos. G,H and K,L are sections after paraffin and gelatin embedding, respectively, of the stained embryos. The arrows in H mark the strong β-gal signal that was detected predominantly in the paraxial mesoderm (PM; F,G) in embryos from the gene trap line XVI-56; the arrowheads indicate a faint signal observed in the intermediate spinal cord columns. The arrowheads in L mark the *lac*Z expression, which was confined to the embryonic vascular system in the gene trap line XVI-168. Bra, branchial arches; Ht, heart; L, liver; Lm, limbs; Ls, lens; SC, spinal cord; Scm, sclerotome; Sn, snout,

ing that the tagged genes might encode for targets for the respective growth/differentiation factors. Detailed molecular, expression, and phenotypic characterization of the trapped genes will be presented in an upcoming study. It is noteworthy that the initial phenotypic analysis on four transgenic lines obtained from ES clones with a prominent in vitro response to growth and differentiation factors revealed a lethal phenotype for the homozygous offspring

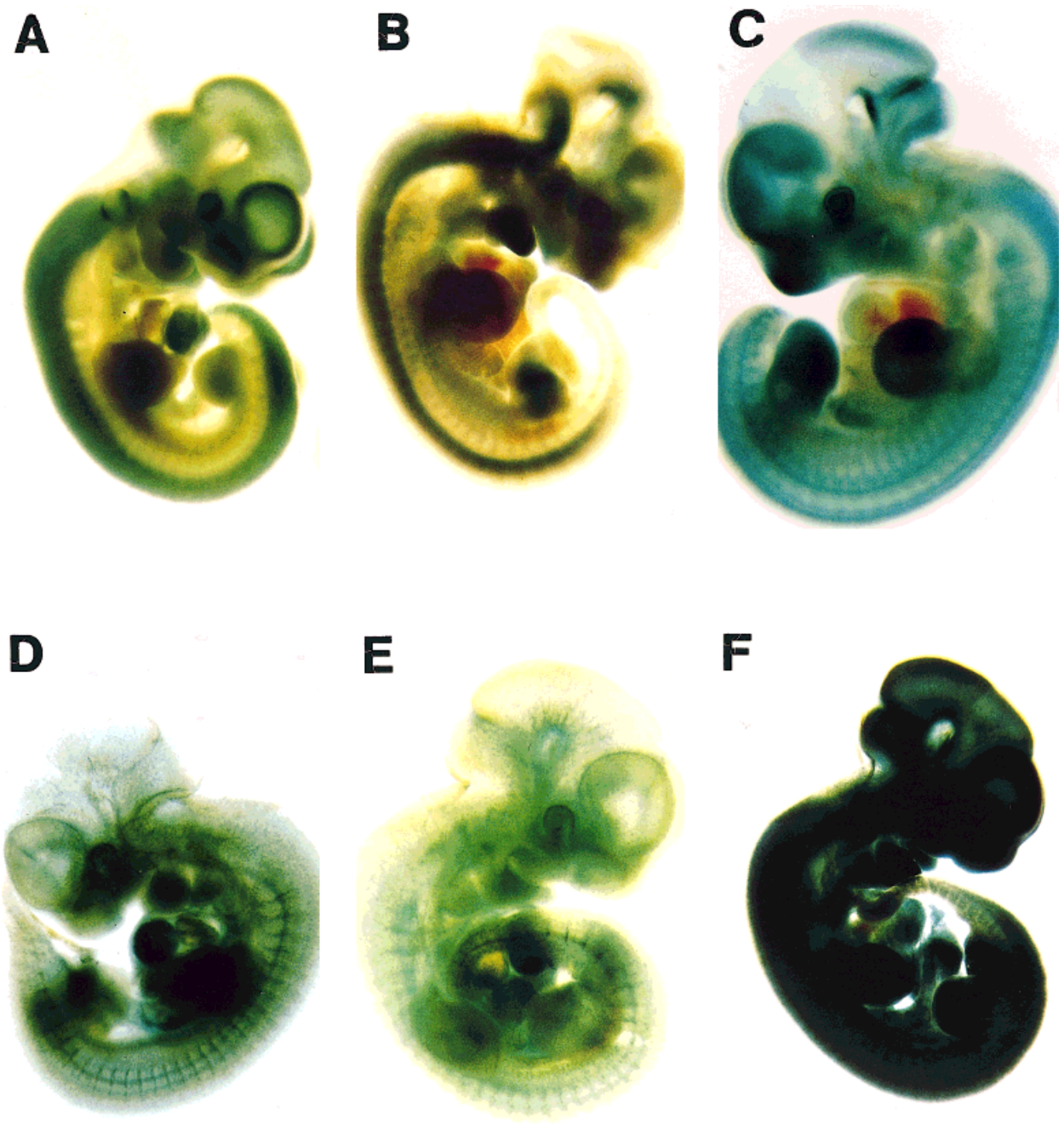

Fig. 3. Examples of whole-mount β-gal staining in heterozygous embryos. **A–C:** Abundant expression in the nervous system as well as faintly to intermediately widespread expression (Table 2, group 2.1), **D,E:** Faintly ubiquitous expression (Table 2, group 2.2). **F:** Strongly ubiquitous expression (Table 2, group 2.3). The embryos were obtained with the gene trap ES cell lines VII-28 (Spnr; A), XIV-138 (B), XVIII-73 (C), XVI-23 (NF-κB; D), XVI-52 (E), and XV-11 (F). Embryos are shown at stages E10.5 (D,E) and E11.5 (A–C,F).

for three of them, as indicated in Table 1. Thus, the careful selection of the trapped cell clones on the basis of their in vitro β-gal staining pattern for the generation of chimeric and heterozygous mice improves significantly the yield of potentially interesting lines for further in vivo analysis.

An important question concerns how the expression of the *lac*Z reporter gene is able to reflect the expression of the trapped endogenous gene. It has been reported that the β-gal staining either matches (Skarnes et al., 1995; Takeuchi et al., 1995; Chen et al., 1996) or is far more restricted (Skarnes et al., 1992; Deng and Behringer, 1995) compared with the expression pattern of the trapped gene. Alternative splicing in different tissues or interruption of regulatory regions of the tagged gene by the vector integration

might result in some inconsistency in the βgal staining detected compared with the real expression of the tagged gene. Comparison of the *lac*Z expression for several known genes captured in our screen with the available expression data revealed a good correspondence in general. This concerned the lines VII-45 (α-E catenin; Torres et al., 1997), XVI-23 (NF-κB; O'Neill and Kaltschmidt, 1977; Schmidt-Ullrich et al., 1996), XVI-78 (MAP-1B; Safei and Fischer, 1989; Black et al., 1994); and XVI-169 (R-PTPκ; Jiang et al., 1993).

