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Genetics

Linkage mapping of a mouse gene, *iv*, that controls left–right asymmetry of the heart and viscera

(mammalian development/cardiac situs/restriction fragment length polymorphism)

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ABSTRACT **Inherited single gene defects have been identified in both humans and mice that lead to loss of developmental control over the left–right asymmetry of the heart and viscera. In mice the recessively inherited mutation *iv* leads to such apparent loss of control over situs: 50% of *iv/iv* mice exhibit situs inversus and 50% exhibit normal situs. The affected gene product has not been identified in these animals. To study the normal function of *iv*, we have taken an approach directed to the gene itself. As a first step, we have mapped *iv* genetically, by examining its segregation in backcrosses with respect to markers defined by restriction fragment length polymorphisms. The *iv* locus lies 3 centimorgans (cM) from the immunoglobulin heavy-chain constant-region gene complex (*Igh-C*) on chromosome 12. A multilocus map of the region suggests the gene order centromere–*Aat* (α_1-antitrypsin gene complex)–(11 cM)–*iv*–(3 cM)–*Igh-C*–(1 cM)–*Igh-V* (immunoglobulin heavy-chain variable-region gene complex).**

The asymmetric left–right position of the heart and viscera of higher vertebrates is a striking developmental feature. The mechanism that determines the so-called situs of an animal is not understood, but study of animals with situs inversus offers an avenue of investigation. Humans with the recessively inherited condition known as Kartagener syndrome frequently exhibit situs inversus (1). Individuals with this syndrome manifest deficient ciliary function in both respiratory epithelium and sperm, associated with a lack of the structures in cilia, dynein arms, that normally connect outer doublet microtubules (2). The association of situs inversus in these individuals with a defect of cilia has led to the hypothesis that normal ciliary function is required for the determination of situs.

Another hereditary condition of situs inversus is provided by a recessive mutation of the mouse, *iv* (3, 4). This mutation was originally detected in several pups from a litter produced by a mating of non-inbred mice: visual inspection revealed the milk-filled stomach on the right side instead of the left, and animals with this transposition were shown on morphologic examination to exhibit inversion of the heart and viscera. In contrast with Kartagener individuals, their respiratory epithelium, sperm, and cilia were structurally normal (5). To produce an inbred strain carrying *iv*, mating of affected brothers and sisters was carried out; two congenic *iv*-containing strains were also derived, by backcross-intercross mating. These matings, producing several thousand progeny animals, established that the situs inversus phenotype was due to the action of a single autosomal genetic locus. It was consistently observed that only approximately 50% of homozygous progeny exhibit situs inversus. This led to the proposal that *iv* behaves as a loss-of-function mutation: the wild-type gene, even in single copy in heterozygous animals, directs determination of normal visceral situs; in its absence, left–right determination becomes random (4).

Loss of *iv* function results not only in randomized cardiac and visceral situs but also in a high frequency of cardiac malformation. Approximately 20% of homozygous *iv/iv* mice exhibit heart defects, regardless of situs. Morphologic examination of developing *iv/iv* embryos suggests that these heart defects arise as the result of defective formation of the cardiac loop, suggesting that in *iv/iv* mice not only does the direction of looping of the primitive midline cardiac tube become randomized, but the very process of looping itself becomes disarrayed (6).

To explore the role of the *iv* gene in cardiac and visceral organogenesis, we are taking a genetic approach. We report here the first step toward the molecular analysis of *iv*, the mapping of the gene to a linkage group in the mouse.

MATERIALS AND METHODS

Mice. Mice of the SJL/J, C57BL/6J, and SI/Col-*iv/iv* inbred strains were obtained from The Jackson Laboratory. SI/Col is an inbred strain derived by brother–sister mating from the outbred stock in which the *iv* mutation was first detected. F_1 and backcross mice were bred at Yale University. Females are named first in all F_1 and cross designations.

