LETTER

An ~1.2-Mb Bacterial Artificial Chromosome Contig Refines the Genetic and Physical Maps of the Lurcher Locus on Mouse Chromosome 6

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Lurcher (*Lc*) is a semidominant mouse mutant that displays a characteristic ataxia in the heterozygous state beginning in the third postnatal week. This symptom results from a neurodegenerative event in the cerebellum: There is a catastrophic loss of Purkinje cells in the heterozygote animal between postnatal days 10 and 15. In an effort to identify the genetic lesion borne by *Lc* mice, we initiated a cloning project based on the position of the *Lc* mutation on mouse chromosome 6. We have extended our previous analysis of the genomic segment containing the *Lc* locus by isolating a set of stable and manipulable genomic clones called bacterial artificial chromosomes (BACs) that cover this region of mouse chromosome 6. These clones provided a good substrate for the isolation of markers that were used to refine the physical map of the locus. Furthermore, 20 of these markers were mapped onto our (B6CBACa-A^{w-J}/A − *Lc* × CAST/Ei)F_1 × B6CBACa-A^{w-J}/A backcross, refining the genetic map and identifying two nonrecombinant markers (*D6Rck354* and *D6Rck355*). These two markers, in conjunction with the closest flanking markers, were used to identify a 110-kb genomic segment that contains all four markers and hence contains the *Lc* locus. This small genomic segment, covered by multiple BACs, sets the stage for the final effort of this project—the identification of transcripts and of the mutation within the *Lc* locus.

[The *Lt1* sequence has been submitted to GenBank as two ESTs; the accession numbers are U89356 and U89357.]

Lurcher (*Lc*) is a semidominant mutation that results in ataxia in the heterozygous state and in death within the first 12 hr of life in the homozygous state (Cheng and Heintz 1997). The heterozygous animal's gross phenotype follows the catastrophic loss of cerebellar Purkinje cells that is observed between postnatal days 10 and 15 (P10 and P15). During this period, ~50% of Purkinje cells degenerate in the *Lc* heterozygote, and by P25, >90% of *Lc*/+ Purkinje have disappeared (Caddy and Biscoe 1979). This degenerative event was studied further and shown to display characteristics that are associated with an apoptotic form of cell death (Norman et al. 1995).

The second postnatal week (P7–P14) is a time of extensive differentiation in the cerebellum; Purkinje cells begin to extend their dendritic arbor and to obtain other morphological features that reflect the terminal phase of differentiation (Dumesnil-Bousez and Sotelo 1992). It is also a time during which there are extensive contacts between the Purkinje cell's dendritic arbor and the granule cell's parallel fibers, facilitating synaptogenesis (Dumesnil-Bousez and Sotelo 1992). Histological and ultrastructural studies of the cerebellum of *Lc* heterozygotes report that their Purkinje cells initiate their terminal differentiation; however, beginning with swollen mitochondria at P8, *Lc* Purkinje cells soon display dysmorphic features such as thickened stem dendrites, aberrant spine distribution on dendrites, and persistent somatic innervation by climbing fibers (Caddy and Biscoe 1979; Dumesnil-Bousez and Sotelo 1992; Rabacchi et al. 1992).

A majority of cerebellar granule cells (90%) and inferior olivary neurons (75%) also degenerate in the *Lc* heterozygote (Caddy and Biscoe 1979), but several experiments analyzing *Lc* ↔ wt (wild type) chimeras demonstrated that the *Lc* mutation has a cell-autonomous mode of action in the Purkinje cells of heterozygous animals (Wetts and Herrup 1982a,b,c, 1983). This finding is important because it yields a key criterion that a candidate gene must meet: It must be expressed within the Purkinje cells of the *Lc* heterozygote at the time these cells die (P10–P15).

The *Lc* mutation was initially described by Phil-

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7:736–746 ©1997 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/97 $5.00

ips (1960) and mapped to linkage group XI, which is now known as mouse chromosome 6. Based on this information, we decided to initiate a positional cloning effort to isolate the gene affected by this mutation. A 504 animal backcross between B6CBACa-*A*$^{w-J}$/*A* − *Lc* and *Mus m. castaneus* (CAST/Ei) (*Lc/cast.* cross) was carried out, and an initial mapping effort led to a high-resolution genetic map of the genomic region containing the *Lc* locus and the construction of a ~3-Mb yeast artificial chromosome (YAC) contig over this region (Norman et al. 1991; Zuo et al. 1995). We decided to use this initial physical map to construct another contig using bacterial artificial chromosome (BAC) clones.

Establishing a BAC contig that covers the *Lc* locus contributes both more information about this segment of mouse chromosome 6 and better tools with which to characterize the locus. The YAC contig assembled earlier (Zuo et al. 1995) provided a physical map of the *Lc* locus and enabled us to refine the locus to a ~300-kb segment flanked by markers *D6Rck326* and *D6Rck329*. However, a substantial portion of this refined locus did not have redundant YAC coverage; it was covered by only one YAC, 157G1. This region of the contig was described as bin VII in Zuo et al. (1995). The lack of redundancy in this region means that a rearrangement could remain undetected.

The BAC contig described here stretches over the nonredundant portion of the YAC contig, yielding a multiply redundant physical map, and reveals a deletion in YAC 157G1. Furthermore, these BACs provide both a good substrate for the construction of a transcript map of this locus and a source of markers that allow further refinement of the genetic map of the *Lc* locus.

RESULTS

Establishing a BAC Contig

Two complementary approaches were used to assemble a BAC contig: chromosome walking and YAC shotgun cloning. The chromosome walk was initiated from *D6Rck329*, the closest flanking telomeric marker (Zuo et al. 1995). Five steps were accomplished, yielding a contig of 15 BAC clones, which stretches to *D6Rck364*. On the other hand, three markers (*D6Rck324*, *D6Rck325*, and *D6Rck327*) derived from the shotgun subcloning of YACs covering the locus (Zuo et al. 1995) were used to pull out 10 BACs centromeric to *Lc*.

In each approach, potential markers, whether end clones or subclones of YACs or BACs, were evaluated before being used in contig construction. The criterion to be met is hybridization to the YAC clones covering the locus. To guard against chimerism in YAC clones, a subset of markers was also hybridized to a panel of somatic cell hybrid lines (SCH); the four centromeric markers and the four telomeric markers used in these experiments all displayed a mouse chromosome 6 pattern upon hybridization to the SCH panel (Table 1). This ensured that our cloning effort resulted in a contig of clones that covers a segment of mouse chromosome 6. A short description of each marker isolated during the construction of the contig is presented in Table 1.