Gene-Trap Lines as In Situ *lac*Z Markers for Patterning of the Developing Nervous System

The usefulness of gene trap lines as in situ markers for defined tissues and cell types has been proposed previously (Korn et al., 1992). The transgenic line XVI-56 (Fig. 2E–H) seems to be useful for lineage analysis of cells with mesodermal origin, especially for those that contribute to the developing paraxial/limb musculature and head mesenchyme. Needless to say, the completely ubiquitous gene trap lines (e.g., XVI-34; ubiquitine hydrolase; XV-11; see Fig. 3F) also have potential for applications in lineage analysis.

Availability of transgenic lines with restricted expression of reporter-gene sequences to distinct domains and cells of the developing nervous system would be of great help in gaining insight into the molecular mechanisms underlying the enormous regional and cellular heterogeneity of the developing brain. In one such mouse line, for example, the *lac*Z gene was found to be expressed specifically in the somatosensory cortex, and transplantation experiments provided evidence that the areal specification of cortex is an early event (Cohen-Tannoudji et al., 1994). Analysis of gene trap lines generated as *lac*Z markers that are confined specifically to the developing nervous system has not been reported previously. Four lines in our gene trap screen appear to be useful tools for neurobiological studies.

The captured gene MAP-1B in the line XVI-78 encodes for the major component of the neuronal cytoskeleton (Chowdhury et al., 1997). MAP-1B is the earliest microtubule-associated protein that is expressed abundantly during the development of the nervous system, and it is concentrated in the distal region of growing axons (Safaei and Fisher, 1989; Black et al., 1994). Similar to the data reported by Easter et al. (1993) for the staining patterns after using the monoclonal antibody TuJ1, in embryos from the gene trap XVI-78, we detected prominent β-gal staining on axons that extended from the mesencephalic tectum and cephalic ganglia as early as stage E9.5 (see Fig. 1F–I). At stage E10.5 and later, the expression strongly increases in the entire developing central and peripheral nervous systems (Fig. 1G–I), delineating axonal pathways. Thus, crossing mutant mice with deficiencies in axonal patterning and cephalic ganglion formation with heterozygous animals of the gene trap line XVI-78 would help in the analysis of the mutant phenotype at any developmental stage with a simple and inexpensive histological procedure.

