

Evidence of a Steroidogenic Enzyme Gene Dose Effect on Adrenal Gene Expression in Hereditary Rabbit Congenital Adrenal Hyperplasia

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ABSTRACT

We previously reported the gene deletion encoding cytochrome P-450 cholesterol side-chain cleavage enzyme (P-450_{SCC}), resulting in complete elimination of the adrenal gene expression and causing congenital adrenal hyperplasia in the rabbit. Using the rabbit congenital adrenal hyperplasia model, we investigated the wild type (wt) P-450_{SCC} gene dose effect on gene expression in three P-450_{SCC} genotype animals [wt/wt, wt/mutant (mt), mt/mt] identified by Southern blot analysis. Northern blots using a rabbit P-450_{SCC} cDNA probe revealed no detectable P-450_{SCC} mRNA in individual adrenals of animals with congenital adrenal hyperplasia (mt/mt) and approximately half or slightly less than half the levels of the mRNA in the pooled adrenals of five heterozygous (wt/mt) newborn animals compared with the mRNA levels in the pooled adrenals of five homozygous normal (wt/wt) newborn animals. Identical P-450_{SCC} mRNA levels were found individual adrenals of adult animals with regard to the P-450_{SCC} genotype, although at a higher expression level than in the newborn animals of the same genotype. Control Northern blots using human CPY21-B cDNA and cytoplasmic actin cDNA probes confirmed the accuracy and integrity of RNA. Western immunoblotting using anti-ovine P-450_{SCC} antibody revealed decreased P-450_{SCC} protein in the adrenals of wt/mt animals at approximately half the level of the P-450_{SCC} protein in the adrenals of the wt/wt animals. Baseline and ACTH-stimulated serum corticosterone (B) levels *in vivo* were similar between the age-matched wt/mt and wt/wt animals, whereas ACTH-stimulated B levels in adult animals were higher than those in

the newborn animals irrespective of P-450_{SCC} genotype. These data demonstrated for the first time the presence of a wt steroidogenic enzyme gene dose effect on gene expression *in vivo* at the levels of the mRNA and enzyme protein. With age, the gene dose-related gene expression was maintained, although at higher expression levels. The normal adrenal B-producing capacity in heterozygous, wt/mt animals despite the markedly decreased P-450_{SCC} gene expression suggests the presence of either a mechanism compensating the availability of mitochondrial cholesterol substrate to overcome the decreased P-450_{SCC} or rate-limiting effect of P-450_{SCC} in the conversion of excess cholesterol substrate available in the adrenal mitochondria. The greater B-producing capacity under ACTH stimulation in the adult animals compared with the newborn animal irrespective of P-450_{SCC} genotype therefore is likely from the greater adrenal receptor capacity for ACTH rather than from the greater P-450_{SCC} gene expression level. (*Pediatr Res* 36: 660-666, 1994)

Abbreviations

CAH, congenital adrenal hyperplasia
P-450_{SCC}, P-450 cholesterol side-chain cleavage enzyme
21-OHase, 21-hydroxylase
wt, wild type
mt, mutant
HET, heterozygous
HOMO NORMAL, homozygous normal
B, corticosterone

CAH is a family of autosomal recessive disorders of adrenal steroidogenesis resulting from deficient activity

of adrenal steroidogenic enzymes essential for glucocorticoid biosynthesis (1, 2). Inadequate glucocorticoid production in CAH causes increased ACTH secretion via the negative feedback mechanism from fetal life, resulting in adrenal cortical hyperplasia at birth (3). Investigation of the gene expression of affected steroidogenic enzyme in an animal model manifesting CAH may provide further understanding of molecular genetics of CAH. We previ-

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ously reported an inherited lipid CAH due to cytochrome P-450_{SCC} deficiency in adrenal and gonadal steroidogenesis in a strain of rabbits (4, 5). The clinical, pathologic, and biochemical manifestations of CAH in the rabbit (4, 5) are nearly identical to the human lipid CAH due to inability to convert cholesterol to steroid molecule in adrenal and gonadal steroidogenesis (6–10). We further defined that P-450_{SCC} deficiency CAH in the rabbit is caused by a deletion mutation of the gene encoding P-450_{SCC} completely eliminating P-450_{SCC} gene expression in CAH adrenals (5).

To date in humans, the effect of wt steroidogenic enzyme gene dose on adrenal gene expression *in vivo* is limited to the findings of the presence or absence of hormonal abnormality to ACTH administration in HET normal subjects from families of patients with CAH disorders (1, 2, 11–14). However, it is not known whether the presence or absence of the hormonal abnormality *in vivo* in the HET subjects results from or is associated with mildly, modestly, or significantly decreased steroidogenic enzyme mRNA and protein levels in the adrenals. In addition, various deleterious mutations of the gene encoding for steroidogenic enzyme in HET subjects for CAH disorders (1, 2, 15) make the assessment of accurate wt gene dose effect on gene expression in adrenals and gonads difficult.

