Activin/inhibin β B subunit gene disruption leads to defects in eyelid development and female reproduction

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Inhibins and activins are dimeric growth factors of the transforming growth factor- β superfamily, a class of peptides that can regulate the growth and differentiation of a variety of cell types. Recently, activins have been implicated in early vertebrate development through their ability to evoke, in *Xenopus* embryo explants, both morphological and molecular changes characteristic of mesoderm induction. To understand these processes further, we have used homologous recombination in embryonic stem cells to create mouse strains carrying mutations in the gene encoding the activin/inhibin β B subunit. These mice are expected to be deficient in activin B (β B: β B), activin AB (β A: β B), and inhibin B (α : β B). Viable mutant animals were generated, indicating that the β B subunit is not essential for mesoderm formation in the mouse. Mutant animals suffered, however, from distinct developmental and reproductive defects. An apparent failure of eyelid fusion during late embryonic development led to eye lesions in mutant animals. Whereas β B-deficient males bred normally, mutant females manifested a profoundly impaired reproductive ability, characterized by perinatal lethality of their offspring. The phenotype of mutant mice suggests that activin β B (1) plays a role in late fetal development and (2) is critical for female fecundity. In addition, we have found that expression of the related β A subunit of activin is highly upregulated in ovaries of mutant females. Altered regulation of β A activin in β B-deficient mice may contribute to the mutant phenotype.

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Candidate genes and proteins that govern the formation and patterning of the mesoderm in vertebrate embryos have recently emerged. Through studies in amphibians came the realization that specific polypeptide growth factors were able to reproduce some aspects of mesoderm induction in embryo explants. These factors include members of the transforming growth factor- β (TGF- β) superfamily such as activins A and B, bone morphogenetic protein 4 (BMP-4) and Vg-1, and members of the fibroblast growth factor (FGF) family such as basic FGF [Kimelman and Kirschner 1987; Slack et al. 1987; Asashima et al. 1990; Smith et al. 1990; Thomsen et al. 1990; van den Eijnden-Van Raaij et al. 1990; Koster et al. 1991; Dale et al. 1992; Jones et al. 1992; Thomsen and Melton 1993]. In addition, other factors that do not have the ability to induce mesoderm from presumptive ectoderm by themselves were found to modify the pattern of mesodermal differentiation evoked by an inducer. These include members of the Wnt family such as *Xwnt-8*, and *noggin* [Smith and Harland 1991, 1992; Sokol et al. 1991; for review, see Kimelman et al. 1992; Sive 1993]. Among mesoderm-inducing molecules, activins are unique be-

cause of their ability to induce a full range of mesodermal fates, from anterior-most mesoderm (organizer) to tail, in a concentration-dependent manner [Green et al. 1992]. Genes induced by activins as an immediate-early response were identified and include the organizer-specific *goosecoid* gene and the *Brachyury* gene [Cho et al. 1991; Smith et al. 1991]. In the *Brachyury* mouse, mesoderm formation is defective. Cloning of the mouse *Brachyury* (*T*) gene [Herrmann et al. 1990] has provided the first identification of a gene located on the pathway of mesoderm formation in vertebrates. As inducers of *Brachyury*, activins are thought to be part of this pathway as well. Animal caps induced by activin also undergo morphogenetic cell movements that are characteristic of gastrulation [Smith and Howard 1992]. Thus histological, molecular, and cell behavior responses of animal cap explants to activin recapitulate many aspects of gastrulation. Hemmati-Brivanlou and Melton (1992) have provided the most compelling evidence of the involvement of an activin-like molecule in mesoderm formation by showing that injection of an mRNA encoding a truncated activin receptor into *Xenopus* embryos in-

terferes with activin signaling and prevents mesoderm induction.

Activins are structurally related to the inhibin class of peptides. Inhibins were initially isolated from mammalian follicular fluid on the basis of their ability to inhibit the release of follicle stimulating hormone (FSH) from anterior pituitary cells (for review, see Vale et al. 1990). In the course of inhibin purification, side fractions that instead stimulated FSH release led to the identification of activins (Ling et al. 1986; Vale et al. 1986). Inhibins and activins are dimers assembled from subunits encoded by three distinct genes: the α , β A, and β B subunit genes. The precursor polypeptides encoded by these genes are proteolytically processed into mature peptides, whose dimers form the biologically active molecules. Inhibins are α : β heterodimers, whereas activins are β : β homo- or heterodimers. The two β subunits, β A and β B, share within a species ~60% sequence identity, whereas the α subunit is more distantly related. Each β subunit is highly conserved across species and >98% identical between mammalian species (Vale et al. 1990). The β B subunit is expressed in *Xenopus* (Thomsen et al. 1990) and chick (Mitrani et al. 1990) late blastula embryos. In the mouse, β subunit mRNAs and β subunit immunoreactivity, in the absence of α subunit expression, were demonstrated during preimplantation stages, indicating the presence of activin in the pregastrulation mouse embryo (van den Eijnden-van Raaij et al. 1992; Albano et al. 1993).

In adult animals, highest expression levels of activin/inhibin subunits are found in the gonads. Recent evidence implicates inhibins and activins as intragonadal paracrine and/or autocrine regulators (for review, see de Jong et al. 1990; Mather et al. 1992; Findlay 1993). The major sites of activin/inhibin expression are the Sertoli cells of the testis and the granulosa cells of the ovary. Inhibins and activins were found to affect both the germ cells and the somatic components of the gonads. Activin can stimulate spermatogonial proliferation, whereas inhibin has the opposite activity. Isotypes of activin receptors were detected on specific populations of male germ cells (Kaipia et al. 1992, 1993), suggesting that these factors play a role in the regulation of spermatogenesis. Furthermore, androgen biosynthesis in testicular Leydig or ovarian thecal cells is inhibited by activin and enhanced by inhibin. In females, activin and inhibin were suggested to either stimulate or inhibit, respectively, oocyte meiotic maturation, and to affect follicular development. The most conclusive evidence for a critical role of inhibin as a regulator of proliferation of somatic components of the gonad was provided by the finding that inhibin-deficient mice generated by α subunit gene targeting develop gonadal stromal tumors at a very early age (Matzuk et al. 1992).

Other studies have revealed extragonadal sources of inhibin and activin synthesis and demonstrated responses in a variety of cell types (Meunier et al. 1988; Vale et al. 1990). Thus, activins have been implicated in inhibition of neural differentiation (Hashimoto et al. 1990; Schubert et al. 1990; van den Eijnden-van Raaij et

al. 1991; Hemmati-Brivanlou and Melton 1992), and induction of erythropoietic differentiation (Murata et al. 1988). In addition to their action on FSH release, their ability to regulate secretion of other hypothalamic or pituitary peptides such as oxytocin, ACTH, and GH was shown (Vale et al. 1990).