The sequence analysis of the integration site in the line XVI-169 indicated that the trapped gene is a known gene encoding the R-PTPκ in mouse (L10106; Chowdhury et al., 1997). Protein tyrosine phosphatases (PTPs) have an action opposite to protein tyrosine kinase (PTKs) in regulating tyrosine phosphorylation and are thereby involved in many aspects of neuronal cell function. As reported by Sap et al. (1994), the inducible expression of the R-PTPκ protein in heterologous cells results in enhanced in vitro aggregation only between the R-PTPκ-expressing cells, suggesting a homophilic binding mechanism. Specification of distinct forebrain regions are preceded by adhesive differences during development (Götz et al., 1996), and similar mechanisms are assumed to be involved in the laminar determination of the cortex and nuclear formation in the developing brain (for review, see Redies, 1995).

The extensive reporter gene expression analysis that was performed for the gene trap line XVI-169 showed a widespread pattern during embryogenesis (see Table 1). The initial expression in the developing brain is moderate and has a mesh-like appearance in the brain (Fig. 4A,B). In agreement with previously reported data from RNA in situ expression analyses (Jiang et al., 1993), we observed a progressive increase of the signal between E9.5 and E14.5, including the dorsal telencephalon (Fig. 4C). In prenatal stage (E18.5), the mesh-like *lac*Z staining in the whole brain increases further (Fig. 4D,E). We assume that this specific pattern reflects the expression of R-PTPκ in the developing brain vascular system. In support of this, in adult brain, we detected a very prominent expression in blood vessels of arachnoidea and choroid plexus in all ventricles (Fig. 4F). A very strong *lac*Z signal of a different type is detected in the dorsal telencephalon that is confined only to the cortical plate cells of the neocortex (Fig. 4D,E). The medial cingulate and the primary olfactory piriform cortex (the paleocortex) show only a diffuse, mesh-like staining of the vascular system, like that seen in all brain regions (Fig. 4E). Thus, the line XVI-169 could be used as a region-specific marker for the developing neocortical compartment. In early postnatal stages (up to 4 weeks after birth), strong expression is observed in the entire depth of the cortex (neocortex, paleocortex, and archicortex), whereas, in adult brain, a prominent signal is detected only in the presumptive layer IV of all isocortical domains (Fig. 4F,G). Therefore, the transgenic line XVI-169 may be of use as a *lac*Z in situ marker for the neocortical compartment of the developing cortex and for granular layer IV of the mature isocortex.

Especially interesting is the finding that the expression of the reporter gene in the developing eye between stages E9.5 and E14.5 is restricted to the dorsal neuroretina (Fig. 4B,C), implicating a role of the R-PTPκ for the dorsal/ventral patterning of the retina. A similar

