Mammalian Genome 5, 814–816 (1994).

Mammalian Genome

High-resolution genetic analysis of a deletion on mouse Chromosome 17 extending over the Fused, tufted, and homeobox *Nkx2-5* loci

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Received: 10 January 1994 / Accepted: 27 July 1994

Lyon and her colleagues described a spontaneous deletion of genetic material that occurred within a *t* haplotype version of mouse Chromosome (Chr) 17 (Lyon and Bechtol 1977). The deletion occurred within a t^6 chromosome which is characterized by an early embryonic lethal phenotype (*tcl-0*). The deleted chromosome was named t^{h20}, and, in initial studies, it was found that the t^{h20} chromosome failed to complement mutations at two Chr 17 loci defined by mutant effects on adult morphology: tufted and Fused. Animals homozygous for a recessive mutation at the tufted (*tf*) locus undergo waves of hair loss throughout their adult lives, and the same phenotype is observed in animals with a tf/t^{h20} genotype (Lyon 1956).

Multiple mutations have been characterized at the Fused locus including Fused (*Fu*), Knobbly (Fu^{Kb}), and Kinky (Fu^{Ki}) (Reed 1937; Jacobs-Cohen et al. 1984; Greenspan and O'Brien 1986). Each has a dominant effect on tail morphology as well as a recessive effect on viability. The t^{h20} chromosome fails to complement the lethal phenotype of mutations at the Fused locus, but t^{h20}/+ heterozygotes have normal tails. This result led to the hypothesis that the previous three mutations recovered at the Fused locus were all "gain of function" defects (Greenspan and O'Brien 1986). To date, there has been no published report of the cloning of either Fused or tufted.

Recently, Harvey and his colleagues recovered a series of novel cDNA clones that contain regions of homology to the classic homeobox domain (Lints et al. 1993). The locus corresponding to one of these clones—*Nkx2-5*—was mapped to the proximal region of mouse Chr 17 with the use of progeny from an interspecific backcross generated by mating (C57BL/6J × *M. spretus*)F_1 females and C57BL/6J males as described previously (Copeland and Jenkins 1991; Copeland et al. 1993). A 3.7-kb *M. spretus*-specific *Hin*cII RFLP was used to follow the segregation of the *Nkx2-5* locus in N_2 mice. The results of this cross indicate a map position for *Nkx2-5* between *Mas1* and *Pim1* as shown in Fig. 1.

In the region of Chr 17 proximal to the *Nkx2-5* locus is the only known inversion polymorphism [*In(17)2*] that distinguishes the *M. spretus* genome from that present in standard laboratory mice of the *M. musculus* species such as B6 (Hammer et al. 1989). Although the *Nkx2-5* locus maps outside the inverted region itself, previous comparative studies have led to the suggestion that suppression of recombination may extend far beyond it in the [(C57BL/6J × *M. spretus*) × B6] cross (Himmelbauer and Silver 1993).

To investigate further the map position of *Nkx2-5,* we typed 374 N_2 offspring from an intersubspecific backcross between two inbred strains representative of different subspecies within *M. musculus*—B6 (*M. m. domesticus*) and CAST/Ei (*M. m. castaneus*)—with the same Chr 17 organization (Himmelbauer and Silver, 1993). The results obtained from typing *Nkx2-5, Pim1* and markers that delineate *Mas1* in this intersubspecific cross are shown in comparison with the results obtained from the interspecific backcross in Fig. 1. The expansion of the map in this region of Chr 17 within the intersubspecific backcross is highly significant. The *Nkx2-5* locus was mapped to higher resolution in this cross with additional markers located between *Mas1* and *Pim1*. The data obtained demonstrate the localization of *Nkx2-5* between *Hba-ps4* and *D17Mit44* with calculated interlocus distances of 1.6 ± 0.6 cM and 1.9 ± 0.7 cM respectively.