To study the physiological role of these factors in mouse development and reproduction, we have used homologous recombination in embryonic stem (ES) cells to create mutant alleles where most of the first coding exon of the β B gene was deleted, and these mutations were introduced into the germ line of mice. Although it remains to be seen whether embryonic development proceeds normally at a molecular level in β B-deficient animals, they are born without major morphological abnormalities, with the exception of a defect in eyelid outgrowth and closure. The generation of viable mutant animals has, however, revealed a critical role for activin/inhibin β B in female reproductive function.

Results

Mice homozygous for a targeted mutation in the activin/inhibin β B subunit gene are viable

The mouse activin/inhibin β B subunit gene was isolated from a genomic DNA library derived from the 129/Sv strain by use of a rat partial cDNA as a probe (rinB-c2-P; K. Mayo, unpubl.). Location of the coding exons is diagrammed in Figure 1, A and B. Two replacement type targeting vectors termed pBB1 and pBB2 (Fig. 1B) were used to disrupt the β B subunit gene by homologous recombination (Thomas and Capecchi 1987). Each vector contained 15.5 kb of homology with the cognate gene. These constructs were designed to replace a 1.3-kb region of the β B subunit locus spanning the translational start codon, the signal peptide coding region, as well as most of exon 1-encoded pro-region sequences with a PGK promoter-driven neo selection cassette containing a PGK poly(A) signal [PGK-NEO-p(A)] (McBurney et al. 1991). The pBB1 construct contained the PGK-NEO-p(A) cassette in the same transcriptional orientation as the β B gene, and the pBB2 construct contained it in reverse orientation. J1 ES cells (Li et al. 1992) were electroporated with linearized DNA and selected with G418. Homologous recombinant ES cell clones were identified by Southern blot analysis of genomic DNA from individual clones (see Materials and methods). Both targeting constructs demonstrated similar, high frequencies of recombination at the endogenous β B subunit locus (Table 1).

Three independent ES clones heterozygous for the BB1 mutation and one clone heterozygous for the BB2 mutation were injected into host blastocysts to generate chimeric founder mice. All four clones contributed to the germ line of chimeric animals, permitting the derivation of several independent mouse lines carrying the mutant alleles. Mice heterozygous for either the BB1 or BB2 mutation did not display an overt phenotype. To evaluate the possibility that the genetic background influences the mutant phenotype, animals heterozygous for the BB1

Figure 1. Mutagenesis at the activin/inhibin βB subunit locus of mice. (A) Exon structure of the βB subunit gene showing the region deleted in the BB1 and BB2 mutant alleles. The transcription start and polyadenylation sites have not been mapped; therefore, the corresponding exon boundaries are indicated with dashes. The hatched box indicates the coding region for the mature βB peptide. Note that the 5'-splice site at the end of the first coding exon is left intact in the BB1 and BB2 recombinant alleles. Not all *NarI* sites within the 1.3-kb deleted fragment are represented. (B) Targeting scheme. A physical map of the locus is shown at the top. The structure of the targeting constructs is indicated below. Dashed lines delineate regions of homology with the chromosome. The new *EcoRI* site introduced by the mutation is indicated in each targeting vector. The pBB2 vector is identical to pBB1, except that the orientation of the neo cassette is reversed. Probe E is the external probe used to screen ES cell colonies and to genotype mice. (rinB-c2-P) The rat cDNA probe used to isolate genomic DNA from the βB locus. (C) Southern blot analysis of DNA from offspring produced by heterozygote intercrosses. DNA was digested with *EcoRI* and hybridized to probe E. Molecular weight markers (in kilobases) are shown at the right. Restriction sites: (B) *BglII*; (R) *EcoRI*; (N) *NarI*; (Nc) *NcoI*.

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mutation were bred to homozygosity on three different genetic backgrounds. Genotype analysis of weanling offspring (Table 2) revealed that homozygous mutant animals with a 129/Sv $\times$ C57BL/6 hybrid genetic background were only marginally compromised in their viability, if at all, as they represented 21% of the animals genotyped, instead of the expected 25%. In the 129/Sv $\times$ BALB/c hybrid background, however, the fraction of homozygous mutants was lower (Table 2), suggesting that alleles in the BALB/c background may interact with the activin mutation and reduce viability. These results therefore indicated that mice homozygous for the BB1 or

BB2 mutation are viable, although their viability may be affected by the genetic background.

Mutant mice lack wild-type mRNA and mature βB peptide

To examine how the BB1 mutation affects transcription at the βB locus, RNA was prepared from ovaries and testes of immature (3-week) or adult animals and subjected to reverse transcription followed by the polymerase chain reaction (RT-PCR). When oligonucleotide primers that span the mature peptide-coding region were

Table 1. Homologous recombination at the activin/inhibin βB locus

Targeting construct	Cells electroporated	G418 ^r colonies	G418 ^r clones screened	Positive clones ^a	Targeting frequency targeted clones/	
					G418 ^r clones	transfected cells
pBB1	5 $\times$ 10 ⁷	5100	172	41	1/4.2	2.4 $\times$ 10 ⁻⁵
pBB2	5 $\times$ 10 ⁷	2900	58	10	1/5.8	1 $\times$ 10 ⁻⁵

^aAs determined by Southern blot analysis using genomic probe E (see Fig. 1).

Table 2. Genotypes at weaning age

Cross (female × male)	Mouse line ^a	Genetic background ^b	Total progeny	βB genotype of progeny		
				+/+	+/m	m/m
(+/m) × (+/m)	129–134	129	40	11	15	14
	BALB-210	(129 × BALB) F ₁	103	30	53	20
	BALB-15	(129 × BALB) N1	60	22	28	10
	B6-134	(129 × B6) F ₁	65	17	35	13
	B6-43	(129 × B6) N1	223	61	114	48
(+/m) × (m/m)	B6-43	(129 × B6) N1	80		40	40
(m/m) × (+/m)	B6-43	(129 × B6) N1	21		11	10

^aAll lines carry the BB1 mutation, except line BALB-210, which carries the BB2 mutation. The number after the dash in a line name refers to the original ES cell clone.