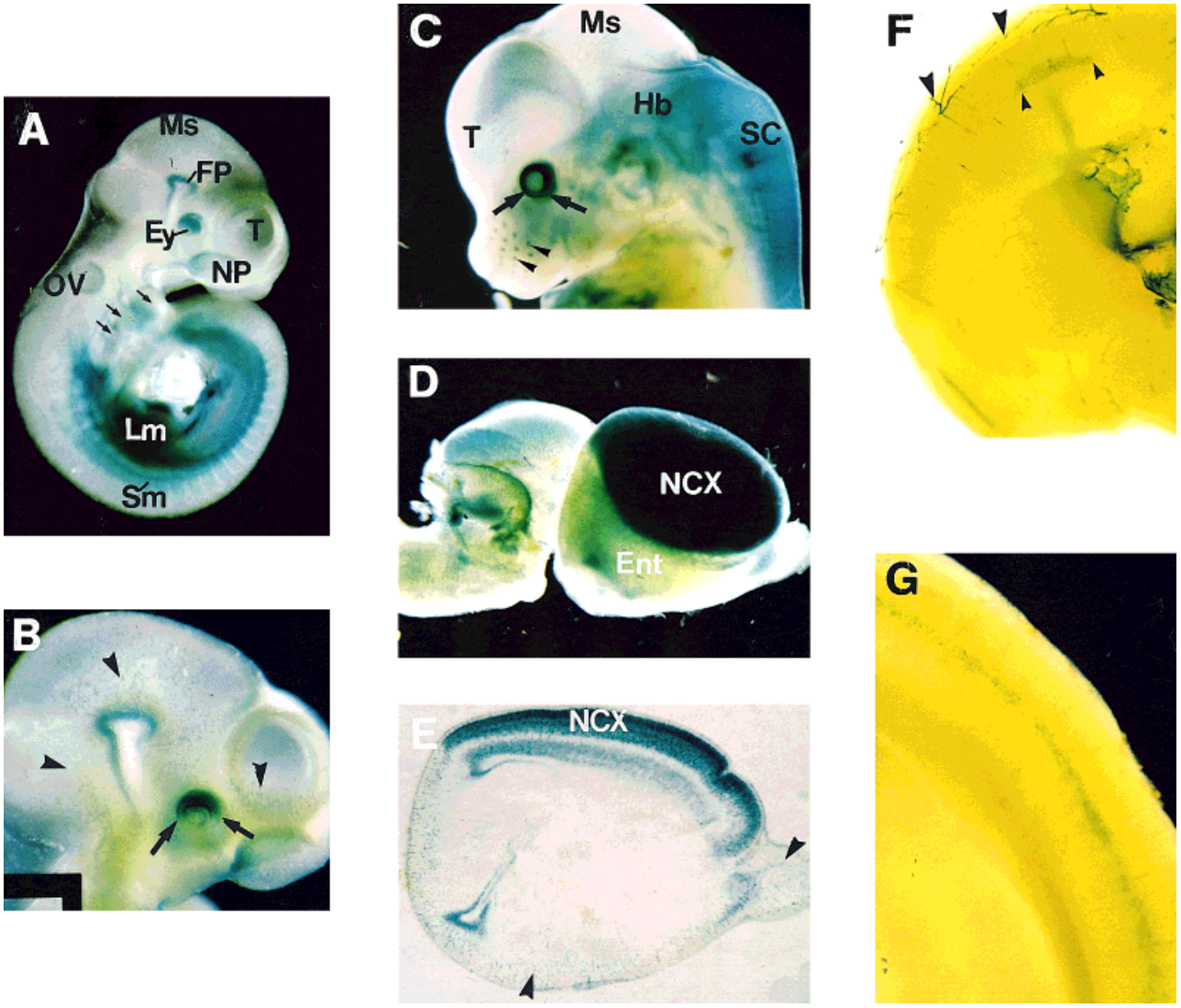

Fig. 4. *Lac*Z expression in developing and adult brain from heterozygous offspring of gene trap line XVI-169. **A–D:** Whole-mount *lac*Z staining of embryos at stages 9.5 (A,B), E14.5 (C), and E18.5 (D). **E:** Sagittal section of gelatin-embedded E18.5 brain after whole-mount *lac*Z staining. **F,G:** Whole-mount *lac*Z staining of 200-μm sections from heterozygous adult brain. The small arrows in A indicate *lac*Z$^+$ cells populating the branchial arches. B is higher magnification of A illustrating the mesh-like staining over the entire brain (arrowheads; also in E) and the restricted expression confined to the dorsal neuroretina (arrows; also in C). The arrowheads in C indicate a signal in the vibrissae. In D and E, note the strong and restricted *lac*Z expression confined only to the neocortical domain (NCX) of the developing cortex. The large and small arrowheads in F indicate the *lac*Z$^+$ vessels and presumptive layer IV of the mature frontal cortex, respectively. In G, note the restricted reporter gene expression in layer IV of the occipital cortex. Ent, entorhinal cortex; Ey, eye; FP, floor plate; Hb, hind brain; Lm, limb; Ms, mesencephalon; NCX, neocortex; NP, nasal pit; OV, otic vesicle; SC, spinal cord.

restricted expression in the developing neuroretina has been reported only for a subtype of the aldehyde dehydrogenases (ADH2; McCaffery et al., 1991).

Our expression analysis further revealed that two novel genes trapped in the lines XVI-129 and XVIII-47 appear to be useful *lac*Z marker for forebrain patterning. The expression in line XVI-129 began around stage E11.5 with a β-gal signal of moderate strength in the developing telencephalon, spinal cord, hindbrain, and heart (Fig. 5A). In later stages, the expression also included the anlage of the olfactory bulb, rhombic lip, neural retina, and dorsal root ganglia (Fig. 5B). At stage E16.5, when the generation of the lower cortical layers (VI, V) is almost completed, a strong signal was detected in the lower part of the cortical plate. Examination of the *lac*Z expression in 200-μm slices from adult brain showed a strong expression in the frontal cortex that was confined mostly to the presumptive layer VI/V, whereas, posteriorly, additional signal was observed in an upper layer (presumptive layer IV; Fig. 5C). Correlated with the expression found in the anlage of cerebellum (rhombic lip) at stage E13.5, the gene was also expressed in adult cerebellum at high or low levels for the Purkinje cell layer or the granular cell layer. Thus,

