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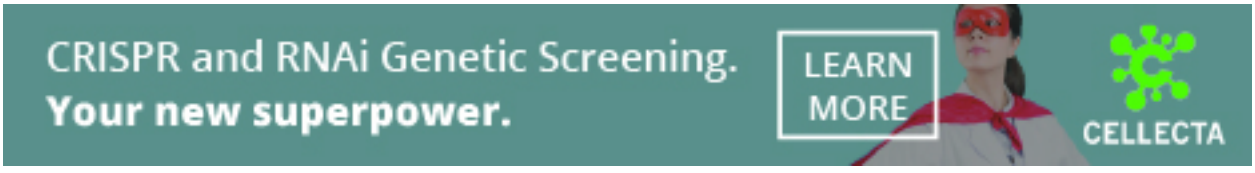

RESEARCH

Generation of a High-resolution Genetic Map and a YAC Contig of the Lurcher Locus on Mouse Chromosome 6

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Lurcher (*Lc*) is a semidominant mouse mutant that displays progressive neurodegeneration during perinatal development. This genetic lesion results in apoptotic neuronal death in a dosage dependent and cell autonomous manner in specific neurons during their terminal differentiation. To understand the molecular basis of the *Lc* mutation, we have adopted a positional cloning approach based on its location on mouse chromosome 6. To define the *Lc* locus, we have extended our previous analysis of an intersubspecific backcross between *Mus m. castaneus* and B6CBACa-A$^{w\text{-}J}$/A-*Lc* consisting of 504 animals (Norman et al. 1991). In addition, 580 animals of a generic backcross between *Mus spretus* and C57BL/6 (The European Collaborative Interspecific Backcross) were utilized for the fine genetic mapping of the *Lc* locus. Using three RFLP markers and nine microsatellite markers in the vicinity of the *Lc* locus, we determined the order and relative genetic distances of these markers at a resolution of 0.1 cM. The *Lc* mutation was mapped between two flanking markers, *D6Mit121* and *D6Mit175*, separated by a genetic distance of 0.5 cM. We then initiated the cloning of the genomic region surrounding these two markers by screening a YAC library and characterizing YAC end sequences for further screening. This effort has resulted in the construction of a YAC contig consisting of 14 YACs and spanning a 3-Mb region. Markers isolated from these YACs were used to further define the *Lc* locus, resulting in a physical map that places the *Lc* gene within an estimated 300-kb interval. This set of YACs and markers will serve as DNA sources for the identification of the *Lc* gene.

Lurcher (*Lc*) is a semidominant mouse neurological mutant that affects cerebellar development (Phillips 1960). Beginning at postnatal day 14 (P14), heterozygous *Lc*/+animals display locomotor difficulties that include an ataxic gait and a tendency to walk backwards. In contrast, homozygous *Lc*/*Lc* animals die within a few hours of birth with no gross anatomic abnormalities (Phillips 1960). The phenotypic consequences of the *Lc* mutation have been studied in detail in *Lc*/+heterozygous animals. Anatomical analysis of adult *Lc*/+heterozygotes reveals a marked reduction in cerebellar volume but no other gross defect in organogenesis. Detailed histological studies of the cerebella of *Lc*/+animals have revealed massive cerebellar cell death and have established a chronology for this neurodegeneration. The Purkinje cells (PCs) degenerate during the second postnatal week, and their disappearance is followed by the progressive loss of ~90% of cerebellar granule cells and 75% of inferior olivary neurons and Bergmann glia (Caddy and Biscoe 1979). Studies of *Lc* and wild-type chimeric mice have defined the action of the *Lc* gene as being cell autonomous; its primary site of action lies within PCs (Wetts and Herrup 1982a,b,c; 1983). The degeneration of other cerebellar cell types is a secondary effect, presumably owing to the lack of essential contacts with PCs. The death of *Lc*/*Lc* homozygotes within a few hours of birth led us to search for lesions in the respiratory, digestive, and nervous systems of the newborn *Lc*/*Lc* mice. Whereas these systems are mostly unaffected, the hindbrain is significantly reduced in both size and cell density at P0. The widespread neuronal cell death that causes this reduction is most clearly illustrated by the loss of large neurons in the motor nuclei of the fifth cranial nerves beginning at embryonic day 16 (E16) (S. Cheng and N. Heintz, in prep.). By combining the results of the analyses of *Lc* heterozygous and

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homozygous animals, we conclude that the *Lc* mutation is involved in the degeneration of specific populations of neurons as they mature in the developing central nervous system and that the dosage of the mutant gene determines which neuronal populations are affected.

Recently, the mechanisms of neuronal death in *Lc* animals have been studied extensively (Norman et al. 1995). Electron microscopy of *Lc*/+PCs at P12 reveals chromatin condensation in the nucleus and membrane blebbing, two ultrastructural hallmarks of apoptotic cell death. In addition, *Lc*/+PCs but not wild-type PCs display double-stranded DNA breaks in their nuclei and expression of sulfated glycoprotein-2, a gene that is up-regulated in certain forms of apoptosis. Therefore, we have concluded that the PCs in *Lc*/+animals die by apoptosis. Furthermore, degeneration of large neurons in the motor nuclei of the fifth cranial nerves in *Lc/Lc* animals is also attributable to apoptosis (S. Cheng and N. Heintz, in prep.). Therefore, the isolation of the *Lc* gene may provide insights into the molecular mechanisms involved in activation of programmed cell death.

To identify the *Lc* gene and to understand its possible roles during the development and degeneration of the mammalian nervous system, we have adopted a positional cloning strategy. This approach takes advantage of mouse genetics and molecular cloning technology to identify the mutant gene based on its chromosomal location. The *Lc* mutation arose spontaneously in 1954 in one of the coat color mutant colonies, Mi^{wh} (Phillips 1960); Mi^{wh} was itself found in a cross between the DBA and C57BL strains (Grobman and Charles 1947). *Lc* was then backcrossed to and maintained on a C3HeB/FeJ strain background in Harwell, England (Caddy and Biscoe 1979). This mutant was then crossed to a B6CBACa-$A^{w\text{-}J}$/A F1 hybrid in the Jackson Laboratory in 1976 and was maintained further in such a hybrid background for >50 generations (H. Sweet, pers. comm.). Therefore, a total of four inbred strains may have contributed to the genetic background in the vicinity of the *Lc* mutation. *Lc* has been genetically mapped to mouse Chromosome 6 and has a single known allele (Phillips 1960). In a previous report, an intersubspecific backcross between *Mus m. castaneus* (CAST/Ei) and B6CBACa-$A^{w\text{-}J}$/A-*Lc* (*Lc/cast.*) was initiated and 504 progeny were analyzed by using six polymorphic DNA markers on Chromosome 6 (Norman et al. 1991). As a result, the *Lc* locus was mapped to an interval of ~2.5 cM. This segment has no known synteny to the human genome. The closest markers that have established human homologs are Ghrhr, ~2 cM centromeric to *Lc*, and Igk, ~2 cM telomeric to *Lc* (Norman et al. 1991; Chua et al. 1993). Ghrhr maps to human 7p14, and Igk maps to human 2p12 (Gaylinn et al. 1994; McBride et al. 1982).