^bTo establish lines B6-43 and BALB-15, heterozygous F₁ animals were first backcrossed once with wild-type C57BL/6 or BALB/c animals, respectively, and the resulting heterozygotes (B6, or BALB, N1 animals) were intercrossed. (129) The 129/Sv strain. (B6) The C57BL/6 strain.

used, a βB-specific band of the expected size was obtained both in mutants and in controls. In addition, primers that span the intron amplified the expected βB-specific band in mutant animal tissue, although with markedly reduced efficiency relative to controls (data not shown). These results indicated that transcription downstream of the neo insertion occurred in the BB1 allele. This is not unexpected as the PGK poly(A) site was shown previously to allow readthrough transcript accumulation (e.g., Lee et al. 1992).

To characterize further the nature of the transcript produced by the BB1 mutated allele, Northern blot analysis was performed (Fig. 2). The two probes used were the mACT10/11,13 probe, a PCR-generated fragment corresponding to the mouse βB mature peptide region (Fig. 2A; see Materials and methods), and a neo probe (Fig. 2B). An ~4.5-kb transcript corresponding to the major wild-type mRNA (Albano et al. 1990; Manova et al. 1992) was detected with the mACT10/11,13 probe in (+/+) and (+/bb1) but not in (bb1/bb1) ovaries. In wild-type animals, a minor ovarian transcript of ~3.5 kb could be detected but not in (bb1/bb1) animals. The 3.5-kb transcript was also seen in wild-type testes, approximately equimolar to the 4.5-kb transcript, as reported previously (Feng et al. 1989a). Neither the 4.5- nor the 3.5-kb transcripts were detected in the (bb1/bb1) mutant testes. The mACT10/11,13 probe, however, detected a novel transcript of ~4.0 kb in both testes and ovaries of animals containing a mutated BB1 allele but not in (+/+) animals. The neo probe also hybridized to a band of the same size. Therefore, the 4.0-kb transcript likely corresponds to a neo-βB chimeric transcript, reading through the PGK poly(A) site and terminating at one of the βB gene endogenous poly(A) sites. Because the promoter(s) and poly(A) site(s) of the mouse βB gene have not been mapped, it is not possible to predict the exact size of such a chimeric transcript. As expected, a major ~1.0-kb band corresponding to a transcript initiated at the PGK promoter and terminated at the PGK poly(A) site was detected by the neo probe in bb1/bb1 and +/bb1 animals. As determined with the neo probe, the 4.0-kb neo-

βB readthrough transcript was ~1/10 as abundant as the neo transcript terminated at the PGK poly(A) site. Examination of RNA from animals homozygous for the BB2 allele likewise showed the absence of the wild-type transcripts and revealed the production from this allele of a new transcript of ~3.0 kb that did not hybridize to the neo probe (Fig. 2), consistent with its initiation within the PGK promoter (used in reverse orientation) and its termination at an endogenous βB poly(A) site.

To establish whether the aberrant transcript made by the BB1 allele could produce βB peptide, we then performed protein blot analysis. βB-Specific antibodies (Vaughan et al. 1989) could readily detect a ~12-kD peptide in ovarian tissue from heterozygous or wild-type weanling females (Fig. 3, top, lanes 6,7,9,10,12–14), but not in tissue from bb1/bb1 mutant females (lanes 4,5,8,11). A polypeptide of ~50 kD, consistent with the βB precursor size, was also detected by the βB-specific antibodies in gonadal extracts from wild-type or heterozygous animals but was absent in homozygous mutants (not shown).

In summary, the results of Figures 2 and 3 indicate that the BB1 mutant allele does not yield productive βB subunit mRNAs capable of generating the mature βB peptide whose dimer with another β subunit or with an α subunit forms the biologically active activin or inhibin ligand. bb1/bb1 mutant animals are therefore expected to lack activins B and AB, as well as inhibin B, but their ability to produce activin A and inhibin A should be preserved.

The βB-deficient ovary up-regulates expression of the βA subunit

Expression of the activin/inhibin βA mature peptide was investigated in total ovarian tissue extract from 3-week-old females. This analysis revealed a 3- to 20-fold increase in levels of βA mature peptide in animals homozygous for the BB1 mutation, relative to their heterozygous or wild-type littermates (Fig. 3, middle, e.g., cf. lanes 4 and 5 with lanes 6 and 7). In the same ovarian

Figure 2. RNA analysis in β B mutant animals. Total RNAs from ovaries (~ 10 μ g, lanes 1–5), testes (20 μ g, lanes 6–8), and uterus (20 μ g, lanes 9–11) from individual animals were examined by RNA blot analysis with a probe corresponding to the β B mature peptide encoding region [mACT1011;13 (A)] or a neo probe (B). Genotypes of animals are indicated at the top, and positions of ribosomal RNAs are shown at the right.

extracts, anti- α inhibin subunit antibodies recognized a ~ 45 -kD polypeptide whose levels were not altered by the β B mutation (Fig. 3, bottom).

Overexpression of the β A subunit in the β B-deficient ovary may reflect altered development and cellular architecture of the mutant tissue, leading to an over-representation of β A-expressing cells. Alternatively, regulatory mechanisms that sense the β B deficiency may exist and cause a compensatory increase in β A subunit expression.

To analyze this effect further, β A subunit RNA expres-

sion was examined by Northern analysis of ovarian extracts from immature animals. No consistent alterations in levels of the ~ 7.0 -kb mRNA were observed in mutant tissue (not shown). Therefore, a post-transcriptional mechanism may operate to up-regulate β A mature peptide production in the β B-deficient ovary.

Failure of eyelid fusion at birth

Upon intercrossing animals heterozygous for the BB1 (or BB2) mutation we observed that a fraction of the neonates in the resulting litters had a distinctive phenotype. Normally, mouse pups are born with closed eyes due to fusion of the eyelids at embryonic day 16 (E16) (Rugh 1990), and eyes open again only at ~ 13 days of postnatal life. In contrast, a fraction of the offspring from heterozygous parents were born with open eyes, as illustrated in Figure 4, A and B. All of the open-eyed neonates derived from four independent ES cell clones carrying either the BB1 or BB2 mutation and genotyped at birth by Southern analysis were homozygous for the mutant allele. Not all homozygotes, however, showed this phenotype, suggesting incomplete penetrance (see below). To determine whether the structure of the eye was otherwise affected in the mutant newborns, histological sections through the heads of bb1/bb1 mutant animals and +/bb1 control littermates were examined (Fig. 4 C–E). The general anatomy of the eye of the mutant newborns appeared normal, indicating a defect confined to the eyelids. Animals born with open eyes quickly developed a number of eye defects, which likely reflected traumatic damage due to the lack of eyelid protection. Hyperkeratinization and squamous metaplasia of the corneal epithelium, accompanied by massive leukocyte infiltration of all cornea layers and eyelids, was observed as soon as the first day of life. During the subsequent days, eyelids often sealed. The timing of eye reopening then appeared delayed in mutants and revealed permanent damage that included ocular dystrophy and opacification of the cornea. This macroscopic eye pathology allowed ready identification of mutant animals during adulthood.

