Genetic analysis of the dominant white-spotting (W) region on mouse chromosome 5: Identification of cloned DNA markers near W

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ABSTRACT We have assigned several mouse cDNA and genomic clones to the W region of mouse chromosome 5, established their position with respect to various marker loci in the region, and provided molecular verification that the W5 mutation is a deletion. Meiotic recombination analysis of an interspecific mouse backcross indicated the following gene order and distances in centimorgans (cM): centromere—Emv—(13 cM)—DS5SC25—(5 cM)—D5SC25—(5 cM)—α-casein—(1 cM)—β-casein—(6 cM)—α-fetoprotein—(18 cM)—β-glucuronidase. D5SC25, an anonymous locus defined by a mouse brain cDNA, maps near the position of W and the breakpoints of the presumed genetic deletion that causes the W5 phenotype. Southern analysis of DNAs of W5/+ and interspecific F1 hybrid mice and somatic cell hybrid lines carrying the W5 deletion chromosome showed the deletion of D5SC25. In fact, analysis of other mutations at or near the W locus, which had been transferred from the strain of origin for many backcross generations, revealed the retention of donor restriction fragment length polymorphisms at the D5SC25 locus. Such evidence confirms close linkage between D5SC25 and W (within 1 cM) and indicates that the D5SC25 cDNA clone could serve as a starting point in a chromosome “walk” to W and other closely linked loci that affect development.

A large number of mutations that affect pigmentation in the laboratory mouse map in the region of the dominant white-spotting (W) locus on mouse chromosome 5. Most of these are allelic with W, but a few are mutations at one of three nearby spotting genes (1–6). Most mutations at the W locus have pleiotropic effects upon hematopoiesis, gametogenesis, and melanoblast development. The two best-studied mutations at this locus, W and W*, exhibit effects that are typical of the majority of W mutations. The W/+ mouse is viable, nonanemic, and fertile and has a white belly spot, white feet, and white tail tip (7). W/W mice usually die late in gestation from a severe macrocytic anemia, but on selected backgrounds a small fraction survives (8). The surviving W/W mice are severely anemic, sterile, and devoid of pigmentation in their skin and coat hairs but have fully pigmented eyes (black-eyed white). The W*, or “viable-W”, allele was so named because some W*/W* homozygotes have normal life-spans. W*/W* mice are black-eyed white, less anemic than W/W animals, and almost always sterile. In addition to the white-spotting pattern characteristic of W/+ mice, W*/+ heterozygotes exhibit dilute pigmentation in the colored areas of their coats and have a mild macrocytic anemia (9).

W5, a more recent, radiation-induced mutation, is a putative deletion (4). Homozygotes die prior to implantation but the heterozygotes survive with a phenotype very similar to that of W/+ mice. W5 does not complement mutations at W or at patch (Ph) (4), a closely linked spotting gene (5, 6), but does complement the mutation rump-white (Rw) (4), which is also closely linked to both W and Ph (5, 6). Ph, Rw, and a third tightly linked locus, recessive-spotting (rs) (10), affect pigmentation. Ph/+ mice have a belt of white fur usually extending from the shoulder to the rump (5). White spotting in Rw/+ mice is limited to the dorsal sacrolumbar region (6). rs/rs animals display a small amount of white spotting (10). While pigmentation is affected in all, effects on blood and germ cells appear to be minimal or nonexistent in patch, rump-white, and recessive-spotting mice. Ph/Ph embryos die in utero with a fetal hydrops (5). Rw/Rw homozygotes also die in midgestation of unknown causes (6).

Although a great deal is known about the developmental biology of the individual tissue defects, the nature of the pleiotropism and the molecular mechanism of W gene action remain obscure (1–3). The isolation of DNA sequences from the W locus could be of great assistance in elucidating W gene action in these tissues. Since the primary gene product of the W locus is unknown, we could not use current techniques to clone this gene directly. Therefore, we attempted to identify cloned sequences that map near enough to be useful in isolating W by chromosome “walking” or other indirect cloning techniques. We have used cDNA and random genomic clones, some of which map to the region of human chromosome 4 known to be homologous to mouse chromosome 5. We have established their positions with respect to markers that flank W on mouse chromosome 5. Finally, we have mapped these clones with respect to mutations at or very near the W locus, including two spontaneous mutations to W, W5 and W*; a putative radiation-induced deletion, W19H; and two of the very closely linked spotting mutations, Rw and rs (2–6, 10, 11). This approach has identified several cDNA clones in the W region, one of which maps very near to W in the W19H deletion.

MATERIALS AND METHODS

Analysis of Genomic DNA. The methods involved in preparing high molecular weight DNA, restriction endonuclease digestion, electrophoresis, Southern transfer, hybridization, and washing of genomic DNA filters have been described (12).

