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The Genes Encoding Gonadal and Nongonadal Forms of 3β-Hydroxysteroid Dehydrogenase/Δ^5–Δ^4 Isomerase Are Closely Linked on Mouse Chromosome 3

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The biosynthesis of steroid hormones in the gonads and adrenal glands requires the activities of the enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3βHSD) which catalyzes the NAD^+-dependent dehydrogenation and subsequent $\Delta^5 \rightarrow \Delta^4$ isomerization of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids. The mouse expresses four isoforms of 3βHSD. 3βHSD I is expressed in gonads and adrenal glands and appears to be the major steroidogenic form, 3βHSDs II and III are expressed in both liver and kidneys, and 3βHSD IV has been detected only in kidneys. To determine the genetic relationship between the 3βHSD isoforms, we have mapped the chromosomal locations of the four genes by linkage analysis using gene-specific probes derived from the 3′ untranslated regions of 3βHSD cDNA clones. The four 3βHSD structural genes (*Hsd3b*) are closely linked within a segment of chromosome 3 that is conserved on human chromosome 1. The order of markers on Chr 3 surrounding *Hsd3b* is: centromere–*Gba*–(4.4 ± 2.2)–*Hsd3b*–(3.3 ± 1.9)–*Tshb*–(6.7 ± 2.7)–*Amy-1*.

INTRODUCTION

The biosynthesis of steroid hormones in the gonads and adrenal glands requires the activity of 3β-hydroxysteroid dehydrogenase/isomerase (3βHSD; EC 1.1.1.145/5.3.3.1), a single polypeptide that catalyzes the NAD^+-dependent dehydrogenation and subsequent $\Delta^5 \rightarrow \Delta^4$ isomerization of the Δ^5-3β-hydroxysteroids, pregnenolone, and dehydroepiandrosterone to the Δ^4-3-ketosteroids, progesterone and androstendione, respectively (Payne *et al.*, 1993). Progesterone is a precursor of the adrenal steroids, cortisol, corticosterone, and aldosterone; androstenedione serves as a precursor of the gonadal steroids, testosterone, and estrogens. Because 3βHSD occupies a central role in steroid hormone biosynthesis, clinical manifestations of 3βHSD deficiency in man are severe and include water and salt imbalance, hypotension, reduced or absent response to stress, pseudohermaphroditism in genetic males, and, in females, mild virilization and irregular or absent ovulation (Bongiovanni, 1981).

Multiple, genetically distinct isoforms of 3βHSD have been characterized in man, rat, and mouse (Lachance *et al.*, 1990, 1991; Lorence *et al.*, 1990, 1991; Zhao *et al.*, 1990). Four distinct isoforms of 3βHSD have been characterized in the mouse (Bain *et al.*, 1991; Clarke *et al.*, 1992a). The first isoform, 3βHSD I, is expressed only in mouse gonads and adrenal glands. Forms II and III, both 83% identical in amino acid sequence to 3βHSD I and 90% identical to each other, are expressed in kidney and liver. We have shown recently that forms I and III, when transiently expressed in COS-1 cells, have the capacity to convert Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids with form I exhibiting lower K_m values for pregnenolone and dehydroepiandrosterone than form III (Clarke *et al.*, 1992b). The fourth form, 3βHSD IV, has only been detected in kidneys and is more distantly related to the other three isoforms, being between 72 and 75% identical on the amino acid level to forms I, II, and III. When transfected into COS-1 cells, form IV does not have the capacity to convert Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids, but can only reduce the 3-keto group of dihydrotestosterone to yield 5α-androstanediol. Unlike forms I, II, and III, which require NAD^+ as a cofactor, form IV requires NADPH (Clarke *et al.*, 1992a).

To determine the genetic relationship between the 3βHSD isoforms, we have mapped the chromosomal location of the genes encoding the four forms by linkage analysis using gene-specific probes derived from the 3′ untranslated regions of the 3βHSD cDNA clones. The four 3βHSD structural genes, for which we propose the designations *Hsd3b-1*, *Hsd3b-2*, *Hsd3b-3*, and *Hsd3b-4*, are closely linked to one another in a region of chromosome (Chr) 3 that is conserved on human Chr 1, suggesting that they arose by tandem gene duplication.