Thus, CAH in the rabbit is an excellent model of natural gene knockout for investigation of a steroidogenic enzyme gene dose effect on gene expression in adrenals and gonads (4, 5). Our previous work in the rabbit demonstrated two levels of adrenal P-450_{SCC} gene expression in phenotypically normal newborn animals and undetectable gene expression in CAH newborn animals (4, 5). We hypothesized that the variable P-450_{SCC} gene expression in the rabbit is due to the effect of a single and double copy of the wt P-450_{SCC} gene in the HET and HOMO NORMAL animals, respectively. We thus investigated P-450_{SCC} gene expression in adrenals of the three P-450_{SCC} genotype animals identified by restriction fragment length polymorphism of the P-450_{SCC} gene from families of CAH animals to determine the gene dose effect on the levels of P-450_{SCC} mRNA and enzyme protein. We further compared the P-450_{SCC} gene expression with adrenal steroid-producing capacity *in vivo* in the animals.

METHODS

Animals background and tissue collection. The animals with the CAH trait were derived by inbreeding and outcrossing known HET animals carrying the mt P-450_{SCC} gene trait as described (4, 5). Animals with the CAH trait (inside strain) were killed at 12 to 24 h of life (newborn animals), and phenotypically normal adult animals were killed at age 9 mo. The age-matched control HOMO NORMAL newborn and normal adult New Zealand White rabbits (unrelated to the CAH pedigree) were purchased from an outside laboratory (outside strain). All

animals were killed by a method approved by the Animal Care Committee of the University of Illinois. The CAH newborn animals were immediately identified by the presence of hyperplastic adrenals at autopsy. Tissues (adrenal, liver, and kidney) obtained from animals were immediately frozen in liquid nitrogen and stored at -70°C until study. One of the adrenals from each animal was used for Northern blotting, and the other adrenal was used for Western immunoblotting in all animals. Body weight and adrenal weight of phenotypically normal newborn and adult animals were similar between the HET and HOMO NORMAL animals.

Southern blot analysis. Genomic DNA was extracted from the kidney or liver tissue from newborn animals and from leukocytes of blood samples obtained via venipuncture in adult rabbits using a lysis buffer and purified by phenol-chloroform and ethanol precipitation as described (5). For genotype identification, purified genomic DNA (15 μg) was digested with *Sst*I and separated on a 0.7% agarose gel by electrophoresis. DNA fragments were transferred to a nylon membrane (Hybond-N Amersham, Arlington Heights, IL) and hybridized in a hybridization solution (Amersham) at 65°C for 3 h in the presence of ^{32}P -labeled rabbit P-450_{SCC} cDNA probe (5). The final washing was carried out in $0.5 \times \text{SSPE}$ ($1 \times \text{SSPE} = 150 \text{ mM NaCl}$, $10 \text{ mM NaH}_2\text{PO}_4$, and 1 mM EDTA at pH 7.4), 0.1% SDS at 65°C . The blot was then exposed to Kodak XAR film and autoradiographed for 18 h at -70°C .

Northern blot analysis. Total RNA was extracted from individual adrenals of adult and CAH newborn animals and from pooled adrenals ($n = 5$) of proven HET newborn animals and HOMO NORMAL newborn animals by restriction fragment length polymorphism analysis of the P-450_{SCC} gene. RNA concentration was determined by the absorbance ratio at 260 and 280 nm as described (4, 5). The pooling of adrenals from HET and HOMO NORMAL newborn animals was necessary to obtain an adequate amount of RNA due to extremely small adrenals in the newborn (1–2 mg/gland). Total RNA was loaded on a 1.2% denaturing agarose gel containing 0.3 M formaldehyde for electrophoresis, stained with ethidium bromide for visual examination of RNA amount equality, transferred to Hybond-N membrane, and hybridized in a hybridizing solution (Amersham) at 65°C for 3 h in the presence of ^{32}P -labeled rabbit P-450_{SCC} cDNA probe, human 21-OHase B cDNA probe, or human cytoplasmic actin cDNA probe. The final washing was carried out in $0.1 \times \text{SSPE}$, 0.1% SDS at 65°C . Autoradiography was performed as in Southern blot analysis. The relative amounts of P-450_{SCC}, 21-OHase, and actin mRNA levels were determined by the band intensity in each autoradiogram and its comparison to the amount of RNA loaded, as well as by scanning densitometry of the autoradiogram of Northern blots of P-450_{SCC}, 21-OHase, and actin at various exposure periods.