Substantial contigs on each flank of the *Lc* locus were isolated using the two different methods (Fig. 1). However, the two contigs did not join at the middle; *D6Rck345* on the centromeric flank and *D6Rck364* on the telomeric flank did not share a common BAC. In addition, *D6Rck364* and *D6Rck365* failed to hybridize to YAC 157G1, revealing a deletion in this YAC. This deletion occurs in bin VII, the region of the YAC contig that was covered only by YAC 157G1 (Zuo et al. 1995).

Completing the Physical Map

The deletion in bin VII prompted us to achieve complete threefold redundancy in the YAC contig over the genomic segment containing the *Lc* locus. Four additional YACs—99B10 197C12, 87E4, and 54H1—were isolated using markers identified during the construction of the BAC contig (*D6Rck344* and *D6Rck365*). These YACs provide at least threefold redundancy in YAC coverage over most of bin VII (Fig. 1); only a small portion of bin VII retains single coverage.

The shotgun subcloning approach was applied again to YACs B10, C12, and E4, and the resulting markers identified five additional overlapping BACs—251E15, 143E21, 226J6, 270L3, and 222L15. Further characterization of these BACs led to the isolation of 124L24 and 344O6, which complete the contig (Fig. 1). The full BAC contig stretches between markers *D6Rck342* and *D6Rck329* and contains the previously described flanking markers *D6Rck325* and *D6Rck329*, which define the genomic segment that contains the *Lc* locus (Zuo et al 1995).

Genetic Mapping of New Markers Within the *Lc* Locus

Using the *Lc/cast.* cross, many of the markers described in Table 1 were also genetically mapped by

Table 1. Summary of the Physical Mapping of the ***Lc*** Locus

					YACs	161F7	188E6.	157G1	197C12	54H1	87E4.	94B10	2H6	162E8.	BACs	22D24	188O8	246H12	185L7	85D8	211C9	13K1	248D20	212A4	57B22	344O6	222L15	251E15.	270L3	226J6	143E21.	124L24	274B6	309C13	182F11	237G19	104A23	540I18	86E20.	403H11	313P7	340 E1.	102I9	339D16	438D17	300G20	513J1
					Size(kb)	620	650	700	800	800	680	630	800	500	Size(kb)	255	100	115	140	95	55	170	155	135	160	110	275	130	65	50	170	100	105	165	112	145	100	100	110	90	120	170	100	130	65	90	280
Marker	Source	Bin	Poly.	SCH																																										
D6Rck342	H12 endclone					•	•	•								•	•	•																												
D6Rck324	G1 subclone	V				•		•	•	•								•	•																											
D6Rck343	L7 endclone	VI					•	•	•	•										•	•	•																								
D6Rck325	E6 subclone	VI	SSCP				•	•	•	•										•	•	•																								
D6Rck327	G1 subclone	VIIa						•	•	•	•												•	•																						
D6Rck344	D20 endclone	VIIa	SSCP					•	•	•	•												•	•																						
D6Rck345	B22 subclone	VIIa	SSCP					•	•	•	•													•	•																					
D6Rck346	C12 subclone	VIIb	SSCP						•	•	•													•	•																					
D6Rck347	C12 subclone	VIIb	SSCP						•	•	•														•																					
D6Rck348	E4 subclone	VIIb	RFLP	Chr.6					•	•	•														•	•																				
D6Rck349	C12 subclone	VIIb	RFLP	Chr.6					•	•	•														•	•																				
D6Rck350	E4 subclone	VIIb	RFLP	Chr.6					•	•	•														•	•																				
D6Rck351	C12 subclone	VIIb	SSCP						•	•	•															•																				
D6Rck352	C12 subclone	VIIb		Chr.6					•	•	•															•	•			•																
D6Rck353	C12 subclone	VIIb	RFLP						•	•	•															•	•			•																
D6Rck354	L3 endclone	VIIc	SSCP						•	•	•	•														•	•	•		•																
D6Rck355	J6 endclone	VIIc	SSCP						•	•	•	•														•	•	•	•	•																
D6Rck356	C12 subclone	VIIc							•	•	•	•														•	•	•	•	•																
D6Rck357	L15 endclone	VIIc	SSCP						•	•	•	•														•	•	•	•	•																
D6Rck358	E15 endclone	VIIc	SSCP						•	•	•	•															•	•	•	•																
D6Rck359	J6 endclone	VIIc	SSCP						•	•	•	•															•	•	•																
D6Rck360	L3 endclone	VIIc	SSCP						•	•	•	•															•	•																		
D6Rck361	C12 subclone	VIIc	SSLP						•	•	•	•																•	•																	
D6Rck362	C12 subclone	VIIc	SSCP	Chr.6					•	•	•	•																•	•																	
D6Rck363	C12 subclone	VIIc		Chr.6					•	•	•	•																•	•																	
D6Rck364	F11 subclone	VIIc	SSCP						•	•	•	•																•	•																	
D6Rck365	I18 endclone	VIIc	SSCP	Chr.6					•	•	•	•																•	•																	
D6Rck366	G1 subclone	VIId						•	•	•	•																	•	•																	
D6Rck367	H11 endclone	VIIe						•				•																																		
D6Rck368	P7 endclone	VIIf	RFLP	Chr.6				•																																						
D6Rck369	I9 endclone	VIIf						•																																						
D6Rck329	H6 endclone	VIII	SSCP	Chr.6				•					•																																	

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 into the bins described previously (Zuo et al. 1995) and six new bins (VIIa–VIIf) derived from the bin described previously as bin VII. The fourth column summarizes polymorphisms that have been tested on the *Lc/cast.* backcross. The fifth column indicates the markers that have been mapped to mouse chromosome 6 by somatic cell hybrid mapping analysis. The top rows indicate the names and insert sizes of the 9 YACs and 32 BACs used in this study. (•) Their marker content, which denotes a positive signal by Southern hybridization.

