

Activin β C and β E Genes Are Not Essential for Mouse Liver Growth, Differentiation, and Regeneration

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The liver is an essential organ that produces several serum proteins, stores vital nutrients, and detoxifies many carcinogenic and xenobiotic compounds. Various growth factors positively regulate liver growth, but only a few negative regulators are known. Among the latter are the transforming growth factor β (TGF- β) superfamily members TGF- β 1 and activin A. To study the function of novel activin family members, we have cloned and generated mice deficient in the activin β C and β E genes. Expression analyses demonstrated that these novel genes are liver specific in adult mice. Here, we show by RNase protection that activin β C transcripts are present in the liver beginning at embryonic day 11.5 (E11.5) whereas activin β E expression is detected starting from E17.5. Gene targeting in embryonic stem cells was used to generate mice with null mutations in either the individual activin β C and β E genes or both genes. In contrast to the structurally related activin β A and β B subunits, which are necessary for embryonic development and pituitary follicle-stimulating hormone homeostasis, mice deficient in activin β C and β E were viable, survived to adulthood, and demonstrated no reproductive abnormalities. Although activin β C and β E mRNAs are abundantly expressed in the liver of wild-type mice, the single and double mutants did not show any defects in liver development and function. Furthermore, in the homozygous mutant mice, liver regeneration after >70% partial hepatectomy was comparable to that in wild-type mice. Our results suggest that activin β C and β E are not essential for either embryonic development or liver function.

Growth factors and hormones play an extremely important role in regulating biological processes from patterning of the early embryo to regulating the function of tissues and organs. The largest family of growth factors is the transforming growth factor β (TGF- β) superfamily of secreted dimeric proteins (12). Members of this family include activins, TGF- β s, bone morphogenetic proteins (BMPs), and growth differentiation factors and demonstrate diverse functions including roles in left-right asymmetry, skeletal development, reproduction, and oncogenesis (29, 52). Both activins (β - β dimers) and inhibins (α - β dimers) have historically been shown to be regulators of follicle-stimulating hormone (FSH) secretion from the pituitary gland (54). These earlier results were later confirmed by *in vivo* analysis of activin β B-, activin receptor type IIA (ActRIIA)-, and α -inhibin-deficient mice (32–34). In studies involving *Xenopus laevis* oocytes (36), activins were tested for their ability to induce mesoderm formation. However, this observation is not true for mice (35), suggesting that the results obtained with *X. laevis* oocyte injection experiments may be due to nonphysiological effects of the activin ligands.

The adult liver detoxifies the blood through the actions of various enzymes, synthesizes normal serum proteins such as

the acute-phase proteins and albumin, and produces bile, which is critical for normal fat absorption (9). Activin A and TGF- β 1 have been shown to affect liver growth and function. These two proteins can inhibit mitogen-induced DNA synthesis in hepatocytes (5, 41, 51, 57), induce hepatocellular apoptosis *in vitro* (7, 21, 42, 48), and stimulate glycogenolysis from cultured hepatocytes (39). *In vivo*, pharmacological levels (e.g., intravenous infusion of recombinant activin A [21, 48]) or pathophysiologically high levels of activins (8, 30, 32) cause a reduction in liver mass by inducing hepatocellular necrosis around the central vein.

Liver regeneration occurs following liver injury that results in loss of liver mass. The liver regenerates by a process of hypertrophy and a near-synchronous proliferation of the remaining cells through several cycles of replication. Several cytokines are thought to play early roles in the regeneration process: interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (reviewed in reference 38). IL-6 has been shown to play a critical role in the progression of liver regeneration but not for initiation (10). While the interleukins and tumor necrosis factor alpha are thought to be important during the early stages of liver regeneration, TGF- β 1 to -3 and activins are thought to be negative regulators during this process (27, 38), since mRNAs encoding these ligands are up-regulated during this process (3, 57). However, this latter hypothesis has not yet been tested *in vivo*.

Recently, our groups (15, 28) and others (19, 43, 47) have cloned three new members of the TGF- β superfamily which demonstrate highest amino acid identity in the mature peptide region to the activin (β A and β B) subfamily. Two of these new members, designated activin β C (*act β C*) and activin β E

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(*actβE*), have been cloned in mammals (15, 19, 28, 47), while the third, activin βD, has been found only in *X. laevis* (43). Weak induction of a secondary axis has been observed when activin βD mRNA is injected into the ventral blastomeres of *Xenopus* embryos (43), but activin βC and βE have not yet been functionally tested by any in vitro or in vivo bioassays. Expression of *actβC* and *actβE* is primarily liver specific in the adult (14, 28, 47), unlike activin βA and activin βB, which are widely expressed in multiple tissues in rodents (16, 37) and humans (53).

Based on the highly restricted tissue expression pattern, we hypothesized that activins βC and βE may play critical roles in liver physiology. To compare the in vivo functions of these novel liver-restricted mammalian activin βC and βE genes to those of the known activin βA and βB genes, we generated null mutations in *actβC*, *actβE*, or both genes in mice. Our studies show that activin βC and βE are not essential for liver development, liver function, or reproduction.

MATERIALS AND METHODS

Construction of targeting vectors and generation of mutant mice. The replacement targeting vector for *actβC* contained 5.7 kb of sequence for the 5' homology arm, a *PGKpr* selectable marker cassette which replaced a 1.7-kb *BamHI-BamHI* region of the locus including exon 2 of the activin βC gene, 4.5 kb of sequence for the 3' homology arm, and an *MC1tk* expression cassette for negative selection (Fig. 1B). Twenty-five micrograms of the *KpnI*-linearized targeting vector was electroporated into 10⁷ AB2.1 embryonic stem (ES) cells (a gift of Allan Bradley, Baylor College of Medicine). ES cells were then selected in medium containing hypoxanthine-aminopterin-thymidine and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5'-iodouracil (FIAU). Culturing of ES cells and collection and injection of blastocysts have been previously described (33). For genomic Southern blot analysis, *HindIII*-digested DNA was transferred to a GeneScreen Plus nylon membrane (NEN Life Science Products, Boston, Mass.) and probed with an external ~300-bp *NcoI-XbaI* fragment (5' probe). An external ~380-bp *StuI-BamHI* fragment (3' probe) was also used to distinguish the wild-type and activin βC null (*actβC*^{mi}) alleles (Fig. 1B).

The *actβE* targeting vector was composed of a 4.85-kb *EcoRI-BamHI* fragment as the 5' homology arm, a *loxP-PGKneo-loxP* selectable marker cassette (a gift from Richard Behringer, University of Texas-M. D. Anderson Cancer Center) that replaced a 1.7-kb *BamHI-BamHI* region of the locus including the entire coding region of activin βE, a 1.8-kb *BamHI-SpeI* fragment as the 3' homology arm, and an *MC1tk* selectable marker cassette. This vector was linearized with *KpnI*, and 25 μg of the linearized plasmid was electroporated into 10⁷ βC45-F1 cells, an AB2.1 ES cell line heterozygous for the *actβC*^{mi} allele, which was generated as described above. The mutant ES cells were selected in G418 and FIAU. *HindIII*, which distinguishes the various targeted and wild-type alleles, was used as the diagnostic restriction enzyme. The probes were a 5' external ~250-bp *BamHI-EcoRI* fragment and an internal ~500-bp *BamHI-XhoI* fragment (Fig. 1D).

Chimeras were generated by blastocyst injections of the mutant ES cells, per standard methods (33). Chimeric males were mated to C57BL/6 females for the generation of F₁ hybrid mice or to 129SvEvBrd female mice to generate F₁ inbred mice.