Hba-ps4 was the first molecular marker demonstrated to lie within the region deleted by t^{h20} (Fox et al. 1984). The demonstration of close linkage between *Hba-ps4* and *Nkx2-5* suggested the possibility that this new locus might also lie within the deletion. To test this possibility, we used *Bgl*II RFLPs to distinguish the *t* haplotype allele of *Nkx2-5* from that present on wild-type *M. musculus* forms of Chr 17 (Fig. 3). DNA samples from two different wild-type strains, B6 and 129/SvJ (129), display two *Bgl*II restriction fragments of 3.6 and 3.8 kb in length. DNA from an animal homozygous for a nonlethal *t* haplotype (t^{w2}) displays a unique restriction fragment of 3.0 kb in length. DNA was obtained from two other animals that are heterozygous with a single 129-derived Chr 17 homolog and second homolog derived from a *t* haplotype (either t^0 or t^6). These two samples display all three restriction fragments identified in the previously described samples. Thus, the 3.0-kb restriction fragment would appear to be characteristic of all *t* haplotypes (Fig. 3).

A fifth DNA sample was obtained from an animal that was heterozygous with a t^{h20} chromosome and a wild-type *M. musculus* chromosome. The only *Nkx2-5* restriction fragments visible in this sample were identical to those seen in the other wild-type samples. The t^{h20} chromosome was recovered as a spontaneous mutation directly from the t^6 chromosome (Lyon and Bechtol 1977), which carries the *t*-specific restriction fragment of 3.0 kb in length. The absence of this band from the $+/t^{h20}$ genotype demonstrates the deletion of the *Nkx2-5* locus from the t^{h20} chromosome.

The intersubspecific backcross data indicate a map position for the *D17Mit44* microsatellite locus that is 1.9 cM distal to *Nkx2-5* (Himmelbauer and Silver 1993). Thus, we sought to determine whether or not the *D17Mit44* locus was also deleted from the t^{h20} chromosome. Unfortunately, the t^6 chromosome does not display a *D17Mit44* allele that is distinguishable from that present on wild-type chromosomes. Thus, the *D17Mit44* primer pair (purchased from Research Genetics, Inc.) was used to recover a 440-kb YAC clone (FANC2) from the Princeton YAC library (Burke et al. 1991; Rossi et al. 1992). Sequence information was obtained from

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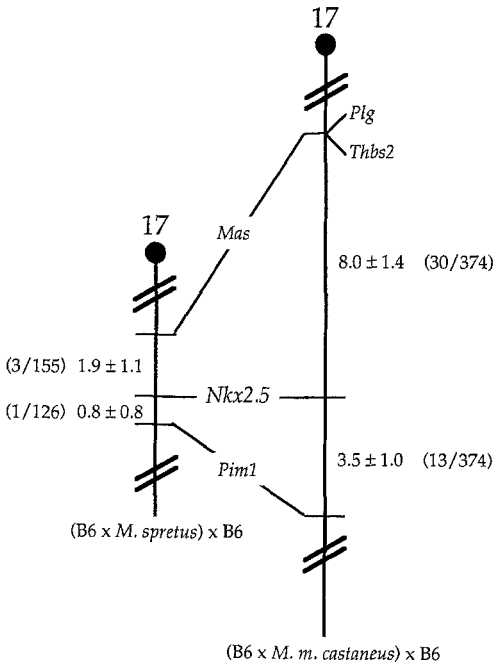

Fig. 1. Linkage maps encompassing the *Nkx2-5* locus on Chr 17. The results of the interspecific backcross (B6 × *M. spretus*) × B6 are shown on the **left.** The results of the intersubspecific backcross (B6 × *M. castaneus*) × *M. castaneus* are shown on the **right.** All loci were typed for RFLPs by Southern blot analysis. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed according to standard protocols. The 1.3-kb *Eco*RI insert from the previously described *Nkx2-5* cDNA clone was used as probe (Lints et al. 1993). A *Hinc*II polymorphism was used to follow the *Nkx2-5* locus in the interspecific backcross, and a *Taq*I polymorphism was used to follow the *Nkx2-5* locus in the intersubspecific backcross. The *Mas1* locus was not typed directly in the *M. castaneus* backcross; instead, two markers known from other data to flank *Mas1* on either side—*Plg* and *Thbs2*—were typed and found to co-segregate with 100% concordance (Himmelbauer et al. 1993; Himmelbauer and Silver 1993).

