Small body and short ear pinnae, a spontaneous mutation on chromosome 4 in the mouse

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Mutation Symbol: *sbse*

Mutation Name: small body and small ear

Strain of Origin: C57BL/6J

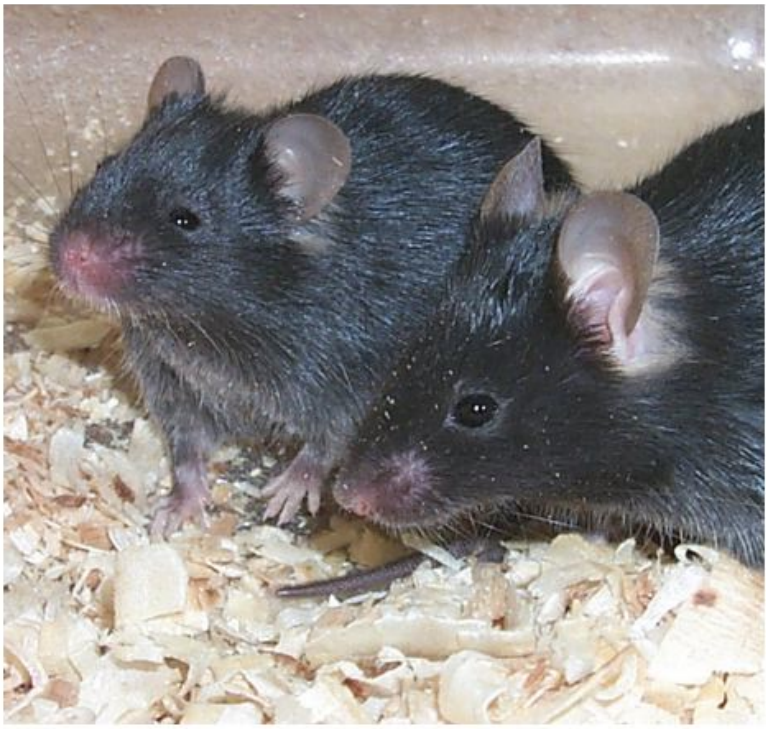
Current Strain Name: C57BL/6J-*sbse*/J

Stock #004246 (jaxmice.jax.org)

Phenotype Category: craniofacial, skeletal, hydrocephaly, hearing

Origin and description

sbse is a spontaneous autosomal recessive mutation that arose on the C57BL/6J background in a production colony at The Jackson Laboratory in July 2001. The phenotype is a smaller body size along with smaller ear pinnae and a domed skull. Homozygotes live normal life spans and are fertile.



Pictured on the left is a homozygous *sbse* mouse with a control littermate on the right

Genetic analysis

sbse is inherited as a recessive mutation as shown by traditional linkage cross analysis. No visible mutants were seen in the F1 generation in which a mutant was mated to an unrelated C57BL/6J (0/29). The F2 generation showed the phenotype about 25% of the time, the expected Mendelian ratio (6/30).

For linkage analysis, a female *sbse/sbse* was mated to a male CAST/Ei. F1s were then intercrossed to produce F2 mutants. Spleens and tail tips were collected from 80 F2 *sbse/sbse* mice and were stored at -70C. DNA was extracted with the standard phenol method and the strain was mapped using the standard mapping protocol of the Mouse Mutant Resource[1]. The mutation is located near the centromere of Chromosome 4. The distal flank is at *D4Mit316* (5.2 Mb) with 2/152 recombinants, or 1.3% recombination. There were no recombinants (0/152) on *D4Mit149* at 3.6 Mb or *D4Mit50* at 3.2 Mb.

A candidate gene *Plag1* is located in our non-recombinant region at 3.8 Mb. *Plag1* homozygous null mice display reduced male fertility, small seminal vesicles and ventral prostate, reduced embryonic and postnatal growth, delayed eyelid opening, and with female breeder mutants, reduced litter size. Our *sbse* exhibits some of these characteristics which are discussed in the Pathology and Biological Characterization sections. Our *sbse* strain has at least one characteristic that differs from the one known allele of *Plag1* (*Plag1*^{tm1Wjmv}); our mutants have a hearing phenotype. Another reported *Plag1* phenotype is a delayed eyelid opening, and this has not been confirmed in the *sbse* colony. We designed primers for the coding region of *Plag1* using Primer3 (Koressaar and Remm, 2007 Bioinformatics 23(10):1289-91). We compared *sbse/sbse* and C57BL/6J control sequence to the mouse sequence publicly available at ensemble.org. PCR products were sequenced at The Jackson Laboratory core sequencing facility, and we did not find a mutation in the coding region.