Mice. Mice carrying the W19H mutation were obtained from Lynn Lamoreux (Texas A & M University). The mutant stock was originally maintained on a C3H/101 hybrid background at Harwell (4) but had been backcrossed to JU for three generations and to C3H/HeJ for a single generation at Texas A & M. We obtained a litter from the backcross to C3H/HeJ. Several W19H/+ JU/C3H females were mated with partially inbred Mus spretus males obtained from The Jackson Laboratory. W19H/+ (M. musculus domesticus × M. spre-
M. spretus) F1 females were backcrossed to C57BL/6J males (The Jackson Laboratory) to produce progeny segregating for W19th.

+/- F1 females from M. musculus × M. spretus matings and their N1 backcross progeny were provided by Lianne Russell (Oak Ridge National Laboratories). Strain 101 mice were purchased from Cumberland View Farms (Clinton, TN). Animals carrying W, Rv, and rs mutations were provided by Jane Barker and David Harrison (The Jackson Laboratory).

Somatic Cell Hybrids. Somatic cell hybrid cell lines were generated by fusion of spleen cells from a W19th/+ × JU/C3H female with a hypoxanthine phosphoribosyltransferase-deficient (HPRT-) hamster fibroblastic line (CHTG49) by PEG fusion and hypoxanthine/aminopterin/thymidine (HAT) selection as described (13). To isolate subclones that retained the W19th deletion chromosome but had segregated the wild-type homologue, we selected for clones that had segregated the active mouse X chromosome to enrich for subclones that had segregated several mouse chromosomes. HAT selection was removed and cells were grown for 3–4 days to allow segregation of mouse chromosomes. HPRT- clones were isolated by growth in normal medium supplemented with thioguanine (5 µg/ml).

DNA Probes. p7D. John Horowitz provided a subcloned (4.2-kilobase (kb)) XbaI fragment of p7D, a clone of the BALB/c Emv-1 ectoecotropic provirus and flanking cellular DNA (14). A 2-kb SacI insert fragment containing flanking cellular DNA detected a Taq I restriction fragment length polymorphism (RFLP) between M. spretus and M. musculus DNAs that segregated in the backcross offspring.

p8MD4. This is an anonymous human genomic clone defining locus D4S76 from the short arm of human chromosome 4 (24). The 2-kb HindIII insert was used to detect a Taq I RFLP between the two mouse species. Hybridizations with this human probe were performed in 30% (vol/vol) formamide at 42°C.

pS25. This is an anonymous cDNA isolated from a mouse brain cDNA library defining the locus D5S25 (24). A 0.4-kb EcoRI–HindII insert fragment of this clone detected a Taq I RFLP segregating in the backcross.

Casein cDNAs. Jeffrey Rosen provided cDNA clones for mouse α-, β-, and γ-caseins cloned into the PstI 1 site of plasmid pBR322 (pCMa11, pCMβ13, and pCMγ19, respectively) (15). The full-length inserts were used as probes. The α- and β-casein cDNA clones detected Taq I RFLPs segregating in the M. spretus backcross offspring. We were unable to identify a RFLP for γ-casein in this cross.

α-Fetoprotein and albumin cDNA. Shirley Tlghman provided cDNA clones for mouse α-fetoprotein (Afp) and albumin (Alb-1), pmAfp-1 and pmAlb-2, respectively (16, 17). HindIII insert fragments of pmAfp-1 and pmAlb-2 were employed as the probes in this backcross analysis. pmAfp-1 revealed a BamHI RFLP segregating in the backcross animals.

β-Glucuronidase cDNA. Roger Genschow provided the mouse β-gluconoridase cDNA clone pGus-1 (18). A 1.5-kb PstI–HindIII insert fragment was used to detect an EcoRI RFLP in the backcross analyses.

RESULTS

Linkage Map of Mouse Chromosome 5 from Meiotic Recombination Frequency. Following a recent innovation in gene mapping in the mouse (19), we mated (M. musculus domesticus × M. spretus) F1 +/− females to M. musculus domesticus +/− males and characterized 85 N1 backcross offspring for the presence of spretus-specific polymorphic restriction fragments segregating at three marker loci (Emv-1, Afp, and Gus) that flank the W region of mouse chromosome 5 (see Fig. 1A). By observing the frequencies of meiotic recombination with these marker loci, we placed three previously unlocalized mouse cDNA clones and one random genomic sequence in the W region on chromosome 5. These are, listed by order of increasing distance from the centromere, D4S76, D5S25, and the casein gene family (Fig. 1B). All these loci map between Emv-1 and Afp. The number of recombinants out of the total number of progeny analyzed for each interval in this multipoint cross is indicated in Fig. 1B. The order of the clones was determined from a simple multipoint analysis selecting for the order requiring the minimum number of multiple crossovers per gamete. The occurrence of a single recombination between α- and β-casein genes established that α-casein is the more proximal member of this gene family. The amino acid homology and tight linkage of the bovine α- and β-casein genes suggest that they derive from a common ancestral gene (21). Therefore, it is of interest that we observed a recombination between these loci in our analysis of a relatively small number of potential recombinants. Unfortunately we were unable to detect a RFLP for γ-casein in this backcross and, therefore, could not order this member of the family.