In this report we have continued our analysis of this set of *Lc/cast.* backcross progeny and of another large set of mice from a generic backcross, the European Collaborative Interspecific Backcross (EUCIB) between *M. spretus* and C57BL/6, with markers in the vicinity of the *Lc* locus. A high resolution genetic map, a YAC contig, and a high density of novel markers in the *Lc* region are reported here. These tools will facilitate the isolation of candidate genes for *Lc*.

RESULTS

A High-Resolution Genetic Map of the *Lc* Region

In a previously established phenotypic backcross between B6CBACa-$A^{w\text{-}J}$/A-*Lc* and *M. m. castaneus*, a total of 504 animals have been genetically typed with markers on either side of the *Lc* locus. This analysis has localized the *Lc* mutation between the centromeric marker *D6Rkf41* and the two telomeric markers, *D6Rkf33* and *D6Rkf39*, which are nonrecombinant with each other (Norman et al. 1991, 1995; J. Zuo and N. Heintz, unpubl.). There are a total of six recombinants between *D6Rkf41* and *D6Rkf33/D6Rkf39*, indicating that the *Lc* region, defined by *D6Rkf41* and *D6Rkf33/D6Rkf39*, has a genetic distance of ~1.2 cM. These recombinants are shown in Figure 1B, which also includes a number of additional markers described later.

To resolve the exact locations of these six crossover events, we utilized the collection of polymorphic markers developed at the Massachusetts Institute of Technology (MIT) (Dietrich et al. 1994). Using eight MIT markers known to map to the appropriate region of mouse Chromosome 6, we first typed 21 animals with recombination events near *Lc*, including the six critical recombinants between *D6Rkf41* and *D6Rkf33/D6Rkf39* (Fig. 1; data not shown). All eight MIT markers are closely linked to *Lc* in these 21 recombinant animals and can be mapped either centromeric or telomeric to *Lc*. Three of the six

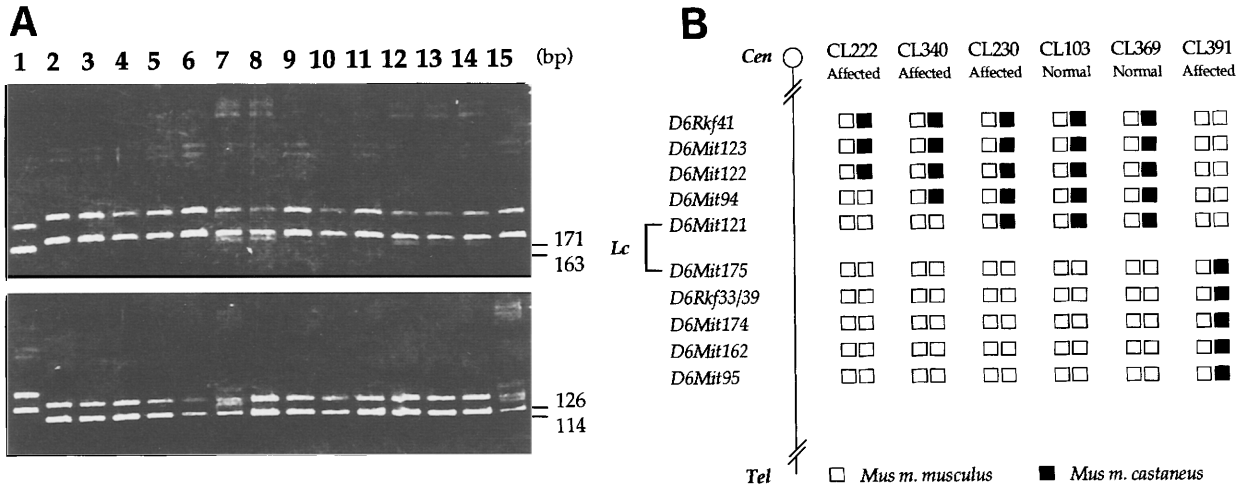

Figure 1 PCR analyses with two MIT markers on DNA samples from the *Lc/cast.* backcross and genotypes of critical recombinant animals in the *Lc* region on Chromosome 6. The *Lc/cast.* backcross was generated by crossing B6CBACa-A$^{w\text{-}J}$/A-*Lc* with *M. m. castaneus*, selecting F_1 progeny displaying the *Lc* phenotype and backcrossing them to B6CBACa-A$^{w\text{-}J}$/A mice. (*A*) Products derived from PCR amplification with two of the MIT markers used, *D6Mit94* (*top*) and *D6Mit95* (*bottom*), following polyacrylamide gel electrophoresis and staining with ethidium bromide. DNA samples (described below) used in each lane are as follows: (Lane *1*) CAST/Ei; (lane *2*) DBA/2J; (lane *3*) C3HeB/FeJ; (lane *4*) C57BL/6J; (lane *5*) B6CBACa-A$^{w\text{-}J}$/A; (lane *6*) *Lc* heterozygote (*Lc*/+); (lane *7*) *Lc/cast.* F1 heterozygote; (lane *8*) CL103; (lanes *9,10*) CL222, (lane *11*), a nonrecombinant sample from *Lc/cast.* backcross with only *M. m. musculus* background; (lane *12*) CL230; (lane *13*) CL340; (lane *14*) CL369; (lane *15*) CL391. Approximately 100 ng of genomic DNA was amplified in each lane. (*B*)Summary of the genotyping of six recombinant animals from the *Lc/cast.* backcross with three gene markers and eight MIT markers in the *Lc* region on Chromosome 6. The six recombinant animals from the *Lc/cast.* backcross are labeled CL222, CL340, CL230, CL103, CL369, and CL391, and their phenotypic status is indicated beneath their names in each column. Both nonrecombinant and recombinant chromosomes are illustrated for each animal. *M. m. musculus* includes one of four possible alleles: C57BL/6J, DBA/2J, C3HeB/FeJ, and CBA/CaGnLe, all of which may be present in the vicinity of the *Lc* locus. The order of markers telomeric to *Lc* cannot be determined in this typing. The markers used and polymorphisms detected are summarized in Table 1 and described in Methods. The relative order of *D6Mit123* and *D6Mit122* was determined later in the text. The location of the *Lc* mutation can be derived from the typing of these recombinant animals as follows: Provided that in this backcross only the recombinant chromosomes could carry the *Lc* mutation, and that their affected status is independently determined, we reasoned that CL222, CL340, and CL230 must have inherited the mutation in their *M. m. musculus* segments. Thus, *Lc* lies telomeric to *D6Mit121*. Similarly CL391 must have inherited the mutation in its *M. m. musculus* segment, placing *Lc* centromeric to *D6Mit175*. Conversely CL103 and CL369 must have inherited the wild-type gene in their *M. m. castaneus* segment, indicating that *Lc* is centromeric to *D6Mit175*.