Pathology

Tissues for histopathological examination[2] were prepared from both *sbse/sbse* and controls. There was severe hydrocephaly in an 11-week-old male mutant and mild hydrocephaly in a ten and 13-week-old mutant that may explain the domed skull. Due to the small bodies of the mutants, the pituitary gland of a 10-week-old mutant brother and sister were examined but no abnormalities were found. A two-month-old *sbse* male had mild testicular degeneration and *Plag1* is reported to have reduced male fertility.

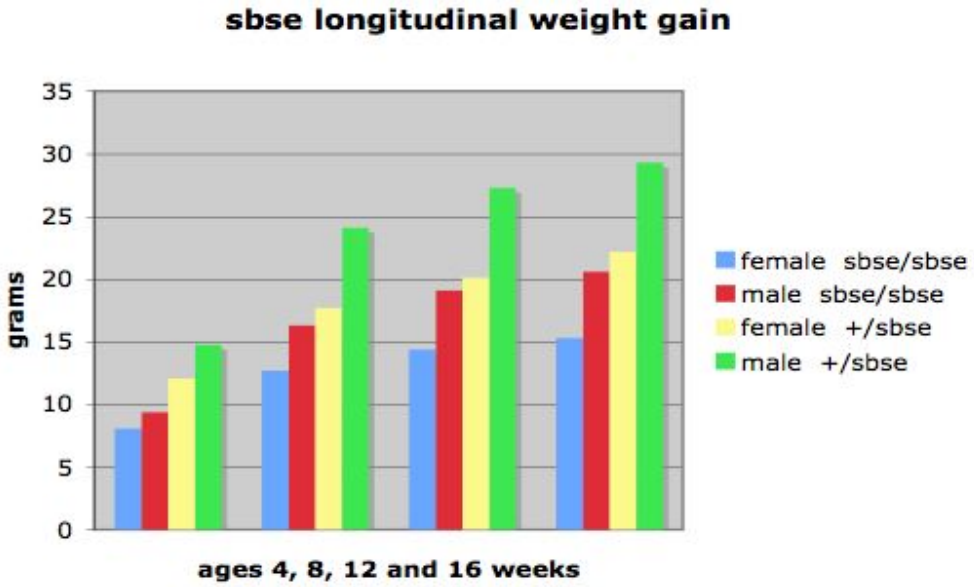
Hearing was assessed by auditory brainstem response threshold analysis[3] (Zheng et al. 1999) on male and female mutants and controls, and mutants had age-related hearing loss. At seven weeks, mutants had 20 db higher thresholds than controls. These same mutants at three months were 30 db higher and were deaf while the controls still had good hearing. Eyes of mutants and heterozygotes from both sexes at 4 weeks old were examined with an ophthalmoscope. There were no abnormalities.

Biological characterization

Reduced litter size: The reduced litter size reported in *Plag1* females is also in our *sbse* strain. Upon examining ten litters from *sbse/sbse* females by +/*sbse* males, there was an average 4.3 pups per litter. To compare, male mutants mated with female carriers produced on average the same number of pups in a litter as heterozygote males mated with heterozygote females with both breeding schemes having 5.4 pups per litter when averaging ten litters from each.

Longitudinal weight study: Mice from eight different litters were collected and weighed at four, eight, 12 and 16 weeks of age (see chart below). Seven mice from each

genotype (+/*sbse* and *sbse/sbse*) and sex (male and female) were represented. At each age, *sbse/sbse* males and females were about 70% the weight of same sex +/*sbse* mice. In conclusion, homozygotes did not catch up in size to the heterozygotes rather they grew within their own parameters. This may be similar to the reduced embryo size and postnatal growth in *Plag1* mice.



DEXA analysis of whole body aBMD and body composition of twelve-week-old mice: Whole body, areal bone mineral density (aBMD), bone mineral content (BMC) and body composition (lean and fat) were assessed by PIXImus densitometry (GE LUNAR, Madison, WI)[4].