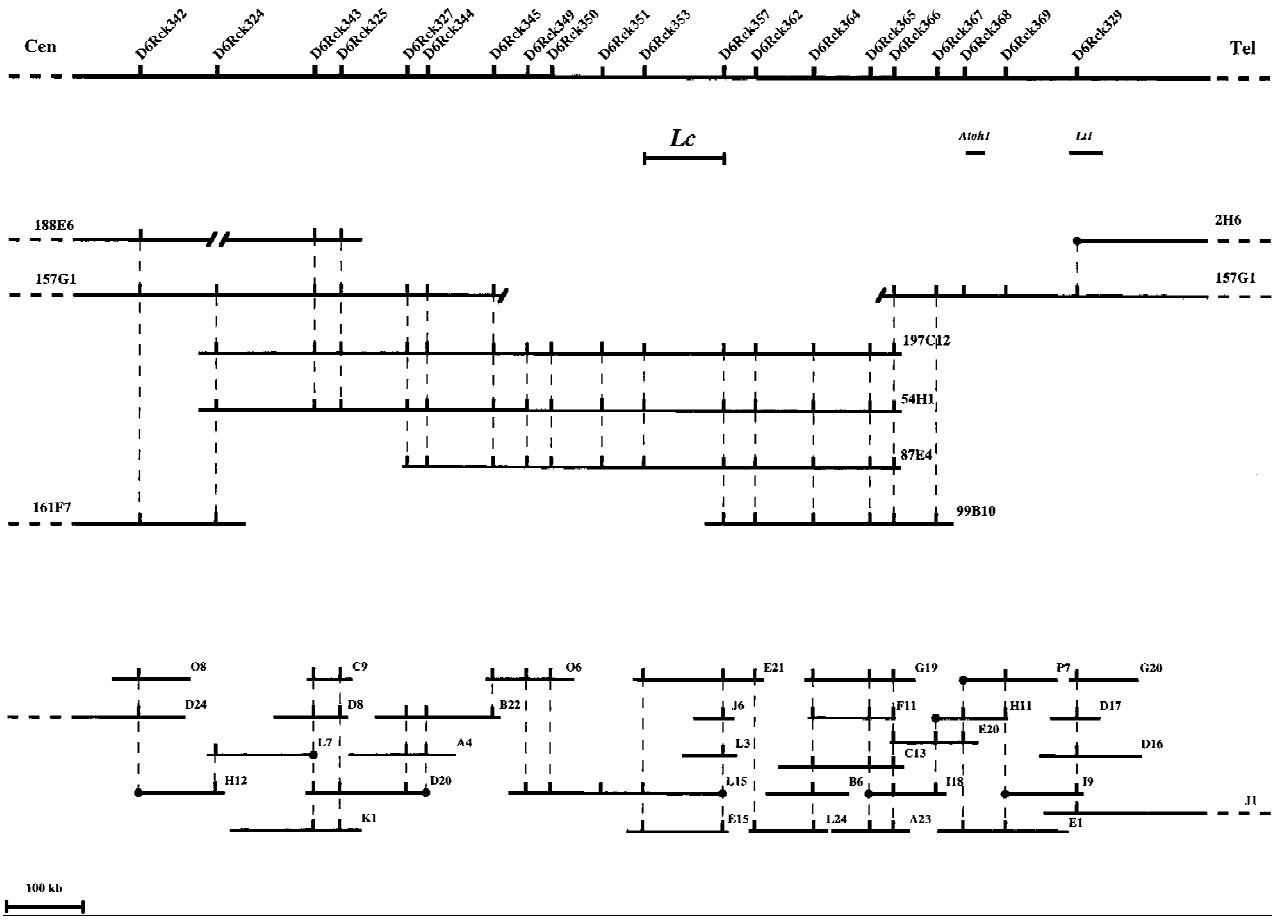

Figure 1 A composite physical map of the *Lc* locus on mouse chromosome 6. The markers presented here are a selection of markers from Table 1 that illustrate the redundancy of the YAC and BAC contigs. (*Top*) A diagram of the chromosomal segment under study showing the relative position of the markers on the chromosome. The YAC contig is shown in the *center;* individual YACs are labeled with their respective names. The BAC contig is found at the *bottom;* individual BACs are labeled with their abbreviated names. The position of each marker is indicated by a bold vertical line on the chromosome, YACs and BACs; markers that are end clones are indicated with a ● on the YAC or BAC from which they were isolated. The BAC contig stretches from *D6Rck342* to *D6Rck329,* covering ~1.2 Mb. The *Lc* mutation is contained within the genomic segment defined by markers *D6Rck353* and *D6Rck357.* Two genes were mapped to positions within the BAC contig; a short line describes the general position of these genes. More specifically, *Lt1* maps to marker *D6Rck329* and *Atoh1* to *D6Rck368.*

taking advantage of the four recombination events that lie within the chromosomal segment defined by *D6Rck325* and *D6Rck329* (Zuo et al. 1995). In total, 20 markers were mapped using restriction fragment length polymorphism (RFLP), simple sequence length polymorphism (SSLP), or single-strand conformation polymorphism (SSCP) (Fig. 2; Table 1); for each marker, three groups of animals were genotyped. The first group consists of the four critical animals—CL103, CL230, CL369, and CL391—which have recombination events within the *Lc* locus; the second consists of CL222 and CL340, which have recombination events within 1 Mb of the locus; and the third consists of 15 control animals from the backcross that have recombination events flanking this 1-Mb locus (Fig. 3).

Among these markers, only two, *D6Rck354* and *D6Rck355,* were found to be nonrecombinant (Figs. 2 and 3); they cosegregated with the mutation in all 504 animals of the *Lc/cast.* backcross. They are flanked by *D6Rck353* on the centromeric side and *D6Rck357* on the telomeric side; the location of these two markers in the physical map was used to identify them as the closest flanking markers, as their position could not be resolved genetically from that of neighboring markers.