Preparation of specific ^{32}P -labeled cDNA probes. A cloned rabbit P-450_{SCC} cDNA fragment was generated using polymerase chain reaction as described previously

in the authors' laboratory (5). Human 21-OHase B cDNA probe was supplied by the American Type Culture Collection (Rockville, MD). Human cytoplasmic actin cDNA probe was provided by Dr. X. Yang at the University of Illinois, Chicago. These cDNA were labeled with ^{32}P by random priming technique using dCTP, the kit reagents, and protocol supplied by Amersham. The probes were further purified by passing them through a spin column (Bio-Rad, Richmond, CA).

Western blot analysis. Protein was extracted from the pooled adrenals of five HET or five HOMO NORMAL newborn animals and individual adrenals of CAH newborn animals and all adult animals. The adrenal tissue was homogenized in lysing solution, then the protein concentration was determined by a BCA protein determination kit (Sigma Chemical Co., St. Louis, MO). Each adrenal homogenate was resolved by one-dimensional electrophoresis on a 7.5% polyacrylamide gel containing 0.1% SDS at 4°C and 12 mA for 18 h. The separated proteins were transferred from the gel to a nitrocellulose filter (Bio-Rad) or an Immobilon-P PVDF filter (Millipore, Bedford, MA) at 4°C and 100 V for 1 h. The filter was preincubated with buffer A [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% NP-40] for 30 min at room temperature, then incubated for 2 h at room temperature with buffer A containing antipolyclonal ovine P-450_{SCC} antiserum specific for cytochrome P-450_{SCC} in several species, including rabbit (4, 16). The filter was washed for 30 min with buffer A and then incubated for 1 h at room temperature with ^{125}I protein A (Amersham) at a concentration of 0.33 $\mu\text{Ci/mL}$ in buffer A. After washing with PBS for 30 min, the protein antibody complexes were visualized by autoradiography. The relative amount of P-450_{SCC} was assessed by both the band intensity of the autoradiogram and the radioactivity of the band detected by an AMBIS radioanalyzer (Bioscan, Inc., Washington, DC).

In vivo adrenal steroid-producing capacity test. Synthetic ACTH 1-24 (Cortrosyn 6 $\mu\text{g/kg}$, Organon, West Orange, NJ) was administered by intramuscular injection to HET adult animals ($n = 9$) and HOMO NORMAL adult animals ($n = 5$) as well as young (2 mo old) HET animals ($n = 2$) and HOMO NORMAL animals ($n = 2$). Blood samples were obtained before and 30 min after ACTH injection. Adrenal steroid-producing capacity was assessed by the levels of serum baseline and ACTH-stimulated corticosterone by an RIA as described previously (4).

RESULTS

Animal P-450_{SCC} genotyping by Southern blot analysis. An example of genotypes of animals determined by DNA analysis is shown in Figure 1. Genomic DNA from a family of an affected offspring revealed two alleles of the P-450_{SCC} gene: one allele revealed a 5.5-kb *Sst*I restriction fragment and the other allele revealed 4.0-kb and 1.5-kb *Sst*I restriction fragments. The HET father dem-

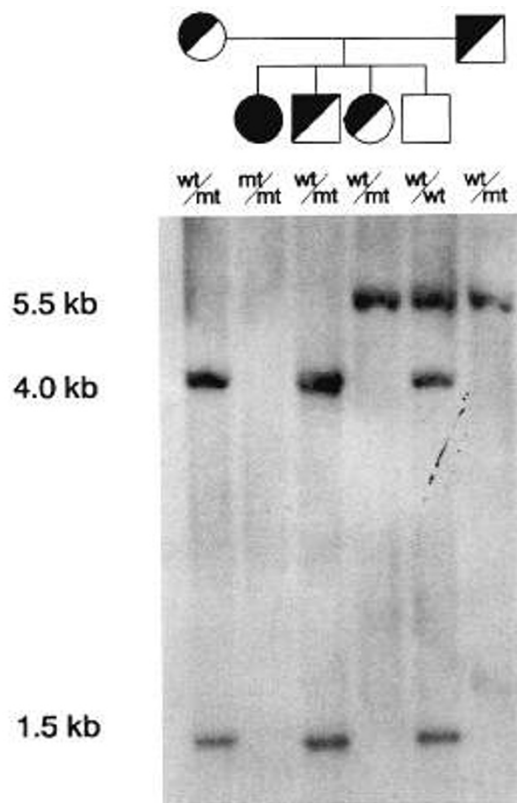


Figure 1. Southern blot analysis of the rabbit P-450_{SCC} genomic DNA digested with *Sst*I. The HET (wt/mt) mother animal carries a wt P-450_{SCC} allele of 4.0- and 1.5-kb *Sst*I fragments. The HET (wt/mt) father animal carries the other wt allele of a 5.0-kb *Sst*I fragment. The CAH offspring (mt/mt) has HOMO deletion of both wt P-450_{SCC} alleles. The genotype of phenotypically normal offspring was identified as wt/mt (HET) when one wt P-450_{SCC} allele was present and wt/wt (HOMO NORMAL) when both wt P-450_{SCC} alleles were present.