both FANC2 YAC end fragments, and this information was used to design PCR primer pairs for the specific amplification of each corresponding genomic fragment. (For the 1089 end, we used primers 5′GTTCCCATGTGGGTGTTAG3′ and 5′AAATGCAGACTGAAGATGTC3′. For the 1091 end, we used primers

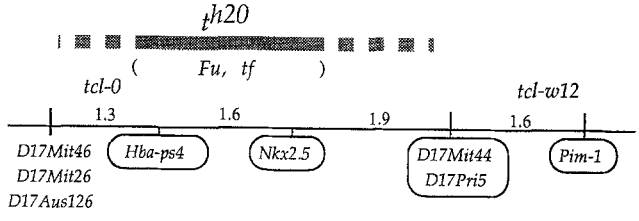

Fig. 2. High-resolution genetic map of the t^{h20} deletion region. Molecular markers are indicated beneath the line, and phenotypically defined loci are above the line. Distances are in centiMorgans and are based on data obtained from the (B6 × CAST) × B6 intercross-backcross. The relative locations of the *t*-haplotype lethal mutations *tcl-0* and *tcl-w12* were determined by Ark and coworkers (1991), and the relative locations of *tf* and *Fu* were determined from data accumulated by Rossi and associates (1992). The t^{h20} deletion definitely extends over *Hba-ps4* and *Nkx2-5*, and does not extend as far as *D17Mit46* or *D17Pri5*. It is impossible to determine whether the *tcl-0* locus is included in the deletion or is just closely linked because the deletion occurred on a chromosome (t^6) that already carried a mutation at this locus. YAC clones have been recovered from all circled loci (Rossi et al. 1992, this report, and unpublished data).

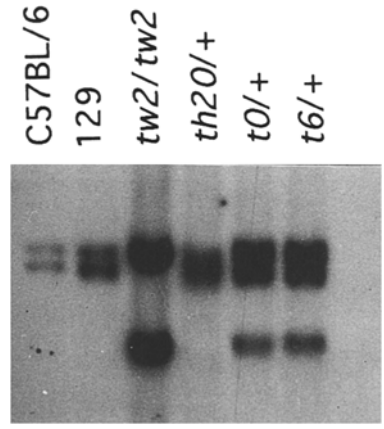

Fig. 3. Restriction fragment length variant analysis at the *Nkx2-5* locus. Samples of liver DNA taken from animals with the genotypes shown above each lane were digested with *Bgl*II and subjected to Southern blot analysis with the *Nkx2-5* cDNA clone. DNA from a mouse of the genotype $t^{h20}/+$ was a gift of Dr. Tom Vasicek (Princeton), who obtained it originally from M.F. Lyon (Harwell, U.K.). Clear evidence that the *Nkx2-5* locus is deleted by the t^{h20} chromosome is provided by an examination of the presence or absence of the *t*-specific 3.0-kb fragment in different genotypes. In addition, there is a second *t*-specific fragment of 3.9 kb in size (just above the top wild-type fragment) that shows complete concordance with the 3.0-kb fragment. The 3.9-kb fragment is difficult to resolve, and thus we did not rely upon it as a means to demonstrate the mapping of *Nkx2-5* to the t^{h20} deletion. However, careful examination of the data indicates the association of only the 3.0-kb and 3.9-kb fragments with *t* haplotypes, and the association of only the 3.6-kb and 3.8-kb fragments with the wild-type chromosome.

5′GTAATGTAGTCTGCCATACATA3′ and 5′TGTTGCCTATCAGTGACATTC3′). Each primer pair was used in a PCR assay with genomic DNA obtained from two somatic cell hybrid lines (R44-1 and 167EJ) that contain Chr 17 along with fragments of other mouse chromosomes on a hamster or human background respectively along with control samples (Smiley et al. 1978; Pickford 1989). The results demonstrate the presence of both YAC end fragment sequences in both Chr 17 cell lines. These data tentatively map the entire FANC2 clone to Chr 17.