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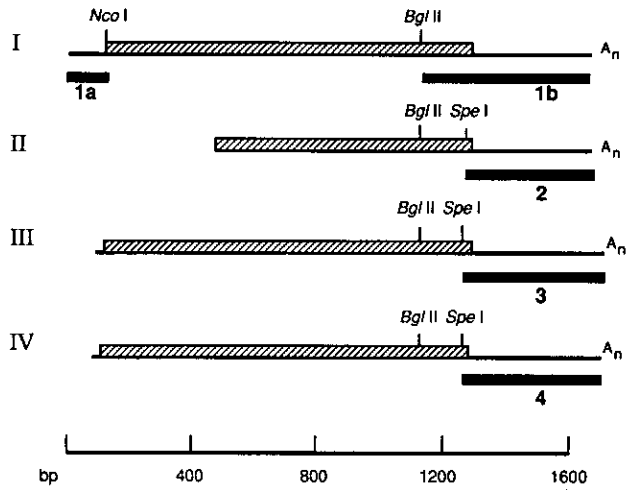

FIG. 1. Hybridization probes used in the linkage analysis of mouse *Hsd3b* genes. Probes are represented by black bars beneath the 3βHSD cDNA clones, identified at the left. Numbers used to denote each probe in the text are indicated. Cross-hatched areas represent coding regions of the cDNA clones. A full-length 3βHSD II cDNA clone has not been isolated.

MATERIALS AND METHODS

Mice. The F_2 progeny of a CAST/Ei × MEV mating were generated at The Jackson Laboratory (Bar Harbor, ME). The CAST/Ei strain was derived from *Mus musculus castaneus.* These mice were typed for *Emv-27* and *Amy-1* as previously described (Dranginis *et al.*, 1984; Taylor and Rowe, 1989). The *Emv-27* genotype was judged by the intensity of an *Emv-27*-specific hybridizing fragment (one copy vs two copies); the possibility of error in such judgements cannot be excluded. The interspecific backcross (C57BL/6J-tg9257 × SPRET/Ei) × C57BL/6J was generated in the Department of Human Genetics, University of Michigan (Ann Arbor, MI). The SPRET/Ei strain was derived from *Mus spretus.* C57BL/6J-tg9257 carries a human amylase transgene insert on Chr 18 (Ting *et al.*, 1992, and unpublished data).

Southern blot analysis. Digested DNA was fractionated through 0.6% agarose and transferred to nylon membranes (ZetaBind, Whatman, Hillsboro, OR, or GeneScreen Plus, New England Nuclear Research Products, Boston, MA) in 0.4 *N* sodium hydroxide. Radiolabeled probes (5.0×10^6 cpm/ml; see below) were hybridized in 50% formamide with 1 *M* sodium chloride, 50 m*M* Tris (pH 7.5), 1% sodium dodecyl sulfate (SDS), 100 μg/ml salmon sperm DNA, and 10% dextran sulfate for 12–16 h at 42°C. The filters were washed twice in 2× SSC/0.1% SDS at room temperature and once at 65°C in 0.1× SSC/0.1% SDS before autoradiography (1× SSC = 150 m*M* sodium chloride, 15 m*M* sodium citrate). When necessary, probes were removed by washing twice in 0.1× SSC/0.1% SDS at 100°C.

Probes were radiolabeled with [α-^{32}P]dCTP (New England Nuclear Radiochemicals, Boston, MA) by the random hexanucleotide primer method to a specific activity of 1×10^8 to 1×10^9 cpm/μg. Probe 1a is a 140-bp *Bam*HI–*Nco*I fragment from a mouse 3βHSD I cDNA clone and includes a small portion of the pBluescript polylinker (Fig. 1). Probe 1b is a 500-bp fragment of 3βHSD I that extends from a *Bgl*II site to a *Sac*I site in the polylinker. Probes 2, 3, and 4 are fragments of 3βHSD II, III, and IV cDNA clones, respectively, that extend from a conserved *Spe*I site to sites within the polylinker (Bain *et al.*, 1991). These fragments are approximately 400 bp in length. The *Tshb* probe was a 1.8-kb fragment of the mouse TSH β subunit promoter generated by PCR (S. Kendall and S. Camper, unpublished data).