onstrated the presence of a 5.5-kb *Sst*I allele and the absence of 4.0-kb and 1.5-kb alleles for the P-450_{SCC} gene. The HET mother revealed the presence of 4.0-kb and 1.5-kb alleles only and the absence of a 5.5-kb *Sst*I allele. The genomic DNA from a CAH newborn offspring showed the absence of both P-450_{SCC} *Sst*I alleles, indicating deletion of the P-450_{SCC} gene. The genotypes of the phenotypically normal newborn sibling rabbits were identified as HET when only one allele P-450_{SCC} *Sst*I fragment was detected and as HOMO NORMAL when both P-450_{SCC} allele fragments were detected by Southern blotting.

RNA dose-related P-450_{SCC} mRNA level. P-450_{SCC} mRNA levels at 4, 2, and 1 μg of adrenal RNA from pooled adrenals of control HOMO NORMAL newborn animals (of outside strain) revealed an RNA dose-related P-450_{SCC} mRNA level based on the band intensity (Fig. 2A). The densitometry scanning revealed nearly identical ratios of P-450_{SCC} to 21-OHase mRNA levels in 1-, 2-, and 4- μg lanes of HOMO NORMAL newborn adrenals (Table 1). The band intensities of P-450_{SCC} mRNA in 4- μg and 2- μg RNA lanes of the HOMO NORMAL newborn adrenals were greater than and similar to, respectively, the band intensity of 4- μg RNA lanes from

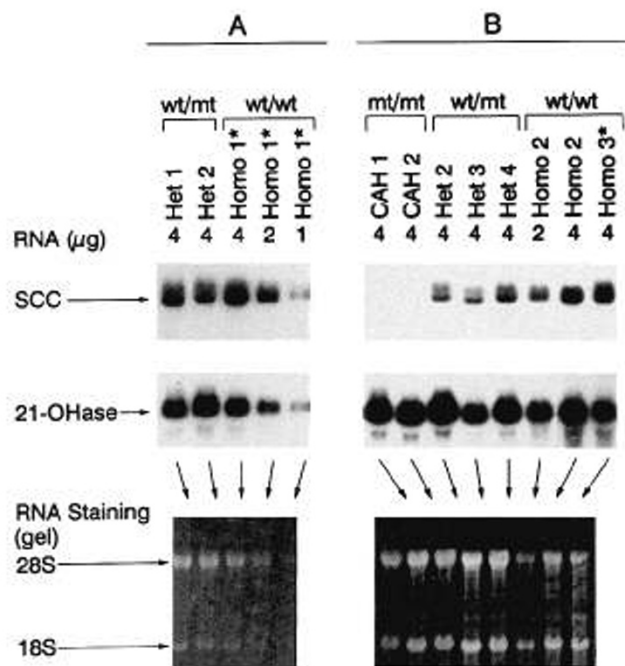


Figure 2. A, RNA dose-related P-450_{SCC} (SCC) mRNA levels based on the band intensity of the autoradiogram in the HOMO NORMAL (*Homo* = wt/wt) newborn animals and its comparison with the mRNA levels in the HET (*Het* = wt/mt) newborn animals. Each lane represents RNA from pooled adrenals of five animals of the same genotypes. 21-OHase mRNA levels represent the control blot study. The number above *Het* or *Homo* indicates group identification of pooled adrenals of animals of the same genotype. An asterisk indicates outside-strain animals. B, Comparison of P-450_{SCC} (SCC) gene expression (mRNA levels) in the three P-450_{SCC} genotypes of newborn animals. 21-OHase mRNA represents the control blot study.

two groups of HET newborn adrenals (Fig. 2A). The band intensity of the control blot study using human 21-B cDNA probe demonstrated the RNA dose-related (4, 2, and 1 μg) 21-OHase mRNA levels of the control HOMO NORMAL newborn adrenals and similar 21-OHase mRNA levels between the 4-μg RNA of the HET and HOMO NORMAL newborn adrenals.