important recombinants centromeric to *Lc* (CL222, CL230, and CL340) do resolve the four centromeric markers, *D6Mit123, D6Mit122, D6Mit94*, and *D6Mit121*, with marker *D6Mit121* being closest to *Lc*. However, the other three recombinants telomeric to *Lc* (CL103, CL369, and CL391) do not resolve the four telomeric markers *D6Mit175, D6Mit174, D6Mit95*, and *D6Mit162*.

To determine the closest marker telomeric to *Lc* and to confirm the order of the centromeric markers, we decided to use a generic backcross with a different strain background. In theory this backcross should provide additional recombination events within the *Lc* region as well as a different distribution of crossover events. We utilized the EUCIB because it is a backcross with a total number of 982 meioses between C57BL/6 and *M. spretus* (The European Backcross Collaborative Group 1994). For convenience, only 580 DNA samples from the London collection (a subset of the 982 DNA samples) were analyzed in this study. To minimize the total amount of DNA typing, we chose only a small set of DNA samples, ~184, that have known crossovers between the two closest Chromosome 6 landmarks, *D6Nds4* and *D6Nds5*. These markers are separated by a

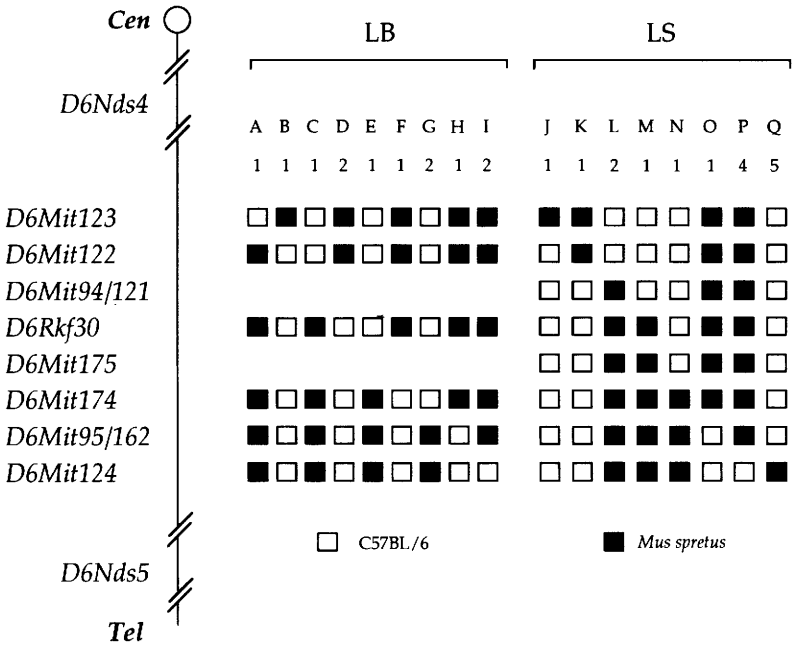

Figure 2 Genotyping of 28 DNA samples from EUCIB with nine MIT markers and a novel YAC end probe in the *Lc* region on Chromosome 6. (*LB*)Samples from the backcross with the C57BL/6 parental strain; (LS) the complementary backcross with the *M. spretus* parental strain. A total of 17 types of recombinant chromosomes (A–Q) from 28 samples are illustrated; the number beneath each letter (A–Q) indicates the number of individuals displaying that type of recombinant chromosome. (*A*) LB437; (*B*) LB596; (*C*) LB159; (*D*) LB346 and LB409; (*E*) LB158; (*F*) LB532; (*G*) LB205 and LB208; (*H*) LB206; (*I*) LB202 and LB420; (*J*) LS786; (*K*) LS869; (*L*) LS216 and LS769; (*M*) LS315; (*N*) LS702; (*O*) LS588; (*P*) LS181, LS191, LS377, and LS830; (*Q*) LS189, LS193, LS378, LS766, and LS872. The typing results of LB samples with *D6Mit94, D6Mit121,* and *D6Mit175* are ambiguous, because these markers failed to amplify the *M. spretus* alleles at these loci. Markers are summarized in Table 1 and described in the text; the novel marker *D6Rkf30* is described later in the text.

genetic distance of ~30 cM and flank the *Lc* locus (The European Backcross Collaborative Group 1994). Based on the known map of the MIT markers, we then typed these 184 samples with two MIT markers, *D6Mit123* and *D6Mit124*, that flank the *Lc* locus and are separated by a relatively large distance. The typing resulted in a total of 28 samples that have recombination events between *D6Mit123* and *D6Mit124*. We then typed these 28 samples with the other seven MIT markers in the *Lc* region to determine the relative order of the markers (Fig. 2). These results confirmed that *D6Mit121* is the closest marker to *Lc* on the centromeric side. More importantly, *D6Mit175* was found to be the closest marker to *Lc* on the telomeric side. *D6Mit121* and *D6Mit175* are separated by a total of five recombinants in 1084 meiosis (four out of 504 in the *Lc/cast.* backcross and one out of 580 in the EUCIB), indicating a relatively small genetic distance of 0.5 cM.

Construction of a YAC Contig Spanning the *Lc* Locus

Using the two closest markers that flank the *Lc* locus *D6Mit121* and *D6Mit175*, we screened a mouse YAC library to begin chromosomal walking. From the centromeric side of *Lc*, we obtained three YACs containing *D6Mit121* (6A12, 23E5 and 140F10), whereas from the telomeric side, three other YACs were obtained containing *D6Mit175* (171F8, 2H6, and 102E8). In addition, we identified four other YACs (37F2, 7G8, 55H7, and 24C8) at the *D6Rkf39* locus. We further characterized these 10 YACs by rescuing their end sequences, determining their insert sizes and analyzing their content of markers (Table 1).