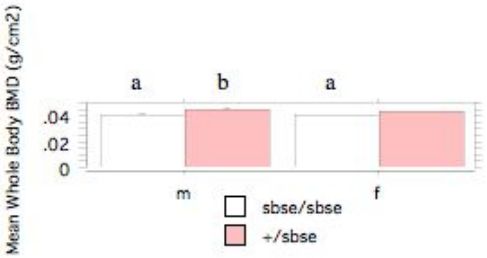
Table 1: PIXImus Densitometric Measurements of Twelve-Week-Old C57BL/6J-*sbse* Mice (n=6; mean±SEM; $^{ab}p \leq 0.05$)

Measurement	Male *sbse/sbse*	Male *+/sbse*	Female *sbse/sbse*	Female *+/sbse*
Whole Body BMD (g/cm^2)	0.0408 ± 0.00038[a]	0.0449 ± 0.00027[b]	0.0397 ± 0.00041[a]	0.0425 ± 0.00079
Whole Body BMC (g)	0.329 ± 0.0170[ab]	0.422 ± 0.0139[b]	0.274 ± 0.0156[a]	0.364 ± 0.0152
Whole Body Lean (g)	13.2 ± 0.59[ab]	18.6 ± 0.51[b]	10.7 ± 0.35[a]	13.9 ± 0.77
Whole Body Fat (g)	2.1 ± 0.14[a]	4.4 ± 0.49	2.1 ± 0.20[a]	3.2 ± 0.16
Total Body Weight (g)	15.3 ± 0.71[ab]	23.0 ± 0.76[b]	12.8 ± 0.45[a]	17.1 ± 0.83
Skull BMD (g/cm^2)	0.1019 ± 0.00136[ab]	0.1113 ± 0.00149	0.1089 ± 0.00277	0.1128 ± 0.00152
Skull BMC (g)	0.197 ± 0.0048[a]	0.253 ± 0.0070	0.204 ± 0.0079a	0.251 ± 0.0096
Skull BMD/Body BMD	2.4983 ± 0.03364[b]	2.4806 ± 0.03719[b]	2.7429 ± 0.05050	2.66 ± 0.053

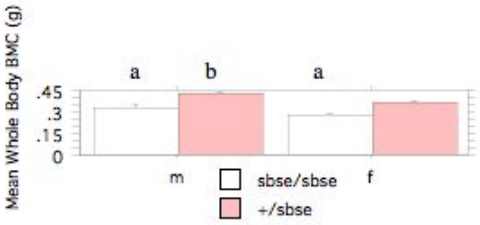
sbse/sbse mice had significantly less fat, whole body aBMD and BMC and skull BMC. Male *sbse/sbse* had significantly lower skull aBMD than both male +/*sbse* and female *sbse/sbse*. Skull to body aBMD ratio was significantly greater with females of

both genotypes compared to males of both genotypes. This skull to body aBMD ratio appears more affected by gender and is due to females having greater skull aBMD than males of the same genotype.

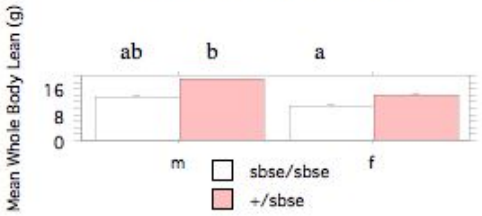
12 Week C56BL/6J-*sbse*
Whole Body BMD (g/cm²)
Split by Sex and Genotype
(n=6;mean ± SEM;[ab]p≤0.05)



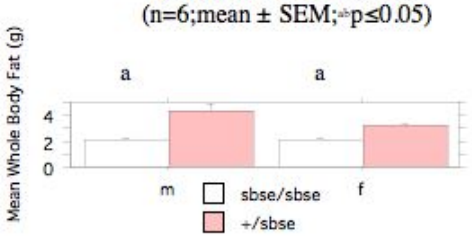
12 Week C57BL/6J-*sbse*
Whole Body BMC (g)
Split by Sex and Genotype
(n=6;mean SEM;[ab]p 0.05)