Genetic Analysis. Backcross progeny were typed for visceral situs by observing the orientation of the milk-filled stomach through the abdominal wall within 4 days of birth. Phenotypically normal progeny were discarded. Three weeks after birth, phenotypically affected progeny were scored for agouti (*a*) or albino (*c*) coat color markers and killed. Inverted situs was in all cases confirmed by dissection. Livers were removed and genomic DNA was extracted by a standard sequence of protease digestion, phenol and chloroform extraction, and ethanol precipitation. DNA was scored by Southern blotting analysis for restriction fragment length polymorphisms (RFLPs). In the initial studies alleles at the locus *Cfh* on chromosome 1 were scored by using the probe Mu23IV and *Eco*RI-digested DNA (7); the locus *Callh* on chromosome 9 was scored by using the probe p36C11 and *Msp* I-digested DNA (P.D., B. Tack, and T. Hunter, unpublished data); and the locus *Hba-4ps* on chromosome 17 was scored by using the probe p$\alpha\psi$-4 and *Taq* I-digested DNA (8). In further studies four markers of chromosome 12 were scored: alleles at *D12Nyu1* were scored by using the probe M13ϕ7-97 and *Msp* I-digested DNA; alleles at *Aat* were scored by using the probe pC1.2 and *Eco*RI-digested DNA; alleles at *Igh-C* were scored by using the probe p3′α and *Bam*HI-digested DNA; and alleles at *Igh-V* were scored by using the probe pVh3609 and *Bam*HI-digested DNA (9, 10).

Computational Analysis. Multilocus linkage maps were constructed by using the BAYLOC algorithm (10) on a VAX

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Abbreviations: RFLP, restriction fragment length polymorphism; cM, centimorgans.

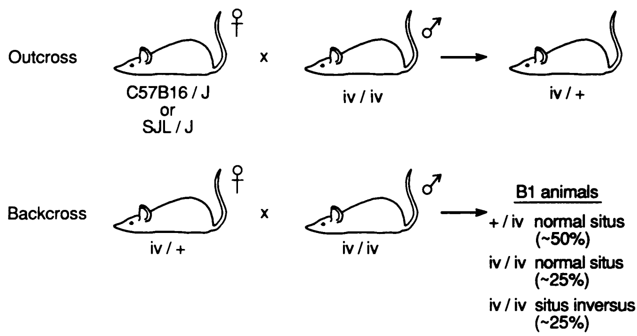

FIG. 1. Backcross mating scheme to map *iv* with respect to known RFLP markers.

11/750 computer (Department of Cell Biology, New York University Medical Center). The program and documentation are available from P.D.

RESULTS

Our strategy for mapping the *iv* mutation (Fig. 1) took advantage of the extensive polymorphism preserved at the level of DNA sequence among inbred strains of mice (11–13). SI/Col-*iv/iv* males were crossed to females of the C57BL/6J and SJL/J inbred strains, to generate F_1 hybrids heterozygous both for *iv* and for many RFLP markers. F_1 females were crossed to SI/Col-*iv/iv* males, to generate backcross progeny that could be tested for cosegregation of *iv* and any of the RFLPs. Distinguishing the *iv/iv* backcross (B_1) progeny from those that were *iv/+* was complicated by the fact that approximately half of all homozygous mutant animals were predicted to exhibit grossly normal visceral situs (3, 4). Therefore, all phenotypically normal backcross progeny were discarded. Eighteen progeny mice that showed visible transposition of the viscera (hence that were genetically *iv/iv*) were scored for inheritance of SI/Col-derived alleles of the coat color markers nonagouti (*a*, chromosome 2) and albino (*c*, chromosome 7) and for RFLPs associated with the loci *Cfh* (chromosome 1; ref. 7), *Callh* (chromosome 9; P.D., B. Tack, and T. Hunter, unpublished data), and *Hba-4ps* (chromosome 17; ref. 8). No concordance was observed beyond that expected by chance (data not shown). However, when the *iv/iv* progeny were scored for alleles of the next marker selected for study, a marker of distal chromosome 12, *Igh-C* (immunoglobulin heavy-chain constant-region gene complex), 17 of 18 mice typed were homozygous for the SI/Col-derived allele (Fig. 2). This degree of concordance indicates odds of linkage between *iv* and *Igh-C* greater than 0.99 (10, 14).