D5S25 Maps in the W19th Deletion. The proximity of these genes to the W locus was established by determining whether the structure of these loci was altered by the W19th mutation, a putative deletion involving 2–7 cM about the W locus (4). A W19th/+ × M. musculus domesticus female was crossed to a +/+ male to produce +/+ and W19th/+ F1 offspring. The DNAs of +/+ and W19th/+ littermate offspring were digested with endonucleases that resolved spretus and domesticus alleles at loci of interest on chromosome 5. Hybridizing restriction fragment(s) derived from the W19th (domesticus) chromosome of the W19th/+ mouse were compared with that of the normal (domesticus) homologue of the +/+ littermate. Any deviation in the size of the W19th/dominesticus-specific band(s) could be attributed to the influence of the W19th mutation. As expected, we failed to detect any alteration associated with W19th at loci that map proximal to Pgm-1 or distal to bl—a, namely, Emv-1 (p7D), D4S76, Alb-1, Afp, and Gus (data not shown). Similarly, the genetic sequences corresponding to β-casein (data not shown) and α-casein (Fig. 2 Upper), which map 5 and 6 cM proximal to Afp, respectively, are unaffected by the W19th mutation.

On the other hand, D5S25 genomic DNA sequences, which map 5 cM proximal to the α-casein gene, are absent from the domesticus chromosome carrying the putative deletion W19th (Fig. 2 Lower). W19th/+ heterozygotes failed to manifest novel pSC25-hybridizing fragments, indicating that D5S25 genomic sequences are confined to the deleted region. The identification of this deleted cDNA clone provides molecular evidence confirming that the W19th mutation is a genetic deletion.

As further confirmation that D5S25 maps within the deletion, a series of somatic cell hybrids, some carrying the deletion chromosome, were derived by fusing CHTG49 hamster fibroblasts with spleen cells from a W19th/+ × C3H/JU female. The W50A2T series of hybrids was particularly interesting because they lack D5S25 sequence (Fig. 3 Lower) while retaining markers that flank W—namely, alb-1 (Fig. 3 Upper) and Emv-1 (p7D), α-, β-, and γ-casein genes, Afp, and Gus (data not shown). Since our meiotic recombination analysis and W19th/+ interspecific hybrid data clearly demonstrate that D5S25 maps between Emv-1 and the α-casein locus and within the W19th deletion, the W50A2T hybrids must have retained the W19th deletion chromosome 5 but segregated the
normal homologue. Therefore, these data confirm that DSSC25 maps within the W<sup>19H</sup> deletion and demonstrate the usefulness of these cell lines for mapping nonpolymorphic loci with respect to the W<sup>19H</sup> deletion.

**Mapping the Distal W<sup>19H</sup> Deletion Breakpoint.** To confirm the position of W<sup>19H</sup> proximal to the α-casein gene and estimate the distance between the α-casein gene and the distal deletion breakpoint, an interspecific F<sub>1</sub> backcross was made between W<sup>19H</sup>/+ (musculus × spreus) females and C57BL/6J males to produce progeny segregating for W<sup>19H</sup>. The map orders of DNA markers in the two types of interspecific crosses are consistent (data not shown). Since no recombination occurred between either the α- or the β-casein gene and W<sup>19H</sup>, the gene order of these loci with respect to Afp was not directly verified in this experiment. However, the presence of the W<sup>19H</sup> deletion did not significantly reduce the recombination between the β-casein gene and Afp (data not shown). Therefore, it is unlikely that the W<sup>19H</sup> deletion maps in this interval. The most likely location for the deletion is proximal to the casein gene family, consistent with the map position of the deleted DSSC25 locus. The most likely gene order then is Emv-1–D4S76–W<sup>19H</sup>–α-casein–β-casein–Afp–Gus. The absence of a crossover between W<sup>19H</sup> and the α-casein gene in 50 potential recombinants indicates that the α-casein gene lies near the distal deletion breakpoint.