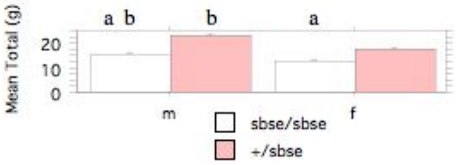
12 week C57BL/6J-*sbse*
Whole Body Lean (g)
Split by Sex and Genotype
(n=6;mean SEM;[ab]p ≤ 0.05)



12 Week C56BL/6J-*sbse*
Whole Body Fat
Split by Sex and Genotype
(n=6;mean ± SEM;[ab]p≤0.05)



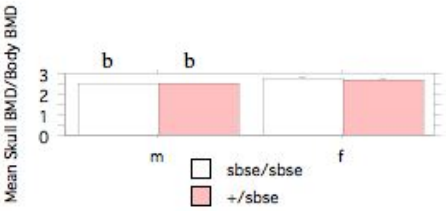
12 Week C56BL/6J-*sbse*
Mean Total (g)
Split by Sex and Genotype
(n=6;mean ± SEM;[ab]p≤0.05)



12 Week C56BL/6J-*sbse*
Skull BMD (g/cm2)
Split by Sex and Genotype
(n=6;mean ± SEM;[ab]p≤0.05)

12 Week C56BL/6J-*sbse*
Mean Skull BMC (g)
Split by Sex and Genotype
(n=6;mean ± SEM;[ab]p≤0.05)

12 Week C56BL/6J-*sbse*
Skull BMD/Body BMD
Split by Sex and Genotype
(n=6;mean ± SEM;[ab]p≤0.05)



Craniofacial morphology of twelve-week-old mice: Skulls were prepared by incomplete maceration in potassium hydroxide, stained with alizarin red, and stored in undiluted glycerin (Green, 1952). Morphological measurements of the skull (Table 2) were made using digital calipers (Stoelting, Wood Dale, Ill) with previously established landmarks (Richtsmeier, 2000)[4]

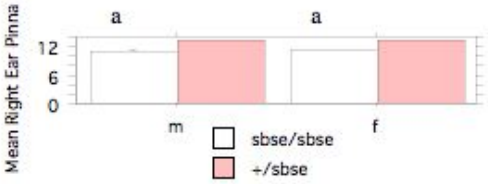
Table 2: Digital Caliper Measurements and Calculated Ratios of Twelve-Week-Old C57BL/6J-*sbse* Skulls Stained with Alizarin Red (n=6; mean±SEM; [ab]p≤0.05)

Measurement	Male *sbse/sbse*	Male *+/sbse*	Female *sbse/sbse*	Female *+/sbse*
Skull Length(mm)	19.87 ± 0.372[a]	22.65 ± 0.098	19.05 ± 0.151[a]	22.37 ± 0.170
Skull Height (mm)	10.17 ± 0.142	10.49 ± 0.085	10.24 ± 0.275	10.36 ± 0.080
Nose Length (mm)	12.81 ± 0.302[a]	15.52 ± 0.164	12.68 ± 0.101[a]	15.37 ± 0.109
Skull Width (mm)	10.30 ± 0.092	10.40 ± 0.091	10.36 ± 0.194	10.18 ± 0.069
Inner Canthal Distance (mm)	5.65 ± 0.093[a]	5.95 ± 0.046[b]	5.88 ± 0.110	5.77 ± 0.067
Upper Jaw Length (mm)	13.77 ± 0.293[a]	16.49 ± 0.059[b]	13.57 ± 0.262[a]	15.89 ± 0.177
Lower Jaw Length (mm)	9.84 ± 0.090[a]	10.78 ± 0.056[b]	9.63 ± 0.072[a]	10.44 ± 0.083
Skull/Nose Length Ratio	1.55 ± 0.012[ab]	1.46 ± 0.017	1.50 ± 0.012[a]	1.46 ± 0.013
Skull Height/Length Ratio	0.51 ± 0.005[a]	0.46 ± 0.006	0.54 ± 0.017[a]	0.46 ± 0.004
Jaw Length Ratio	1.40 ± 0.024[a]	1.53 ± 0.008	1.41 ± 0.024[a]	1.52 ± 0.016
Skull Length/Width Ratio	1.93 ± 0.031[a]	2.18 ± 0.025	1.84 ± 0.042[a]	2.20 ± 0.017
Skull Height/Width Ratio	0.99 ± 0.012	1.01 ± 0.006	0.99 ± 0.009[a]	1.02 ± 0.007
Right Ear Pinna (mm)	11.08 ± 0.270[a]	13.23 ± 0.118	11.19 ± 0.083[a]	13.07 ± 0.098