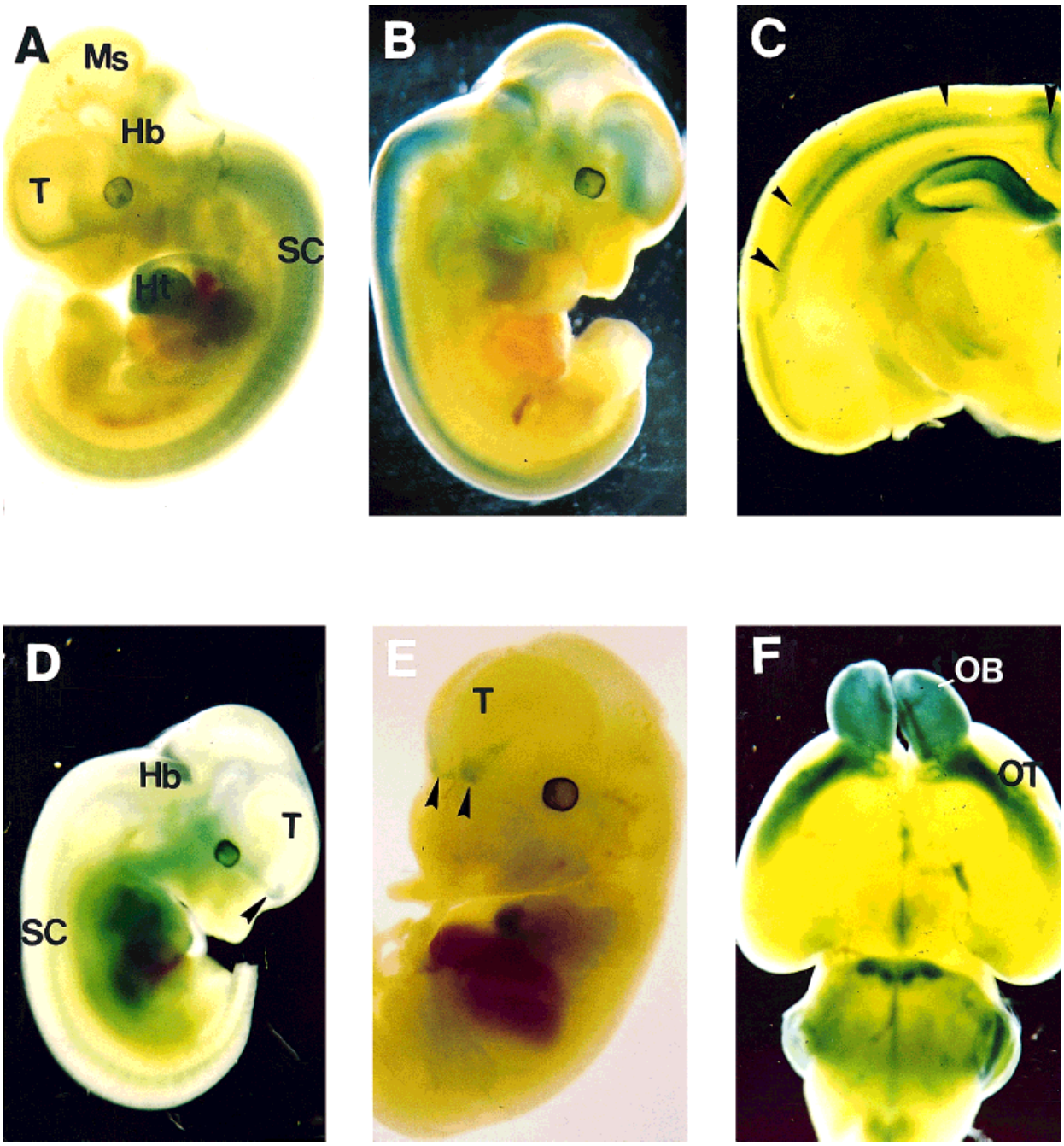

Fig. 5. **A–C:** *Lac*Z expression in embryos at stages E11.5 (A) and E13.5 (B) and in a section through adult brain (C) of heterozygous offspring from the gene trap line XVI-129 after whole-mount *lac*Z staining. The large and small arrowheads in C point to restricted *lac*Z expression domains in the presumptive layers V and VI and layer IV, respectively. **D–F:** Whole-mount *lac*Z staining of embryos at stages E12.5 (D), E14.5 (E) and in isolated brain at stage postnatal day 2.5 (F) of heterozygous offspring from the gene trap line XVIII-48. The arrowheads in D and E indicate the *lac*Z signal in the anlage of the olfactory bulb. Hb, hindbrain; Ms, mesencephalon; OB, olfactory bulb; OT, olfactory tract; SC, spinal cord; T, telencephalon.

the gene trap line XVI-129 could be useful not only as an in situ *lac*Z marker for cortical layering but also as a cell-specific in situ marker for the developing cerebellum.