**Lack of Recombination Between DSSC25 and Alleles at W, rs, and Rw.** To obtain direct evidence concerning the proximity of DSSC25 and W, we tested several W-region-congenic mouse lines for the occurrence of recombination between
these loci during transfer of the selected W mutation onto a different genetic background. The strain of origin was either C57BL/6J (B6) or C3H/HeJ (C3H). The congenic lines are designated by the strain onto which the locus has been transferred followed by the strain of origin. Both W congenic lines tested, B6.C3H-W44 and C3H.B6-W39 (produced at The Jackson Laboratory by 40 and 50 generations of backcrossing, respectively), had segregated donor alleles at Pgm-1, 3 cM proximal to W (data not shown), but retained the donor alleles at the D5SC25 locus (Fig. 4). Therefore, the D5SC25 locus has been cotransferred with selected mutations at W for 90 backcross generations. The mean length(s) of the donor chromosomal segment that accompanies a selected locus during the generation of congenic strains has been estimated by Johnson (22) as $1/n$, where $n$ = the number of generations of backcrossing beyond $F_1$. The 95% confidence interval is $(-0.975)/n < 1/n < (0.025)/n$. Therefore, the cosegregation of D5SC25 with the selected W mutations for 90 generations indicates that D5SC25 is within 1 cM of the W locus.

Furthermore, the B6.C3H-rs line, produced by ≈30 generations of backcrossing, retained the donor allele at D5SC25 (Fig. 4). D5SC25 has also cosegregated with Rw for three backcross generations involved in the generation of a novel congenic line carrying this mutation (data not shown). These observations further validate our assertion that W and D5SC25 are very close.

**DISCUSSION**

We have identified cloned cDNAs that are closely linked to the white-spotting gene complex on mouse chromosome 5, established the map positions of these loci with respect to known markers, and confirmed that $W^{10H}$ is a deletion. One of the new markers, pSC25, an anonymous mouse brain cDNA, maps within 1 cM of W and within the $W^{10H}$ deletion. The α-casein gene maps just distal to the $W^{10H}$ deletion. These clones should be extremely useful for investigating the relationship among the several tightly linked developmental loci in this region, as well as the molecular basis of their control of embryonic development and differentiation.

Due to its close proximity to the map position of the W locus, it is possible that the pSC25 cDNA represents the transcript of one of the white-spotting genes such as W, Ph, or rs. All three are distinct with respect to their phenotypic effects but have been shown to be tightly linked by recombination analyses. The rs locus maps within 1 cM of W with no recombination events so far observed (10, 11). Recombination frequencies between Ph and W alleles vary from 0.07 to 0.35, depending on the W allele tested (3). It is unlikely that the deleted pSC25 cDNA represents the Rw transcript since complementation data of Lyon et al. (4) demonstrate that the $W^{10H}$ deletion does not extend to Rw.

pSC25 was obtained from a mouse brain cDNA library, and although mice carrying mutations at Ph, W, and rs have no obvious neuropathology, we cannot preclude the possibility that these genes are expressed in the brain. Deletions confined to individual genes would be very useful for generating a long-range restriction map of the D5SC25 and casein-gene regions and identifying genomic rearrangements asso-
ciated with mutations at one or more of these white-spotting loci. Some W alleles may represent deletions confined to W. Mutations of this type would be expected to exhibit the null phenotype represented by the classic W allele. Unfortunately, the only known deletion in this region, W\textsuperscript{199}, spans W, Ph, and \textsuperscript{15-17} (4), a recessive embryonic lethal mutation (23). The original complementation data indicated that this deletion encompasses between 2 and 7 cm distal to \textit{Rw} (4). The data presented in the current paper allow us to further define the extent of the \textit{W}\textsuperscript{199} deletion. \textit{D5SC25} maps within the \textit{W}\textsuperscript{199} deletion, and the distal deletion breakpoint maps between \textit{D5SC25} and the a-casein gene, a distance of \textasciitilde5 cm. Since the published genetic map indicates that \textit{W} is 9 cm proximal to \textit{Afp} (20) and we have shown that the a-casein gene maps 6 cm proximal to \textit{Afp} and distal to the \textit{W}\textsuperscript{199} deletion, the maximum size of the deletion is \textasciitilde3 cm. Since \textit{D5SC25} maps 5 cm proximal to a-casein, \textit{D5SC25} should map very near the proximal deletion breakpoint. As this breakpoint separates \textit{Rw} from the closely linked markers \textit{W} and \textit{Ph}, \textit{D5SC25} should be near all three spotting loci.

Cloned genomic sequences from the \textit{W} region could be used to identify additional expressed sequences that map (i) within the \textit{W}\textsuperscript{199} deletion and (ii) within the region of the donor chromosome retained by the \textit{W}-congenic lines. Candidate \textit{W} sequences could be identified by their differential expression in developing neural crest, germ cells, and hematopoietic tissues or from transcripts of altered size or concentration in the tissues of \textit{W} mutants. Ultimately, the verification of a \textit{W}-locus clone will rely on its ability to ameliorate one or more of the \textit{W}-locus tissue defects when introduced into developing \textit{W}-mutant embryos.

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