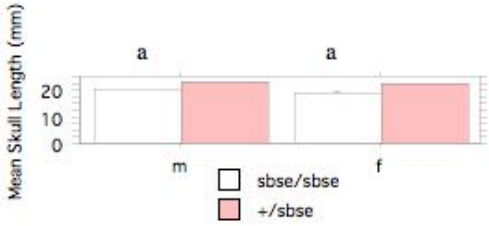
*Female *sbse*/*sbse* n=5

Skull and nose lengths, lower and upper jaw lengths, ear pinnae, and skull length-to-width ratios were all significantly lower in *sbse/sbse* males and females than same sex +/*sbse,* illustrating an overall smaller head in mutants. Additionally, the inner canthal distance of male homozygotes was significantly smaller than male heterozygotes indicating a slightly more proportionate smaller skull than females. However, skull to nose length ratio was greater in *sbse/sbse* mice compared to controls due to disproportionately shorter noses when compared to total skull length. Skull height to length ratios were significantly greater in *sbse/sbse* mice compared to same sex heterozytoges due to skull height in mutants being nearly the same as controls. Mutants heads are overall smaller but are disproportionate.

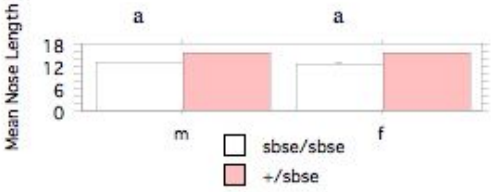
12 Week C56BL/6J-*sbse*
Right Ear Pinna Length (mm)
Split by Sex and Genotype
(n=6*;mean ± SEM;ab p≤0.05)



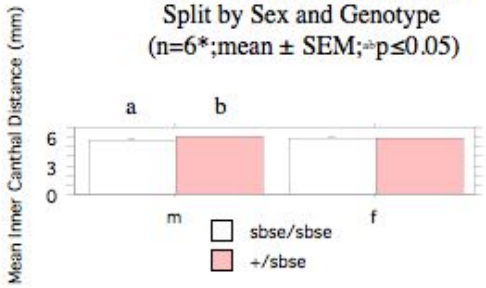
12 Week C56BL/6J-*sbse*
Skull Length (mm)
Split by Sex and Genotype
(n=6*;mean ± SEM;ab p≤0.05)



12 Week C56BL/6J-*sbse*
Nose Length (mm)
Split by Sex and Genotype
(n=6*;mean ± SEM;ab p≤0.05)

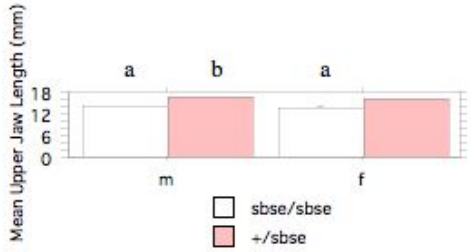


12 Week C56BL/6J-*sbse*
Inner Canthal Distance (mm)
Split by Sex and Genotype
(n=6*;mean ± SEM;ab p≤0.05)



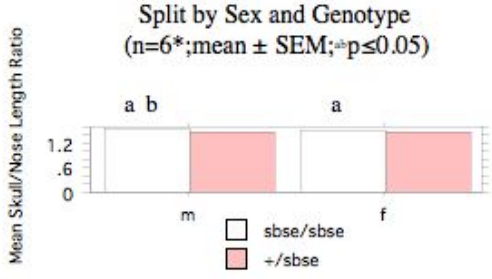
12 Week C56BL/6J-*sbse*
Lower Jaw Length (mm)
Split by Sex and Genotype
(n=6*;mean ± SEM;ab p≤0.05)

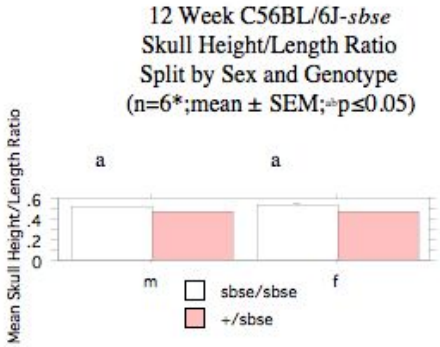
12 Week C56BL/6J-*sbse*
Upper Jaw Length (mm)
Split by Sex and Genotype
(n=6*;mean ± SEM;ab p≤0.05)

