The Genes Encoding Gonadal and Nongonadal Forms of 3β-Hydroxysteroid Dehydrogenase/Δ⁵-Δ⁴ Isomerase Are Closely Linked on Mouse Chromosome 3

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INTRODUCTION

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 Δ⁵ → Δ⁴ isomerization of Δ⁵-3β-hydroxysteroids to Δ⁴-3-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 ± 1.0)–Tshb–(6.7 ± 2.7)–Amy–1. © 1993 Academic Press, Inc.

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 Δ⁵-3β-hydroxysteroids to Δ⁴-3-ketosteroids with form I exhibiting lower Kₘ 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 Δ⁵-3β-hydroxysteroids to Δ⁴-3-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.
FIG. 1. Hybridization probes used in the linkage analysis of mouse Hsd3b genes. Probes are represented by black bars beneath the 3HSD 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 3HSD II cDNA clone has not been isolated.

MATERIALS AND METHODS

Mice. The F\textsubscript{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 Emu-27 and Amy-1 as previously described (Dranginis et al., 1984; Taylor and Rowe, 1989). The Emu-27 genotype was judged by the intensity of an Emu-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 spreitus. 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, Hillscoro, OR, or GeneScreen Plus, New England Nuclear Research Products, Boston, MA) in 0.4 M sodium hydroxide. Radiolabeled probes (5.0 × 10\textsuperscript{6} cpmp/\mu g; see below) were hybridized in 50% formamide with 1 M sodium chloride, 50 mM Tris (pH 7.5), 1% sodium dodecyl sulfate (SDS), 100 \mu g/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 mM sodium chloride, 15 mM sodium citrate). When necessary, probes were removed by washing twice in 0.1× SSC/0.1% SDS at 100°C.

Probes were radiolabeled with [\alpha,\textsuperscript{32}P]dCTP (New England Nuclear Radiochemicals, Boston, MA) by the random hexanucleotide primer method to a specific activity of 1 × 10\textsuperscript{6} to 1 × 10\textsuperscript{7} cpmp/\mu g. Probe 1a is a 140-bp BamHI-Neol fragment from a mouse 3HSD I cDNA clone and includes a small portion of the pBluescript polylinker (Fig. 1). Probe 1b is a 500-bp fragment of 3HSD I that extends from a BglII site to a StuI site in the polylinker. Probes 2, 3, and 4 are fragments of 3HSD II, III, and IV cDNA clones, respectively, that extend from a conserved SpeI site to sites within the polylinker (Bain et al., 1991). These fragments are approximately 400 bp in length. The Tshh probe was a 1.8-kb fragment of the mouse TSHβ subunit promoter generated by PCR (S. Kendall and S. Camper, unpublished data).

Polymerase chain reaction. The sequences of the Amy-1 primers were 5'-GAAACATATGGTAAAGTAAATGTTAC-3' and 5'-GATTATTATTCCATTAAGGGTGTTAG-3' (Meisler and Selkind, 1991). The Mg\textsuperscript{2+} concentration was 2 mM, and primers were annealed to 600 ng of template at 35°C. For Gbo, the sequences of the primers were 5'-GAAGGGAAGGATTCGTAC-3' and 5'-GGCTCTGGCTCTCTAATGCTAC-3' (Hearne et al., 1991). The Mg\textsuperscript{2+} concentration was 1.5 mM, and primers were annealed to 600 ng of template at 55°C. For both markers, primers were included at a concentration of 0.5 \mu M, each deoxyribonucleoside triphosphate was present at 0.2 mM, and 25 rounds of amplification were performed. Taq polymerase was purchased from Perkin-Elmer Cetus (Norwalk, CT). Amplification products were fractionated through 10% polyacrylamide (Gbo) 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. spreitus allele and do not amplify the C57BL/6J allele. The Gbo primers amplify two products of 200 and 180 bp from the M. spreitus 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 \mu l) of digested DNA were diluted to 4 ml with a solution containing 50 mM sodium phosphate (pH 7.4), 2 M sodium chloride, and 1 \mu g/ml bisbenzamide H 33342 (Caliobichem, 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)\textsuperscript{F\textsubscript{2}} mice. Probe 1a (Fig. 1) hybridizes with a 5.3-kb HindIII 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/
EI × MEV)F₂ 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 Emu-27 (endogenous ectropic 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)−(Emu-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 PouII 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. Haplotype 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)F₂ 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 EcoRI fragments specific for Hsd3b-2 and Hsd3b-3. The 3' untranslated region probes for Hsd3b-2 and -2 hybridize intensely with EcoRI fragments of 8 and 7 kb, respectively (arrows, Figs. 2E and 2F). Probe 1b, from a 3βHSD 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, and that the 3' region of this gene is highly diverged from inbred domestic strains. When Psal-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, lane 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 Psal fragment, genomic DNA was quaniticated 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.

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. 3. Linkage of Hsd3b to Chr 3 markers. Columns represent haplotypes inherited from the F₁ parents for the indicated loci. The number of individuals with each haplotype is indicated under each column. (A) CAST/Ei × MEV)F₂;

Hsd3b-1

Amy-1

Emu-27

Gba

Hsd3b-1

Tshb

Amy-1

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(A) Hsd3b-1 (B) Interspecific Backcross

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(B) Interspecific Backcross
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 investiga-
tors attempting to identify and isolate structural genes in this region of Chr 1 by positional cloning.

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 BglII 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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