RNA isolation. Timed matings of wild-type C57BL/6 mice were established, and embryonic livers were collected at various time points for RNA expression analysis. RNA collected at embryonic day 11.5 (E11.5) was liver enriched by using only trunk tissue (i.e., rostral and caudal tissues were excluded). Adult tissues from wild-type 129/SvEv-C57BL/6 hybrid mice were also collected. When necessary, tissues from several mice were pooled. Tissues were immediately homogenized in RNA STAT-60 (Leedo Medical Laboratories, Houston, Tex.) using a Tissue Tearor electronic homogenizer (Biospec Products, Bartlesville, Okla.). RNA was extracted per Leedo Medical Laboratories' instructions and stored under 70% ethanol at -80°C until required. Some samples were stored in RNA Later (Ambion, Austin, Tex.) until required for processing in accordance with the manufacturer's instructions.

RNase protection assay. Probe plasmids were obtained to analyze the RNA expression levels of *actβC*, *actβE*, β-actin (Ambion), and *junB* (Ambion) and β2-microglobulin levels using RNase protection assays. β2-Microglobulin was used as a quantitative control since its expression is constant during liver regeneration (17). The plasmids containing probe DNA fragments generated in the laboratory were sequenced bidirectionally for accuracy. The mouse activin βC probe was composed of a *BamHI-EcoRI* fragment from the 3' untranslated region (+1184 to +1434 of the cDNA; GenBank accession no. U40773) cloned from a 129/SvEv genomic library as previously described (28). The mouse activin βE probe fragment was derived from exon 2 (+750 to +972 of the cDNA; GenBank accession no. U96386) and was generated using PCR primers *actβE-F2* (5'-GAGACCACTATGTAGACTTCC) and *actβE-R4* (5'-AGAGA

GAGGCTTCGTGCAGT). Two different mouse β2-microglobulin probes were used. For embryonic studies, the β2-microglobulin probe β2-MG3 (+1 to +317 of the cDNA) was derived from pHuActβ2m (a gift from Elizabeth Bikoff, Harvard University [2]). For all other experiments, a second β2-microglobulin probe (β2-MG5, +114 to +306 of the cDNA; GenBank accession no. X01838) was isolated by reverse transcription-PCR using forward primer β2-MGF1/H3 (5'-ACTGCAAGCTTAACACAG-3'), which inserts a *HindIII* site at the *SnaBI* site, and the reverse primer β2-MGR1 (5'-TAACCTCTGCAGGCGTATG-3').

Linearized probe plasmids were transcribed in vitro to produce antisense RNA using the Ribomax in vitro transcription kit (Promega, Madison, Wis.). Since some messages are more abundant than others, cold UTP was added to all reactions to limit the protected band intensities to approximately the same range. The amount of cold UTP added was as follows: *actβC*, *actβE*, and *junB*, 0.1 nmol of cold UTP; β-actin and β2-microglobulin, 2.5 nmol of cold UTP. The [³²P]UTP-radiolabeled RNA probes were purified by gel electrophoresis and eluted at 37°C overnight in elution buffer as described by the manufacturer (RPAII kit; Ambion).

The RPAII RNase protection assay kit (RPAII kit) was used as described by the manufacturer with minor modifications. In brief, 2.5 μl of total RNA (2 μg/μl) and 3 μl of probe mix were added to the hybridization buffer. The mixture was denatured at 95°C for 3 min. The reaction mixtures were then incubated overnight at 45°C followed by digestion with a 1:100 dilution of the RNase cocktail, containing RNase A and RNase T₁, at 37°C for 1 h. The protected RNA-RNA hybrids were precipitated and separated on a nondenaturing 5% acrylamide gel except for the *junB* probe, which was separated on an 8% acrylamide gel. Gels were vacuum dried at 80°C, exposed, and visualized by autoradiography.

Following autoradiography, dried gels were also exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, Calif.). Exposed screens were then read with a Storm 860 scanner (Molecular Dynamics), and resulting images were analyzed by ImageQuant version 4.2a software (Molecular Dynamics). Data, following integration, were analyzed by Student's *t* test using Microsoft Excel98 (Microsoft Corp., Redmond, Wash.) for statistical significance. A *P* value of <0.05 was considered significant for all statistical analyses. For RNase protection assays of RNA collected after partial hepatectomy, a minimum of three mice were used at each time point, and each sample was analyzed in duplicate.

Serum analysis. Adult 129/SvEv-C57BL/6 hybrid mice at 42 to 45 days were anesthetized using Metofane (Schering-Plough Animal Health, Union, N.J.), and blood was obtained by closed cardiac puncture. Serum was separated in Microtainer tubes (Becton Dickinson, Franklin Lakes, N.J.) and stored at -20°C. Serum samples were analyzed for alanine aminotransferase, aspartate aminotransferase, albumin, random glucose, and total protein by the Comparative Pathology Laboratory (Baylor College of Medicine) on a Roche Cobas MIRA analysis machine. Serum FSH levels were measured by a rat FSH radioimmunoassay with a sensitivity of 10 ng/ml using a National Institute of Diabetes and Digestive and Kidney Diseases kit (National Hormone and Pituitary Distribution Program, National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health) as previously described (26). Sera from five to six male mice of each genotype were analyzed for FSH levels.

Morphological and histological analysis. Mice were anesthetized and sacrificed by cervical dislocation, and tissues were harvested immediately. The wet weights of relevant tissues from mice 42 to 45 days of age were recorded. For testis weights, 11 to 37 mice were analyzed per genotype. All tissues except for testis were fixed overnight at 4°C in 10% buffered formalin (pH 7.2). The testis samples were fixed overnight in Bouin's reagent and washed extensively in 70% ethanol. In some cases, prostate glands were also collected from >1-year-old mice and placed into formalin. The fixed tissue was then embedded in paraffin and sectioned at 5 μm for staining in hematoxylin and eosin for the liver. The ovaries and testes were stained with hematoxylin-periodic acid-Schiff reagent. Embedding and staining were performed per standard procedure by the Baylor College of Medicine Pathology Core Services Laboratory.

Partial hepatectomy. The partial hepatectomy procedure was performed as described previously with minor modifications (18). Adult 129 × Sv/Ev-C57BL/6 hybrid female mice, 9 to 12 weeks of age and weighing 18 to 22 g, were included in the experiment. IsoFlo (isoflurane; Abbott Laboratories, North Chicago, Ill.) was used as the anesthetic. The partial hepatectomy procedure removes the left medial, right medial, and left lateral lobes. The right half of the medial lobe was removed with one ligature. Both the left half of the medial lobe and the left lateral lobe were removed with a second ligature. During the procedure, care was taken to prevent damage to the gall bladder and the surrounding ducts. Only mice that had >70% of the liver removed were used in our analyses.

RESULTS

Expression of two novel activins in the embryonic liver. The cloning and tissue expression patterns of the *actβC* and *actβE* genes in adult mice have been previously reported by us and others (14, 28, 47). Since both genes appear to be primarily liver specific, we analyzed the temporal expression profiles of

TABLE 1. Genotype distribution from crosses of various mutant mice

Mutant cell line	Data for mouse group					
	+/- \times +/-			Avg litter size (total progeny/litter no.) ^b	Male/female ratio (no. male/female)	-/- \times -/-; avg litter size (total progeny/litter no.) ^b
	No. with genotype (%)					
Wild type	Heterozygous	Homozygous				
β C45-F1 ^a	92 (26.5)	159 (46.0)	95 (27.5)	9.18 (349/38)	1.06 (181/171)	8.03 (233/29)
β E82-A3	81 (27.1)	152 (50.8)	66 (22.1)	8.82 (300/34)	1.20 (163/136)	8.65 (173/20)
β E82-C12	58 (25.9)	117 (52.2)	49 (21.9)	8.30 (224/27)	0.95 (109/114)	7.20 (123/17)
β C β E82-A9	72 (29.9)	110 (45.6)	59 (24.5)	8.03 (241/30)	0.85 (111/130)	— ^c
β C β E82-G12	64 (25.8)	128 (51.6)	56 (22.6)	8.55 (248/29)	0.89 (116/130)	7.40 (140/19)

^a Data for β C46-H3 are not shown due to small sample size and few F₂ progeny.