Our initial *lac*Z expression analysis of the gene trap line XVIII-47 during embryogenesis showed that, until stage E12.5, the expression pattern is restricted mainly outside of the developing nervous system (see Table 1). It was interesting to see, however, that the only positive region in the brain at stage E12.5 was in the anlage of the olfactory bulb (Fig. 5D). At this stage, the formation of the olfactory bulb begins with the production of the

early mitral and tufted neuronal cells. The expression in the olfactory bulb increases progressively in later developmental stages. From stage E16.5 onward, in addition to the olfactory bulb, the olfactory tract, which is formed by the growing axons of mitral cells, becomes strongly positive (Fig. 5F). In prenatal and early postnatal stages, the germinative zone of the anterior forebrain (the ventricular and subventricular zones) produces the later generated periglomerular, granular, and glial cells that populate the main olfactory bulb even postnatally (Luskin, 1993). In the postnatal brain, the corresponding cell layers (mitral and periglomerular layers) in the main olfactory bulb are strongly positive (not shown). Taken together, these results show that the developmental expression of the reporter gene in the brains of embryos from line XVIII-47 correlates not only with the generation of the early (mitral and tufted) and late (periglomerular and granular) neurons from the telencephalic germinative zones but also with the establishment of the afferent axonal pathway from the olfactory bulb.

Mutational Analysis of the Homozygous Mouse Lines

Previously, we have shown that the integration of the gene trap vector into the mouse genome is essentially random and that the insertion event can create complete or partial knockouts (Chowdhury et al., 1997). It also seems likely that there are certain hot spots of recombination in the ES cell genome. For instance, by using the IRESβgeo vector, we captured the gene R-PTPκ, which has also been trapped in another gene trap screening by using a vector specifically designed to capture the N-terminal signal sequences (Skarnes et al., 1995). Similarly, *jumonji*, a new gene involved in neural development, has been captured by two groups using different vectors (Takeuchi et al., 1995; Baker et al., 1997).

The preliminary phenotypic analysis performed during our screen was initially aimed only at obtaining information on the viability and fertility or presence of overt phenotype in the homozygous offspring after birth (and, in several cases, during embryogenesis). From 21 mouse lines that were bred to homozygosity (see Table 1), 16 lines showed potentially interesting *lac*Z expression pattern in the developing nervous system, whereas five of them (XIV-109, XVI-52, XVI-56, XVI-60, and XVIII-72) were restricted predominantly outside of the nervous system. Interestingly, from the first group of gene trap lines, eight lines (50%) displayed overt (lethal) phenotype, whereas, in three lines (19%), live and fertile homozygous animals were present in a normal Mendelian ratio and without any obvious morphological abnormality. Because, sometimes, no unequivocal conclusion can be drawn from the genotyping by quantitative Southern analysis, in most cases, we confirmed the presence of viable homozygotes by test breeding (see Experimental Procedures). It should be noted, however, that it is possible that the integration site may be located at an extreme downstream position and may produce completely functional protein. Such mice are likely to lack any phenotype. More detailed molecular analyses are needed to resolve this issue. The number of the homozygous newborn pups from five gene trap lines (31%) was less than expected according to the Mendelian ratio, thus these lines are presently considered as sublethal or presumably sublethal, although, in few cases, additional numbers of determinations may be needed (designated Live HM* in Table 1). Notably, from this group, homozygous pups from the line VII-28 were reduced in weight threefold compared with the wild-type litter mates in the early postnatal period (Pires da Silva, unpublished observation). Even without considering the number of the phenotypes designated as sublethal, our results indicate that the gene trap screening is a suitable approach for the mutational analysis of the mouse genome. Various examples for different frequencies of phenotype obtained in a gene trap screen have been reported in the literature (1 of 4: Forrester et al., 1996; 2 of 4: von Melchner et al. 1992; 2 of 3: Skarnes et al., 1992). Another study (Gasca et al., 1995) failed to observe any phenotype in homozygous lines. This indicate that not every mouse line generated with a splice acceptor-βgeo type of vector will display an overt phenotype. One likely explanation for this is the possible integration of the vector into an intron. Even a reduced amount of splicing around the vector-containing intron might generate sufficient wild-type message and protein necessary for proper functioning of the mouse. In other cases, the mutated gene product might be compensated for by other redundant members of the same gene family. However, low levels of wild-type transcript do not always lead to the absence of phenotype (Serafini et al., 1996). If one is able to trap a hypomorphic allele, then a reduced wild-type transcript level will be sufficient to generate a phenotype.