P-450_{SCC} gene dose effect on adrenal gene expression in newborn animals of the three P-450_{SCC} genotypes. P-450_{SCC} mRNA levels in adrenals of CAH animals (mt/mt alleles),

HET animals (wt/mt alleles), and HOMO NORMAL animals (wt/wt alleles) of the inside and outside strains were compared by band intensity in relation to the amount of RNA loaded (Fig. 2B). P-450_{SCC} mRNA was not detected in two CAH adrenals. P-450_{SCC} mRNA levels in adrenals of three groups of HET newborn animals at a 4-μg RNA dose were similar to those of HOMO NORMAL newborn adrenals of the inside strain at a 2-μg RNA dose and were markedly decreased compared with those of HOMO NORMAL newborn adrenals of either the inside- or outside-strain animals at a 4-μg RNA dose. P-450_{SCC} mRNA levels were similar between the inside strain and outside strain of HOMO NORMAL animals. These data indicate the presence of a wt P-450_{SCC} gene dose effect at the mRNA level *in vivo*. 21-OHase mRNA levels determined by the band intensity were detected in CAH adrenals and were also similarly expressed in the HET or HOMO NORMAL adrenals, except for somewhat low expression in one 4-μg RNA lane of HET adrenals and one 2-μg RNA lane of HOMO NORMAL adrenals. RNA staining in the latter two lanes revealed the presence of 4 μg of RNA in the lane of the HET adrenals and an unequivocally low degree of staining in the 2-μg RNA lane in the HOMO NORMAL adrenals. The densitometry scanning of the lanes of HET adrenals revealed one-half ratios of P-450_{SCC} to 21-OHase mRNA levels and P-450_{SCC} to actin mRNA levels of the HOMO NORMAL adrenals, whereas the ratios of 21-OHase to actin mRNA levels were similar between the HET and HOMO NORMAL adrenals (Table 1).

P-450_{SCC} gene expression in adult HET (wt/mt) and HOMO NORMAL (wt/wt) animals. The mRNA levels were determined by the band intensity in relation to the amount of RNA loaded (Fig. 3). P-450_{SCC} mRNA levels at a 4-μg RNA dose from three HET adult adrenals for the P-450_{SCC} mt gene were similar to or slightly lower than the mRNA levels at a 2-μg RNA dose from adrenals of three HOMO NORMAL adult animals of either the inside or outside strain. Control 21-OHase and actin mRNA levels at a 4-μg RNA dose in HET and HOMO NORMAL adrenals were similar. 21-OHase and actin

Table 1. Adrenal mRNA quantification by densitometry scanning on Northern blot analysis in three genotypes of P-450_{SCC} in newborn animals and two genotypes of P-450_{SCC} in adult animals

| | Newborn animals | | | | | Adult animals | | |
|--------------------------------------|-----------------|-------|--------------|-----------------|-----------------|---------------|--------------|-----------------|
| | wt/wt | | | wt/mt (4 μg) | mt/mt (4 μg) | wt/wt | | wt/mt (4 μg) |
| | 1 μg | 2 μg | 4 μg | | | 2 μg | 4 μg | |
| mRNA: P-450 _{SCC} /21-OHase | 1.0* | 0.8* | 1.02 ± 0.06† | 0.48 ± 0.07† | 0* | 0.82 ± 0.04† | 1.14 ± 0.08† | 0.53 ± 0.09† |
| <i>n</i> | 1 | 1 | 5 | 7 | 2 | 3 | 5 | 5 |
| mRNA: P-450 _{SCC} /actin | | 1.0* | 1.1* | 0.51 ± 0.06† | 0* | 0.97 ± 0.03† | 1.65 ± 0.17† | 0.87 ± 0.07† |
| <i>n</i> | | 1 | 1.3* | 3 | 0* | 3 | 5 | 5 |
| mRNA: 21-OHase/actin | | 1.33* | 1.45* | 1.13 ± 0.38† | 1.25* | 1.16 ± 0.01† | 1.62 ± 0.09† | 2.16 ± 0.09† |
| <i>n</i> | | 1 | 1.27* | 3 | 1.33* | 3 | 5 | 5 |

* Individual values.
† Mean ± SEM.

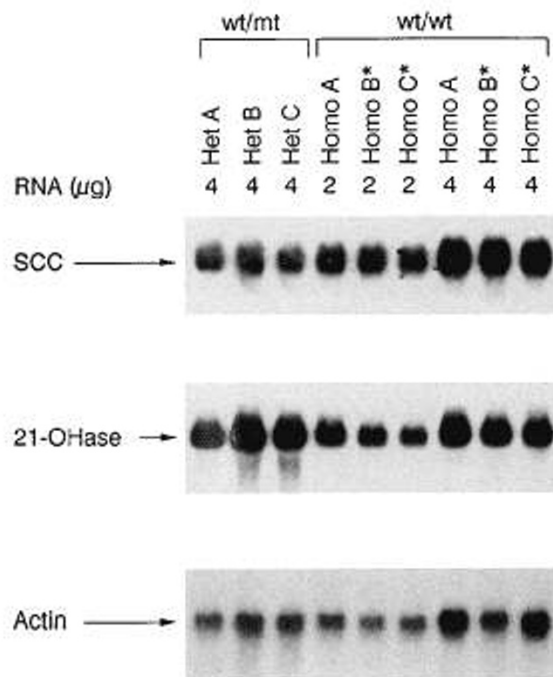


Figure 3. P-450_{SCC} (*Scs*) mRNA level based on the band intensity of the autoradiogram in adult animals. *Het*, HET (wt/mt) animals; *Homo*, HOMO NORMAL (wt/wt) animals. The letters *A*, *B*, and *C* above *Het* or *Homo* indicate the individual animal's identification. An asterisk indicates outside-strain animals. 21-OHase mRNA and actin mRNA levels represent the control blot studies.