The open eyelid phenotype of the BB1 mutation was found to depend on the genetic background (Table 3). In the original mouse line 43, F₁ animals had been backcrossed once to C57BL/6 and then intercrossed. In this line, the fraction of homozygous mutant animals exhibiting obvious eye defects during adulthood was 0.84 ($n = 89$). A similar phenotype was observed in a 129/Sv $\times$ BALB/c hybrid genetic background, although with reduced penetrance. In contrast, when the BB1 mutation was present in a 129/Sv inbred background, the failure of eyelid fusion was never observed: None of the pups born from heterozygote intercrosses ($n = 137$), or born from homozygous mutant males mated to heterozygous females ($n = 69$), had open eyes at birth; and among 14 adult mutant animals, none had eye defects. This suggests that the 129/Sv inbred strain carries a gene (or genes) that suppresses the open eyelid phenotype caused by the BB1 mutation. Segregation of such 129/Sv-specific suppressor genes may also explain the incomplete

Figure 3. Animals homozygous for the BB1 mutation lack mature β B peptide and up-regulate β A peptide expression. Ovarian extracts from individual immature (21-day-old) females were run on a SDS-PAGE gel under reducing conditions and examined by immunoblot analysis with anti- β B antibodies (*top*). These antibodies recognize a peptide in the carboxy-terminal region of the β B subunit. The same blot was stripped and reanalyzed using anti- β A antibodies (*middle*) or anti- α antibodies (*bottom*). Lanes 1–3 contain 1 ng each of human recombinant inhibin A (lane 1), activin A (lane 2), and activin B (lane 3). Lanes 4–14 contain 100 μ g each of total protein from ovary. Genotypes are indicated at the *top*. Lanes 13 and 14 represent a control wild-type 129/Sv and a control wild-type C57BL/6 female, respectively. Extracts in lanes 4–7 originate from siblings with a 129/Sv $\times$ BALB/c hybrid genetic background (BALB-N2 backcross), lanes 8–10 are from siblings with a 129/Sv $\times$ C57BL/6 background (B6-N1), and lanes 11 and 12 are from siblings from a (129/Sv $\times$ C57BL/6) N4 backcrossed generation. Migrations of molecular mass markers (in kilodaltons) are shown at *left*.

penetrance of the phenotype in the two hybrid genetic backgrounds. Furthermore, the expressivity of the phenotype varied among affected individuals, with various degrees of completion of eyelid fusion at birth or, occasionally, a single eye being affected.

Mutant females manifest a reproductive impairment

Activins and inhibins are thought to be regulators of reproductive functions through their influence on both the pituitary and the gonads. Therefore, we then evaluated the fertility of the BB1 homozygous mutant animals. The viability of homozygous mutant embryos had also raised the possibility that maternal supply of β B peptide might rescue the potential defect in mutant embryos. If this were the case, mutant females might be expected to be impaired in sustaining normal pregnancy and delivery of live pups.

To assess reproductive performance, we bred *bb1/bb1* mutant animals of either gender with heterozygous mates and intercrossed homozygous mutant animals or heterozygous littermates. The average number of viable offspring per breeding pair and per month of breeding was determined for each type of cross. Figure 5 summarizes the reproductive performances observed. Homozygous mutant males bred as well as their heterozygous littermates when mated to heterozygous females. In sharp contrast, homozygous mutant females mated to either heterozygous or homozygous mutant males manifested a profoundly reduced ability to generate live offspring as compared with their heterozygous littermates. Histological examination of ovaries from β B-deficient fe-

Figure 4. Failure of eyelid closure at birth. (A) A heterozygous control pup showing fused eyelids covering the eye. (B) Homozygous mutant littermate with open eyelids. Photographs in A and B were taken within a few hours of birth. (C–E) Transverse sections through the eyes of a heterozygous control neonate (C); an arrow indicates the site of eyelid fusion, a mutant neonate a few hours after birth (D), before inflammation is evident, and a mutant neonate at ~0.5–1 day of age (E), displaying acute leukocyte infiltration in the cornea and eyelids, corneal edema, and corneal hyperkeratinization. Objective magnification was 10 $\times$.

Table 3. The eyelid closure defect: phenotypic variation with genetic background

Cross (female × male)	Mouse line	Genetic background	oeb/Total ^a (neonates)	Penetrance ^b (adults)
(+/m) × (+/m)	129-134	129	0.00 (n = 137)	0.00 (n = 14)
	BALB-210	(129 × BALB) F ₁	0.04 (n = 90)	0.21 (n = 24)
	BALB-15	(129 × BALB) N1	0.12 (n = 102)	N.D.
	B6-134	(129 × B6) F ₁	0.11 (n = 299)	0.33 (n = 21)
	B6-43	(129 × B6) N1	0.20 (n = 367)	0.84 (n = 89)
(+/m) × (m/m)	B6-43	(129 × B6) N1	0.46 (n = 225)	
(m/m) × (+/m)	B6-43	(129 × B6) N1	0.53 (n = 36)	
(m/m) × (m/m)	B6-43	(129 × B6) N1	0.86 (n = 78)	

^aoeb/Total, fraction of total offspring with one or both eye(s) open at birth or displaying resulting eye defects as observed within 1 week of life. The total number of offspring examined for each cross is indicated in parentheses. All pups whose eyes could be examined are entered irrespective of their viability.

^bFor each mouse line, the penetrance is calculated as the fraction of adult homozygous mutant animals displaying obvious eye defects (ocular dystrophy and/or corneal opacification) of one or both eyes, as determined by simple visual inspection. The total number of homozygous mutant animals examined is in parentheses. (N.D.) Not determined.

males did not show overt abnormalities (data not shown). The reproductive impairment of mutant females did not result from defective oogenesis, inability to undergo fertilization, or development of embryos in utero, because they became pregnant at a frequency similar to their heterozygous littermates. Reduced fertility appeared rather to result from perinatal loss of the progeny. Offspring were born from mutant females but rarely survived beyond 24 hr postpartum. A common observation on the day of delivery was death of an entire normal-sized litter.