Polymerase chain reaction. The sequences of the *Amy-1* primers were 5′-GAACATATGTGTAAGTAAAATGTAC-3′ and 5′-GATTTTAATTCATTAATTAAGGGTTAG-3′ (Meisler and Seldin, 1991). The Mg^{2+} concentration was 2 m*M*, and primers were annealed to 500 ng of template at 50°C. For *Gba,* the sequences of the primers were 5′-GAAGGAAAGGACTTAGTCTACC-3′and5′-GGCCTTGGCTCTGTTATTCTGT-3′ (Hearne *et al.*, 1991). The Mg^{2+} concentration was 1.5 m*M*, and primers were annealed to 500 ng of template at 55°C. For both markers, primers were included at a concentration of 0.5 μ*M*, each deoxyribonucleoside triphosphate was present at 0.2 m*M*, and 25 rounds of amplification were performed. *Taq* polymerase was purchased from Perkin–Elmer Cetus (Norwalk, CT). Amplification products were fractionated through 10% polyacrylamide (*Gba*) or 1.2% agarose (*Amy-1*) and visualized by staining with ethidium bromide. The *Amy-1* primers amplify a 190-bp product from the *M. spretus* allele and do not amplify the C57BL/6J allele. The *Gba* primers amplify two products of 200 and 180 bp from the *M. spretus* allele and two products of 210 and 190 bp from the C57BL/6J allele.

DNA assay. For some experiments, the concentration of genomic DNA was measured by a fluorometric assay after digestion with restriction endonucleases. Aliquots (1 to 5 μl) of digested DNA were diluted to 4 ml with a solution containing 50 m*M* sodium phosphate (pH 7.4), 2 *M* sodium chloride, and 1 μg/ml bisbenzimide H 33342 (Calbiochem, La Jolla, CA). Fluorescence was measured with a Perkin–Elmer model LS-5 fluorescence spectrophotometer. DNA concentrations were estimated by comparison with standards of calf thymus DNA.

RESULTS

Initial chromosomal localization of a *Hsd3b* locus was obtained by linkage analysis using a panel of (CAST/Ei × MEV)F_2 mice. Probe 1a (Fig. 1) hybridizes with a 5.3-kb *Hin*dIII fragment in CAST/Ei DNA and a 5.2-kb fragment in MEV DNA; heterozygotes contain both fragments (Fig. 2A). The *Hsd3b* genotypes of 57 (CAST/

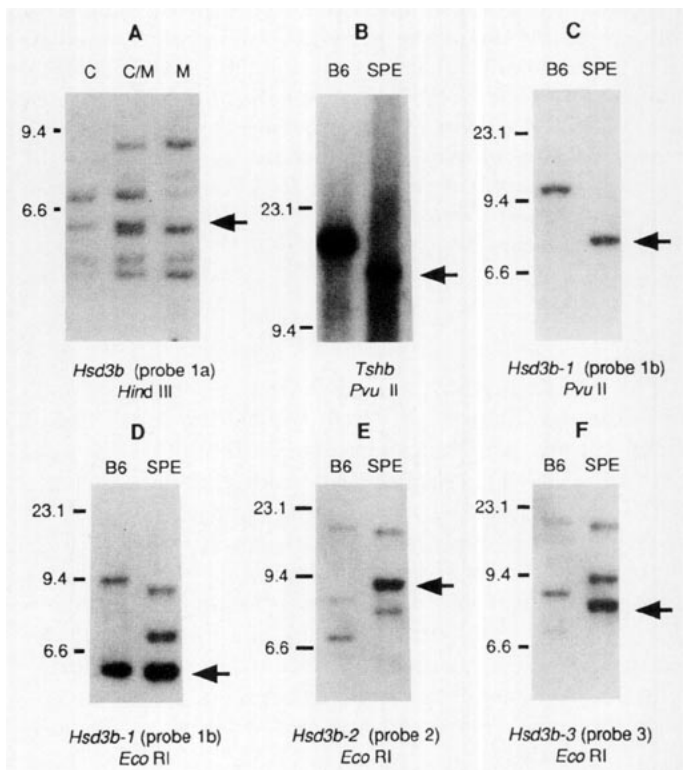

FIG. 2. Restriction fragment length variation. The source of DNA is indicated at the top of each panel, and the probe and restriction endonuclease are identified at the bottom. Molecular weight markers (kb) are shown at the left of each panel. **(D–F)** One blot hybridized sequentially with probes 1b, 2, and 3. Arrows indicate unique CAST/Ei or SPRET/Ei fragments. C, CAST; M, MEV; C/M, CAST × MEV heterozygote; B6, C57BL/6J; SPE, SPRET/Ei.