mRNA levels at a 2- μ g RNA dose of HOMO NORMAL adrenals were approximately 50% lower than the mRNA levels at a 4- μ g RNA dose of both HET and HOMO NORMAL adrenals. P-450_{SCC} mRNA levels were similar in the adrenals of HOMO NORMAL animals of inside and outside strains. These data indicate the presence of the same effect of wt P-450_{SCC} gene dose at the level of P-450_{SCC} mRNA in adrenals of adult animals *in vivo* as in the newborn animals. The densitometry scanning of lanes of adult HET adrenals revealed approximately one-half ratios of P-450_{SCC} to 21-OHase mRNA and P-450_{SCC} to actin mRNA levels of HOMO NORMAL adrenals, whereas the ratios of 21-OHase to actin mRNA levels did not differ significantly between the HET and HOMO NORMAL adrenals (Table 1).

Comparison of adrenal P-450_{SCC} gene expression between newborn and adult animals with regard to the P-450_{SCC} genotype. P-450_{SCC} mRNA levels in adrenals of adult animals were greater than those in newborn animals of the same P-450_{SCC} genotype by the band intensity (Fig. 4). This finding was verified by several other Northern blot analyses (not shown). P-450_{SCC} mRNA levels in adrenals of the HET animals (wt/mt) were lower than the mRNA levels in the age-matched HOMO NORMAL animals (wt/wt), as shown in Figures 2 and 3 and Table 1. 21-OHase mRNA levels assessed by the band intensity were similarly detected in adrenals of both newborn and adult animals irrespective of age.

P-450_{SCC} protein level in adrenals from the three P-450_{SCC} genotypes of newborn animals and from the two genotypes of

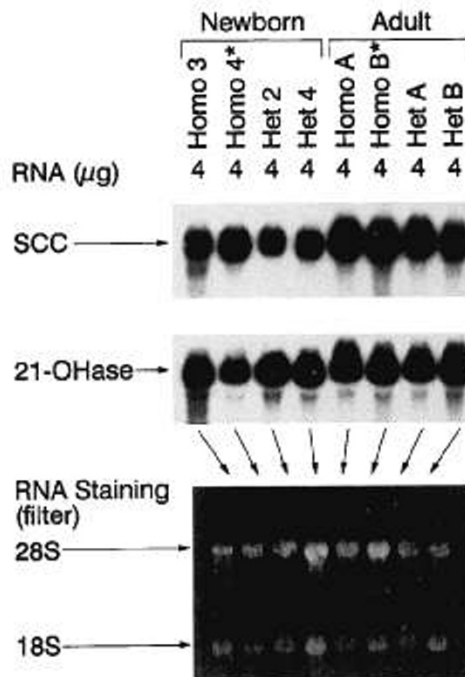


Figure 4. Comparison of P-450_{SCC} (*SCC*) mRNA level based on the band intensity of the autoradiogram between newborn and adult animals of the same genotypes. *Het*, HET animals; *Homo*, HOMO NORMAL animals. The number above *Het* or *Homo* indicates group identification of newborn animals whose adrenals were pooled. The letter above *Het* or *Homo* indicates identification of adult individual animals. An asterisk indicates outside-strain animals.

adult animals. Based on the band intensity of newborn animals (Fig. 5A), P-450_{SCC} protein was not detected in adrenals of two CAH animals. P-450_{SCC} protein levels by the band intensity in 100 μ g of adrenal protein from the three groups of HET newborn animals were slightly and markedly lower than the levels in the 50 μ g and 100 μ g of adrenal protein, respectively, from the two groups of HOMO NORMAL animals. In adult animals (Fig. 5B), P-450_{SCC} protein levels (by the band intensity) in 100 μ g of adrenal protein in three HET animals appeared to be lower than the level found in 100 μ g of adrenal protein of three HOMO NORMAL animals and were similar to or slightly lower than the level found in 50 μ g of adrenal protein in the same HOMO NORMAL animals. The ¹²⁵I radioactivity of the P-450_{SCC} band detected by an AMBIS radioanalyzer revealed 1432–1457 net counts in the 100- μ g protein bands of all HET animals, 1660–1693 net counts in the 50- μ g protein bands of HOMO NORMAL adrenals, and 2504–3019 net counts in the 100- μ g protein bands from the HOMO NORMAL animals. Radioactivity in the lanes of CAH animals were not different from the background.