There was no excess of phenotypically mutant animals among the pups dying perinatally. Consistent with

this, we observed no distortion of genotypes among the rare pups that survived the perinatal period: Of 21 weanling mice born from bb1/bb1 mutant females mated to heterozygous males, 10 were bb1/bb1 and 11 were +/bb1. Thus, neonatal lethality appears unaffected by the genotype of the offspring but rather reflects a maternal defect.

Female reproductive failure was observed in lines of mice derived from three independent ES cell clones and was manifest in the three genetic backgrounds tested: In inbred 129/Sv background and in the two hybrid backgrounds (129/Sv × C57BL/6 and 129/Sv × BALB/c, after one backcross to C57BL/6 or BALB/c, respectively). 129/

Figure 5. Reproductive performance of animals heterozygous or homozygous for the BB1 mutation. The number of viable progeny per breeding pair and per month of breeding was averaged for each type of cross in each genetic background. Genetic background is indicated at the top, and the number of breeding pairs (n) is indicated at the bottom. Several m/m (129 × BALB) females died during their first pregnancy from failure to deliver and were not included.

Sv×BALB/c hybrid mutant females appeared the most seriously affected: A total of 10 pregnancies carried by four different females failed to generate live offspring.

Mutant females show increased gestation time and decreased nursing ability

In staged matings where the day of vaginal plug (E0.5) was recorded, β B-deficient females were observed to show an increase in the average duration of gestation. Among 13 control sibling females (heterozygous or wild type), 8 had delivered the morning of day E19.5 and all but one had delivered by the afternoon of that day. In striking contrast, none of 12 mutant females delivered on day E19.5. Most mutant females delivered on day E20.5 and some in the morning of day E21.5. Occasionally, initiation of labor appeared to fail altogether, resulting in sickness of the gravid mother and death of fetuses in utero.

Delivered babies that were about to die were morphologically unremarkable but were cold and had empty stomachs, suggesting that their death was due to a maternal failing. Lactogenesis, however, occurred in mutant females. Histological analysis of mammary glands in a pair of control and mutant siblings at ~0.5 day postpartum is shown in Figure 6. Accumulation of milk in

lobules and ducts of mutant tissue is evident. These observations suggest that either milk let-down is impaired or that the babies fail to suckle. The former possibility is supported by preliminary data showing that offspring born from mutant females and not nursed can be rescued by fostering to a normal female (not shown).

FSH measurements in mutant animals

To analyze further the cause of the reproductive defect in mutant females and to determine whether pituitary function was altered in β B-deficient animals, serum FSH levels were measured (Table 4). The interpretation of these data was complicated by the facts that (1) FSH values fluctuate widely within an experimental group and (2) an age-dependent increase in FSH values was evident in adult animals from 11 to 24 weeks of age. Nevertheless, this study revealed an ~20% increase in average serum FSH levels in mutant animals relative to heterozygotes, and the increase was consistent in all groups analyzed. It thus appears that in the context of a deficiency in both activins B, AB (which enhance FSH release in vitro) and inhibin B (which decreases it), the effect of inhibin deficiency is slightly dominant. The physiological significance of this modest elevation in circulating FSH is unknown.

Discussion

We have created mouse lines carrying two different mutant alleles for the gene that encodes the activin/inhibin β B subunit. In both alleles the coding capacity (in exon 1) for approximately one-third of the β B precursor polypeptide was deleted at its amino terminus. Gray and Mason (1990) have shown that the pro-region of the activin A precursor is essential for the intracellular assembly of the homodimer and secretion of biological activity. It is not known where the dimerization activity resides within the pro-region and whether the exon 2-encoded pro-region would suffice in this function. It is likely, however, that the alteration generated loss-of-function alleles because the signal sequence and approximately half of the propeptide sequence were deleted. Protein analysis confirmed the lack of mature β B peptide in mutant tissue. The mutation, therefore, should prevent the synthesis of activins B, AB and of inhibin B. Nevertheless, mutant mice completed embryonic development and were viable. Their birth with open eyes indicated, however, that some aspects of development were altered in the absence of a wild-type β B subunit gene. The generation of viable mice has furthermore uncovered a maternal effect by which maternal β B chain expression is required for efficient perinatal survival of newborns. As a result, β B-deficient females had an extremely low fecundity, despite their ability to sustain gestation up to the time of birth.

Recent evidence implicates an activin-like molecule as a patterning morphogen during early vertebrate development (for review, see Kimelman et al. 1992; Sive 1993). Remarkably, interference with the activin path-

Figure 6. Postpartum histology of mammary glands in mutant and control heterozygote siblings. (A,C) Control tissue from a heterozygous female. (B,D) Tissue from a mutant β B-deficient sibling. Tissue was taken ~0.5 days after delivery. Note the increased glandular/adipose tissue ratio, the accumulation of milk and the vacuolation of lobules in mutant mammary tissue. The mutant animal had delivered a litter that was not nursed. Both animals were from a BALB-N1F2 generation. Objective magnification was 4 $\times$ (A,B), and 20 $\times$ (C,D).

Table 4. Serum FSH values in β B-deficient mice

Mouse line	Genetic background	Gender	Age (weeks)	FSH (ng/ml)		
				+ / +	+ / m	m / m
129-134	129	male	11–16	N.D.	90.4 $\pm$ 14.6 (n = 9)	105.7 $\pm$ 18.5 (n = 6)
B6-43	(129 $\times$ B6) N1	male	11–25 ^a	141.6 $\pm$ 33.2 (n = 8)	172.0 $\pm$ 42.8 (n = 14)	206.0 $\pm$ 57.7 (n = 14)
			6.5–14.5 ^a	124.1 $\pm$ 62.6 (n = 6)	110.6 $\pm$ 33.8 (n = 11)	171.0 $\pm$ 111.4 (n = 11)
B6-43	(129 $\times$ B6) N1	female	12–24	78.9 $\pm$ 22.4 (n = 6)	73.2 $\pm$ 18.1 (n = 9)	86.4 $\pm$ 27.4 (n = 11)

^aTwo experimental groups of males of the B6-43 line had FSH values determined in separate experiments.