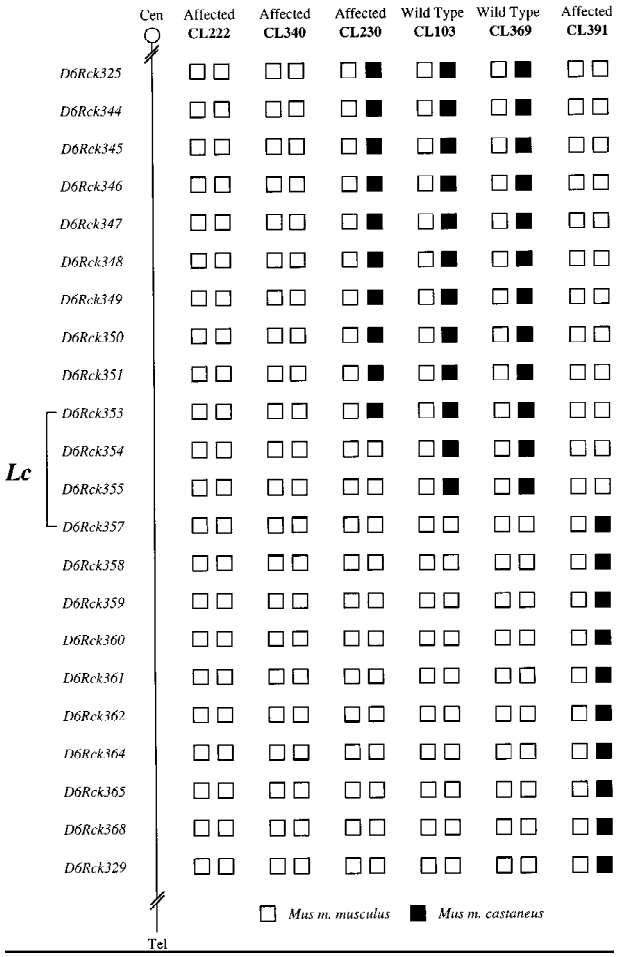

Figure 2 Genotyping of the six critical animals with markers in the *Lc* locus of mouse chromosome 6. These animals—CL103, CL222, CL230, CL340, CL369, and CL391—have recombination events within 1 Mb of the *Lc* locus; however, CL222 and CL340 have recombination events that map outside the *D6Rck342–D6Rck329* genomic segment that is under study. The phenotypic status of each animal is indicated beneath the name of the animal. The markers used in this study are listed in a column to the *left* of the diagram of mouse chromosome 6. The two chromosomes of each animal are illustrated to the *right* of the diagram. The polymorphism used to genotype each marker is described in Table 1. Because the position of most of these markers cannot be resolved genetically, their position relative to each other was determined using the physical map (Table 1). The location of the *Lc* mutation can be inferred by looking for nonrecombinant markers, markers that cosegregate with the mutation in all 504 animals of the *Lc/cast.* backcross. We would expect all affected animals to have a *musculus* allele at the nonrecombinant marker [four different strains may have contributions to the genomic segment containing the *Lc* locus (Zuo et al. 1995): DBA/2J, C3HeB/FeJ, C57BL/6J, and B6CBACa-A^{w-J}/A]. In addition, all wild-type mice should have the wild-type allele, the CAST/Ei allele, at the nonrecombinant marker. This pattern is seen at two markers: *D6Rck354* and *D6Rck355.* The closest flanking markers *D6Rck353* and *D6Rck357* thus define the genomic segment containing the *Lc* locus.

A Restriction Map of the *Lc* Locus

The two nonrecombinant markers (*D6Rck354* and *D6Rck355*) and the two flanking markers (*D6Rck353* and *D6Rck357*) were used to construct a restriction map of the region surrounding the *Lc* locus. The 143E21 BAC was used as the primary substrate in this study; the enzymes used alone and in combination to produce partial digests of this BACs are *Bss*HII and *Sfi*I. Analysis of Southern hybridization experiments to the restriction-digested BAC resulted in the identification of a 110-kb genomic fragment that contains all four markers (Fig. 4). This restriction map was confirmed using the 222L15 BAC as a substrate. Thus, the *Lc* locus must be <110 kb and, as seen in Figure 1, has at least threefold coverage in BAC clones.

A Partial Transcript Map of the *Lc* Locus

In the course of our study, one YAC end clone, *D6Rck329,* was seen to strongly hybridize to two independent bands in genomic DNA. Furthermore, upon hybridization to a zoo blot, *D6Rck329* hybridized to genomic fragments in mouse, rat, and human genomic DNA (J. Zuo, unpubl.). Screening of a mouse P6 cerebellar cDNA library with this marker yielded five different clones that were organized into two families based on sequence homology of their 3′ end. When primer pairs derived from the cDNA sequence are used to map the location of a cDNA on the physical map by PCR, one family of cDNA clones clearly originates from the terminal portion of YAC 2H6; the other does not. The former family of clones identify the location of a gene found within our YAC contig; thus, we name this gene lurcher transcript 1 (*Lt1*) (Fig. 1). Sequence derived from these clones was analyzed; it did not present significant homology to any known gene but has a high degree of homology to several mouse, rat, and human ESTs found in the GenBank database