The 1089 end of the FANC2 clone was used to retrieve a genomic clone (44/1089) from a lambda library of mouse genomic DNA. A 2.2-kb *Bam*HI fragment was subcloned from this lambda clone and used as a probe to detect polymorphisms among different forms of Chr 17. This new DNA marker is named *D17Pri5*. Analysis of the BXD set of RI strains shows a strain distribution pattern for *D17Pri5* that is indistinguishable from that associated with the *Pim-1* locus. This result confirms the integrity of the FANC2 YAC from *D17Pri5* to *D17Mit44*. A *D17Pri5* polymorphism was detected between the t^6 chromosome and the wild-type form of Chr 17. The Southern blot pattern obtained with $+/t^{h20}$ DNA was the same as that obtained with $+/t^6$ DNA. Thus, the *D17Pri5* locus is not deleted from the t^{h20} chromosome.

The accumulated data on the delineation of the t^{h20} deletion are shown in Fig. 2. The deletion has minimum size of 1.6 cM, since it includes the two molecular markers *Hba-ps4* and *Nkx2-5*. More proximal (*D17Aus126*) and more distal (*D17Pri5*/*D17Mit44*) markers are not removed by the deletion. Thus, the maximum width of the deletion is 4.8 cM.

To further characterize the *Nkx2-5* region at the physical level, we sought to recover YAC clones that extended over this locus. The Princeton YAC library (Burke et al. 1991; Rossi et al. 1992) was screened with primers derived from the *Nkx2-5* coding sequence (5′GGCCCAATGGCAGGCTGAAT3′ and 5′CTCTCTGCCCTGGATGTGGC3′), and a single 230-kb clone was recovered (C199F2). One end of this clone (defined by the primer 1089)

was recovered and mapped tentatively to Chr 17 by a PCR screen of the same genomic DNA samples used to analyze the ends of the *D17Mit44* YAC clone. (The end-fragment specific primers were: 5′TAAAGGAAGCTTTGAAGGATG3′ and 5′GGTTCAGATACTTCCATAACT3′). The other end of C199F2 was amplified and found to contain only repetitive sequences, which precluded its chromosomal assignment. Thus, the possibility of chimerism with C199F2 cannot be ruled out.

The *Nkx2-5* gene was identified originally on the basis of the presence of a homeobox-like motif. During early embryonic development, the *Nkx2-5* gene is expressed in myocardiogenic progenitor cells and the heart, as well as the pharynx, thyroid, tongue, spleen, and stomach (Lints et al. 1993). In addition, targeted disruption of the *Nkx2-5* gene results in an embryonic lethal phenotype without the tissue hyperplasia or twinning seen sometimes in embryos homozygous for some mutant Fused alleles (I. Lyons and R. Harvey, unpublished data). These data make it seem unlikely (although not impossible) that *Nkx2-5* is actually equivalent to either Fused or tufted. The t^6 lethality also occurs much earlier than expected if this locus were equivalent to *Nkx2-5*.

Since homeobox genes are often found in clusters, we sought to determine whether *Nkx2-5*-related genes might be located nearby to this locus as well. To test this possibility, we prepared a Southern filter with *Eco*RI-digested DNA from the *Nkx2-5* YAC-clone C199F2, along with DNA from two unrelated YAC-clones, DNA of the yeast host strain AB1380, and B6 DNA as controls. This blot was hybridized to a radiolabeled *Nkx2-5* cDNA clone and washed under low stringency conditions (2× SSC, 0.1% SDS, 65°C, 30 min). We did not see any bands in the *Nkx2-5*-containing YAC clone other than the single 9-kb *Eco*RI fragment that is also found in mouse genomic DNA. These data suggest that the *Nkx2-5* gene is not present within a cluster of related sequences. However, we can not rule out the possibility that the 9-kb-long *Eco*RI fragment contains additional *Nkx2-5*-like genes. Furthermore, we have not precisely determined the localization of *Nkx2-5* on our YAC-clone. Thus, it is formally possible that *Nkx2-5* could be located close to one end of C199F2 and be the last gene present in a presumptive cluster in a manner that would preclude our detection of other family members.

Acknowledgments. This research was supported by National Institutes of Health grant HD20275 (L.M. Silver), and by the National Cancer Institute through DHHS contract N01-CO-74101 with ABL (N.G. Copeland and N.A. Jenkins).

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