^b Total litter size was used, including nongenotyped mice.

^c —, not determined.

act β C and *act β E* in the embryonic liver using RNase protection (Fig. 1A). Expression of *act β C* was readily detectable by E11.5 and reached a maximum in the adult at 9 weeks. In contrast, *act β E* was first detectable at E17.5 and appeared to be maximally expressed at birth (Fig. 1A).

Targeting of the *act β C* and *act β E* genes. To determine the function of activin β C and activin β E, we generated ES cell lines containing single mutations in each of the two genes and a linked (double) mutation in both genes. A serial targeting strategy was employed since the *act β C* and *act β E* genes are separated by 5.5 kb on mouse chromosome 10 (14, 47). Initial targeting deleted the entire coding region of *act β C* exon 2, which encodes the mature (active) peptide domain (*act β C^{ml}*), to produce a putative null allele (Fig. 1B and C). For the second targeting event, in which a putative null mutation in activin β E (*act β E^{ml}*) was generated, we used ES cells carrying the *act β C^{ml}* allele instead of wild-type ES cells (Fig. 1D and E). Targeting of the *act β C^{ml}* heterozygous cell line with the activin β E targeting vector would generate two sets of chromosomes, a *cis* set and a *trans* set, with respect to the *act β C^{ml}* and *act β E^{ml}* alleles. If targeting of the *act β E* locus occurs on the same chromosome as the previously targeted *act β C* locus, then a *cis* set of chromosomes will be generated (*act β C^{ml}*, *act β E^{ml}/+*, +), eventually allowing us to generate double-homozygous mutant mice. Conversely, if targeting of the *act β E* locus occurred on the wild-type chromosome (*trans*; *act β C^{ml}*, +/+, *act β E^{ml}*), then the two mutations segregated and permitted us to generate homozygous mice carrying only the *act β E^{ml}* allele. The *act β E^{ml}* allele is also predicted to be a null allele since both coding exons have been replaced (Fig. 1D). Utilizing the above strategy, these constructs were electroporated into ES cells, and subsequently, chimeric mice were generated. Germ line transmission of two cell lines for each mutation (*act β C^{ml}*, *act β E^{ml}*), and the double mutant *act β C^{ml}*-*act β E^{ml}* was achieved (Fig. 1C and D).

General phenotypes. Heterozygous mice were intercrossed, and all lines were bred separately. The offspring from the heterozygous crosses were genotyped at 3 weeks of age, and all lines genotyped showed the expected Mendelian ratio of 1:2:1 (Table 1). Therefore, activin β C and/or activin β E is not required during embryogenesis. At 42 days, homozygous mutant mice from all three mutations were grossly indistinguishable from their control littermates, indicating that growth and development were unaffected by the individual or combined null mutations.

Since *act β C* and *act β E* are closely linked on the same chromosome, the integration of a selectable marker cassette into one locus could have effects on the transcription of the oth-

er locus. We examined the expression of both genes in the *act β C^{ml}/act β C^{ml}*, *act β E^{ml}/act β E^{ml}*, and double-homozygous mutant mice. The expression of *act β C* was not changed in *act β E^{ml}/act β E^{ml}* mice (Fig. 2A and C). However, there appeared to be a slight elevation of *act β E* message in the *act β C^{ml}/act β C^{ml}* mice, although the difference was not statistically significant ($P > 0.22$) when data were quantitated because of the high variability of expression of *act β E* in wild-type mice (Fig. 2B and D). Homozygous mutant mice lacked any detectable expression from the corresponding gene, further demonstrating that our gene targeting strategy produced *act β C* and *act β E* null alleles.

Despite the restricted expression pattern of both *act β C* and *act β E* genes, liver lobular morphology and histology appeared normal at the light microscopy level in the homozygous mutant mice (Fig. 3). We analyzed if there were changes in liver growth in the null mice by comparing the ratio of wet liver mass to body mass. Even though related members such as TGF- β 1 and activin A have been implicated in growth regulation of hepatocytes (5, 7, 21, 41, 42, 48, 51, 57), mice deficient in either *act β C* or *act β E* or both show no statistically significant differences in liver mass/body mass ratio compared to their wild-type littermates (Fig. 4).

Liver function analysis. Although there appeared to be no morphological or histological differences between the wild-type and the homozygous mutant mice, a defect in liver function could still exist. Activin A has been shown to affect glycogenolysis in cultured hepatocytes (39). To test whether the liver-restricted activin β C and activin β E genes may function similarly in the liver, various components in the serum obtained from 42-day-old male and female mice were analyzed (Table 2). Markers for hepatocellular damage, such as alanine aminotransferase and aspartate aminotransferase, were not significantly different between wild-type mice and null mice. Female *act β C^{ml}/act β C^{ml}* mice demonstrated a significant decrease in serum albumin levels ($P < 0.04$). In contrast, both *act β C^{ml}/act β C^{ml}* males and *act β C^{ml}*-*act β E^{ml}* double homozygous females have normal albumin levels. There were no differences in random glucose levels among the groups (Table 2). These results suggest that adult mice at 42 days lacking activin β C and β E have normal liver function.

To check which phenotypes, if any, would eventually develop later at adult stages in the mutant mice, mice were observed for more than 6 months. Weekly weight data were considered as a gross indicator of overall health. There were no significant differences between wild-type mice and any of the homozygous mutant mice for up to 6 months (data not shown). Homozygous mutant mice survived for more than 1 year and were

