

Fate mapping of the mouse midbrain–hindbrain constriction using a site-specific recombination system

Dawn L. Zinyk, Eric H. Mercer, Esther Harris, David J. Anderson and Alexandra L. Joyner

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Materials and methods

Construction of plasmids

The *En2-cre* transgene was constructed from the plasmids pB9 [S1], pKS(*Sal*)₂ (compliments of M. Hanks), pMC12 (compliments of D.L. Song), and pBS185. The *En2* minimal promoter [S2], obtained as a 2.3 kb *Bam*HI–*Sma*I fragment from pB9, was fused with the 3.0 kb *Bam*HI–*Sma*I vector backbone from pKS(*Sal*)₂. The *En2* embryonic enhancer [S2,S3] obtained as a 2.6 kb *Bam*HI fragment from pMC12, was inserted into the *Bam*HI site upstream of the *En2* promoter. The construct was completed by inserting the *cre* coding region with a metallothionein polyadenylation signal, obtained as a blunted 2.4 kb *Hind*III–*Xho*I fragment from pBS185, into the blunted *Clal* site downstream of the *En2* promoter.

The cβ–STOP–*lacZ* reporter transgene consists of the following elements, in 5′ to 3′ order: (1) a 3.1 kb fragment of the chick β-actin promoter including the first exon, first intron, and the 5′ untranslated portion of the second exon; (2) a *loxP* site; (3) a STOP sequence [S4] containing an inverted *his3* element with a false translational stop, and an SV40 splice donor/poly(A)-addition site; (4) a second *loxP* site; (5) the *lacZ* coding sequence containing a nuclear localization signal (*nlacZ*); (6) the 3′ untranslated region (UTR) of the mouse protamine-1 (*mPrrm-1*) gene, included to improve expression of the transgene. Briefly, this construct was generated by first cloning the *Sal*I/*Bgl*II fragment of the plasmid p*nlacF* into the *Sma*I site of plasmid pBSlox², thereby placing the *nlacZ* coding and *mPrrm-1* 3′ UTR sequences downstream of tandem *loxP* sites separated by a polylinker region containing *Xho*I, *Bgl*II and *Hind*III sites, in an intermediate plasmid called pX²Z3 (E.M., unpublished). A 1.3 kb *Bam*HI–*Hind*III fragment containing the STOP sequence from plasmid pBS241 [S4] was then inserted between the *Bgl*II and *Hind*III sites flanked by the tandem *loxP* sites in pX²Z3, to generate pXSTOPXnZ. Finally, a 3.1 kb *Sal*I–*Xba*I fragment from the plasmid pβact-1 containing the chick β-actin promoter was cloned between *Sal*I and *Xba*I sites located upstream of the 5′-most *loxP* sites in pXSTOPXnZ, to generate the final plasmid pβact-XSTOPX-*nlacZ* (referred to in this study as cβ–STOP–*lacZ*). Transgenic mice were generated by injecting the 8.2 kb *Sal*I–*Asp*718 fragment from this plasmid into zygotes. A detailed restriction map of this plasmid is available on request.

To generate the mouse (m) β–STOP–*lacZ* targeting vector to insert a (*loxP*-STOP-*loxP*-*nlacZ*) reporter cassette into the second exon of the mouse β-actin locus, the following steps were performed, in brief. A 3.9 kb *Sal*I–*Xho*I fragment containing the 3′ arm of the mouse β-actin gene was cloned into *Sma*I-digested pUC19, to generate pUC-Act3′-2. In parallel, a fragment containing the bacterial neomycin-resistance gene (*neo*^r) followed by the SV40 intron and three consecutive SV40 poly(A)-addition sites [S5] was inserted between the tandem *loxP* sites in the plasmid pX²Z3, to generate the intermediate pXneoXnZ. The 3′ β-actin arm, with the *Eco*RV site inactivated, was then transferred from pUC-Act3′ to a position immediately downstream of the *lacZ* coding sequence, to generate pXneoXnZac3′. A fragment containing the PGK-tk cassette fused to the 5′ arm of the mouse β-actin gene (a 1.2 kb *Sal*I–*Xba*I fragment with the initiator codon deleted) was ultimately inserted upstream of the 5′-most *loxP* site to generate the final targeting construct. The 5′ and 3′ arms of the mouse β-actin gene were the generous gift of Richard Behringer.