***In vivo* serum.** Baseline serum B levels in nine adult HET animals (1.24 ± 0.52 μ g/dL) were similar to the B levels in five adult HOMO NORMAL animals (1.52 ± 1.03 μ g/dL). ACTH-stimulated B levels rose similarly in both genotypes of animals (HET, 21.0 ± 8.0 μ g/dL; HOMO NORMAL, 19.9 ± 7.9 μ g/dL). In two young HET animals, baseline and ACTH-stimulated B levels

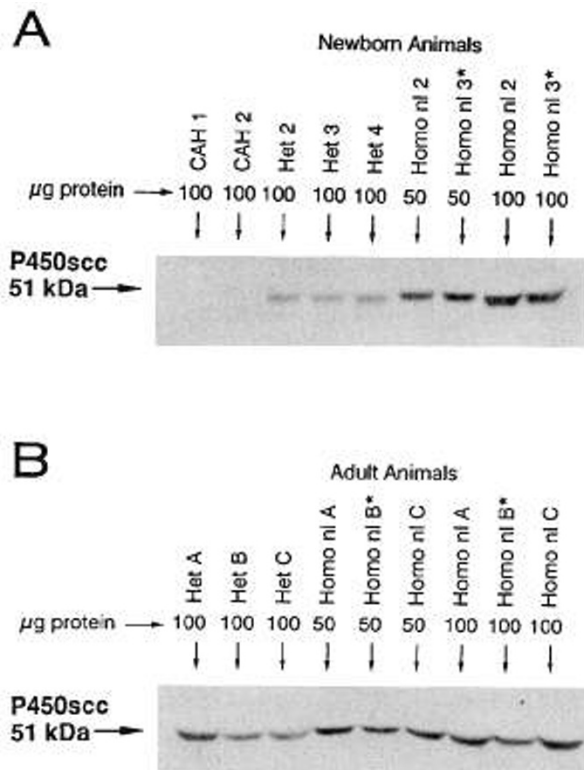


Figure 5. *A*, P-450_{scc} protein levels in the three P-450_{scc} genotypes of newborn animals. *Het*, pooled adrenals (*n* = 5) of five HET newborn animals; *Homo nl*, pooled adrenals (*n* = 5) of five HOMO NORMAL newborn animals. The number above *Het* or *Homo nl* indicates identification of the pooled adrenals. An asterisk indicates outside-strain animals. *B*, P-450_{scc} protein levels in the *Het* and *Homo nl* adult animals. The letter above *Het* or *Homo nl* indicates identification of the individual adrenal of the animal. An asterisk indicates outside-strain animals.

were 1.48 and 1.44 μg/dL and 7.51 and 8.35 μg/dL, respectively. In two young HOMO NORMAL animals, the baseline B levels (2.19 and 1.15 μg/dL) and ACTH-stimulated B levels (8.39 and 6.68 μg/dL) were similar to those in the young HET animals. These hormonal data indicate that adrenal steroid-producing capacity *in vivo* is similar in HET and HOMO NORMAL animals.

DISCUSSION

It is now well known that human CAH disorders result from various deleterious mutations of each specific gene encoding the adrenals or gonadal steroidogenic enzyme (1, 2, 15). Various deleterious mutations of the gene would likely affect gene expression to various degrees. Thus, investigation of the wt gene dose effect on adrenal gene expression based on *in vivo* hormonal response in the human HET subjects may not accurately reflect the effect of wt gene dose on the gene expression. Moreover, an ACTH stimulation test *in vivo* in proven human HET subjects for CAH disorders demonstrated both the presence and absence of mildly decreased enzyme activity in the heterozygous state (11–14). In 21-OHase deficiency, mildly increased ACTH-stimulated precursor hormone (17-hydroxyprogesterone) levels in proven HET subjects

suggested the presence of decreased gene function on the enzyme activity (11, 12). In 11β-hydroxylase deficiency (13) and 3β-hydroxysteroid dehydrogenase deficiency (14), the proven HET subjects exhibited no adrenal hormonal abnormality under ACTH stimulation. Furthermore, it is not known whether the mildly decreased 21-OHase activity in HET subjects proven for 21-OHase deficiency and the normal 11β-hydroxylase and 3β-hydroxysteroid dehydrogenase activities in the HET subjects for enzyme deficiency result from the same or slightly or markedly decreased adrenal steroidogenic enzyme mRNA and protein levels. Thus, investigation into the functional relationship of single and double doses of steroidogenic enzyme gene from the gene expression (mRNA and protein level) to gene product level (hormonal secretion) will further aid in understanding of the molecular genetics of CAH.