A total of 15 YAC ends were isolated by a combination of the inverse PCR and homologous recombination methods (Hermanson et al. 1991; Silverman et al. 1991; Zuo et al. 1992). For each YAC end isolated, we first confirmed that it was from Chromosome 6 by using either a panel of somatic cell hybrid (SCH) cell lines (Fig. 3), or a panel of these 10 YACs isolated from this region and described above. By these analyses, we found that, among the 15 YAC ends isolated, 10 are from Chromosome 6 (Table 1) and two are from regions other than Chromosome 6 (J. Zuo, P. De Jager, and N. Heintz, unpubl.). In addition, two other YAC ends are repetitive, and one remains uncharacterized. All three, therefore, have not been assigned to Chromosome 6 (described later in Fig. 6).

To confirm their close linkage to the *Lc* locus, we utilized important polymorphic portions of these YAC end probes (*D6Rkf15*, *D6Rkf29*, and *D6Rkf30*) to type the 21 recombinant animals near the *Lc* locus, including the 6 important recombinants between *D6Rkf41* and *D6Rkf33/D6Rkf39* (Fig. 4 and Table 1). The YAC end probe,

Table 1. Summary of the genetic and physical mapping of the *Lc* region

					YAC Size (kb)	119G7 700	6A12 450	23 E5 250	140F10 200	157G1 700	188 E6 650	161F7 620	171F8 900	2H6 800	102 E8 500	37F2 1000	7G8 480	55H7 920	24C8 600
Marker	**Source**	**Bin**	**Polymorphism**	**Somat.**	**RI/D3(kb)**														
D6Rkf1	E5 end clone			chr.6	2/	x	x	x											
D6Rkf2	E5 subclone	I			1.3/4		x	x											
D6Rkf3	E5 subclone	I			4.6/5.5		x	x											
D6Mit121	MIT marker	II	SSLP				x	x	x										
D6Rkf4	E5 subclone	II			0.45/6		x	x	x										
D6Rkf5	E5 subclone	II			2.1/		x	x	x										
D6Rkf6	E5 subclone	III			2.8/5.8			x			x	x							
D6Rkf7	E6 subclone	III			2/			x			x	x							
D6Rkf8	E6 subclone	III			1.5/4.1			x			x	x							
D6Rkf9	E6 subclone	III			2.9/3.5			x			x	x							
D6Rkf10	E6 subclone	III			2.8/2.5			x			x	x							
D6Rkf11	E6 subclone	III			1.7/1			x			x	x							
D6Rkf12	E6 subclone	III			2.6/2.7			x			x	x							
D6Rkf13	E6 subclone	III			2.7/6.5			x			x	x							
D6Rkf14	IRS-PCR	III	SSLP		7/			x			x	x							
D6Rkf15	E5 end clone	IV	RFLP(Hae III)	chr.6	4.6/			x		x	x	x							
D6Rkf16	E6 subclone	V			1.6/2.9					x	x	x							
D6Rkf17	E6 subclone	V	SSCP		1.8/1.7					x	x	x							
D6Rkf18	G1 subclone	V			0.9/2.8					x	x	x							
D6Rkf19	G1 subclone	V			1.2/10					x	x	x							
D6Rkf20	G1 subclone	V			0.7/2.8					x	x	x							
D6Rkf21	G1 subclone	V			0.45/3					x	x	x							
D6Rkf22	G1 subclone	V			0.5/11					x	x	x							
D6Rkf23	G1 subclone	V			0.5/7.5					x	x	x							
D6Rkf24	G1 subclone	V	RFLP(Pst I)		1.4/11					x	*	x							
D6Rkf25	E6 subclone	VI	SSCP		1.2/2.6					x	x								
D6Rkf26	G1 subclone	VI			2.9/4					x	x								
D6Rkf27	G1 subclone	VII			0.8/7.5					x									
D6Rkf28	G1 subclone	VII			2.2/4.8					x									
D6Rkf29	H6 end clone	VIII	SSCP	chr.6	3.8/					x			x	x					
D6Rkf30	E8 end clone	IX	Novel	chr.6	8.8/					x			x	x	x				
D6Rkf31	G1 subclone	IX			1.4/11					x			x	x	x				
D6Rkf32	IRS-PCR	IX								x			x	x	x				
D6Mit175	MIT marker		SSLP										x	x	x				
D6Rkf33	Gene marker		RFLP(Rsa I)	chr.6									x	x	x				
D6Mit174	MIT marker		SSLP										x	x	x				
D6Rkf34	F2 end clone			chr.6	4/								x	x		x			
D6Mit162	MIT marker		SSLP											x		x			
D6Rkf35	G8 end clone				5/									*		x	x		
D6Rkf36	H7 end clone													*		x	x	x	
D6Rkf37	H6 end clone				1.1/									x		x	x	x	
D6Rkf38	C8 end clone				4/											x	x	x	x
D6Rkf39	Gene marker		RFLP(Rsa I)	chr.6												x	x	x	x
D6Rkf40	F2 end clone			chr.6	2.8/											x		x	x

All markers used in this study are listed in the first column. The second column indicates their source. In the third column, markers that hybridize to the same set of overlapping YACs are grouped together to form nine bins, I to IX. The fourth column summarizes polymorphisms that have been tested on the *Lc/cast.* backcross (see Methods). The fifth column indicates the markers that have been mapped to Chromosome 6 by somatic cell hybrid mapping analysis. The sixth column indicates the size (in kb) of *Eco*RI (RI) and *Hin*dIII (D3) genomic fragments to which the markers hybridize. The top rows indicate the names and the insert sizes of the 14 YACs used in this study. Their probe content is as follows: (x) Positive signals by both Southern blot hybridization and PCR analysis; (*) failure to detect positive signal by Southern blot hybridization or PCR amplification, possibly because of a rearrangement within the YAC.