Ei × MEV)F_2 progeny were compared with those for previously mapped markers. Linkage was observed between *Hsd3b* and two loci on distal Chr 3, *Amy-1* (amylase-1) and *Emv-27* (endogenous ecotropic murine leukemia virus-27). Maximum likelihood estimates of recombination frequencies were calculated using the computer program LINKAGE (Green, 1985). The data indicate the following gene order (with recombination frequencies): centromere–*Hsd3b*–(0.061 ± 0.023)–*Amy-1*–(0.018 ± 0.012)–(*Emv-27*) (Fig. 3A). Several invariant fragments were also present on the Southern blots, reflecting the presence of multiple related 3βHSD genes.

To map each of the 3βHSD genes, we analyzed 90 progeny from the interspecific backcross (C57BL/6J-tg9257 × SPRET/Ei) × C57BL/6J. *Hsd3b* and *Tshb* were typed by Southern blot (Figs. 2B and 2C). The 6.5-kb *Pvu*II fragment specific for *Hsd3b-1* was detected with probe 1b (Fig. 1) and is not recognized by 3′ probes from any other 3βHSD cDNA clone (data not shown). *Gba* and *Amy-1* were typed by PCR (see Materials and Methods). At each locus, progeny were scored as either homozygous for the C57BL/6J allele or heterozygous for C57BL/6J and SPRET/Ei alleles. Haplotypes of the backcross progeny are presented in Fig. 3B. Minimizing the number of crossovers among the loci results in the following gene order (with recombination frequency): centromere–*Gba*–(0.044 ± 0.022)–*Hsd3b-1*–(0.033 ± 0.019)–*Tshb*–(0.067 ± 0.027)–*Amy-1*. The distance between *Amy-1* and *Hsd3b* observed in the backcross is consistent with the data from the (CAST/Ei × MEV)F_2 mice.

To determine whether *Hsd3b-2* and *Hsd3b-3* are closely linked to *Hsd3b-1*, 84 progeny of the backcross were examined for the inheritance of *Eco*RI fragments specific for *Hsd3b-2* and *Hsd3b-3*. The 3′ untranslated region probes for *Hsd3b-2* and *-3* hybridize intensely with *Eco*RI fragments of 8 and 7 kb, respectively (arrows, Figs. 2E and 2F). Probe 1b, from a 3βHSD I cDNA clone, hybridizes intensely to a 5-kb fragment that is not variant between C57BL/6J and SPRET/Ei (Fig. 2D). Among the 84 progeny examined, there was no recombination among *Hsd3b-1*, *Hsd3b-2*, or *Hsd3b-3*, indicating that the three genes are very closely linked.

The *Hsd3b-4* probe did not hybridize with SPRET/Ei DNA after digestion with any of four restriction endonucleases (Fig. 4A and data not shown). Although 3βHSD IV is abundantly expressed in the kidneys of CD-1 and C57BL/6J mice, RNA from adult *M. spretus* kidney could not protect 3βHSD IV antisense RNA probes from ribonuclease digestion (Clarke *et al.*, 1992a; P. A. Bain and A. H. Payne, unpublished data). It appears that *M. spretus* lacks a 3βHSD IV gene, or that the 3′ region of this gene is highly diverged from inbred domestic strains. When *Pst*I-digested DNA from backcross animals was hybridized with the *Hsd3b-4*-specific probe, a twofold difference in intensity of a 7-kb fragment was observed. The difference in intensity is consistent with the lack of hybridization with *M. spretus* DNA, so that backcross animals are either hemizygous (one copy, low intensity) or homozygous (two copies, high intensity) for the C57BL/6J allele (Fig. 4B, lanes 5–15). We examined 17 backcross progeny, including all seven progeny with crossovers between *Gba* and *Tshb* (Fig. 3B). To reduce the possibility of error in judging the intensity of the *Pst*I fragment, genomic DNA was quantitated with a fluorometric assay after endonuclease digestion and before electrophoresis. The blot was also rehybridized with probe 1b to verify the precision of loading and the *Hsd3b-1* genotypes of the mice (Fig. 4B). The relative intensity of the fragments was judged visually. No recombination was observed between *Hsd3b-4* and the other *Hsd3b* loci. Since the remaining 73 mice were not recombinant between *Gba* and *Tshb*, if we assume no double recombinants, there were 0/90 recombinants between *Hsd3b-4* and the other three *Hsd3b* loci, indicating close linkage of these genes.