To confirm the assignment of *iv* to chromosome 12, backcross progeny were tested for inheritance of three additional informative markers of chromosome 12. As illustrated in Fig. 2 and summarized in Table 1 (cross 2), 28 *iv/iv* progeny from the SJL/J backcross were typed for alleles of the following informative markers, ordered proximal to distal by other mating experiments (refs. 9 and 10; P.D. and V. Villani, unpublished work): *D12Nyu1* (defined by an anonymous DNA clone), *Aat* (α_1-antitrypsin gene complex), *Igh-C*, and *Igh-V* (immunoglobulin heavy-chain variable-region gene complex). Similarly, 17 *iv/iv* progeny from the C57BL/6J backcross were typed for inheritance of informative alleles of *Igh-C* and *Igh-V* (Fig. 2; Table 1, cross 1). Among the 45 total *iv/iv* backcross progeny, 44 were homozygous for the SI/Col-derived allele of *Igh-C*, indicating a recombination fraction between *iv* and *Igh-C* of 0.0222, with 95% binomial confidence limits of 0.0006 and 0.1177 (15, 16). Only one recombination was observed between *Igh-C* and *iv*, in a C57BL/6J backcross animal. When the same backcross progeny were typed for alleles of *Igh-V*, the results obtained were identical (Table 1). In particular, the single animal that had been identified as recombinant between *iv* and *Igh-C* was also the only animal exhibiting recombination between *iv* and *Igh-V*.

In the study of 28 C57BL/6J backcross progeny informative for *Aat*, 27 *iv/iv* animals were found to be homozygous

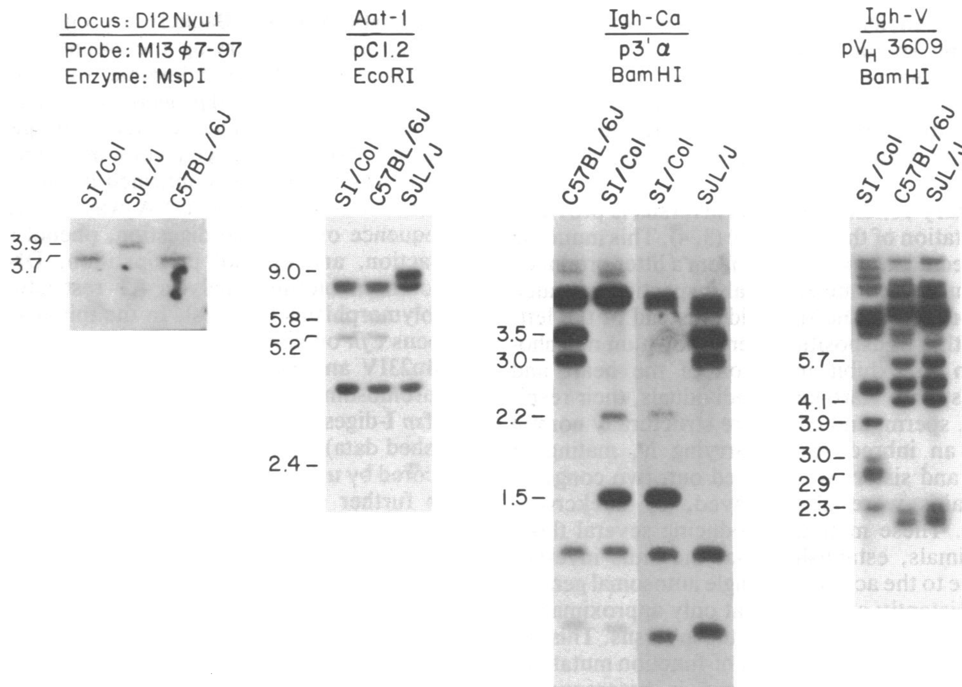

FIG. 2. RFLPs associated with markers of chromosome 12. Ten-microgram quantities of liver genomic DNA from SI/Col, C57BL/6J, and SJL/J were digested with the designated restriction enzymes and analyzed by Southern blot hybridization with the probes indicated above each panel. The sizes of polymorphic DNA fragments, in kilobases, were calculated from their mobilities relative to fragments generated by *Hin*dIII digestion of λ phage DNA.