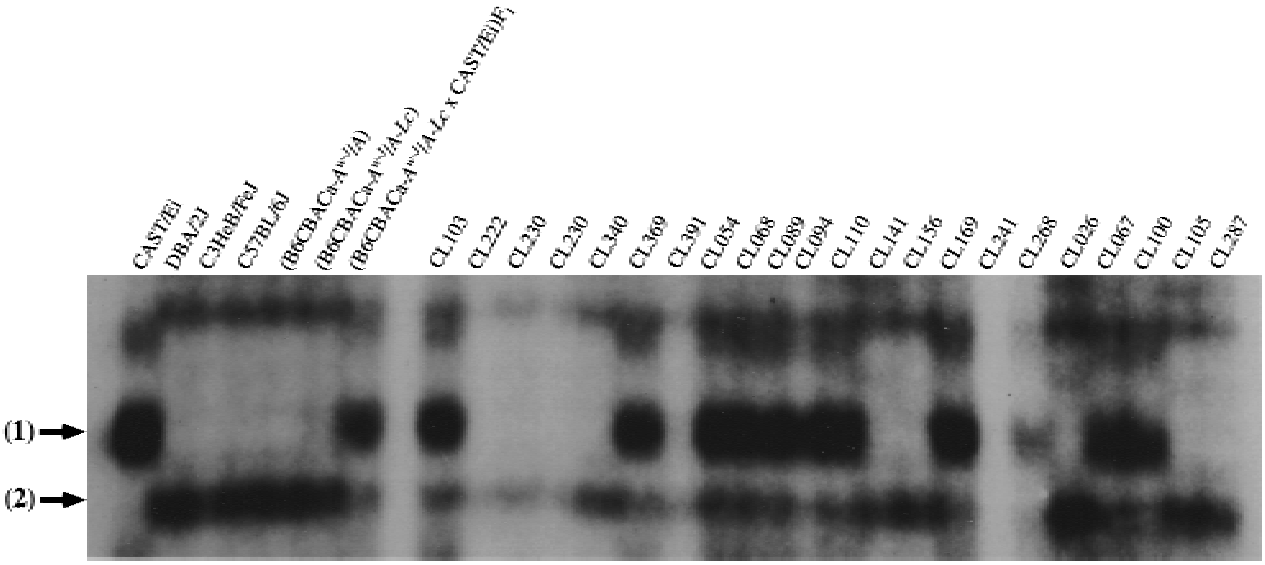

Figure 3 Genetic mapping of the nonrecombinant marker *D6Rck354* by SSCP. The first seven lanes contain a panel of control reactions; the origin of the substrate genomic DNA is indicated at the *top* of each lane. The following seven lanes contain the products of the reactions using the genomic DNA of the critical animals as substrate. These six animals are CL103, CL222, CL230 (two lanes), CL340, CL369, and CL391. The phenotypic status of these animals is described in Fig. 2. Finally, the last 15 lanes contain the products of reactions amplified from the genomic DNA of 15 control animals from the backcross. None of the latter animals has a recombination event within 1 Mb of the *Lc* locus. Two bands are highlighted with arrows. The higher band (*1*) is CAST/Ei-specific; the lower band (*2*) is specific to the inbred strains tested here (DBA/2J, C3HeB/FeJ, C57BL/6J, and B6CBACa-A^{w-J}/*A*). Some of the lanes—especially animals CL222, CL230, CL340, and CL391—in this gel appear to have a signal strength that is much weaker than that of the control lanes; however, because other bands in the affected lanes have a proportionally lower signal, the difference among the lanes probably arises from differences in the efficiency of certain PCR reactions. A longer exposure of this gel presents the expected pattern of a nonrecombinant marker.

shown). Further characterization of these clones and of the putative family member encoded by the other group of clones was not pursued further.

In addition, the *Atoh1* gene was genetically mapped by Ben-Arie et al. (1996) and found to be nonrecombinant with *Etl1,* a locus on the telomeric flank of the *Lc* locus (Zuo et al. 1995). Physical mapping of the *Atoh1* gene placed it within our BAC contig, where it colocalized with marker *D6Rck368* (Fig. 1).

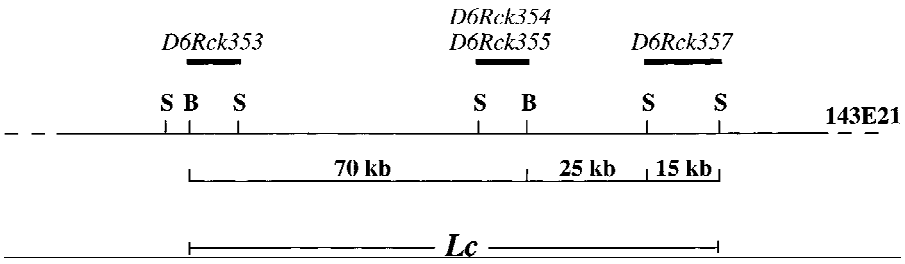

Figure 4 Restriction map of the *Lc* locus. Partial digests of BACs 143E21 and 222L15 with either *Bss*HII (B) or *Sfi*I (S) and with both *Bss*HII and *Sfi*I were separated by PFGE. The two nonrecombinant markers, *D6Rck354* and *D6Rck355,* and the two flanking markers, *D6Rck353* and *D6Rck357,* were hybridized to the resulting blots and defined a 110-kb segment that contains all four markers. The positions of both the markers and the restriction sites are noted on a diagram of 143E21; the genomic segment containing the *Lc* locus is outlined below.

DISCUSSION

We have accumulated the two elements necessary to undertake the final steps of positional cloning: We have both a small, genetically defined interval and a multiply redundant physical map over this interval.

Using an average of 3.3 markers per BAC, we have constructed a contig of 32 BACs that have an average insert size of 130 kb. These BACs provide an average of three-

fold coverage over the genomic segment defined by *D6Rck342* and *D6Rck329*, and coverage varies from a single BAC in one location, a segment of 222L15, to six BACs in another region of the contig (Table 1). Markers isolated from the BACs were mapped onto the *Lc/cast.* cross in an effort to isolate nonrecombinant markers; once such markers—*D6Rck354* and *D6Rck355*—were found, they allowed the characterization of a 110-kb genomic segment that contains both the telomeric (*D6Rck353*) and the centromeric (*D6Rck357*) flanking marker as well as the two nonrecombinant markers. The *Lc* locus is thus <110 kb, and there is at least threefold BAC coverage of this genomic segment (Fig. 1).

Based on the size of the BACs, their overlapping pattern, and the size of the overlapping YACs, we estimate that the distance between markers *D6Rck342* and *D6Rck329* is ~1.2 Mb. The deletion in YAC 157G1 is ~400 kb and has proven to be critical, as the *Lc* locus (*D6Rck353–D6Rck357*) is contained within the deletion. However, the four additional YACs described here complemented the deletion well and were essential in the isolation of the critical BACs.

BACs were isolated using one of two strategies during the construction of the contig: chromosome walking and YAC shotgun subcloning. Chromosome walking yields a highly redundant (four to five times) contig, but it is rather slow. On average, a turnaround time of 2.5 weeks can be expected for each walking event; one walk is defined here as the time between the receipt of a BAC to the receipt of additional BACs that contain the end clones of the initial BAC. Chromosome walking requires the isolation of the ends of the BAC insert under study. Because there was no established method of BAC end isolation at the time this study was performed, we used three different methods to isolate BAC ends: concatamer rescue (CR)–PCR, colony hybridization of shotgun subclones, and direct sequencing of BAC substrates. We prefer the latter method because it proved to be the most rapid, robust, and versatile one; a detailed comparison of the three protocols can be found in Methods.

The other approach used in establishing a BAC contig is YAC shotgun subcloning into Lambda Zap vector, which provides an abundant source of markers spread over a large region. The preparation is more arduous and ultimately yields a less redundant map in our hands, but the coverage is much greater relative to the amount of time spent screening the libraries. Six weeks of library construction, clone characterization, and BAC library screening yielded ~600 kb of coverage. Furthermore, this approach is less likely to become bogged down in regions that are difficult to clone because of repetitive element content or other reasons. A coverage/time comparison shows us the following: walk, 52 kb/week (130 kb/2.5 weeks), versus shotgun, 100 kb/week (600 kb/6 weeks). The best strategy thus appears to have two steps: an initial YAC shotgun subcloning yielding coverage of the whole region, followed by a more limited BAC end clone characterization to increase redundancy over the region of interest.