Transgenic CMV–*cre* transgenic mice were generated from a linearized fragment derived from plasmid pBS185 (the generous gift of Brian Sauer), which contains the CMV promoter-enhancer, the *cre* coding sequence, and introns and the 3′ UTR from the metallothionein-I (MT-I) gene. The position of insertion and number of transgene inserts (30–40) in line 32-3 does not appear to be deleterious, as homozygous cβ–STOP–*lacZ* mice appear healthy.

Screening of transgenic mice

Transgenic mice were generated [S6] and identified by Southern blot analysis. For *En2-cre* mice, tail DNA was digested with *Eco*RI and probed with a 683 bp *Bam*HI–*Mlu*I *cre*-containing fragment from pBS185. For cβ–STOP–*lacZ* mice, tail DNA was digested with *Eco*RV and probed with a 1.1 kb *Eco*RI–*Eco*RV *nlacZ*-containing fragment from the original transgene vector. After founders were established, genotyping was usually done by three-primer PCR. Yolk sac or tail DNA from *En2-cre* (0.18 μg) or cβ–STOP–*lacZ* (0.06 μg) was amplified for 27 cycles (1 min at 94°C, 1 min at 62°C, 1.5 min at 72°C) on a thermal cycler. The 5′ *cre* primer was 5′-CATTTCGTGATGAATGCCAC-3′, the 5′ endogenous mouse metallothionein primer (which excludes sequence in common with the mouse metallothionein polyadenylation site downstream of *cre*) was 5′-CCGTAGCTCCAGCTTCACC-3′, and the common 3′ MT primer was 5′-ACTTGCAGTTCTTGCAGGC-3′. The 5′ *lacZ* primer was 5′-GGTCGCTACCATTACCAGTTG-3′, the 5′ endogenous mouse protamine-1 primer (which excludes sequences in common with the mouse protamine-1 polyadenylation site downstream of *lacZ*) was 5′-AGGTGGTGTCCCTGCTC-3′ and the common 3′ mouse protamine-1 primer was 5′-ACGCAGGAGTTTTGATGGAC-3′. Half of the 20 μl reaction volume was analyzed on a 2% agarose gel. With the *En2/cre* primers, the wild-type allele is indicated by a 588 bp band, whereas the *En2/cre* transgene produces a 668 bp band. With the cβ–STOP–*lacZ* primers, wild type has a 332 bp band, whereas the cβ–STOP–*lacZ* transgene produces a 349 bp band.

Detection of β-galactosidase activity

E9.5 embryos were dissected into cold phosphate-buffered saline (PBS) and transferred as dissected into PBS on ice. When all embryos were dissected, they were changed into cold paraformaldehyde (PFA) 4%/PBS and rocked at 4°C for 1 h. After fixation, embryos were washed three times for 30 min with wash buffer (2 mM MgCl₂, 0.02% Nonidet-P40, 0.1 M phosphate buffer pH 7.3) once at 4°C and twice at room temperature. Embryos were then transferred to prewarmed X-gal solution (0.1 mg/ml X-gal/DMF, 2.12 mg/ml potassium ferrocyanide and 1.64 mg/ml potassium ferricyanide in wash buffer) and placed at 37°C overnight. The next day, they were washed twice with PBS then placed in 10% formalin at 4°C overnight. Embryos were then washed twice with PBS and stored in 70% ethanol at 4°C.

E14.5 embryos to be stained were dissected into cold PBS, their heads pinched off with forceps as far down the shoulders as possible without taking the arms, and transferred as dissected into 50 ml PBS on ice (≤6 heads per tube). When all embryos were dissected, they were changed into cold PFA 4%/PBS and allowed to rock at 4°C for 4 h. Following fixation, embryo heads were either cut sagittally at the midline with a scalpel blade and washed in PBS or were transferred into cold 30% sucrose/PBS and allowed to sit at 4°C overnight until they sank. Such embryo heads were dried on lens paper and mounted in OCT. A cryostat was used to cut sagittally to midline. Three to four

head-containing blocks were placed in 50 ml tubes and allowed to rock in 40 ml of wash buffer at room temperature for 15 min and then in fresh 40 ml wash buffer for 30 min. Three to four brains, cut with a scalpel or cryostat, were then put into 15–20 ml of prewarmed X-gal solution and allowed to stand covered at 37°C overnight. After one PBS rinse, heads were placed in 40 ml 10% formalin for 4 h to overnight. Heads were then rinsed with PBS and stored in 70% ethanol at 4°C.