Feasibility of Large-Scale Gene Trap Screening

Our results show that the combination of an appropriate vector together with a careful in vitro and in vivo selection based on the reporter-gene expression of the trapped genes allows the identification of potentially important developmental genes. The principal aim of our large-scale gene trap screen was the identification of new genes that might have important roles for the development of the nervous system. We found that, for such a screen, a more extensive in vivo *lac*Z expression analysis is necessary and contributes to a significant increase in the numbers of potentially interesting trapped genes: those that either exert an overt phenotype or offer useful tools as region- or cell-specific in situ *lac*Z markers.

One of the most important questions that biologists would like to answer within the next few decades is that regarding the structure and function of the entire vertebrate genome. This, in turn, raises the question about the approaches currently available. Both chemical mutagenesis of the murine genome and individual gene knock outs by homologous recombination are

extremely cumbersome and time consuming. In the first case, the mutated genes can be isolated only by the positional cloning method, whereas the knock out can be done only after the isolation and characterization of individual, known genes. Our present and previous results (Chowdhury et al., 1997) indicate that both βgeo- and IRES βgeo-type vectors can be used successfully for trapping genes with interesting patterns in vivo, for cloning by 5′-RACE-PCR, and for producing mutants. Another kind of vector has been described in which the transcriptional termination poly-A signal has been replaced by a splice-donor element (Yoshida et al., 1995; Salminen et al., 1998). However, sufficient cloning and mutational screening data are still not available in order to draw a firm conclusion about the efficiency of this kind of vector. Based on specifically constructed vectors, strategies have been proposed for trapping retinoid-responsive genes (Forrester et al., 1996), genes encoding secreted molecules (Skarnes et al., 1995), or genes that are involved in apoptosis (Russ et al., 1996). Recently, Hicks at al. (1997) reported on the feasibility of a large-scale gene trap screen using splice-acceptorless, exon-trap vectors that contain heterologous exon sequences attached to the reporter gene. The expression of the reporter gene requires integration into a genomic exon, thus improving the frequency of the mutagenesis. If methods for direct genomic sequencing starting from the heterologous sequence could be developed, then the identification of a very large number of trapped exon would be achieved easily.

In conclusion, our data clearly support the view that large-scale gene trapping is feasible as an attractive alternative for isolation of new genes and the functional analyses of the murine genome. Saturation tagging of the ES cell genome and cloning of the corresponding sequences can be performed in the initial phase. For the mutational screen, a large and concerted international effort will be necessary in the near future.

EXPERIMENTAL PROCEDURES

Gene Trap Vectors

Two different reporter vectors were used: 1) the βgeo vector (pGT1.8geo; kindly provided by Dr. W.C. Skarnes), which contains the splice-acceptor sequence from the mouse *En-2* gene (Gossler et al., 1989) attached upstream of promoterless βgeo reporter-selectable marker and is activated only when a correct integration within a transcriptionally active endogeneous gene occurs (Friedrich and Soriano, 1991). This vector was applied in all electroporations up to number XVI (Table 1). 2) IRESβgeo vector (Chowdhury et al., 1997) in which the internal ribosomal entry site (IRES) from the *emc* virus (Kim et al., 1992) is introduced after the splice acceptor site, thus providing a cap-independent translation of the reporter gene from fusion transcripts.

Electroporation and Screening of ES Cells

Gene trap vectors were introduced into R1 ES cells (Nagy et al., 1993) and cultured on a feeder layer of mitomycin C-treated primary embryonic fibroblasts in ES medium that contained Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM 2-mercaptoetahanol, 1,000 U/ml leukemia-inhibitory factor (LIF; ESGRO), and 20% heat-inactivated fetal calf serum (FCS; GIBCO, BRL, Gaithersburg, MD). For electroporation, 10^7 ES cells were electroporated with 30 μg linearized pGT1.8geo or IRESβgeo vector DNA in 1 ml phosphate-buffered saline (PBS) by applying a single pulse at 250 V, 500 μF in a gene pulser (Bio-Rad, Cambridge, MA). After electroporation, cells were seeded on a monolayer of G418-resistant primary embryonic fibroblasts at 3 / 10^6 cells/100-mm dish. Selection with G418 (GIBCO-BRL) at 250 μg/ml was started 24 hr after electroporation. After 7–10 days of selection, single G418-resistant colonies were picked, trypsinized, plated into 96-well dished for 2–4 days, and then processed for freezing of cells at −80°C and for β-gal staining.