Investigation of the P-450_{scc} gene expression in adrenals of the three P-450_{scc} genotypes of animals demonstrated unequivocally the presence of the wt P-450_{scc} gene dose effect on the adrenal mRNA and protein levels but not at the hormonal product level. P-450_{scc} mRNA in adrenals was consistently absent in CAH animals and decreased in the HET animals at approximately half the level of the mRNA in the HOMO NORMAL animals. The levels of the P-450_{scc} mRNA were similar among animals of the same genotype and were also nearly identical between the HOMO NORMAL animals of the inside and outside strains. This gene dose-related gene expression was present in both newborn and adult animals, although at higher levels of expression in adult animals than in newborn animals of the same genotype. These data confirm the presence of a P-450_{scc} gene dose effect on adrenal P-450_{scc} gene expression at the mRNA level and suggest increasing P-450_{scc} gene expression with age. The findings of the steroidogenic enzyme gene dose-related expression at the mRNA level in the rabbit are similar to the heterozygous expression in mice carrying the photoreceptor-specific rd allele, H beta 58 allele, or Snap gene allele (17–19). The P-450_{scc} protein level in adrenals was not detected in CAH animals and was decreased in the HET animals at approximately one half of the enzyme protein detected in the HOMO NORMAL animals. This indicates that there is a parallel relationship in the mRNA levels and the enzyme protein levels. This further confirmed the presence of P-450_{scc} gene dose effect at the level of gene product, the enzyme protein. P-450_{scc} gene expression is steroidogenic tissue specific (16, 20–25). Recent reports suggest that P-450_{scc} activity in human and bovine adrenal and bovine and rat gonadal cells may be dependent on the overall amount of enzyme present and changes in the level of P-450_{scc} mRNA (16, 21–23, 26). Thus, if P-450_{scc} mRNA levels are decreased, it would likely result in decreased P-450_{scc} protein and decreased activity.

However, despite markedly decreased adrenal P-450_{scc} mRNA and protein levels in the HET animals, nearly identical baseline and ACTH-stimulated serum B

levels in the HET animals compared with the HOMO NORMAL animals indicate the presence of normal adrenal steroidogenic function in the HET animals and the presence of similar P-450_{SCC} activity in HET and HOMO NORMAL animals. P-450_{SCC} is a rate-limiting enzyme in the biosynthetic pathway; the amount of cholesterol available for cleavage determines the level of B synthesis and secretion, and the transfer of cholesterol substrate across the mitochondrial membrane is regulated at a number of steps (15, 27–29). Thus, similar adrenal steroid-producing capacity despite the low P-450_{SCC} gene expression in HET animals and the twice greater level of P-450_{SCC} gene expression in HOMO normal animals *in vivo* may result from the rate-limiting effect of P-450_{SCC} in converting the possibly excess cholesterol substrate available in the mitochondria. Alternatively, a compensatory mechanism(s) may be available for provision of adequate cholesterol substrate across the mitochondrial membrane to maintain normal steroidogenic function in the presence of low P-450_{SCC} in the HET animals. In addition, the greater B-producing capacity under ACTH stimulation by the adult adrenals than in newborn adrenals irrespective of the P-450_{SCC} genotype appears to result from the larger adrenal receptor capacity for ACTH rather than the higher P-450_{SCC} gene expression level in adult animals. The findings of P-450_{SCC} gene expression in the three genotypes of animals suggest that in human HET CAH subjects a compensatory mechanism exists to maintain adequate adrenal steroidogenic function in the presence of low levels of the steroidogenic enzyme mRNA and enzyme protein levels in the adrenals.

CAH disorders in humans often result from the compound HET mutation of the gene encoding the enzyme (12, 15). Thus, evaluation of gene dose effect on gene expression in family members of patients with CAH resulting from compound HET mutations may not provide the linear relationship of gene dose function in the gene expression. The rabbit model of P-450_{SCC} deficiency CAH due to P-450_{SCC} gene deletion resulting in identical manifestation of human lipoid CAH allowed examination of molecular genetics of the compound HOMO affected state, HET normal state, and HOMO NORMAL state of a steroidogenic enzyme gene at the levels of mRNA, enzyme protein, and hormonal production. In conclusion, this study using the natural gene knockout model of CAH in the rabbit demonstrates for the first time the presence of a wt P-450_{SCC} enzyme gene dose effect on gene expression *in vivo* at the levels of the mRNA and enzyme protein. The possible mechanism by which normal adrenal cortical function is maintained in the HET animals despite markedly decreased P-450_{SCC} gene expression is speculated.

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