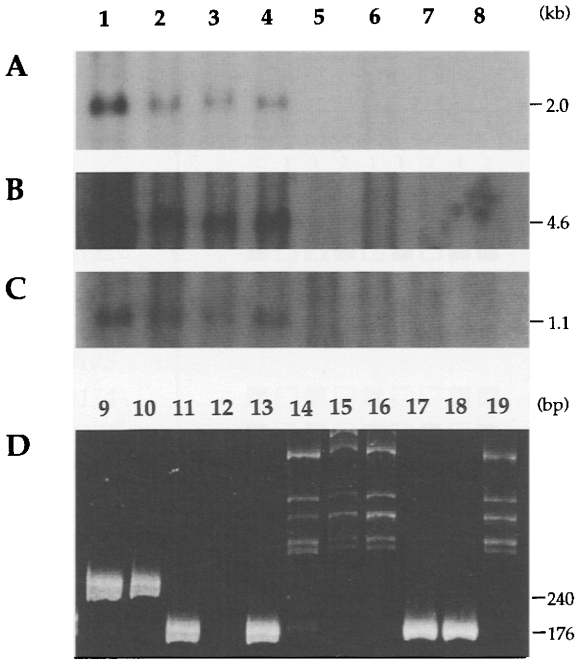

Figure 3 Examples of the localization of YAC end probes on mouse Chromosome 6 using a somatic cell hybrid mapping panel. (*A–C*) Autoradiographs of Southern blots made from SCH lines. The probes used in *A–C* YAC end probes, *D6Rkf1*, *D6Rkf15*, and *D6Rkf37*, respectively. Approximately 5–10 μg of genomic DNA was digested with *Eco*RI and loaded into each lane in *A–C*. (*D*) PCR amplification from ~100 ng of genomic DNA with primers derived from YAC end probe *D6Rkf30*. The DNA samples are as follows: (Lane *1*) *Lc*/+heterozygote; (lanes *2,13*) 2A2B1; (lane *3,17*) 2A2H3; (lanes *4,18*) BEM1-4; (lanes *5,16*) E36; (lanes *6,15*) 2A2C2; (lanes *7,19*) ECM4C; (lanes *8,14*) R2-24; (lane *11*) C57BL/6J; (lane *10*) CAST/Ei; (lane *9*) SPRET/Ei; (lane *12*) control without DNA. Positive signals in hybrids 2A2B1, 2A2H3, and BEM1–4 (lanes *2,3,4,13,17,18*) as well as in genomic DNA (lanes *1,9,10,11*) in the absence of signal in any other hybrid lines indicate a Chromosome 6 location.

D6Rkf15, is one recombination event centromeric to *Lc* and nonrecombinant with *D6Mit121*; the other two YAC ends, *D6Rkf29* and *D6Rkf30*, are three recombination events telomeric to *Lc* and nonrecombinant with *D6Mit175*. Although these polymorphic YAC end probes have not crossed any of the four crucial recombination breakpoints, they do provide closer flanking markers which refine the *Lc* locus.

Using two of these YAC end probes, *D6Rkf15* and *D6Rkf30*, we screened the YAC library again and identified a common YAC, 157G1, containing both probes. In addition, two other YACs, 188E6 and 161F7, were identified from *D6Rkf15*, and another YAC, 119G7, was isolated from *D6Rkf1*. We therefore have identified a total of 14 YACs that span a large genomic interval, from *D6Rkf1* to *D6Rkf40*, in the vicinity of the *Lc* mutation (Table 1). The *Lc* locus, as defined by the two flanking markers *D6Rkf15* and *D6Rkf30* is spanned by five YACs with threefold redundancy in most regions. A single YAC with a 700-kb insert, 157G1, spans the entire *Lc* candidate region between markers *D6Rkf15* and *D6Rkf30*.

Subcloning YACs to Obtain High Density Markers from the *Lc* Region

To refine the genetic and physical maps of the *Lc* locus, we chose to subclone our YAC DNA into a smaller and more convenient cloning system to obtain more markers. Sublibraries were made from YACs 157G1, 188E6, and 23E5 (see Methods), and a total of 26 plasmid clones were mapped to the YAC contig spanning the *Lc* locus. These subcloned probes, in addition to YAC end probes, gene markers, and MIT markers, can be further categorized into groups or "bins" (Zuo et al. 1993). Each bin consists of markers that hybridize to the same set of YACs (Table 1). An example of such a hybridization with four probes is shown in Figure 5. In this analysis, we also found that nine out of 19 subcloned probes from YAC 188E6 are not from Chromosome 6, suggesting that this YAC is chimeric. Similarly, as all 11 subcloned probes isolated from YAC 157G1 (bin IV–IX in Table 1) are mapped to Chromosome 6, we assumed that the YAC 157G1 is a nonchimeric YAC.

Some of these subcloned probes were developed into polymorphic markers to refine the genetic map of the *Lc* locus. *D6Rkf17* and *D6Rkf24* in bin V, and *D6Rkf25* in bin VI, were such polymorphic markers and were utilized in genetic typing of the 21 recombinant animals in the *Lc* region as described above (Fig. 4). In addition, interspersed repetitive sequence PCR was used to isolate additional internal probes from these YACs, such as the polymorphic marker *D6Rkf14* (Fig. 4; Table 1). Combining the genetic mapping and YAC hybridization binning data, the *Lc* locus was refined and now consists of bins VI and VII between *D6Rkf25* and *D6Rkf29*, the two closest flanking markers (Fig. 6).