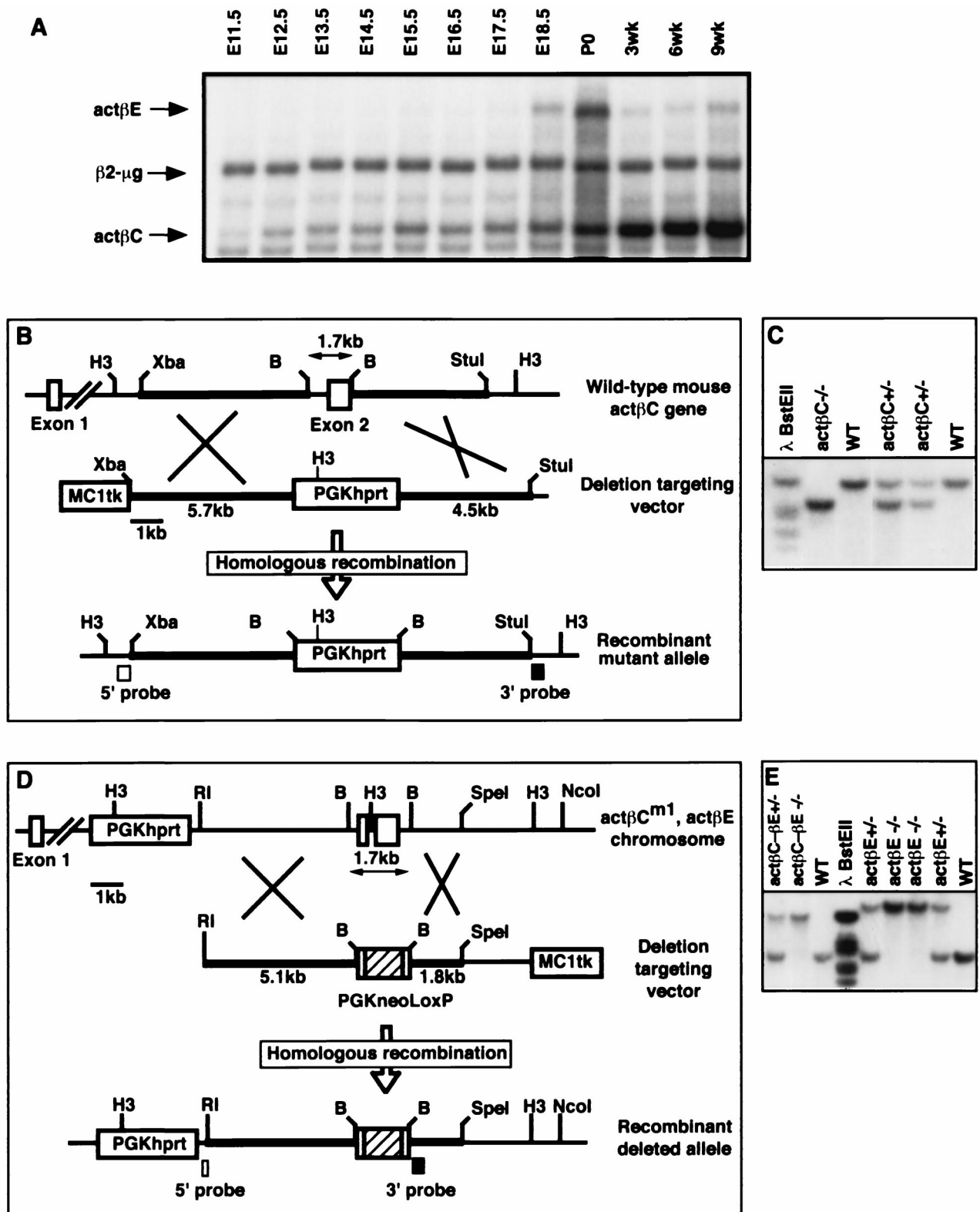


FIG. 1. Expression of activin β C and activin β E in the livers of embryos and adult mice and gene targeting constructs for activins β C and β E. (A) Expression of *act* β C and *act* β E in mouse embryonic and adult livers was detected using RNase protection assays. β 2-Microglobulin was used as the internal control. Five micrograms of total RNA from C57BL/6 mice was hybridized to probes specific for activin β C, activin β E, and β 2-microglobulin. Shown is a representative autoradiograph repeated in four independent experiments. (B) The targeting strategy used to delete exon 2 of the mouse activin β C gene. The construct contains an *MC1tk* expression cassette for negative selection and a *PGKhppt* expression cassette for positive selection. Homologous recombination will delete all of the coding sequence in exon 2. (C) Southern blot analysis of tail DNA from F₂ mice at weaning. The 3' probe identifies the wild-type 13.8-kb band and the 8.3-kb mutant band. (D) The targeting construct to delete all of the coding exons of the *act* β E gene. Homologous recombination within the homology arms will replace the entire coding region of activin β E with a floxed (vertical-box-flanked) *PGKneo*-positive selectable marker cassette. The *MC1tk* cassette is used for negative selection. Due to the close physical proximity of activin β C and β E loci on the same chromosome, ES cells carrying the *act* β C^{m1} mutation (cell line β C45-F1, which was used previously for germ line transmission of *act* β C^{m1}) were electroporated. (E) Southern blot analysis showing weaned F₂ mice homozygous for the *act* β E^{m1} mutation (19.2-kb band) or the double mutant allele (*act* β C^{m1}-*act* β E^{m1}; 13.8-kb band). The wild-type band migrates at 5.3 kb. H3, *Hind*III; B, *Bam*HI; Xba, *Xba*I; RI, *Eco*RI; WT, wild type.

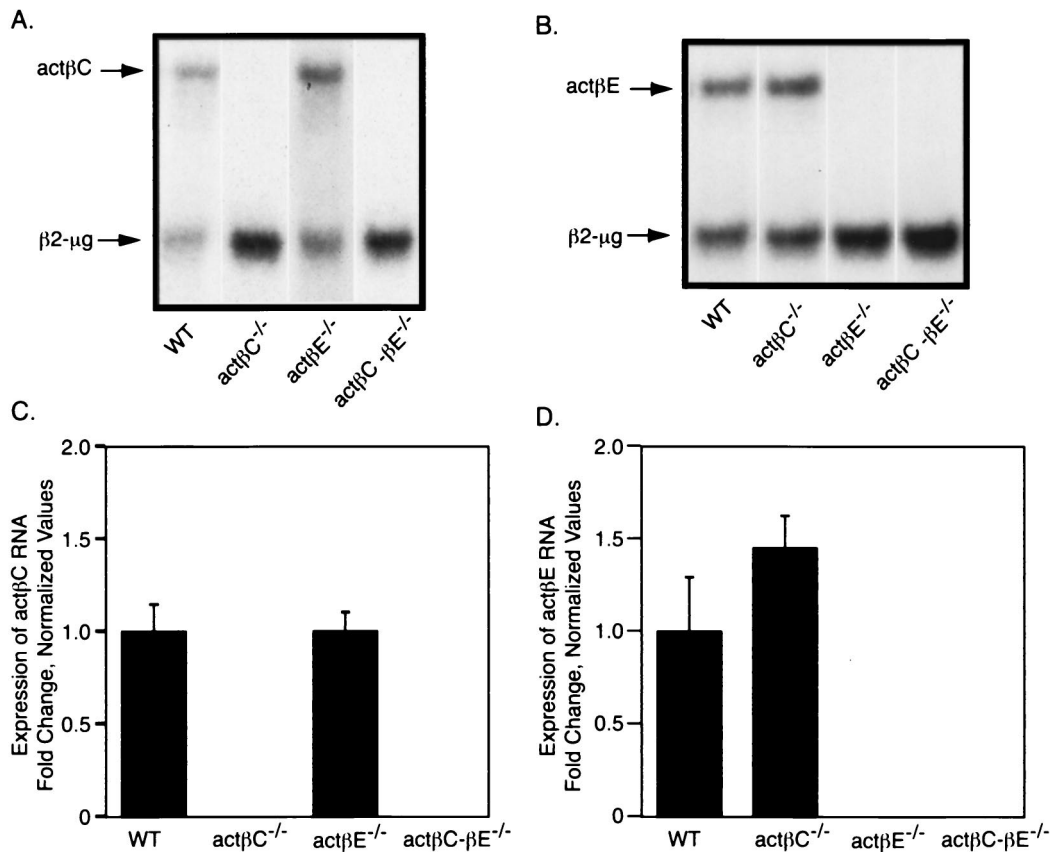


FIG. 2. Analysis of activin β C and β E expression in *actβC^{ml}/actβC^{ml}*, *actβE^{ml}/actβE^{ml}*, and *actβC^{ml}-actβE^{ml}* homozygous mice. RNase protection was used to examine the expression of *actβC* (A) and *actβE* (B) RNA expression in *actβC^{ml}/actβC^{ml}*, *actβE^{ml}/actβE^{ml}*, and *actβC^{ml}-actβE^{ml}* homozygous mutant mice. β 2-Microglobulin was used as the internal control. Representative lanes from a single experiment are shown. Phosphorimaging was used to quantitate the expression of *actβC* (C) and *actβE* (D). All values were normalized to the average ratio of activin subunit expression to β 2-microglobulin expression in wild-type mice. The bars show means \pm standard errors of the means. Values were not statistically significant ($P > 0.05$) compared to the wild type. $n = 5$ for all genotypes. WT, wild type; *actβC*, activin β C; *actβE*, activin β E; *actβC-βE*, activin β C- β E; β 2- μ g, β 2-microglobulin.

indistinguishable from wild-type cage mates (data not shown). This suggests that activin β C and activin β E do not play an essential role in regulating systemic processes related to liver metabolism.