Two transcripts were mapped onto our contig during the construction process. *Lt1* maps to *D6Rck329*, a marker that is ~500 kb from *D6Rck357*, the flanking marker telomeric to the *Lc* locus. *Lt1* is thus a poor candidate for the *Lc* gene, as it maps far from the *Lc* locus and is separated from the locus by another gene, *Atoh1*. *Atoh1* maps to *D6Rck368*, which is ~400 kb from *D6Rck357*. This is still quite a great distance from the *Lc* locus, and although no other gene has been described in the *D6Rck357–D6Rck368* interval so far, it is unlikely that *Atoh1* is a serious candidate. This hypothesis is supported by in situ studies showing that *Atoh1* expression in the cerebellum is restricted to the external granular layer (Akazawa et al. 1995); this finding is inconsistent with the well-documented findings supporting a cell-autonomous mode of action for the *Lc* gene in Purkinje cells (Wetts and Herrup 1982a,b,c, 1983). In addition, Ben-Arie et al. (1996) sequenced the *Atoh1* coding region of a *Lc* animal and screened the *Lc* genome for rearrangements in the vicinity of the *Atoh1* gene. Both approaches failed to demonstrate a mutation in the *Atoh1* gene of *Lc* animals. Although the possibility of a mutation in the regulatory region of *Atoh1* remains, the distance that separates this gene from the *Lc* locus makes this hypothesis unlikely.

Atoh1 and its human homolog *HATH1* are useful in another manner: They establish synteny between a segment of mouse chromosome 6 and 4q22 in the human genome (Ben-Arie et al. 1996). Previously, the *Lc* locus was in a segment of unknown synteny lodged between two large segments of synteny to human chromosomes 2 (telomerically) and 7 (centromerically) (Zuo et al. 1995). Because we do not have a syntenic marker centromeric to *Lc*, we cannot say that the human homolog of the *Lc* gene lies in human 4q22. However, the proximity of the syntenic region encourages us to search for candidate genes in this segment of human chromosome 4.

Our efforts are currently focused on isolating transcripts from the genomic segment containing the *Lc* locus itself. The BACs are a good substrate for

all approaches undertaken in this effort—exon trapping, cDNA selection, and shotgun sequencing. We are favoring the shotgun sequencing approach and aim at only 80%–90% sequence coverage of the locus, as our aim is to find genes using sequence analysis algorithms.

METHODS

Mice

Mice used in this study were purchased from the Jackson Laboratory and maintained at the Specific Pathogen Free facility at the Rockefeller University Laboratory Animal Research Center under standard procedures. The *Lc* mutation is maintained in a B6CBACa-A^{w-J}/A background.

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. The standard 10× PCR buffer (Perkin Elmer Cetus) was used in all reactions. All PCR reactions were carried out in a 25 µl volume. Samples were processed through an initial denaturation (94°C for 4 min), then 35 cycles of denaturation (94°C, 30 sec), annealing (30 sec), and elongation (72°C, 30 sec), followed by 10 min of elongation at 72°C and storage at 4°C. The annealing temperature for each PCR primer pair is listed in Table 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 or BMR Kodak films were exposed to the filters at −70°C for 10–48 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.

BAC templates were treated slightly differently than other plasmids. After the initial isolation of DNA using standard protocols, the DNA was purified by polyethylene glycol (PEG) precipitation: Two-thirds volume of the stock PEG solution (2.5 M NaCl, 20% PEG-8000) was added to 1 volume of the BAC DNA solution and mixed gently; the precipitated DNA was then spun down at 13,000 rpm for 15 min (4°C); finally, the pellet was washed once with 70% ethanol, dried, and resuspended in distilled H_2O. The concentration of DNA was then estimated by comparing a *Hin*dIII digest of the BAC DNA to a 1-kb ladder. The cycle sequencing protocol for BACs consists of an initial 2-min denaturation at 96°C, followed by 30 cycles of three-step PCR (96°C, 10 sec; 50°C, 5 sec; 60°C, 4 min). One microgram of template and 160 ng of primer were used in every reaction. All reactions were performed using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS, and were carried out on a Perkin Elmer Thermal Cycler 9600.

Sequence homology searches were performed using the NCBI database.

Pulsed-Field Gel Electrophoresis

All pulsed-field gel electrophoresis (PFGE) analyses were performed on a Bio-Rad Chef DR II under conditions that fractionate the DNA from 50 to 1000 kb range as recommended by the vendor. The Lambda ladder from GIBCO BRL and the Mid-range PFG marker I from New England Biolabs (NEB) were used to size the BACs and YACs.

Table 2. Sequence and Characteristics of Primer Pairs Used in This Study

Marker	Primer Pair	Product Size
D6Rck344	A: 5'- TACCATGCATGGGTACACAAAGC	297 bp
	B: 5'- AGTGTACATACAGCGTTCAGCAC	
D6Rck345	A: 5' - CAATTCACCTAAATTGTCACCCAC	301 bp
	B: 5' - GCAGACTAAATACTTGGTTAATTTCAG	
D6Rck346	A: 5'- GCTGCAGTGGCAGCAGACAAG	349 bp
	B: 5'- GAAAAGATCTGAAAGGAGTTGG	
D6Rck347	A: 5'- CCTGTGAACTGAACTGCTTACTTC	219 bp
	B: 5'- AAAGCCTTAATGAAGTAACAGC	
D6Rck351	A: 5'- CCATGTCTTTGTGGACTTGTTTCC	324 bp
	B: 5'- CTATTGGTAGTCTGTCCTCACATC	
D6Rck354	A: 5'- ATCTCATATCCTCTATTGACTC	328 bp
	B: 5'- GGCAGACAAGAAACAGATGTGGTC	
D6Rck355	A: 5'- AATAATGCTCAACTGGGACTGGC	327 bp
	B: 5'- GTTGAGAAAGGTGAGACAACCAAG	
D6Rck357	A: 5'- ATCATGGTGAGAATTTGAATTGGC	263 bp
	B: 5'- TTCTGACATCTCTAGCTAACAGG	
D6Rck358	A: 5'- AAGCTTTGGCTGGTCCCTCGGG	354 bp
	B: 5'- GTGGTCACATAACTTCAGGACAG	
D6Rck359	A: 5'- AAGCTTTGGCTGGTCCCTCGGG	281 bp
	B: 5'- CCATCCCAGACACTCCTCCGTGC	
D6Rck360	A: 5' - GTTTCCGTTACTCTTCAAATGTC	262 bp
	B: 5' - TTTAGGAAATATCTGAACAGTC	
D6Rck361	A: 5'- GTTGCATACATGAATTATTACACC	200 bp
	B: 5'- GACTGGACATTGAAACTGGCTA	
D6Rck362	A: 5'- GAAAAGTCTGCAATGGATCTCAG	295 bp
	B: 5'- GAATAGGAGCAGGGAAACTAG	
D6Rck364	A: 5' - ACATTCACTGCCAGGAAGCCCTG	278 bp
	B: 5' - GAAGGCCACACTTGTTACAGTGC	
D6Rck365	A: 5' - GTTTCAAATTATGCTACGAAGAAATTGG	117 bp
	B: 5' - CACCCAAGAGCCAAGAAAGGC	

All PCR reactions were carried out with an annealing temperature of 55°C, and all polymorphisms between CAST/Ei and B6CBACa-A^{w-J}/A displayed by these PCR products were SSCPs except for the *D6Rck361* product, which has an SSLP.