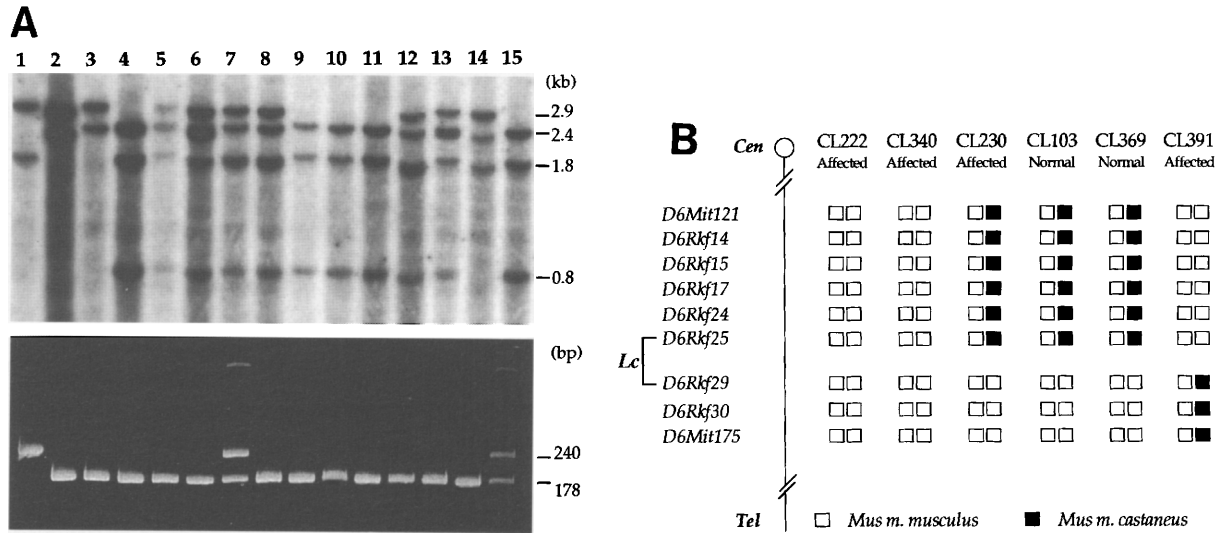

Figure 4 Genotyping of the six recombinant animals from the *Lc/cast.* backcross with markers within a refined *Lc* region on Chromosome 6. DNA samples used in *A* are the same as described in Fig. 1A. (*A*) (*top*) Autoradiograph of a genomic Southern blot hybridized with the YAC end probe *D6Rkf15*. Approximately 5–10 μg of genomic DNA was digested with *Hae*III and loaded into each lane. *D6Rkf15* detects three different alleles: a *M. m. castaneus* allele of 2.9-kb and 1.8-kb fragments, a C57BL/6J allele of 2.4-, 1.8- and 0.8-kb fragments, and a common allele between DBA/2J, C3HeB/FeJ, and CBA/CaGnLe of 2.9- and 2.4-kb fragments. (*Bottom*) The products of PCR amplification with the primer pair derived from the polymorphic YAC end marker *D6Rkf30*. Approximately 100 ng of genomic DNA was used for amplification in each lane. (*B*) Summary of the genotyping of the six recombinant animals in the *Lc/cast.* backcross with polymorphic markers that map between *D6Mit121* and *D6Mit175*. These markers include YAC end probes and YAC subclones and their order was determined as described later in the text. The six recombinant animals and their other features are as described in Fig. 1B. None of the polymorphic markers from the *Lc* region, except *D6Rkf15*, were able to distinguish definitively among the four inbred strain alleles, DBA/2J, C3HeB/FeJ, CBA/CaGnLe, and C57BL/6J.

DISCUSSION

As an important step towards the isolation of the *Lc* gene, we report here the construction of a high resolution genetic map and a YAC contig with a high density of markers in the *Lc* locus.

The YAC contig reported here consists of 14 YACs with an average size of 630 kb and a threefold redundancy. The most centromeric and telomeric markers of this contig, *D6Rkf1* and *D6Rkf40*, have been confirmed to be from Chromosome 6 by SCH mapping analysis. Evenly distributed within this contig, a large number of markers have been extensively characterized by a combination of SCH mapping analysis, genetic linkage analysis, and YAC content mapping. This set of independent analyses are consistent with each other and therefore confirm the continuity of the YAC contig. Although YAC 157G1 has a small internal segment (bin VII) not contained in any other redundant YAC clones, it is nevertheless likely to be colinear with genomic DNA; *D6Rkf15* and *D6Rkf30*, which both map to this 700-kb YAC, also share one 600-kb *Sal*I fragment and another 1-Mb partial *Sal*I fragment in genomic pulsed-field gel electrolphoresis (PFGE) analysis (J. Zuo, P. De Jager, and N. Heintz, unpubl.).

Based on the sizes of the nonchimeric YACs, their overlapping pattern, and the physical distances between markers, we estimate that the distance between *D6Rkf1* and *D6Rkf40* is ~2.6 Mb. With additional YACs isolated from *D6Rkf1* and *D6Rkf40*, our YAC contig spans ⩾3 Mb of genomic DNA. Assuming a random distribution of the subcloned probes over a YAC, we were able to refine the physical distances between these markers. The Chromosome 6 portion (bin III–VI in Table 1) of 650-kb YAC 188E6, for example, is estimated to be ~310 kb as 10 of 19 subcloned probes are mapped within bin III–bin VI of this contig. Similarly, among 11 157G1 subclones that are mapped to this contig, three are within bins VI and VII. Based on the 700-kb size of the 157G1 YAC, the estimated size of bins VI and VII

is ~300 kb. Therefore, the minimal candidate region of the *Lc* locus is further defined by the two closest flanking markers, *D6Rkf25* and *D6Rkf29*, which are contained within a genomic segment of ~300 kb. This refined physical map will greatly facilitate the establishment of a P1 or BAC contig of the *Lc* region and the analysis of candidate genes isolated from this relatively small genomic region.

In the *Lc* phenotypic backcross between B6CBACa-$A^{w\text{-}J}$/A-*Lc* and *M. m. castaneus*, there are a total of four recombination events out of 504 meioses in the *Lc* region smaller than 600 kb. In the EUCIB backcross between *M. spretus* and C57BL/6, however, the crossovers seem to be distributed evenly in this region of the chromosome. Statisitical analyses suggest that these two recombination frequencies in the *Lc* region (4/504 and 1/580) are not significantly different ($P > 0.25$, df = 1) from the mean value (5/1084) (Reeves et al 1990). In addition, we have not found any differences between wild-type and *Lc*/+genomic DNA in the long range restriction maps of the region (J. Zuo et al., unpubl.). Therefore, the *Lc* mutation is unlikely to involve any gross genomic rearrangements.

Many approaches have been described for the recovery of polymorphic probes from YACs (Chen et al. 1995). The strategy we used here is simple and efficient. It consists of subcloning YAC DNA into the λ-Zap phagemid followed by direct PCR amplification of recombinant subcloned fragments. These PCR products can be directly sequenced and further developed into sequence-tagged sites (STSs). Furthermore, most of these STSs can be easily used as polymorphic markers by single-strand confirmation polymorphism (SSCP). Finally, these clones can be directly used as hybridization probes, to identify conserved sequences by "zooblot", or to screen P1 or BAC libraries.

Given the progress now evident in the mouse genome project, it seems reasonable that the approach we have taken to establishing genetic and physical maps of mutant loci can be broadly applied. The construction of a high resolution genetic map of a desired locus can be established using the available MIT polymorphic markers on a generic backcross such as EUCIB. Simultaneously, one or two phenotypic crosses specific for the desired mutant can be typed with these same MIT markers. The use of one or two subspecific strains different from those involved in the generic cross will ensure the even distribution of informative crossovers. In addition to a comparative map of the region of interest, these phenotypic crosses will order the markers that flank the mutant locus. The two closest flanking polymorphic markers can then be used to type pups before they are weaned, so that only those pups with informative crossovers will be kept for later phenotypic studies. This measure can significantly decrease the cost of maintenance of large numbers of offspring. It is after this step that YACs should be isolated from the two closest flanking markers, and a YAC contig can then be constructed with minimal effort. With the availability of MIT markers and EUCIB DNA samples, the initial genetic analysis necessary for physical mapping can be made significantly more efficient.

METHODS

DNA Markers and Sequences

Two known gene markers, Cbl-1 (*D6Rkf41*) and Etl-1 (*D6Rkf33*), were described previously (Norman et al. 1991, 1995). A third gene marker, Jrl (*D6Rkf39*), is an 880-bp genomic fragment isolated from pJrl-1, kindly provided by Dr. J. R. Hansbrough (Washington University, St. Louis, MO). MIT markers or Map-pairs were purchased through Research Genetics, Inc. Novel PCR primers and markers used in this study are listed in Table 1 and 2.