Table 1. Genetic linkage of *iv* and markers of distal chromosome 12

Cross 1: (C57BL/6J × SI/Col)F_1 × SI/Col				Cross 2: (SJL/J × SI/Col)F_1 × SI/Col					
iv	*Igh-C*	*Igh-V*	n	*D12Nyu1*	*Aat*	*iv*	*Igh-C*	*Igh-V*	n
s	s	s	16	s	s	s	s	s	21
s	· b	b	1	j	· s	s	s	s	6
s	s	· b	0	j	j	· s	s	s	1
				s	s	s	· j	j	0
				s	s	s	s	· s	0

Backcross progeny that showed phenotypic visceral situs inversus (hence were *iv/iv*) were typed for RFLPs associated with markers of chromosome 12 (Fig. 1). Numbers, n, of each segregant type among the progeny are listed. "s" is used generically to indicate an SI/Col-derived allele, "b" a C57BL/6J allele, and "j" an SJL/J allele. Assuming the gene order shown here, no progeny chromosomes showed more than one recombination event over the portion of chromosome 12 examined. Dots indicate recombination between neighboring markers.

for the SI/Col-derived allele of *Aat*. The one *Aat* heterozygote was also heterozygous for *D12Nyu1* but was homozygous for the SI/Col alleles of *Igh-C* and *Igh-V* (Table 1). The gene order that best fits all of the observations is centromere–*D12Nyu1–Aat–iv–Igh-C–Igh-V*.

To refine the localization of *iv* with respect to these markers of distal chromosome 12, a multilocus map-building scheme was used to analyze both the foregoing data and data from previous experiments that measured recombination fractions among *D12Nyu1*, *Aat*, *Igh-C*, and *Igh-V* (refs. 10, 17–22; P.D. and V. Villani, unpublished work). Although the observed recombination fractions between pairs of these loci varied by as much as a factor of 2 from experiment to experiment, 2 × 2 contingency tests (23) of these recombination fractions indicated that the differences were no greater than could be expected from sampling fluctuation alone. All of the data were therefore pooled (Table 2) and the BAYLOC algorithm (10) was used to construct a multilocus linkage map of distal chromosome 12 (Fig. 3). Assuming *Aat* and *Igh-C* are 14 cM apart, the most likely position of *iv* is 11 cM from *Aat* and 3 cM from *Igh-C*; the 95% confidence limits for the position extend from 4 cM distal to *Aat* to 0.4 cM proximal to *Igh-C*.

DISCUSSION

The utility of RFLPs as markers in linkage maps of humans and mice is well established. In many instances, in carrying out crosses of mice aimed at enabling linkage analysis, mouse geneticists have pursued a strategy of crossing standard laboratory strains of mice, derived largely from the species *Mus domesticus*, with the genetically dissimilar wild-derived individuals of the species *Mus spretus* (24). However, recent analysis of DNA from these standard laboratory strains, using both cloned genes and anonymous probes, suggests that this may not be necessary, because a large percentage of DNA markers are polymorphic between the various laboratory strains (13). This genetic variation fixed between standard laboratory strains is not surprising, because the ancestors of these strains were themselves diverse. In the present study a lucky choice of the order in which RFLPs were tested resulted in a limited survey. However, Eppig (13) has documented the existence of a sufficient number of RFLPs, widely enough distributed, so that a limited number of backcrosses between a mutant stock and standard laboratory mouse strains can be used to survey the entire genome for linkage to the mutation.