Detection of Polymorphisms

For SSLPs, a standard three-step PCR cycle was used to amplify the desired genomic segment. Amplified products were separated in a 15 × 20-cm 10% acrylamide gel (30:1 acrylamide to bis-acrylamide) in 0.5 × TBE at 200 mV for 2.5 hr. For RFLPs, 5–10 µg of genomic DNA from different parental mouse strains was digested with a variety of enzymes. Labeled probes were hybridized to Southern blots of the digested DNAs. Observed RFLPs were further used to genotype backcross DNA samples. Five markers displayed RFLPs: *D6Rck348, Eco*RI; *D6Rck349, Hae*III; *D6Rck350, Bam*HI; *D6Rck353, Pst*I; and *D6Rck368, Bam*HI. 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.

Somatic Cell Hybrid Mapping Panel

Somatic cell hybrid 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, BCM1-4, ECM4C, and R2-24, were obtained and analyzed further for their mouse chromosomal content by karyotypic analysis. Mouse chromosome 6 is only present in lines 2A2B1, 2A2H3, and BCM1-4 but not in lines 2A2C2, ECM4C, and R2-24. These cell lines were kindly provided by Dr. Jeffrey Friedman (HHMI, Rockefeller University).

Genomic Libraries and Screening

The pooled YAC library distributed by Research Genetics, Inc., was screened by PCR, and the BAC library, also distributed by Research Genetics, Inc. (Shizuya et al. 1992), was screened by both PCR and Southern hybridization to isolate BACs present in the *Lc* locus. These two screening techniques complemented each other well; at least one of the two techniques worked for each marker that was used to screen the BAC library.

YAC Shotgun Subcloning

This protocol was described in detail earlier (Zuo et al. 1995). In short, PFGE-isolated YAC DNA was digested, subcloned into the Lambda Zap II cloning vector (Stratagene), and packaged. The resulting phage clones were isolated, and their inserts were mapped onto the YAC contig.

BAC End-Clone Isolation

BAC end clones were isolated using one of three methods: direct sequencing of the BAC, CR–PCR, and colony hybridization of shotgun subclones. The sequencing approach was described above and proved to be a rapid and robust method to isolate BAC ends. With purified BAC DNA, 14 of 16 BAC ends were sequenced successfully (88% success rate) after an initial attempt. The remaining two ends were sequenced in subsequent attempts. The rate-limiting step in this method proved to be the sequencing process, as DNA isolation and purification were completed readily. The additional benefit of having this sequence is that primer pairs can be readily designed for the purpose of mapping a BAC end by SSCP.

CR–PCR, which is described below in detail, proved to be rapid because the BAC DNA does not need to be purified and the ligation step requires only 3 hr. This ligation reaction can be considered the rate-limiting step of this method, although the subsequent PCR amplification step and separation of products on a 10% acrylamide gel (as for the SSLP above) require a similar amount of time. Initially, five of eight BAC ends attempted (63%) were isolated using this method, and the average end-clone size was 200 bp. However, because of considerable background in some of the PCR reactions, the 63% success rate, the small insert size, and the success of our sequencing effort, we did not investigate this method further.

The third method—selecting BAC shotgun subclones that contain flanking sequences from the vector—is described below in detail. This method is slower than either sequencing or CR–PCR; the rate-limiting step is the required colony hybridization with an oligonucleotide. The advantage of this method is that it is very robust—all BAC ends can be isolated if sufficient numbers of clones are screened—and isolates large end clones. The size of the end clones can be important if the BAC insert sequence flanking the vector is repetitive; in this case, isolating an end clone by this method usually allows one to get beyond the repetitive element. We used this technique on 8 BACs and recovered 6 ends after screening 50 subclones from each BAC.

Thus, we recommend direct BAC sequencing as the best method for isolating BAC ends, both because of the rapidity and robustness of this method and because it yields information that can be used for various purposes. CR–PCR does not present any advantage over direct sequencing, but subclone selection proved to be a useful backup method to extract end clones from certain BACs.

CR–PCR

In the CR–PCR method, a BAC clone is digested with a restriction enzyme that cuts frequently (four-cutter); the resulting fragments are ligated to themselves. A fraction of the ligated products are concatamers of identical fragments that are ligated in an antiparallel orientation. If such a concatamer contains a known sequence, such as Sp6 or T7, the BAC end fragments flanking such sequences can be selectively amplified by using a single primer, Sp6 or T7.

With the pBeloBAC11 vector, a *Hae*III digestion yields insert-end fragments that contain Sp6 or T7 sequences. Thus, a single ligation reaction (at least 3 hr at 14°C) can be used as a substrate in the two amplification reactions (2 min at 94°C of denaturation, and 35 cycles of 94°C, 55°C, and 72°C each for 30 sec). The end fragments trapped in the concatamer are quite small (75–300 bp with an average of ~200 bp); however, two copies of the end fragment are present in the PCR product, making it an efficient substrate for random-primed labeling reactions.

Colony Hybridization in BAC End-Fragment Isolation

Colony hybridization also takes advantage of sequences flanking the cloning site to isolate the end fragments of the BAC; the Sp6 sequence of the pBeloBAC11 vector was used in this protocol. The T7 sequence could also be used to isolate the other end of the BAC; one would only have to substitute a T7 sequence-less plasmid for the pBluescript II vector

(Stratagene) in the protocol detailed below. A number of enzymes or enzyme combinations can be used to prepare a shotgun-subcloned library of a BAC; we chose the *Eag*I–*Eco*RI combination for two reasons. First, there is a convenient *Eag*I site 248 bp distal to the *Hin*dIII cloning site on the Sp6 side; thus, the vector contribution to the end-clone insert is minimal. Second, *Eag*I cuts more rarely than many six-cutters because its target sequence is GC-rich; for example, *Eco*RI cuts, on average, three times more frequently than *Eag*I (1/5 kb and 1/15 kb, respectively) (NEB 1996/1997 catalog, p. 253). Thus, especially if the BAC of interest has few *Eag*I sites, the complexity of an *Eag*I/*Eco*RI library will be less than that of an *Eco*RI library (on average, one-third less), facilitating the screening process. In addition, the *Eco*RI enzyme reduces the size of the *Eag*I fragments, yielding a more manageable set of subclones. The ends rescued by this protocol are quite large (4 to >12 kb, with an average of 9 kb), providing good probes for Southern hybridization.