Regulation of FSH secretion. Since activin β A and β B are known regulators of the hypothalamic-pituitary-gonadal axis, we analyzed the serum FSH and reproductive tracts from homozygous mutant mice. Serum FSH levels in male *actβC^{ml}/actβC^{ml}* (30.1 ± 2.9 ng/ml), *actβE^{ml}/actβE^{ml}* (35.5 ± 5.1 ng/ml), and *actβC^{ml}/actβE^{ml}* homozygous (31.2 ± 2.1 ng/ml) mice were not statistically different ($P > 0.05$ by Student's *t* test) from those of male wild-type mice (27.7 ± 2.7 ng/ml). Morphology and histology of the gonads from the null mice were normal (data not shown). Testis weights at 6 weeks of age were not significantly different ($P > 0.05$) between wild-type (82.3 ± 1.4 mg) and *actβC^{ml}/actβC^{ml}* (86.7 ± 2.5 mg), *actβE^{ml}/actβE^{ml}* (79.7 ± 2.0 mg), and *actβC^{ml}-actβE^{ml}* homozygous (79.2 ± 2.4 mg) mice. Furthermore, the male and female homozygous mutant mice bred normally, showed no reproductive defects, and produced normal litter sizes (Table 1). Thus, in contrast to activin β A and β B, activin β C and activin β E are not essential regulators of FSH secretion or reproductive function.

Partial hepatectomy in activin β C and β E knockout mice. Since there was no gross liver phenotype associated with the loss of *actβC* and/or *actβE* under normal physiological condi-

tions, we performed 70% partial hepatectomy on these homozygous mutant mice to create a physiological stress paradigm and induce liver regeneration. To estimate the mass of the liver prior to the partial hepatectomy procedure, the average liver mass/body mass ratio in hybrid female mice for each mutation was calculated (wild-type; 0.040 ± 0.004 , $n = 31$; *actβC^{ml}/actβC^{ml}*, 0.041 ± 0.003 , $n = 31$; *actβE^{ml}/actβE^{ml}*, 0.043 ± 0.004 , $n = 22$; and *actβC^{ml}-actβE^{ml}* $-/-$; 0.043 ± 0.001 , $n = 21$). We analyzed the expression of *actβC*, *actβE*, β -actin, and *junB* at various time points (0, 3, 6, 12, 24, 48, and 72 h) after partial hepatectomy.

In wild-type (Fig. 5A and C) and activin β E knockout (Fig. 5B and C) mice, *actβC* expression tended to be reduced 3 to 6 h following partial hepatectomy (Fig. 5C). By 12 h, activin β C expression appeared to be induced and appeared to peak at 24 h after partial hepatectomy. By 72 h, the *actβC* RNA was near basal levels (Fig. 5C). On the other hand, *actβC* levels in *actβE^{ml}/actβE^{ml}* mice did not show a trend of increased induction (Fig. 5C). Though there was a trend toward lowered induction kinetics of *actβC* expression in *actβE^{ml}/actβE^{ml}* mice, the values were not statistically significant (e.g., wild type at 0 h versus wild type at 24 h, $P > 0.18$).

Next, we examined the expression of *actβE* during liver regeneration following partial hepatectomy (Fig. 6). Like activin β C expression, *actβE* expression tended to be reduced 3 h after partial hepatectomy in both wild-type (Fig. 6A and C)