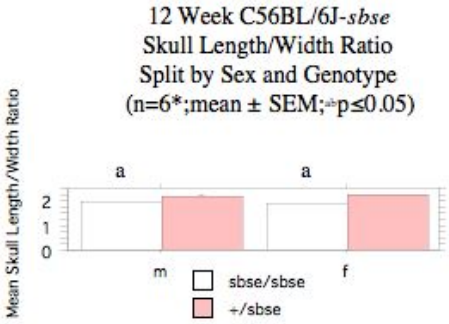


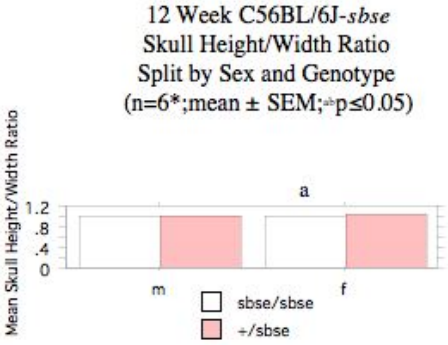
12 Week C56BL/6J-*sbse*
Jaw Length Ratio
Split by Sex and Genotype
(n=6*;mean ± SEM;ab p≤0.05)

12 Week C56BL/6J-*sbse*
Skull/Nose Length Ratio
Split by Sex and Genotype
(n=6*;mean ± SEM;ab p≤0.05)









[a] *+/sbse* vs. *sbse/sbse* within sex
[b] male vs. female within genotype

Discussion

While we cannot confirm our *sbse* strain is a new allele of *Plag1* without further sequencing and/or an allele test, *sbse* mice exhibit characteristics indicative of a mutation in this gene.

Acknowledgements

We would like to thank Sharon Colson for discovering the mutation; Coleen Kane for preparation of tissues for histological assessment; Rod Bronson, Ph.D, for pathological evaluation; Heping Yu for ABR analysis; Norm Hawes for clinical eye evaluation.

Footnotes

[1] **Standard Mapping Protocol used in The Mouse Mutant Resource**

Linkage crosses

To map new mouse mutations we use CAST/Ei, an inbred strain of *Mus musculus castaneus*, as our standard linkage testing strain. In some cases, because of breeding difficulties or reduced phenotypic penetrance, we use other strains. An intercross of F1 hybrids is usually used to analyze linkage of recessive mutations and a backcross to analyze linkage of dominant mutations. Our goal is to produce enough informative mice from each mapping cross to test the recombinational products from at least 100 meioses.

DNA isolation

DNA is extracted from the frozen tail tips of mutant (homozygous) F2 mice or backcross progeny by a standard hot sodium hydroxide and Tris (Hot SHOT) procedure (Truett, et al., 2000) or from spleens using standard phenol extraction methods.

Polymerase chain reaction

PCR primer pairs (MapPairs, from Research Genetics, Huntsville, Ala., or from Integrated DNA Technologies, Coralville, Ia.) are used to type MIT microsatellite markers positioned throughout the genome. PCR reactions contain 20 ng genomic DNA in 10 ul containing 50 mM KCL, 10 mM Tris-HCL (pH 9.0 at 250C), and 0.01% Triton-X-100, 2.25 mM MgCl2, 100 nM of each primer (forward and reverse), 100 uM of each of four deoxyribonucleoside triphosphates, and 0.5 Units of Taq DNA polymerase (Amplitaq from Applied Biosystems #N808-0145). Amplification consists of one cycle of

denaturation at 940C for 3 minutes followed by 30 cycles, each consisting of 940C for 15 sec. denaturation, 550C for 2 minutes of annealing, and 720C for 2 minutes of extension. After the 30 cycles, the final product is extended for 7 minutes at 720C. The PCR products are run on 2.5% Metaphor agarose gels or 6% polyacrylamide (non-denaturing) gels. The gels are then stained with ethidium bromide, destained with distilled water, visualized on a UV light table, and photographed.