A BAC clone is digested simultaneously with *Eco*RI and *Eag*I and shotgun-subcloned into the pBluescript II vector (Stratagene). In the ligation reaction, ~40 ng of the digested BAC DNA is ligated into an *Eco*RI–*Eag*I-digested pBluescript II vector (~200 ng) [calf intestinal phosphatase (CIP)-treated for 30 min at 37°C]. Following transformation using XL-1 Blue cells and selection with β-galactosidase, colonies containing insert are organized in a grid, grown, and lifted onto nitrocellulose filters (Schleicher & Schuell). The filters are then denatured (0.5 M NaOH, 1.5 M NaCl) for 5 min, neutralized (0.5 M Tris at pH 8.0, 1.5 M NaCl) for 5 min, and rinsed twice in 2× SSPE (5 min each). Colonies were probed by Southern hybridization with a labeled Sp6 oligonucleotide.

The Sp6 oligonucleotide was kinased using polynucleotide kinase (PNK) (Boehringer Mannheim) and hybridized to the filters overnight at 40°C. They were then washed twice in 6× SSC for 30 min each time. The filters exposed film for 6 hr, and positive colonies were picked for further characterization.

Nomenclature Change

The nomenclature committee altered the name of the markers used to characterize the *Lc* locus; the names were changed from *D6Rkf* to *D6Rck*. In addition, a third digit, a 3, was added so that numbers went from 01 to 301. For example, one of the markers used in this study changed from *D6Rkf29* (Zuo et al. 1995) to *D6Rck329*.

ACKNOWLEDGMENTS

We thank colleagues in our laboratory for their support and comments, particularly Wei Hu for outstanding technical support and Xiangdong W. Yang for his comments. We also acknowledge Drs. Jeffrey Friedman, Claude Desplan, and Andrew Peterson for advice and are grateful to Dr. Friedman for somatic cell hybrid lines. P.L.D. is supported by the National Institutes of Health/ National Institute of General Medical Sciences (NIH/NIGMS) training grant 5T32GMO7739; J.Z. is a postdoctoral associate and N.H. an Investigator of the Howard Hughes Medical Institute.

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REFERENCES

Akazawa, C., M. Ishibashi, C. Shimizu, S. Nakanishi, and R. Kageyama. 1995. A mammalian helix-loop-helix factor structurally related to the product of Drosophila proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system. *J. Biol. Chem.* 270: 8730–8738.

Bahary, N., J. E. Pachter, R. Felman, R. L. Leibel, K. Albright, S. Cram, and J. M. Friedman. 1992. Molecular mapping of mouse Chromosomes 4 and 6: Use of a flow-sorted Robertsonian chromosome. *Genomics* 13: 761–769.

Ben-Arie N., A.E. McCall, S. Berkman, G. Eichele, H.J. Bellen, and H.Y. Zoghbi. 1996. Evolutionary conservation of sequence and expression of the bHLH protein Atonal suggests a conserved role in neurogenesis. *Hum. Mol. Genet.* 5: 1207–1216.

Caddy, K.W. and T.J. Biscoe. 1979. Structural and quantitative studies on the normal C3H and lurcher mutant mouse. [Review]. *Phil. Trans. Roy. Soc. Lond. Ser. B. Biol. Sci.* 287: 167–201.

Cheng, S. and N. Heintz. 1997. Perinatal death of homozygous lurcher mice results from massive loss of hindbrain neurons during embryonic development of homozygous lurcher mice. *J. Neurosci.* 17: 2400–2407.

Dumesnil-Bousez, N. and C. Sotelo. 1992. Early development of the lurcher cerebellum: Purkinje cell alterations and impairment of synaptogenesis. *J. Neurocytol.* 21: 506–529.

Norman, D.J., C. Fletcher, and N. Heintz. 1991. Genetic mapping of the lurcher locus on mouse Chromosome 6 using an intersubspecific backcross. *Genomics* 9: 147–153.

Norman, D.J., L. Feng, S.S. Cheng, J. Gubbay, E. Chan, and N. Heintz. 1995. The lurcher gene induces apoptotic death in cerebellar Purkinje cells. *Development* 121: 1183–1193.

Phillips, R.J.S. 1960. ''Lurcher'', a new gene in linkage group XI of the house mouse. *J. Genet.* 57: 35–42.

Rabacchi, S.A., Y. Bailly, N. Delhaye-Bouchaud, K. Herrup, and J. Mariani. 1992. Role of target in synapse elimination: Studies in cerebellum of developing lurcher mutants and adult chimeric mice. *J. Neurosci.* 12: 4712–4720.

Shizuya, H., B. Birren, U.-J. Kim, V. Mancino, T. Slepak, Y. Tachiiri, and M. Simon. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc. Natl. Acad. Sci.* 89: 8794–8797.

Vidal-Puig, A. and D.E. Moller. 1994. Comparative sensitivity of alternative single-strand conformation polymorphism (SSCP) methods. *BioTechniques* 17: 490–492.

Wetts, R. and K. Herrup. 1982a. Cerebellar Purkinje cells are descended from a small number of progenitors committed during early development: Quantitative analysis of lurcher chimeric mice. *J. Neurosci.* 2: 1494–1498.

———. 1982b. Interaction of granule, Purkinje and inferior

olivary neurons in lurcher chimaeric mice. I. Qualitative studies. *J. Embryol. Exp. Morphol.* 68: 87–98.

———. 1982c. Interaction of granule, Purkinje and inferior olivary neurons in lurcher chimeric mice. II. Granule cell death. *Brain Res.* 250: 358–362.

———. 1983. Direct correlation between Purkinje and granule cell number in the cerebella of lurcher chimeras and wild-type mice. *Brain Res.* 312: 41–47.

Zuo, J., P.L. De Jager, D.N. Norman, and N. Heintz. 1995. Generation of a high-resolution genetic map and a YAC contig of the lurcher locus on mouse Chromosome 6. *Genome Res.* 5: 381–392.

Received February 18, 1997; accepted in revised form May 20, 1997.