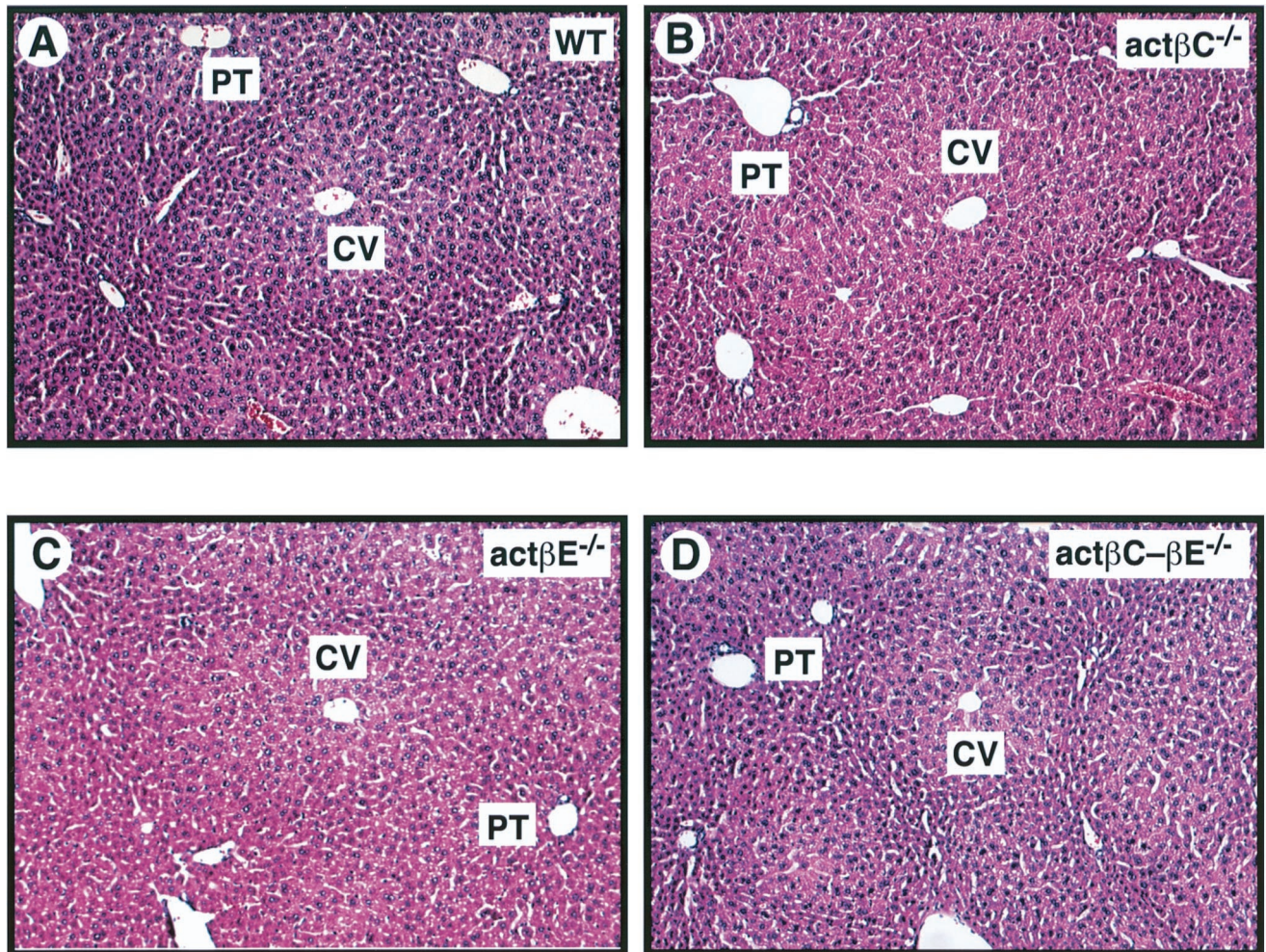


FIG. 3. Histological analysis of livers from 42-day-old wild-type and null mice. Liver tissue from C57BL/6-129/SvEv hybrid mice was formalin fixed and stained with hematoxylin and eosin. The liver histology of the homozygous mutant mice compared to that of wild-type mice showed the expected liver lobular organization of the hepatic parenchyma. All sections were photographed at a magnification of $\times 100$. (A) Wild-type mouse (WT); (B) $act\beta C^{m1}/act\beta C^{m1}$ mouse ($act\beta C^{-/-}$); (C) $act\beta E^{m1}/act\beta E^{m1}$ mouse ($act\beta E^{-/-}$); (D) $act\beta C^{m1}-act\beta E^{m1}$ ($act\beta C-\beta E^{-/-}$) homozygous mutant mouse. CV, central vein; PT, portal triad.

and $act\beta C^{m1}/act\beta C^{m1}$ mice (Fig. 6B and C). Activin βE was rapidly induced and appeared to peak around 6 h following partial hepatectomy in wild-type mice. Expression of $act\beta E$ was still high at 12 h and dropped to near-basal levels by 48 h in wild-type mice. However, in $act\beta C^{m1}/act\beta C^{m1}$ mice, $act\beta E$ expression appeared to peak between 6 and 24 h after the surgery (Fig. 6C).

In addition, we examined the expression of $junB$ (Fig. 7), an immediate-early gene that has been previously characterized in the regenerating liver of wild-type C57BL/6 mice (10). Basal expression of $junB$ in the whole liver was barely detectable in $act\beta E^{m1}/act\beta E^{m1}$ and $act\beta C^{m1}-act\beta E^{m1}$ mutant mice compared to wild-type or $act\beta C^{m1}/act\beta C^{m1}$ mice (Fig. 7C and D). However, during liver regeneration, the kinetics of $junB$ expression were similar between wild-type (Fig. 7A and E) and homozygous mutant (Fig. 7B, C, D, and E) mice. Interestingly, a second low-level $junB$ expression peak was seen at the 48-h point in $act\beta E^{m1}/act\beta E^{m1}$ and $act\beta C^{m1}-act\beta E^{m1}$ homozygous mutant mice (Fig. 7C and D).

We also characterized the expression of β -actin, a growth-related gene (17), during liver regeneration in wild-type mice. In general, β -actin expression in the null mice was very similar

compared to its expression in wild-type mice from 0 to 72 h after partial hepatectomy (data not shown).

DISCUSSION

In this study, we have used gene targeting to determine the essential *in vivo* functions of the novel activin subunits, activin βC and activin βE . Mice deficient in these two genes, either singly or in combination, appear grossly normal. Despite the dynamic embryonic expression patterns of these two subunit mRNAs, mice with either single or double mutations in these genes were recovered in the expected Mendelian ratios, showing that these genes are dispensable for normal embryonic and postnatal development. Since there was no embryonic or perinatal lethality and no sign of mandibular defects, these data strongly suggest that lack of activin βC and/or activin βE signaling is not the cause of the perinatal defects seen in ActRIIA knockout mice (34). Deleting $act\beta C$ and/or $act\beta E$ also does not produce phenotypes similar to those of activin receptor type IIB-deficient mice, the majority of which die perinatally from axial and left-right asymmetry defects (44). The receptors that transduce the activin C and/or activin E signals are not known.

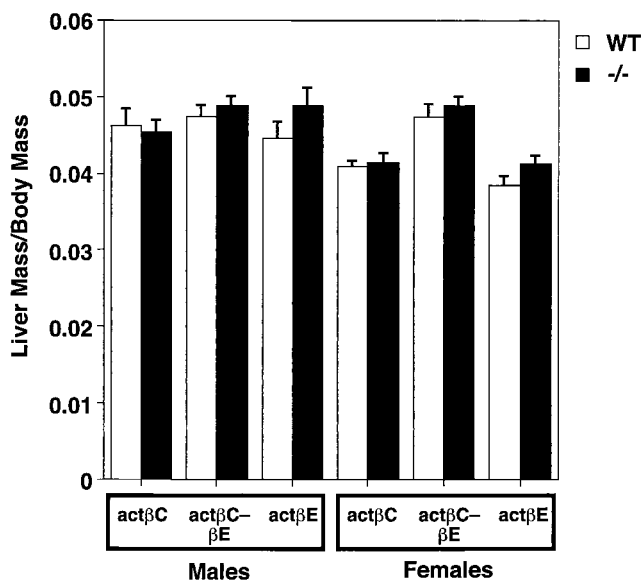


FIG. 4. Liver mass/body mass ratios of male and female knockout mice compared to those of wild-type littermates at 42 to 45 days of age. Each point represents the mean \pm standard error of the mean. There was no ratio from the homozygous mutant groups that statistically differed significantly from the wild-type ratio ($P > 0.05$). Mice analyzed in this study are as follows: activin β C, wild-type males, $n = 7$; $act\beta C^{m1}/act\beta C^{m1}$ males, $n = 16$; wild-type females, $n = 6$; $act\beta C^{m1}/act\beta C^{m1}$ females, $n = 5$; activin β E, wild-type males, $n = 9$, $act\beta E^{m1}/act\beta E^{m1}$ males, $n = 13$; wild-type females, $n = 7$; $act\beta E^{m1}/act\beta E^{m1}$ females, $n = 15$; $act\beta C^{m1}-act\beta E^{m1}$, wild-type males, $n = 10$; $act\beta C^{m1}-act\beta E^{m1} -/-$ males, $n = 17$; wild-type females, $n = 6$; $act\beta C^{m1}-act\beta E^{m1} -/-$ females, $n = 12$.

Our mutant mice can be a good resource, combined with recombinant activin C, E, or CE, to identify their cognate receptors, assuming that they will be up-regulated in the absence of ligands.

Loveland et al. (31) have shown that $act\beta C$ is expressed in human and rat gonads, and others have shown that human activin β C is expressed in prostate, kidney, heart, and ovary among other tissues listed in the UniGene database (<http://www.ncbi.nlm.nih.gov/UniGene/>). However, our studies using Northern blot analysis (28) and RNase protection have not detected any $act\beta C$ message in mouse ovary or testis. Histological analysis of other tissues, including the heart, testis, ovary, and prostate, did not show any gross differences. Additional phenotypes were not seen in homozygous mutant mice over 1 year of age, suggesting that activin β C and β E are not critical regulators of any systemic processes.

We tested whether activin β C and activin β E play essential roles in the regulation of FSH secretion from the pituitary gland since activin β A and β B were originally isolated for this activity. We did not detect any differences in the serum FSH levels among the various mutants. This is in contrast to the results we have observed with other knockout models of activins, inhibins, and the activin signaling pathway (33, 34, 55). As predicted by the current model, mice lacking α -inhibin and activin β B have higher serum FSH levels (33, 55), while ActRIIA knockout mutant mice show a decrease in serum FSH levels (34). Therefore, unlike activins A and B, activin β C and activin β E are not essential (positive) regulators of pituitary FSH.