Adult brains were dissected out dry, cooled at 4°C for 3 min and sliced sagittally into 2 mm slices. The slices were transferred to room temperature PFA 4%/PBS and then placed on ice until all brains were finished. The brain slices were changed into fresh cold PFA 4%/PBS and placed at 4°C for 1 h: 15 min standing, 45 min rocking. After fixation, brains were washed three times for 30 min with wash buffer, once at 4°C and twice at room temperature. They were then changed into prewarmed X-gal solution and placed at 37°C overnight. The next day, brains were washed twice with PBS then placed in 10% formalin at 4°C overnight. Brains were then washed twice with PBS and stored in 70% ethanol at 4°C.

RNA in situ hybridization

Whole-mount RNA *in situ* procedures were carried out according to Conlon and Rossant [S7].

Production of targeted mice: ES cell culture, screening for homologous recombinants and chimera production

The m β -STOP-*lacZ* targeting construct was generated by homologous recombination in embryonic stem (ES) cells of the m β -STOP-*lacZ* targeting construct into the second exon of the mouse β -actin gene [S8]. The m β -STOP-*lacZ* targeting construct was released from the plasmid by *SalI* digestion and then introduced into ES cells by electroporation. The R1 ES cell line [S9] was propagated, electroporated and selected as described by Wurst and Joyner [S8]. After positive-negative selection with G418/ganciclovir-containing media, surviving ES cell clones were analyzed by genomic Southern blot analysis using two external probes, a 5' 0.37 kb *EcoRV* fragment and a 3' 0.48 kb *XhoI*-*BamHI* fragment. Genomic DNA digested with *EcoRV* and probed with the 5' probe gave a 1.4 kb wild-type and 5.3 kb mutant fragment, and probed with the 3' probe gave a 8.2 kb wild-type and 12.5 kb mutant band. Southern blot analysis identified 3 of 55 neomycin- and ganciclovir-resistant clones as being correctly targeted. Two of the targeted lines were used to produce chimeras through aggregation with morulae [S10]. Three chimeras from one line were found to transmit the embryonic stem cell genotype to heterozygous mice after breeding with CD1 mice, referred to as β -actin^{neolacZ}. Homozygous β -actin^{neolacZ} mice were never obtained, suggesting that disruption of the β -actin gene results in embryonic lethality. Tail or yolk-sac DNA (~10 μ g) was digested with *EcoRV* and run on a 1% agarose gel. A 370 bp *EcoRV*-*XhoI* β -actin-containing fragment of the original targeting construct was used as the hybridization probe.

To determine the potential for *lacZ* expression from the β -actin locus, β -actin^{neolacZ} mice were mated with mice carrying a CMV-*cre* transgene, a different line than the one used to study the STOP-*lacZ* mice. From this cross, 4 of 11 progeny were identified as carrying both the targeted β -actin allele and the CMV*cre* construct. Two of the double transgenics carried the β -actin^{lacZ} allele generated by Cre-mediated deletion of *neo* in most cells. One of the β -actin^{lacZ} females was crossed with a CD1 male and killed when the embryos reached E11.5. Various tissues were removed from the mother and half of each adult tissue was used for either β gal staining or northern blot analysis with a *lacZ* probe. As a control for *lacZ* expression, tissues from a mouse carrying the ROSA26 gene trap insertion that expresses *lacZ* ubiquitously [S11] were also analyzed. Whereas every cell type in all ROSA tissues analyzed expressed *lacZ*, only the brain of the adult β -actin^{lacZ} mouse showed any staining, primarily limited to the cortex, granular cells of the cerebellum and hippocampus (data not shown). Northern blot analysis did not detect a *lacZ* transcript in any of the β -actin^{lacZ} tissues whereas