To identify gene trap lines that were activated or repressed by specific growth/differentiation factors (follistatin, NGF, retinoic acid), a simple differentiation protocol was used (Bonaldo et al., in preparation). Briefly, after electroporation, single resistant colonies were grown into duplicate 96-well dishes—one for freezing and the other for screening. The cells from the screening plate were split into individual dishes and cultured under different conditions in ES medium without LIF in the presence of 1% FCS (for retinoic acid and NGF treatment) or 20% FCS (for the follistatin treatment). The clones were selected for their responsiveness (activated or repressed) to the applied soluble factors. After the expansion of the clones on 35-mm plates, the responsiveness of selected gene trap lines was also tested. These lines were then used for generating mouse chimeras.

The screening for β-gal activity in the G418-resistant ES cell lines was done by histochemical staining as described by Gossler and Zachgo (1993). According to their in vitro β-gal staining, the clones were classified as ubiquitous (similar staining in all cells of the clone), restricted (with a low levels of β-gal staining in most cells and a much stronger staining in few cells), very restricted (with a faint, dot-like expression in few cells), and regulated (activated or repressed by a certain factor with either ubiquitous, restricted, or very restricted β-gal pattern). ES cell lines of each group were selected and proceeded further for in vivo analysis.

Generation and Analysis of Mice

The selected ES cell lines were used for generating mouse chimeras by morula aggregation according to the method of Nagy and Rossant (1993). Chimeric mice were checked for germ-line transmission and used for generation of transgenic mice that are heterozygous for the tagged gene.

The spatiotemporal expression pattern of the "trapped" gene was analyzed routinely by whole-mount

β-gal staining (Gossler and Zachgo, 1993) of heterozygous embryos in at least two litters at each of the following three developmental stages: E9.5/10.5, E11.5/12.5, and E13.5/14.5. Embryos dissected until stage E12.5 were washed in ice-cold PBS and fixed for 20 min at 4°C in a fixative (1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP40 in PBS). Older embryos were cut in sagittal or cross sections and fixed for 30–40 min in the same fixative containing 0.2% NP40 and 0.1% sodium deoxycholate. After two washes for 30 min, the specimens were stained in a PBS solution containing 1 mg/ml X-gal (Bethesda Research Laboratories, Bethesda, MD), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, ad 2 mM $MgCl_2$ at 30°C overnight. For embryos older than E13.5, the staining solution contained 0.2% NP40. After staining, the embryos were washed twice for 30 min in PBS, dehydrated in glycerol with gradually increasing concentrations, and kept in 80% glycerol/PBS at 4°C. The negative lines were also analyzed at later developmental stages and, in some cases, after birth. If necessary, after extensive washing, the whole-mounted, stained embryos were fixed in 4% paraformaldehyde overnight and proceeded for gelatin or paraffin sectioning. In several cases, isolated adult brains were cut into 200-μm slices and processed for βgal staining as described above.

Genotyping by Quantitative Southern Blotting

For the initial phenotypic analysis of the mutant mice (assessment of the lethality of the created mutation), heterozygous F1 generation mice with mixed, out-bred backgrounds (129/Svx NMRI) were crossed to homozygosity. Usually, these crosses have been done after two or three back crosses to NMRI background. The genotyping of DNA isolated from newborn offspring or embryos was done by quantitative Southern blotting. Briefly, DNAs were prepared from equal sizes of cut tail, and approximately 5 μg DNA were digested with BamHI, and, after electrophoresis, transferred to nylon membranes (Qiagen, Chatsworth, CA). The membranes were hybridized according to the manufacturer's protocol with a ^{32}P-labeled mixed probe containing equal amounts of LacZ and Fkf5 DNA (gift of R. Wehr) sequences. The later is a single-copy gene and was used as an internal control to normalize the amount of DNA loaded in each lane. The intensities of both bands in each lane were quantitated on autoradiographs by visual estimation and by counting on an Instant Imager (Packard Instruments, Meridian, CT). The availability of live homozygous animals was also confirmed by test breeding for 100% transmissibility of the transgene in the offspring litter, which was checked for *lac*Z expression at stage E11.5.

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