Normally, liver size is proportional to body habitus. Numerous studies have previously shown in different organisms (including humans), that, upon transplantation, the liver will modulate its size to "fit" the body (27). Another good example of this modulation is the restorative hyperplasia that occurs during liver regeneration (18, 27, 38). Many positive growth factors for hepatocytes such as hepatocyte growth factor/scatter factor, epidermal growth factor, insulin-like growth factor 1, and norepinephrine are known, but very few negative regulators have been discovered (27, 38). TGF- β 1 has been shown to negatively regulate hepatocyte proliferation (5, 41, 50, 51), although TGF- β 1 is inhibitory only 72 h after partial hepatectomy (46). Activin A has also been implicated as a negative regulator since injection of recombinant activin A intraportally into the regenerating liver delayed liver regeneration. In contrast, follistatin, an activin β -subunit binding protein, injected into the portal vein accelerated liver regeneration (24). Both activin β A and TGF- β 1 genes are induced during liver regeneration, but with different kinetics. Expression of activin β A is low in the quiescent liver but is rapidly up-regulated by 24 h after partial hepatectomy (57). TGF- β 1 expression is also low in the quiescent liver, but during liver regeneration induced by partial hepatectomy, expression increases from 3 h to peak at 48 to 72 h (3). These initial studies suggested a negative regulatory role for the TGF- β superfamily ligands during liver regeneration. We have also observed an increase in $act\beta C$ and $act\beta E$ message levels in α -inhibin knockout mice (data not shown), further suggesting that these genes play roles during liver regeneration. We did not observe an increased rate of liver regeneration in the activin β C and β E mutant mice, but the induction patterns of activin β C and β E appeared to be affected in the corresponding null mice (Fig. 5 and 6). This suggests that the induction of each of these genes is mutually dependent on that of the other. However, this dependency occurs only during liver regeneration, since basal levels of

TABLE 2. Serum analysis of wild-type and homozygous mutant mice to study liver function^a

Genotype	Glucose (mg/dl)	Albumin (g/ml)	AST (IU/liter)	ALT (IU/liter)	Total protein (g/dl)
Male					
Wild type	199 \pm 15	3.31 \pm 0.12	103 \pm 16	21.3 \pm 1.6	4.87 \pm 0.11
$act\beta C^{m1}$	212 \pm 15	3.26 \pm 0.12	128 \pm 33	19.7 \pm 3.3	4.96 \pm 0.05
$act\beta E^{m1}$	193 \pm 8	3.26 \pm 0.10	136 \pm 22	26.3 \pm 3.4	4.87 \pm 0.09
$act\beta C^{m1}-act\beta E^{m1}$	201 \pm 11	3.53 \pm 0.10	104 \pm 10	24.4 \pm 2.2	5.10 \pm 0.16
Female					
Wild type	195 \pm 12	3.69 \pm 0.09	135 \pm 28	16.3 \pm 2.3	4.95 \pm 0.06
$act\beta C^{m1}$	216 \pm 14	3.31 \pm 0.14 ^b	84 \pm 11	21.0 \pm 3.4	5.1 \pm 0.09
$act\beta E^{m1}$	164 \pm 15	3.79 \pm 0.09	114 \pm 27	20.6 \pm 2.2	4.98 \pm 0.10
$act\beta C^{m1}-act\beta E^{m1}$	185 \pm 10	3.62 \pm 0.12	141 \pm 40	22.8 \pm 2.3	4.81 \pm 0.16

^a Mice at 42 to 45 days of age were used. For all data points, $n = 8$ to 10. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

^b Student's t test, $P < 0.04$; $P > 0.05$ for all other values in comparing mutant groups to wild-type mice.

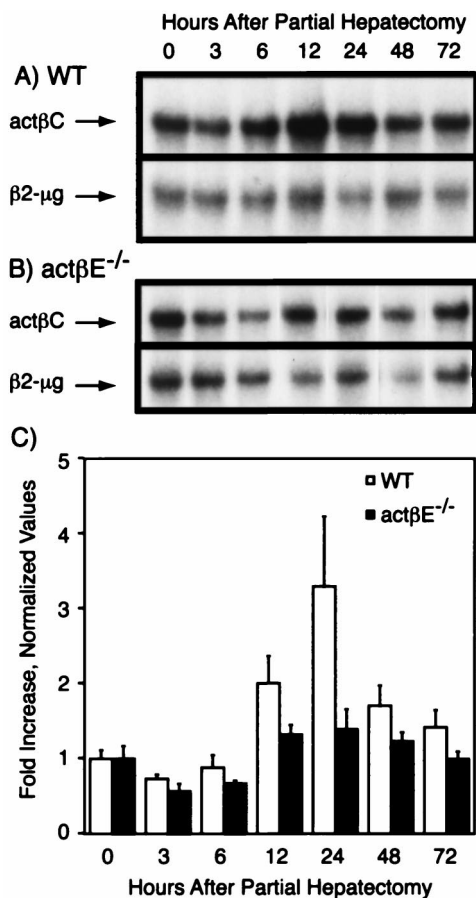


FIG. 5. Expression of *actβC* before and after partial hepatectomy. (A and B) Activin β C expression levels in the whole or remnant livers were determined before partial hepatectomy (0 h) and at several time points (3, 6, 12, 24, 48, and 72 h) after partial hepatectomy in wild-type (WT) (A) and *actβE*^{m1}/*actβE*^{m1} (*actβE*^{-/-}) (B) mice. Representative autoradiographs are shown. (C) Autoradiographs were quantitated and visualized as fold increase over the 0-h point \pm standard error of the mean for each genotype. There was no significant difference between time zero and all other time points ($P > 0.05$). The following numbers of mice were analyzed per time point: three to five (wild type) and three to four (*actβE*^{m1}/*actβE*^{m1}). β 2- μ g, β 2-microglobulin.

activin β C and β E are normal in the corresponding mutant mice (Fig. 2). Despite the change in activin β C and β E expression profiles during liver regeneration, the mRNA expression profiles of *junB* and β -actin suggest that liver regeneration initiates and progresses normally in the homozygous mutant mice.

Since the liver is the major site for the production of acute-phase proteins, activin β C and activin β E may play a role during the acute-phase response. TGF- β superfamily ligands have also been shown to affect inflammation and the acute-phase response. One of the phenotypes seen in the TGF- β 1 knockout mouse is a hyperstimulated inflammatory response (49). Activin A has also been shown to affect inflammatory response through inhibition of IL-6 action (45, 58) and was isolated biochemically based on this function (4). Activin A may play a role in wound repair since activin β A and β B mRNA is induced at the site of wounding (20) and skin wound healing is enhanced if activin β A is expressed from the human keratin K14 promoter in the skin (40). Further experiments are needed to understand what role(s) *actβC* and *actβE* may play during acute-phase response and wound repair.

The expression of many members of the TGF- β superfamily and their signaling components in the liver suggests highly redundant roles. Expression of TGF- β 1, TGF- β 2, TGF- β 3 (22), BMP-6 (23), BMP-9 (6), growth differentiation factor 10 (11), and activin β A (16, 37) among others has all been detected in the liver. BMP-9 has also been shown to be a liver-restricted gene (6). Previous studies have suggested a degree of promiscuity between different receptor and ligand combinations (1, 13, 56). This suggests possible large overlaps between the functions of multiple members of the TGF- β superfamily and their downstream signaling components, especially in such an important organ as the liver.