Pooled DNA Method (Taylor et al 1994).

In the case of an intercross, a pool of DNA prepared from 25-30 mutant F2 mice is compared with DNA from F1 hybrids. In the case of a backcross, a DNA pool from 25-30 mutant N2 mice is compared with a pool from 25-30 unaffected N2 mice. These DNA samples are typed by PCR for MIT markers located throughout the genome. For linked markers, the mutant strain allele will predominate in the DNA pool from mutant mice compared with controls. When a particular marker indicates linkage by analysis of the pooled sample, individual DNA samples are typed with that marker and additional markers in the same region to confirm linkage. Once a linkage is confirmed, additional DNAs from individual mice are typed to obtain a finer map position.

Linkage analysis

Gene order and recombination frequencies are calculated with the Map Manager computer program (Manley1993, 2001), a MacIntosh program for storage and analysis of genotyping data.

References

Manley KF (1993) A MacIntosh program for storage and analysis of experimental mapping data. Mamm Genome 4: 303-313.

Manly KF, Cudmore RH Jr, and Meer JM (2001) Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome12: 930-932.

Taylor BA, Navin A, and Phillips SJ (1994) PCR-amplification of simple sequence repeat variants from pooled DNA samples for rapidly mapping new mutations of the mouse. Genomics 21: 626-32.

Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, and Warman ML(2000) Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and Tris (HotSHOT). Biotechniques 29:52-54

[2]**Standard Histology Protocol used in The Mouse Mutant Resource**

For fixation of tissues, mice were deeply anesthetized with tribromoethanol (avertin) until they no longer displayed a withdrawal reflex in the hind limbs and then perfused intracardially with Bouin's fixative following a flush of the vasculature with saline solution. After soaking in Bouin's for one week to demineralize bones, tissues were dissected. Six segments of spine with axial muscles and spinal cord in situ, representing cervical, thoracic and lumbar spinal segments, were dissected. The brain was removed and sliced into 6 cross sectional pieces at the levels of olfactory lobes, frontal cortex, striatum, thalamus, midbrain, rostral and caudal medulla with cerebellum. Midsagittal slices of hind leg through the knees were prepared. Slices of basal skull through the pituitary and inner ears were taken. Both eyes, salivary glands and submandibular lymph node, trachea plus thyroid and sometimes parathyroid were removed and cassetted. A longitudinal slice of skin from the back was removed. The thymus, slices of lung, and a longitudinal slice of heart were cassetted. Similarly slices of liver through gall bladder, kidney with adrenal attached, pancreas and spleen were prepared. The stomach was sliced longitudinally to include both squamous and glandular portions. Loops of small intestine from 3 levels and slices of large intestine and cecum were removed, as were slices of urinary bladder. The whole uterus, with ovaries attached, was taken. In males testes were sliced longitudinally. The accessory male organs including seminal vesicles, coagulating gland and prostate were removed en block. Altogether in most cases all tissue fit into a total of 10 cassettes. The cassettes were processed in an automatic tissue processor to dehydrate tissues which were then embedded in paraffin. Six micron sections were cut and stained with hematoxylin and eosin (H&E). Sections of brain and spinal cord in vertebral bones also were stained with luxol fast blue (LFB) for myelin and cresylecht violet (CV) for cellular detail.

[3]**ABR thresholds** in mice are determined using a semi-automated computer system (Intelligent Hearing Systems, Miami, Florida). Subdermal needle electrodes are inserted at the vertex and ventrolaterally to both ears of anesthetized mice. Specific auditory stimuli from 10-100 dB SPL are delivered binaurally through plastic tubes from high frequency transducers. ABR thresholds are obtained, in an acoustic chamber, for clicks and for 8, 16, and 32 kHz pure-tone pips.

[4]**Standard Protocols and Procedures of The Jackson Laboratory Craniofacial Mutant Resource:**

Mouse Colony Maintenance
Craniofacial Resource mice are housed in 51 square inch polycarbonate boxes, on bedding composed of sterilized shavings of Northern White Pine, under 14:10 hour light:dark cycles. A diet of autoclaved NIH 31 (6% fat diet, Ca:P of 1.15:0.85, 19% protein, vitamin and mineral fortified; Purina Mills International, Richmond, IN) and water acidified with HCl to achieve a pH of 2.8-3.2 (which prevents bacterial growth) are freely available. Mouse colony maintenance and use is reviewed and approved by The Jackson Laboratory Institutional Animal Care and Use Committee and is in accordance with The National Institutes of Health guidelines for the care and use of animals in research.