way by injection of an mRNA encoding a truncated activin receptor into *Xenopus* embryos prevents mesoderm induction and formation of axial structures (Hemmati-Brivanlou and Melton 1992). Of the two activin genes, the β B subunit gene is the first expressed in *Xenopus* (Thomsen et al. 1990; Dohrmann et al. 1993) and chick embryos (Mitrani et al. 1990), which has led to the hypothesis that activin B may be involved in mesoderm induction. Activin activity is already detectable in the unfertilized *Xenopus* egg (Asashima et al. 1991), but zygotic expression occurs only at the late blastula stage (Thomsen et al. 1990). Thus, it is not clear whether activins are involved in the induction of mesoderm itself or, rather, in patterning and regionalization of already induced mesoderm. In the mouse embryo, activin β B-specific RNA and activin β subunit protein have been detected in blastocysts and at cleavage stages, respectively (van den Eijnden-van Raaij et al. 1992; Albano et al. 1993). Survival of β B mutant mice to adulthood, therefore, indicates that activins B, AB and inhibin B are dispensable for embryonic development in the mouse. Matzuk et al. (1992) have shown previously that α subunit-deficient mice, lacking inhibins A and B, develop to term. These molecules may not play a function in early mouse development, or overlapping pathways may exist allowing compensation for their deficit at these stages. The β A subunit gene in particular may cover the β B deficiency in our mutants. Our finding of up-regulated expression of the β A subunit in the β B-deficient ovary possibly reflects a regulatory mechanism that also operates in the mutant embryo and provides a means for normal morphogenesis in the absence of activin B. Generation of an activin-less mutant by targeting the β A gene and intercrossing β A and β B mutant mice should provide definitive answers to some of these questions. In addition, examination of the expression of activin immediate-early response genes, as *Brachyury* and *gooseoid*, in the β B-deficient embryos should help to determine whether development proceeds normally, both temporally and spatially.

Although β B subunit mutant embryos develop to term, the deficiency in either activin B, activin AB, or inhibin B affects prenatal development, as revealed by a defect in eyelid fusion at birth. Inhibin-deficient mice

are born with closed eyes, suggesting that the open eye phenotype specifically results from activin deficiency. Unfused eyelids at birth could result from either premature (prenatal) eye opening or, perhaps more likely, from failure of fusion at E16. The event of eyelid closure depends on the proliferation of mesenchymal cells to effect apposition and eventual fusion of the eyelids at E16. These cells are neural crest derivatives that populate the first branchial arch (see Juriloff and Harris 1993). The significance of the open eye phenotype is unclear. A variety of mutations are known to cause birth with open eyes in mice (Lyon and Searle 1989). Some reside at unknown loci and others result from targeted disruption of specific genes. The latter category includes mutants in the *c-abl* and *TGF- α* genes (Schwartzberg et al. 1991; Luetke et al. 1993; Mann et al. 1993). The variety of primary defects causing open eyes at birth suggests an unspecific nature to this phenotype. The process of eyelid outgrowth and fusion may be especially sensitive to sublethal developmental perturbations. For example, it may rely on events that can only occur in a narrow window of time. Alternatively, as suggested by the fact that the genes for both the β B subunit and an activin receptor are expressed in the eye anlagen of *Xenopus* embryos (Dohrmann et al. 1993), a more specific effect of β B on the eye may be operating. A different mutation in the β B subunit gene, which results in deletion of part of the mature peptide-encoding region, was also found to affect eyelid fusion in mouse embryos (H. Schrewe and T. Gridley, pers. comm.).

Expression of the eye phenotype was affected by the genetic background. The defect was never observed in 129/Sv inbred mice but was seen when the mutation was introduced into either of two hybrid genetic backgrounds (129/Sv $\times$ C57BL/6 or 129/Sv $\times$ BALB/c). The incomplete penetrance observed in hybrid mice may therefore be due to the existence of suppressor allele(s) contributed by the 129/Sv genome. Further backcrosses of the mutant animals to C57BL/6 or BALB/c mice will clarify this question. In addition, the occasional individuals displaying a single affected eye suggest that not all variation is attributable to genetic factors. In contrast to the eye phenotype, the reproductive failure of mutant females (see below), appears fully penetrant in the 129/

Sv inbred genetic background, suggesting that the suppressor(s) carried by the 129/Sv genome act(s) specifically during embryonic development.

A large body of evidence exists for the relevance of activins and inhibins in the biology of reproduction of both males and females. Initially discovered as antagonistic regulators of FSH secretion from anterior pituitary cells, activins and inhibins were also shown to exert a variety of local effects in the gonads, their primary site of expression in adults (for review, see Vale et al. 1990; Mather et al. 1992). The decidual tissue, forming in the uterine wall in response to nidation of an embryo, has also been found to express activin/inhibin β subunits. Expression of the β A subunit mRNA has been demonstrated in the mouse uterine decidua shortly after implantation (Manova et al. 1992), and β B subunit expression in human decidua was reported to increase in the course of pregnancy (Petraglia et al. 1990). It seemed possible, therefore, that maternal peptide secreted by the decidua functions in the developing embryo to promote its normal development to birth. We have thus tested the reproductive capacity of mutant animals. Histological examination of testes and ovaries from β B-deficient animals had failed to detect overt abnormalities. However, when the fertility of mutant animals mated to heterozygous partners was assayed, it became apparent that mutant males bred indistinguishably from heterozygous controls, but mutant females suffered an impaired reproductive performance. Although mutant females were able to become pregnant and to carry pregnancies to the end of gestation, they failed to raise their offspring normally. Perinatal lethality appeared to affect equally homozygous mutant and heterozygous neonates. This demonstrated that embryonic development can be completed in the absence of both maternal and fetal β B peptide. The viability of β B-deficient embryos is therefore not dependent upon maternal supply of β B peptide. Our experiments have, however, revealed a critical role for maternal β B expression in perinatal survival of the newborns.

The causes that underlie the reproductive failure in mutant females are not resolved. The newborn pups appeared morphologically normal, suggesting that their death was due to a defect in the delivery process or in maternal nursing behavior. β B-deficient mothers may manifest a defect in parturition, in postpartum events such as uterine remodeling, lactation, or of the hormonal milieu affecting maternal behavior. Examination of mammary tissue indicated that lactogenesis was not impaired in mutant females. The abnormal accumulation of milk in mutant mammary glands suggests, however, that milk let-down might be impaired. Oxytocin is a hypothalamic nonapeptide that is very potent in inducing uterine contractions and controlling milk ejection. It is significant that Sawchenko et al. (1988) have demonstrated β subunit immunoreactivity in the central neural pathways involved in oxytocin secretion. Furthermore, it was reported that local administration of activin in the hypothalamus of rats leads to a rapid increase in plasma oxytocin level and infusion of anti- β subunit serum was

reported to inhibit milk ejection (Vale et al. 1990). A local source of oxytocin expression by the epithelium of the uterine endometrium peaks at parturition (Lefebvre et al. 1992) and is a potential target of the β B mutation as well. Preliminary analysis of postpartum uteri suggests morphological alterations in mutant females (A. Vassalli and H. Gardner unpubl.). It is interesting to note that the mutation of another pleiotropic cytokine, leukemia inhibitory factor (LIF), was shown to affect specifically the reproductive function of females by interfering with the ability of the mutant uterus to allow implantation of blastocyst embryos (Stewart et al. 1992). Various cytokines may be involved in the timely regulation of the critical physiological changes that occur in the uterus during pregnancy.