In conclusion, we have generated and characterized mutant mice lacking *actβC* and *actβE* genes. Targeted overexpression of activin β C and β E in the livers of transgenic mice and the

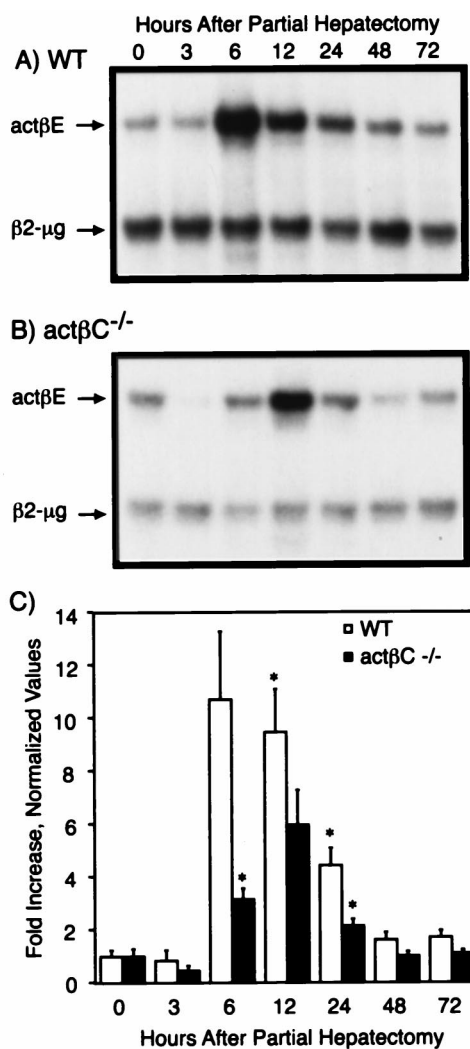


FIG. 6. Expression of *actβE* before and after partial hepatectomy. (A and B) Remnant or whole livers from wild-type (WT) (A) and *actβC*^{m1}/*actβC*^{m1} (*actβC*^{-/-}) (B) mice were collected at 0, 3, 6, 12, 24, 48, and 72 h after partial hepatectomy. Representative autoradiographs are shown. Expression of *actβE* in the livers was assayed by RNase protection with β 2-microglobulin (β 2- μ g) as the internal control. (C) The protected bands were quantitated and visualized as fold increase over the 0-h point \pm standard error of the mean for each genotype. An asterisk denotes time points where expression of *actβE* in *actβC* knockout or wild-type mice was statistically significantly different from the time zero value for the same genotype ($P < 0.05$) by Student's *t* test. The numbers of mice analyzed at each time point were three to four for wild type and *actβC*^{m1}/*actβC*^{m1}.

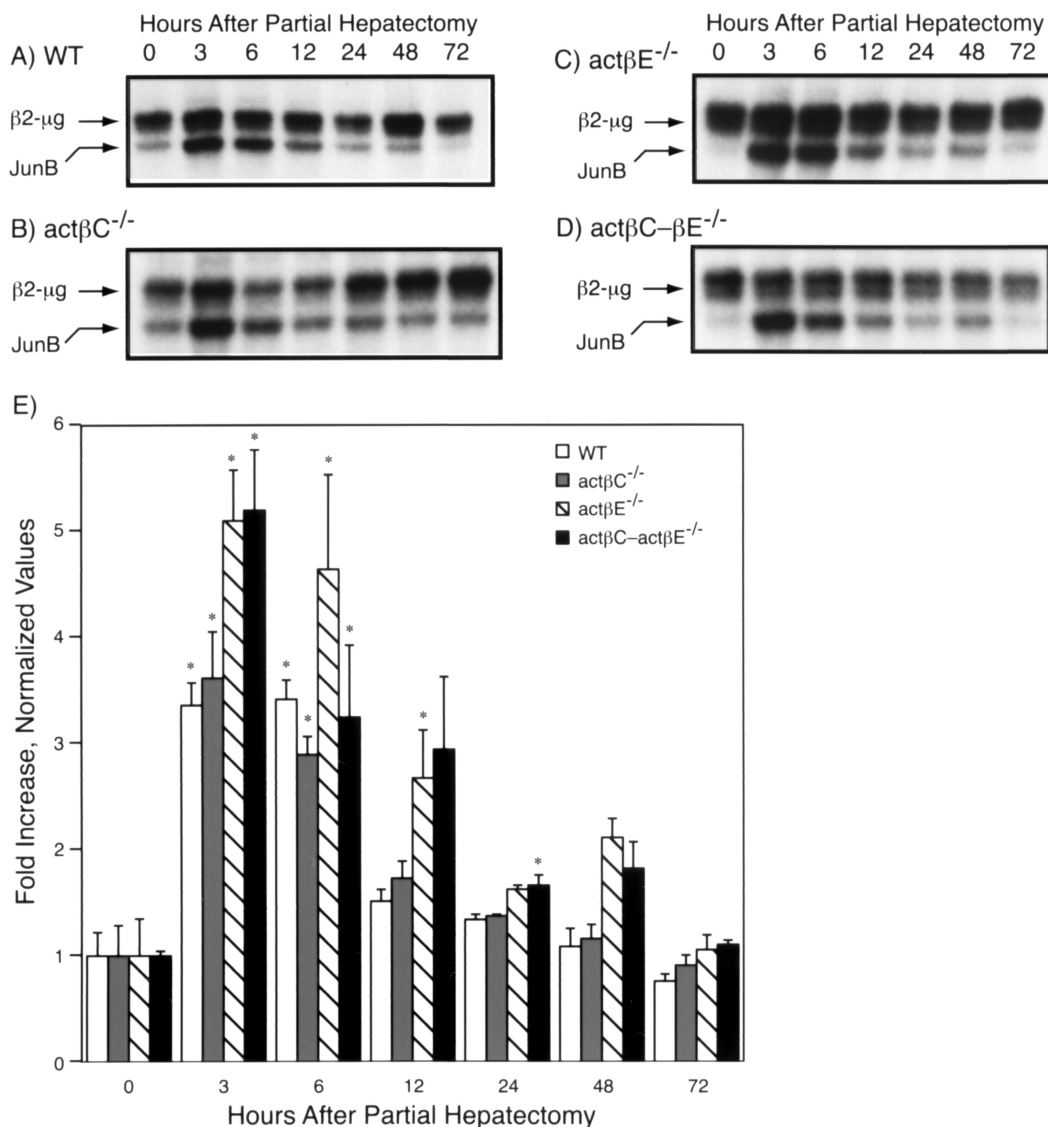


FIG. 7. Expression of *junB* before and after partial hepatectomy. The expression of *junB* and the internal control β 2-microglobulin (β 2- μ g) in the liver was analyzed by RNase protection. (A to D) Representative autoradiographs from wild-type (WT) (A), *act β C^{m1}/act β C^{m1}* (*act β C^{-/-}*) (B), *act β E^{m1}/act β E^{m1}* (*act β E^{-/-}*) (C), and *act β C^{m1}-act β E^{m1}* knockout (*act β C- β E^{-/-}*) mice (D) are shown. (E) Quantitative results were obtained by normalizing values to the ratio of *junB* to β 2-microglobulin at time zero for each genotype (mean \pm standard error of the mean). Asterisks denote values that were statistically significantly different from the value for time zero of the same genotype ($P < 0.05$). The number of mice was three to four for all genotypes at all time points.

production of functional recombinant activin C, E, and CE proteins will be invaluable in the future to study gain-of-function effects of these two genes in vivo and in vitro. Kron et al. have recently reported the production of recombinant human activin β C protein using an insect expression system (25). Purified recombinant active protein can be tested in several functional assays such as hepatocellular apoptosis. Production of anti-activin β C and anti-activin β E antibodies is under way, and the antibodies will be useful for immunohistochemistry to localize the protein. We are also interested in analyzing the circulating levels of activins C and E and identifying the tissues to which these novel activins specifically bind.

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