Southern Blot Hybridization

All probes were labeled by random priming. Hybridization was performed using standard procedures at 65°C in a Hybridization Incubator (Robbins Scientific model 400) and filters were then washed at 65°C in 0.2 × SSC and 0.1% SDS for 2 × 30 min. Finally, XAR Kodak films were exposed to the filters at –70°C for 24–72 hr.

Sequencing and Homology Searches

DNA sequencing was performed on either ABI 370A or 373A automated sequencers (Applied Biosystems, Inc.) using both PCR products and plasmids as templates, or by manual procedures using plasmids as templates. Sequence homology searches were performed using the National Center for Biotechnology Information (NCBI) data base.

PCR Amplification

DNA Thermal Cycler, GeneAmp PCR System 9600 (Perkin-Elmer Cetus) and DNA Engine (M.J. Research, Inc.) were used for PCR amplification in this study. Four different buffer conditions were used: TNK25, TNK50, TNK100 (Zuo et al. 1992) and 10 × PCR buffer (Perkin-Elmer Cetus). All PCR reactions were carried out in 25-μl volume. Samples were processed through an initial denaturation (94°C for 4

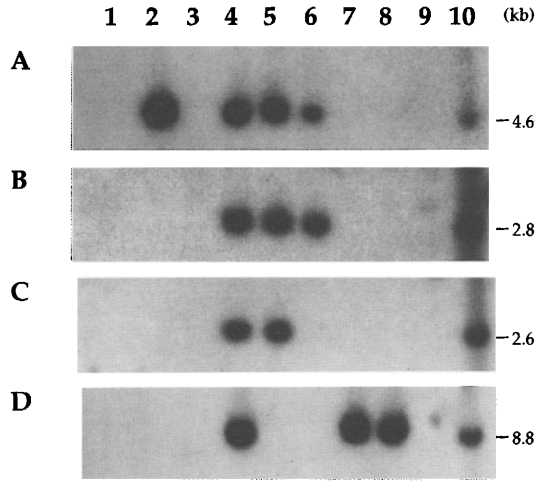

Figure 5 Overlap of YAC clones by Southern blot hybridization analyses with four DNA markers in the refined *Lc* region between *D6Mit121* and *D6Mit175* on Chromosome 6. A Southern blot of *Eco*RI-digested DNA from nine YACs in the *Lc* region, as well as mouse genomic DNA, was sequentially hybridized with two YAC end probes, *D6Rkf15* (*A*) and *D6Rkf30* (*D*). Another Southern blot of the same DNA samples digested with *Hin*dIII was sequentially hybridized with two subcloned probes, *D6Rkf18* (*B*) and *D6Rkf25* (*C*). (Lane *1*) YAC 6A12; (lane *2*) YAC 23E5; (lane *3*) YAC 140F10; (lane *4*) YAC 157G1; (lane *5*) YAC 188E6; (lane *6*) YAC 161F7; (lane *7*) YAC 171F8; (lane *8*) YAC 2H6; (lane *9*) ADH-G1, a YAC isolated from *D6Rkf41*; (lane *10*) B6CBACa-A^{w-J}/A genomic DNA. YAC agarose blocks with ~1×10^7 cells/block, and genomic agarose blocks with ~1×10^6 cells/block were digested and analyzed in each lane.

min), then 35 cycles of denaturation (94°C, 1 min), annealing (1 min), and elongation (72°C, 3 min), followed by 10 min elongation at 72°C and stored at 4°C. The annealing temperature for each PCR primer pair is listed in Table 2.

YAC Isolation and Characterization

The YAC library purchased from Research Genetics, Inc. was screened by PCR using standard procedures. Positive clones were purified; both agarose blocks (5×10^7 cells/block) and liquid DNA were made as described previously (Zuo et al. 1992). The sizes of YAC inserts were determined by PFGE as described below. YAC agarose blocks were digested according to the protocol from New England Biolabs and analyzed by regular gel electrophoresis, or PFGE in a Bio-Rad CHEF DRII apparatus in 0.5 × TBE at 14°C at 6V/cm for 24–36 hr under conditions to fractionate DNA from 50 to 1500 kb. Gels were then acid-nicked and transfered to GeneScreenPlus or Hybond-N^+ membranes (Amersham). Both the inverse PCR and homologous recombination methods were used to rescue the end sequences of YACs; procedures and sequences have been described elsewhere (Hermanson et al. 1991; Silverman et al. 1991; Zuo et al. 1992).

Mice

Mice used in this study were purchased from the Jackson Laboratory and maintained at the Specific Pathogen Free

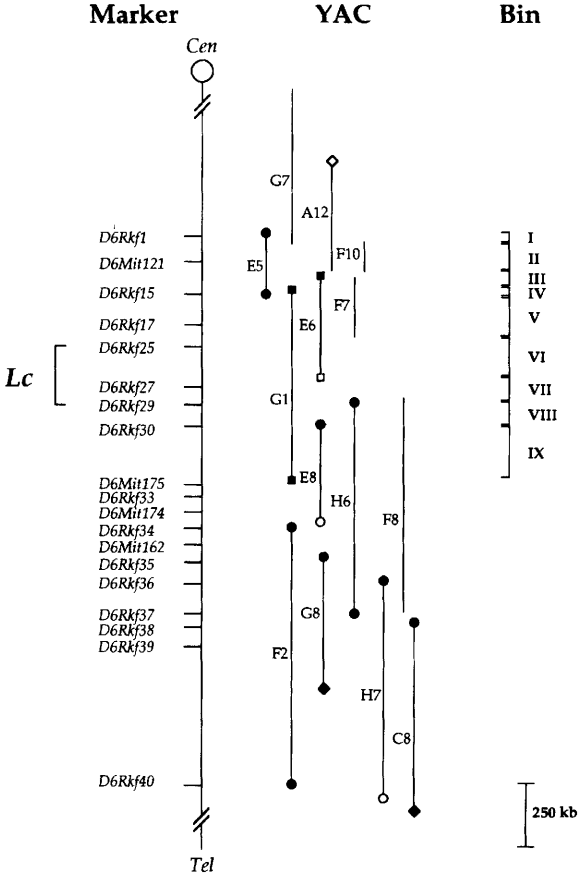

Figure 6 A composite map of the *Lc* region on Chromosome 6. The markers presented here are described in Table 1. One marker from each bin is included in this diagram. The 14 YACs are labeled with their abbreviated names. (●) Nonchimeric Chromosome 6 YAC ends; (○) chimeric YAC ends; (■) assumed to be nonchimeric Chromosome 6 YAC ends; (□) assumed to be chimeric YAC ends; (♦) repetitive YAC ends; (◇) YAC ends that have not been characterized. Based on the meiotic recombination events, the sizes of nonchimeric YACs, their overlapping pattern, estimation of the sizes of bins, and physical distances between markers, these 14 YACs can be assembled into a contig and the order of markers can be determined. The contig starts from *D6Rkf1* and ends at *D6Rkf40*, covering ~3 Mb of genomic DNA that contains the *Lc* locus. The *Lc* mutation is mapped between the two closest polymorphic markers, *D6Rkf25* and *D6Rkf29*; this region is estimated to be 300 kb in size (see Discussion).