Whether the β B subunit gene is active in pathways of early development that display redundancy can best be addressed by studying the interaction of the mutated gene with other, early-acting mutations. For example, the presence of the β B mutation may alter the phenotype of embryos heterozygous or homozygous for mutations that affect mesoderm formation, such as the *Brachyury* mutation at the *T* locus (Lyon and Searle 1989) or the 413.d mutation in the *nodal* gene (Zhou et al. 1993). When mutated in mice, the *T* gene causes axial deficiencies whose severity are dependent on the dose of the residual wild-type *T* function (MacMurray and Shin 1988). Because activin is an inducer of *T*, and levels of *T* expression affect development in a concentration-dependent manner, it is possible to envisage that activin B deficiency would cause an altered, perhaps enhanced, *T* mutant phenotype.

β B-deficient mice may also have relevance to the study of tumorigenesis. α subunit mutant mice (inhibin deficient) develop gonadal tumors early in life with complete penetrance (Matzuk et al. 1992). In contrast the β B subunit mutant mice do not develop such tumors. Inhibin deficient mice would be expected to have increased levels of effective activin activity due to the lack of antagonist activity. The involvement of the β B gene in the mechanisms of tumorigenesis can be studied in α/β B double mutants.

In summary, the mutations in the β B subunit gene indicate that activins B, AB and inhibin B are dispensable for embryonic development in the mouse. The gene is, however, active during prenatal development, as revealed by the failure of eyelid closure in mutant neonates. Secondly activins B, AB and/or inhibin B function(s) later in adult female life to regulate events that surround parturition. A deeper understanding of the causes underlying these phenotypes may allow future study of processes relevant to both development and reproduction.

Materials and methods

Cloning of the mouse activin/inhibin β B subunit gene and construction of targeting vectors

To clone the mouse β B subunit gene, a 129/Sv mouse strain genomic DNA library derived from the D3 ES cell line (Doet-

schman et al. 1985; gift of Drs. En Li and Doug Gray, Whitehead Institute) was screened with a partial rat β B cDNA fragment (rinB-c2-P, gift of Dr. Kelly Mayo, Northwestern University, Evanston, IL). A total of 23 kb of genomic DNA, centered on the β B subunit-coding regions, were isolated in overlapping phages and restriction-mapped. Exons were mapped by Southern blot hybridization with exon-specific probes (see PCR cloning of mouse β B subunit exon probes). To construct the targeting vectors, the 17-kb λ 1 phage insert was cloned into pBlue-script(KS-) (Stratagene), digested with *NarI* enzyme, and religated, yielding a construct designated p λ 1 Δ NarI. This results in the deletion of a 1.3-kb *NarI* fragment that comprises the mouse-coding regions homologous to the coding regions for amino acids 1–137 of the rat precursor chain (Feng et al. 1989b). For positive selection, a PGK-NEO-p(A) cassette derived from p(KJ1) (gift of Dr. Michael Rudnicki, Whitehead Institute; Tybulewicz et al. 1991) and ligated to *Clal* linkers, was inserted into the *NarI* site of p λ 1 Δ NarI. The resulting targeting vector containing the neo gene in the same transcriptional orientation as the β B subunit gene is termed pBB1, and pBB2 contains the neo cassette in reverse orientation. Both vectors have the same arms of homology, of 9.5 kb (5') and 6.0 kb (3').

The 3' breakpoint of the Δ NarI deletion was confirmed by PCR amplification on tail DNA of a BB1 allele-specific fragment, "010"/ACT15b, that flanks the breakpoint of the deletion, using a PGK p(A) primer ("010", 5'-AACGAGATCAG-CAGCCTCTG-3') and an exon 1 β B primer (ACT15b) [see PCR cloning of mouse β B subunit exon probes]. This yielded the expected fragment of 127 bp in animals carrying a BB1 mutant allele but not in +/+ littermates. Moreover, the ACT16 primer (5'-ATGGTCACGGCCCTGCGCAA-3'), which lies within the Δ NarI deletion, was used in conjunction with the ACT15b primer to amplify a 138-bp wild-type allele-specific fragment in +/+ and +/bb1 but not in bb1/bb1 animals, further confirming the 3' breakpoint of the deletion.

PCR cloning of mouse β B subunit exon probes

A mouse exon 1 probe, mACT14/15, and a mouse exon 2 probe, mACT10/11;13, were cloned from D3 genomic DNA with the use of the PCR. Published rat (Esch et al. 1987; Feng et al. 1989b) and human (Mason et al. 1989) sequences were used in the choice of primers. Limited degeneracy was introduced in the primer sequences. For mACT14/15, 700 ng of D3 genomic DNA was amplified using ACT14 (5'-CGAATTCACGACACCTGTACGTCGTG-3') and ACT15 (5'-GCGGATC-CCTCTGCAAAGCTGATGAT(CT)TC-3') primers (0.2 μ M each) in a 50- μ l PCR reaction comprised of 10 mM Tris-HCl (pH 9.0), at 25°C, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 100 μ g/ml of gelatin, 200 μ M dNTPs, and 1.5 units of *Taq* polymerase (Cetus). Cycling parameters were 95°C for 1 min, 56°C for 30 sec, and 72°C for 1 min (5 cycles) followed by 40 cycles of 95°C for 1 min and 72°C for 2 min. The 318-bp PCR product was gel purified, digested with *EcoRI* and *Bam*HI, and cloned into pKS. All 12 clones analyzed were identical and contained the *NarI* site at the homologous position to codons for amino acids 137–138 in the rat (Feng et al. 1989b). For mACT10/11;13, ACT10 [5'-CGGATCCGGC(CT)TGGAGTG(CT)GA(CT)GG-3'] and ACT11 primers [5'-CGAATTCACACTCCTCCAC(AGT)AT-CAT-3'] (0.5 μ M each) were used with the same reaction components as above, and amplification was carried out by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The 350-bp PCR products were purified and cloned into pKS as above. The ACT10 and ACT11 primers amplify both β A and β B gene fragments (see Mitrani et al. 1990; Thomsen et al. 1990), which are distinguishable by their restriction maps.