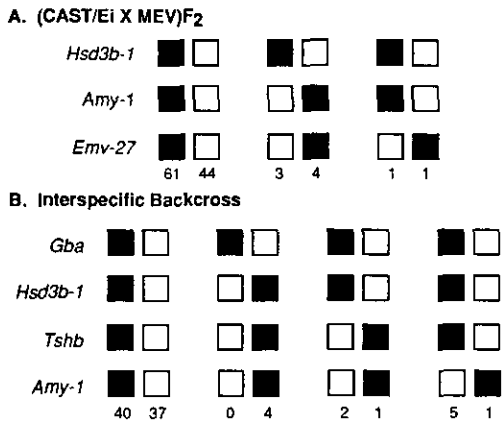

FIG. 3. Linkage of *Hsd3b* to Chr 3 markers. Columns represent haplotypes inherited from the F_1 parents for the indicated loci. The number of individuals with each haplotype is indicated under each column. (**A**) (CAST/Ei × MEV)F_2. Haplotypes of certain progeny were inferred by assuming the absence of multiple crossovers within the 7.9-cM interval between *Hsd3b-1* and *Emv-27*. (**B**) Interspecific backcross (C57BL/6J-tg9257 × SPRET/Ei) × C57BL/6J. Solid boxes, CAST/Ei and SPRET/Ei alleles; open boxes, MEV and C57BL/6J alleles.

DISCUSSION

The results of our analysis demonstrate close linkage between four members of the mouse 3βHSD gene family and localize them to Chr 3 between *Tshb* and *Gba* at approximately 49 cM on the Chr 3 composite map (Meisler and Seldin, 1991). The 95% upper confidence limit of the maximum genetic distance among *Hsd3b-1*, *Hsd3b-2*, and *Hsd3b-3*, based on 0/84 recombinants, is 3.5 cM (Green, 1981; Friedman *et al.*, 1991). For the interval *Hsd3b-4* to *Hsd3b-1*, the comparable figure is 3.3 cM, based on 0/90 recombinants and assuming no double recombination. The close linkage of the four genes suggests that the mouse 3βHSD gene family exists as a tandem cluster of related genes similar to the amylase or globin gene families and is likely to have arisen through duplication and divergence of a single ancestral gene (Collins and Weissman, 1984; Samuelson *et al.*, 1990).