Table 2. Sequences of primers used in this study and their PCR conditions

Marker	Primer pair	Anneal. temp (°C)	Product size (bp)
D6Rkf1	1: 5'-ATC TGC CTT TAC ACA TTG CC-3'	55	245
	2: 5'-TTC TCT AGT GAT GCA AAT GAA TG-3'		
D6Rkf14	L: 5'-ACC TCA AAC TAG ACC CTG AAG-3'	58	231
	R: 5'-TAA AGA AAA TGT ATT GAT CC-3'		
D6Rkf15	1: 5'-GCT CAT GCA TGG CAA AGA AG-3'	55	167
	2: 5'-CAA CTA TTG AGA ATC TCT GTT AG-3'		
D6Rkf17	B: 5'-TTC ATT GAT GAG TTG AAG CCC-3'	58	284
	C: 5'-AGG AGA TGA GTG AGC AGG ACA-3'		
D6Rkf24	B: 5'-CTG AGC AGC AAT CAC GTT GT-3'	58	309
	C: 5'-TCA TGC TGA CCC AGA CTC TG-3'		
D6Rkf25	A: 5'-GAA GGC ACA CAT TCC TAT CTC C-3'	58	250
	B: 5'-GAT AGA CTG GGA GAT GAG TGG-3'		
D6Rkf29	3: 5'-TCG GTG TGA TTA GCA TTC ATG-3'	58	157
	4: 5'-CTG TGG AGG TTG GAC ACT AGC-3'		
D6Rkf30	1: 5'-GTT CCC TAA GCC CTA GGT GG-3'	58	178
	2: 5'-TAG GCC ATA GTC TTT GGG GA-3'		
D6Rkf33	1: 5'-TGC CAG GAA TTT GCA GGT-3'	55	260
	3: 5'-CAA CAC CAC AAC CAA TGA GC-3'		
D6Rkf34	1: 5'-GAC TAA CAG TAA CGT TTA AAG TG-3'	58	220
	2: 5'-GTA TTA GTA TGT ATT TTT GAC-3'		
D6Rkf39	1: 5'-CTA CCA CTT CTG TTG ATC TGT TG-3'	55	310
	2: 5'-AAG TAA ATA GAG CCT TAG AAA TGG-3'		

facility at the Rockefeller University Laboratory Animal Research Center under standard procedures.

Mouse Genomic DNA Agarose Block and Liquid DNA Preparation

Mouse spleens were homogenized and embedded in low melting agarose (5×10^6 cells/block). These genomic agarose blocks were then treated with Proteinase K and *N*-lauroylsarcosine (Zuo et al. 1992), digested with different restriction enzymes according to the manufacturer's instructions (New England Biolabs), and then analyzed by regular gel electrophoresis or PFGE as described above. Liquid genomic DNA was prepared from homogenized spleens, kidneys, or tails (Lovell-Badge 1987). Genomic DNA samples of EUCIB were obtained from Human Genome Mapping Project (HGMP) Resource Center (UK).

Detection of Polymorphisms

For simple sequence length polymorphisms (SSLPs), MIT map-pairs were purchased from Research Genetics, Inc. and used for PCR amplification under the recommended conditions. For the novel polymorphism at the *D6Rkf30* locus, the same procedures were used by PCR as the MIT map-pairs. Amplified products were separated in a 15 × 20cm 10% acrylamide gel (30:1 acrylamide to bisacrylamide) in 0.25 × TBE at 200 V for 2.5 hr. For restriction fragment length polymorphisms (RFLPs), 5–10 μg of genomic DNA from different parental mouse strains was digested with a variety of enzymes. Southern blots of the digested DNAs were hybridized with labeled probes. Any RFLPs observed were further used to genotype backcross DNA samples. SSCP analysis was used to genotype backcross DNA samples following a protocol described by Vidal-Puig and Moller (1994) using large acrylamide gels with glycerol. The polymorphism for each probe used in this study is listed in Table 1.

Making YAC Sublibraries

YAC DNA was excised from low melting agarose gel after separation from endogenous yeast chromosomes by PFGE. Purified YAC DNA (~500ng) was digested with restriction enzyme *Eco*RI in the low melting agarose, purified by glass

wool centrifugation or β-agarase I treatment followed by ethanol precipitation, and finally ligated into 1 μg of predigested λ Zap II/*Eco*RI/CIAP cloning vector (Stratagene). The ligation mix was packaged (Gigapack Gold; Stratagene) and analyzed under conditions recommended by the manufacturer. A large number of white plaques or recombinant clones were picked into 96-well plates and directly PCR amplified by T3 and T7 vector primers. Each product was analyzed further for development of new STSs and to determine which YACs they map to.

Interspersed Repetitive Sequence PCR (IRS-PCR)

YACs were amplified using TNK 50 buffer (described above) and 0.5 μM of a B1 repeat-specific primer, B1.1 (Simmler et al. 1991). Samples were processed through an initial denaturation (94°C, 4 min), then 35 cycles of denaturation (94°C, 1 min), annealing (45°C, 2 min), and elongation (72°C, 3 min), followed by 10 min elongation at 72°C and storage at 4°C. PCR reactions were analyzed on 1.6% regular agarose gels in 1 × TAE buffer, and individual bands were excised. PCR products were cloned into the pCR II vector (Invitrogen) following isolation from the gel block by passage through glass wool during centrifugation at 12,000 rpm. Partial sequencing of the clones yielded one dinucleotide (CA) stretch; marker *D6Rkf14* was developed by making primers flanking this repeat (Table 2).

Somatic Cell Hybrid Mapping Panel

SCH cell lines have been described in detail elsewhere (Bahary et al. 1992). Briefly, macrophages from A/He mice or L cells from a C3H background were fused with the Chinese hamster cell line E36. Six such hybrid lines, 2A2B1, 2A2C2, 2A2H3, BEM1-4, ECM4C, and R2-24, were obtained and analyzed further for their mouse chromosomal content by karyotypic analysis. Chromosome 6 is present only in lines 2A2B1, 2A2H3, and BEM1-4 but not in lines 2A2C2, BEM1-4, and R2-24. These cell lines were kindly provided by Dr. Jeffrey Friedman (Rockefeller University, New York, NY).

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