Transfection and selection of ES cells

The J1 ES cell line (Li et al. 1992) was established from a male 129/Sv embryo and grown essentially as described by Robertson (1987). ES cells were cultured on a feeder layer of γ -irradiated embryonic fibroblasts (EFs) in DMEM supplemented with 15% heat-inactivated fetal bovine serum (Hyclone), nonessential amino acids (GIBCO), 10^{-4} M β -mercaptoethanol and antibiotics. Leukemia inhibitory factor (LIF) was added to the medium at 200–500 U/ml. G418^r EFs were prepared from E13.5 embryos carrying a targeted mutation in the β_2 -microglobulin gene (Zijlstra et al. 1989).

Two T75 flasks of subconfluent J1 cells at passage 9–10 were trypsinized and resuspended at 2.5×10^7 ml⁻¹ in PBS. Linearized pBB1 plasmid DNA was added to the cell suspension at a concentration of 30 μ g/ml, and cells were electroporated at 250 V and 250 μ F by use of a BTX 300 electroporator. Electroporated cells were plated on feeder layers of G418^r EF cells at a density of 5×10^6 cells per 9-cm plate. Selection with G418 at 350 μ g/ml (dry powder, GIBCO) was initiated 20–30 hr later and carried out for 7–10 days. At that time individual G418^r colonies were picked, dissociated in trypsin, and plated on wells of feeder-covered 24-well plates. After a 3- to 4-day expansion, two-thirds of the cells in each well were frozen, and the rest further expanded in the absence of feeders for DNA preparation.

The pBB2 targeting plasmid was electroporated in similar conditions, but the cells were resuspended at 5×10^7 /ml in the presence of 25 μ g of linearized DNA and electroporated in one cuvette of a Bio-Rad Gene Pulser set at 800 V and 3 μ F.

Screening of recombinant ES clones and animal genotyping

ES cell and tail genomic DNAs were prepared according to Laird et al. (1991), digested with *EcoRI*, and submitted to Southern blot analysis, using a 850-bp *Bgl*III–*Bam*HI genomic fragment as a probe (E in Fig. 1), which lies adjacent 3' to the right arm of the targeting constructs. Of 172 G418^r ES clones transfected with the pBB1 vector, 41 were homologous recombinants, as evidenced by the presence of an 8.2-kb recombinant *EcoRI* restriction fragment in addition to the ~20-kb wild-type fragment. Further characterization of the mutated alleles in 22 targeted clones was done by use of probe E on *Bam*HI or *Hind*III genomic digests, and by use of an internal probe corresponding to the other end of the targeting vectors (probe A, Fig. 1), as well as a neo probe. Probe A is a 1.5-kb *Sal*I–*Bgl*III fragment corresponding to the 5' extremity of the left arm of the vectors. These analyses confirmed that 20 of the 22 clones had undergone the predicted homologous recombination event with no additional event. The same screening strategy was employed in the pBB2 transfection experiment. Ten ES clones among 58 that were screened contained the predicted 6.4-kb recombinant *EcoRI* fragment, indicative of the reverse orientation of the PGK-NEO-p(A) cassette in this allele. Final washes with both probes A and E were in 0.2 $\times$ SSC, 0.5% SDS at 65°C.

Blastocyst injections and breeding of chimeras

Embryo manipulations were carried out as described by Bradley (1987). Subconfluent ES cells were trypsinized, resuspended in injection medium (45% DMEM, 45% HEPES-buffered saline, supplemented with 10% fetal bovine serum, 10^{-4} M β -mercaptoethanol, and nonessential amino acids), and injected into either C57BL/6 or BALB/c blastocyst embryos. Resulting chimeric animals of both genders were bred to C57BL/6 or BALB/c mates. Both female and male chimeras transmitted the mutated allele to some of their progeny. To obtain the BB1 mutation in

an inbred genetic background, a chimeric male that had demonstrated high chimerism of the germ line was bred to 129/Sv females.

RNA analysis

RNA was isolated from tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987), electrophoresed on a 2.2 M formaldehyde, 1% agarose gel and transferred onto Zetabind membrane. Blots were hybridized to the mACT10/11;13 probe (see above) or a neo probe, consisting of a 600-bp *Pst*I fragment of p(KJ1).

Protein analysis

Ovaries from individual 3-week-old females were homogenized in 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, in the presence of 2 mM PMSF, 5 µg/ml of chymostatin, 5 µg/ml of pepstatin A, 5 µg/ml of leupeptin, and 25 µg/ml of aprotinin. Protease inhibitors were from Sigma. Extracts were spun and total protein in supernatants was quantitated by BCA assay (Pierce). Extracts were run in reducing conditions on 15% polyacrylamide-SDS gels and transferred to nitrocellulose. Equal loading of protein was verified by Ponceau S staining of the membrane after transfer. All subsequent incubations and washes were done at room temperature. Blots were blocked with 5% Carnation nonfat dry milk, 0.2% NP-40 in Tris-buffered saline [TBS; 50 mM Tris-HCl (pH 7.4), 150 mM NaCl] for 30 min, and then incubated overnight in primary antibodies diluted in blocking buffer. After being washed once in 0.2% NP-40 in TBS and twice in 0.1% Tween 20 in TBS, blots were blocked again, incubated for 1 hr in anti-rabbit HRP-conjugate antibodies (Sigma), that had been diluted (1:10,000) in blocking buffer. Blots were then washed as above, rinsed in TBS, and detected by enhanced chemiluminescence (Amersham).

Anti-peptide affinity purified rabbit antibodies were a generous gift of Dr. Wylie Vale (Vaughan et al. 1989). The βB-specific antibodies were directed against peptide (80-112)-NH₂ of the mature βB subunit, and the βA-specific antibodies recognize peptide (81-113)-NH₂ of the mature βA subunit. Both antibody samples were used at a dilution (1:300). The anti-α subunit antibodies were directed against a human inhibin α(1-25)-Gly-Tyr peptide and were used at a (1:150) dilution. Specificity was confirmed using human recombinant activins A and B, and inhibin A as standards run in parallel in Western blotting assays. Human recombinant proteins were kindly provided by Drs. Jennie Mather and Lynne Krummen (Genentech).

Histological analysis

Neonatal mice were fixed in 10% buffered formalin. Heads were dehydrated, paraffin embedded, and serially sectioned in the transverse plane. Sections were stained with hematoxylin and eosin. Abdominal mammary glands were processed similarly and sectioned longitudinally.

FSH measurements

Blood (0.5 ml) was taken by retro-ocular bleeding of animals that had been ether anesthetized. All mice used in the study shown in Table 4 were nonbreeding animals. Serum was prepared and FSH values were determined by radioimmunoassay, as described by Matzuk et al. (1992).

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