a transcript was detected in all of the ROSA26 tissues (data not shown). The fact that a *lacZ* transcript was not detected in the brain tissues of the β -actin^{lacZ} mouse in spite of β gal activity probably reflects the differing sensitivities of the two assays. Five of the nine E11.5 embryos recovered from the β -actin^{lacZ} mouse carried the β -actin^{lacZ} allele and they exhibited only scattered β gal staining in cells in the heart and, in one embryo, also in the spinal cord (data not shown). Similar results were obtained with embryos from the other β -actin^{lacZ} mouse. Targeting elsewhere in the β -actin gene might produce more robust *lacZ* expression, although at least one other attempt to produce a gene trap in the first intron did not result in ubiquitous expression [S12].

References

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Table S1

Percentage of animals expressing β gal in various regions of CMV-cre;c β -STOP-lacZ adult brains.

Brain region	% β gal staining ($n = 20$)		
	+	\pm	-
Olfactory bulbs	85	15	0
Cortex	70	30	0
Ventral telencephalon	40	30*	30
Hypothalamus	20	15 [†]	65
Thalamus	65	35 [‡]	0
Hippocampus	85	5	10
Tectum	30	40 [§]	30
Tegmentum	25	5	70
Cerebellum	40	50 [¶]	10
Hindbrain	25	50	25

+ denotes strong expression in most or all cells; \pm denotes mosaic staining; - denotes expression in no or very few cells. *Ventral telencephalon staining is strongest in the striatum. [†]Only a small group of cells express in the hypothalamus. [‡]Thalamus stains most strongly anteriorly. [§]Often layer-specific expression in the tectum. [¶]Expression in many animals was stronger in the posterior cerebellum. ^{||}Expression only in the pontine nucleus of the metencephalon.

Table S2

Percentage of animals with β gal staining in various brain regions of *En2-cre;c β -STOP-lacZ* double-transgenic E14.5 embryos.

Brain region	Line 3 ($n = 8$)			Line 4 ($n = 8$)			Line 6 ($n = 4$)			Line 11 ($n = 8$)			Line 22 ($n = 3$)		
	+	\pm	-	+	\pm	-	+	\pm	-	+	\pm	-	+	\pm	-
Cortex			100		50	50			100			100			100
Ventral telencephalon			100		12	88			100			100			100
Dorsal diencephalon			100		12	88			100			100			100
Ventral diencephalon		100			12	88			100			100			100
Superior colliculus		100			100			50	50		62	38			100
Inferior colliculus		100			100			50	50		62	38			100
Tegmentum		100			50	50		50	50		12	88			100
Cerebellum		100		50	50		25	75		12	88		33		67
Metencephalon		100			62	38		25	75			100			100

+ denotes strong staining in most or all cells; \pm denotes mosaic staining; - denotes no or few cells staining.

Table S3

Percentage of animals with β gal staining in various brain regions of *En2-cre;c β -STOP-lacZ* double-transgenic adults.

Brain region	Line 3 (n = 4)			Line 4 (n = 3)			Line 6 (n = 5)			Line 11 (n = 4)			Line 22 (n = 4)		
	+	±	-	+	±	-	+	±	-	+	±	-	+	±	-
Cortex			100		67	33			100			100			100
Ventral telencephalon			100			100			100			100			100
Thalamus			100			100			100			100			100
Hypothalamus	50	50				100			100			100			100
Hippocampus		100*			100*			100*			100*				100*
Superior colliculus medial	75	25			100			20	80			100		50	50
lateral		75	25		33	67			100			100		100	
Inferior colliculus medial	75	25			100			60	40		25	75		75	25
lateral		75	25		67	33			100			100		100	
Tegmentum medial		100			33	67		20	80			100		50	50
lateral		100			33	67			100			100		100	
Cerebellum medial	75	25		33	67 [†]			100 [†]			100 [†]		100		
lateral		100			100			40 [†]	60		50	50		50	50
Hindbrain		100				100			100			100			100

+ denotes strong staining in most or all cells; ± denotes mosaic staining;
 - denotes no or few cells staining. *Weak and variable staining was

observed in control reporter animals. [†]Expression was strongest in the
 posterior cerebellum. Olfactory bulbs did not stain in all samples.