PIXImus Densitometry
PIXImus scans (PIXImus, LUNAR, Madison, WI) which provide skeletal and body composition data such as Bone Mineral Density (aBMD, g/cm^2), Bone Mineral Content (BMC, g/cm2), body mass (g), lean mass (g), fat mass (g), and % fat mass, are completed on groups of 6 male and 6 female 12 week old mutant and control mice. The skulls and bodies are scanned separately to provide independent data on skull aBMD and BMC and body aBMD and BMC. The PIXImus small animal densitometer (DEXA) has a resolution of 0.18 x 0.18 mm pixels and is equipped with software version 1.46 . The PIXImus is calibrated routinely with a phantom utilizing known values, and a quality assurance test is performed daily. The variability in precision for measuring total body aBMD is, less than 1%, and approximately 1.5% for specialized regions such as the skull. The correlation between PIXImus aBMD measurements of 614 lumbar vertebrae compared to peripheral quantitative computerized tomography (pQCT) measurements was found to be significant ($p<0.001$; $r=.704$) (Donahue, 1999).

Faxitron X-rays
X-rays at 5X magnification of the skull and at 3X magnification of the body of a male and female mutant and control at 12 weeks of age are obtained using a Faxitron MX20 cabinet X-ray (Faxitron X-Ray Corp., Wheeling, IL. USA) and Kodak Min-R 2000 mammography film (Eastman Kodak Co., Windsor, CO, USA). X-rays are then analyzed to determine the specificity of the skeletal phenotype.

Skull Preparation
Skulls of 6 male and 6 female mutants and controls are collected at 12 weeks of age, prepared by incomplete maceration in potassium hydroxide, stained with alizarin red, and stored in undiluted glycerin (Green, 1952). During the collection process, right ear pinnae are measured with digital hand calipers (Stoelting, Wood Dale, IL, USA).

Hand Caliper Skull Measurements
Seven measurements taken with hand held digital calipers are used routinely to define skull morphology at the Lab's craniofacial resource. These measures have a high degree of accuracy and precision in our hands and are able to discriminate differences between mutant and control skull characteristics. Our linear measures have been added to those illustrated by Dr. Joan Richtsmeier in her paper characterizing craniofacial differences in mouse models of Down Syndrome using three dimensional anatomical landmarks (Richtsmeier, 2000. Dev. Dyn. Feb; 217(2):137-45). Skulls are cleared with potassium hydroxide and stained with alizarin red dye in preparation for caliper measurements to be taken.

Skeletal Preps
In many cases whole skeletons of mutant and control mice are cleared in 1% KOH, stained with alizarin, stored in glycerin (Green, 1952) and then evaluated for skeletal malformations. Malformations found can indicate that the craniofacial phenotype is part of a greater syndrome.

Data Analysis
Hand caliper skull measurements and PIXImus skeletal and body composition data are evaluated using StatView 4.5 software (Abaccus Cary, NC) for Macintosh computers. Differences are considered significant when $p < 0.05$.

Molecular Mapping
Molecular mapping is completed using DNA extracted from tail or spleen through a Hot Sodium and Tris (HotSHOT) protocol (Truett, 2000). Primer pairs (MapPairs, Research Genetics, Huntsville Ala.) of microsatellite markers are used to establish and refine the initial mapping location. PCR products are visualized and scored via gel electrophoresis and ethidium bromide staining. Linkage analysis and recombination frequencies are calculated via the Map Manager computer program (Manly, 2001) and compared to known and predicted gene location data and marker location data published in the Celera and Ensembl genome databases and the Mouse Genome Informatics website.

Sequencing
Primers are designed using published cDNA sequences from the Ensemble or Celera mouse genome databases. DNA is amplified through PCR and separated via gel electrophoresis. The resultant products are

then removed and purified with QIAquick Gel Extraction Kits (Qiagen, Inc., Valencia, CA, USA) and sequenced using an Applied Biosystems 373A DNA Sequencer and an optimized DyeDeoxy Terminator Cycle Sequencing method.

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