Crosses between laboratory strains of mice have several advantages over interspecies crosses. First, both sexes of F_1 animals are fertile, in contrast with the usual F_1 male infertility of interspecies matings. Second, there are significant differences in gene spacing and possibly also gene order between *M. domesticus* and *M. spretus* (22, 25). While such differences are quantitatively small and would affect chromosome regions of only a few centimorgans, they could be expected to complicate the refined genetic and physical mapping of at least a number of chromosomal regions. Third, the visible phenotypes associated with many mouse mutations are strongly affected by the genetic background in which these mutations are expressed. Thus, for example, the fraction of mice homozygous for the *gld* mutation that show

Table 2. Recombination fractions among markers of mouse chromosome 12

Locus	*D12Nyu1*	*Aat*	*iv*	*Igh-C*	*Igh-V*
D12Nyu1	—	126/460*	7/28	192/460*	115/248
Aat	6/28	—	1/28*	113/834*	44/248*
iv	7/28	1/28*	—	1/45*	1/45*
Igh-C	7/28	1/28*	1/45*	—	3/265*
Igh-V	7/28	1/28*	1/45*	0/45*	—

Each entry is the number of recombinant chromosomes found for the total number of backcross progeny examined. Entries below the diagonal are derived from Table 1. Entries above the diagonal are summed from all backcross experiments informative for these markers (10, 17–22). Recombination fractions that represent significant deviations from 1:1 segregation (10, 14) are flagged: *, $P < 0.001$. For all unflagged recombination fractions, $P > 0.05$.

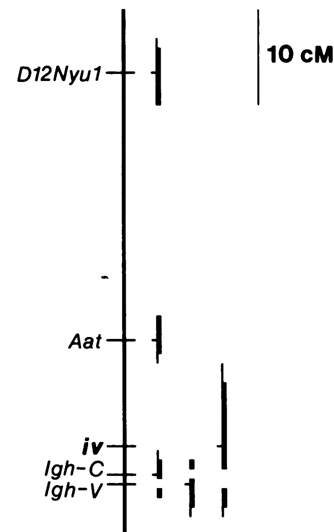

FIG. 3. Multilocus linkage map of distal chromosome 12. The BAYLOC algorithm (10) was applied to the data shown in Table 2 above the diagonal, in order to position the marker loci *D12Nyu1*, *Aat*, and *Igh-C*. The algorithm was then applied to position *iv* in this map. The 95% (thick vertical bar) and 99% (thin vertical bar) confidence intervals associated with the placement of each locus, contingent on the placements of the other markers, are shown. cM, centimorgans.

signs of lymphoproliferative disease fell from 100% on a C3H/HeJ inbred background to 66% among progeny of a backcross between *M. spretus* and C3H/HeJ-*gld*/*gld* mice (26).

In the present study, the complexity of the DNA polymorphisms associated with the chromosome 12 marker loci is striking (Fig. 2). However, this complexity did not interfere with the interpretation of the results, because each of these complex physical units behaves as a single genetic locus. For example, *Aat* is a complex locus encoding at least six serum protease inhibitors. These genes show considerable sequence homology, and several RFLPs associated with the complex have been defined (27, 28). Yet all of these RFLPs behave as markers of a single genetic locus, whose position on chromosome 12 in the mouse is now definitively established through studies of somatic cell hybrids, recombinant inbred strains of mice, and backcross progeny (9, 10, 21). Similarly, the gene complexes visualized by the p3′α and pVh3609 probes, *Igh-C* and *Igh-V* respectively, have likewise been analyzed at both the molecular and the genetic level, and again, the RFLPs associated with each complex have been shown to define a single genetic locus on distal chromosome 12 (refs. 9, 10, 21, and 29; P.D., unpublished). The crucial point is that in each case the RFLP cosegregates, defining a single Mendelian locus.

The linkage analysis carried out here provides information with which to focus further genetic and physical mapping studies that may ultimately permit isolation of the *Iv* gene. The analysis may also prove revealing for the study of inherited human situs inversus (1, 2). Linkage groups are well conserved between humans and mice, and the group that spans from *Fos* to *Igh* on mouse chromosome 12 is found in the human on chromosome 14q (21, 30, 31). Specifically, *Aat* and *Igh* map to human chromosome band 14q32 (32). At least one available inbred human pedigree displays apparent recessively inherited situs inversus in multiple generations (33). This family can now be examined with probes from the 14q32 region to test whether situs inversus in this pedigree is indeed a human equivalent of the disease in mice.

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