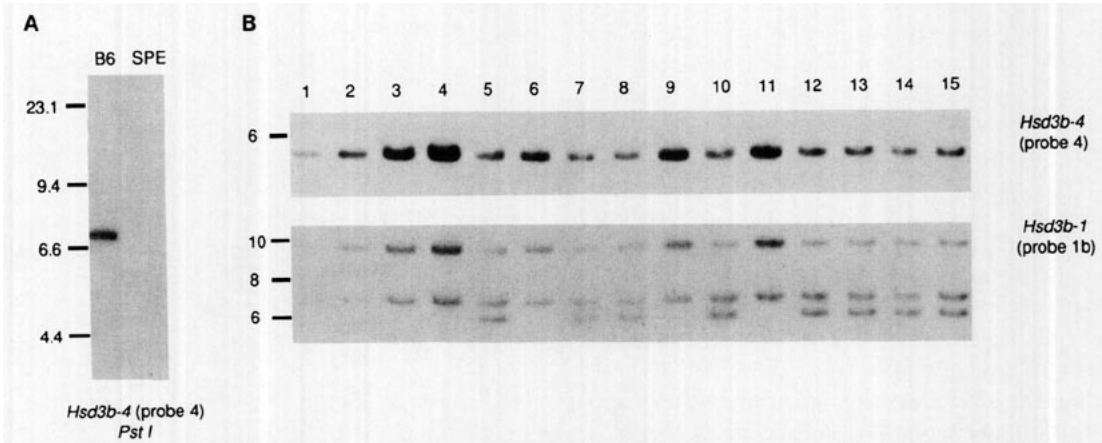

FIG. 4. Inheritance of the C57BL/6J allele of *Hsd3b-4* in interspecific backcross progeny. (**A**) Absence of hybridizing DNA in *Mus spretus* (SPE). Probe 4 (Fig. 1) recognizes a single fragment in C57BL/6J (B6) DNA and does not hybridize to DNA from *Mus spretus*. (**B**) Inheritance of one or two copies of the C57BL/6J allele in interspecific backcross progeny. Top and bottom panels represent the same Southern blot hybridized sequentially with probe 4 and probe 1b. Lanes **1–4** contain 1.8, 3.5, 7.0, and 10 μg of C57BL/6J DNA. Lanes **5–15** contain DNA (7.0 μg) from representative backcross progeny. Molecular weight (kb) markers are at the left.

The mouse 3βHSD genes map to a segment of mouse Chr 3 that shows conservation of gene order and physical distance with the centromeric region of human Chr 1 (Moseley and Seldin, 1989; Kingsmore *et al.*, 1990). The conserved region spans about 15 cM of the central portion of mouse Chr 3 from *Gba* to *Amy-1* (Fig. 5). On human Chr 1, this region extends from the *AMY* genes at 1p21, across the centromere, to *GBA* at 1q21. Human 3βHSD has been mapped to 1p13 by *in situ* hybridization (Bérubé *et al.*, 1989; Morrison *et al.*, 1991). Our results suggest that all of the human 3βHSD genes will be found on the short arm of human Chr 1, proximal to *TSHB*. This prediction may prove useful to investigators attempting to identify and isolate structural genes in this region of Chr 1 by positional cloning.

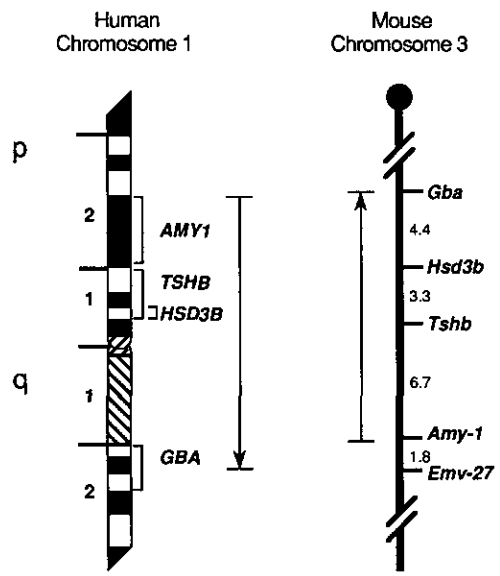

FIG. 5. Maps of the conserved linkage group on human Chr 1 and mouse Chr 3. A portion of each chromosome is illustrated. The mouse map is based on data presented in this paper; the human map is from Bruns and Dracopoli (1991). The sex-averaged distance between human *AMY1* and *TSHB* is 19 cM (Dracopoli and Meisler, 1990). Arrows indicate the relative orientation of the linkage group on the two chromosomes.

Two human 3βHSD genes have been identified: 3βHSD I, which encodes a form expressed predominantly in skin and placenta, and 3βHSD II, which encodes a gonadal and adrenal 3βHSD isoform (Lachance *et al.*, 1990, 1991). The presence of additional human genes is suggested by the existence of hepatic and renal isoforms of 3βHSD in rodents (Zhao *et al.*, 1990; Bain *et al.*, 1991) and the number of restriction fragments in human DNA that hybridize with human 3βHSD probes (Lorence *et al.*, 1990; Rhéaume *et al.*, 1991). Recently, cosegregation of a *Bgl*II polymorphism in the human 3βHSD I gene with a defective allele of human 3βHSD II has been observed in three families with 3βHSD deficiency (Rhéaume *et al.*, 1992), suggesting close linkage of these two human genes. Our demonstration of close linkage among the mouse genes predicts that all of the human 3βHSD genes, like the mouse genes, are closely linked.

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